The biosynthesis of ribonucleic acid in virus-infected and uninfected cells

by

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The biosynthesis of ribonucleic acid (RNA) was studied in Krebs II ascites tumour cells and in Krebs II ascites tumour cells infected with encephalomyocarditis (EMC) virus. Two approaches were adopted to investigate this problem.

1. The enzyme systems which catalyse the synthesis of polyribonucleotides in extracts of infected and uninfected cells were studied.
2. RNA was extracted from infected and uninfected cells and subjected to fractionation and analysis.

In order to study the enzyme systems, conditions were determined for the incorporation of (^{32}P)UMP from (^{32}P)UTP into polyribonucleotides by extracts of uninfected cells. Two such systems were present, one dependent on deoxyribonucleic acid (DNA) as primer and the other dependent on RNA as primer.

The activity of the DNA-dependent RNA nucleotidyltransferase was at a maximum at pH 7.5, was absolutely dependent on Mg^{2+} ions and was stimulated more by native DNA than by DNA which had been denatured by heat. Addition of Mn^{2+} ions, 2-mercaptoethanol and ATP, CTP and GTP stimulated the reaction

while the removal of any one or all three of the triphosphates markedly reduced incorporation. Analysis of the reaction products showed that (^{32}P)UMP residues were incorporated adjacent to AMP, GMP and CMP residues, thus suggesting synthesis of polyribonucleotides containing each of the four ribonucleotides. Cell fractionation experiments showed that the DNA-dependent enzyme was located almost exclusively in nuclear preparations.

The RNA-dependent RNA nucleotidyltransferase activity was at a maximum between pH 8.5 and 10.0, was absolutely dependent on Mg^{2+} ions and was inhibited by Mn^{2+} ions. Addition of ribonucleoside 5'-triphosphates inhibited incorporation of (^{32}P)UMP and analysis of the products of the reaction demonstrated the predominant formation of polyuridylic acid, but there were also indications of the simultaneous formation of polyribonucleotides containing all four ribonucleotide residues. Cell fractionation experiments showed that the enzymes catalysing the RNA-dependent incorporation of (^{32}P)UMP into polyribonucleotides were derived principally from the cytoplasm.

No changes were observed in the levels of DNA nucleotidyltransferase or of the DNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells following infection with EMC virus.

However, the RNA-dependent RNA nucleotidyltransferase activity was increased following infection with EMC virus. This increase was associated with cytoplasmic rather than nuclear enzymes and analysis of the reaction products suggested the presence of a new enzyme.

A method was developed for the extraction of all the RNA of infected and uninfected Krebs II ascites tumour cells in an undegraded form. Under the conditions employed for sedimentation analysis of RNA in sucrose density gradients, RNA extracted from uninfected cells was resolved into three ultraviolet-absorbing components; the two components of ribosomal RNA had sedimentation coefficients of 30s and 19s, while the soluble RNA (sRNA) had a sedimentation coefficient of 4s. Following brief exposure of the cells to small amounts of ^{3}H-uridine of high specific activity, a rapidly-sedimenting, radioactive RNA component (sedimentation coefficient about 40s) was demonstrated which formed DNA-RNA hybrids with homologous DNA but not with heterologous DNAs. A small amount of labelling was also found associated with sRNA.

On more prolonged exposure of the cells to ^{3}H-uridine, the radioactivity associated with the ribosomal RNA components increased. Similar patterns were observed in experiments with ^{32}P-orthophosphate, but the appearance of radioactive component was somewhat later than in experiments with ^{3}H-uridine.

Exposure of the cells to high concentrations of actinomycin D eliminated the incorporation of ^{3}H-uridine or ^{32}P-orthophosphate into all RNA, except sRNA. When lower concentrations of the antibiotic were employed, there was limited incorporation into rapidly-sedimenting RNA components and ribosomal RNA, in addition to sRNA.

Infection of Krebs II ascites tumour cells with EMC virus produced no marked qualitative changes in the sedimentatio patterns in sucrose density gradients of the RNA extracted after incubation of the cells with ^{3}H-uridine or ^{32}P-orthophosphate. Analysis of the RNA extracted from actinomycin treated cells infected with EMC virus showed that a rapidly-sedimenting, radioactive component appeared about 4 hr. after infection. No such fraction could be demonstrated in uninfect controls. Fractionation of the subcellular components of cells infected with EMC virus, followed by extraction and analysis of the RNA, showed that the RNA synthesised in the presence of actinomycin D was associated with the cytoplasmic components of the cell. No evidence was obtained for the occurrence of either a double-stranded RNA or of detectable amounts of an RNA complementary in base composition to viral RNA during the replication cycle.

