Studies on the biosynthesis of Ribonucleic Acid.

by

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Abbreviations

The following abbreviations will be used in this thesis:-

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
UR	uridine
CR	cytidine
AMP	adenosine-5' monophosphate
AMP-3'(or2')	adenosine-3'(or2') monophosphate
ADP	adenosine-5' diphosphate
ATP	adenosine-5' triphosphate
CMP	cytidine-5' monophosphate
CMP-3'(or2')	cytidine-3'(or2') monophosphate
CDP	cytidine-5' diphosphate
CTP	cytidine-5' triphosphate
GMP	guanosine-5' monophosphate
GMP-3'(or2')	guanosine-3'(or2') monophosphate
GDP	guanosine-5' diphosphate
GTP	guanosine-5' triphosphate
UMP	uridine-5' monophosphate
UMP-3'(or2')	uridine-3'(or2') monophosphate
UDP	uridine-5' diphosphate
UTP	uridine-5' triphosphate
IMP	Inosine-5' monophosphate
dAMP	deoxyadenosine-5' monophosphate

dATP	deoxyadenosine-5' monophosphate
dCMP	deoxycytidine-5' monophosphate
dCTP	deoxycytidine-5' triphosphate
dGMP	deoxyguanosine-5' monophosphate
dGTP	deoxyguanosine-5' triphosphate
TMP	thymidine-5' monophosphate
TTP	thymidine-5' triphosphate
^{32}P-UTP	uridine-5' triphosphate labelled with a radioactive phosphorus atom. (see text)
^{32}P-UDP	uridine-5' diphosphate labelled with a radioactive phosphorus atom. (see text)
^{32}P-ATP	adenosine-5' triphosphate labelled with a radioactive phosphorus atom. (see text)
^{14}C-UTP	uridine-5' triphosphate labelled at the C6 position with a radioactive carbon atom. (see text)
CEP	2-cyanoethyl phosphate.
^{32}P-CEP	2-cyanoethyl phosphate containing a radioactive phosphorus atom.
DCC	dicyclohexylcarbodiimide.
DCU	dicyclohexylurea.
NAD	nicotinamide-adenine dinucleotide.
$NADH_2$	reduced nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
P_i	inorganic orthophosphate
PP_i	inorganic pyrophosphate
Tris	tris(hydroxymethyl) aminomethane

Contents

SECTION I

INTRODUCTION.

1.1 The Nucleic Acids.

To state that the heart of present day biochemistry lies in the field of nucleic acids and their derivatives is probably no exaggeration. Nevertheless the combination of chemical, cytological and genetic studies has now led to the realisation that nucleic acids are of fundamental importance in controlling the metabolism, reproduction and growth of living systems.

The discovery of the nucleic acids was the result of the investigations of Miescher whilst in the laboratory of Hoppe-Seyler in Tubingen (Miescher, 1871). From the nuclei of pus cells he isolated a substance which he termed "nuclein" - now known to be nucleoprotein. Due to its high phosphorus content and its acidity it warranted special attention, because at that time the only known phosphorus containing organic compound in tissue was lecithin. His work was soon to be repeated and confirmed by others including Hoppe-Seyler himself, who eventually published the results in his own journal. It was Altmann, one of Miescher's students, in 1889 who, having developed methods for the isolation of protein-free "nuclein", called this substance nucleic acid (Altmann, 1889). Another of Miescher's students was Piccard who first isolated the purine bases guanine and hypoxanthine from an acid hydrolysate of salmon sperm nucleic acid (Piccard, 1874), and Miescher's

successor, Kossel, another of Hoppe-Seyler's pupils, was responsible for the isolation of xanthine from yeast and adenine from beef pancreas (Kossel, 1879). His splendid example was quickly followed by other groups, and around the turn of the century other pyrimidine bases such as thymine (Kossel and Neuman, 1894), uracil (Ascoli, 1900-1901), and cytosine (Kossel and Steudel, 1902-03; Levene, 1902-03) were identified in nucleic acid hydrolysates. However, it was Kossel (1891) who first detected carbohydrate in a yeast nucleic acid hydrolysate. This was subsequently identified by one of his students as D-ribose, a hitherto unknown pentose. Levene and Jacobs (1909) went on to demonstrate D-ribose as a component of the nucleoside adenosine.

This work of Miescher, which amply demonstrated the polymeric character of nucleic acid, was regrettably overlooked by later workers, who made use of material which had been extracted from tissues by methods involving heat treatment and the use of acid or alkali for investigation, using the degradative methods of organic chemistry. For example, in 1899 Neuman described a variation of the Altmann procedure for the isolation of nucleic acid involving heating of the minced tissue in a 3% solution of sodium hydroxide. Undoubtedly the product was suitable for degradative studies, but it bore little resemblance

to the native nucleic acid. Such extraction procedures set the pattern for the ensuing thirty years or so, and were almost certainly responsible for the acceptance of a highly erroneous value for the molecular weight of the nucleic acid. Although the complexity and lability of nucleic acid were appreciated by Miescher and Kossel, later workers, using degraded material, were led to fallacious conclusions, some of which seriously hindered the development of ideas on the structure and function of nucleic acid. An outstanding example of this was the original conception of a nucleic acid molecule as a tetranucleotide. This was based on such misleading molecular weight studies on degraded material, and the fact that a hydrolysate of the same material contained four nucleotides in approximately equimolar proportions, which appeared to indicate a molecule composed of four nucleotides.

However, despite this obvious drawback, the hydrolytic studies involving the classical methods of organic chemistry were of fundamental importance in the determination of the elementary molecules which together constitute nucleic acid. Indeed, by 1930, a definite picture had emerged of two distinct types of nucleic acid. One of these, the nucleic acid from yeast, on hydrolysis yields the bases adenine, guanine, cytosine and uracil,

along with phosphoric acid and a pentose, which had been previously identified by Levene and Jacobs (1909) as D-ribose. The other, the nucleic acid from thymus gland, yields adenine, guanine, cytosine, thymine, phosphoric acid and a deoxypentose, which was later shown to be D-deoxyribose (Levene, Mikeska and Mori, 1930). As a result of these studies, the two nucleic acids were named ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively. Moreover, since most nucleic acid of animal origin was similar to that of thymus gland, whilst that of plant origin resembled the nucleic acid of yeast, it was believed for a time that RNA only occurred in plant tissues, and DNA only in animal tissues. However, within a short time, many exceptions to this classification came to light. By 1924, the idea began to develop that RNA was also distributed in animal tissues. Conclusive evidence of this came from the histochemical studies of Brachet (1933; 1937; 1940a,b), and the ultraviolet spectrophotometric examination of tissues, pioneered by Caspersson, which demonstrated the presence of RNA in the cytoplasm of rapidly proliferating cells (Caspersson, 1936; 1940; 1941; Caspersson and Schultz, 1939; 1940; Caspersson and Thorell, 1941; Caspersson, Nystrom and Santesson, 1942). These data were soon confirmed chemically by Davidson and Waymouth (1943; 1944a). From these results it was concluded that RNA, whilst mainly found

in plants, could also be detected in the embryonic tissues of higher animals. Therefore it seemed reasonable to postulate that the occurrence of RNA was characteristic of rapidly proliferating tissues, such as those of embryonic origin. However, Davidson and Waymouth (1944b,c) were soon to isolate RNA from various adult tissues, and so that theory had to be abandoned.

Originally, the nucleic acids were thought to be essentially nuclear constituents, however RNA was for some time suspected to occur also in the cytoplasm. The development of the histochemical tests such as the Feulgen stain (Feulgen and Rossenbeck, 1924), specific for DNA, has been of considerable value in deciding the location of the nucleic acids within the cell. By this means, for instance, it was demonstrated that DNA is confined to the cell nucleus of plants and higher animals, and indeed it appears exclusively associated with the chromosomes, or chromatin material. Similarly the Brachet histochemical test, involving ribonuclease, has demonstrated RNA in the cell cytoplasm, as mentioned earlier. The general conclusion from such researches is that the cytoplasmic components contain RNA, whilst the nuclei contain DNA and a small amount of RNA.

The concept that the carrier of genetic information is nucleic acid arises from the discovery of Avery et al. (Avery, MacLeod and McCarty, 1944) that the 'transforming

principle", first discovered by Griffith in 1928 (Griffith [illegible], 1928), which will induce the transformation of unencapsulated pneumococci into fully capsulated cells, is a highly polymerised DNA. Outstanding as this work appeared, its importance was not fully realised at the time, as ideas concerning the structure of nucleic acids were highly prejudiced in favour of the tetranucleotide theory. Nevertheless, this discovery did well to demonstrate that milder extraction methods produced a more highly polymerised material, which was biologically active, and it soon encouraged a slow but sure return to milder extraction methods, which eventually made it possible to estimate the molecular weight of nucleic acids on a more realistic basis.

Concomitant with this growing realisation was the development of paper and ion-exchange chromatographic techniques, such that very precise analyses of the chemical constituents of the nucleic acids could be carried out. As a result, it was soon evident that nucleic acids in their native state are indeed highly polymerised, and most certainly did not exist as tetranucleotides.

With such a stimulus, research in the nucleic acid field was greatly accelerated and with continued investigation using these new techniques it was soon recognised that the RNA exists in the cell in combination with protein

as part of the nucleus, microsomes and cell sap, and can be extracted in a relatively undegraded state with a variety of gentle procedures (Magasanik, 1955). Structurally RNA is basically a large linear copolymer built up from the four ribonucleotide units, namely the 5'-monophosphates of adenosine, guanosine, cytidine and uridine. (AMP, GMP, CMP and UMP) in 3'-5' phosphodiester links, so that the complete molecule could be termed more accurately a polyribonucleotide (Markham, 1957). Hydrolysis by dilute alkali yields 2 isomers of each nucleotide, the 2'-phosphate and the 3'-phosphate (Cohn 1950; 1951). The involvement of C'_5 in the internucleotide linkage was further confirmed by Cohn and Volkin (1953) who treated polyribonucleotide material with snake venom diesterase and obtained a mixture of 5'-phosphates of all four ribonucleosides. The possibility that the linkage is C'_2-C'_5 rather than C'_3-C'_5 is excluded by the observation that hydrolysis with spleen diesterase yields nucleoside 3'- phosphates (Whitefield, Heppel and Markham, 1955).

As mentioned previously, RNA exists as nucleoprotein in the cell, and it is very probably heterogeneous in character, varying from one subcellular component to another. Moreover, since it is highly conceivable that many preparations of RNA consist of fragments torn out of the cell, some degradation is inevitable, and so it is not surprising

that estimates of molecular weight vary over quite a large range. Values of between 20,000 and 2,000,000 have been recorded using various techniques, such as sedimentation, intrinsic viscosity, diffusion and light scattering (Ochoa and Heppel, 1957), whilst a particular type of RNA, termed soluble RNA, found in the cytoplasm of mammalian cells, such as liver cells, has a particularly low molecular weight of around 10,000 (Zamecnik, Stephenson and Hecht, 1958). However confusion still exists, due to the varying conditions that are presently used. For instance, Jordan (1952) has pointed out that the diffusion constant and the sedimentation constant of RNA vary quite considerably with concentration and with the ionic strength of the solvent, so that it is extremely difficult to obtain reliable figures. Also it is clear that for highly accurate molecular weight determinations an analysis for heterogeneity and a fractionation must be carried out. Very recent data indicate that RNA, although linear in part, may well have a secondary structure consisting of small helical regions involving up to half its nucleotides (Doty, 1961).

From the functional point of view, it has become abundantly clear that RNA is intimately concerned in the process of cellular protein biosynthesis. This arose from the work of Caspersson (1950) and Brachet (1950) who demonstrated, using histochemical techniques, that RNA is

particularly abundant in cells engaged in the synthesis of protein for growth and secretion. Thus RNA can be found in fairly high concentration in embryonic tissues, in tumours and in rapidly growing bacterial cultures. In microorganisms, a correlation has been demonstrated between growth rate and RNA content per cell (Caldwell and Hinshelwood, 1950). Gale and Folkes (1953) found that the synthesis of protein by disrupted staphylococci depends on their nucleic acid content. Later work showed that ribonuclease inhibits the incorporation of amino acids into cytoplasmic components of animal cells (Allfrey, Daly and Mirsky, 1953).

Nevertheless, it is important to consider the formation of protein from the genetic point of view. From the work of Avery et al. (1944), already discussed, there is evidence that in bacterial transformation DNA acts as a hereditary determinant in producing a permanent change in the inherited characteristics of the cell similar to that produced by a mutation. Histochemical evidence demonstrates that in higher plants and animals DNA is localised on the chromosomes, or chromatin material, and bacteria are known to contain nuclear material and a genetic system analagous to that found in higher organisms (Robinow, 1947). Therefore, it seems reasonable to interpret bacterial transformation as indicating that DNA is the active material of the gene; that

it can be extracted and purified while still retainings its genetic function and that it can enter a homologous cell and become a permanent part of the genetic equipment of that cell. It was suggested that if DNA were the active material of the gene, then in any given species the DNA content per set of chromosomes should be constant. Boivin, Venderly and Venderly (1948) showed that while the mean amount of DNA in the nucleus varies quite widely from species to species, it is apparently constant for the nuclei of the different somatic tissues of a given species. On the other hand, the amount of DNA in sperm nuclei, which contain the haploid number of chromosomes, is approximately half that found in the somatic cell nuclei of the same species. Further evidence for the genetic function of DNA comes from a study of the metabolism of DNA. Although DNA may not be completely inert, it is much more stable than other components of the cell (Smellie, 1955). According to Hughes (1959), this is precisely what one would expect if DNA were the hereditary material of the cell.

If DNA is to convey genetic information, then it must have some sort of code incorporated into its chemical structure. DNA, like RNA, is a linear copolymer, made up of four nucleotide units in C'_3-C'_5 phosphodiester linkages. In the case of DNA, these are the 5'-monophosphates of thymidine, deoxyadenosine, deoxycytidine and deoxyguanosine

(TMP, dAMP, dCMP and dGMP), and it is conceivable that the genetic code comprises the sequential variation of these compounds along the length of the polydeoxyribonucleotide chain. However it is difficult to visualise DNA in the role of genetic transfer in the form of a single stranded polynucleotide, since any structural model must provide for (a) the coding of genetic information (b) its re-duplication at each cell division and (c) the translation of information into protein structures.

On the basis of X-ray analysis techniques, Watson and Crick in 1953 described a structure which is now widely accepted as the basic structure of DNA. This structure would appear to fulfil the above requirements. They envisaged DNA as comprising two complementary polydeoxy-ribonucleotide chains, which are coiled around one another and held together in the form of a double helix through specific hydrogen bonds between their respective bases. By constructing scale models they were able to demonstrate that the bases could only fit if they were arranged in pairs, one purine base opposite one pyrimidine base. Under these circumstances, when the formation of hydrogen bonds between the pairs of bases was considered in detail, it became evident that the only pairs which would fit together were adenine with thymine and guanine with cytosine. According to Delbruck and Stent (1957), shortly before cell division

the two strands unwind and each strand subsequently acts as a template for the formation of a new helix, such that the DNA content of the cell nucleus at this stage is approximately doubled.

If it can be assumed, in accordance with the generally accepted view first put forward by Beadle (1951), that the synthesis of each enzyme (or for that matter protein) is controlled by a particular gene, and if it is assumed that the active material of the gene is DNA, it follows that any theory of protein synthesis must account for the participation not only of RNA but also of DNA. By 1957 it was commonly supposed that specific DNA molecules, corresponding to genes, give rise to the formation of complementary molecules of RNA, which in turn induce the synthesis of specific proteins (Brachet, 1958). In other words, it is possible that the information contained in DNA is transferred to the nucleotide sequence of RNA, and it is this secondary code which controls the synthesis of protein.

By using only four nucleotides, Crick found it theoretically possible to code the twenty or so amino acids found in normal proteins, if three successive bases on the chain are required to code each amino acid (Crick, 1958). Nevertheless, the precise mechanism by which DNA exercises its influence on the cell is still obscure, although the

Watson and Crick model for DNA is supported from several fields of study, such as hydrolytic investigations, X-ray crystallography and ultracentrifugation methods (Jordan 1960). Moreover the X-ray patterns obtained from intact biological material suggest that the helical structure is present not merely in DNA extracted from the cell but in the living cell itself. From the genetic point of view it is of considerable interest to note that if the number of genetic units in a bacteriophage is determined by the classical methods of genetics and compared with the number of nucleotide pairs available in the DNA of the organism, rough calculation indicates that there are only a few nucleotide pairs per genetic unit (Benzer, 1957).

However it has only been in very recent years that any real advance has been made towards understanding the enzymes, or enzyme systems, involved in the biosynthesis of RNA and DNA by living organisms. Such studies have contributed greatly to our understanding of the intricate processes through which the nucleic acid molecules exert their control over the growth, metabolism and reproduction of living cells. It is the purpose of this thesis to deal with one important aspect of the biosynthetic problem, namely the building up of RNA molecules by enzymes of mammalian cells. At the same time, an attempt will be made to illustrate the effect that DNA has on this process, and its bearing on protein synthesis.

1.2 Biosynthesis of purines and their derivatives.

For a very long time it has been known that higher organisms are capable of synthesing purines de novo from smaller molecules, but the most important work on the biosynthesis of the purine ring system has been carried out during the last ten years by Buchanan and Greenberg and their colleagues. The work was initiated by studies on the distributions of ^{13}C within the uric acid molecule as excreted by pigeons after the administration of small molecules labelled with ^{13}C.

Buchanan, Sonne and Delluva (1948) worked out a series of reactions for the degradation of the uric acid molecule in such a way that the individual carbon atoms of uric acid could be isolated and assayed for isotope content. By this means it was found that CO_2 was incorporated chiefly into carbon 6 of the purine ring, the carboxyl group of glycine into carbon 4, and that formate was utilised almost exclusively for carbons 2 and 8 (Sonne, Buchanan and Delluva, 1948). The observations have been amply confirmed by other workers (Elwyn and Sprinson, 1950; Sakami, 1948; Sprinson and Rittenberg 1952; Krasna, Peyser and Sprinson, 1952). Most of the subsequent work was performed on pigeon liver preparations, in which the mechanism of biosynthesis of hypoxanthine was studied, there being no xanthine oxidase present to degrade the hypoxanthine to uric acid. Greenberg, using a pigeon liver system,

observed that ^{14}C-formate, ^{14}C-bicarbonate and ^{14}C-glycine could be incorporated into the hypoxanthine molecule (Greenberg, 1948; Greenberg 1950). Subsequent work with extracts of pigeon liver confirmed that glycine, CO_2 and formate provide the precursor units from which hypoxanthine is synthesised (Greenberg, 1941a; Schulman, Sonne and Buchanan, 1952), combining in the molar proportions 1:1:2 respectively.

It has only been recently that the precursors of the nitrogen atoms of the purine ring have been established conclusively. Shemin and Rittenberg (1947) found that the nitrogen 7 of the uric acid molecule was derived from glycine and this was confirmed by Buchanan et al. (1948). Further investigations revealed that the nitrogenous precursor of positions 3 and 9 was the amide group of glutamine and that either glutamic acid or aspartic acid donated the nitrogen of position 1 in hypoxanthine synthesis in extracts of pigeon liver (Sonne, Lin and Buchanan, 1953; Sonne, Lin and Buchanan, 1956). Recently Buchanan, Flaks, Hartman, Levenberg, Lukens and Warren (1957) conclusively demonstrated aspartic acid as the specific donor of nitrogen atom 1.

During the time when the precursors of the purine ring system were being investigated, other relevant observations were made. For instance, in 1945, Stetten and Fox, using E. coli grown in the presence of sulphonamides, isolated from

the culture medium a diazotisable amine, which was later shown by Shive, Ackerman, Gordon, Getzendener and Eakin (1947) to be 4-amino-5-imidazole carboxamide. On account of the resemblance of this compound to the purines, it attracted much attention, in view of the possibility that it might function as a precursor of the complete purine ring system. Indeed, it was found to serve as a precursor of rat nucleic acid purines (Miller, Gurin and Wilson, 1950), and it was subsequently demonstrated that it combined with formate in equimolar proportions to form hypoxanthine (Schulman and Buchanan, 1952). However, Greenberg (1951b) and Schulman and Buchanan (1952) conducted experiments to show that 4-amino-5-imidazole carboxamide itself was not on the direct pathway of purine biosynthesis. In view of this finding, and from evidence from Greenberg (1950, 1951a) that inosine 5'-monophosphate (IMP) was formed before hypoxanthine in pigeon liver extracts, it became evident that 4-amino-5-imidazole carboxamide ribotide (AICAR) was an intermediate in the synthesis of IMP from small molecule precursors and that IMP was the first purine compound to be formed, being converted to inosine, and subsequently to hypoxanthine in the liver extracts.

With the discovery of a new intermediate in nucleotide biosynthesis, 5-phosphoribosyl pyrophosphate (PRPP) by

Kornberg, Lieberman and Simms (1954,1955a,1955b) and Lieberman, Kornberg and Simms (1954), the pathway of formation of IMP began to take shape. PRPP was found to combine with purines in an enzyme catalysed reaction to form nucleotides according to the general reaction:

$$\text{Purine} + \text{PRPP} \rightleftharpoons \text{Purine nucleoside 5'-monophosphate} + \text{inorganic pyrophosphate.}$$

PRPP was also shown to be utilised by a purified pigeon liver system in the presence of glutamine to form 5'-phosphoribosylamine (PRA), which in turn could then condense with glycine to form glycinamide ribotide (GAR) (Hartman, Levenberg and Buchanan, 1955, 1956; Goldthwait, Peabody and Greenberg, 1955, 1956; Hartman, 1956). The GAR is then formylated to give formyl glycinamide ribotide (FGAR) (Goldthwait, Peabody and Greenberg, 1954, 1956; Hartman, Levenberg and Buchanan, 1956; Warren and Flaks, 1956), using N^5,N^{10}- anhydro formyl tetrahydrofolic acid as formyl donor (Hartman and Buchanan, 1959). The following stage of the enzymic reaction sequence was found by Levenberg and Buchanan (1956) to be the conversion of FGAR to 5-amino-imidazole ribotide (AIR) in the presence of glutamine with the intermediate formation of formyl-glycinamide ribotide. The AIR is then converted to 4-amino-5-imidazole carboxamide ribotide (AICAR) in the presence of CO_2 and aspartate (Lukens and Buchanan, 1956), 4-amino-5-imidazole-(N-succinyl) carboxamide having been tentatively identified as the intermediate in this

Figure 1. The enzymic synthesis of inosinic acid (IMP) de novo.

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

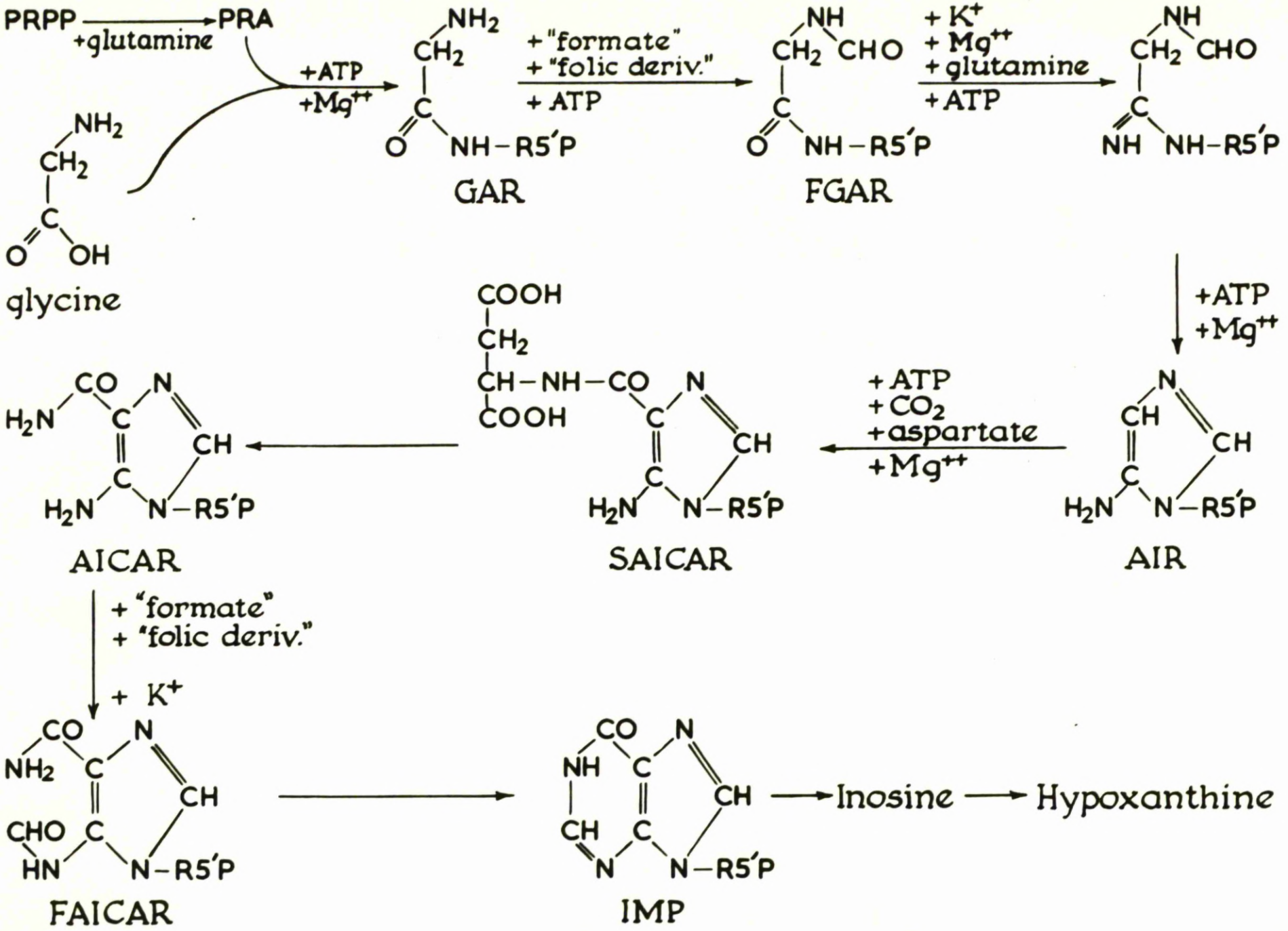

PRPP
PRA
+glutamine
+ATP
+Mg++
glycine
GAR
+"formate"
+"folic deriv."
+ ATP
FGAR
+ K+
+ Mg++
+glutamine
+ATP
+ATP
+Mg++
AIR
+ATP
+ CO2
+aspartate
+Mg++
SAICAR
AICAR
+ "formate"
+ "folic deriv."
+ K+
FAICAR
IMP
Inosine
Hypoxanthine

Figure 1

transformation. The introduction of the final carbon atom is brought about by the formylation of AICAR by N^{10}-formyl tetrahydrofolic acid (Hartman and Buchanan, 1959). The formyl-AICAR is finally cyclised to IMP. Thus the mode of IMP biosynthesis has been clarified by the use of pigeon liver extracts and is illustrated in Fig.1.

Further in vitro work illustrated how AMP and GMP, nucleotide constituents of RNA, could be derived from IMP. AMP is produced by the reaction of IMP with L-aspartate to form the intermediate adenylosuccinic acid (Carter and Cohen, 1956; Abrams and Bentley, 1955; Lieberman, 1956a, b and c) guanosine 5'-triphosphate (GTP) is required as a cofactor. The adenylosuccinic acid formed thus then breaks down to AMP and fumarate (see Fig.2.)

The first step in the enzymic conversion of IMP to GMP involves the oxidation of IMP to xanthosine 5'-monophosphate (XMP) in the presence of nicotinamide-adenine dinucleotide (NAD). The XMP is then enzymically aminated in the presence of glutamine and adenosine 5'-triphosphate to yield GMP. (see Fig.2.)

1.3. Biosynthesis of the pyrimidines and their derivatives.

The biosynthetic pathway to the pyrimidines (see Fig.3) has been elucidated mainly on the basis of work involving microorganisms, but the same system appears to operate in mammalian systems.

Figure 2. Enzymic synthesis of adenylic and guanylic acids from inosinic acid.

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

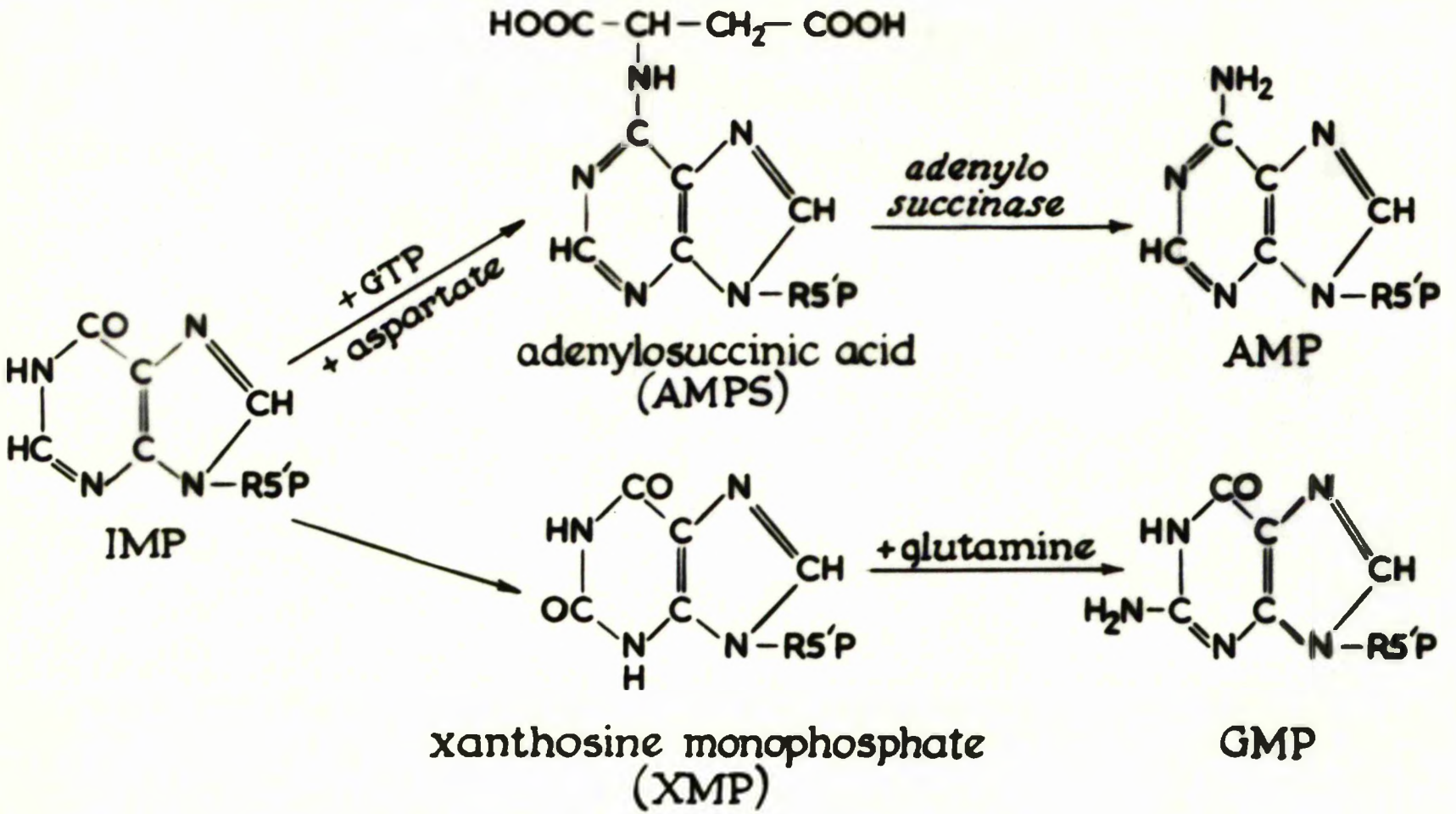

HOOC – CH – CH2 – COOH
NH
adenylo succinase
NH2
+GTP
+aspartate
adenylosuccinic acid
(AMPS)
AMP
IMP
+glutamine
xanthosine monophosphate
(XMP)
GMP

Figure 2

Degradation of labelled pyrimidines has shown that N1 of the pyrimidine ring is derived from NH_3, C2 from CO_2 and C4, C5 and N3 from aspartic acid. Experiments involving the incorporation of possible labelled precursors have revealed that ureidosuccinic acid (carbamyl aspartic acid) and orotic acid (uracil 4-carboxylic acid) lie on the biosynthetic pathway (Reichard, 1955; Lieberman and Kornberg, 1954; Cooper, Wu and Wilson, 1956; Wu and Wilson, 1956.)

The first step involves the interaction of CO_2 and NH_3 under the influence of ATP to form carbamylphosphate (CP) (Jones, Spector and Lipmann, 1955). The carbamylphosphate then reacts with aspartic acid under the influence of aspartate carbamyl transferase to form ureidosuccinate (US) (Schulman and Badger, 1954; Smith and Stetten, 1954; Heinrich, Dewey and Kidder, 1954). Then, under the influence of the enzyme dihydroorotase, which has been isolated by Lieberman and Kornberg (1954), ring closure of US is effected to yield dihydroorotic acid (DHO), which in turn is oxidised by dihydroorotic dehydrogenase to yield orotic acid (OA). Under the influence of orotidine 5'-phosphate pyrophosphorylase and magnesium ions, OA reacts with PRPP to form orotidine 5'-monophosphate (UMP), which, according to Hurlbert and Reichard (1954), is either degraded to uridine and uracil, or phosphorylated to uridine 5'-pyrophosphate compounds.

Although formate does not serve as a precursor of

Figure 3. The pathway of <u>de novo</u> synthesis of pyrimidines.

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

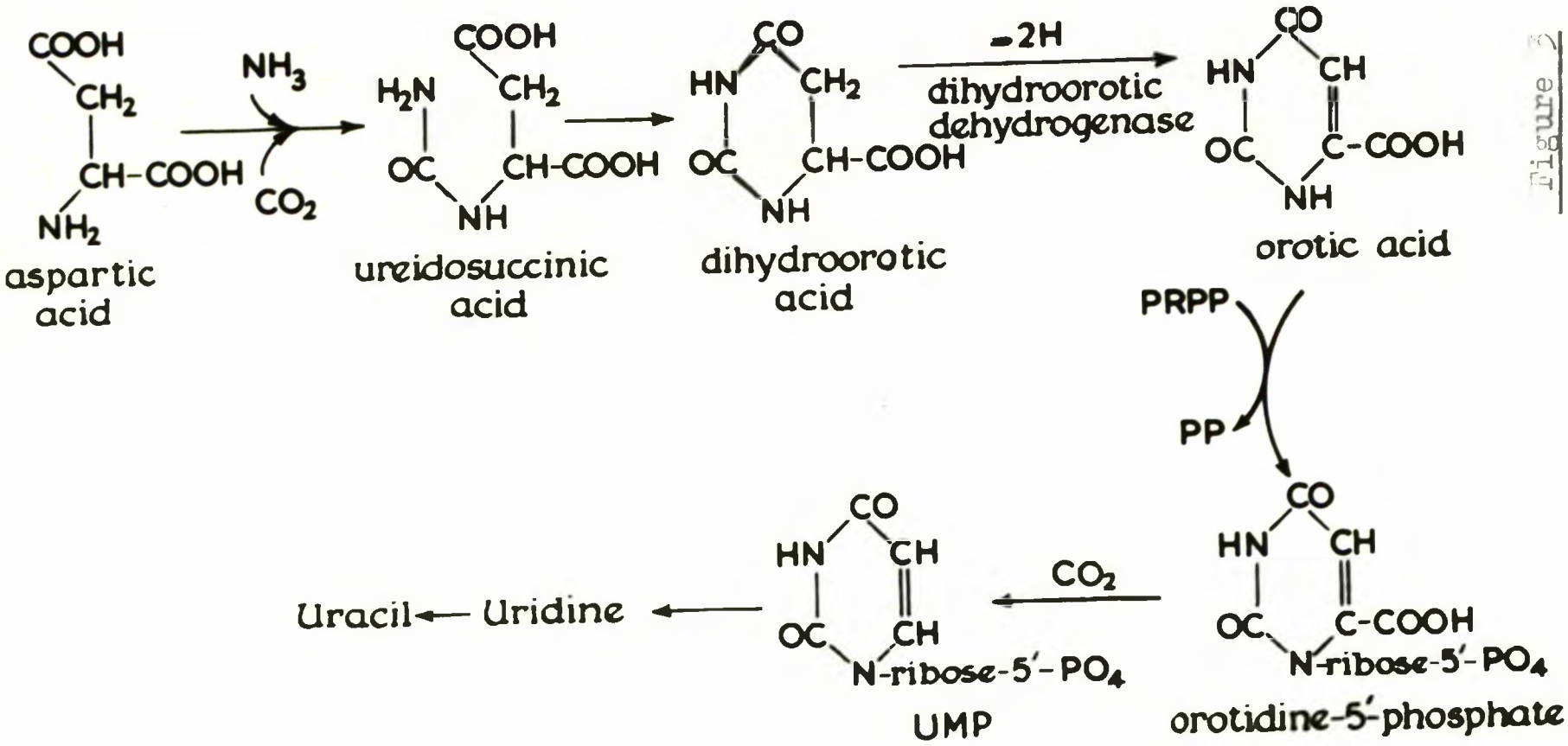

COOH
CH2
CH-COOH
NH2
aspartic acid
NH3
CO2
H2N
CH2
OC
CH-COOH
NH
ureidosuccinic acid
HN
CH2
OC
CH-COOH
NH
dihydroorotic acid
-2H
dihydroorotic dehydrogenase
CO
HN
CH
OC
C-COOH
NH
orotic acid
PRPP
PP
N-ribose-5'-PO4
orotidine-5'-phosphate
CO2
HN
CH
OC
CH
N-ribose-5'-PO4
UMP
Uridine
Uracil

Figure 2

nucleic acid uracil, it does serve as a precursor for the 5-methyl group of thymine and the recent work of Kornberg (1957) implicated hydroxymethyltetrahydrofolic acid in this context. From a review by Crosbie (1961) it appears that uridine serves as a precursor of the thymine ring and there is evidence to suggest that the glycosidic linkage remains intact during conversion to the deoxyriboside which is then methylated in the 5 position with the involvement of this folic acid derivative, hydroxymethyltetrahydrofolic acid.

1.4. Biosynthesis of Nucleic acids.

(a) General considerations.

With the knowledge that purine and pyrimidine derivatives can be synthesised from small molecules, many of the early investigations in this field were carried out using small molecules such as $^{32}PO_4^{3-}$, ^{14}C-formate and ^{14}C-glycine as isotopic tracers (Brown and Roll, 1955). Although the present knowledge of the mechanisms by which RNA and DNA are synthesised *in vivo* is derived almost entirely from experiments using cell-free systems, the early work performed on whole animals and surviving tissue preparations has been of great value.

Much of the basic work in the field of polynucleotide biosynthesis was carried out by Brown and his collaborators (Brown, 1954; 1955; 1956) who used isotopically labelled bases, nucleosides and nucleotides to investigate the manner in

which various organisms can utilise exogenous supplies of these compounds for the synthesis of RNA and DNA. Their experiments indicated that in the rat, for example, adenine is incorporated into polynucleotide adenine and guanine, whilst adenosine and AMP are utilised to a lesser degree. On the other hand guanine and guanosine are not incorporated to an appreciable extent although GMP is moderately well utilised. Free pyrimidines are not utilised extensively in polynucleotide biosynthesis but their nucleosides and nucleotides appear to be readily incorporated.

The work of Rose and Schweigert (1953) using cytidine uniformly labelled with ^{14}-C demonstrated that the ribose was incorporated into RNA as extensively as the base, and they concluded that the nucleoside was incorporated without breakage of the glycosidic bond.

Because nucleosides are so readily incorporated into nucleic acid, isotopically labelled thymidine has been used widely in studies of the biosynthesis of DNA. Labelled thymidine has been shown to be readily incorporated into DNA in the rat (Reichard and Estborn, 1951), the chick embryo (Friedkin, Tilson and Roberts, 1956), onion root tips (McQuade, Friedkin and Atchison, 1956) and tissue culture (Lu and Winnick, 1954).

However although bases and nucleosides are readily incorporated into nucleic acid, the mechanism of these

reactions is uncertain. In 1954, Marrian reported that 8-^{14}C-adenine injected into rats was rapidly and extensively incorporated into the AMP, ADP and ATP of liver, intestine and muscle, thereby confirming the observations of Goldwasser (1953) on pigeon liver homogenates and by Bennet (1953) with mice. Further work of a similar nature (Bennet and Kreuckel, 1955) in which 4,6-^{14}C-adenine was administered to mice, proved that the labelled adenine was extensively utilised for the formation of acid-soluble AMP, ADP, ATP and for the formation of RNA and DNA in such a manner as to suggest that the acid-soluble nucleotides serve as precursors of the nucleic acids. Thus it became of interest to ascertain, if possible, the level of phosphorylation at which a nucleotide would become a direct nucleic acid precursor.

Attempts to incorporate nucleotides labelled isotopically in all three components, that is base, ribose and phosphate ester group, demonstrated that they were extensively dephosphorylated after injection into the rat (Roll, Weinfeld, Carroll and Brown, 1956), and no evidence could be obtained for the incorporation of intact nucleotides into RNA. However, despite this, the adenosine moiety of labelled AMP is largely incorporated intact into RNA, and the conversion of adenosine into RNA guanosine and DNA deoxyguanosine occurs without the rupture of the glycosidic link. Leibman and Heidelberger (1955) incubated ^{32}P-labelled ribonucleoside 5'-monophosphates

with rat liver, Flexner-Jobling carcinoma slices, and suspensions of Ehrlich ascites carcinoma cells. A study of the distribution of the isotope in the acid-soluble and nucleic acid fractions proved that the nucleotides were dephosphorylated and that the random ^{32}P incorporation into the nucleic acids was due to the uptake of labelled inorganic phosphate resulting from nucleotide breakdown.

(b) Polynucleotide phosphorylase.

In 1956, the first indications of a reaction which could lead to the enzymic synthesis of polyribonucleotides were obtained through the notable researches of Ochoa and his colleagues (Grunberg-Manago, Ortiz and Ochoa, 1956; Ochoa and Heppel, 1957). These workers found that the synthesis of polyribonucleotide material can occur by condensation of ribonucleoside 5'-diphosphates under the influence of the enzyme polynucleotide phosphorylase, which they prepared from Azotobacter vinelandii. This enzyme requires the presence of magnesium ions and proceeds with the liberation of inorganic orthophosphate, according to the following equation: where R stands for ribose, PP for pyrophosphate, P for orthophosphate and B for one of the following bases; adenine, guanine, uracil, cytosine or hypoxanthine.

$$nBRPP \rightleftharpoons (BRP)_n + nP_i$$

By incubating the enzyme with the appropriate ribonucleoside 5'-diphosphates homopolymers containing AMP, UMP, CMP and IMP have been obtained, but polymers containing GMP are much more

difficult to prepare. Degradative studies of these synthetic polymers have shown that like RNA they consist of ribonucleoside 5'-monophosphate units linked together by 3', 5'-phosphodiester bonds.

This enzyme appears to be mainly of bacterial origin, occurring in a variety of microorganisms (Littauer and Kornberg, 1957; Beers, 1957; Olmsted and Lowe 1959), although its presence has been reported in spinach leaves (Brummond, Staehlin and Ochoa, 1957), and in a rather tentative way in Ascaris lumbricoides (Entner and Gonzalez, 1959), atypical epithelioma of the rat (Yagi, Ozawa and Konogi, 1959) and in nuclei prepared from guinea pig liver (Hilmoe and Heppel, 1957). Such work suggested that the ribonucleoside 5'-diphosphates might be the direct precursors of RNA in mammalian cells. Hurlbert (1954), Schmitz et al. (1954) and Brumm, Potter and Siekevitz (1956) carried out in vivo studies on the livers of rats which had received 6-^{14}C-orotic acid, 1-^{14}C-glucose and ^{32}P as inorganic phosphate prior to killing. The results of specific activity determinations on the isolated acid-soluble nucleotides indicated that the labelling of base, ribose and ester phosphate is very rapid, and nearly the same for all members of each ribonucleotide series, so that it was not possible to establish the ribonucleoside 5'-diphosphates as the proximal precursors of the RNA.

(c) Biosynthesis of DNA.

As mentioned in a previous section (1.4a) isotopically labelled thymidine has been used extensively in studies on the biosynthesis of DNA in intact cells. Subsequently it was extended to studies with cell-free preparations. In 1956 Kornberg and his associates, using cell free extracts prepared from E.coli demonstrated that ^{14}C-thymidine in the presence of ATP and inorganic ions was a specific precursor of DNA. This process required the prior conversion of the thymidine to thymidine 5-triphosphate (TTP), and it was evident that these crude extracts contained a kinase system capable of catalysing this conversion. In later work TTP was used as substrate, and this made possible the purification of the enzyme responsible for its incorporation into DNA. This enzyme, termed DNA polymerase, or, using systematic nomenclature, deoxynucleoside-triphosphate : DNA deoxynucleotidyltransferase, requires the presence of primer DNA, magnesium ions and the 5'-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine (dATP, dGTP, dCTP and TTP) for optimal activity. Net synthesis of up to twenty times the amount of primer added has been obtained (Kornberg, Lehman, Bessman and Simms, 1956; Kornberg, 1959; Lehman, Bessman, Simms and Kornberg, 1958). The overall reaction catalysed by this enzyme can be represented as follows :

$$\left.\begin{array}{l} n.\ dATPP \\ n.\ dGPPP \\ n.\ dCPPP \\ n.\ dTPPP \end{array}\right\} + DNA \rightleftharpoons DNA - \left[\begin{array}{l} dAP \\ dGP \\ dCP \\ dTP \end{array}\right] + 4(n)PP_i$$

From this work it seemed reasonable to postulate that the biosynthesis of DNA takes place by three steps: (i) formation of deoxynucleosides, or their monophosphates (ii) their polymerisation to the triphosphate level by the appropriate kinase system (iii) the enzymic polymerisation of the triphosphates to form DNA. The DNA so prepared *in vitro* using the purified enzyme from *E. coli* has the same physical and chemical characteristics as the calf thymus DNA used as primer (Kornberg, 1959). If *E. coli* DNA is used as primer, the newly synthesised DNA has the relative proportion of bases characteristic of *E. coli* DNA.

During 1957-60, several groups demonstrated similar systems in cell-free extracts of regenerating rat liver (Bollum and Potter, 1958; Mantsavinos and Canellakis, 1959) and Ehrlich ascites carcinoma of the mouse (Davidson, Smellie, Keir and McArdle, 1958; Smellie, Keir and Davidson, 1959; Smellie, Gray, Keir, Richards, Bell and Davidson, 1960). Bollum (1960a, 1960b) whilst studying this type of system in calf thymus extracts observed that the priming ability of many DNA samples can be enhanced by heating to 100° for 10 minutes. Such

treatment is known to bring about the dissociation of the DNA double helix into two single strand polynucleotides (Doty, 1961), and it seems likely, therefore, that single stranded DNA may be the true priming agent. This is confirmed by the observation that the single stranded DNA of bacteriophage ØX174 is a particularly good primer of the polymerase reaction.

(d) RNA biosynthesis in animal tissues.

Up till 1959, the mechanism of RNA biosynthesis in animal tissues was still obscure. As discussed in Section 1.4(a), early research involving whole animals and intact cells seemed discouraging, since such workers as Leibman and Heidelberger (1955) and Roll, Weinfeld, Carroll and Brown (1956) had shown that intact nucleotides were not incorporated into RNA. However, research in this field received a stimulus when Heidelberger, Harbers, Leibman, Takagi and Potter (1956) showed that cell-free preparations of rat liver incorporate AMP specifically into RNA without randomisation of its phosphate group, so confirming earlier observations by Goldwasser (1954; 1955) on cell-free preparations from pigeon liver. Thus, it appeared that with the intact cell, the access of intact nucleotides to the cell may be prevented due to permeability difficulties, with the result that the phosphate ester group is split from the nucleoside at the cell membrane and appears randomly

distributed among all four nucleotides subsequently prepared from the RNA.

This work was extended by Harbers and Heidelberger (1959) who used rat liver homogenates to study the incorporation of isotopically labelled AMP into RNA. Degradation of the RNA so labelled suggested a preferential linkage AMP to CMP in the RNA. Similar results have been obtained from a variety of other biological systems (Paterson and Lepage, 1957; Canellakis, 1957; 1959; Edmonds and Abrams, 1957; Herbert, 1958; Preiss and Berg, 1960; Hurwitz, Bresler and Kaye, 1959). These observations, together with the finding that most of the incorporated adenine is released as the nucleoside on alkaline hydrolysis (see Results section) indicate that the AMP has been added in a terminal location to the RNA chain. The significance of these findings has been elucidated by Zamecnik, Hoagland and their colleagues, working with soluble or transfer RNA (sRNA) of rat liver cytoplasm, which has the peculiar capacity to act as acceptor for the terminal attachment ribonucleotide units (Hecht, Zamecnik, Stephenson and Scott, 1958; Hecht, Stephenson and Zamecnik, 1959; Zamecnik, 1960; Hoagland, 1960). Such terminal attachment reactions are an obligatory prelude to amino acid attachment in the process of protein synthesis. The precursors of the terminally added nucleotide units are the ribonucleoside 5'-triphosphates, and their

attachment involves a reversible pyrophosphate splitting. Inorganic pyrophosphate inhibits the attachment, and Herbert (1959) found that it accumulated during the incorporation of nucleotides.

Although the complete base sequence of s-RNA is unknown, Zamecnik, Stephenson and Hecht (1956) described reactions whereby one or two CMP residues and an AMP residue are successively attached to the end of an existing molecule of sRNA. The reactions are as follows :

$$sRNA + CTP \longrightarrow sRNA\text{-}CMP + PP_i$$

$$sRNA\text{-}CMP + CTP \longrightarrow sRNA\text{-}CMP\text{-}CMP + PP_i$$

$$sRNA\text{-}CMP\text{-}CMP + ATP \longrightarrow sRNA\text{-}CMP\text{-}CMP\text{-}AMP + PP_i$$

SRNA with such a nucleotide end sequence can then act as acceptor for the transfer of aminoacyl residues from their respective activating enzymes to the site of incorporation into protein (Hoagland, 1960).

However, such limited terminal incorporation can hardly be regarded as representing true RNA biosynthesis and is quite different from the extensive polymerisation reaction catalysed by polynucleotide phosphorylase, an enzyme not widely distributed in mammalian tissue.

Thus, when the present study was undertaken, the mechanis of true biosynthesis in mammalian cells was far from clear, an so the initial aim of the experiments was to obtain from mammalian tissue a cell-free system capable of RNA synthesis

and to study the mechanisms involved. Previous work has shown that Ehrlich ascites tumour cells readily utilise ^{14}C-formate, ^{14}C-adenine and ^{32}P-inorganic phosphate for the biosynthesis of RNA (Smellie, Thomson and Davidson, 1958; Thomson, Smellie and Davidson, 1958). The work described in this thesis demonstrates that extracts of ascites tumour cells, and other mammalian cells, besides containing enzymes responsible for the phosphorylation of uridine to the triphosphate level appear to have an enzyme capable of polyribonucleotide biosynthesis which will use ribonucleoside 5'-triphosphates as substrates. Thus for the incorporation of UMP units into polyribonucleotide material this enzyme will utilise UTP as substrate. The conditions and characteristics of these reactions are examined in an attempt to elucidate the precise mechanism of RNA biosynthesis in mammalian cells.

1.5. The intracellular location of RNA synthesising systems.

While the main bulk of cellular RNA is found in the cytoplasmic fractions (mitochondria, microsomes and cell sap) a small but important part is present in the cell nucleus (Smellie, 1955). Since these morphological fractions probably play very different roles in the functioning of the cell it is important to consider the relation between the RNA of these different sources especially in connection with its biosynthesis.

When Bergstrand et al. (1948) administered ^{15}N-glycine to rats, the isotope content of the RNA from the liver cell nuclei was greatly in excess of that in the RNA of the cytoplasm. Using radioactive phosphate, Marshak (1941; 1943) showed that the RNA of the nucleus assimilated ^{32}P much more than did the RNA of the cytoplasm. Moreover, while the radioactivity of the nuclear RNA rose quickly after administration of the isotope and fell rapidly afterwards, the activity of the cytoplasmic RNA increased and reached a maximum level much later than the nuclear RNA. This type of finding has been confirmed by a number of workers (Barnum and Huseby, 1950; Barnum, Huseby and Vermund, 1953, Davidson, McIndoe and Smellie, 1951; Smellie, McIndoe, Logan, Davidson and Dawson, 1953; McIndoe and Davidson, 1952). Such an approach has been extended using labelled precursors of the purine and pyrimidine bases. Potter, Recknagel and Hurlbert (1951) and Hurlbert and Potter (1952) have investigated the incorporation of 6-^{14}C-orotic acid into the RNA of the nucleus and cytoplasm of rat liver. They found that the uptake of ^{14}C by the pyrimidines was much more rapid in the nuclear RNA than in cytoplasmic RNA. Using ^{14}C-formate Smellie, McIndoe and Davidson (1953) also observed greater assimilation of isotope into the RNA of the nucleus than into that of the cytoplasm. Since the amount of RNA in the nucleus is small, these isotope incorporation experiments

indicate that it must have quite a high metabolic activity.

Recent investigations, using microautoradiographic techniques suggest that the nucleus and the nucleolus might be the major site of the synthesis of RNA for both the nucleus and cytoplasm. Prescott(1957) in experiments with amoeba demonstrated that labelling of the nucleus with ^{14}C-uracil precedes the appearance of isotopic label in the cytoplasm. The experiments of Goldstein and Plaut (1955) have shown the movement of newly synthesised RNA from the nucleus to the cytoplasm in amoeba. ^{3}H-uridine was employed by Zalokar (1959) in studies of neurospora hyphae. He showed that labelling appeared initially in the nucleus and subsequently migrated to the microsomes. Sholdssek, Schneider and Potter (1958) and Schneider (1959) carried out experiments on a much larger scale and observed an RNA in the nuclei of rat liver cells which is rapidly transferred to the cytoplasm.

Within the nucleus, the RNA particularly associated with the nucleolus shows a rapid rate of labelling. In the nuclei isolated from thymus tissue a fraction, said to contain nucleolar RNA, incorporates acid-soluble RNA precursors faster than any other RNA containing fraction from these nuclei (Allfrey and Mirsky, 1957). Indeed evidence for the existence of at least two types of nuclear RNA ($nRNA_1$ and $nRNA_2$) has been obtained by several authors (Logan and Davidson, 1957; Vincent, 1957a; Osawa, Takata and Hotta,1958).

According to these authors one of the nuclear ribonucleic acids, $nRNA_2$, can be tentatively identified with the RNA associated with the nucleolus.

That nucleolar RNA can be a precursor of at least a fraction of the RNA in the cytoplasm has been indicated from work on Acetabularia (Stich and Hammerling, 1953) salivary glands of Drosophila (Taylor, McMaster and Cayuela, 1955), starfish oocytes (Vincent, 1957), bean root tips (Woods and Taylor, 1959) and pancreas cells (Fitzgerald and Vinijchaikul, 1959). By enucleation with ultraviolet rays, Perry and Errara (1960) found that in HeLa cells two-thirds of the incorporation of precursors into RNA is nucleolar dependent. Woods (1960) is of the opinion that any incorporated isotopic label passing from the nucleolus to the cytoplasmic RNA does so via soluble intermediates which do not exchange with the pool of acid-soluble RNA precursors. Until the nuclear or cytoplasmic origin of these intermediates is known, any claim that all the cytoplasmic RNA comes from the nucleus must remain open.

Considerable attention has been focussed on the very small amount of RNA on the chromosomes. Autoradiographic evidence suggests that chromosomal RNA could be the precursor of a fraction of nucleolar RNA in the starfish oocyte (Fiq, 1955), bean root tips (Woods, 1960), HeLa and leukaemic cells (Feinendegen, Bond, Shreeve and Painter, 1960) and in liver and pancreatic cells of mice (Amano and Leblond, 1960).

Even by 1960, despite the obvious abundance of autoradiographic evidence, there was little real evidence at the enzymic level to support these claims. Thus it was the purpose of the present study, not only to elucidate the enzymic mechanism of RNA biosynthesis in mammalian cells, but also to determine the intracellular location of the process.

SECTION II

EXPERIMENTAL

2.1 Biological.

Ehrlich ascites carcinoma, originally supplied by Dr. G. Popjak of Hammersmith Hospital, London, was propagated by serial transplantation in albino mice of the departmental colony. Neither the strain nor sex of the mice used was critical, and a pure cross-bred strain derived from Strong A was found very suitable. For the growing of this tumour it was advantageous to use mice aged from eight to ten weeks and weighing between 25g and 30 g, since younger mice had a high mortality rate and older mice produced ascitic fluid containing relatively few cells.

Tumour for transplantation was withdrawn by syringe under sterile conditions from mice inoculated seven days previously. 0.2 ml. of tumour cell suspension, containing in the region of $1x10^7$ cells, was inoculated intraperitoneally into the recipient mice and gave a high yield of ascitic fluid within seven to eight days. For experimental purposes, ascitic fluid was withdrawn from mice after seven days. The usual yield of fluid was within the range 5 to 10 ml., and the cell count about 5 to $10x10^7$ per ml.

The Landschutz ascites carcinoma was obtained from Dr.L. Crawford, Department of Virology, University of Glasgow. This tumour was propagated by serial transplantation in a pure Porton strain of mice. When inoculated with about $2x10^7$ cells, these mice, weighing between 40g and 50g gave a maximum yield of ascitic

fluid (about 5 ml.) after nine to ten days. The cell population of the ascitic fluid from these tumours was substantially greater than with the Ehrlich ascites carcinoma and the tumour was much less susceptible to contamination with erythrocytes.

For the experiments on immature rabbit tissues, young rabbits weighing from 1500g. to 2000g. were selected from the departmental colony.

2.2 Preparation of crude cytoplasmic extracts from mammalian cells.

(a) from ascites carcinoma cells.

Crude cytoplasmic extracts were prepared from both Ehrlich and Landschutz ascites carcinoma cells by the method of Smellie, Keir and Davidson (1959). The appropriate ascitic fluid was withdrawn from several mice under aseptic conditions, pooled and the suspension of cells centrifuged at low speed (200-300xg) for five minutes at 0° to separate the cells from the ascitic plasma. The sediment was resuspended in 5-10vol. of chilled 0.1M phosphate buffer pH 8.1 and again centrifuged at 200-300x g for five minutes at 0° to separate tumour cells from erythrocytes. This washing procedure was repeated several times until the sediment of tumour cells was freed from contamination with erythrocytes.

The cells were then disrupted using an osmotic procedure. For this the washed cells were resuspended in chilled 0.1M phosphate buffer and centrifuged at 600 x g to pack them tightly in the centrifuge tube. 10-12 vol. ice-cold distilled

water were then added and the suspension gently homogenised in a precooled Potter type homogeniser (Potter and Elvehjem, 1936). Three or four passes of the material were sufficient for adequate dispersion. Microscopic examination of wet smears with the aid of 1% crystal violet in 0.1M citric acid was used to control this process which appeared to rupture most of the cells without causing much nuclear breakdown. The resulting suspension was centrifuged in the Spinco Model L preparative ultracentrifuge at 105,000 x g for 60 minutes at 0° to yield a clear cytoplasmic extract.

(b) from tissues of immature rabbits.

Extracts of immature rabbit tissues were prepared from thymus, spleen, brain, intestine, testes, bone marrow, appendix, kidney and muscle of young rabbits by homogenising the appropriate tissue for 2 minutes in 10vol. ice-cold distilled water using a cooled Potter type homogeniser, and centrifuging the resulting suspension for 60 minutes at 105,000 x g.

2.3 Preparation of subcellular fractions from ascites cells.

For some experiments, ascites carcinoma cells were fractionated into subcellular fractions (nuclei, mitochondria and microsomes) in order to conduct incorporation studies on these individual cell fractions.

(a) Nuclei.

Because both the Ehrlich and Landschutz ascites tumour cells are relatively robust, homogenisation in a Potter type

homogeniser with a sucrose-calcium chloride medium was found insufficient to cause their disruption. Therefore before nuclei could be isolated from these ascites cells the methods of Hogenboom, Schneider and Striebich (1952) and Allfrey, Mirsky and Osawa (1957) for the isolation of nuclei from rat liver cells and thymus gland cells respectively, had to be modified. The initial step in such a modification was to subject the ascites cells, of the appropriate type, to osmotic disruption in precisely the manner described in 2.2(a). This treatment leaves the nuclei intact. The suspension of osmotically disrupted cells was then centrifuged at 600 x g for ten minutes at 0°. The supernatant fluid was then removed for the subsequent preparation of mitochondrial and microsomal fractions and the sediment, which contains nuclei heavily contaminated with cytoplasmic material, was suspended in 5 vols. ice-cold sucrose-calcium chloride (0.25M - 0.003M) by gentle homogenisation in a Potter homogeniser. This suspension was centrifuged at 600 x g for 10 minutes to collect the nuclei. Further washing of the nuclei was achieved by resuspension in 5 vol. ice-cold sucrose-calcium chloride (0.25M - 0.003M) followed by centrifugation. One to two washings was usually sufficient, as the supernatant was quite clear by that time. Such a procedure yielded nuclei free from any obvious cytoplasmic contaminants as judged by microscopic examination of wet smears stained with 1% crystal violet in 0.1M citric acid.

(b) Mitochondria, microsomes and cell sap.

After the removal of the nuclear fraction from the suspension of osmotically disrupted cells, by centrifuging at 600 x g (see 2.3), the supernatant fluid was then made 0.25M with respect to sucrose and 0.0033M with respect to calcium chloride. A mitochondrial fraction was prepared from this by centrifugation at 10,000 x g for 10 minutes at 0^{o} and was washed by resuspension in sucrose- calcium chloride (0.25M - 0.0033M) followed by centrifugation.

After the removal of mitochondria from this suspension, microsomes were collected by further centrifugation at 105,000xg in the Spinco Model L preparative ultracentrifuge. The supernatant fraction from this centrifugation was considered to be free from most of the cytoplasmic particulate material and to consist mainly of the soluble components of the cell sap.

(c) Nucleoli and chromatin material.

An attempt was made to isolate nucleoli from ascites cell nuclei applying the principle used successfully by Monty, Litt, Kay and Dounce (1956) for the isolation of nucleoli from rat liver cells. The ascites cell nuclei (see 2.3a) from 20ml. packed cells were suspended in approximately 35 ml. of 1% gum arabic (w/v) pH 6.3, and the pH of the resulting suspension readjusted to pH 6.25 - 6.30 with very dilute NaOH. Careful stirring was required to avoid local excesses. This operation

and subsequent operations were carried out as close to 0° as possible.

The suspension was then exposed in batches of 10-15ml. to vibration by a Mullard ultrasonic drill (50W. ; 20kc.) for 7 to 7.5 minutes. The material after ultrasonic vibration was centrifuged in two 50ml. centrifuge tubes (each tube containing about 18ml.) for 20 minutes at 400 x g in an International refrigerated centrifuge. The supernatant fluid contained the bulk of the chromosomal material, much of which was in a very finely divided form. It was set aside for further fractionation.

The sediment from the first centrifugation was next suspended in 25 ml. of ice-cold distilled water, and allowed to sediment by gravity in a 50ml. measuring cylinder for 15 hours. The top 15ml. were withdrawn carefully with a Pasteur pipette, leaving nuclear membrane material, unbroken nuclei, fibrous material and other unidentifiable fragments in the remaining 2ml. The top 23ml. was carefully and evenly resuspended (without further change in volume) and again subjected to sedimentation by gravity in a 50 ml. cylinder, this time for 7 hours. The top 21ml. were then drawn off and transferred to a 50ml. centrifuge tube, and the concentration with respect to gum arabic adjusted to 2% by the addition of the appropriate amount of 10% gum arabic solution at pH 6.0. This suspension was centrifuged at 300 x g for 20 minutes. The pellet was

suspended in 8 to 10ml. of 2% gum arabic at pH 6.0, and the resulting suspension centrifuged in a 15ml. conical centrifuge tube for 15 minutes at 200 x g. These centrifugations in gum arabic caused the sedimentation of the nucleoli, leaving the finely divided chromosomal contamination in suspension. The gum arabic was removed from the nucleoli by two washings with small volumes of water. Microscopic examination of the wet smears of these nucleoli, using 1% crystal violet in 0.1M citric acid and pyronine methyl green stain (B.D.H.), suggested that while sonication of the nuclei had broken off the main bulk of the chromosomes from the nucleoli, traces of chromosomal material still remained attached to the nucleoli.

The original supernatant suspension remaining after the first centrifugation of nucleoli was fractionated in an arbitrary method by centrifugation at 25,000 x g for 30 minutes at 0°. The sediment, termed "chromosomal fraction" is said by Monty, Litt, Kay and Dounce (1956) to consist mainly of whole and fragmented chromosomes, chiefly the latter. The supernatant fluid, which had a slightly milky appearance, is believed to contain finely divided chromosomal material in colloidal suspension and presumably a small amount of material brought into solution by sonic treatment. No particulate matter could be seen on microscopic examination.

The "chromosomal fraction" and nucleoli were then suspended in 0.01M Tris buffer at pH 7.5 for subsequent experimental work.

2.4 Preparation of fractions from ascites carcinoma cell nuclei.

(a) Soluble fraction (or nuclear extract).

Nuclei, prepared from the appropriate variety of ascites cells as described previously (2.3 a), were suspended in three times their volume of ice-cold 0.01M Tris buffer pH 7.4 by gentle homogenisation in a Potter homogeniser. The suspension of nuclei was then subjected to ultrasonic vibration, using a Mullard ultrasonic drill (50W. ; 20kc.). Batches of nuclear suspension of about 15ml. were exposed to vibration for periods of about two minutes. The length of exposure was determined by following nuclear disintegration in wet smears stained with crystal violet. The disintegrated nuclear suspension was then centrifuged in a Spinco Model L preparative ultracentrifuge for 60 minutes at 105,000 x g to yield a clear nuclear extract, containing 1-3mg. protein/ml.

For use in some experiments, the sediment obtained after ultracentrifugation, was suspended in 3vol. of chilled 0.01M Tris buffer at pH 7.5 and divided into two portions. One of these was termed "nuclear sediment" and the other (1ml.) after treatment with 1mg. crystalline deoxyribonuclease in the presence of 2μmoles of $MgCl_2$ for 4 hours at 37°, was termed "DNase treated nuclear sediment".

(b) An aggregate fraction.

By modifying the method of Weiss (1960) an aggregate enzyme fraction was prepared from nuclei of ascites cells.

Packed ascites cell nuclei were suspended in twenty times their volume of ice-cold 0.05M Tris buffer at pH 7.4 and exposed in small batches of 10ml. to sonic vibration by a Mullard ultrasonic drill (50W. ; 20kc.) for periods of 2 minutes. This gave a suspension of disrupted nuclei which was centrifuged at 10,000 x g for 10 minutes at 0°. The sediment obtained in this manner was suspended in 0.05M Tris buffer of pH 7.4 to a final volume of 16ml. 2M KCl was carefully added to the suspension, with adequate stirring, to a final concentration of 0.4M KCl. Within a few minutes a white aggregate formed which was washed twice with 0.05M Tris buffer pH 7.4 - 0.4M KCl medium and finally suspended in 0.05M Tris buffer of pH 8.1 by vigorous homogenisation in a Potter homogeniser, to give a protein concentration of 8-10mg. per ml.

2.5 Isolationof nucleic acids and nucleoproteins from ascites cells.

(a) RNA from whole cells.

Washed ascites cells were packed and suspended in 10vols. ice-cold distilled water and subjected to osmotic disruption as described in 2.2a. RNA was then extracted from this disrupted cell preparation using a modification of the Kirby procedure (Kirby, 1956).

An equal volume of 90% (w/v) phenol (B.D.H.) was added quickly to a portion of the disrupted ascites cell preparation. The mixture was shaken mechanically for 60 minutes, and then

centrifuged at 600 x g for 60 minutes at 0°. The cloudy aqueous layer was removed by suction and the residue washed twice with water. The aqueous layer was separated each time by centrifuging for 45 minutes and removed by suction. The aqueous extracts were pooled and extracted five times with ether to remove phenol. Nitrogen was blown through the solution to evaporate the ether and the solution was made up to 2% with respect to potassium acetate. The RNA was precipitated by the addition of 2 vol. of ethanol, washed twice with ethanol and twice with ether and allowed to dry before storing at -10°. Prior to use it was dissolved in distilled water or in 0.01M NaCl to give a concentration in the region of 2mg. RNA/ml. No DNA could be detected in this preparation.

(b) <u>RNA from cytoplasm, nucleus and 105,000 x g sediment.</u>

Using precisely the method described above, RNA was extracted from crude cytoplasmic extracts of ascites cells, and termed <u>cytoplasmic RNA.</u> Similarly the method was directly applied to the sediment obtained after centrifuging suspensions of osmotically disrupted ascites cells at 105,000 xg. To facilitate the extraction the sediment was suspended in 5 vol. of distilled water. The RNA thus extracted was referred to as <u>105,000 x g sediment RNA</u>.

To prepare RNA from ascites cell nuclei, the packed nuclei were first suspended in three times their volume of ice-cold distilled water and subjected to ultrasonic vibration for 2

minutes as described in 2.4a. A portion of this suspension of sonically disrupted nuclei was treated with phenol exactly as described above and the RNA extracted in precisely the same manner. Although this RNA (2mg/ml. distilled water or 0.01M NaCl) was then referred to as nuclear RNA it must be emphasised that such a phenol extraction procedure failed to remove all the RNA from the ascites cell nucleus. Such a finding is consistent with the observations of Sibatani, Yamana, Kimura and Takahashi (1960) that treatment of ascites cell nuclei with phenol will release only one type of RNA, while another type remains unaffected in the residue.

(c) Two distinct types of ribonucleoprotein from ascites cell nuclei.

Two types of ribonucleoprotein were prepared from ascites cell nuclei by application of the methods previously described by Osawa, Takata and Hotta (1958) and Logan and Davidson (1957). Packed ascites cell nuclei were homogenised for 1 minute in five times their volume of ice-cold 0.1M potassium phosphate buffer at pH 7.1 and the resulting suspension centrifuged at 20,000 x g for 20 minutes in a refrigerated Servall centrifuge. The opalescent supernatant fraction obtained was termed the "pH 7.1 fraction" and contained type I ribonucleoprotein. The sediment was resuspended in the same buffer, homogenised and centrifuged as before. After discarding the supernatant fluid, the residual pellet was suspended in 30 vols. of ice-cold 1M NaCl, homogenised with careful cooling in an M.S.E. Nelco blender

at top speed for 30 seconds, and stirred vigorously with a mechanical stirrer for 2 hours, again with careful cooling. The resulting viscous solution was centrifuged at 30,000 x g for 60 minutes at 0° in a Spinco Model L ultracentrifuge. After discarding the viscous supernatant fluid, which contains most of the cellular deoxyribonucleoprotein, the residual pellet was resuspended in 10 vols. of cold 1M NaCl, using the Nelco blender and centrifuged as before. The pellet finally obtained was referred to as the "1M NaCl fraction" and contained type II ribonucleoprotein. For experimental use this pellet was dispersed in 5 vols. of ice-cold distilled water by vigorous homogenisation in a cooled Potter homogeniser. In some experiments ^{3}H-uridine was incorporated into this ribonucleoprotein (see 2.8)

(d) nRNA$_1$ and nRNA2 from ascites cell nuclei.

Having prepared two types of ribonucleoprotein from ascites cell nuclei, an attempt was made to isolate the RNA from each. Thus type I ribonucleoprotein would be expected to yield nRNA$_1$, and type II ribonucleoprotein, nRNA$_2$. This was of particular interest because according to Allfrey, Mirsky and Osawa (1957), the RNA associated with type II ribonucleoprotein was at least partly derived from the nucleoli. While the use of the phenol technique (see 2.5) was successful for the isolation of RNA (nRNA$_1$) from type I ribonucleoprotein, it proved useless for the isolation of RNA from type II

ribonucleoprotein.

In order to obtain RNA ($nRNA_2$) from type II ribonucleoprotein it was necessary to treat the suspension not with phenol, but with an equal volume of 10% NaCl at 60° for 20 minutes. The results of Eigner, Boedtker and Michaels (1961) suggest that longer treatment will destroy the RNA. The resulting mixture was centrifuged at 20,000 x g for 10 minutes at 0° and the supernatant fluid containing $nRNA_2$ dialysed for 18 hours against two 7l. changes of distilled water at 4° to remove as much salt as possible. At this stage it was found that the $nRNA_2$ contained a large amount of DNA as contaminant. Treatment with phenol and subsequent extraction of the RNA failed to remove this contamination. Even treatment with DNase (as described in 2.4a for nuclear sediment) followed by subsequent treatment with phenol and precipitation with alcohol in the presence of potassium acetate failed to remove DNA entirely.

For experimental purposes both $nRNA_1$ and $nRNA_2$ were dissolved in distilled water to give a concentration of 2mg. RNA/ml. based on ultraviolet and pentose estimations.

(e) Native and denatured DNA.

DNA was prepared from ascites cells following the method of Kay, Simmonds and Dounce (1952). 100ml. of ascites carcinoma cell suspension was centrifuged at 1,500 x g for 10 minutes to pack the cells, and the supernatant fluid discarded.

The cells were washed repeatedly with 0.1M phosphate buffer, pH 8.0, to remove contaminating red blood cells and were lysed by the addition of twelve times the packed cell volume of distilled water followed by homogenisation in a Potter homogeniser. The resulting suspension was centrifuged at 4,500 x g for 30 minutes. The supernatant fluid was discarded and the residue was homogenised in 0.9% sodium chloride - 0.01M sodium citrate solution and made up to 200ml. with same solution. The resulting mixture was centrifuged at 900 x g for 10 minutes and the supernatant fluid discarded. This was repeated three times. The sediment finally obtained was suspended in 0.9% NaCl, homogenised in a Potter homogeniser and made up to 300ml. with 0.9% NaCl. 27ml. of Duponal (5% sodium dodecyl sulphate in 45% ethanol) were added with stirring. This stirring was continued for 30 minutes, and then 16.5g. of NaCl were added to give a 1M solution. Stirring was continued until all the NaCl had dissolved and then the mixture was centrifuged at 20,000 x g for 30 minutes. The supernatant fluid was decanted and 2 vol. of ethanol added with swirling. The DNA, which precipitated in thread-like strands, was collected on a glass rod, washed three times with ethanol, three times with acetone and allowed to dry. The DNA thus obtained was further purified. Firstly it was cut into small pieces and dissolved in 300ml. of water at room temperature using a Vibromix vibratory stirrer. Then, 27ml. of Duponal were added and the

mixture stirred for 30 minutes before 20 - 25g. of NaCl were added. The stirring was continued until the NaCl had dissolved and then the mixture was centrifuged at 20,000 x g for 30 minutes. The DNA was precipitated, washed and dried as before. Then the DNA obtained in this way was redissolved again, this time in 200ml. of water, and 1.8g. of NaCl were added and dissolved. The resulting mixture was centrifuged as before and 10g. of NaCl dissolved in the supernatant fluid. 2vol. of ethanol were added to precipitate the DNA which was collected, washed and dried, as before. The procedure yielded about 120mg. of DNA which were dissolved in distilled water to a concentration 2mg. DNA/ml.

A 200 ml. portion of this solution was then incubated with 400µg. RNase (crystalline) and 20ml. of 1M Tris buffer pH 7.6 for 2 hours at 37°. An equal volume of water saturated phenol was then added and the mixture gently shaken for 60 minutes at 20°. The supernatant aqueous layer which was obtained on centrifuging the mixture at 2,000 x g for 20 minutes was drawn off, leaving the phenol layer as a thin interfacial film of insoluble material, which was carefully removed. The phenol layer was treated twice more with equal volumes of distilled water in the manner described above, the aqueous layer being collected. The pooled aqueous layers were extracted three times with equal volumes of ether, to remove the phenol. The viscous solution was then made 1.0M with respect to NaCl,

and DNA was precipitated by the addition of ethanol to 5% (v/v). The precipitate was washed three times with ethanol, and three times with acetone, dried in air and stored at -10°. Prior to use, it was dissolved in 0.01M NaCl and dialysed for 24 hours at 4° against four 4l. changes of 0.01M NaCl, the final concentration being 2.0mg. DNA/ml.

For some experiments, solutions of DNA were subjected to heat treatment. This was carried out by immersing 1ml. portions contained in 15ml. conical centrifuge tubes in a water-bath at 100° for ten minutes, and rapidly cooling these solutions by shaking the tubes in an ice-bath. This treatment is referred to as thermal denaturation of DNA, and DNA not submitted to this heating process is termed native DNA.

2.6 Fractionation of nuclear and cytoplasmic extracts of ascites cells.

(a) With ammonium sulphate alone.

All steps were carried out between 0° and 4°. The extract, nuclear or cytoplasmic (40ml.), was brought to 0.20 saturation by the slow addition, with stirring, of 4.4g. of ammonium sulphate. The solution was allowed to stand for 30 minutes, with continuous stirring, and the precipite was removed by centrifuging at 8,000 x g for 8 minutes. This process was repeated five times, the degree of saturation with ammonium sulphate being adjusted to 0.30, 0.40, 0.50, 0.60, and 0.70 saturation by the successive addition of 2.8g, 2.8g, 2.8g, 3.2g and

3.2g of solid ammonium sulphate. After each addition the solution was stirred for 30 minutes and the precipitate collected by centrifugation. Each of the precipitates was dissolved in ice-cold distilled water to give a protein concentration in the region of 2-4 mg./ml, and the solutions were dialysed for 18 hours at 4° against two 1l. changes of distilled water to remove ammonium sulphate.

This procedure was subsequently modified when it became evident that only two protein fractions, those obtained between 0.30 - 0.40 and 0.55 - 0.70 saturation were active. In this modified procedure all steps were carried out between 0° and 4°. 40ml. of the appropriate extract were brought to 0.30 saturation by the slow addition, with constant stirring, of 7.2g. ammonium sulphate. The solution was allowed to stand for 30 minutes, with continuous mechanical stirring and the precipitate removed by centrifuging at 8,000 x g for 30 minutes and discarded. The supernatant fluid was then brought to 0.40 saturation by the addition of 2.8g. ammonium sulphate and after stirring for 30 minutes the precipitate was collected by centrifuging as above. It was dissolved in 0.01M Tris buffer pH 9.5 to give a protein concentration of 2-3mg./ml. and dialysed for 12 hours against two 1l. changes of 0.01M Tris buffer pH 9.5 to yield either nuclear or cytoplasmic Fraction A, depending on the source of the initial extract. The supernatant fluid after the removal of Fraction

A at 0.40 saturation was brought to 0.55 saturation by the addition of 4.8g. ammonium sulphate and the precipitate discarded. The supernatant fluid from this step was brought to 0.70 saturation by the addition of 4.4g. ammonium sulphate. The precipitate was collected, dissolved in 0.01M Tris buffer pH 7.7 to give a protein concentration of 2 - 3mg./ml. and dialysed against two 1l. changes of the same buffer for 12 hours to yield nuclear and cytoplasmic Fraction B as the case may be. Although both types of fraction could be stored at -10° and retained their activities for a few days, they were none the less highly susceptible to any freezing and thawing.

(b) <u>With ammonium sulphate, acetone and calcium phosphate gel.</u>

In order to achieve partial purification of a polyribonucleotide synthesing system, cytoplasmic extracts were fractionated with ammonium sulphate, acetone and calcium phosphate gel. As before, unless otherwise specified, all steps were carried out between 0° and 4°. A crude cytoplasmic extract was prepared from a preparation of osmotically disrupted Landschutz ascites cells, following the method described in 2.2a. This cytoplasmic extract (CE) was then made 0.01M with respect to Tris buffer at pH7.5, and brought to 0.25 saturation by the slow addition with continuous mechanical stirring of the appropriate volume of saturated ammonium sulphate solution, previously adjusted to pH 7.5. The

solution was allowed to stand for 30 minutes, with continuous stirring, and the precipitate was removed by centrifugation at 8,000 x g for 8 minutes, and discarded. The supernatant fluid was then brought to 0.45 saturation by the addition of a further amount of saturated ammonium sulphate (pH 7.5). After stirring for 30 minutes, this solution was centrifuged at 8,000 x g for 8 minutes, and the resulting sediment dissolved in 0.01M Tris buffer at pH 7.5 to give a protein concentration between 6 and 10mg./ml. and dialysed overnight against two 7l. changes of the same buffer. The protein fraction was termed the ammonium sulphate fraction (AMSF) and to every 10ml. was added 1ml. of 1M Tris buffer at pH 6.5. Then, after immersion in a bath of ethylene glycol at -15° enough acetone (previously cooled to -15°) was added to make the fraction 15% with respect to acetone. The solution was stirred gently for 5 minutes at -15°, and then spun at 1,500 x g for 20 minutes at -15° to remove the precipitate which was discarded. Again at -15°, the supernatant fluid was made 25% with respect to acetone by the further addition of the appropriate volume of acetone (cooled to -15°). As before, the solution was stirred for 5 minutes at -15° and then centrifuged. The resulting sediment was dissolved in 0.01M potassium phosphate buffer at pH 6.8 and dialysed against two 2l. changes of the same buffer for 4 hours (or in one experiment, 18 hours). The fraction was termed

the acetone fraction (ACF) and contained in the region of 4 - 6mg. protein/ml.

The next step involved the use of calcium phosphate gel, which was prepared following the method of Keilin and Hartree (1938). 150ml. calcium chloride solution (132g. $Na_3PO_4.12H_2O$ per litre). The mixture was brought to pH 7.4 with dilute acetic acid and the precipitate washed three or four times by decantation with large volumes of distilled water (15 - 20l.). The precipitate was finally washed with distilled water by centrifugation. At this stage the yield was around 9g. dry weight. The precipitate was then suspended in distilled water and allowed to stand for about a month. After removing the clear water layer, the gel was well shaken and the dry weight per ml. determined, and was found to be 18mg./ml. The gel was then ready for use, and was stored in the dark.

For use in these experiments, the gel was initially equilibrated with 0.01M potassium phosphate buffer at pH 6.8 by repeated suspension and centrifugation, followed finally by suspension in the original volume of buffer. A portion of the dialysed acetone fraction was mixed with an equal volume of this suspension of calcium phosphate gel, now containing 18mg. dry material/ml. of potassium phosphate buffer at pH 6.8. The mixture was then centrifuged at 1,000 x g for 5 minutes and the supernatant fluid discarded. The gel was then extracted with 0.05M potassium phosphate buffer at pH 6.8 for ten

minutes with stirring. The volume of buffer used was equivalent to the volume of acetone fraction originally submitted to this process. This extract was removed by centrifugation at 1,000 x g for 5 minutes and discarded. Using the same procedure, the gel was further extracted with 0.1M potassium phosphate buffer at pH 6.8. The extract was collected and dialysed for 4 hours against two 1l. changes of 0.01M potassium phosphate pH 6.8 and was referred to as the calcium phosphate gel fraction (CPgelF).