Some preliminary experiments performed to examine polyribosomes in Krebs II ascites tumour cells showed that

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Thesis presented for the degree of

Doctor of Philosophy,

The University of Glasgow

April, 1964

after the cells were incubated in a buffered salt solution (Earle's medium), polyribosomes could no longer be demonstrated.

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ACKNOWLEDGEMENTS

I am most indebted to Professor J. N. Davidson for the opportunity to carry out this work and for the excellent facilities available. I express my sincere gratitude to Dr. R. M. S. Smellie for invaluable help and guidance in the course of this research. I am most grateful to Dr. M. J. Cline, of the Department of Medicine, Salt Lake General Hospital, Salt Lake City, Utah, U.S.A., for the stimulation he provided and for fruitful collaboration during part of this work. I also wish to thank Miss Sheena Bowcott for efficient and extensive technical assistance. I am grateful to Mr. D. Breese, Department of Virology, for assistance in the initial stages of this work. I am indebted to Mr. H. J. Gray for proof reading and for his help in general. I would also like to thank Miss Sheila Simpson for typing.

I acknowledge with thanks the receipt of a Medical Research Council Scholarship during the first two years of this work.

ABBREVIATIONS

The following abbreviations will be used in this thesis:

DNA	Deoxyribonucleic acid.
RNA	Ribonucleic acid.
sRNA	Soluble RNA or transfer RNA.
AMP	Adenosine 5'-monophosphate.
3'(2')AMP	Adenosine 3'(2')-monophosphate.
ATP	Adenosine 5'-triphosphate.
CMP	Cytidine 5'-monophosphate.
3'(2')CMP	Cytidine 3'(2')-monophosphate.
CTP	Cytidine 5'-triphosphate.
GMP	Guanosine 5'-monophosphate.
3'(2')GMP	Guanosine 3'(2')-monophosphate.
GTP	Guanosine 5'-triphosphate.
UMP	Uridine 5'-monophosphate.
3'(2')UMP	Uridine 5'(2')-monophosphate.
UTP	Uridine 5'-triphosphate.
dATP	Deoxyadenosine 5'-triphosphate.
dCTP	Deoxycytidine 5'-triphosphate.
dGTP	Deoxyguanosine 5'-triphosphate.
TMP	Thymidine 5'-monophosphate.
TTP	Thymidine 5'-triphosphate.
(^{32}P)UMP	Uridine 5'-monophosphate labelled with a radioactive phosphorus atom.

$(\alpha\text{-}^{32}P)$UTP	Uridine 5'-triphosphate labelled with a radioactive phosphorus atom in the α position.
(^{32}P)TMP	Thymidine 5'-monophosphate labelled with a radioactive phosphorus atom.
$(\alpha\text{-}^{32}P)$TTP	Thymidine 5'-triphosphate labelled with a radioactive phosphorus atom in the α position.
CEP	2-cyanoethyl phosphate.
(^{32}P)CEP	2-cyanoethyl phosphate labelled with a radioactive phosphorus atom.
DCC	Dicyclohexylcarbodiimide.
DCU	Dicyclohexylurea.
tris	2-amino-2-hydroxymethylpropane-1,3-diol.
tris-HCl buffer	tris buffer adjusted to the required pH with concentrated HCl.
EDTA	Ethylenediamine tetraacetic acid.
HMC	5-hydroxymethylcytosine.
HMC-DNA	DNA containing 5-hydroxymethylcytosine in place of cytosine.
$NADPH_2$	Reduced nicotinamide-adenine dinucleotide phosphate.
TCA	Trichloroacetic acid.
PBS	Phosphate-buffered saline.
TMV	Tobacco mosaic virus.
TMV-RNA	RNA from tobacco mosaic virus.
EMC	Encephalomyocarditis.

ME	Mouse encephalomyelitis.
NDV	Newcastle disease virus.
poly A, etc.	Polyribonucleotide stands containing only adenylate residues; similarly for the other synthetic polyribonucleotides mentioned in the text.
pCpCpA	A trinucleotide sequence with a 5'-phosphate terminal group.
A-T, etc.	An adenine residue linked to a thymine residue by hydrogen bonds; similarly for the other base-pairs referred to in the text.

CONTENTS

Experimental

INTRODUCTION

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

1. The development of studies on the mechanism of virus infection

From the time of their discovery, when the Russian botanist Ivanowski (1892) observed that a filter-passing agent from tobacco plants having a typical mosaic disease was capable of infecting healthy plants, the viruses were something of an enigma. The nature of the disease-causing agent was not at first clear and it was commonly believed that the factors were completely soluble, or, since they failed to grow on media known to support many forms of microorganism, that the viruses were an extreme form of microorganism unable to reproduce in the normal way because of some restriction imposed by their small size. Since it is now known that the size of the viruses ranges from 20 mμ to 300 mμ, such confusion is not surprising. This uncertain situation persisted until the 1930's when tobacco mosaic virus was obtained in sufficient amount to enable the particles to be characterised (Stanley, 1935).