2.7 Chemical synthesis of isotopically labelled ribonucleoside 5'-triphosphates.

(a) ^{32}P-labelled UTP and UDP

Both UTP and UDP labelled with ^{32}P in the α-phosphorus (uridine-^{32}P-P-P and uridine-^{32}P-P) were prepared from ^{32}P-labelled UMP using a modification of the procedure of Smith and Khorana (1958). The UMP labelled with ^{32}P was prepared by reacting 2',3'- isopropylidene uridine with ^{32}P-labelled cyanoethyl phosphate according to the method of Tener (1961).

Thus in a typical preparation the initial step was the preparation of ^{32}P-labelled cyanoethyl phosphate (CEP). The reactions involved can be outlined as follows :

$$2CN.CH_2.CH_2.OH + H_3{}^{32}PO_4 \xrightarrow[\text{pyridine}]{\text{DCC in anhyd.}} (CN.CH_2.CH_2.O)_2\overset{O}{\underset{|}{P}}.O.\overset{O}{\underset{|}{P}}(O.CH_2.CH_2.CN)_2$$

$$(CN.CH_2.CH_2.O)_2\overset{O}{\underset{|}{P}}.OH \xrightarrow{\text{water, heat}} CN.CH_2.CH_2.O.\overset{O}{\underset{O\,Ba}{P}}.O \xleftarrow{Ba(OH)_2}$$

A carrier free solution of disodium hydrogen phosphate (PBS.1.,1.5ml. and 25mc at time of dispatch from The Radiochemical Centre, Amersham.) was mixed with 2mmoles of unlabelled orthophosphoric acid and concentrated to dryness *in vacuo* at 40° to remove traces of hydrochloric acid. Anhydrous pyridine (20ml., dried with CaH_2) and 2-cyanoethanol(2ml.) were added and the solution concentrated *in vacuo* at 40° to an oil. The crystalline pyridinium phosphate which sometimes separated, redissolved as the solution became more concentrated. A second portion of anhydrous pyridine (20 ml.) was added and the solution again concentrated to an oil. Anhydrous pyridine (10ml.) and 4.2g. dicyclohexylcarbodiimide(DCC) were added and the reaction mixture set aside at room temperature overnight. 10ml. of distilled water were then added to stop the reaction and the resulting solution was heated in a water bath at 100° for 30min. The solution was then concentrated to dryness *in vacuo* and 20ml. distilled water and 20ml. barium hydroxide added to the residue. After 5min. at room temperature the solution was adjusted to pH 7.5 with acetic acid (a few drops of glacial acetic acid were found to suffice), and filtered to remove dicyclohexyl urea(DCU) and barium phosphate. Two volumes of ethanol were added to precipitate the barium 2-cyanoethyl phosphate which after standing for

1hr., was collected by centrifugation. The crystals were redissolved in water (5ml., plus a trace of glacial acetic acid to aid solution), centrifuged to remove a trace of insoluble material and recrystallised by adding 10ml. ethanol. The product was then collected by centrifugation, washed with ethanol, acetone and finally ether. The yield after air drying was 411mg. (1.28mmoles), and equivalent to 64%. Yields as high as 74% have been achieved.

For use in the phosphorylation of 2',3'-isopropylidene ribonucleosides the barium salt of C$\overset{32}{P}$ was dissolved in distilled water (5ml., plus trace of glacial acetic acid) and passed through a column of Dowex-50-hydrogen resin (3x 0.8cm). The column of resin was washed with distilled water until all the radioactivity was removed. 8ml. anhydrous pyridine was added to the effluent and the mixture was taken down to dryness (<u>in vacuo</u> at 40°) giving the pyridinium salt of C$\overset{32}{P}$. This was used for subsequent phosphorylation reactions.

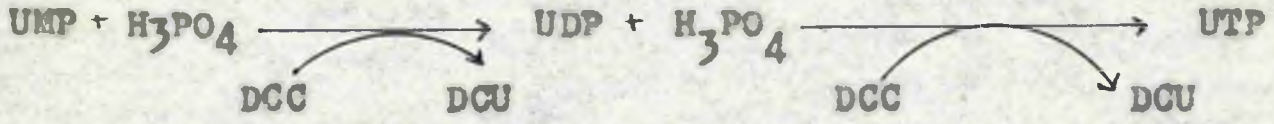
For the phosphorylation of 2',3'-isopropylidene uridine to 2',3'-isopropylidene U$\overset{32}{P}$, 1mmole of the pyridinium salt of ^{32}P-C$\overset{32}{P}$ was used. 2mmoles of 2',3'-isopropylidene uridine (568mg.) were dissolved in anhydrous pyridine (10ml.) and 1mmole of the pyridinium salt of ^{32}P-C$\overset{32}{P}$ was added. The solution was concentrated to an oil <u>in vacuo</u> at 40° and then anhydrous pyridine(10ml.) was added and the solution again concentrated to dryness. The process was repeated once more

and the residue was dissolved in 5ml. anhydrous pyridine and 620mg. DCC added. The solution was kept in a well stoppered flask for 17hr. at room temperature, after which 1ml. distilled water was added. After 1hr. the solution was concentrated to dryness in vacuo and the residue hydrolysed for 90min. in 40ml. 10% acetic acid at 100° to remove isopropylidene groups. The acetic acid was removed by evaporating the solution to dryness, dissolving the residue in distilled water and evaporating for a second time. The residue was then heated with 40ml. 9N ammonium hydroxide at 60° for 90min. to cleave the phosphoamide bonds that may have formed during the course of the reaction. The ammonia was removed by concentrating the mixture to dryness. 10ml. distilled water were added to the residue and the insoluble DCU removed by filtration, care being taken to wash the precipitate with water.

The filtrate was adjusted to pH 7.5, diluted to approximately 200ml. and applied to a column (2.2x16cm.) of Dowex-1-chloride resin. The column was washed with distilled water to remove unreacted uridine, the elution being followed by measurement of the optical density of the effluent at 262mμ. 0.01N HCl was used to displace any ^{32}P-orthophosphate from the column, and this was followed by 0.1N HCl to elute the ^{32}P-UMP. The yield was usually in the region of 327μmoles. Thus about 33% of the uridine was converted to

^{32}P-UMP i.e. a 66% yield based on ^{32}P- CEP. Chromatography on paper using the isobutyrate solvent (see 2.10a) indicated that the material in the water wash was uridine, and the 0.1N HCl eluate contained UMP free from 2',3'-isopropylidene UMP. Routinely the paper chromatograms were dipped as described in 2.10b to test for the presence of contaminating inorganic phosphates. Such a procedure showed the UMP to be free from contaminating phosphates.

The ^{32}P-UMP was then used to prepare ^{32}P-UDP and ^{32}P-UTP. The sequential phosphorylation of ^{32}P-UMP by non-radioactive inorganic orthophosphate to form high energy pyrophosphate bonds occurs by a condensation reaction under the influence of DCC, and tri-n-butylamine which, according to Smith and Khorana (1958), is also necessary. The reaction may be tentatively represented thus :



To the 327μmoles ^{32}P-UMP, previously prepared, were added 19.2ml. anhydrous pyridine, 9.6g. DCC, 3.82ml. tri-n-butylamine and 0.64ml. 85% orthophosphoric acid. The reaction was shaken mechanically for 48hr. at room temperature, after which the reaction was stopped by the addition of an equal volume of distilled water. The mixture, after shaking, was allowed to stand at 0° for 30min and the precipitated DCU removed by filtration. The filtrate and

water washings were extracted with ether (5x20ml.) to remove pyridine. The ether extracts were washed with water and the washings combined with the aqueous layer which was then concentrated by freeze drying. The resulting concentrate, which was approximately pH 7 and did not smell of pyridine, was applied to a Dowex-50-sodium column (15x4cm.) to remove tri-n-butylamine. The column was washed with distilled water until the effluent, examined at 262mμ, was free from any ultraviolet absorbing material to ensure that all the ribonucleotides had been eluted from the resin. The effluent after dilution to about 400 ml. was applied to a column (2x20cm.) of Dowex-1-chloride resin(AG-1-X8,100-200 mesh). The column was washed with water to remove any uridine that might have been formed during the reaction and ^{32}P-UMP, ^{32}P-UDP and ^{32}P-UTP were eluted by gradient elution using a HCl-lithium chloride mixture. Initially the mixing vessel contained 1.5l. 0.01N HCl and the reservoir 2l. 0.3M lithium chloride in 0.01N HCl. Eluates were collected in an automatic fraction collector, the extinction at 262mμ of alternate tubes being measured. The elution was continued until three peaks of absorbing material had been eluted from the resin. The largest and last of these three peaks to be eluted from the column corresponded to ^{32}P-UTP, and it was evident that a considerable amount of ^{32}P-UDP was formed as a by-product of the reaction (see Fig.4.)

Figure 4. Separation of ^{32}P-uridine nucleotides by anion exchange chromatography on Dowex-1-chloride column using HCl-lithium chloride gradient elution (for details see text).

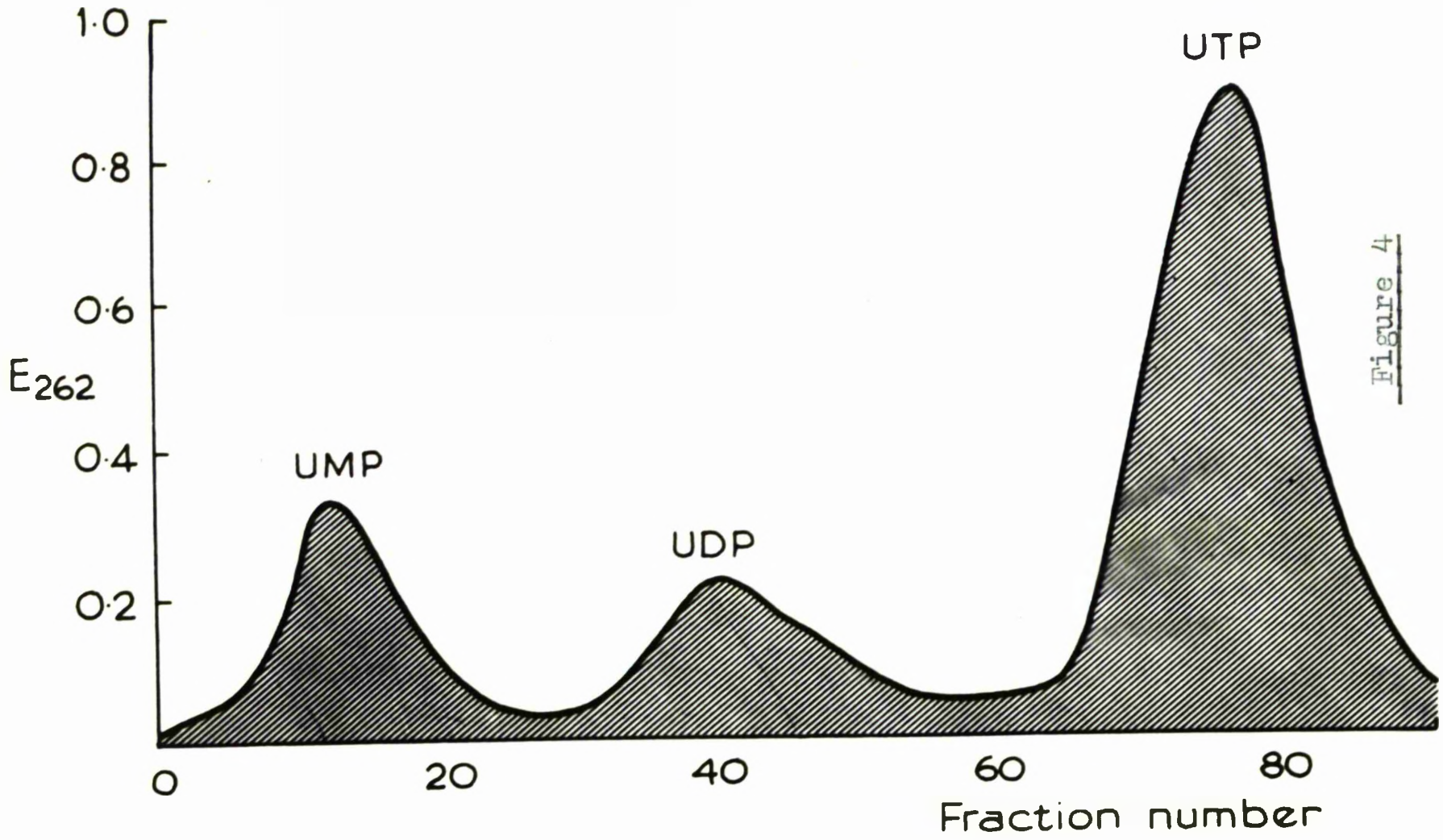


Figure 4

The fractions corresponding to ^{32}P-UTP were pooled and immediately neutralised to pH 7 with lithium hydroxide, and the procedure repeated for fractions corresponding to ^{32}P-UDP.

The procedures involved in the subsequent purification of ^{32}P-UTP were also applicable to the purification of other 5'-ribonucleotides such as ^{32}P-UDP, and they will therefore be described as a general technique. Charcoal columns were prepared using "20/60 grist" charcoal obtained from British Charcoals and MacDonalds, Greenock. This charcoal was ground and sieved to 60-100 mesh and stirred with 5N HCl until evolution of H_2S ceased. It was then poured into a wide chromatography column, washed with more HCl(5 col.vol.) and then with water till neutral. A large excess (20-30 col. vol.) of 0.01M sodium bicarbonate was then passed through the column followed by water until the effluent was neutral again. The charcoal was then washed with 70% ethanol-0.14M ammonia solution (about 50col.vol.), and once more with water until the effluent was neutral. After washing the column with 1N HCl and then with water the column was ready for use.

The pooled ribonucleotide fraction from the Dowex-1-chloride was adjusted to pH 3 and poured on to a column of charcoal (about 10ml. wet bed vol. per 100µmoles of nucleotide) prepared in the manner described above. The

charcoal was washed with water until no chloride could be detected in the effluent (using silver nitrate test) and then with 0.01M sodium bicarbonate (about 10-15col.vol.) to remove the inorganic phosphates which adhere to the charcoal before such treatment. One or two column volumes of distilled water were then passed through the column to remove any remaining bicarbonate and the ribonucleotide was then eluted with 70% ethanol-0.14M ammonia solution (Hecht, Zamecnik, Stephenson and Scott, 1958). This eluate was concentrated in vacuo at 40° to remove ethanol and ammonia, and then diluted to about 100ml. with distilled water. The nucleotide was then converted to its sodium salt by passage through a column of Dowex-50 resin in the sodium form. The column was washed with water until no further ultraviolet absorbing material was eluted and the eluate freeze dried. The ^{32}P-nucleotide was stored at -70° and before use was dissolved in an appropriate amount of distilled water.

Paper chromatography in the isobutyrate system (see 2.10a) of the ^{32}P-UTP and ^{32}P-UDP so prepared showed them to be identical with pure commercial samples of non-radioactive UTP and UDP and no contamination with ortho- or pyrophosphate could be detected by means of the phosphorus dip procedure. A sample of each nucleotide was diluted with distilled water and taken for measurement of extinction at 262mμ in a 1cm. light path to estimate the yield. From 327μmoles of

^{32}P-UMP, 72μmoles ^{32}P-UTP and 50μmoles ^{32}P-UDP were obtained (i.e. 21% and 15% yields respectively. The specific activities of the nucleotides were in the region of 2-4x10^{6} counts/min./μmole.

In some cases, samples of ^{32}P-UTP and ^{32}P-UDP were taken for estimation of base(2.12e) and phosphorus content (2.12d). The sample of ^{32}P-UTP gave a base to phosphorus ratio of approximately 1 to 3, and the ^{32}P-UDP a ratio close to 1 to 2.

(b) ^{32}P-labelled ATP

^{32}P-ATP was prepared from ^{32}P-orthophosphate in a manner analagous to that used for ^{32}P-UTP. Gradient elution of ^{32}P-AMP, ^{32}P-ADP and ^{32}P-ATP was carried out using a mixing vessel containing 21.0.01N HCl and a reservoir containing 0.2M lithium chloride. The fractions corresponding to ^{32}P-ATP(see Fig.5) were pooled and neutralised to pH7 with lithium hydroxide. Lithium chloride and inorganic phosphate were removed from this solution using exactly the same charcoal procedure as previously described for UTP purification(2.7a). Finally the ^{32}P-ATP was converted to its sodium salt by passage through a Dowex-50-sodium column. Paper chromatographic and ultraviolet techniques showed the final product to be free of contamination and to behave as pure ATP. A sample was diluted in distilled water and the amount of ATP synthesised was calculated on the assumption

Figure 5. Separation of ^{32}P-adenosine nucleotides by anion exchange chromatography using HCl-lithium chloride gradient elution (for details see text).

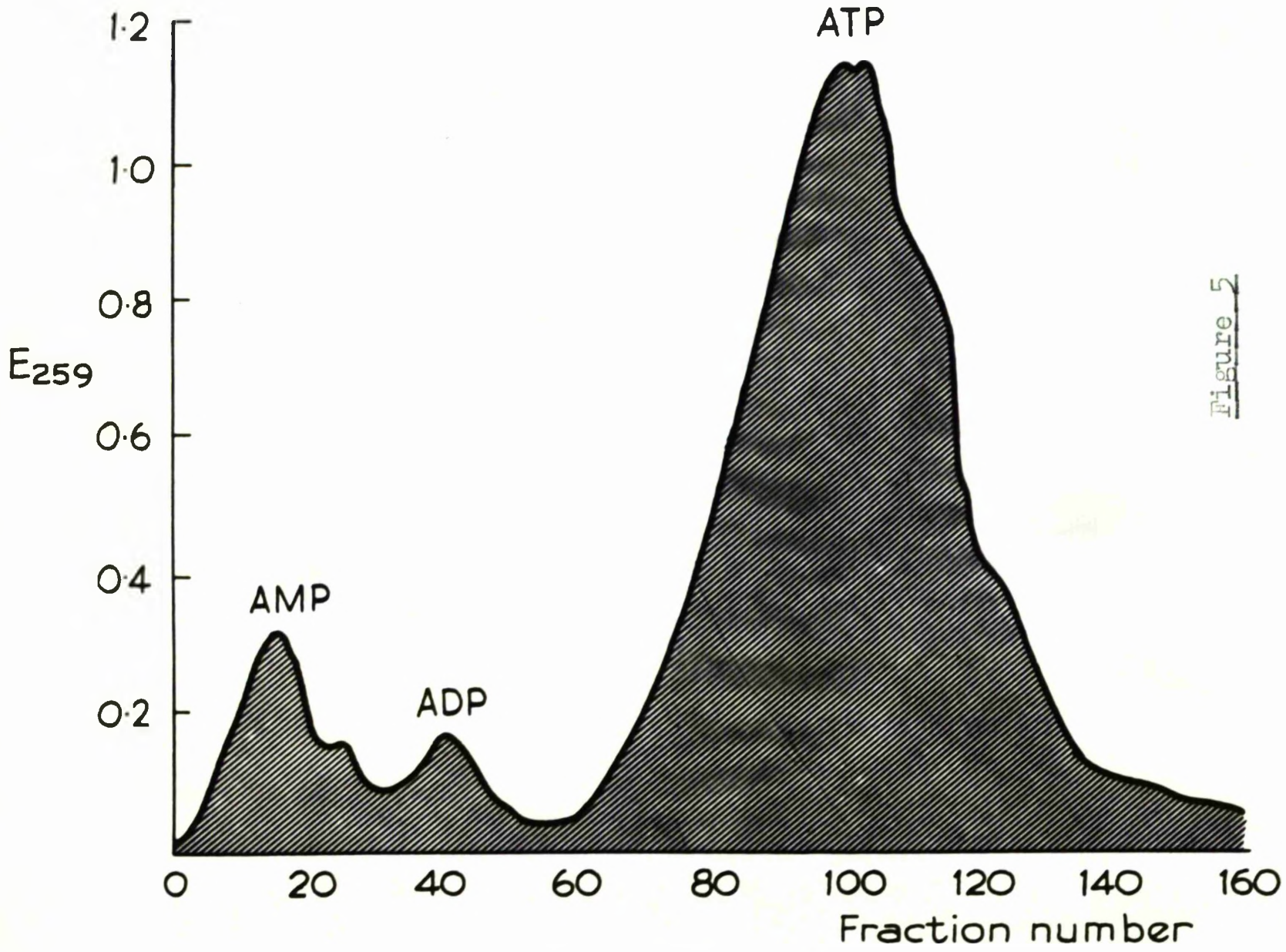

1·2
1·0
0·8
0·6
0·4
0·2
E259
AMP
ADP
ATP
0
20
40
60
80
100
120
140
160
Fraction number

Figure 5

that the molar extinction coefficient at E_{257} for ATP was 14,000 in acid solution (Specifications and Criteria for Biochemical compounds, 1960). The yield of ^{32}P-ATP from 750μmoles ^{32}P-AMP was 260μmoles, or 34.6%. The specific activity was 1.25×10^6 counts/min./μmole. Estimation of base and phosphorus content gave a base to phosphorus ratio of approximately 1 to 3.

(c) ^{14}C-labelled UTP

6-^{14}C-UTP was prepared from 6-^{14}C-UMP by the methods outlined in 2.7a. The 6-^{14}C-UMP was a gift from Dr. E.Reid, Chester Beatty Institute, London. The initial reaction mixture contained 46μmoles 6-^{14}C-UMP, 3ml. anhydrous pyridine, 1.5 g. DCC, 0.1ml. orthophosphoric acid and 0.75ml. tri-n-butylamine. After isolation and purification, the ^{14}C-UTP was subjected to paper chromatography and compared with pure samples of non-radioactive UTP. All the radioactivity was confined to the UTP spots and no contamination with inorganic ortho- or pyrophosphate could be detected. Assuming the molar coefficient for UTP at 262mμ to be 10×10^3, the net yield of ^{14}C-UTP from 46μmoles ^{14}C-UMP was 9.4μmoles, or 20.5%. The specific activity was 1.906×10^6 counts/min./μmole. According to Specifications and Criteria of Biochemical Compounds (1960) the same molar extinction coefficient as used for UTP estimation must be used for UDP (see also 2.7a).

2.8 Incubations.

Incubation of extracts and enzyme fractions with the appropriate additions was carried out in air, in stoppered reaction vessels at 37° with continuous shaking. Where the total reaction volumes were 5ml., the incubations were carried out in 25ml. Erlenmeyer flasks. Other reaction mixtures of 1ml. volume were incubated in 15ml. graduated round bottomed centrifuge tubes, and for the reaction mixtures of 0.25ml., incubation was carried out in 4" x ½" rimless test tubes. All reactions were terminated by immersion of the vessels in a mixture of solid carbon dioxide and ethanol.

In more specialised experiments where it was the aim to incorporate ^{3}H-uridine into the nuclear ribonucleoproteins of intact ascites tumour cells, a more complex incubation procedure was necessary. Enough ascites cells were washed (according to the method outlined in 2.2a) to give a packed cell volume of 5ml. These cells were then suspended in 0.9% NaCl to final volume of 25ml. This ascites cell suspension was then incubated, without any additions, in a 100ml. Erlenmeyer flask at 37° with shaking for 2 minutes. Then ^{3}H-uridine was quickly added with swirling to a final concentration of 10μc/ml., and the incubation continued for a further 2 minutes. The reaction mixture was quickly cooled to 1° and nuclei prepared (see 2.3a) for the subsequent

isolation of tritium labelled nuclear ribonucleoproteins (see 2.5c).

2.9 Analytical procedures.

(a) For ^{3}H-uridine incorporation experiments.

The frozen 5 ml. incubation mixtures were allowed to thaw at 0° and were transferred to 15ml. centrifuge tubes in which subsequent procedures were carried out. The material was first treated with 0.5vol. ice-cold 2.1N perchloric acid to precipitate protein and nucleic acid, and was centrifuged. The supernatant fluid was decanted and the sediment extracted twice more with ice-cold 0.7N perchloric acid. The extracts were combined, neutralised carefully with 7N potassium hydroxide, centrifuged at 0° for 45 minutes to remove the potassium perchlorate, and were termed the acid-soluble fraction. The sediment after acid extraction was treated successively with acetone, ethanol, ethanol-chloroform (3:1), ethanol-ether (3:1) and ether to remove lipid material. The residue consisting of protein and nucleic acid was then suspended in 1ml. of 0.3N potassium hydroxide and incubated for 18 hours at 37° to hydrolyse the RNA to its constituent mononucleotides. After incubation, the material was acidified to pH 1 in the cold with 2.1N perchloric acid, to precipitate protein and DNA, and was centrifuged. The supernatant fluid, containing the RNA in the form of ribonucleoside 3'(or 2')-monophosphates, was

removed, neutralised to pH 7 with potassium hydroxide and centrifuged at low speed at 0° for 60 minutes to remove as much of the potassium perchlorate as possible. The supernatant fluid was decanted into a clean tube and diluted with distilled water to 10ml. 0.2ml. portions of this labelled fraction (corresponding to RNA) were taken for radioactivity measurements (see 2.14) while a second portion was taken for measurement of the extinction at 268mμ in a 1-cm. light path using a Beckmann spectrophotometer. The RNA content of the material was then calculated on the assumption that a solution of RNA containing 1μg. RNA phosphorus/ml. would give an E_{268} of 0.285 (Paul, unpublished results).

In some experiments portions of the labelled RNA hydrolysate were subjected to paper chromatography to separate the pyrimidine nucleosides and nucleotides (see 2.10b), and in others portions of the acid-soluble fractions were taken for paper chromatography to separate uridine, UMP, UDP and UTP (See 2.10a)

As mentioned in 2.8, more specialised experiments were carried out in which ^{3}H-uridine was incorporated into nuclear ribonucleoproteins of intact ascites cells. After carrying out the incubation as described, the two ribonucleoproteins labelled with ^{3}H were isolated from ascites cell nuclei according to the method described in 2.5c.

For the determination of the specific activity of the RNA in each of these two ribonucleoproteins, samples were treated in the same manner as cells taken for autoradiography (see 2.15). Thus, nucleoprotein was precipitated from 2ml. samples by treatment with an equal volume of ice-cold 4N perchloric acid-absolute ethanol (1:1) for ten minutes. After centrifugation, the precipitate was washed twice with 70% ethanol, then successively with 50% ethanol, 25% ethanol and distilled water, followed by three extractions with ice-cold 0.7N perchloric acid. After perchloric acid extraction, the precipitate was successively washed with distilled water, 25% ethanol, 50% ethanol, 70% ethanol, 95% ethanol, absolute ethanol, ethanol- chloroform (3:1), ethanol-ether (3:1), ether, absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 25% ethanol, distilled water. The precipitate was extracted once more with ice-cold 0.7N PCA and washed successively with distilled water, 25% ethanol, 50% ethanol, 70% ethanol, 95% ethanol, absolute ethanol, ether and allowed to dry in air. The residue was then digested in 1ml. 0.3N potassium hydroxide for 18 hours at 37°. Protein and slight traces of DNA were removed by acidification to pH 1 with perchloric acid, followed by centrifugation. The supernatant, containing the RNA hydrolysate was neutralised carefully with potassium hydroxide, and the potassium perchlorate removed by centrifuging as already described. Since the

specific activity of the RNA was expected to be very low, the supernatant fraction containing the ribonucleotides from the RNA was diluted with distilled water to 2ml. rather than 10ml. 1ml. portions of this were taken for the estimation of radioactivity (2.14) and 0.2ml. samples used for the determination of RNA content by ultraviolet absorption measurements.

(b) For ^{32}P-UTP, ^{32}P-UDP, ^{32}P-ATP and ^{14}C-UTP incorporation experiments.

For reaction mixtures of total volume 5ml. the analytical procedure was identical to that employed for ^{3}H-uridine incorporation experiments. When the reaction volumes were only 1 ml., the same analytical procedure was used, but suitably scaled down.

Pilot experiments were carried out in order to determine how much radioactivity was incorporated from ^{32}P-UTP and ^{32}P-ATP into DNA during the course of these incubations. As the amount was found to be negligible, a more rapid analytical procedure was devised, requiring only small volume reaction mixtures (0.25ml).

After incubation of these small reaction mixtures, 1ml. of yeast RNA solution (2mg./ml.) was first added to act as a carrier of RNA in the subsequent precipitations. Immediately thereafter, 1.25ml. of 10% (w/v) trichloroacetic acid was added to precipitate the proteins and nucleic acids of the reaction mixture, along with the carrier RNA. The

mixture was thoroughly stirred and centrifuged at 600 x g in the cold for 15 minutes. The sediment was further extracted twice with 5ml. quantities of 5% (w/v) trichloroacetic acid to remove all acid-soluble material. Thereafter the sediment was extracted with ethanol and ether to remove trichloroacetic acid, and allowed to dry at room temperature. The dry residue, containing the radioactive RNA plus protein and DNA was then dissolved in 1ml. of 0.1N sodium hydroxide. 0.4ml. samples of this solution were then taken for measurement of radioactivity. The even spreading of this solution on planchettes was facilitated by the use of discs of lens paper.

From time to time a more rapid, but less sensitive, procedure was also employed. This was based on that originally described by Bollum (1959) for the assay of DNA polymerase. 50µl. samples of the incubation mixture (0.25ml.) were pipetted on to numbered discs of Whatman No.1 filter paper, 2.5cm. in diameter. The discs were dried and dropped into a large beaker containing 1ml. ice-cold 5% (w/v) trichloroacetic acid per disc. After 3 minutes the discs were washed by swirling in the beaker and the trichloroacetic acid removed by suction. Fresh trichloroacetic acid was added and the procedure repeated twice more. The discs were finally washed in the beaker with ethanol and ether, and after drying were assayed for radioactivity. For reasons

which will be explained in 2.14 this method is only suitable for use with ^{32}P labelled RNA.

2.10 Use of paper chromatographic techniques.

(a) Isolation of radioactive acid-soluble components of incubation mixtures.

As mentioned in 2.9a and 2.9b, samples of the acid-soluble fractions were subjected to paper chromatography to separate the acid-soluble nucleotides. 0.2ml. portions of the acid-soluble fractions were applied to sheets of Whatman No.1 chromatography paper. Since the quantity of uridine nucleotides was too small to detect by scanning the paper in ultraviolet light, a mixture of non-radioactive uridine, UMP, UDP and UTP was applied to these spots, so that the regions containing these substances could subsequently be located. The composite spots were dried in cold air, and the paper developed for 18 hours as a descending chromatogram in the solvent of Krebs and Hems (1953), modified by Keir and Smellie (1959) to give a pH of 4.6. The solvent was made up as follows : 100ml. isobutyric acid, 55.8ml. distilled water, 4.2ml. 35% (w/v) ammonia solution and 1.6ml. of ethylene diamine tetraacetic acid. The R_f values of some nucleosides and nucleotides are given in Table 1.

Spots were located by scanning the paper in a Hanovia Chromatolite. Those spots corresponding to the non-radioactive markers were cut out and eluted from the paper with water by capillary flow. A volume of 0.5ml. was collected

Table I

Compound	R_f value in isobutyrate solvent
uridine	0.49
cytidine	0.67
adenosine	0.85
UMP-3'(2')	0.42
CMP-3'(2')	0.58
ATP	0.34
ADP	0.42
AMP	0.34
UTP	0.18
UDP	0.24
UMP	0.32
2', 3'-isopropylidene adenosine	0.95
2', 3'-isopropylidene uridine	0.86
inorganic orthophosphate	0.38 (slight streaking)
inorganic pyrophosphate	0.23 (-do-)
inorganic tripolyphosphate	0.17 (-do-)

from each spot, and 0.2ml. samples of the eluate were taken for the measurement of radioactivity.

(b) Analysis of the products at various stages in the chemical synthesis of labelled nucleotides.

Paper chromatograms, using the isobutyric solvent, were run at various stages in the chemical synthesis of labelled ribonucleotides. (see 2.7). In Table 1 are the R_f values of compounds of interest in this respect.

As mentioned, sections of these chromatograms were dipped to test for the presence of inorganic phosphate as a possible impurity. Such a reagent for the detection of phosphate on chromatograms was made up according to Burrows, Grylls and Harrison (1952). 1g. ammonium molybdate was dissolved in 8ml. water and to this were added 3ml. concentrated HCl, 3ml. 12N perchloric acid and 86ml. acetone. The chromatogram was dipped in this reagent, allowed to dry and exposed to U.V. light for at least 30 minutes, after which the phosphate containing spots show up as blue areas on a white ground.

(c) The isolation of products from alkaline hydrolysates of labelled RNA.

As mentioned in 2.9a and 2.9b, portions of the alkaline hydrolysates of the labelled RNAs were subjected to paper chromatography in order to isolate the pyrimidine nucleosides and nucleotides. 0.2ml. portions of the hydrolysates were applied to the papers and a mixture of non-radioactive

uridine, cytidine, UMP-3' (or 2') and CMP-3' (or 2') was applied with each hydrolysate to act as internal markers. The papers were then developed in two solvents in the same direction; firstly butanol-H_2O (86:14) (Markham and Smith, 1949) for 48 hours to separate the nucleotides (R_f= 0) from the nucleosides, uridine (R_f=12) and cytidine (R_f= 17) as in Fig.6A, followed by the isobutyrate solvent (see 2.10a) for 18 hours to complete the separation uridine, cytidine, UMP-3'(2') and CMP-3'(2') (see Fig.6B). The spots corresponding to the markers were located using the Hanovia Chromatolite, cut out and eluted with water by capillary flow. 0.5ml. eluates were collected and 0.4ml. samples of these were taken for the measurement of radioactivity.

(d) <u>The isolation of RNA bases</u>.

Solutions containing RNA, or the nucleotides derived from RNA by alkaline hydrolysis followed by acidification, were evaporated to dryness <u>in vacuo</u> over concentrated sulphuric acid. The dry residue was then treated with 0.1ml. of 12N perchloric acid at 100° for 1 hour. The resulting hydrolysate was adjusted to pH 8 with 7N potassium hydroxide, acidified to pH 2 with HCl and centrifuged. A portion of the supernatant fluid was then applied to Whatman No.1 chromatography paper for two dimensional chromatography. The spots were applied 12cm. from the edge on the long axis and 4cm. from the edge on the short axis. The solvents

Figure 6. A. Paper chromatographic separation of UMP-3'(2') and CMP-3'(2') from uridine and cytidine using butanol-water as solvent (see text).

B. Completion of paper chromatographic separation of UMP-3'(2') and CMP-3'(2') from uridine and cytidine using the isobutyrate solvent (see text).

C. Separation of AMP-3'(2'), GMP-3'(2'), CMP-3'(2') and UMP-3'(2') by paper ionophoresis.

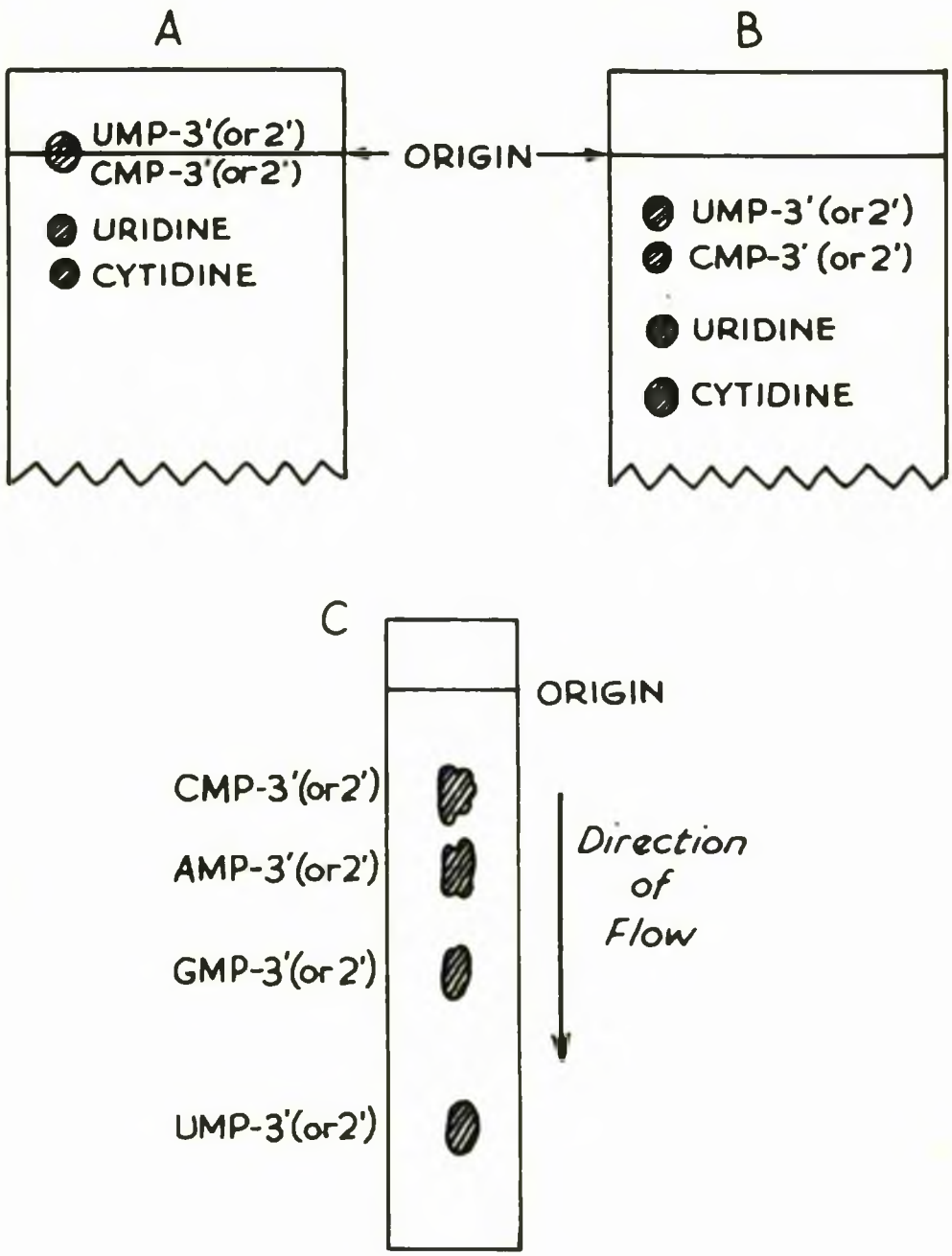

A
B
UMP-3'(or 2')
CMP-3'(or 2')
ORIGIN
URIDINE
CYTIDINE
UMP-3'(or 2')
CMP-3'(or 2')
URIDINE
CYTIDINE
C
ORIGIN
CMP-3'(or 2')
AMP-3'(or 2')
GMP-3'(or 2')
UMP-3'(or 2')
Direction of Flow

Figure 6

employed were isopropanol/HCl (descending; 170ml. isopropanol, 41ml. concentrated HCl plus water to 250ml.) (Wyatt, 1951) and butanol/NH_3 (ascending; 100ml. n-butanol saturated with water at room temperature, plus 1ml. 15N ammonium hydroxide) (MacNutt, 1952.) The chromatograms were run in both directions such that the solvent reached about 10cm. from the end of the paper. The areas corresponding to the bases (see Fig.7) were cut out after location in ultraviolet light, and eluted by capillary flow using 0.1N HCl for adenine, cytosine and uracil, and 1.6N HCl for guanine. 0.5ml. eluates were collected in each case and 0.2ml. samples of these taken for the measurement of radioactivity, or diluted for spectrophotometric examination as the experiment demanded.

2.11 Paper ionophoresis

For the isolation of ^{32}P-labelled AMP-3'(or 2'), GMP-3' (or 2'), CMP-3' (or 2') and UMP-3' (or 2') from alkaline hydrolysates of ^{32}P-labelled RNA the technique of paper ionophoresis, as described by Davidson and Smellie (1952), was employed. A portion (0.5ml.) of the radioactive hydrolysate was applied in a narrow band of almost 3cm. wide 5cm. from one end of a strip of Whatman 3MM filter paper, 72cm. x 7cm. To the band of hydrolysate was added a mixture of non-radioactive AMP-3'(or 2'), GMP-3'(or 2'), CMP-3' (or 2') and UMP-3' (or 2') to act as markers. The paper

Figure 7. Paper chromatographic separation of the RNA bases adenine, guanine, uracil and cytosine.

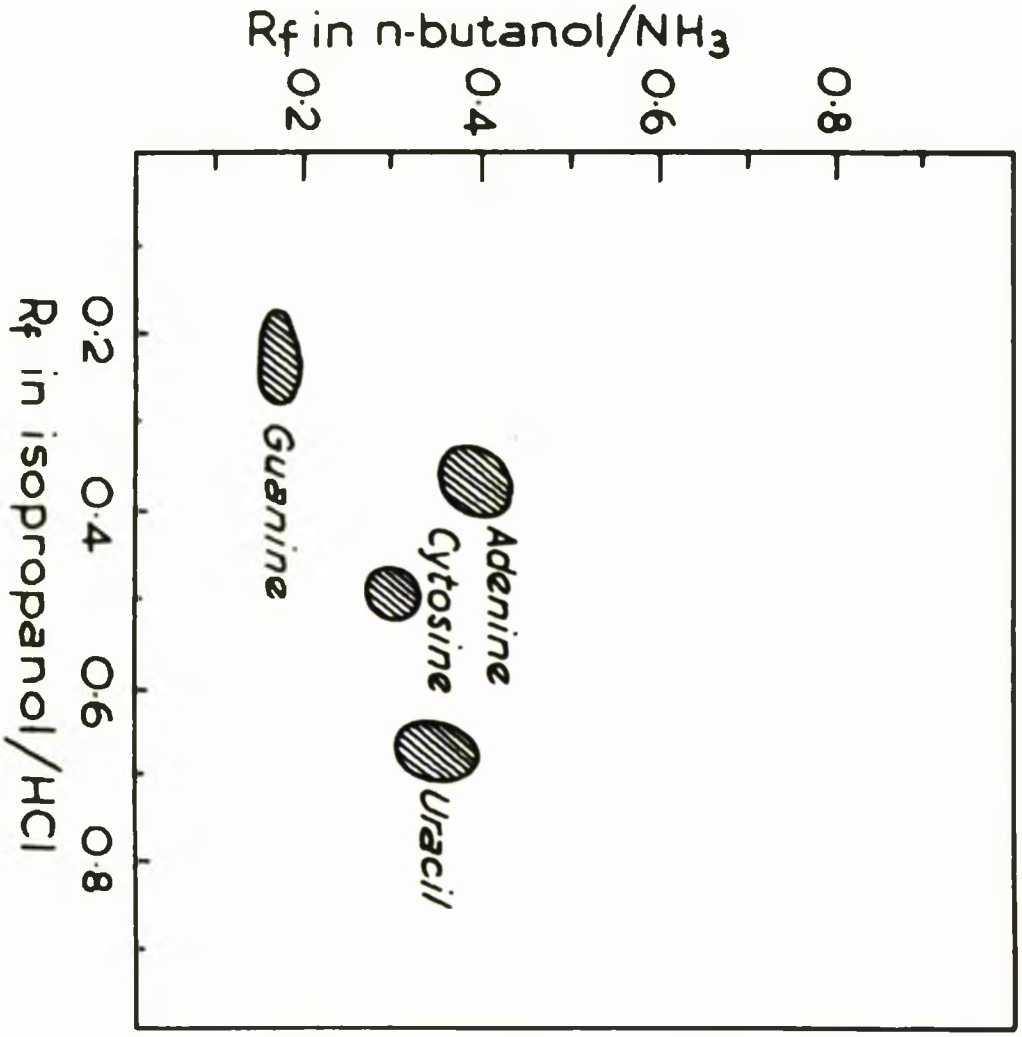

Rf in n-butanol/NH3
0·2
0·4
0·6
0·8
Rf in isopropanol/HCl
0·2
0·4
0·6
0·8
Guanine
Adenine
Cytosine
Uracil

Figure 7

was then moistened with 0.02M citric acid - trisodium citrate buffer at pH 3.5, and suspended over a glass rod so that the two ends were immersed to a depth of 1cm. in two separate glass dishes containing the same buffer. Carbon electrodes, placed in each dish, were connected to a source of direct current so that the anode was in the vessel further away from the spot on the paper. Complete separation of adenylic acid, cytidylic acid, guanylic acid and uridylic acid was obtained by running at a potential gradient of 11v/cm. for 18 hours, the current flowing being about 0.5 to 1.0ma./cm.

On completion of the run the paper was dried by exposure to a pair of 250W. 125V. infra-red lamps. Spots were located in a Hanovia chromatolite, and appeared as indicated in Fig.6c. Once the spots were located, they were cut out and eluted with water by capillary flow, 0.5ml. of eluate being collected. 0.2ml. samples were used for the measurement of radioactivity.

2.12 Estimations.

(a) Protein.

Estimation of protein was carried out by the method of Lowry, Rosebrough, Farr and Randall (1951). This involves the use of a modified type of biuret reagent and the method is in fact a modification of one first introduced by Wu (1922). The reagents used were as follows :

A : 2% sodium carbonate in 0.1N sodium hydroxide.

B : 0.5% copper sulphate in 1% sodium or potassium tartrate.

C : 50ml. of A mixed with 1ml. of B.

D : Folin-Ciocalteau reagent (Folin and Ciocalteau, 1927).

The Folin-Ciocalteau reagent obtained from B.D.H. was titrated with sodium hydroxide to a phenolphthalein end-point. On the basis of this titration the Folin reagent was diluted to make it 1N with respect to acid. The standard used was bovine serum albumin (Armour Pharmaceuticals, Eastbourne).

To a sample of 5 to 400 µg. of protein in 1ml. of solution was added 5ml. of reagent C. The solution was mixed well, and allowed to stand for 10 minutes at room temperature. 0.5ml. of reagent D was added very quickly and mixed within a few seconds. After 30 minutes the colour intensity of the sample was read in a spectrophotometer. For solutions containing low amounts of protein it was desirable to take readings at 750mµ., the absorption maximum. For stronger solutions, readings were taken at 500mµ., which allows a greater working range.

(b) RNA.

RNA was determined spectrophotometrically on the assumption that a solution of RNA containing 1µg. RNA phosphorus per ml. would give an extinction of 0.285 at 268mµ. in a

1cm. cell (Paul, unpublished results)

RNA was also estimated by the orcinol reaction for pentose (Kerr and Seraidarian, 1945). The orcinol reagent, which must be made up just prior to use, contained 0.6mg. orcinol dissolved in 100ml. 0.02% (w/v) $FeCl_3.6H_2O$ in concentrated HCl. 3ml. of ribose containing solution (in the case of RNA, containing in the region of 1 - 6 µg. RNA phosphorus/ml.) were treated with 3ml. of fresh orcinol reagent in a clean pyrex test tube. This mixture was then placed in a boiling water bath for 30 minutes, cooled rapidly, and the colour intensity measured at 665µm. A blank was prepared similarly using distilled water in place of RNA hydrolysate. As standard, a solution of ribose, containing 10µg. per ml. was prepared and this was equivalent to 4.13µg. RNA phosphorus/ml.

(c) DNA

DNA was determined spectrophotometrically on the assumption that a solution of DNA containing 1µg. DNA phosphorus per ml. would give an extinction of 0.230 at 268mµ in a 1cm. cell (Ceriotti, 1955)

DNA was also estimated quantitatively using the indole reaction of Ceriotti (1952, 1955). To 2ml. of a solution containing from 2.5 to 15µg. DNA per ml. was added 1ml. of 0.04% (w/v) indole solution and 1ml. concentrated HCl. The tubes were then immersed in boiling water for 10 minutes

and then cooled under running tap water. The cooled solutions were then extracted three times with 4ml. portions of chloroform, the chloroform extract being discarded each time. The yellow colour remaining in the aqueous phase was due to the DNA pigment and was measured in a spectrophotometer at 490mμ against a blank, containing water instead of DNA solution, prepared in a similar manner. Standard DNA solutions were prepared in the department from calf thymus DNA.

(d) Inorganic and Organic Phosphorus.

Phosphorus was estimated by a modification of the method of Allen (1940). The first step in this procedure was to convert all phosphorus to inorganic orthophosphate by digestion with sulphuric acid and hydrogen peroxide. However, when an estimate of inorganic phosphate alone was required, this digestion step was omitted, although sulphuric acid was routinely added.

A sample of the solution to be assayed containing 4 - 30μg. phosphorus was pipetted into a pyrex tube, and 0.24ml. of 10N sulphuric acid were added. The resulting solution was heated in a sand bath at about 130° until the volume had dropped to around 0.1ml. The solution was cooled and 2 drops of hydrogen peroxide (M.A.R., 100 vols.) were added. The solution was heated for a further 30 minutes after which it was cooled and a small portion of water, 2 - 3ml., was added. If the experiment only demanded the estimation of

inorganic phosphorus in the solution, it was necessary only to add 0.24ml.10N sulphuric acid to the solution, no subsequent digestion being required.

0.2ml. 8.3% (w/v) solution of ammonium molybdate and 0.4ml. 1% (w/v) solution of 2:4 diamino-phenol hydrochloride (Analar) in 20% (w/v) sodium metabisulphite solution were added, and the mixture allowed to stand for 10 minutes at room temperature before the optical density was measured at 640mμ, against a reagent blank. A standard phosphorus solution was prepared by dilution of a stock phosphate standard (Analar ; 2.193g. KH_2PO_4 in 500ml. water. i.e. 1ml.= 1mg. P).

(c) RNA bases.

After isolation by chromatography (see 2.10d) and elution from the paper in dilute HCl, samples of the eluate were diluted with HCl of the appropriate concentration for spectroscopic estimation.

Adenine was estimated in 0.1N HCl at 262mμ and the molar extinction coefficient used 13,100.

Uracil was estimated in 0.1N HCl at 260mμ and the molar extinction coefficient used 8,200.

Cytosine was estimated in 0.1N HCl at 275mμ and the molar extinction coefficient used 10,400.

Specifications and Criteria for Biochemical compounds, 1960)

2.13 Enzyme assays.

(a) Ribonuclease.

A rough method for the assay of ribonuclease was devised which depended on the degradation of yeast RNA (Nutritional Biochemicals) to acid-soluble fragments which are then assayed spectrophotometrically. A solution of yeast RNA (2mg. per ml. of 0.01M NaCl) was prepared and dialysed for 24 hours against a large excess of 0.01M NaCl. The reaction mixture (1.0ml) containing 0.25 ml. of this RNA solution, 0.15ml. 1M Tris buffer of the appropriate pH value and 0.6 ml. of the enzyme fraction to be assayed was incubated for 30 minutes at 37°. 0.5ml. of ice-cold 1N perchloric acid was added to precipitate nucleic acid and proteins, which were removed by centrifugation in the cold. The supernatant fluid was diluted with 5 vol. distilled water and the optical density at 260 mμ determined.

(b) Phosphatase.

The assay depends on the estimation of the phosphatase catalysed release of inorganic phosphate from a solution of ribonucleoside triphosphates. The reaction mixtures (1.0) contained 1μmole each of UTP, CTP, ATP and GTP, 2μmoles $MgCl_2$, 0.6ml. of the enzyme fraction to be assayed and was 0.1M with respect to Tris buffer of the appropriate pH. Incubation was carried out at 37° for 30 minutes after which 0.5ml. 1N perchloric was added. The precipitated nucleic

acid and proteins were removed by centrifugation. The inorganic phosphate present in the supernatant fluid was measured as in 2.13d, giving an estimate of phosphatase activity.

2.14. Measurement of radioactivity.

Except in one specific case, measurements of radioactivity were carried out using a Nuclear Chicago scaling unit and automatic sample changer fitted to a windowless gas flow counter operating in the Geiger region.

The counting of ^{3}H and ^{14}C was carried out on samples which had been plated, with drying under infra-red lamps, on 1.25" diameter aluminium planchettes which were first thoroughly cleaned by rubbing with "Ajax" cleaner. The major difficulty in counting ^{3}H and ^{14}C is that of self-absorption. Preliminary experiments indicated that counting of ^{3}H samples at infinite thickness could give rise to very large errors due to non-uniform distribution of material on the planchettes. At the same time, it was almost impossible to achieve infinitely thin ^{3}H containing samples. However, by serial dilution it proved possible to attain samples that approached infinite thinness and this technique was adopted for ^{3}H and ^{14}C containing samples. As a further safeguard all experiments were carried out in duplicate or triplicate, repeat dilutions and counts always being performed where any serious disagreement between

duplicates was observed. The greatest care had to be taken to remove as much potassium perchlorate as possible from solution before preparing the samples on planchettes for counting. Under these conditions an efficiency for ^{3}H of 34% was achieved. For ^{14}C the efficiency achieved was somewhat greater, being in the region of 40%. In all cases, at least 1000 counts were collected.

For the counting of ^{32}P the same procedure was adopted although the problems of self-absorption and efficiency were less critical. When the ^{32}P samples were dissolved in 0.1N NaOH, stainless steel planchettes were used, and even spreading was achieved with the use of 1" diameter discs of lens tissue. ^{32}P labelled, trichloroacetic acid washed, filter paper discs were placed on clean stainless planchettes prior to counting.

In one particular series of experiments where it was necessary to measure very low levels of ^{3}H in RNA samples from ribonucleoproteins of ascites cell nuclei (2.9m) a Nuclear Chicago Model 6000 (50 cycle) Dynacon Electrometer was employed. This is a dynamic condenser electrometer designed for precise measurement of small ion currents originating in an ionisation chamber. Measurement of radioactivity was carried out on a rate of change basis. For ^{3}H at infinite thinness the ionisation chamber constant was 4×10^{-13} amperes/μc $\pm$ 2%, and each sample was measured at least six

times for maximum accuracy.

2.15 Microautoradiography.

After intact ascites cells had been allowed to incorporate ^{3}H-uridine over a period of 2 minutes (see 2.9a), a sample of cells was withdrawn for autoradiography. The cell suspension was treated with an equal volume of a mixture of ice-cold 4N perchloric acid - absolute ethanol (1:1). After 10 minutes treatment with this fixative in the cold, the cells were washed successively with 70% ethanol, 50% ethanol, 25% ethanol and water. This was followed by two 3 minute extractions with ice-cold 0.7N perchloric acid to remove any radioactive acid-soluble material. The cells were further washed with 25% ethanol, 50% ethanol and 70% ethanol at which stage they could be conveniently stored.

The next step was to embed the cells in paraffin wax prior to sectioning. Thus it was necessary to dehydrate the cells further in order to infiltrate them with paraffin wax. The suspension of cells in 70% ethanol was centrifuged and resuspended in 95% ethanol, followed by absolute ethanol, absolute ethanol-xylene (3:1), absolute ethanol-xylene (1:1), absolute ethanol-xylene (1:3) and then xylene alone. The cell suspension in xylene was allowed to warm in an oven at 61^{o} and then an equal volume of molten, filtered paraffin wax (M.P.58^{o}) was added. The cells were allowed to sit until the paraffin wax (which partially solidifies) re-melts.

Then the cells were mixed as thoroughly as possible in the paraffin wax - xylene mixture at 61°. A convenient technique was to suck up the clear mixture on top with a previously heated Pasteur pipette, and quickly eject this back into the tube, thus mixing the cells which have settled to the bottom. It is important, from the point of view of suspending cells during paraffin wax infiltration to use tubes which, although conical, are not too sharply pointed at the bottom. The cells were then left overnight at 61° in the paraffin wax - xylene mixture. On the following day the paraffin wax - xylene was removed from the cells which had settled to the bottom, and fresh paraffin wax added. During the day the paraffin wax was changed twice more. Finally when the cells had settled to a pellet at the bottom of the tube the embedding was completed by removing the tube and placing in an ice-bath. The embedded cells were then sectioned. 3μ sections were taken which gave 50-60% compression (thinner sections gave even greater compression.).

The glass slides on which these sections were mounted were "subbed" to ensure good wet adhesion of the film emulsion when this was applied. Clean glass slides were dipped in a 0.5% (w/v) solution of gelatine containing 0.05% (w/v) of chrome alum. The slides were allowed to dry after which they were ready for use.

The sections prepared from the embedded cells were then mounted on the "subbed" sides and washed successively with xylene, xylene - absolute ethanol (3:1), xylene - absolute ethanol (1:1) xylene - absolute ethanol (1:3), absolute ethanol, boiling absolute ethanol - ether (3:1) for 5 minutes to remove absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 35% ethanol, water, ice-cold 0.7N perchloric acid for 3 minutes, and finally twice with water. The sectioned cells were then covered with AR 10 autoradiographic stripping film (Kodak Ltd., London), by the light of a Wratten Series 1 red safelight.

Using a sharp scalpel blade, a cut was made in the emulsion layer of the AR 10 plate around an area sufficient to cover the whole of a specimen with a margin of at least a quarter of an inch all round. The slide bearing the sectioned cells was then placed in a bath of distilled water at least one inch deep. With the tip of the scalpel blade, the emulsion layer was stripped from the plate, turned over, and thrown on to the surface of the water so that the sensitive side of the emulsion layer was facing downwards. The emulsion swells, crumples and then stretches out tight and flat. It was left for two minutes more and lifted from the water by raising the slide underneath it. By holding the slide at 30° from the horizontal one edge touched the emulsion first and the emulsion then

draped itself snugly over the specimen and most of the water drained away as the slide was gradually lifted clear of the water. The cell sections with emulsion superimposed were then dried overnight in a drying box. Once dry, the slides were placed in a black light-tight box containing silica gel as desiccant. Before exposure at 3 - 5° (in refrigerator) this box was carefully sealed in several layers of thick paper.

After an exposure time of 2-3 weeks, the slides were then processed with the emulsion layer in permanent contact with the specimen. After letting the slides come to room temperature they were allowed to develop in undiluted D.19b Kodak developer (300ml./10 slides) for 6 minutes at 18° with the minimum of red light. The slides were then rinsed in tap water for 30 seconds and treated with Kodak acid fixer (diluted 1 part to 2 parts with water) for 8 minutes at 18°. After rinsing in tap water for 2 hours, they were finally washed with distilled water. However before mounting the specimen it was necessary to dehydrate the film. This was achieved by treating the slide successively with 50% ethanol, absolute ethanol, absolute ethanol - xylene (1:1), absolute ethanol - xylene (1:3) and xylene. The cell sections and superimposed emulsion were then mounted using Depex mounting medium. (G.T.Gurr Ltd., London).

2.16 Materials.

Nominally 5,6 - ^{3}H - uridine of specific activity 62μC/μmole, and carrier free disodium hydrogen phosphate (PBS.1,1.5ml. and 25mC) of specific activity greater than 1mC/mg. were purchased from the Radiochemical Centre, Amersham.

2',3'-isopropylidene uridine and 2',3'-isopropylidene adenosine were obtained from the Aldrich Chemical Co., Inc.; NAD, NADP, $NADH_2$ from C.F. Boehringer und Soehne ; uridine, cytidine UMP-3'(2'), CMP-3'(2') AMP-3'(2'), GMP-3'(2'), crystalline DNase, crystalline RNase and yeast RNA from Nutritional Biochemicals Corporation. Other ribonucleoside 5'-phosphates were obtained from the Sigma Chemical Company or Pabst Laboratories.

SECTION III

RESULTS

3.1 Incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of ascites carcinoma cells.

The incorporation of ^{3}H-uridine into the RNA of intact Ehrlich ascites cells *in vitro* is illustrated in Table II. Osmotic disruption of the cells produced a preparation which showed diminished incorporation, but the ability of this preparation to incorporate uridine was found to be markedly increased by supplementation of the incubation medium with ATP, magnesium ions, NAD and glucose. High speed centrifugation of the osmotically disrupted cell suspensions gave rise to a crude cytoplasmic extract which when supplemented as above readily incorporated labelled uridine into RNA and a sediment which showed little activity in this respect. Examination of the acid-soluble fractions showed that, with the exception of the 105,000 x g sediment, all the fractions shown in Table II were capable of phosphorylating uridine to UMP, UDP and UTP. The 105,000 x g sediment appeared to lack the kinases required for this process.

These preliminary results were obtained using a reaction medium buffered with 0.1M Tris pH7.9. A series of incubation mixtures were set up to determine the optimum pH for the incorporation of uridine into RNA and the results shown in Fig. 8 indicate an optimal pH of 7.9. Analysis of the acid-soluble fractions from incubations carried out at pH6.0 indicated that there was little phosphorylation of uridine: at pH 7.9 however there was extensive conversion to UMP, UDP

Table II

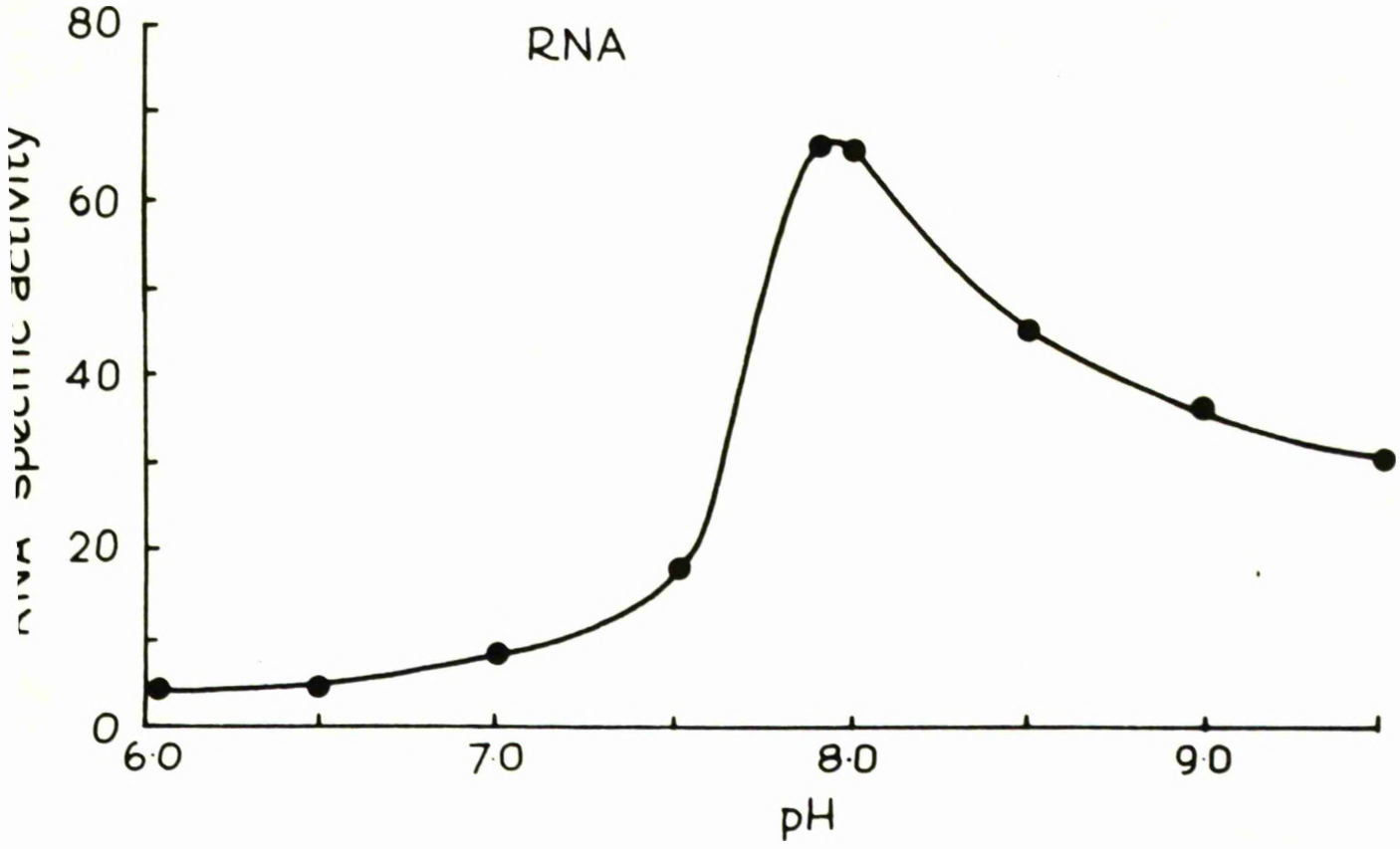
Incorporation of ^{3}H-uridine into the RNA of fractions of Ehrlich ascites carcinoma cells

Fraction	RNA specific activity (Counts/min./μmole RNA-P)
Intact ascites cells	56,600
Osmotically disrupted ascites cell preparation	5,700
Osmotically disrupted cell preparation plus ATP, Mg^{++}, NAD and glucose.	26,000
Cytoplasmic extract (105,000xg supernatant fraction from disrupted cells) plus ATP, Mg^{++}, NAD and glucose.	20,600
Sediment (105,000xg) plus ATP, Mg^{++}, NAD and glucose.	1,900

The reaction mixtures were 0.1M with respect to Tris buffer at pH 7.9, and contained where indicated 5μmoles ATP/ml., 5μmoles $MgCl_2$/ml., 1μmole NAD/ml., 5μmoles glucose/ml. and 3μc ^{3}H-uridine/ml. Incubation time was 2 hr.

Figure 8. The effect of variation in pH on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells, and on the distribution of radioactivity in the acid-soluble fractions.

The medium was 0.1 M with respect to tris buffer and contained 5 µmoles ATP/ml., 5 µmoles $MgCl_2$/ml., 1 µmole NAD/ml., 5 µmoles glucose/ml. and 3 µC ^{3}H-uridine/ml.
Incubation time : 2 hours.
RNA specific activity expressed as counts/min./ µmole RNA-P x 10^{-3}.


RNA
80
60
40
20
0
6·0
7·0
8·0
9·0
pH

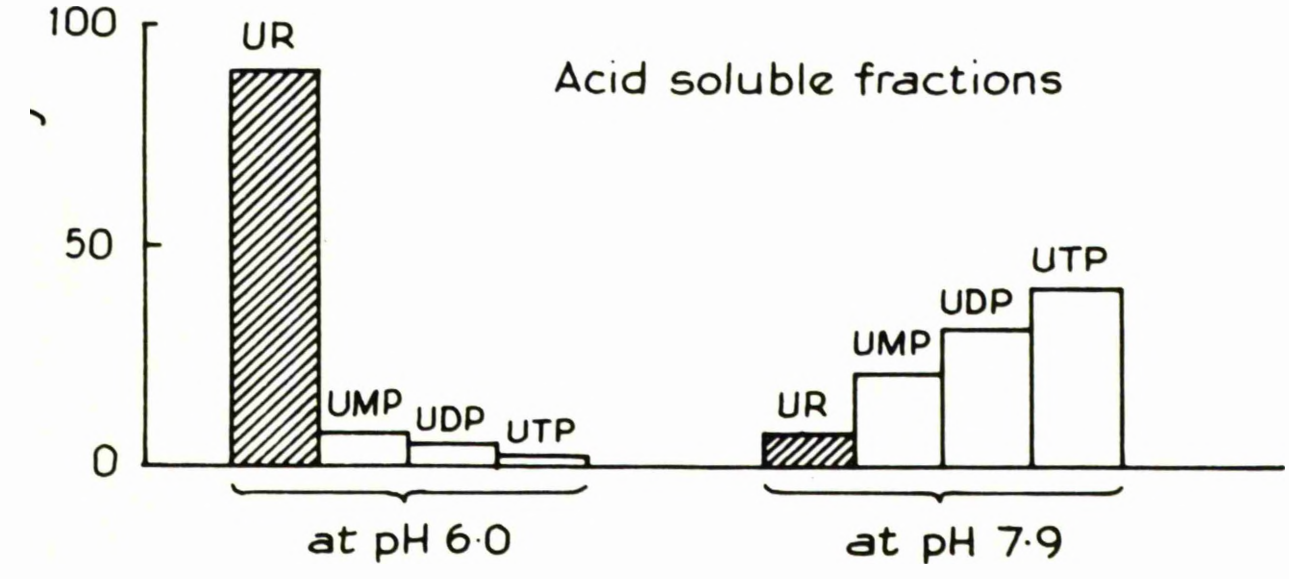

100
50
0
UR
UMP
UDP
UTP
Acid soluble fractions
UR
UMP
UDP
UTP
at pH 6·0
at pH 7·9

Figure 8

and UTP (Fig.8).

Examination of the other cofactors required showed that ATP is required in concentrations of at least 2μmoles per ml. (Fig.9). In the absence of ATP negligible amounts of uridine were phosphorylated and there was a corresponding reduction in the amount of ^{3}H-uridine incorporated into the RNA.

The effect of inorganic cations is shown in Fig.10. The only combination more effective than magnesium ions alone was magnesium ions plus potassium ions. All other combinations were less effective especially those containing calcium ions which were somewhat inhibitory.

If the incorporation of uridine into RNA by these cytoplasmic extracts involves the addition of UDP to an existing polyribonucleotide with the concomitant liberation of inorganic orthophosphate, the addition of inorganic orthophosphate to the reaction medium should depress the reaction. Similarly inorganic pyrophosphate might be expected to inhibit any reaction involving the addition of UTP with the release of inorganic pyrophosphate. The effect of adding inorganic ortho-or pyrophosphate to the incubation mixture (Fig.11) was to inhibit uridine incorporation into RNA. Together inorganic ortho- and pyrophosphate had an even greater inhibitory effect on this reaction but either singly or together they had little influence on the

Figure 9. The effect of varying concentrations of ATP on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells, and on the distribution of radioactivity in the acid-soluble fractions.

The medium contained 5 µmoles $MgCl_2$/ml., 1 µmole NAD/ml., 5 µmoles glucose/ml., 3 µC ^{3}H-uridine/ml. and was 0.1 M with respect to tris buffer at pH 7.9. Incubation time : 2 hours. RNA specific activity expressed as counts/min./µmole RNA-P x 10^{-3}.

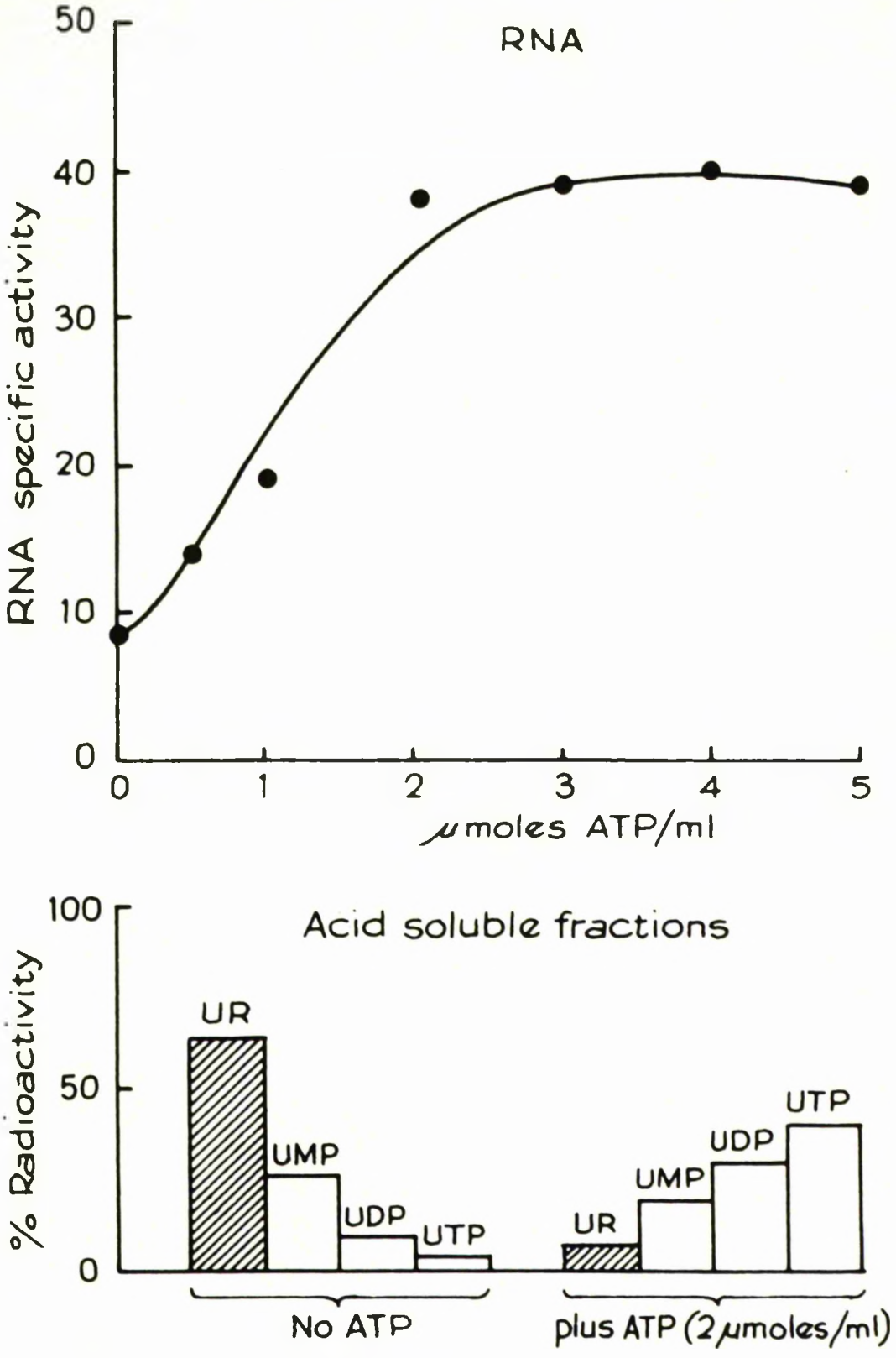
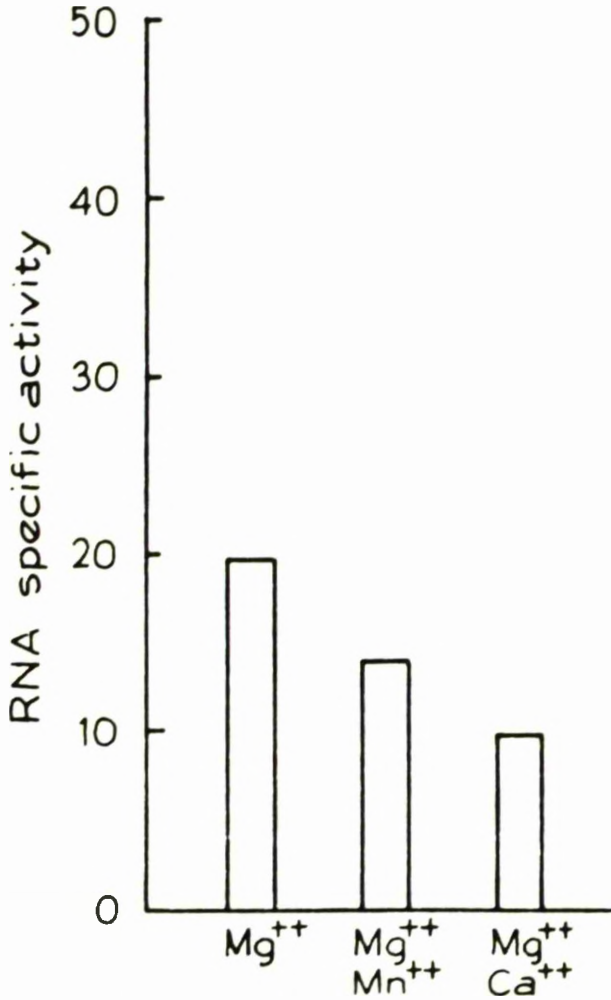

RNA
RNA specific activity
50
40
30
20
10
0
0
1
2
3
4
5
μmoles ATP/ml
Acid soluble fractions
% Radioactivity
100
50
0
UR
UMP
UDP
UTP
UR
UMP
UDP
UTP
No ATP
plus ATP (2 μmoles/ml)

Figure 9

Figure 10. The effect of different ions on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

The medium was 0.1 M with respect to tris buffer at pH 7.9 and contained 5 µmoles ATP/ml., 1µmole NAD/ml., 5µmoles glucose/ml. and 3 µC ^{3}H-uridine/ml. $MgCl_2$, KCl, $MnCl_2$ and $CaCl_2$ when added were at a concentration of 5 µmoles/ml. Incubation time : 2 hours. RNA specific activity expressed as counts/min./ µmole RNA-P x 10^{-3}.


RNA specific activity
50
40
30
20
10
0
Mg++
Mg++ Mn++
Mg++ Ca++

RNA

Figure 10

Figure 11. The effect of addition of inorganic ortho- or pyrophosphate on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

The medium was 0.1 M with respect to tris buffer at pH 7.9 and contained ATP, $MgCl_2$, KCl and glucose each at a concentration of 5 μmoles/ml., 1 μmole NAD/ml., 3 μC ^{3}H-uridine/ml. and where indicated inorganic ortho- and pyrophosphate at a concentration of 20 μmoles/ml.

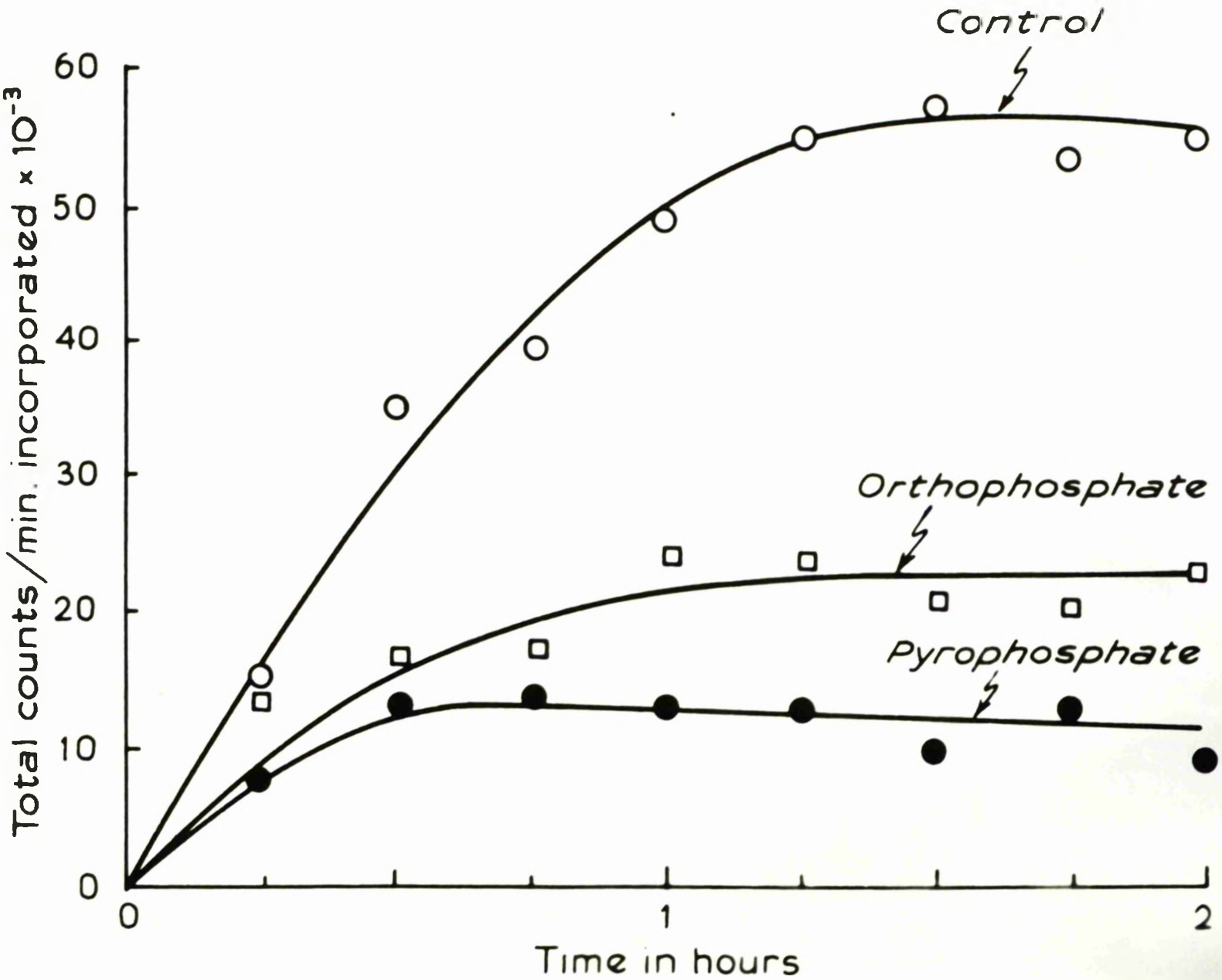

Control
Orthophosphate
Pyrophosphate
Total counts/min. incorporated × 10⁻³
60
50
40
30
20
10
0
0
1
2
Time in hours

Figure 11

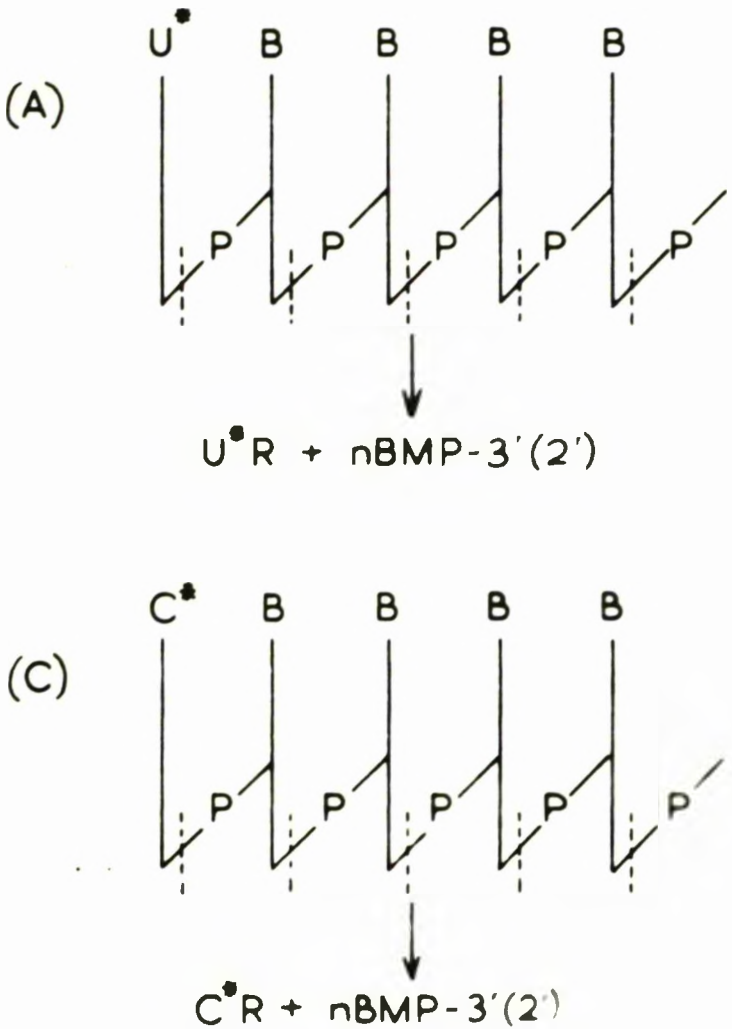
phosphorylation of uridine to UDP or UTP.