The virus particle, or virion, is composed of a protein coat, or capsid, comprised of subunits, or capsomeres, which surrounds the genetic material (Lwoff, Anderson & Jacob, 1959). They do not possess the enzymes required for intermediary metabolism and energy production and are therefore obligatory cell parasites. On infection, the virus particle enters the cell where it gives rise to the formation of new virus particles which are ultimately liberated into the environment. Within recent years, a very great effort has been expended to elucidate the mechanism

of intracellular replication of viruses from widely differing sources.

In 1928, Griffith observed that mice, which had been injected with an attenuated, unencapsulated strain of pneumococci along with a virulent, encapsulated strain which had been killed by heating, developed pneumococcal peritonitis from which the encapsulated, virulent strain could be cultured. Some factor in the dead cells had therefore "transformed" the harmless organisms into the virulent type. Efforts to identify the transforming factor demonstrated that it was sensitive to treatment with deoxyribonuclease, gave a positive diphenylamine reaction for deoxyribonucleic acid (DNA) and exhibited other properties which proved decisively that it was DNA (Avery, MacLeod and McCarty, 1944). Transformation of many types of bacteria has confirmed the process of genetic transfer by purified preparations of DNA (Hotchkiss, 1952).

A great body of evidence is available to confirm the genetic importance of DNA.

DNA is metabolically more stable than other components of the cell (Brown, Roll, Flentl & Cavalieri, 1948; Furst, Roll & Brown, 1950), it is a principle component of the chromosomes (Mirsky & Pollister, 1942; Mirsky & Ris, 1950) and the DNA content of virtually all cells is constant (Boivin, Vendrely & Vendrely, 1948). When microorganisms are irradiated with ultraviolet light, the production of mutant forms is greatest at that wavelength which is absorbed by the nucleic acids (Emmons & Hollaender, 1939; Hollaender & Emmons, 1941; Kaplan, 1932). In the process of bacterial mating by strains of Escherichia coli, DNA

has been implicated in transference of genetic characteristics (Jacob & Wollman, 1955, 1956).

From the point of view of virus replication, the important and definitive work performed by Hershey and Chase (1952; review by Hershey, 1957) showed that DNA of T2 bacteriophage is the genetic determinant of all the phenotypic characters of the mature virus particles. This has been confirmed with other bacteriophages such as T5 (Luria & Steiner, 1954; Lanni, 1954) and ØX 174 (Sinsheimer, 1959a,b). When T2 bacteriophage, which has been cultivated in E. coli cells which can synthesise thymine, infects a thymineless mutant of E. coli, it can induce the bacterial host to form thymine (Barner & Cohen, 1954). The acquisition of metabolic function is a result of the transduction of genetic material by the bacteriophage from one host cell to the other.

Another basic landmark in determining the importance of the nucleic acids in viral infection came from the discovery that ribonucleic acid (RNA) prepared from tobacco mosaic virus could itself produce infective lesions (Gierer & Schramm, 1956). If the tobacco mosaic virus particle is treated with alkali, groups of capsomeres become detached from the rod-shaped virus structure. When the pH is reversed, the capsomeres reaggregate and reconstitute the rod (Fraenkel-Conrat & Singer, 1957). If the nucleic acid and protein constituents are separated from one another after treatment with alkali and mixed with protein and nucleic acid respectively from another strain of tobacco mosaic virus which has been similarly treated, stable rods are formed. The progeny of the

reconstituted rods exhibit the genetic specificity of the virus from which the nucleic acid had been derived.

In recent years, infective viral RNA has been extracted from tissues infected with animal viruses and from the viruses themselves, as in the case of Mengo encephalitis virus, poliomyelitis virus (Colter, Bird & Brown, 1957), equine encephalomyelitis virus (Wecker, 1959), encephalomyocarditis virus (Huppert & Sanders, 1958) and foot and mouth disease virus (Brown, Sellers & Stewart, 1958). Therefore RNA must also be capable of transmitting genetic information.

Similarly, the infectivity of DNA extracted from rabbit papilloma virus (Ito, 1961), polyoma virus (di Mayorca et al., 1959), Simian virus (Gerber, 1962) and the bacteriophages ØX 174 (Guthrie & Sinsheimer, 1960), T1 (Evans, Mackal & Coleman, 1962) and λ (Meyer, Mackal, Tao & Evans, 1961) has been demonstrated. In each case, the progeny virus particles were found to be indistinguishable from the virus from which the infective nucleic acid had been derived.