Fig.12 illustrates the possible localisation of ^{3}H in RNA labelled in these experiments either (a) in UMP moieties incorporated terminally, so that alkaline hydrolysis (indicated by the broken lines) would give rise to ^{3}H-uridine or (b) in UMP moieties located in non-terminal positions so that alkaline treatment would yield ^{3}H-UMP-3' (or 2'). The possibility must also be considered that during incubation some uridine is converted to cytidine derivatives (Lieberman, 1955; 1956; Hurlbert and Kammen; 1960) in which case ^{3}H would also be found in the CMP moieties of the polynucleotide chain attached terminally(c) or non-terminally (d) and would give rise to ^{3}H-labelled cytidine and CMP-3'(or 2') respectively on alkaline hydrolysis.

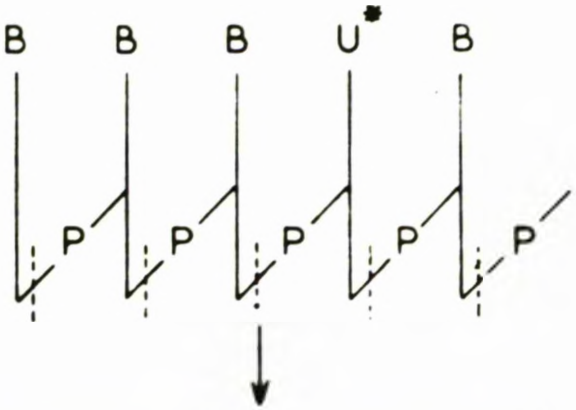
Table III shows the distribution of radioactivity between the ribonucleoside and ribonucleotide fractions formed during alkaline hydrolysis of the RNA. ^{3}H-uridine was incorporated into both terminal and non-terminal positions but occupied principally a non-terminal position. In the presence of inorganic pyrophosphate the proportion of total RNA radioactivity recovered as UMP-3'(or 2') was greatly increased indicating that the terminal incorporation of uridine had been greatly depressed. Inorganic orthophosphate seemed to have an opposite effect; since a higher proportion of the

Figure 12. Possible locations of ^{3}H in RNA after incubation with ^{3}H-uridine (see text).

U and C represent uracil and cytosine respectively, and B any other ribonucleotide base. The vertical lines represent ribose moieties and the oblique lines, 3'-5' phosphodiester linkages.

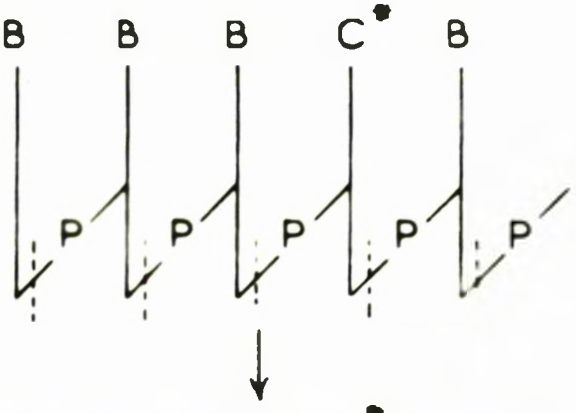

(A)
U*
B
B
B
B
P
P
P
P
P
U*R + nBMP-3'(2')
(C)
C*
B
B
B
B
P
P
P
P
P
C*R + nBMP-3'(2')

(B)



BR + nBMP-3'(2') + U*MP-3'(2')

(D)



BR + nBMP-3'(2') + C*MP-3'(2')

Figure 12

Table III

The effect of addition of ortho- and pyrophosphate on the localization of ^{3}H in alkaline hydrolysate of RNA.

Additions	Per cent total RNA radioactivity			
	Uridine	Cytidine	UMP-3' (or 2')	CMP-3' (or 2')
Nil	24	6	56	14
Inorganic pyrophosphate	12	0	82	6
Inorganic orthophosphate	65	13	12	10

Incubation conditions same as those for Fig.11.

Incubation time : 2 hours.

activity was recovered as uridine, inhibition of non-terminal incorporation must have occurred. Since approximately 20% of the total radioactivity of RNA was recovered as cytidine or CMP-3'(or 2'), appreciable conversion of uridine to cytidine derivatives took place during incubation.

The inclusion of non-radioactive UDP or UTP in the incubation mixtures at concentrations of 1μmole per ml. in an attempt to dilute the pool of RNA precursors led to enhanced uptake of uridine into the RNA, while together they had little effect (Table IV). Since the system under investigation was probably quite complex, these observations could be due to the sizes of the UDP and UTP pools influencing the ultimate incorporation into RNA, or to some coenzymic role of the uridine nucleotides. To investigate the former possibility, experiments were set up with varying concentrations of added UDP or UTP. The results are illustrated in Fig.13 and show that at concentrations above 2μmoles per ml., UDP and UTP diluted the incorporation of ^{3}H-uridine into RNA but enhanced the uptake at lower concentrations, having optimal effect at 1μmole per ml. In view of the fact that uridine nucleotides might be concerned with the availability of the various forms of pyridine nucleotides, experiments were carried out in which the requirements for glucose, NAD, $NADH_2$, NADP, UDP and UTP were followed. From the results presented in Table V, it was clear that the optimal incorporation

Table IV

The effect of UDP and UTP onthe incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

Additions (1 µmole/ml. of each)	RNA specific activity (counts/min./umole RNA-P)
Nil	19,000
UDP	31,500
UTP	36,000
UDP and UTP	15,000

The reaction mixtures were 0.1M with respect to Tris buffer at pH 7.9 and contained 5 µmoles ATP/ml., 5 µmoles $MgCl_2$/ml., 5 µmoles KCl/ml., 1 µmole NAD/ml., 5 µmoles glucose/ml. and 3 µC ^{3}H-uridine/ml.
Incubation time : 2 hours.

Figure 13. The effect of added UDP and UTP on the uptake of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

The medium was 0.1 M with respect to tris buffer at pH 7.9 and contained ATP, $MgCl_2$, KCl and glucose each at a concentration of 5 µmoles/ml., 1 µmole NAD/ml., and 3 µC ^{3}H-uridine/ml.

Incubation time : 2 hr. RNA specific activity expressed as counts/min./µmole RNA-P x 10^{-3}.

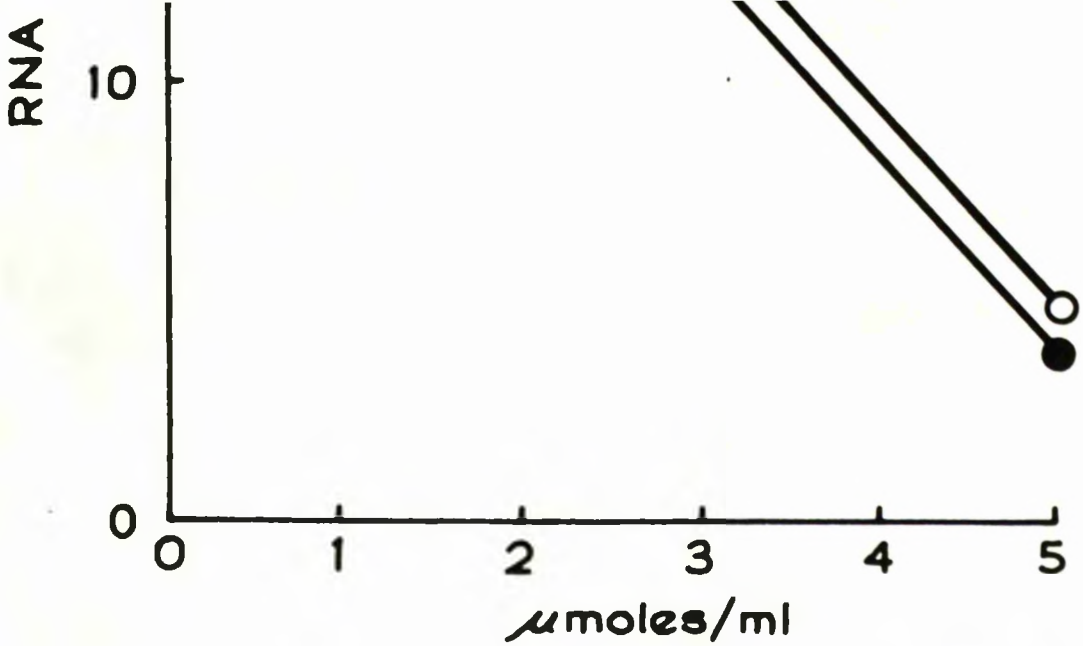


Figure 13

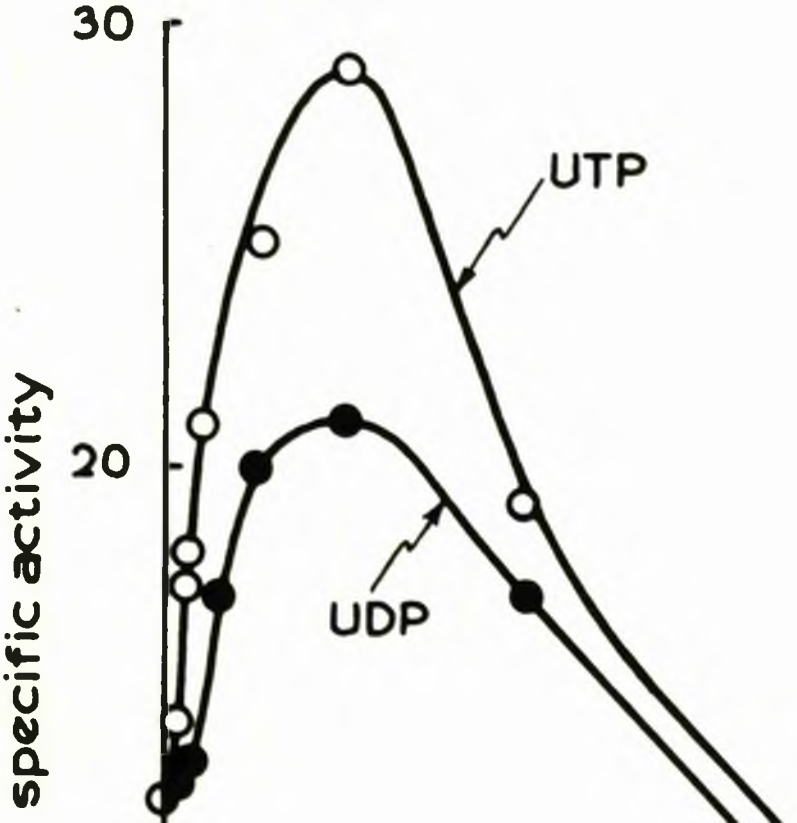

30
20
UTP
UDP
specific activity

Table V

The effect of glucose, NAD, $NADH_2$, NADP and UTP on the incorporation of ^3H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

Additions	RNA specific activity (counts/min./μmole RNA-P)
Nil	8,300
glucose	11,000
NAD	18,500
glucose plus NAD	20,000
glucose plus UTP	16,500
NAD plus UTP	28,000
UTP plus NAD plus glucose	39,600
$NADH_2$	27,000
NADP	30,000
NADP plus $NADH_2$	37,700

The reaction media were 0.1M with respect to tris buffer at pH 7.9 and contained 5 μmoles ATP/ml., 5 μmoles $MgCl_2$/ml., 5 μmoles KCl/ml. and 3 μC ^3H-uridine/ml., and also where indicated 5 μmoles glucose/ml., 1 μmole NAD/ml., 1 μmole UTP/ml., 1 μmole $NADH_2$/ml. and 1 μmole NADP/ml. Incubation time : 2 hours.

of ^{3}H-uridine into RNA required either a mixture of glucose, NAD and UTP or an equimolar mixture of NADP and $NADH_2$. In the prdsence of these two substances, UDP or UTP added to the reaction mixture at concentrations of 1µmole per ml. depressed the incorporation of uridine into RNA (Table VI).

Analysis of the products of alkaline hydrolysis of RNA showed that the increased incorporation of uridine obtained in the presence of NADP and $NADH_2$ was due to an increase in the amount of ^{3}H-uridine attached terminally to the RNA (Table VII) and investigation of the acid-soluble (Table VIII) demonstrated that NADP and $NADH_2$ promoted the formation of larger amounts of UTP from ^{3}H-uridine.

If the incorporation of ^{3}H-uridine represents true synthesis of RNA, it must be assumed that the other three ribonucleotides are present in the tissue extract or can be formed during the incubation. In either event, the availability of these might limit the extent of the reaction. As might be expected, therefore, supplementation of the reaction mixture with low concentrations of ADP, GDP and CDP, or of GTP and CTP, stimulated the incorporation of ^{3}H-uridine (Table IX).

While the crude cytoplasmic extracts contained appreciable quantities of RNA, the addition of further amounts of RNA to incubation mixtures stimulated the uptake of uridine (Fig.14). This was particularly true of the RNA (microsomal

Table VI

The effect of UDP and UTP on the uptake of ^{3}H-uridine into RNA, by cytoplasmic extracts of Ehrlich ascites carcinoma cells, in the presence of $NADH_2$ and NADP.

Additions (1 µmole/ml. of each)	RNA specific activity (counts/min./µmole RNA-P
Nil	23,500
UDP	17,500
UTP	15,000
UDP plus UTP	10,500

The reaction mixtures were 0.1M with respect to tris buffer at pH 7.9 and contained 5 µmoles each of ATP, $MgCl_2$ and KCl/ml., 1 µmole each of $NADH_2$ and NADP/ml. and 3 µC ^{3}H-uridine/ml.

Incubation time : 2 hours.

Table VII

The effect of $NADH_2$ and NADP on the localization of ^3H in alkaline hydrolysates of RNA.

Additions	Per cent total RNA radioactivity			
	uridine	cytidine	UMP-3'(or 2')	CMP-3'(or 2')
Nil	28	2	66	4
1 µmole each of $NADH_2$ and NADP	48	2	48	2

Incubation conditions same as in Table V.

Table VIII

The effect of NADP and $NADH_2$ on the distribution of radioactivity in the uridine derivatives of the acid-soluble fraction after incorporation of ^3H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

Additions	Per cent of acid-soluble radioactivity			
	Uridine	UMP	UDP	UTP
Nil	6	10	26	58
1 µmole each of $NADH_2$ and NADP	3	2	15	80

Incubation conditions same as in Table V.

Table IX

The effect of adding di- and triphosphates of adenosine, guanosine and cytosine, on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

Additions	RNA specific activity (count/min./µmole RNA-P
Nil	20,500
ADP, GDP and CDP	33,500
GTP and CTP	43,000
ADP, GDP, CDP, GTP and CTP	27,000

The reaction mixtures were 0.1M with respect to tris buffer at pH 7.9 and contained ATP, $MgCl_2$, KCl each at a concentration of 5 µmole/ml., also 1 µmole NADP/ml., 1 µmole $NADH_2$/ml., 1 µmole 2-mercaptoethanol/ml. and 3 µC ^{3}H-uridine/ml. Where indicated the ribonucleoside di- and triphosphates were present at a concentration of 1 µmole/ml. Incubation time : 2 hours.

Figure 14. The effect of adding varying amounts of ascites RNA on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites carcinoma cells. The medium was 0.1 with respect to tris buffer at pH 7.9 and contained ATP, $MgCl_2$, KCl and glucose each at a concentration of 5 µmoles/ml., 1 µmole NAD/ml. and 3 µC ^{3}H-uridine/ml. Incubation time : 2 hrs.

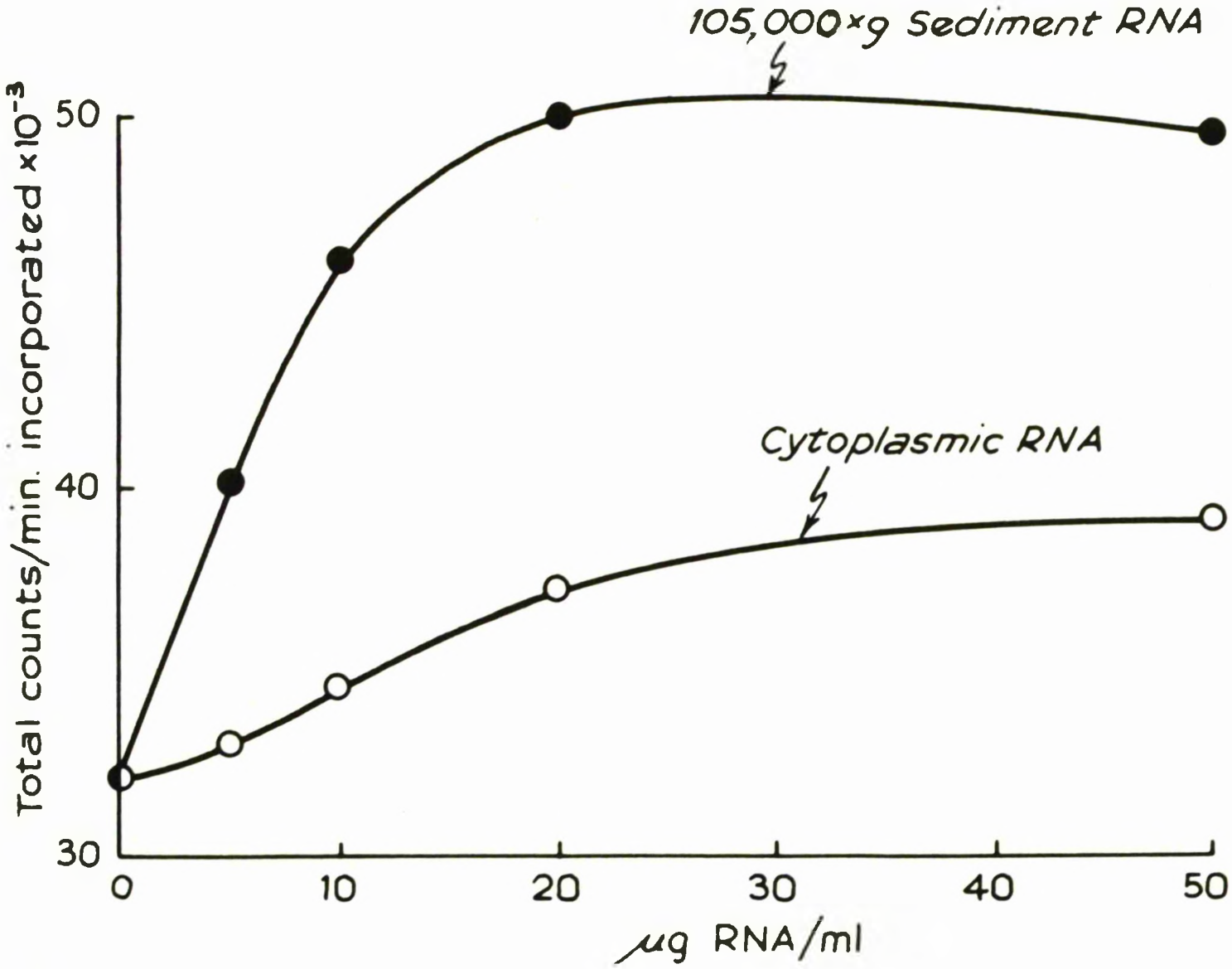

105,000×g Sediment RNA
Cytoplasmic RNA
Total counts/min. incorporated ×10⁻³
50
40
30
0
10
20
30
40
50
μg RNA/ml

Figure 14

and nuclear) prepared from the 105,000xg sediment of the osmotically disrupted ascites cells; the addition RNA prepared from the cell sap had a similar but less pronounced effect.

Dialysis of the crude cytoplasmic extracts against 0.01M Tris buffer pH 7.9 for 18hr. at 4° had little influence on their ability to catalyse the incorporation of uridine into RNA. Prior incubation of the extracts at 37° for 30 minutes was inhibitory (Table X).

3.2 Incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of immature rabbit tissues

The results described so far have been obtained with cytoplasmic extracts of ascites cells. Cytoplasmic extracts, prepared in a similar fashion from a series of immature rabbit tissues, showed considerable variation in the ability to catalyse the incorporation of uridine into RNA (Table XI). In most tissues the ratio of uridine incorporated in a terminal/position to that in a non-terminal location was very low (about 0.1), but in brain and muscle a very much higher proportion of terminal addition was observed (Table XII).

3.3. Synthesis of polyribonucleotide material by cytoplasmic extracts of ascites carcinoma cells.

Evidence that cytoplasmic extracts of ascites cells are capable of net synthesis of small amounts of polyribonucleotide material is presented in Table XIII. In the

Table X

The effect of dialysis and of preincubation at 37° for 30 min. on the incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts of Ehrlich ascites tumour cells.

Type of extract	RNA specific activity (counts/min./μmoles RNA-P)
normal	33,600
dialysed	32,100
preincubated	7,600

The reaction mixtures were 0.1 M with respect to tris buffer at pH 7.9 and contained 5 μmoles ATP/ml., 5 μmoles $MgCl_2$/ml., 5 μmoles KCl/ml., 1 umole NADP/ml., 1 μmole $NADH_2$/ml., 1 μmole 2-mercaptoethanol/ml. and 3 μC ^{3}H-uridine/ml.

Incubation time : 2 hours.

Table XI

The incorporation of ^{3}H-uridine into RNA by cytoplasmic extracts prepared from immature rabbit tissues.

Cytoplasmic extract	counts/min. incorporated/mg. protein
thymus	14,000
spleen	13,000
brain	10,800
intestine	8,500
testes	7,800
bone marrow	6,500
appendix	5,200
liver	4,200
kidney	4,000
muscle	1,600

The reaction mixtures were 0.1M with respect to tris buffer at pH 7.9 and contained 5 µmoles ATP/ml., 5 µmoles $MgCl_2$/ml., 5 µmoles KCl/ml., 1 µmole NAD, 5 µmoles glucose/ml., 1 µmole 2-mercaptoethanol/ml. and 3 µC ^{3}H-uridine/ml. Incubation time : 2 hours.

Table XII

The localization of ^{3}H in alkaline hydrolysates of RNA from cytoplasmic extracts of various rabbit tissues after incorporation of ^{3}H-uridine.

Cytoplasmic extract	Per cent total RNA radioactivity			
	Uridine	Cytidine	UMP-3' (or 2')	CMP-3' (or 2')
thymus	13	9	67	11
spleen	5	4	68	23
brain	27	23	27	23
intestine	9	2	81	8
testes	5	5	77	13
bone marrow	8	14	63	15
appendix	12	6	72	10
liver	6	3	82	9
kidney	11	7	79	3
muscle	54	10	25	11

Incubation conditions same as for Table XI.

Table XIII

Synthesis of polynucleotide by cytoplasmic extracts of Ehrlich ascites carcinoma cells.

Additions	RNA/reaction vessel (µg.)
nil (control)	30.6
ADP, GDP, UDP and CDP	34.3
ATP, GTP, UTP and CTP	38.9

The reaction mixtures were 0.1M with respect to tris buffer at pH 7.9 and contained 5 µmoles $MgCl_2$/ml., 1 µmole $NADH_2$/ml., 1 µmole NADP/ml., 1 µmole 2-mercaptoethanol/ml. and the di- and triphosphates of adenosine, guanosine, uridine and cytidine where indicated 1 µmole/ml. Incubation time : 4 hours.

presence of magnesium ions, NADP, $NADH_2$, ATP, GTP, UTP and CTP about 20% more RNA was recovered than in controls from which ATP, GTP, UTP and CTP were omitted. In similar experiments in which the four ribonucleoside triphosphates were replaced by the four ribonucleoside 5'-diphosphates, ADP, GDP, UDP and CDP, a much smaller difference was observed between test and control tubes.

3.4. Incorporation of UTP into RNA by enzyme fractions prepared from cytoplasmic extracts of ascites carcinoma cells.

When cytoplasmic extracts of ascites carcinoma cells were fractionated with ammonium sulphate (2.6a) and the fractions subsequently assayed for ability to incorporate ^{32}P-UTP into RNA, two main active fractions were obtained (Fig.15). However neither these fractions, nor any other ammonium sulphate fractions, were capable of incorporating ^{32}P-UDP.

Fig.16 shows the pH activity of these fractions, A and B, from which it is clear that the optimum pH for the incorporation of ^{32}P-UTP by Fraction A was in the region of 9.5 while that for Fraction B was much lower, around pH 7.7.

In the case of either fraction, the uptake of labelled UTP could represent the incorporation of all four ribonucleoside moieties (adenosine, guanosine, cytidine and uridine) forming a new section of polyribonucleotide or merely, the terminal addition of uridylate residues to an existing

Figure 15. The incorporation of ^{32}P-UTP into RNA by fractions prepared from cytoplasmic extracts of Ehrlich ascites cells by ammonium sulphate fractionation.

The assay mixtures, of total volume 0.25ml., contained 0.5μmole $MgCl_2$, 0.25μmole NAD, 50μg. ascites cell RNA, 0.1 μmoles ^{32}P-UTP, approximately 750μg protein and were 0.1M with respect to Tris buffer at pH 7.9. All tubes were incubated at 37° for 10 minutes.

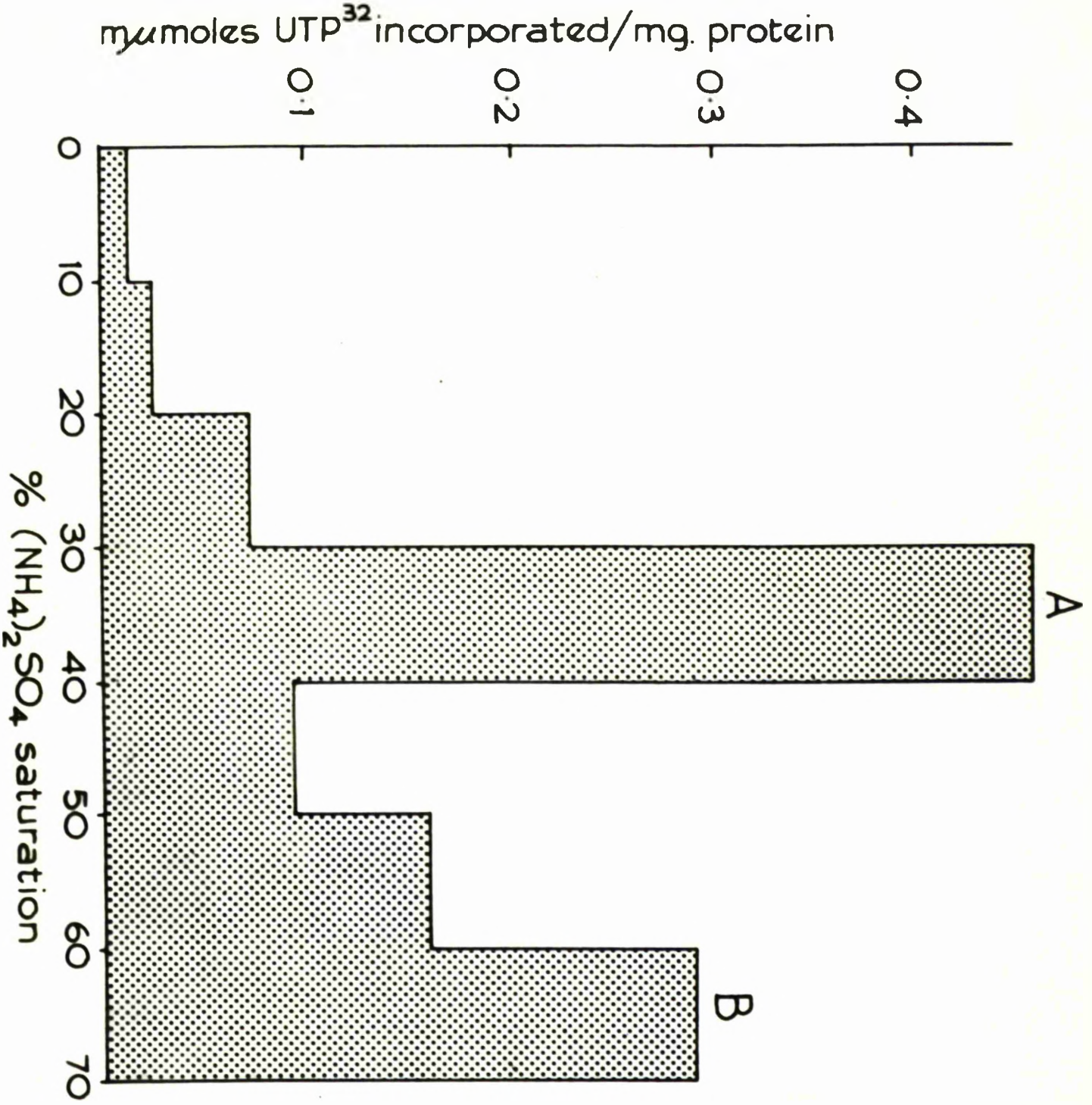

mμmoles UTP32 incorporated/mg. protein
0·1
0·2
0·3
0·4
0
10
20
30
40
50
60
70
% (NH4)2SO4 saturation
A
B

Figure 15

Figure 16. The effect of variation in pH on the incorporation of ^{32}P-UTP into polyribonucleotides by cytoplasmic enzyme fractions from Ehrlich ascites cells.

In this experiment both enzyme fractions were dissolved in ice-cold distilled water and dialysed against distilled water for 18 hr. The assay medium for both fractions was 0.1M with respect to Tris buffer and contained 0.5 µmole $MgCl_2$, 0.25 µmole NAD, 50 µg whole ascites cell RNA, 100 mµmoles ^{32}P-UTP and 750 µg protein. In each case, the total reaction volume was 0.25 ml. and incubation was carried out at 37° for 10 min.

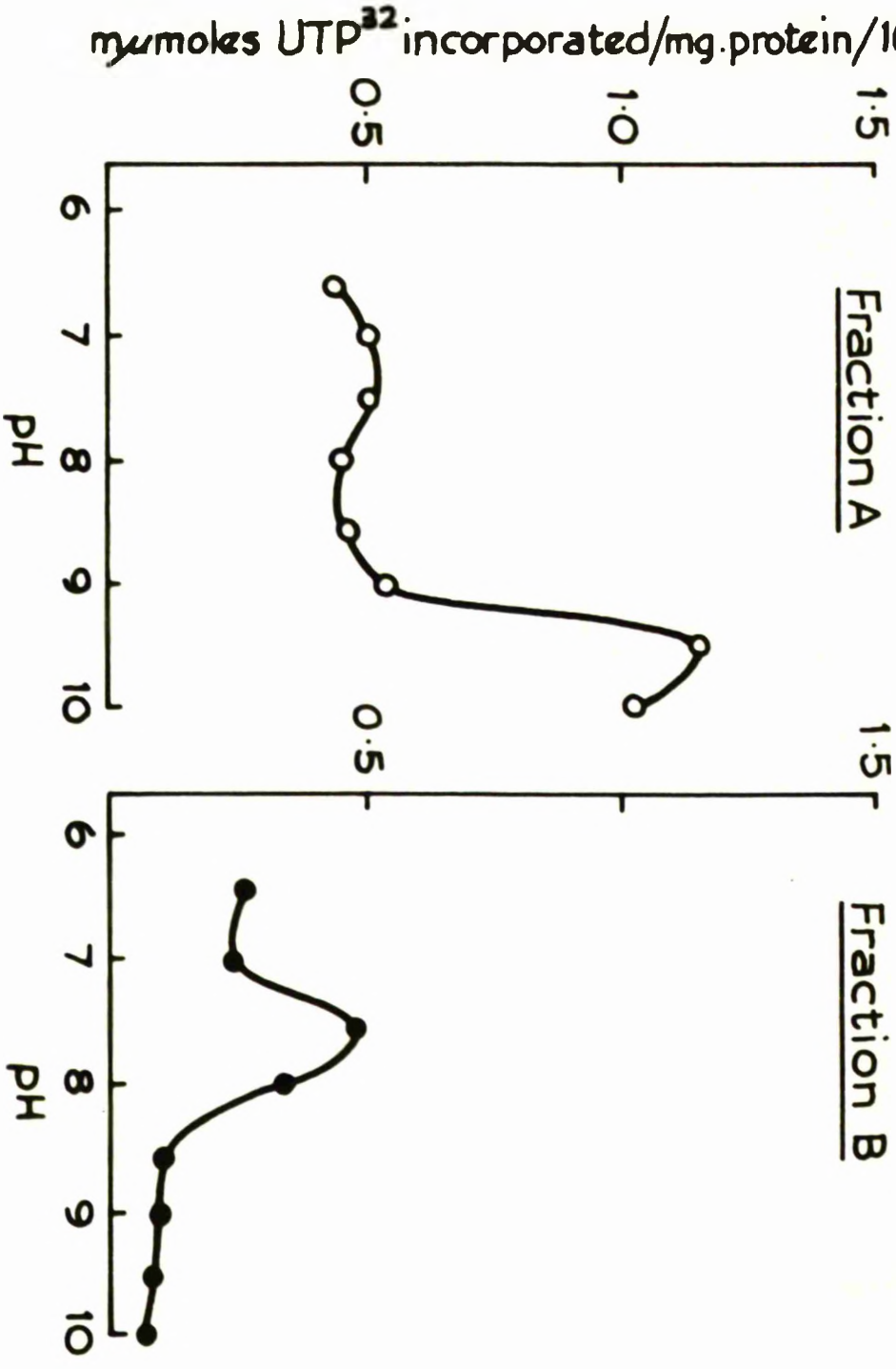

mμmoles UTP32 incorporated/mg.protein/10 min.
0·5
1·0
1·5
6
7
8
9
10
pH
Fraction A
Fraction B

Figure 16

polyribonucleotide chain. As shown in Table XIV, supplementation of the reaction mixture with small quantities of ATP, GTP and CTP stimulated the incorporation of UTP by Fraction A but not by Fraction B while the addition of ribonucleoside 5'-diphosphates (ADP, GDP and CDP) had no effect on UTP incorporation by either fraction. In subsequent experiments a mixture of ATP, GTP and CTP was added in the assay of Fraction A, but not in the assay of Fraction B. These preliminary experiments suggested that the incorporation of UTP catalysed by Fraction A might represent true synthesis of polyribonucleotide, while that catalysed by Fraction B might only indicate the terminal addition of uridylic acid residues.

The mechanism of incorporation of UTP into polyribonucleotide by these fractions probably proceeds by the attachment of UMP to the nucleoside end of an existing polyribonucleotide chain with the elimination of inorganic pyrophosphate. The addition of inorganic pyrophosphate to the incubation mixture would therefore be expected to depress UTP incorporation. Similarly the addition of inorganic orthophosphate would be likely to depress any reaction involving the addition of UDP to the end of a polyribonucleotide chain with the elimination of inorganic orthophosphate. The effects of inorganic ortho- and pyrophosphate on the incorporation of UTP by Fractions A and B are shown in Table XV. While pyrophosphate strongly inhibited UTP utilisation by both

Table XIV

The effect of ribonucleoside 5'-triphosphates on the incorporation of ^{32}P-UTP into polyribonucleotide by cytoplasmic enzyme fractions from Ehrlich ascites cells.

Fraction	Additions	^{32}P-UTP incorporated (mμmoles/mg.protein/10 min.)
Fraction A	Nil	0.64
	ATP GTP CTP	1.17
Fraction B	Nil	0.25
	ATP GTP CTP	0.19

For the assay of Fraction A, the reaction medium in a total vol. of 0.25 ml. was 0.1M with respect to Tris buffer pH 9.5 and contained 0.25 μmole $NADH_2$, 0.5μmole $MgCl_2$, 50 μg whole ascites cell RNA, 100 mμmoles ^{32}P-UTP and 1250 μg protein. For Fraction B, the medium also in a total vol. of 0.25 ml was 0.1M with respect to Tris buffer pH 7.7 and contained 0.25 μmole NAD, 0.5 μmole $MgCl_2$, 50 μg whole ascites cell RNA, 100 mμmoles ^{32}P-UTP and 1800 μg protein. Where indicated 0.25 μmole each of ATP, GTP and CTP were added. The tubes were all incubated at 37° for 10 min.

Table XV

The effect of inorganic ortho- and pyrophosphate on the incorporation of ^{32}P-UTP into polyribonucleotides by cytoplasmic enzyme fractions from ascites cells.

Fraction	Additions	^{32}P-UTP incorporated. (mμmoles/mg. protein/10 min.)
Fraction A	Nil	0.64
	orthophosphate	0.51
	pyrophosphate	0.06
Fraction B	Nil	0.25
	orthophosphate	0.23
	pyrophosphate	0.11

Assay conditions for fraction A and fraction B as in Table XIV. Where indicated 5 μmoles of inorganic ortho- or pyrophosphate were added.

fractions, orthophosphate had little effect on either.

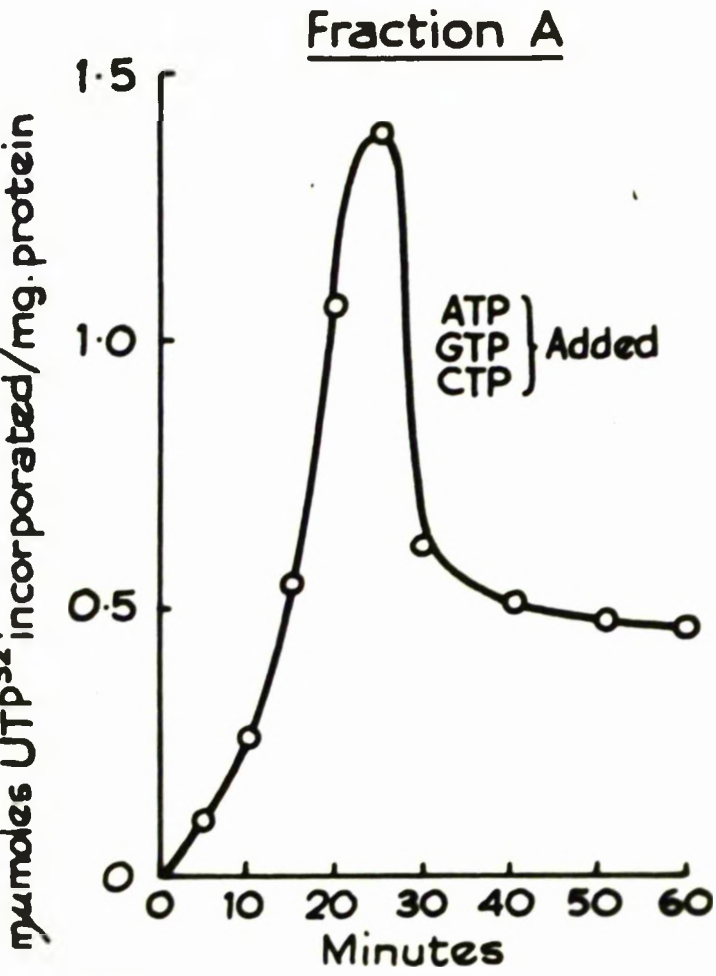
Fig.17 shows the time course of the incorporation of UTP by Fractions A and B and it is clear that maximal incorporation occurred in about 25 minutes for Fraction A and in about 20 minutes for Fraction B. These times proved to be rather variable, the older the enzyme preparation the shorter the optimal time of incorporation. The extent of the reaction was also reduced with older enzyme fractions. The sharp fall in the amount of uridine nucleotide incorporated after 25 minutes by Fraction A was at first puzzling; however this fraction showed marked ribonuclease activity (which declined on increase of pH of incubation medium) and contained phosphatases which attack UTP with the formation of UDP and UMP. The explanation of the peak seems to be that uridine nucleotides are incorporated until the supply of UTP is exhausted (by the synthetic reaction, or by phosphatase action) and thereafter the attack of ribonuclease on the polynucleotide results in the breakdown of the newly formed polynucleotide.

The effect of varying magnesium ion concentration on the two fractions is shownin Fig.18. The optimum concentration for Fraction A was about 3μmoles per ml. while that for Fraction B was rather lower.

Since previous experiments on the incorporation of ^{3}H-uridine into RNA by crude cytoplasmic extracts of ascites

Figure 17. The time course of ^{32}P-UTP incorporation into polyribonucleotide by cytoplasmic enzyme fractions of Ehrlich ascites cells.

For the assay of Fraction A, the incubation medium was 0.1M with respect to Tris buffer pH 9.5 and contained 0.25 µmole each of $NADH_2$, ATP, GTP and CTP, 9.5 µmole $MgCl_2$, 50 µg ascites cell RNA, 100 mµmoles ^{32}P-UTP and 790 µg enzyme in a total volume of 0.25 ml. For Fraction B, the assay medium was 0.1M with respect to Tris buffer pH 7.7 and contained 0.25 µmoles $MgCl_2$, 50 µg. ascites cell RNA, 100 mµmoles ^{32}P-UTP and 520 µg. protein in a total volume of 0.25 ml. All incubations were carried out at 37°.