The role which viruses play in the field of molecular biology has been compared to the role of the beams of particles employed by physicists to probe into the organisation of atoms and nuclei as a tool of the greatest power in analysing cellular organisation (Luria, 1959). It is probably true to say that the insight one gains into a problem is a function of the methods available for its study, and it can be clearly seen that the importance of virology today is attributable to the precision introduced into experimentation by the remarkable technological

and methodological advances of recent years. A minor branch of bacteriology a few decades ago, virology has developed and expanded to embrace and integrate biophysics, biochemistry and genetics and has assumed a central position in molecular biology. To investigate viral growth, a present day biochemist must study simultaneously a range of biochemical problems each expanding rapidly in its own right.

2. The structure of the nucleic acids

1. Deoxyribonucleic acid

DNA exists intracellularly in combination with protein (Chargaff, 1955) and, by taking careful precautions to avoid breakdown, has been isolated with a molecular weight of 5-10 million (Cavalieri, Rosenberg & Deutsch, 1959; Cavalieri, Finston & Rosenberg, 1961; Davison, 1959; Zubay & Doty, 1959). T2 and T4 bacteriophage deoxyribonucleic acids have been isolated with molecular weights of about 130 x 10^6 (Levinthal & Davison, 1961).

The basic components of DNA are an organic base, a sugar component and phosphoric acid. The sugar, which has been identified as 2-deoxyribose, and the phosphate group occur in equal numbers and are arranged in long chains consisting of alternate phosphate groups and sugar groups. The organic bases, of which there are commonly four kinds, guanine, adenine, cytosine and thymine, are attached to the sugar molecules (Fig. 1). Other bases, 5-methylcytosine and 5-hydroxymethyl cytosine, also occur in DNA, the latter replacing cytosine in the DNA of T-even coliphages

FIGURE 1

A section of the polynucleotide chain in the DNA molecule.

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

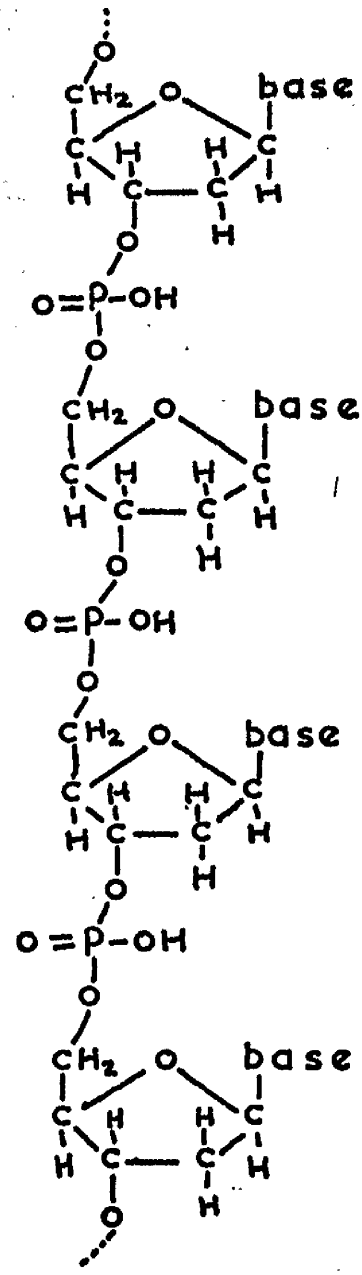


FIGURE 1.

(Wyatt & Cohen, 1953).

It was first considered that the four main bases were present in equal amounts. However, the molar proportion of bases vary according to the origin of the DNA. Analysis of samples of DNA from a variety of sources has revealed that there is an equivalent amount of adenine and thymine and of guanine and cytosine (Chargaff, 1950, 1951). The reason for this became clear when it was discovered that DNA was made up of two strands in which adenine on one strand was paired with thymine on the other and guanine was similarly paired with cytosine (Fig. 2). The two strands are wound around each other to form a double helix (Wilkins, Stokes & Wilson, 1953; Watson & Crick, 1953), consisting of ten nucleotides in each turn of the helix, and are held together by the hydrogen bonds of the adenine-thymine (A-T) and guanine-cytosine (G-C) base pairings (Fig. 3). The constituent polynucleotide chains of the double helix are arranged in an antiparallel manner so that there is a narrow and a deep groove running up the length of the molecule. Deoxyribonucleic acids isolated from a very wide range of organisms, although varying widely in base composition, appear to exist in the form of double helices (Hamilton et al., 1959).

As a consequence of this structure, the order in which the bases occur in one chain automatically determines the order in the other chain which is its complement. Support for the double-stranded nature of DNA comes from the study of thermal denaturation of DNA. When DNA is heated and cooled rapidly, single-stranded DNA is formed with half the

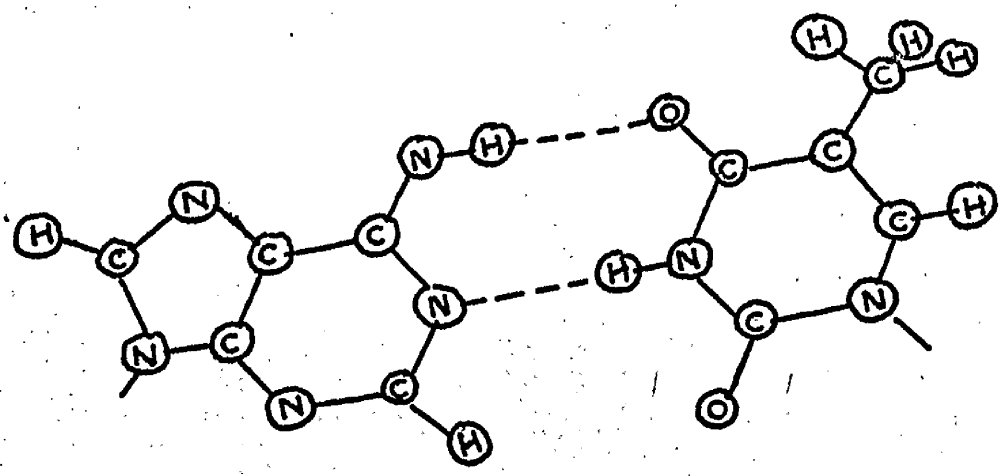
FIGURE 2

The pairing of adenine and thymine and of guanine and cytosine. Dotted lines indicate the hydrogen bonds.

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

Adenine

Thymine



Guanine

Cytosine

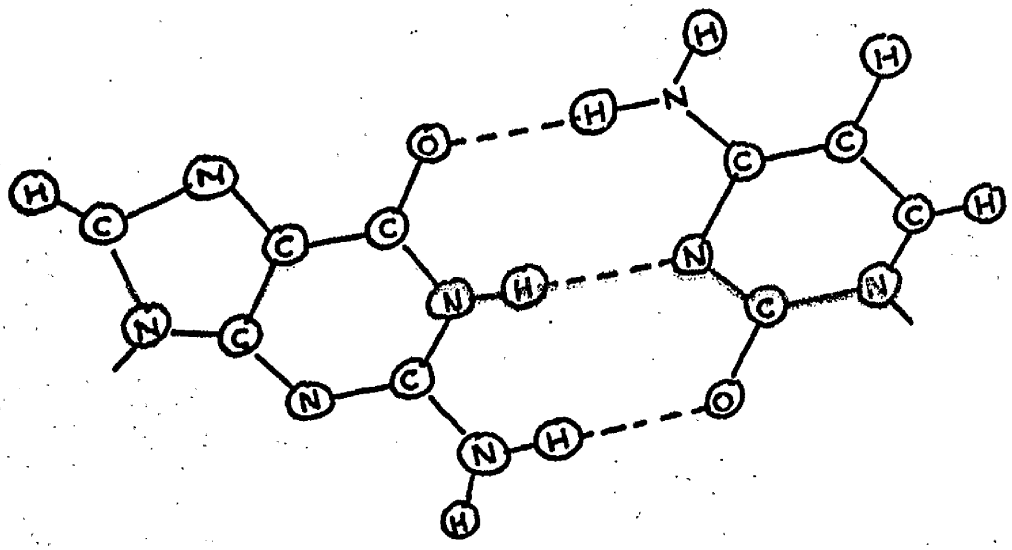


FIGURE 2

FIGURE 5

A model of the structure of DNA.

(After Feughelman, M., Langridge, R., Seeds, W. E., Stokes, A. R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., Barclay, R. K. & Hamilton, L. D. (1955). Nature, Lond., 175, 834.).