Fraction A
1·5
1·0
0·5
0
μmoles UTP32 incorporated/mg. protein
ATP
GTP
CTP
Added
0
10
20
30
40
50
60
Minutes

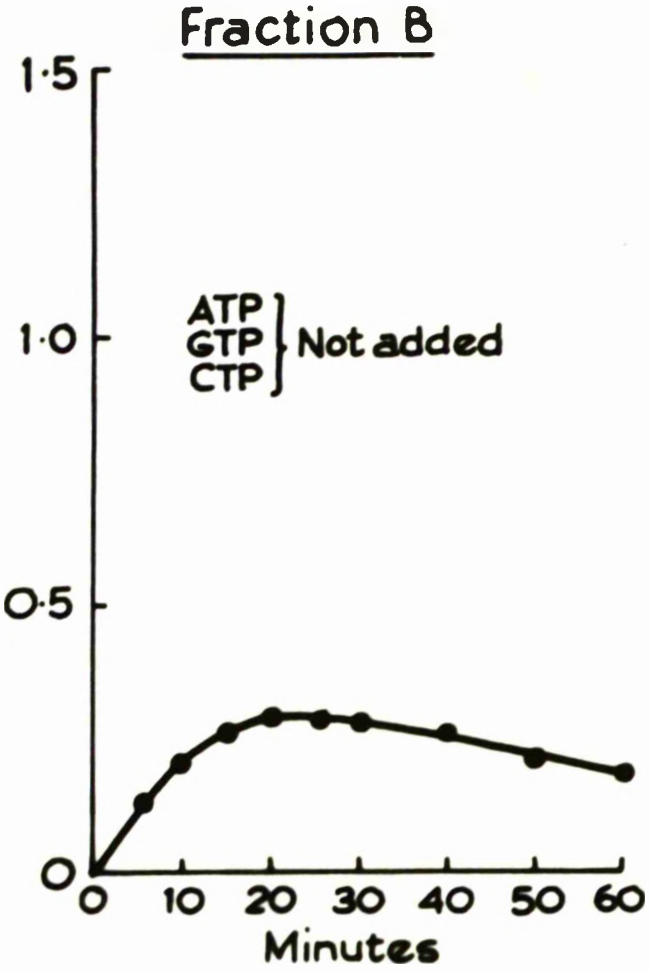

Fraction B
1·5
1·0
0·5
0
ATP
GTP
CTP
Not added
0
10
20
30
40
50
60
Minutes

Figure 17

Figure 18. The effect of variation in Mg^{++} ion concentration on the incorporation of ^{32}P-UTP into polyribonucleotides by cytoplasmic enzyme fractions of Ehrlich ascites cells.

Fraction A was assayed in a medium which was 0.1M with respect to Tris buffer pH 9.6 and contained 0.25µmole each of $NADH_2$, ATP, GTP and CTP, 50 µg ascites cell RNA, 100 mµmoles ^{32}P-UTP and 520 µg enzyme in a volume of 0.25 ml.

Fraction B was assayed in a medium 0.1M with respect to Tris buffer pH 7.7 and contained 0.25µmole NAD, 50µg ascites cell RNA, 100 mµmoles ^{32}P-UTP and 380 µg protein in a volume of 0.25 ml. Incubations were carried out at 37° for 10 minutes.

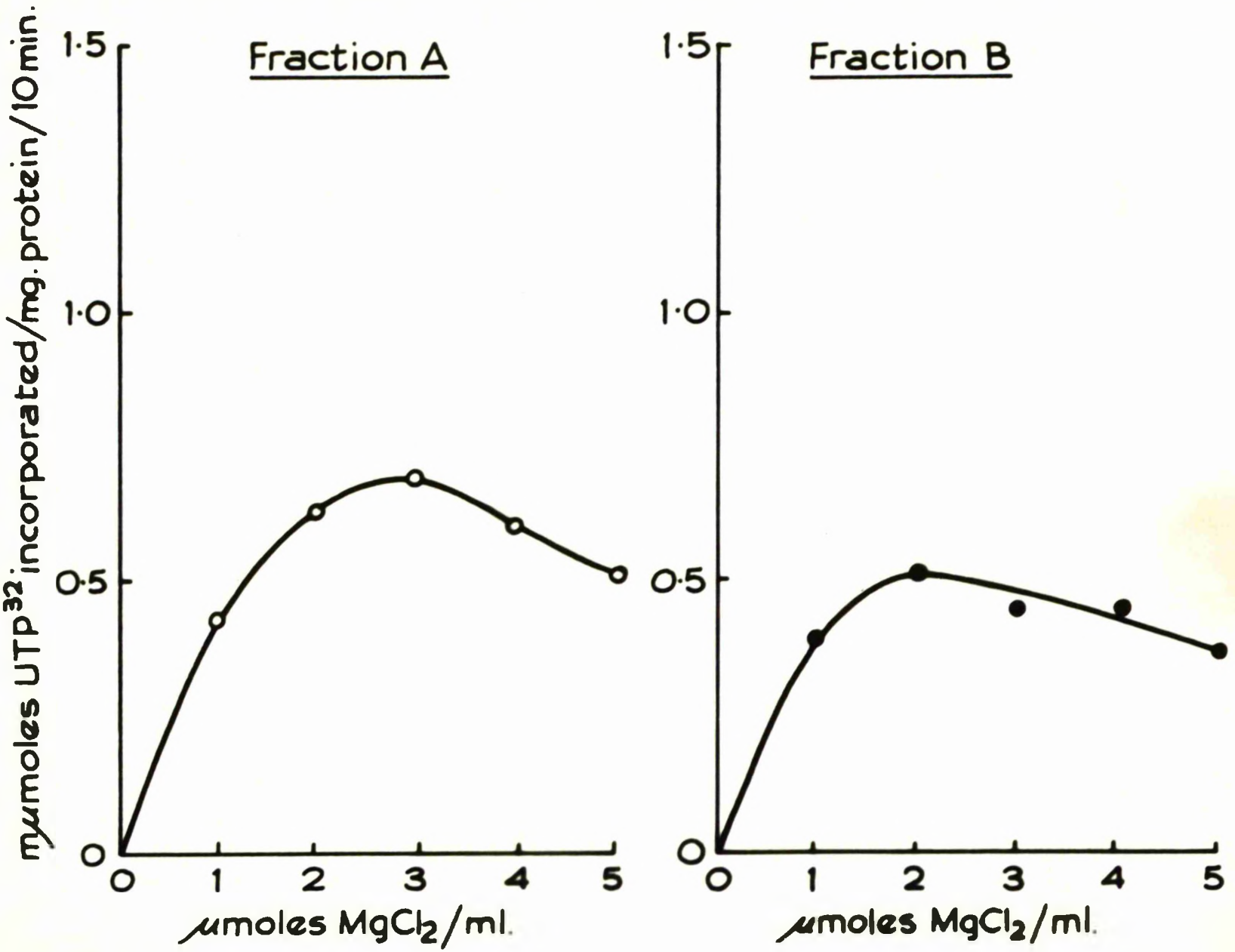

Fraction A
Fraction B
1·5
1·0
0·5
0
0
1
2
3
4
5
μmoles MgCl2/ml.
μmoles UTP32 incorporated/mg. protein/10 min.

Figure 18

carcinoma cells had shown a requirement for $NADH_2$ (see 3.1), the effects of NAD and $NADH_2$ on these fractions were tested. It was found that Fraction A was stimulated about fourfold by $NADH_2$ at 1µmole per ml., while Fraction B was stimulated twofold by NAD at 1µmole per ml. Both fractions contain small amounts of RNA (1-3% of protein concentration), but the addition of RNA from whole ascites cells was found to enhance the incorporation of UTP by both fractions (Fig.19), presumably by acting as a primer.

In order to determine the intramolecular location of the uridine nucleotides incorporated into the RNA, the distribution of ^{32}P amongst the ribonucleoside-3' (or 2') monophosphates obtained on alkaline hydrolysis of the polyribonucleotides, labelled in such experiments, was determined. When UTP labelled in the α-phosphorus with ^{32}P is incorporated into a polyribonucleotide, the ^{32}P-UMP residue may appear adjacent to other ^{32}P-UMP residues as a polyuridylic acid extension (Fig.20a), or may be randomly distributed in the polyribonucleotide (Fig.20b). The effect of alkaline hydrolysis of these hypothetical polynucleotides is indicated by the broken lines. If UTP has been incorporated as a polyuridylic acid extension to an existing chain (Fig. 20a) the radioactivity of the ribonucleotides obtained on alkaline hydrolysis will be predominantly in UMP-3' (or 2'). If, however, the UTP has been incorporated randomly (Fig.20b),

Figure 19. The effect of adding ascites cell RNA on the incorporation of ^{32}P-UTP into polyribonucleotide by cytoplasmic enzyme fractions from Ehrlich ascites cells.

Fraction A was assayed in a medium 0.1M with respect to Tris buffer pH 9.6 containing 0.25 µmole each of $NADH_2$, ATP, GTP and CTP, 0.5 µmole $MgCl_2$, 100 mµmoles ^{32}P-UTP and 270µg fraction A in a volume of 0.25 ml. Fraction B was assayed in a medium 0.1M with respect to Tris buffer pH 7.7. containing 0.25 µmole each of NAD and $MgCl_2$, and 100 mµmoles ^{32}P-UTP and 160 µg enzyme in a total volume of 0.25 ml. Where indicated 50 µg ascites cell RNA were added. Incubation was carried out at 37°.

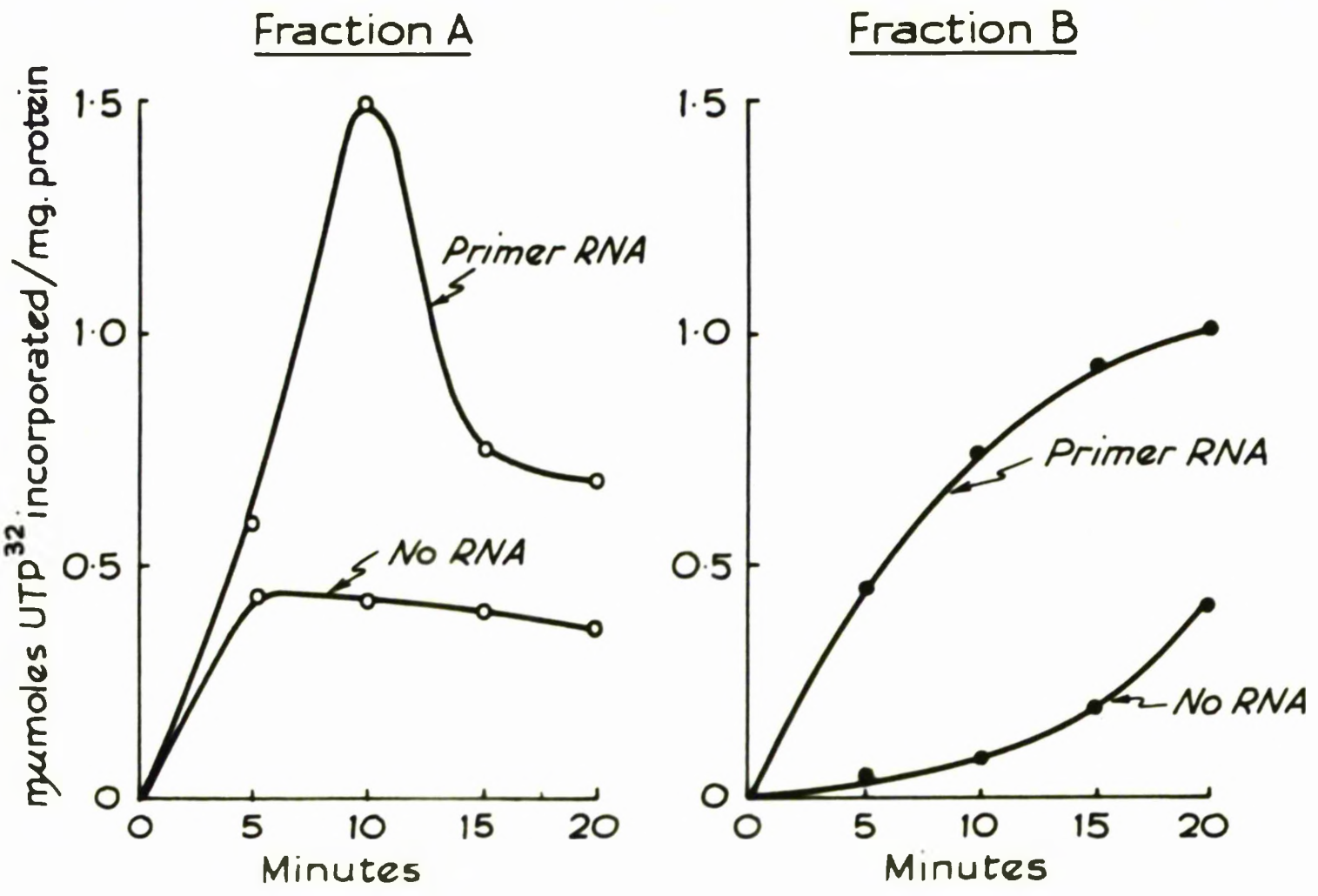

Fraction A
Fraction B
mμmoles UTP32 incorporated/mg. protein
1·5
1·0
0·5
0
Primer RNA
No RNA
0
5
10
15
20
Minutes

Figure 19

Figure 20. Possible locations of ^{32}P in polyribonucleotides following the incorporation of ^{32}P-UTP. U, C, A and G represent uracil, cytosine, adenine and guanine respectively. The vertical lines represent ribose moieties and the oblique lines, 3'-5' phosphodiester linkages.

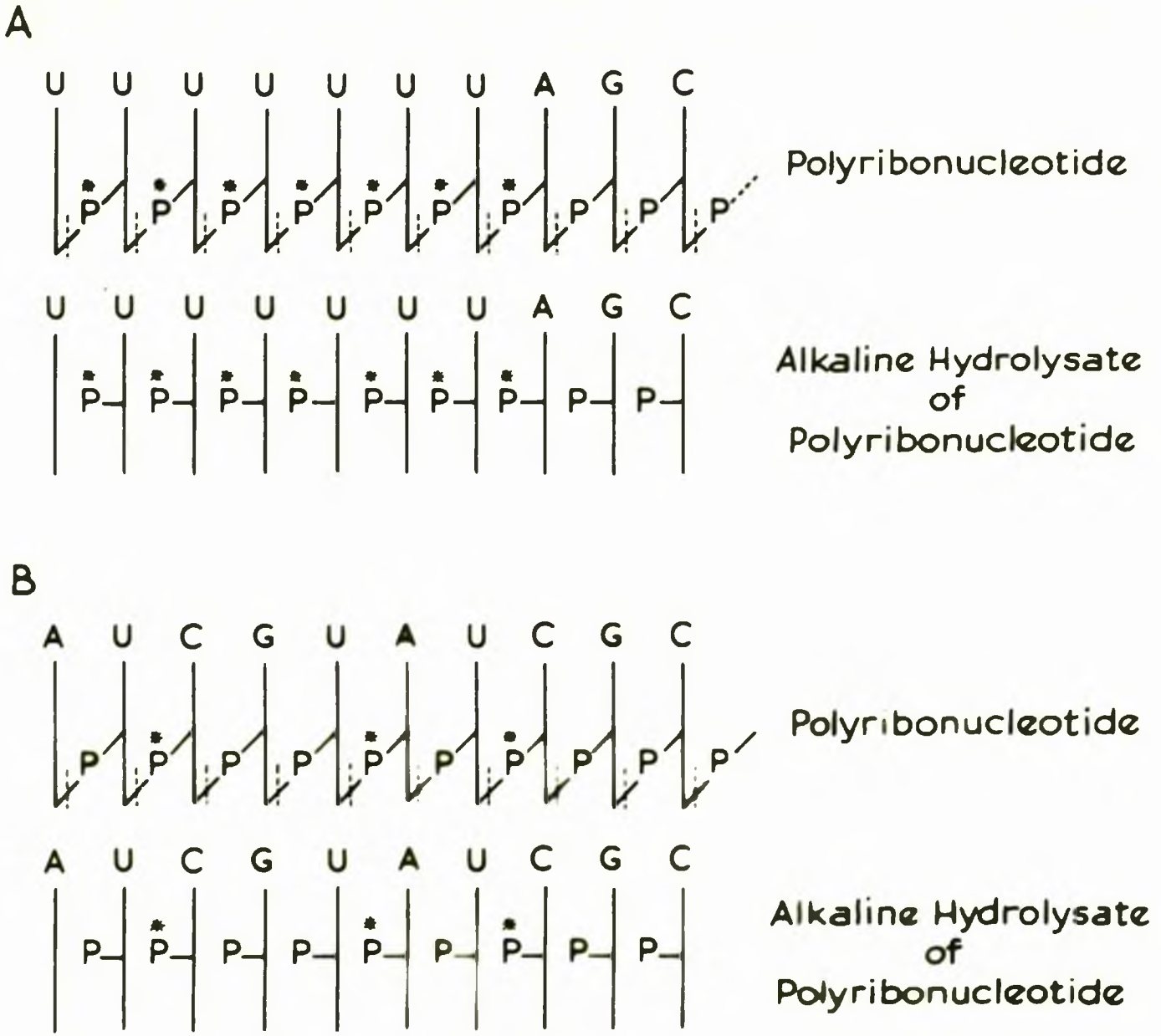

A
U U U U U U U A G C
Polyribonucleotide
U U U U U U U A G C
Alkaline Hydrolysate of Polyribonucleotide
B
A U C G U A U C G C
Polyribonucleotide
A U C G U A U C G C
Alkaline Hydrolysate of Polyribonucleotide

Figure 20

the radioactivity will be distributed amongst all four ribonucleotides in the alkaline hydrolysate. Table XVI shows the distribution of radioactivity in the nucleotides obtained from alkaline hydrolysates of RNA labelled in experiments with Fraction A in the presence and absence of ATP, GTP and CTP, and with Fraction B in the absence of ATP, GTP and CTP. With Fraction A in the absence of the other three triphosphates, the bulk of the radioactivity was recovered in UMP-3' (or 2') indicating that most of the incorporated uridine residues in the polynucleotide are adjacent to one another. About 20% of the activity is associated with CMP-3' (or 2') showing that about a fifth of the uridine residues are adjacent to cytidine nucleotides. When ATP, GTP and CTP were added to the incubation mixture along with the ^{32}P-UTP, however, there was a marked shift in the distribution of radioactivity amongst the nucleotides of the alkaline hydrolysate; much more activity was recovered in CMP-3'(or 3'), GMP-3' (or 2') and AMP-3' (or 2'), indicating a more random distribution of the uridine residues. With Fraction B on the other hand, most of the recovered radioactivity was present in AMP-3' (or 2') and a smaller proportion was found in the UMP-3' (or 2'). No activity was associated with CMP or GMP. Thus in this instance 60% of the incorporated UMP was adjacent to AMP residues, 40% was adjacent to UMP and none was adjacent to CMP or GMP.

Table XVI

The distribution of ^{32}P amongst the nucleoside-3' (or 2') monophosphates formed on the alkaline hydrolysis of RNA after the incorporation of ^{32}P-UTP by cytoplasmic enzyme fractions from ascites cells.

Fraction	Additions	Per cent total polynucleotide radioactivity			
		UMP	CMP	AMP	GMP
Fraction A	^{32}P-UTP	73	21	5	1
	^{32}P-UTP, ATP, GTP and CTP	52	26	16	6
Fraction B	^{32}P-UTP	40	0	60	0

9.6 mg. of Fraction A were incubated for 10 min. at 37° in 5 ml. reaction medium 0.1M with respect to Tris buffer pH 9.5, which contained 5 µmoles $NADH_2$, 10 µmoles $MgCl_2$ and 800 µg ascites cell RNA: 5.1 mg. Fraction B were incubated for 10 min. at 37° in 5 ml. reaction medium, 0.1M with respect to Tris buffer pH 7.7 which contained 5 µmoles NAD, 5 µmoles $MgCl_2$ and 800 µg ascites cell RNA. Where indicated 5 µmoles each of ^{32}P-UTP, ATP, GTP and CTP were added.

Similar experiments were carried out using ^{14}C-UTP. In this case, uridine nucleotides incorporated at the end of a chain would be released as nucleosides on alkaline hydrolysis while those located in non-terminal positions would be recovered as UMP-3'(or 2') (see 3.1). The results of these experiments (Table XVII) showed that with Fraction A, 80% of the ^{14}C-uridine nucleotide was located in non-terminal positions in the polyribonucleotide chain and only a small proportion was terminal. With Fraction B on the other hand, there was a much higher proportion of terminal uridine. There was also evidence of considerable conversion of uridine to cytidine nucleotides, much of which is incorporated in non-terminal positions.

The possibility has been considered that the mechanism of attachment of uridine nucleotides to polyribonucleotides might be concerned with a role related to amino acid transfer. The effects of adding a mixture of amino acids to the systems were therefore examined. Fig.21 shows that Fraction A was unaffected by such an addition whereas the incorporation of UTP catalysed by Fraction B was greatly enhanced in the presence of amino acids.

About the time of these experiments, an infection developed in the departmental mouse colony which effected the supplies of the Ehrlich ascites tumour. As a result, it was necessary to use a different strain of mice which would

Table XVII

The distribution of ^{14}C in alkaline hydrolysates of RNA after the incorporation of ^{14}C-UTP by cytoplasmic enzyme fractions from ascites cells.

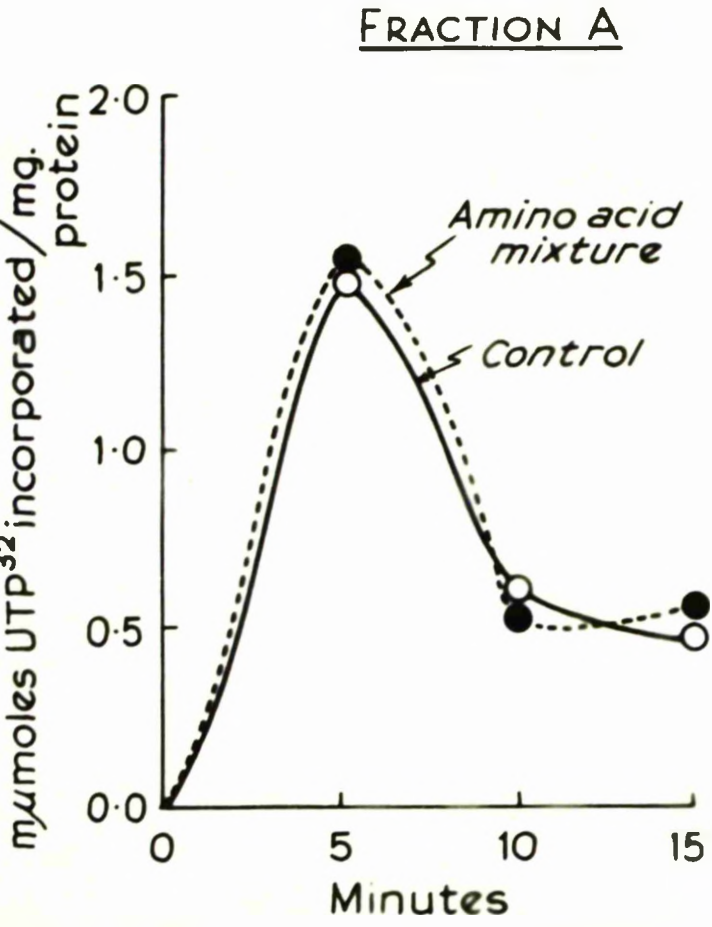
Fraction	Per cent total polyribonucleotide radioactivity.			
	Uridine	Cytidine	UMP-3'(or 2')	CMP-3' (or 2')
Fraction A	4	6	80	10
Fraction B	37	11	26	26

In the experiment with Fraction A, 720 µg ascites cell RNA were incubated with 19.5 mg. fraction A and 0.86 umole ^{14}C-UTP (specific activity 1.9×10^6 counts/min./µmole) at 37° for 10 min. with the following additions: 5 µmoles each of ATP, GTP, CTP and $NADH_2$, 4.14µmoles UTP, 10 µmoles $MgCl_2$ and 500 µmoles Tris buffer pH 9.5 in a total volume of 5 ml.
For Fraction B, 520 µg ascites cell RNA were incubated with 14.1 mg. fraction B and 0.86 µmoles ^{14}C-UTP (specific activity as above) at 37° for 10 min. with the following additions: 5µmoles NAD, 10 µmoles $MgCl_2$, 4.14 µmoles UTP and 500 µmoles Tris buffer pH 7.7 in a total volume of 5 ml.

Figure 21. The effect of an amino acid mixture on the incorporation of ^{32}P-UTP into polyribonucleotide by cytoplasmic enzyme fractions from Ehrlich ascites cells.

The reaction mixture for Fraction A contained 620 µg enzyme protein, 0.25 µmole each of $NADH_2$, ATP, GTP and CTP, 0.5 µmole $MgCl_2$, 50 µg ascites cell RNA, 100 mµmoles ^{32}P-UTP and 25 µmoles Tris buffer pH 9.5 in a total volume of 0.25 ml.

For Fraction B, the reaction mixture contained 390 µg. enzyme protein 0.25 µmole each of NAD and $MgCl_2$, 50 µg ascites cell RNA, 100 mµmoles ^{32}P-UTP and 25 µmoles Tris buffer pH 7.7 in a volume of 0.25 ml. Incubations were carried out at 37°, and where indicated a mixture containing 0.3 µmole each of the following amino acids was added : arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan and valine.


FRACTION A
mμmoles UTP32 incorporated/mg. protein
2·0
1·5
1·0
0·5
0·0
0
5
10
15
Minutes
Amino acid mixture
Control

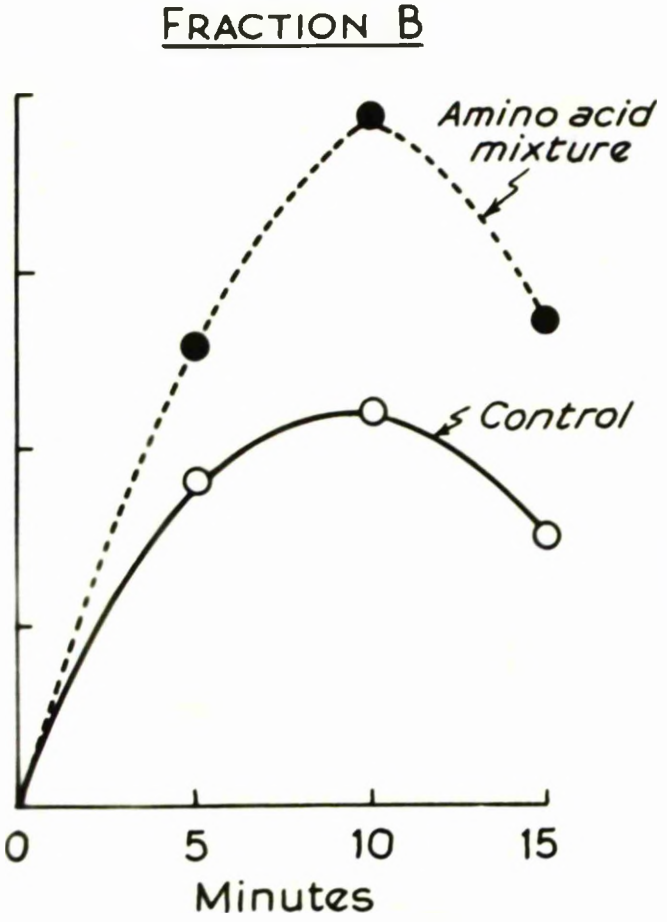


Figure 21

be more resistant to infection. Also it was decided to propagate a different variety of ascites tumour to avoid any further inconvenience due to the possible transmission of any latent virus infection via the tumour cells themselves.

Pilot experiments, similar to those described above, were carried out with fractions prepared from Landschutz ascites cells which are quite similar to the Ehrlich cells. The results of such experiments indicated that the only differences between the systems from these two tumour cell varieties were quantitative rather than qualitative, in as much as the enzymes from the Landschutz ascites tumour always seemed to be a little less active than those from the Ehrlich ascites tumour.

3.5 Incorporation of ^{32}P-UTP into RNA by enzyme fractions prepared from ascites cell nuclei.

As mentioned in a previous section (2.2a), the osmotic disruption of ascites cells does not appear to damage many of the cell nuclei, and the extract prepared by high-speed centrifugation may be regarded as being essentially cytoplasmic in origin. However, in view of the possibility that the enzymes responsible for the incorporation of uridine nucleotides might be of nuclear origin, soluble extracts were prepared from ascites cell nuclei (2.4a). These nuclear extracts were fractionated with ammonium sulphate (2.6a) and each fraction was assayed for ability to incorporate ^{32}P-UTP into RNA. As can be seen in Fig. 22,

Figure 22. The incorporation of ^{32}P-UTP into RNA by fractions prepared from nuclear extracts of Landschutz ascites cells by ammonium sulphate fractionation.

The assay mixtures, of total volume 0.25ml., contained 0.5μmole $MgCl_2$, 0.25μmole $NADH_2$, 50μg. ascites cell RNA, 0.1μmole ^{32}P-UTP, approximately 750μg. protein and were 0.1M with respect to Tris buffer at pH 9.5. All tubes were incubated at 37° for 10 minutes.

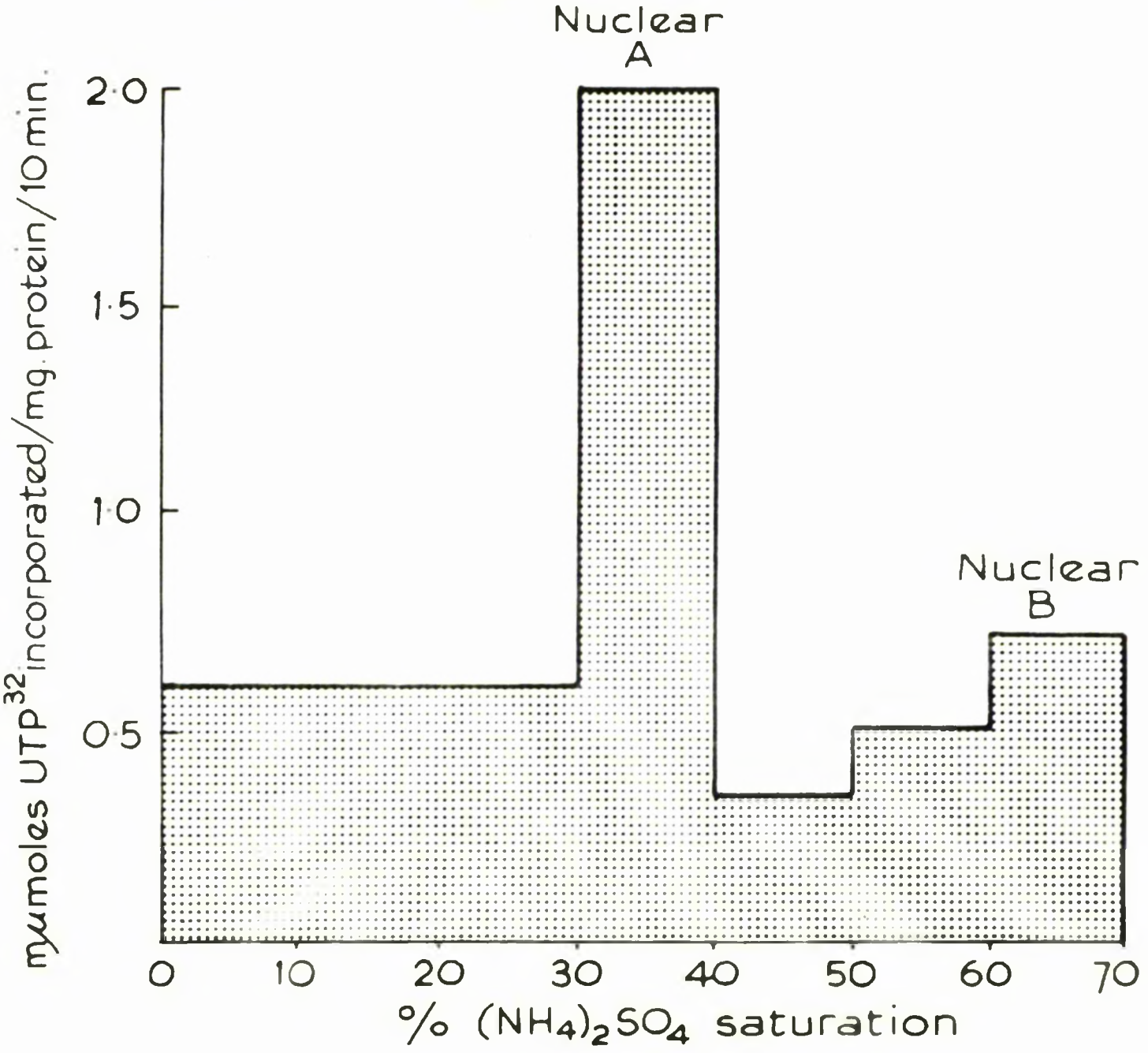

Nuclear
A
Nuclear
B
2·0
1·5
1·0
0·5
0
10
20
30
40
50
60
70
mμmoles UTP^{32} incorporated/mg. protein/10 min.
% $(NH_4)_2SO_4$ saturation

Figure 22

this procedure gave rise to a nuclear Fraction A and a nuclear Fraction B both capable of incorporating ^{32}P-UTP into RNA. These nuclear A and B Fractions were found to have similar properties to the cytoplasmic A and B Fractions: UTP uptake by the nuclear A Fraction was optimal at pH 9.5, stimulated by the addition of a mixture of ATP, GTP and CTP and inhibited by inorganic pyrophosphate, whereas the nuclear Fraction B incorporated UTP best at pH 7.7, in the absence of added ribonucleoside 5'-triphosphates. However, from the results illustrated in Fig.23, it can be seen that the nuclear Fraction A was much more active than the corresponding A Fraction from the cytoplasm, under the same assay conditions. Also under assay conditions appropriate for B Fractions, it was clear that the nuclear Fraction B was slightly less active than the cytoplasmic Fraction B (Fig.23).

In order to determine the extent to which these uridine nucleotide incorporating systems had been solubilised by the process of sonic disruption of the ascites cell nuclei in dilute buffer, the sediment obtained after high-speed centrifugation of a suspension of sonically disrupted ascited cell nuclei (2.4a) was assayed. This sediment was also found to be capable of incorporating ^{32}P-UTP into RNA in the presence of added ATP, GTP and CTP at pH 9.5 but to a much lesser extent than the soluble nuclear fractions. However,

Figure 23. The incorporation of ^{32}P-UTP into polyribonucleotide by nuclear and cytoplasmic fractions of Landschutz ascites cells.

The reaction mixtures for the assay of the crude extracts and A Fractions contained between 300 and 400µg. protein, 0.25µmole each of $NADH_2$, ATP, GTP and CTP, 0.5µmole $MgCl_2$, 50µg. ascites cell RNA, 0.1µmole ^{32}P-UTP and 25µmoles Tris buffer pH 9.5 in a total volume of 0.25ml. For B Fractions, the assay medium contained between 200 and 300µg. protein, 0.25µmole each of NAD and $MgCl_2$, 50µg. ascites cell RNA, 0.1µmole ^{32}P-UTP and 25µmoles Tris buffer pH 7.7 in a total volume of 0.25ml. All incubations were carried out at 37° for 10 minutes.

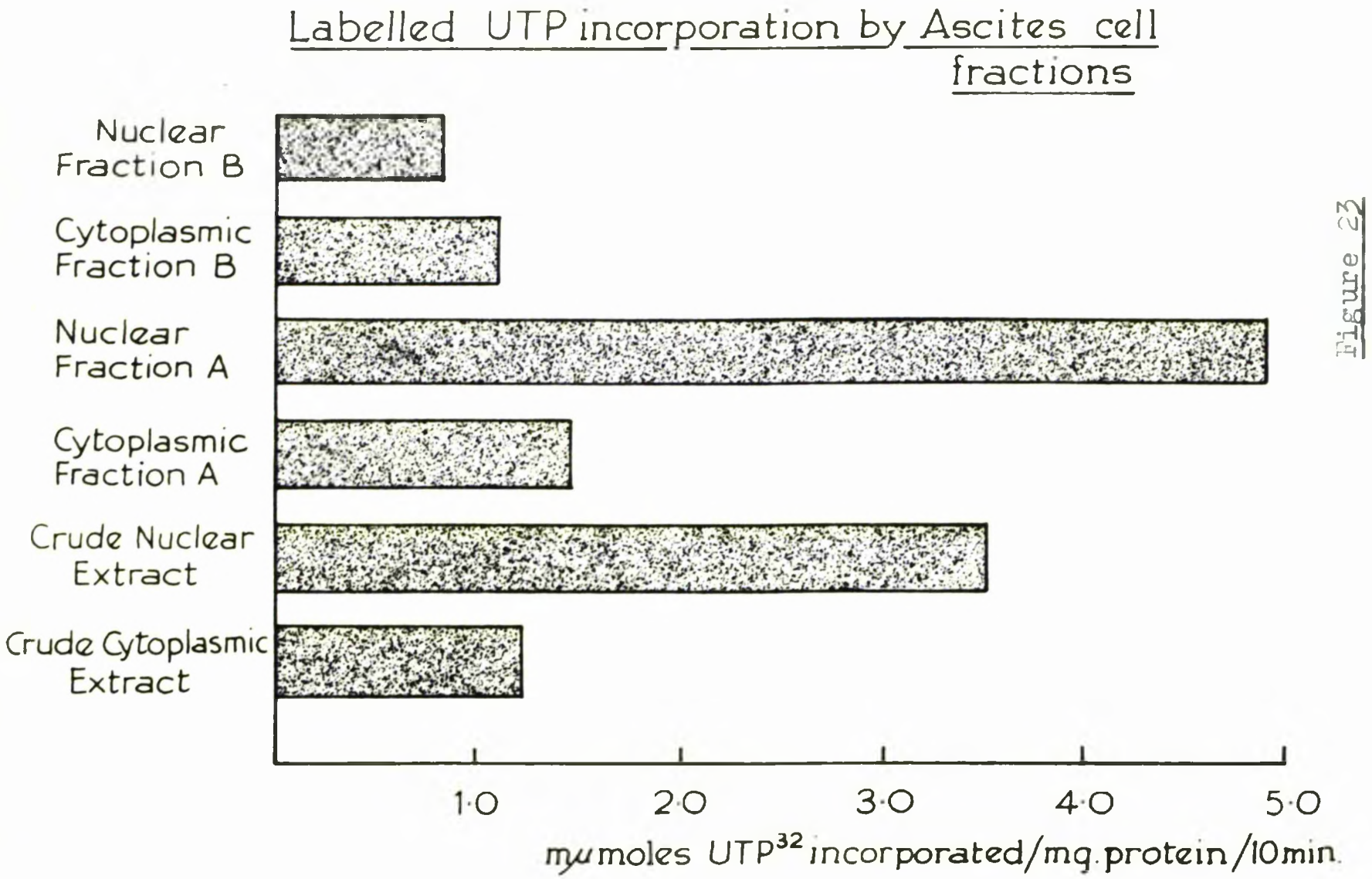

Labelled UTP incorporation by Ascites cell fractions
Nuclear Fraction B
Cytoplasmic Fraction B
Nuclear Fraction A
Cytoplasmic Fraction A
Crude Nuclear Extract
Crude Cytoplasmic Extract
1·0
2·0
3·0
4·0
5·0
mμ moles UTP32 incorporated/mg. protein/10min.

Figure 23

preincubation of the sediment with DNase (see 2.4a), resulted in a loss of approximately 80% of the original UTP incorporating activity. Thus these results, while suggesting that sonic disintegration effectively solubilised most of the enzymes capable of ^{32}P-UTP incorporation into polyribonucleotide material, revealed a possible dependence on DNA for this process. Indeed similar experiments with, and without, DNase carried out on the "aggregate" enzyme from ascites cell nuclei, known to contain appreciable amounts of DNA, substantiated this hypothesis.

3.6 Nucleic acid requirements of UTP incorporating systems.

From the results presented in 3.4, it was evident that both cytoplasmic Fraction A and Fraction B showed a requirement for RNA. However, the precise nature of this requirement for RNA was not clear. Indeed the question of possible DNA involvement, as outlined above (3.5), was another complicating feature. Experiments carried out using cytoplasmic Fraction A showed nuclear RNA, long recognised as a metabolically active RNA (see Introduction), to be a much better primer than RNA derived from the ascites cell cytoplasm. (see Fig.24). The same was true in the case of UTP uptake catalysed by nuclear Fraction A (see Fig.24). Nevertheless DNA also plays a very important role in the uptake of UTP by nuclear Fraction A. From Table XVIII, it is evident that in the case of nuclear Fraction A the addition of heat-denatured

Figure 24. The effect of nuclear and cytoplasmic RNA on ^{32}P-UTP incorporation into polyribonucleotide by nuclear and cytoplasmic A Fractions prepared from Landschutz ascites cells.

The reaction mixtures in a total volume of 0.25ml. contained 0.25μmole each of $MnCl_2$, ATP, GTP and UTP, 0.5μmole $MgCl_2$, 30μg. RNA as indicated, 100mμmoles ^{32}P-UTP, 25μmoles Tris buffer pH 9.5 and 350μg protein. All tubes were incubated at 37° for 10 minutes.