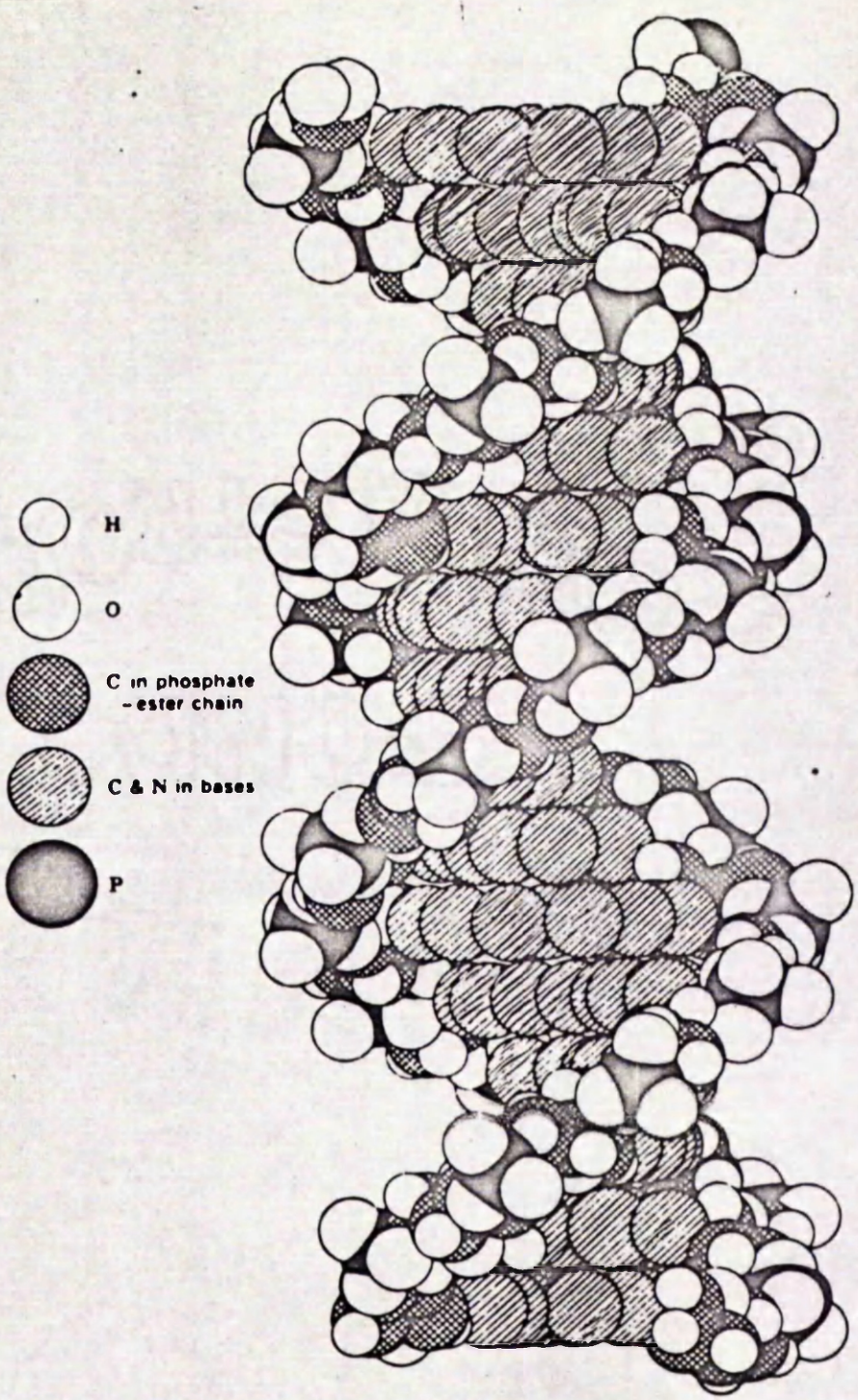


FIGURE 3.

molecular weight of the original DNA. On slow cooling, the single strands link by complementary base pairing to form renatured DNA (Doty, Marmur, Eigner & Schildkraut, 1960).

The double helix is not universal since the DNA from bacteriophage ØX 174 was found to be single-stranded (Sinsheimer, 1959a,b).

The sequence in which the bases are arranged within the polynucleotide chain has not yet been determined because of the complexity of the problems involved.

An unusual DNA which contains uracil in place of thymine, but which has a DNA-like structure, has been observed in Bacillus subtilis bacteriophage PBS 2 (Takahashi & Marmur, 1963).

2.2. Ribonucleic acid

It was originally considered that the nucleic acids were confined to the nucleus of the cell, but histochemical methods demonstrated that there was present in the cytoplasm a nucleic acid component which proved to be RNA (Caspersson & Schultz, 1938, 1939). Two species of RNA, ribosomal RNA and soluble RNA (sRNA), have been identified in the cytoplasm while in the nucleus, although the picture is not quite so well defined, RNA has been associated with the nucleolus, the chromatin material and the nuclear sap.

In RNA, the pentose sugar is ribose which alternates with a phosphate group to form a long chain as in DNA. The constituent bases of RNA are mainly adenine, guanine, cytosine and uracil, but, especially in sRNA, there are additional components such as 5-methylcytosine, 6-methylaminopurine

1-methylguanine and 2-methylamino-6-hydroxypurine. The nucleoside 5-ribosyluracil, or pseudouridine, has also been identified as a minor constituent.