Ribonucleic Acid Requirement

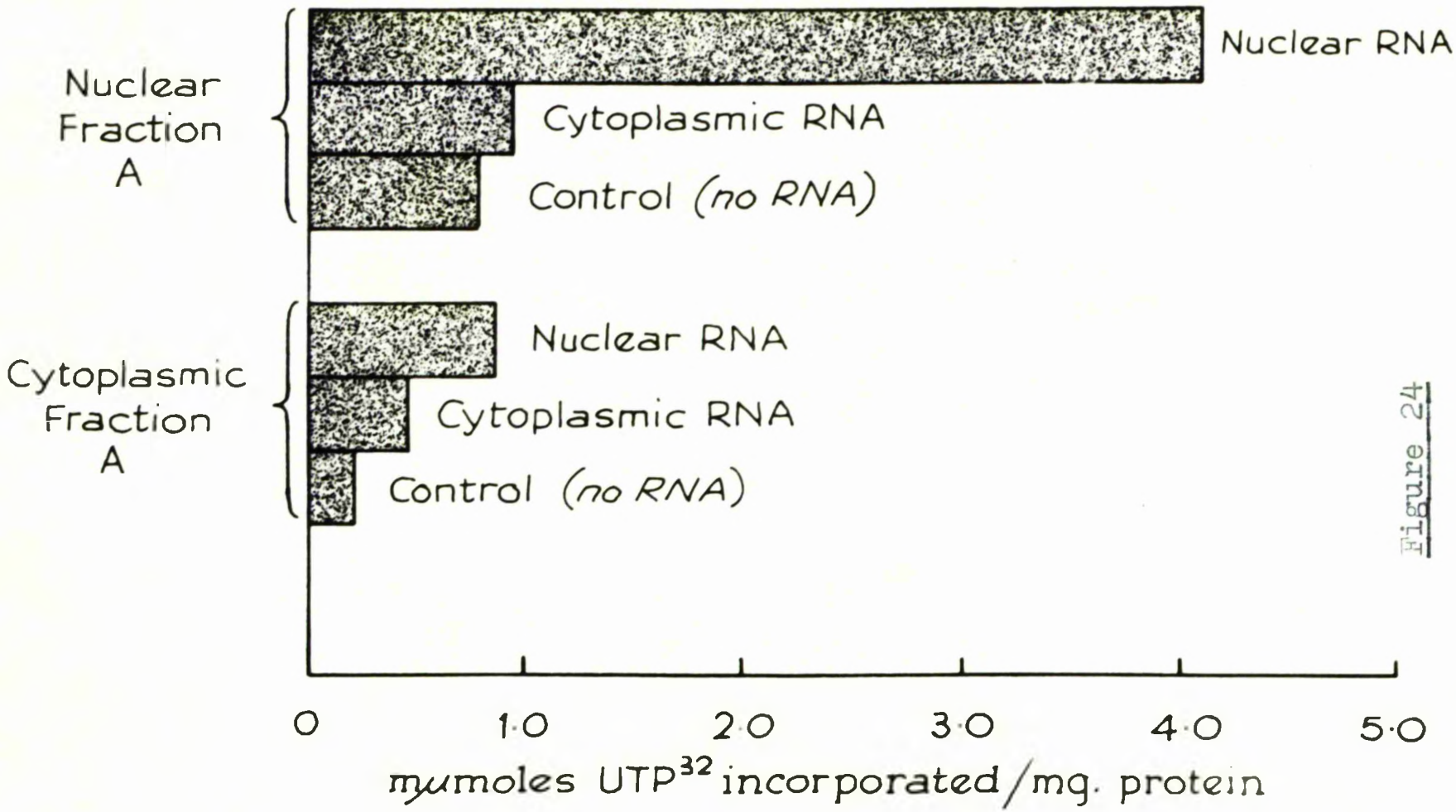


Figure 24

Table XVIII

The effect of DNA on the incorporation of ^{32}P-UTP into polyribonucleotide by nuclear Fraction A prepared from Landschutz ascites carcinoma cells.

Addition	^{32}P-UTP incorporated (mμmoles/mg. protein/10 mins.)
nil (control)	1.01
native DNA	1.15
heat-treated DNA	1.63
DNase treated DNA **	0.63

The assay medium in each case was 1ml. and contained 2μmoles $MgCl_2$, 1μmole each of ^{32}P-UTP, $NADH_2$, ATP, GTP and CTP, 3.4mg. protein and were 0.1M with respect to Tris buffer at pH 9.5. Where indicated 200μg of ascites cell DNA were added. All tubes were incubated at 37° for 10 minutes.

** DNase treatment was carried out as described in Table XIX.

ascites cell DNA to the incubation mixture will cause a marked stimulation to UTP uptake, even in the absence of nuclear RNA. In this respect native DNA or DNase treated DNA proved less effective.

On the other hand, the situation appears to be somewhat different in the case of cytoplasmic Fraction A. With this cytoplasmic fraction, DNA seemed only able to exert an effect on UTP incorporation in the presence of added nuclear RNA (Table XIX). In this connection, it seems that the cytoplasmic system requires the native, double-stranded molecule rather than the single-stranded, heat-denatured molecule.

3.7 Incorporation of ^{32}P-ATP into polyribonucleotide by an enzyme fraction from ascites tumour cell nuclei.

Fig.25 shows the time course (indicated by the continuous line) of ^{32}P-ATP incorporation into polyribonucleotide at pH 9.5 when incubated at 37° with the ascites nuclear Fraction A in the presence of added nuclear RNA, magnesium ions, $NADH_2$, and the three ribonucleoside 5'-triphosphates (GTP, CTP and UTP). ATP uptake appeared to occur, under these conditions, at a fairly linear rate, reaching a maximum level after about 10 minutes. Subsequently the amount of radioactivity in the isolated polyribonucleotide material decreases in a similar manner to that found in the experiments previously described with ^{32}P-UTP. If the incubation was continued for a further 30 minutes, a second period of

Table XIX

The effect of RNA and DNA on the incorporation of ^{32}P-UTP into polyribonucleotide by cytoplasmic Fraction A prepared from Landschutz ascites carcinoma cells.

Additions	^{32}P-UTP incorporated (mμmoles/mg.protein/10mins.)
nil (control)	0.21
nuclear RNA	0.42
native DNA	0.15
heat-treated DNA	0.06
DNase treated DNA **	0.25
nuclear RNA native DNA	0.63
nuclear RNA heat-treated DNA	0.45
nuclear RNA DNase treated DNA **	0.36

The reaction mixtures (total volume 1ml.) contained 2μmoles $MgCl_2$, 1μmole each of ^{32}P-UTP, ATP, GTP, CTP and $NADH_2$, 2.12mg. protein and were 0.1M with respect to Tris buffer at pH 9.5. Where indicated 200μg ascites cell DNA and 160μg ascites cell nuclear RNA were added. All tubes were incubated at 37° for 10 mins.

** 1ml. DNA solution containing 2mg./ml. was treated with 10μg crystalline DNase in the presence of 2μmoles $MgCl_2$ for 4hr. at 37°, the reaction medium being 0.01M with respect to Tris buffer at pH7.5.

Figure 25. The time course of ^{32}P-ATP incorporation into polyribonucleotide at pH 9.5 by nuclear Fraction A from Landschutz ascites cells.

The reaction mixtures, of total volume 0.25ml., contained 0.5μmole $MgCl_2$, 0.25μmole each of $NADH_2$ and ^{32}P-ATP, 50μg. ascites nuclear RNA, 290μg. protein and were 0.1M with respect to Tris buffer at pH 9.5. Where indicated 0.1 umole each of GTP, CTP and UTP were added. Incubation was carried out at 37°.

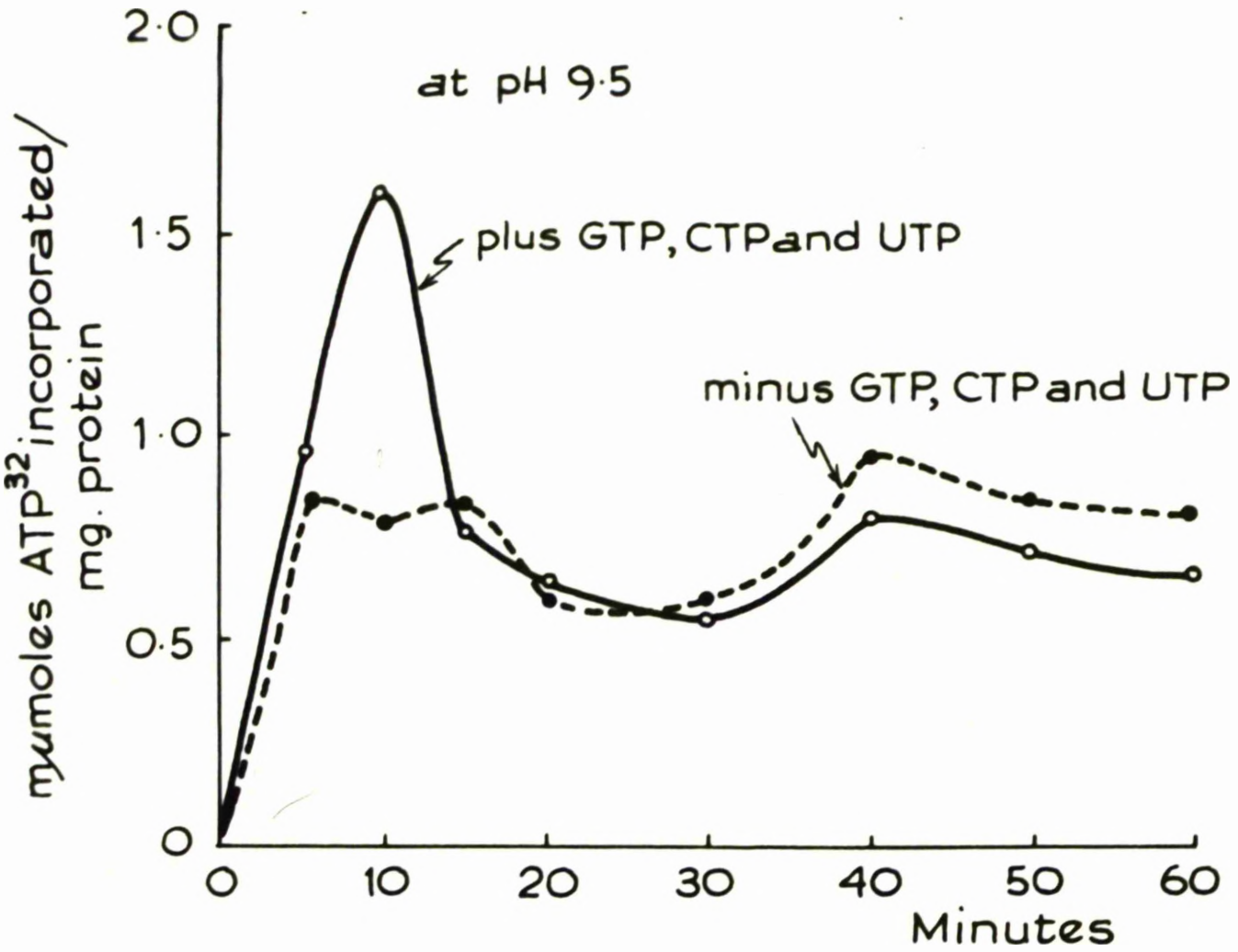

at pH 9·5
plus GTP, CTP and UTP
minus GTP, CTP and UTP
mμmoles ATP32 incorporated/ mg. protein
2·0
1·5
1·0
0·5
0
0
10
20
30
40
50
60
Minutes

Figure 25

increased ATP incorporation occurred. On the other hand, when the time course of ^{32}P-ATP incorporation was followed under the same conditions, but in the absence of GTP, CTP and UTP (see Fig.25, broken line), the first peak of activity was somewhat depressed.

A series of incubation mixtures were then set up to determine the optimum pH values for the incorporation of ATP into polyribonucleotide by nuclear Fraction A after 10 minutes incubation, and after 40 minutes incubation. The results shown in Fig.26B illustrate that when the incubation was carried out for 40 minutes in the absence of GTP, CTP and UTP, pH 8.0 was optimal. However for incubation in the presence of GTP, CTP and UTP for 10 minutes, pH 9.5 was optimal (Fig.26A). The time course of ATP incorporation was then followed at pH 8.0 (Fig.27). In the absence of added GTP, CTP and UTP, the level of ATP incorporation after 40 minutes is greatly enhanced (indicated by broken line). Addition of GTP, CTP and UTP mixtures to the incubation medium is found to depress this activity (indicated by continuous line).

Thus it appears that two systems are operative in nuclear Fraction A. One of these catalyses the initial uptake of ATP into polyribonucleotide (occurring in the first 10 minutes of the incubation), which is stimulated in the presence of the other three ribonucleoside 5'triphosphates

Figure 26. The effect of variation in pH on ^{32}P-ATP incorporation into polyribonucleotide in the presence and absence of a mixture of GTP, CTP and UTP, by nuclear Fraction A prepared from Landschutz ascites cells.

In A, the reaction mixtures (0.25ml.) contained 0.5μmole $MgCl_2$, 0.25μmole each of $NADH_2$ and ^{32}P-ATP, 50μg. ascites nuclear RNA, 305μg protein and were 0.1M with respect to Tris buffer. Where indicated 0.1μmole each of GTP, CTP and UTP were added. Incubation was carried out at 37° for 10 minutes. The situation in B was identical with that in A, except that incubation was carried out at 37° for 40 minutes, rather than 10 minutes.

Figure 26

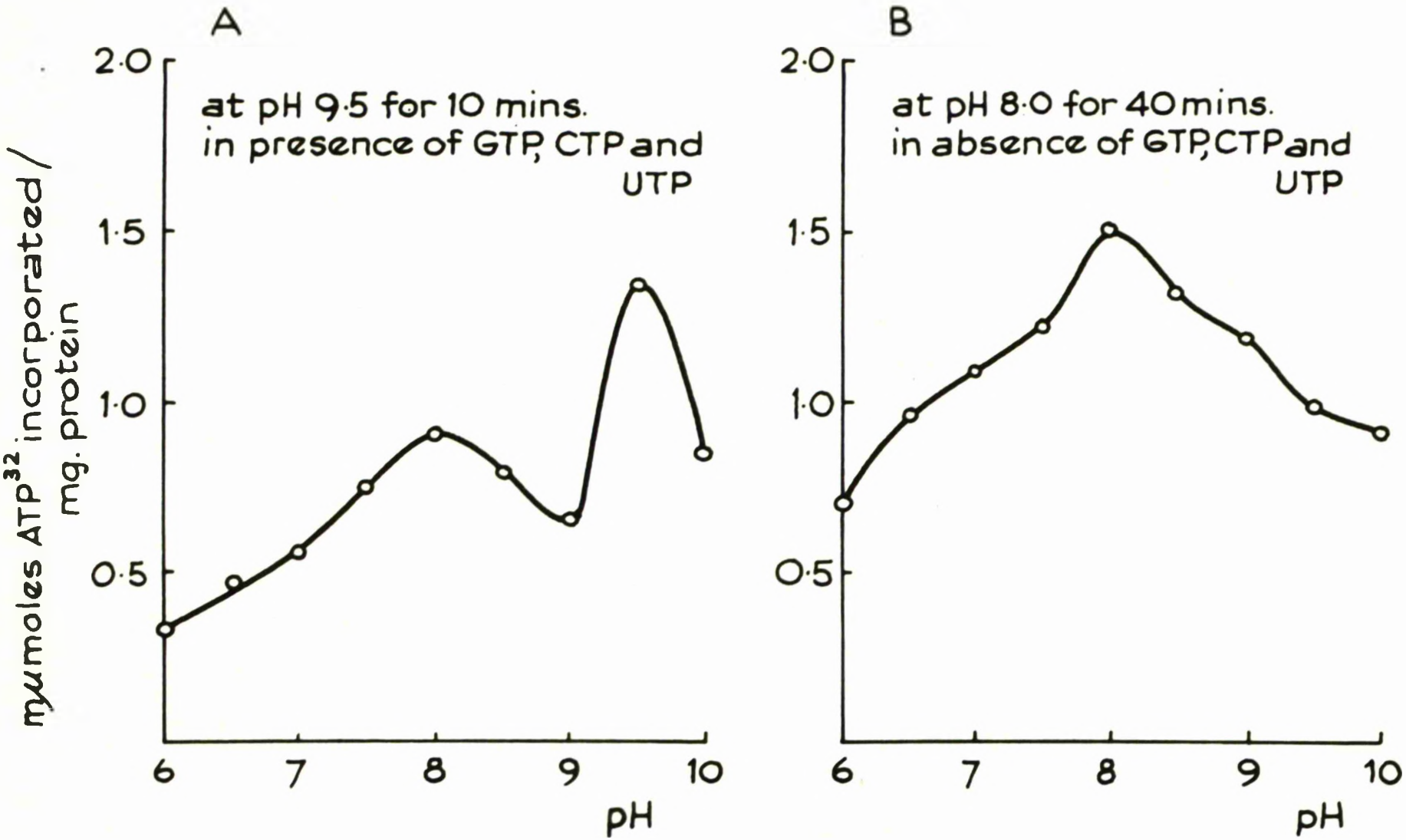

A
B
2·0
1·5
1·0
0·5
at pH 9·5 for 10 mins.
in presence of GTP, CTP and UTP
at pH 8·0 for 40 mins.
in absence of GTP, CTP and UTP
mμmoles ATP32 incorporated / mg. protein
6
7
8
9
10
pH

Figure 27. The time course of ^{32}P-ATP incorporation into polyribonucleotide at pH 8.0 by nuclear Fraction A from Landschutz ascites cells.

The reaction mixtures, of total volume 0.25ml., contained 0.5µmole $MgCl_2$, 0.25 µmole each of $NADH_2$ and ^{32}P-ATP, 50µg. ascites nuclear RNA, 210µg. protein and were 0.1M with respect to Tris buffer at pH 8.0. Where indicated 0.1µmole each of GTP, CTP and UTP were added. Incubation was carried out at 37°.

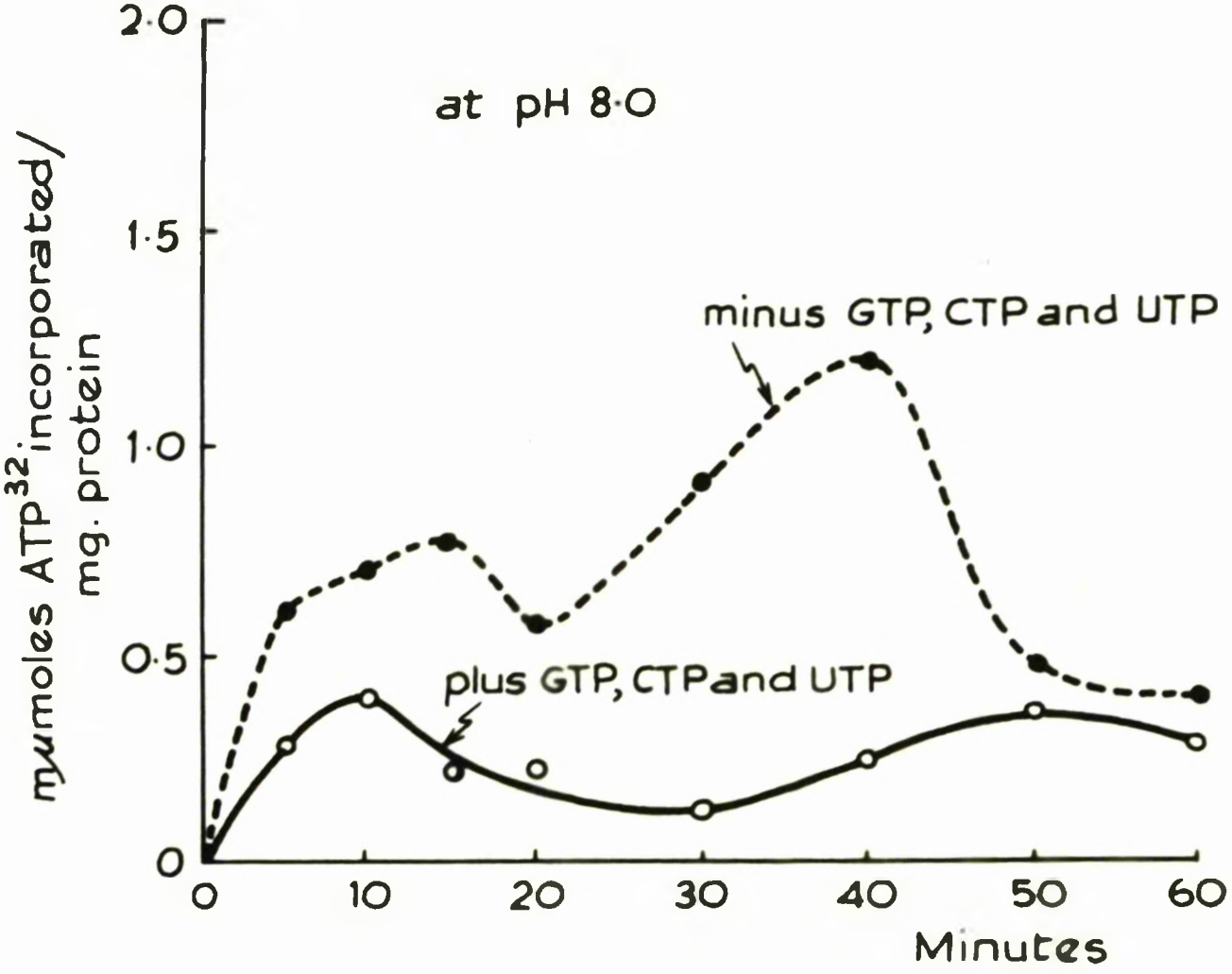

at pH 8·0
minus GTP, CTP and UTP
plus GTP, CTP and UTP
mμmoles ATP32 incorporated/ mg. protein
2·0
1·5
1·0
0·5
0
0
10
20
30
40
50
60
Minutes

Figure 27

(GTP, CTP and UTP) and proceeds best at a pH value in the region of 9.5. The second of these systems, which shows a pH optimum of 8.0 catalyses the uptake of ATP with a peak incorporation after 40 minutes incubation and is markedly inhibited (60%) by the presence of a mixture of GTP, CTP and UTP. Close examination of this inhibitory effect of ribonucleoside 5'-triphosphates on ATP uptake by the second system was carried out. The results in Table XX indicate that it was UTP alone which was inhibitory rather than the mixture of GTP, CTP and UTP.

To determine the intramolecular location of the adenosine nucleotides incorporated into the polyribonucleotides by these systems the distribution of ^{32}P amongst the ribonucleoside 3' (or 2') monophosphates obtained on alkaline hydrolysis of the polyribonucleotides labelled in the above experiments was studied. Table XXI shows the distribution of radioactivity in the nucleotides obtained from the alkaline hydrolysates of polyribonucleotides following experiments with ^{32}P-ATP under the influence of nuclear Fraction A at pH 8.0 in the absence of GTP, CTP and UTP (after 40 minutes incubation), and at pH 9.5 in the presence of GTP, CTP and UTP (after 10 minutes incubation). These results are compared with those obtained using a cytoplasmic Fraction A.

After incubation at pH 8.0 for 40 minutes in the absence

Table XX

The effect of GTP, CTP and UTP on the incorporation of ^{32}P-ATP into polyribonucleotide at pH 8.0, after 40 minutes incubation, catalysed by nuclear Fraction A prepared from Landschutz ascites carcinoma cells.

Additions	^{32}P-ATP incorporated (mμmoles/mg.protein/40mins.)
nil (control)	1.31
UTP	0.41
CTP	1.59
GTP	1.74
UTP+CTP+GTP	0.39

The reaction mixtures, of total volume 1ml., contained 2μmoles $MgCl_2$, 160 μg ascites cell nuclear RNA, 1μmole ^{32}P-ATP, 1μmole $NADH_2$, 3.65mg. protein and were 0.1M with respect to Tris buffer at pH 8.0. Where indicated 1μmole each of UTP, GTP and CTP were added. All tubes were incubated at 37° for 40 minutes.

Table XXI

The distribution of ^{32}P amongst the ribonucleoside 3' (or 2')-monophosphates found on alkaline hydrolysis of polyribonucleotides after the incorporation of ^{32}P-ATP by cytoplasmic and nuclear A Fractions prepared from Landschutz ascites carcinoma cells.

Fraction	pH	Incubation time(min.)	Additions	% of total polyribonucleotide radioactivity			
				AMP	GMP	CMP	UMP
Cytoplasmic A	9.5	10	GTP CTP UTP	13	32	12	43
Nuclear A	9.5	10	GTP CTP UTP	11	23	23	43
Nuclear A	8.0	40	nil	57	16	14	13

In each case 9.8 mg. protein were incubated with 5μmoles ^{32}P-ATP at 37° in 5ml. reaction mixtures containing 5μmoles $NADH_2$, 10μmoles $MgCl_2$, 800μg ascites cell nuclear RNA and 0.1M with respect to Tris buffer. Where indicated 5μmoles each of GTP, CTP and UTP were added.

of GTP, CTP and UTP, the bulk of the radioactivity in the polynucleotide was recovered in AMP-3' (or 2'), indicating that at least 54% of the incorporated adenosine residues in the polynucleotide chain are adjacent to one another. On the other hand after incubation at pH 9.5 in the presence of GTP, CTP and UTP, there was a marked shift in the distribution of radioactivity amongst the nucleotides of the alkaline hydrolysate; much more activity was recovered with the UMP-3' (or 2') and CMP-3' (or 2') fractions suggesting a tendency for the adenosine residues to be incorporated adjacent to UMP and CMP units. Thus these results are further evidence to substantiate the possibility that two separate ATP incorporating systems exist in the nuclear Fraction A, one of which has characteristics similar to the system already described (3.5) in nuclear Fraction A responsible for UTP incorporation.

Although the precise nucleic acid requirement of the second type of ATP incorporating system in the nuclear Fraction A was not determined, a definite requirement for nuclear RNA could be demonstrated.

3.8 Incorporation of ^{32}P-UTP into RNA by mitochondria and microsomes of ascites cells.

Although a detailed study of ^{32}P-UTP incorporation into RNA by microsomal and mitochondrial fractions from ascites cells was not carried out, preliminary experiments indicated that both of these fractions were active in this respect,

the mitochondrial fraction being slightly more active than the microsomal fraction.

3.9 Incorporation of ^{32}P-UTP into RNA by isolated nucleoli and chromosomal material of ascites cells.

Nucleoli and chromosomal material were isolated from sonically disrupted ascites tumour cells by the method of Monty, Litt, Kay and Dounce (1956), and assayed for ability to incorporate ^{32}P-UTP into RNA in the presence of a mixture of ATP, GTP and CTP. The results are indicated in Table XXII from which it is evident that the most active fraction in this respect was the so-called "chromosomal supernatant". This fraction was supposed to contain chromosomes in a very finely divided form (2.3c). Although the isolated ascites cell nucleoli also showed considerable activity, analysis showed them to contain a considerable quantity of DNA (about 25%), which is suggestive of considerable chromosomal contamination. Thus, the results of these experiments, whilst possibly indicative of a trend, should be interpreted with great caution.

3.10 Incorporation of ^{3}H-uridine into nuclear components of intact ascites tumour cells

Intact ascites tumour cells were incubated for 2 minutes in a medium containing ^{3}H-uridine, after which two samples of the cells were taken, one for autoradiography (2.15a), and the other for isolation (at 0°) of the nuclear ribonucleoprotein types I and II (2.5c). These ribonucleoproteins were then

Table XXII

Incorporation of ^{32}P-UTP into RNA by fractions prepared from Landschutz ascites carcinoma cell nuclei.

Nuclear fraction	RNA specific activity (counts/min./μmole RNA-P)
Nucleolar fraction	1,437
"Chromosomal fraction"	798
"Chromosomal supernatant"	1,985

Each reaction mixture, of total volume 1ml., contained 2μmoles $MgCl_2$, 1μmole each of $NADH_2$, ATP, GTP, CTP and ^{32}P-UTP, approximately 5mg protein and were 0.1M with respect to Tris buffer at pH 9.5. All tubes were incubated at 37° for 10 minutes.

washed and extracted using a procedure (2.9a) analagous to that used for the whole cells prior to autoradiography, and the specific activity of their RNA components estimated. As shown in Table XXIII, $nRNA_2$ (derived from ribonucleoprotein type II), which is said to be associated with the cell nucleoli, has the higher activity.

The process was also followed by autoradiographic techniques which showed that during the short 2 minutes exposure of the intact ascites cells to ^{3}H-uridine incorporation takes place mainly in the nucleus (see Fig. 28). Although some nucleoli are labelled, it would be erroneous to suggest that there was no labelling of other nuclear components, such as chromosomes.

3.11 Effect of $nRNA_1$ and $nRNA_2$ on ^{32}P-UTP incorporation into RNA by cytoplasmic fractions of ascites cells.

Time only permitted preliminary experiments to be carried out in an attempt to obtain more precise information on the actual type of nuclear RNA which acted as primer in the incorporation of UTP by cytoplasmic Fraction A. The effect of addition of either $nRNA_1$ or $nRNA_2$ on ^{32}P-UTP incorporation by cytoplasmic Fraction A, in the presence of ATP, GTP and CTP, is shown in Table XXIV. Both types of RNA were found to stimulate the reaction to approximately the same extent.

Table XXIII

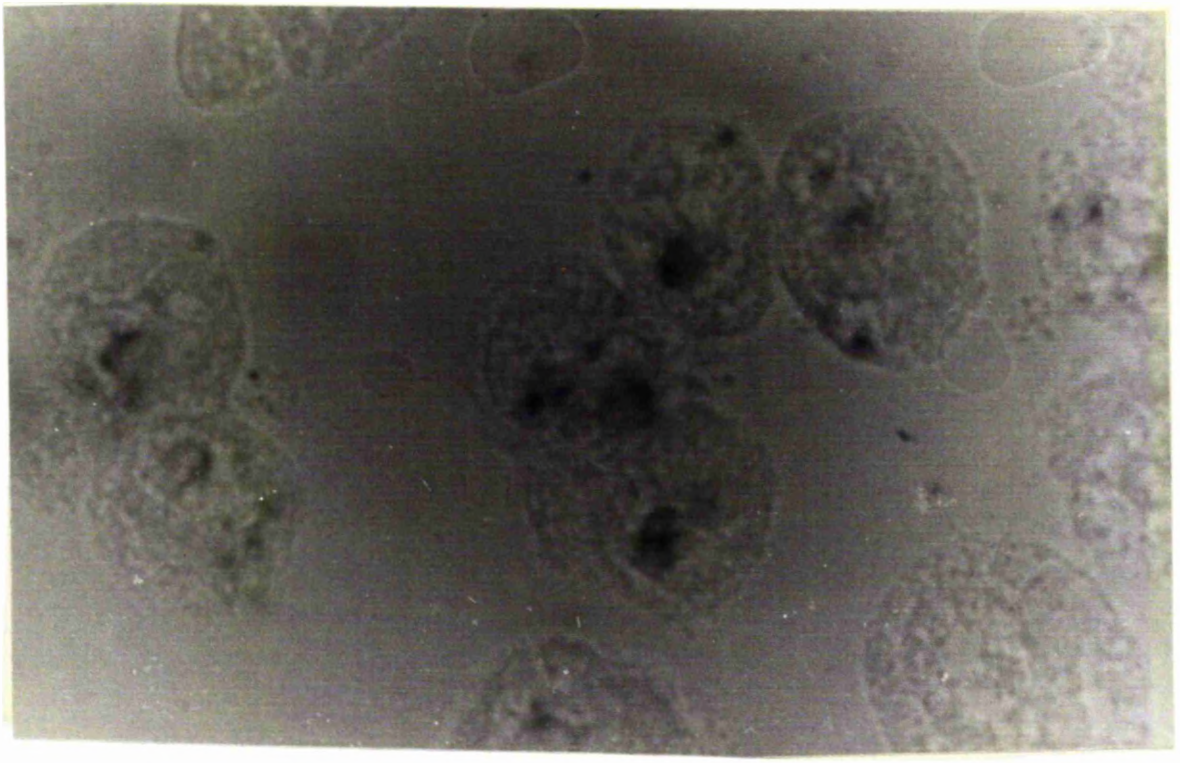
The incorporation of ^{3}H-uridine into nuclear ribonucleic acids of intact Landschutz ascites carcinoma cells.

Type	Specific activity
$nRNA_1$	0.091mμc/μgRNAphosphorus
$nRNA_2$	0.548mμc/μgRNAphosphorus

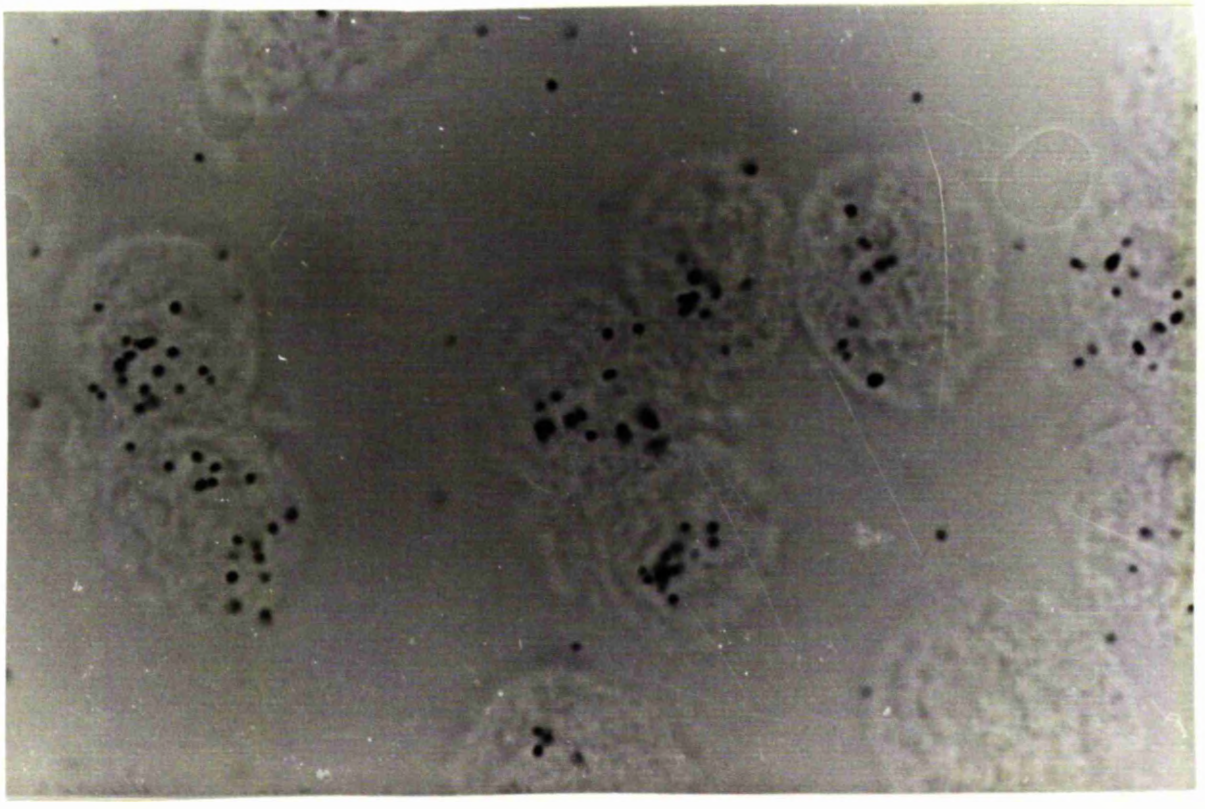
Incubation of intact cells with ^{3}H-uridine, isolation of nuclear ribonucleic acids and their analysis was carried out as described in the experimental section.

Figure 28. Phase contrast photomicrograph of a 3μ section (with corresponding autoradiograph) of Lanschutz ascites carcinoma cells treated with ^{3}H-uridine for 2 minutes (a) in the focal plane of the cells, (b) in the focal plane of the emulsion over cells shown in (a). Emulsion exposed for 14 days.

Figure 28.



(a)



(b)

TABLE XXIV

The effect of $nRNA_1$ and $nRNA_2$ derived from ascites cell nuclei on the incorporation of ^{32}P-UTP into RNA by cytoplasmic Fraction A prepared from Landschutz ascites cells.

Addition	^{32}P-UTP incorporated (μmoles/mg.protein/10mins.)
nil(control)	1.14
$nRNA_1$	1.97
$nRNA_2$	2.02

The reaction mixtures of total volume 1ml. each contained 2μmoles $MgCl_2$, 1μmole each of $NADH_2$, ATP, GTP, CTP and ^{32}P-UTP, 1.9mg. protein and were 0.1M with respect to Tris buffer at pH 9.5. Where indicated, 480μg RNA were added (see 2.5d). All tubes were incubated for 10 mins. at 37°.

3.12 Partial purification of a cytoplasmic enzyme catalysing the RNA dependent incorporation of ^{32}P-UTP into RNA requiring the presence of ATP, GTP and CTP

Having gathered some information concerning the characteristics of ^{32}P-UTP incorporation as catalysed by cytoplasmic Fraction A, attempts were made to purify the enzymes responsible for this process. The procedure employed involved the successive use of ammonium sulphate fractionation, acetone fractionation followed by adsorption and elution from calcium phosphate gel, as is described elsewhere (2.6b).

Ammonium sulphate fractionation was retained as an initial step because of the ease with which the separation of the enzyme under investigation from the other UTP incorporating system, in cytoplasmic Fraction B, could be achieved. However, from the point of view of enzyme recovery, it was found preferable to add saturated ammonium sulphate solution of pH7.5 to the cytoplasmic extract already adjusted to the same pH, and to collect the precipitate between 0.25 and 0.45 saturation with respect to ammonium sulphate. The yields of enzyme were greatly improved over those obtained by fractionation with solid ammonium sulphate. Moreover the procedure using saturated ammonium sulphate solution was much more reproducable, presumably because of the more controlled conditions.

The second step involved the use of acetone (chilled to -15° to minimise denaturation of the enzyme) and proved more troublesome, being more difficult to reproduce. The solution appeared to lie in the control of the ionic strength of the ammonium sulphate fraction on each occasion just prior to the addition of the cold acetone. This procedure facilitated the precipitation of the enzyme at comparatively low concentrations of acetone and so minimised the danger of the enzyme being denatured by acetone. However one disadvantage with this procedure lay in the fact that it was necessary to remove the ammonium sulphate by dialysis before a final adjustment of the ionic strength could be made, thereby lengthening the procedure. However repeatable results could be obtained if the ionic strength of the dialysed ammonium sulphate fraction was adjusted by the addition of 1ml. 1M Tris buffer pH 6.5 for each 10ml.

As soon as the acetone fraction was collected, it was dissolved in 0.01M potassium phosphate buffer pH 6.8 so as to dilute any acetone present in the precipitate, and dialysed against 0.01M potassium phosphate buffer pH 6.8 for 4hr. Any further dialysis appeared to influence the subsequent behaviour of the enzyme with respect to calcium phosphate gel.

The enzyme, dissolved in 0.01M potassium phosphate buffer pH 6.8, was completely adsorbed on to the calcium phosphate gel which had been previously equilibrated with the same buffer.

Elution of the gel with 0.05M potassium phosphate buffer pH 6.8 succeeded in removing a fair quantity of inactive protein material leaving the enzyme still adsorbed to the gel. The protein fraction containing the enzyme was then eluted with 0.1M potassium phosphate buffer pH 6.8. This purification step, involving the use of calcium phosphate gel, proved to be extremely efficient and gave a very high yield of enzyme.

A typical purification is shown in Table XXV, the overall purification of the enzyme being in the region of 32-fold and 25% of the enzyme was recovered. Whilst both the ammonium sulphate and acetone fractions still contained appreciable quantities of nucleic acid, fractionation with calcium phosphate gel effectively removed most of the nucleic acid from the enzyme, suggesting that the enzyme is not a nucleoprotein.

Table XXV

Purification of a cytoplasmic enzyme catalysing the RNA dependent incorporation of ^{32}P-UTP into RNA, requiring the presence of ATP, GTP and CTP.

Purification step	Volume (ml.)	Activity (units)	Specific activity (units/mg. of protein)	Yield (%)
1. Cytoplasmic extraction	135	390	0.44	100
2. Ammonium sulphate fractionation	30	240	2.58	61
3. Acetone fractionation	15	109	2.85	28
4. Calcium phosphate gel fractionation	15	102	14.10	26

The assay mixtures of total volume 1ml. contained 2μmoles $MgCl_2$, 1μmole each of $NADH_2$, ATP, GTP, and ^{32}P-UTP, 200μg. ascites nuclear RNA, 1 to 4mg. protein and were 0.1M with respect to Tris buffer at pH 9.5. All tubes were incubated at 37° for 10 minutes.

SECTION IV

DISCUSSION

4.1 The use of ^{3}H-uridine in a preliminary study of RNA biosynthesis in mammalian cells.

It is apparent that the material into which the ^{3}H-uridine is incorporated by cytoplasmic extracts of ascites tumour cells is RNA since it is insoluble in acid and liberates on alkaline hydrolysis the four ribonucleoside -3'(or 2') monophosphates. These may be degraded to yield the four bases adenine, guanine, cytosine and uracil of which only cytosine and uracil are radioactive. The amount incorporated into DNA is negligible.

The evidence available from several laboratories suggests that the biosynthesis of RNA occurs in two steps: phosphorylation of ribonucleosides or ribonucleoside-5' monophosphates to the corresponding di- or triphosphates followed by condensation of either the di- or triphosphate to form part of a polynucleotide chain (Khorana, 1960).

Several workers have shown that the four ribonucleoside-5' mono-, di- and triphosphates are widely distributed in biological material (Schmitz, Potter, Hurlbert and White, 1954; Hurlbert, Schmitz, Brumm and Potter, 1954; Schmitz, Hurlbert and Potter, 1954; Bergkvist and Deutsch, 1954a,b) and these could therefore act as a source of RNA precursors.

Enzymes catalysing the phosphorylation of uridine and uridine nucleotides have been observed in cytoplasmic fractions from rat liver cells (Canellakis, 1957a,b; Cardini, Paladini, Caputo and Leloir, 1950; Paege and Schlenk, 1954; Herbert, Potter and Takagi, 1955), yeast cells (Lieberman, Kornberg and Simms, 1955; Berg and Joklik, 1954), and Ehrlich ascites carcinoma cells (Reichard and Skold, 1957; Skold, 1960). AMP, GMP, CMP and UMP kinases have been demonstrated in extracts of acetone powder of calf liver cells (Strominger, Heppel and Maxwell, 1959; Heppel, Strominger and Maxwell, 1959), and similar CMP and GMP kinases have been found in cell fractions of rat liver (Herbert and Potter, 1956). Other workers (Canellakis, Gottesman and Kammen, 1960) have studied the four ribonucleoside monophosphate kinases in preparations from brewers' yeast and E. coli while the deoxyribonucleoside monophosphate kinases have been observed in cytoplasmic extracts of Ehrlich ascites carcinoma cells (Weir and Smellie, 1959).