The precise secondary structure of RNA has not been determined as in the case of DNA (Rich & Watson, 1954). However, amino acid transfer RNA isolated from yeast has recently been shown to possess a double-stranded helical structure similar to, but not precisely the same as, that of DNA (Spencer, Fuller, Wilkins & Brown, 1962). Furthermore, ribonucleic acids from other sources, such as ribosomal RNA and viral RNA, appear to have regions which possess a similar structure. It has been demonstrated that tobacco mosaic virus RNA, high molecular weight cellular RNA and sRNA consist of single polynucleotide chains (Gierer, 1957, 1958; Ginoza, 1958; Gavrilova & Spirin, 1959; Hall & Doty, 1959; Habermann, 1960; Allen, Glassman, Cordes & Schweet, 1960; Singer & Cantoni, 1960). The secondary structure of the RNA molecules therefore arises from interaction within the chain by hydrogen bonding between guanine-cytosine and adenine-uracil pairs forming numerous short helices.

3. The biochemical events in bacteriophage infection

1. Introduction

Until quite recently, no reliable methods were available to study the replication of animal viruses since a system was required which could be manipulated under precisely controlled conditions. Such a system was available in bacteriophage infection of *E. coli*. The mechanisms

of virus infection were therefore elucidated originally in this system which is now regarded as the model for problems in virus infection. We shall consider the infection of E. coli by the virulent coliphages, that is, by those bacteriophages which produce an infection that is invariably followed by lysis of the host bacterium and liberation of progeny bacteriophage particles.

3.2. The replication of T-even bacteriophages

The T coliphages are composed of approximately equal amounts of DNA and protein and contain neither RNA or lipid (see Stent, 1958). The DNA, together with a little protein, is enclosed within a protein envelope to form the head of the bacteriophage (Hershey, 1955) to which is attached a structurally complicated tail (Fig. 4) (Brenner et al., 1959). Infection is initiated by the tail of the bacteriophage adhering to the cell-wall of the host bacterium. A bacteriophage enzyme is then released which acts on the molecular groupings of the cell wall to open a gate for infection (Weidel & Primosigh, 1958; Barrington & Kozloff, 1954).

The classical "blendor" experiment of Hershey and Chase (1952) illustrated that the whole bacteriophage particle does not enter the host cell. T2 bacteriophage particles labelled either with phosphorus-32 exclusively in the DNA or with sulphur-35 only in the protein were prepared. A culture of E. coli B was then infected with one or other of the labelled bacteriophage preparations and the attached particles removed by violent agitation in a blendor. It was found by examining

FIGURE 4

A representation of the structure of bacteriophage T2.