There is little doubt that, in the case of cytoplasmic extracts of ascites tumour cells, phosphorylated derivatives of uridine are the precursors of RNA uridine since in conditions in which uridine is not phosphorylated, such as low pH (Fig.8) or in the absence of ATP (Fig.9) there is little incorporation of uridine into RNA.

Such findings would appear to indicate the existence of kinases responsible for the formation of UMP, UDP and UTP from uridine in cytoplasmic extracts of ascites tumour cells and it seems likely that the corresponding enzymes required for GDP and GTP, CDP and CTP and ADP and ATP formation are also present. However these kinases are absent from the mitochondrial, microsomal and nuclear fractions of ascites tumour cells and thus a limit is imposed on the usefulness of ^{3}H-uridine for the investigation of RNA biosynthesis in these cells.

The work of Ochoa and others on polynucleotide phosphorylase (see 1.4b) suggests that RNA biosynthesis might proceed by condensation of ribonucleoside-5' diphosphates under the influence of this particular enzyme. As already mentioned, polynucleotide phosphorylase has been shown to occur in a variety of microorganisms, in plants, in yeasts and, in a rather tentative way, in a limited number of mammalian tissues. It seemed quite likely therefore polynucleotide phosphorylase might occur in cytoplasmic extracts of Ehrlich ascites cells. These cytoplasmic extracts are certainly capable of forming UDP from uridine and the observation that uridine incorporation into RNA is inhibited by inorganic orthophosphate appears to lend support to this view.

An alternative mechanism by which RNA biosynthesis

might be achieved involves the terminal addition of ribonucleoside-5' triphosphates. This type of reaction and its relation to protein biosynthesis have already been discussed (see 1.4d). Extracts of Ehrlich ascites cells (Edmonds and Abrams, 1957; Hecht, Zameenik, Stephenson and Scott, 1958), embryonic chick liver and heart (Chung and Mahler, 1958), rat liver (Heidelberger, Harbers, Leibman, Takagi and Potter, 1956; Harbers and Heidelberger, 1959; Canellakis, 1957c; Herbert, 1958) and E.coli (Preiss and Berg, 1960) can catalyse the incorporation of an AMP residue into RNA at a terminal position.

Other workers have demonstrated systems promoting the terminal incorporation of CMP residues from CTP into RNA in calf thymus nuclei (Hurwitz, Bresler and Kaye, 1959; Krakow and Kammen, 1960; Edmonds and Abrams, 1960a; Hurwitz and Bresler, 1961) and in the nuclear fraction of rat liver (Weiss and Gladstone, 1959). In most of these studies inorganic pyrophosphate was found to inhibit the reaction. In the case of cytoplasmic extracts of Ehrlich ascites cells, the observation that a proportion of the ^{3}H-uridine incorporated into the RNA is located terminally and that this reaction is subject to inhibition by inorganic pyrophosphate suggests that this pathway too may play a part in the observed incorporation of ^{3}H-uridine.

However the possibility exists that there may well be

other mechanisms for the incorporation of ribonucleotides into RNA in mammalian systems. Canellakis (1957b) has reported the non-terminal incorporation of ^{14}C-UMP into the RNA of rat liver cell cytoplasm and Edmonds and Abrams (1960) have described a system which promotes the non-terminal incorporation of an AMP moiety derived from ATP. In the chick embryo system described by Chung and Mehler (1959) there appears to be one mechanism for the terminal incorporation of adenine nucleotides into RNA and another for the non-terminal incorporation. While incorporation of ribonucleotide units on the ends of existing RNA chains clearly does not represent true polyribonucleotide biosynthesis, incorporation of ribonucleotides into non-terminal positions in polyribonucleotide chains must represent a more radical extension of the chains or formation of new chains.

The results presented in the previous section (3.1) suggest that there might be two pathways in cytoplasmic extracts of Ehrlich ascites cells, one inhibited by inorganic orthophosphate, catalysing the non-terminal incorporation of UMP into RNA, and the other, inhibited by inorganic pyrophosphate, catalysing the terminal addition of UMP to RNA. In the presence of either inhibitor, incorporation proceeds predominately by the alternative pathway. Since the polymerisation of ribonucleoside-5' diphosphates by polynucleotide phosphorylase is inhibited by inorganic orthophosphate it

was thought that the non-terminal incorporation of UMP might be brought about by such an enzyme. Similarly the inhibition by inorganic pyrophosphate suggests that the terminal addition is promoted by an enzyme utilising ribonucleoside triphosphates. However the enzyme system employed in these experiments is complex and it would be scarcely justifiable to draw such conclusions. Indeed the work of Mackenzie (1960) demonstrated the absence of the enzyme polynucleotide phosphorylase from cytoplasmic extracts of Ehrlich ascites tumour cells.

The requirement for glucose, NAD and UTP or $NADH_2$ and NADP is extremely difficult to explain. It may well be that in this complex system UDP and UTP are preferentially required in small quantities for a series of enzymic conversions leading to the formation of $NADH_2$ and NADP and it might be expected therefore that low concentrations would fail to dilute the incorporation of uridine into RNA. The addition of $NADH_2$ and NADP would obviate the need for such reactions and the added UTP and UDP would increase the pool of RNA precursors leading to dilution of the uridine incorporated. The possibility cannot be excluded however that the addition of UDP and UTP facilitatesthe incorporation of uridine by increasing the RNA precursor pools to an optimal size.

If the incorporation of ^{3}H-uridine by cytoplasmic

extracts of ascites cells represents true biosynthesis of RNA it must be assumed that the other three ribonucleotides are present in the tissue extract or can be formed during the incubation. The stimulation of uridine uptake by the addition of a mixture of ADP, GDP and CDP or GTP and CTP supports this view, and the greater stimulation obtained by addition of the ribonucleoside triphosphates suggests that the triphosphates might be the more important precursors of RNA in the system.

While dialysis of cytoplasmic extracts of ascites tumour cells has little effect on the uptake of uridine into RNA, preincubation is inhibitory (Table X). Hecht, Stephenson and Zamecnik (1959) have shown that the RNA isolated from a freshly prepared pH 5 precipitate of the soluble fraction of Ehrlich ascites cells generally contains the cytosine and adenine nucleotide end groups and is at least partially saturated with amino acids. On preincubation however, the terminal nucleotides and amino acids are removed from the RNA. It may be that in the ascites cytoplasmic system a terminal nucleotide sequence exists on the RNA which allows UMP residues to be incorporated initiating the synthetic process and that on preincubation this terminal arrangement of the RNA is lost.

The distribution of enzymes responsible for the incorporation of ^{3}H-uridine (Table XI) is quite different

from the distribution of enzymes responsible for the synthesis of DNA (Smellie, Keir and Davidson, 1959). This is consistent with results of earlier experiments on the in vivo incorporation of $^{32}PO_4^{3-}$ which have shown that many rabbit tissues are capable of RNA synthesis and that there is little correlation between synthesis of RNA and DNA (Smellie, Humphrey, Kay and Davidson, 1955).

4.2 Attempts to demonstrate net synthesis of polyribonucleotide by cytoplasmic extracts of ascites tumour cells.

Net synthesis of very small amounts of polyribonucleotide has been demonstrated only in the presence of $NADH_2$, NADP, magnesium ions and ATP, CTP, GTP and UTP or ADP, GDP, CDP and UDP. One of the effects of $NADH_2$ and NADP is to increase the formation of UTP (Table XIII) and since the highest figures for net synthesis are obtained on adding the ribonucleoside-5'triphosphates rather than the corresponding diphosphates it seemed possible that the system utilising the ribonucleoside-5'triphosphates is of considerable importance in the synthesis of RNA in this cytoplasmic extract. It could be that the utilisation of the ribonucleoside-5'diphosphates for net synthesis is due merely to the conversion of these to the corresponding triphosphates by nucleoside diphosphate kinases. However more satisfactory results from these attempts to demonstrate net synthesis of polyribonucleotide could not be obtained

due to the high nuclease content of these cytoplasmic extracts.

4.3 The incorporation of ^{32}P-labelled uridine nucleotides into RNA by ammonium sulphate fractions prepared from cytoplasmic extracts of ascites cells.

From the results described in the previous section (3.4), it is clear that cytoplasmic extracts contain at least two different systems catalysing the incorporation of ^{32}P-UTP into RNA. These two systems are obtained under different fractionation conditions with ammonium sulphate and show quite different characteristics with respect to the reactions they catalyse. Thus the optimum pH (Fig.16), the influence of a mixture of ATP, GTP and CTP (Table XIV), the time course of the reaction (Fig.17), the intramolecular location of the incorporated radioactivity (Tables XVI and XVII) and the influence of amino acids (Fig.21) is quite different for the two fractions, A and B. The only similarities between the two fractions lie in the utilisation of the same precursor (Table XIV), the inhibition of both systems by inorganic pyrophosphate (Table XV) and the stimulation by added RNA.

No evidence has been obtained in any of these experiments for a system utilising UDP as a precursor of polyribonucleotides. Such a mechanism cannot however be excluded completely since inorganic orthophosphate failed to produce

any substantial inhibition in these experiments whereas it had previously been observed to inhibit the incorporation of ^{3}H-uridine by cytoplasmic extracts of Ehrlich ascites cells (3.1).

The results in Tables XIV, XVI and XVII suggest that Fraction A is concerned in a system which incorporates all four ribonucleoside moieties into polyribonucleotides since it is stimulated by the addition of ATP, GTP and CTP and in the presence of these nucleotides the uridine moiety is more randomly distributed in the polyribonucleotide than in their absence, when it is incorporated as polyuridylic acid. The observation (Table XIV) that Fraction B is not stimulated by the addition of ATP, GTP and CTP and that most of the uridine moieties incorporated are adjacent to either uridine or adenosine nucleotides in the polynucleotide, suggests a different function for this system, and this is borne out by the finding that Fraction B is stimulated by the addition of an amino acid mixture (Fig. 21).

The reaction catalysed by Fraction A prepared from cytoplasmic extracts of Ehrlich ascites tumour cells closely resembles that of the Type I enzyme of Chung, Mahler and Evans (1960) prepared from the cytoplasmic fraction of chick embryo cells. This enzyme catalyses the incorporation of ^{14}C-ATP into internucleotide linkages in the presence of GTP, CTP and UTP and RNA at a pH between 9.0 and

9.5. Similar systems utilising ribonucleoside-5'triphosphates have since been described in extracts of E.coli (Stevens, 1960), in the soluble fraction of pea embryos (Huang, Maheshwari and Bonner, 1960) and in pigeon liver microsomes (Straus and Goldwasser, 1961). On the other hand, the results show that Fraction A is also capable of attaching UMP units derived from UTP preferentially to UMP residues in polyribonucleotides as a polyuridylic acid sequence (Table XVI) and thus also resembles a more highly purified UTP incorporating system recently obtained by Klemperer and Kammen (1962) from the pH 5 supernatant fraction of a rat liver homogenate subjected to centrifugation at 105,000 x g. This cytoplasmic enzyme system requires the presence of added RNA for the incorporation of uridine nucleotides as a polyuridylic acid sequence. Thus, even in cytoplasmic Fraction A itself, there may actually be two systems responsible for the UTP uptake; one catalysing the random incorporation of UTP into polyribonucleotide in the presence of ATP, GTP and CTP indicative of possible true RNA synthesis and the other responsible for the formation of polyuridylic acid sequences in polyribonucleotide chains.

Such cytoplasmic polyribonucleotide synthesising systems are of particular interest since Sutter, Whiteman and Webster (1961) suggest, on the basis of work with Acetabularia, that the RNA of ribosomes is synthesised in

the cytoplasm and the cell nucleus was not directly required. However as will be discussed later (4.7) the ribosomal RNA, even if formed under the influence of cytoplasmic enzymes, probably has a nucleotide sequence which is dictated in some manner by the cell nucleus.

The reaction catalysed by cytoplasmic Fraction B more closely resembles that of the Type II enzyme of Chung, Mahler and Errione (1960). It is not stimulated by the addition of the other three ribonucleoside-5' triphosphates (Table XIV) and a considerable proportion of uridine nucleotide is incorporated at terminal positions of polyribonucleotides (Table XVII). It appears therefore, that the reaction catalysed by Fraction B whilst giving rise to a product of possible importance in the transfer of amino acyl residues from their respective activating enzymes to the site of their final incorporation into protein, cannot be regarded as responsible for true RNA biosynthesis.

4.4 Incorporation of ^{32}P-UTP into polyribonucleotide by fractions prepared from isolated ascites cell nuclei.

The major limitation in the use of aqueous media for the isolation of cell nuclei is that both high and low molecular weight material is undoubtedly extracted from the nuclei during the course of their isolation (Dounce,1955). However the degree of permeability of the nuclear membrane is still a matter of some controversy. Also according to

Dounce (1955), when nuclei are prepared in aqueous media, in the absence of nuclear autolysis, the nuclei should be capable of forming a peculiar type of structural gel upon the addition of alkali or strong sodium chloride solution. This gel formation is considered by Dounce (1949) to be an indication that little or no autolytic degradation of nuclear structure has occurred.

By 1950, it was found possible to isolate liver cell nuclei in isotonic sucrose to which calcium chloride had been added. This use of calcium chloride was first introduced by Schneider and Peterman (1950) whose example was soon followed by Hogeboom, Schneider and Striebich (1952). Apparently calcium chloride inhibits autolytic degradation and permits the isolation of liver cell nuclei at pH values sufficient to allow the formation of structural gels with alkali or strong saline. Concentrations of calcium chloride as low as 0.0018M suffice under certain conditions, but higher concentrations are desirable for obtaining clean nuclei. For example, Allfrey, Mirsky and Osawa (1957) used 0.0033M to isolate nuclei from calf thymus tissue. For the isolation of ascites tumour cell nuclei this higher concentration of calcium chloride was employed.

From the results described in the previous section (3.5), it is clear that nuclear extracts prepared from isolated ascites tumour cell nuclei contain at least two

systems catalysing the incorporation of ^{32}P-UTP into RNA. These two systems, namely nuclear Fraction A and nuclear Fraction B, are obtained under the same fractionation conditions with ammonium sulphate as are the A and B Fractions from cytoplasmic extracts. As with the cytoplasmic A and B Fractions, the nuclear A and B Fractions show quite different characteristics with respect to the incorporation of ^{32}P-UTP into RNA, although the reactions catalysed by the A Fractions from nuclear and cytoplasmic extracts resemble one another, as do the reactions catalysed by the B Fractions.

Reactions similar to those catalysed by nuclear A Fraction, involving the incorporation of any one labelled ribonucleotide in the form of the triphosphate which are greatly augmented by the presence of the other three have been described in nuclear fractions from rat liver cells (Weiss, 1960), in isolated nuclei of pea seedlings (Rho and Bonner, 1961) and in the nuclear fractions from HeLa cells and mouse fibroblasts (Goldberg, 1961). However, unlike nuclear Fraction A, these enzyme preparations, although derived from cell nuclei, are particulate in nature. In the case of ascites tumour cell nuclei it is necessary to employ sonic vibration as a means of causing their disruption and the results suggest that it is this process of sonic disintegration which is responsible for making

soluble most of the enzyme which subsequently appears in nuclear Fraction A, although a certain amount of RNA synthesising activity still remains in particulate form.

The fact that nuclear Fraction A has an activity four to five times greater than that of cytoplasmic Fraction A (Fig.23) does not necessarily imply that the nucleus is the only site of RNA biosynthesis, and that any activity occurring in the cytoplasm is due to the enzymes responsible for this process being leached from the nucleus during cell disruption in aqueous media.

The occurrence of a ^{32}P-UTP incorporating system in nuclear Fraction B with characteristics similar to cytoplasmic Fraction B is at first sight puzzling when the cytoplasm is considered by many to be the site of protein synthesis (Hoagland, 1960). However Allfrey (1960) has described not only amino activating systems in nuclei isolated from calf thymus tissue but also the transfer of these activated amino acids to carrier ribonucleic acids of nuclear origin. Furthermore he has provided some evidence to suggest that RNA-rich particles which exist in calf thymus nuclei are sites of active amino acid incorporation into protein. Indeed the isolation from calf thymus nuclei of ribonucleoprotein particles, thought to be nuclear ribosomes, has recently been achieved by Wang (1961).

4.5 Formation of a sequence of adenylate units from ATP by an enzyme fraction from ascites tumour cell nuclei.

The characteristics of ^{32}P-ATP incorporation into polyribonucleotide by nuclear Fraction A prepared from ascites tumour cell nuclei have been described (3.7). The results suggest that in this ammonium sulphate fraction there are two discrete systems responsible for ATP incorporation into polyribonucleotide. One of these systems promotes the incorporation of ATP into polyribonucleotide at pH 9.5 and requires the presence of the other three ribonucleoside-5' triphosphates (GTP, CTP and UTP). In this respect it closely resembles the system already discussed (4.4) which exists in nuclear Fraction A and requires the presence of ATP, GTP and CTP for the optimal incorporation of ^{32}P-UTP into polyribonucleotide at pH 9.5. However only further purification of these systems will reveal whether or not they are under the contrd of one and the same enzyme.

Nevertheless the other system which promotes the incorporation of ATP into polyribonucleotide in the absence of added ribonucleotides, at positions adjacent to other AMP residues, is fundamentally different. The function of such a system is at present difficult to rationalise but a similar system capable of forming a sequence of adenylate units from ATP has been described

in a soluble preparation from calf thymus nuclei (Edmonds and Abrams, 1960b).

4.6 The dependence of ascites tumour cell ^{32}P-UTP incorporating systems upon the presence of DNA.

From the results in section 3.6 it is clear that the incorporation of ^{32}P-UTP into polyribonucleotide catalysed by nuclear and cytoplasmic A Fractions shows a requirement for DNA. However, whereas UTP uptake in the presence of the other three ribonucleoside-5' triphosphates by nuclear Fraction A is enhanced by heat-denatured (single-stranded) DNA in the presence, or absence, of added nuclear RNA (Table XVIII), the incorporation of UTP in the presence of ATP, GTP and CTP by cytoplasmic Fraction A is enhanced by native (double-stranded) DNA only in the presence of added nuclear RNA (Table XIX). Such an observation seems to strengthen the view that the system synthesising polyribonucleotide in the cytoplasm is somewhat different from that synthesising polyribonucleotide in the nucleus, and is not merely a portion of the nuclear system leached into the cytoplasmic preparation during the isolation of the subcellular fractions from ascites tumour cells.

Such a requirement for DNA in RNA biosynthesis has been reported by other workers. For instance the inhibition of

the biosynthesis of RNA in cell-free systems by the enzyme DNase has been reported for a system in calf thymus nuclei (Krakow and Canellakis, 1961), for a soluble system in E.coli (Hurwitz, Bresler and Diringer, 1960), for systems in L.arabinosus and A.vinelandii (Ochoa, Burma, Kroger and Weill, 1961) and for systems from rat liver nuclei (Weiss, 1961a) and M.lysodeikticus (Weiss and Nakamoto, 1961a). Correspondingly the presence of DNA is reported to be essential for RNA synthesis by enzymes in rat liver nuclei (Weiss, 1961a and b) in M.lysodeikticus (Weiss and Nakamoto, 1961a and b) in E.coli (Stevens, 1961a and b; Hurwitz, Bresler and Diringer, 1960) and in both L.arabinosus and A.vinelandii (Ochoa, Burma, Kroger and Will, 1961; Burma, Kroger, Ochoa, Warner and Weill, 1961). According to some of these authors DNA must apparently be in the native form; heating and partial degradation with DNase abolishes its activity. However, Weiss (1961a) suggests that DNA need not necessarily be double-stranded.

Using partially purified preparations from M.lysodeikticus Weiss and his co-workers (Weiss and Nakomoto, 1961b and c; Geiduschek, Nakomoto and Weiss, 1961) carried out further investigations of the involvement of DNA in RNA biosynthesis. Initially, they were able to show that "primer" DNAs from different sources altered the position of cytidylate residues in the newly assembled RNA chain and that the polyribonucleotide had an average base composition similar

to the "primer" DNA used. Further work on nearest neighbour base frequencies suggested that the sequential arrangement of nucleotides in the RNA is probably analagous to that in the "primer" DNA. More conclusive evidence came from caesium chloride density gradient centrifugation which demonstrated that the RNA synthesised can form specific complexes with the DNA "primer". According to Hall and Spiegelman (1961) such an interaction between DNA and RNA indicates that there are entire nucleotide sequences in the DNA and RNA chains that are simultaneously capable of binding to each other in the face of the competing tendency of complimentary sequences on DNA strands to recombine. Accordingly it appears therefore that RNA strands are synthesised with a base sequence "complementary" to the DNA "primer" and the term "complementary" is defined as a specific fit of portions of "primer" and product by appropriate base interactions.

Other evidence of the directing role of DNA in RNA synthesis has been obtained with purified preparations of E. coli in the presence of the four ribonucleoside-5' triphosphates (Furth, Hurwitz and Goldmann, 1961a, b and c). When DNA preparations of widely varying base composition (T2-DNA, calf thymus gland DNA, E. coli DNA and M. lysodeikticus DNA) were added to the system, in all cases the base ratios of the RNA produced was determined by the DNA added and the results were consistent with the

hypothesis that the incorporation of ribonucleotides is determined by the ability of the new polymer to form hydrogen bond pairs with the bases in the "primer" DNA so that the product is some sort of DNA-RNA complex. This view is supported by the observation that when polydeoxythymidylate is used as "primer" in place of DNA only ATP is used for polyribonucleotide synthesis and the product is a polydeoxythymidylate-polyadenylate hybrid. With the copolymer of deoxyadenylate and deoxythymidylate the product was a polymer made up of AMP and UMP units in alternating sequence. Hence not only do the deoxyribonucleotides in the "primer" determine the nature of the ribonucleotides in the RNA produced, but the sequence of the deoxyribonucleotides in the "primer" determines the sequence of the ribonucleotides in the RNA produced.

Such results with partially purified enzyme systems suggest that part of the product may exist at least temporarily as a complex between RNA and DNA. Several researchers have chemical evidence that such complexes can occur (Rich, 1960; Schildkraut, Marmur, Fresco and Doty, 1961). Hall and Spiegelman (1961) showed that infection of E. coli with T2-bacteriophage results in the production of a fraction of RNA with an apparent base ratio analagous to that of the T2-bacteriophage DNA. This RNA, which is synthesised immediately following infection, shows a specific

complex formation with single-stranded T2-bacteriophage DNA whereas no hybrid formation occurs with heterologous DNA even if it has the same base composition as T2-DNA. It is presumably because T2-DNA and T2-specific RNA possess "complementery" nucleotide sequences that they form hybrids. Recently Schulman and Bonner (1962) have demonstrated a DNA-RNA complex to occur naturally in Neurospora crassa.

Having discussed the directing role of DNA in RNA synthesis it is of considerable interest that more evidence is accumulating to strengthen the concept that DNA is the active material of the gene directing protein synthesis. For some time it has been known that irradiation of organisms with ultraviolet light will induce mutations (Srb and Owen, 1958). In 1961, Litman was able to demonstrate that treatment of isolated "transforming" DNA of pneumococcus with ultraviolet light results in its chemical and genetic alteration. However even more striking evidence for a DNA mediated genetic control over protein synthesis has been demonstrated in a cell-free system from E.coli previously induced to synthesise de novo the enzyme β-galactosidase (Novelli, Eisenstadt and Kameyama, 1961). When the system is supplemented with inducer, amino acids, a suitable energy source and nucleoside triphosphates, it is capable of effecting an increase

in β-galactosidase activity. An inhibition of enzyme synthesis occurs upon treatment of the system with DNase or with ultraviolet light. This inhibition can be overcome by addition of native DNA from normal cells to the system.

Thus, from the point of view of genetic control over protein synthesis, it is possible to envisage a single strand of DNA directing the synthesis of a single strand of RNA (uracil pairing with adenine and cytosine with guanine etc.) so as to form a hybrid duplex. In the living cell this might then dissociate into a single strand of DNA, which could repeat the process, and a single strand of RNA of complementary base composition which would presumably act as a "messenger" RNA directing the assembly of amino acids into the protein molecules. Evidence for such a "messenger" RNA in E.coli has recently been obtained (Brenner, Jacob and Meselson, 1961; Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson, 1961). The results of experiments on E.coli indicated that a very small RNA fraction, characterised by a high rate of removal and a base composition analagous to E.coli DNA, associates specifically with certain ribosomal particles, the site of protein synthesis. After infection of the E.coli cells with T2-bacteriophage only phage proteins are synthesised and according to Monod, Jacob and Gros (1961) it is possible to demonstrate that although the actual

synthesis of phage protein takes place in the ribosomes of the bacteria the "messenger" RNA has a base ratio similar to that of the T2-bacteriophage DNA. They conclude that the "messenger", which carries the genetic information from the DNA of the genes to protein synthesising centres, is a short lived intermediate and cannot be identified with ribosomal RNA.

However it must be appreciated that at present these theories are only applicable to certain bacterial systems. The situation is still far from clear when mammalian cells like ascites tumour cells are considered in this respect. The possible function of single-stranded (heat-denatured) DNA in the nuclear polyribonucleotide synthesising system could be envisaged in terms of promoting the formation of a DNA-RNA hybrid with the subsequent production of a "messenger" RNA but work along these lines is hindered because the enzymes are difficult to purify and normally have quite low activities. However the situation in the cytoplasmic fraction of ascites tumour cells seems quite different. As already mentioned, DNA only exerts an effect in the presence of added nuclear RNA (Table XIX) and thus it could be the case that DNA only acts indirectly in the cytoplasmic system, possibly functioning as an *in vitro* inhibitor of endogenous RNase present in the cytoplasmic extracts. However other explanations must

be considered. Double-stranded DNA could direct the synthesis of RNA as a third strand. Such a triple-stranded molecule has been envisaged by Zubay (1958). Alternatively, in the cytoplasm, it may be the case that the RNA double helices do not need to unwind completely in serving as templates for RNA synthesis. Nevertheless theories based on the direct participation of DNA, double or single-stranded, in cytoplasmic RNA synthesis are difficult to rationalise since DNA does not occur normally in the cytoplasm.

4.7 <u>The dependence of ascites tumour cell ^{32}P-UTP incorporating systems upon the presence of added RNA.</u>

The results presented in the previous section (3.6) indicate that ^{32}P-UTP uptake into polyribonucleotide by nuclear and cytoplasmic A Fractions is augmented not only by the addition of the other three ribonucleoside-5' triphosphates but also by the addition of RNA derived from the nuclei of ascites tumour cells (Fig.24) by the process of phenol extraction (2.5b). The addition of RNA derived from ascites cell cytoplasmic extracts has a similar but much less pronounced effect (Fig.24). However it must be emphasised that phenol extraction does not remove all the RNA from the ascites tumour cell nuclei (Sibatani, Yamana, Kimura and Takahashi, 1960). In this respect it appears that the RNA which is extracted from

ascites cell nuclei is similar to $nRNA_1$, whilst that which remains unextracted is similar to the RNA in the "1M NaCl fraction", namely $nRNA_2$.

The RNA dependent incorporation of ribonucleotides has since been described in other laboratories. For example, Hurwitz, Furth, Anders, Ortiz and August (1961) demonstrated a system in ribosomes of E.coli leading to the incorporation of all four ribonucleotides. This reaction, they found, was inhibited by RNase but not by DNase. In addition, Reddi (1961) has described a cytoplasmic system from spinach leaves which is capable of polyribonucleotide biosynthesis in the presence of added RNA and as already mentioned Klemperer and Kammen (1962) have described a system capable of incorporating UTP into polyribonucleotide as polyuridylate and have found it to be dependent on the presence of added RNA.

However as already discussed (4.6), the addition of single-stranded (heat-denatured) DNA can also augment ^{32}P-UTP incorporation into polyribonucleotide catalysed by nuclear Fraction A. Thus in ascites tumour cells there probably exists in the nucleus two RNA synthesising systems, one dependent on single-stranded DNA and the other dependent on a type of RNA derived from the nucleus. In the cytoplasm there appears to be a system which is also dependent on nuclear RNA, although this system could conceivably have

been leached from the nucleus during the preparation of the cytoplasmic extract.

It is possible to envisage a system operating in ascites cells whereby the native DNA double helix in the chromosomal material of the cell nucleus unwinds with the simultaneous formation of RNA chains on both single strands of DNA so formed. Such a process could be brought about by the DNA dependent RNA synthesising system in nuclear fraction A. This process would give rise to two RNA-DNA hybrid molecules. At present there is no conclusive evidence for the existence of such hybrids in ascites cells. However in the experimental section (2.5d) it was mentioned that the RNA which was not extracted from the nuclei by the phenol process, namely $nRNA_2$, always contained a proportion of DNA which could not be removed by DNase treatment. Such a finding is of interest as Schildkraut, Marmur, Fresco and Doty (1961) have demonstrated that their DNA-RNA complex was resistant to pancreatic DNase. Indeed it is this $nRNA_2$ which is most highly labelled with ^3H after a two minute exposure of intact ascites tumour cells to ^3H-uridine (Table XXIII; 4.8).

Each of the RNA-DNA hybrid molecules in turn could give rise to a single strand of DNA and a single strand of RNA which could act as a "template" in the subsequent

synthesis of RNA in the cytoplasm or nucleus. The existence of such a "template" is implied in the theories of Leslie (1961) for the transference of information from nucleus to cytoplasm, although he considers that the formation of single-stranded "template" RNA in mammalian cells involves the genetic activity of the double helix form of DNA.

It is conceivable that this RNA "template" could remain in the nucleus and there direct the synthesis of RNA for the formation of nuclear ribosomes, the process being catalysed by the RNA dependent polyribonucleotide synthesising system present in nuclear Fraction A. On the other hand, the RNA "template" could pass into the cytoplasm rather like a "messenger" and there direct the cytoplasmic synthesis of any ribosomal RNA. However although nuclear and cytoplasmic extracts prepared from ascites tumour cells almost certainly contain trace amounts of ribosomes, or ribonucleoprotein particles, further work is required to determine whether a "template" type of RNA will associate with them or not. In fact attempts to discover which variety of nuclear RNA proved most effective in the cytoplasmic RNA synthesising system were unsuccessful (Table XXIV) probably due to DNA contaminated preparations of $nRNA_2$.

4.8 The initial site of RNA synthesis in the nucleus of ascites tumour cells.

As mentioned in the Introduction (1.5) there remains considerable controversy as to the actual site of initial RNA synthesis in the cell nucleus. Using microautoradiographic procedures many workers are of the opinion that the nucleolus is the primary site of RNA synthesis (for references see 1.5), however others using similar techniques are convinced that RNA is first built up in intimate association with the chromosomes and could be a precursor of a fraction of nucleolar RNA (for references see 1.5). Such a theory, based on microscopy and autoradiography, that RNA is built up in close association with the genetic material is consistent with the results discussed in the previous sections (4.6 and 4.7).

Attempts to isolate and study the enzymes of mammalian cell nucleoli have not met with much success. In 1952, Krakauer reported that nucleoli could be concentrated from homogenates of liver cells made in very strong sucrose solution. Photographs were not given and the degree of purity of these nucleolar fractions could not be stated with any certainty.

Subsequently Vincent (1952) reported the isolation of nucleoli from the eggs of starfish. Photographs of

the isolated material showed a very high degree of purity. However the size and morphology of the starfish egg nucleoli are such as to preclude the use of Vincent's procedure for the isolation of nucleoli from mammalian cells.

Nevertheless Monty, Litt, Kay and Dounce (1956) described a method whereby nucleoli could be obtained from rat liver nuclei. However analysis of these "nucleoli" showed them to contain a high proportion of DNA and a low proportion of RNA and in composition they resembled whole chromosomes (Thorell, 1955).

Reliable information concerning the nucleolus of the cell is scanty. According to Caspersson (1950) and Schultz (1947) nucleoli are formed near "chromocentres", which consist of "heterochromatin" or portions of chromosomes, which tend not to disperse during interphase, but remain in a more or less condensed state. Thus according to these early theories, the nucleolus appears to be structurally independent of chromosomes but probably dependent on "heterochromatin" for the synthesis of some of its constituents such as RNA.

Evidence is available that the eggs of amphibia possess large numbers of nucleoli which migrate to the nuclear membrane to empty their contents through the membrane into the cytoplasm (Dounce, 1955). However

in the nucleoli of mammalian cells the situation is more complex. No nucleolar migration has apparently been observed in mammalian somatic cells and the number of nuclear inclusion bodies commonly termed nucleoli depends on the degree of polyploidy of the cell (Biesele, Poyner and Painter, 1942), since a relationship exists between the degree of polyploidy and the number of nucleoli. Lewis (1940) carried out cinephotographic studies on the nucleoli of rat fibroblasts and found that nucleoli are intregal parts of chromosomes, and that they become dispersed during mitosis and reappear in condensed form during interphase when chromosomes are dispersed. Thus in mammalian somatic cells, nucleoli may be defined as intranuclear inclusion bodies which are attached to chromosomes .

It is therefore not surprising that the nucleoli isolated by Monty, Litt, Kay and Dounce (1956) resemble whole chromosomes in composition since such nucleoli appear to be special parts of chromosomes. Nucleoli isolated by a similar method from nuclei of ascites tumour cells also have a high DNA content (3.9) and microscopic examination reveals an association with chromosomal material (2.3c).

The possibilities have been mentioned by Vincent (1957a) and Woods and Taylor (1959) that the nucleolus

functions as a mass-production and finishing machine for different RNA "templates" from the chromosomes. The results of Schultz, Caspersson and Aquilonius (1940) and Lin (1955) suggest that the nucleoli are attached to certain chromosomes, the so-called "nucleolar organisers" which determine their composition. Prenucleolar bodies have been found amidst chromosomes and these bodies eventually combine to form nucleoli at the sites of these organising chromosomes (Lafontain, 1958; Tadler, 1959). In mutant cells lacking "nucleolar organiser" chromosomes these prenucleolar bodies remain dispersed (Elsdale, Fischberg and Smith, 1958). It is likely therefore that chromosomal products such as RNA "templates" become associated with the prenucleolar bodies, and a function of the "nucleolar organisers" is to "organise" parts contributed from various chromosomes into a nucleolus.

Table XXII shows that the "chromosomal supernatant" fraction obtained from ascites tumour cell nuclei (a fraction consisting of highly fragmented chromosomal material) incorporates ^{32}P-UTP into RNA to a slightly greater extent than the nucleoli themselves. This is of interest in view of observation that $nRNA_2$, the type of RNA in the nucleus in apparent association with DNA, becomes most extensively labelled when intact ascites tumour cells are exposed to ^{3}H-uridine for short

periods such as two minutes. Indeed autoradiography of intact ascites tumour cells, after the same length of exposure to ^{3}H-uridine, would seem to support the concept that the nucleoli are not the initial site of RNA synthesis, but rather that other structures of the nucleus, such as chromosomes, may also be involved. In the ascites cells the initial site of RNA synthesis might well be at the chromosomes. The fact that nucleoli also incorporate ^{32}P-UTP could be either due to contamination, or to an ability to modify, or complete, molecules of RNA initially produced in association with the chromosomes. However this is obvious speculation and much work has yet to be done to clarify this situation.

4.9 Synthesis of polyuridylate moieties by cytoplasmic Fraction A and its possible bearing on the genetic code.

From the results in section 3.3 it is evident that cytoplasmic Fraction A can catalyse the incorporation of uridylate units into polyribonucleotide in such a fashion that several uridylate units are adjacent to one another. The very recent work of Nirenberg and Matthaei (1961) indicated that a synthetic polyuridylic acid "template" will direct the inclusion of polyphenyalanine in protein material by a cell-free system from E.coli. Polyphenyalanine-sRNA has been isolated and shown to be an intermediate in this process (Nirenberg, Matthaei and Jones, 1962). These

results imply that a sequence of uridylate units in an RNA "template" will direct the inclusion of a phenylalanine residue in a polypeptide chain. The genetic studies of Crick, Barnett, Brenner and Watts-Tobin (1961) suggest that only a triplet of adjacent uridylate units is required for this purpose and this is substantiated by the more biochemical studies of Lengyel, Speyer and Ochoa (1961) and Speyer, Lengyel, Basilio and Ochoa (1962). Thus it is of interest that in ascites tumour cells there is a system which is capable of forming a sequence of uridylate units (Table XVI) although its significance is as yet far from clear.

SUMMARY

1. Cytoplasmic extracts of Ehrlich ascites tumour cells are capable of incorporating 5,6-^{3}H-uridine into ribonucleic acid (RNA). Adenosine-5' triphosphate (ATP), magnesium ions, potassium ions, nicotinamide-adenine dinucleotide (NAD), glucose and added RNA are required, and the process involves the formation of radioactive uridine-5' mono-, di- and triphosphate (UMP, UDP and UTP). Alkaline degradation of the RNA reveals that about 20% of the radioactivity is in terminal uridine residues and about 55% in uridine located non-terminally. Inorganic pyrophosphate inhibits terminal incorporation and inorganic orthophosphate depresses non-terminal incorporation.

The substitution of reduced nicotinamide-adenine dinucleotide ($NADH_2$) and nicotinamide-adenine dinucleotide phosphate (NADP) for NAD and glucose in the reaction mixture leads to increased terminal incorporation of uridine and stimulates UTP formation. The incorporation of uridine is also stimulated by supplementation with guanosine-5' triphosphate (GTP) and cytidine-5' triphosphate (CTP), and to a lesser extent by a mixture of adenosine-5' diphosphate, guanosine-5' diphosphate and cytidine-5' diphosphate. Dialysis of the extracts has little effect on the reaction and preincubation

inhibits uridine incorporation.

2. The presence of similar uridine incorporating systems in cytoplasmic extracts of a number of tissues of immature rabbits has also been demonstrated.

3. Evidence is presented that cytoplasmic extracts prepared from Ehrlich ascites tumour cells are capable of net synthesis of small amounts of polyribonucleotide material in the presence of all four ribonucleoside-5' triphosphates (ATP, GTP, CTP and UTP), magnesium ions, $NADH_2$ and NADP.

4. Fractionation of cytoplasmic extracts of Ehrlich (or Landschutz) ascites tumour cells with ammonium sulphate yields two fractions which will catalyse the incorporation of UTP labelled with ^{32}P or ^{14}C into RNA. With one of these, cytoplasmic Fraction A, the incorporation of UTP is greatly increased by the addition of a mixture of ATP, GTP and CTP and in these conditions most of the incorporated radioactivity is distributed randomly in internucleotide linkages. In the absence of the other three ribonucleoside-5' triphosphates UMP units derived from UTP are incorporated adjacent to one another in the form of a polyuridylic acid sequence.

Cytoplasmic Fraction B catalyses less extensive incorporation of UTP much of which is located terminally. This reaction is unaffected by the addition of a mixture of ATP, GTP and CTP but is stimulated by a mixture of amino acids.

Both systems are inhibited by inorganic pyrophosphate and require the presence of added RNA for optimal activity.

5. Fractionation of soluble extracts prepared from Landschutz ascites tumour cell nuclei also yields two fractions capable of the incorporation of ^{32}P-UTP into RNA. These are termed nuclear Fraction A and nuclear Fraction B and have similar properties to the cytoplasmic A and B Fractions: UTP uptake by the nuclear Fraction A is stimulated by a mixture of GTP, CTP and UTP whereas UTP incorporation by nuclear Fraction B is unaffected by the addition of such a mixture of GTP, CTP and UTP. Nuclear Fraction A is four to five times as active as cytoplasmic Fraction A under the same assay conditions and under assay conditions appropriate for B Fractions nuclear Fraction B was slightly less active than the cytoplasmic B fraction.

6. Whereas UTP uptake into RNA by nuclear Fraction A can

be augmented by either RNA derived from ascites tumour cell nuclei or single-stranded ascites cell DNA, UTP incorporation into RNA by cytoplasmic Fraction A stimulated by native DNA only in the presence of RNA.

7. Two enzyme systems are present in nuclear Fraction A which catalyse the incorporation of ^{32}P-ATP into polyribonucleotide. One of these catalyses the initial uptake of ATP into polyribonucleotide (occurring in the first 10min. of incubation) which is stimulated by the presence of a mixture of GTP, CTP and UTP. The second system catalyses the incorporation of ATP after about 40 min. incubation and is markedly inhibited by the presence of a mixture of GTP, CTP and UTP. Whereas the first system in the presence of GTP, CTP and UTP, incorporates the ATP in a random fashion, the second system incorporates the ATP as a sequence of adenylate units.

8. Both nucleoli and finely divided chromosomal material, isolated from ascites cell nuclei, will incorporate ^{32}P-UTP into RNA, the latter to a greater extent than the former.

9. Exposure of intact Landschutz ascites tumour cells to

^{3}H-uridine for 2min. results in extensive labelling of $nRNA_2$, the type of RNA of the nucleus which cannot be extracted with phenol and is always contaminated with a small amount of DNA which is resistant to DNase treatment.

10. Autoradiography of intact Landschutz ascites cell exposed to ^{3}H-uridine for 2min. suggests that nuclear structures other than nucleoli are involved in the initial synthesis of RNA in the ascites cell nucleus.

11. Attempts to purify the cytoplasmic enzyme catalysing the RNA dependent incorporation of ^{32}P-UTP requiring the presence of ATP, GTP and CTP resulted in only a 32-fold purification.

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