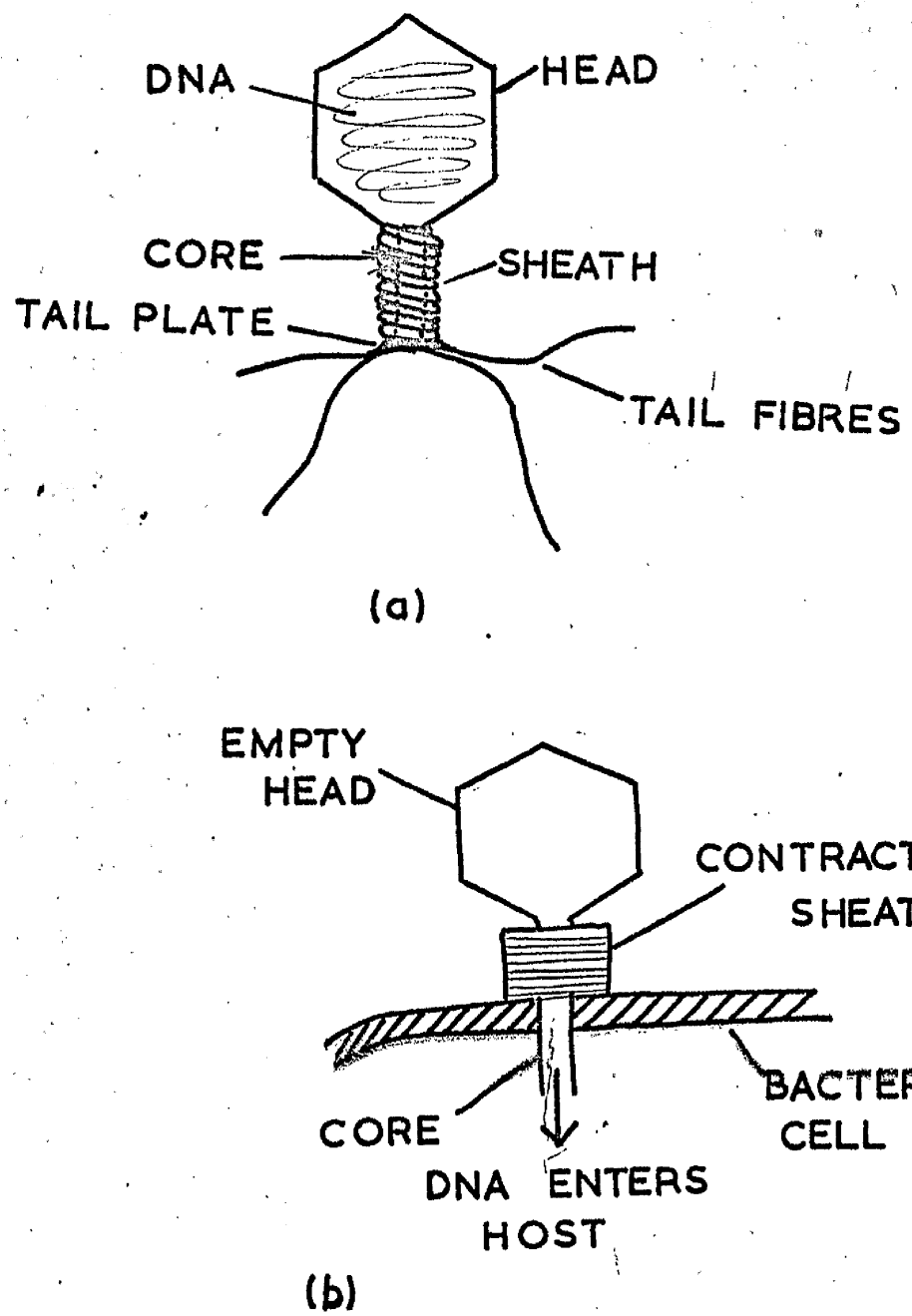

DNA
HEAD
CORE
SHEATH
TAIL PLATE
TAIL FIBRES
(a)
EMPTY HEAD
CONTRACTED SHEATH
BACTERIAL CELL WALL
CORE
DNA ENTERS HOST
(b)

FIGURE 4.

the distribution of ^{32}P and ^{35}S between the cells and the medium that most of the ^{35}S could be removed from the infected cells but that very little of the ^{32}P was liberated by the blender treatment. It is therefore evident that the bacteriophage DNA is injected into the bacterium while its protein coat remains outside. This conclusion has since been confirmed by electron micrographs of infected cells which show that the heads of many of the attached particles are empty (Anderson, 1953).

The standard procedure for studying the kinetics of bacteriophage multiplication in infected bacteria is the one-step growth experiment of Ellis and Delbrück (1939). Bacteriophage particles are added to the host organism, allowed to adsorb for a few minutes then bacteriophage antiserum is added to remove unadsorbed particles. The mixture is then diluted with warm medium to prevent progeny bacteriophage particles adsorbing to other bacteria and incubated at 37°. Samples are withdrawn at intervals from an infected culture and plaque counts are performed to determine the number of plaque-forming units present. A latent phase of some twenty-five minutes occurs during which the plaque count remains constant, thereafter the plaque count rises sharply until a plateau is reached 35-45 minutes after infection. The latent phase represents the shortest time that elapses between infection and bursting of the host to liberate progeny bacteriophage particles and the rise in plaque titre is due to progressive bursting of the infected cells until a plateau is reached when all the progeny have been released.

If infected cells are artificially lysed at intervals after infection

(Doermann, 1952), infective particles are not detectable until about twelve minutes after infection, that is, until about halfway through the latent period. This interval is called the eclipse phase (Wollman & Wollman, 1957). Study of the eclipse phase has produced results of great significance in molecular biology.

3.3. The discovery of "messenger" RNA

A fundamental problem in biology is the way in which the information contained in the DNA is made available and functionally expressed in the cell. Such mechanisms have been elucidated by studying the way in which the invading DNA of bacteriophage particles affects host cell metabolism and directs the synthesis of bacteriophage components.

Following infection with bacteriophage, the normal high rate of bacterial RNA synthesis is rapidly arrested and measurements of the incorporation of ^{32}P-orthophosphate have demonstrated the synthesis of new RNA with a high rate of metabolic turnover. Furthermore, the base composition of this new RNA was found to be complementary to that of the infecting bacteriophage DNA (Volkin & Astrachan, 1956; Volkin, Astrachan & Countryman, 1958; Astrachan & Volkin, 1958; Astrachan & Fisher, 1961; Nomura, Hall & Spiegelman, 1960). The complementary nature of the newly synthesised RNA was confirmed by hybrid formation with T2 DNA (Hall & Spiegelmen, 1961; Spiegelman, Hall & Storck, 1961).

This RNA therefore corresponds to the "messenger" RNA proposed by Jacob and Monod (1961) as an agent for the transfer of genetic information from DNA to the site of protein synthesis.

3.4. The genetic code and protein synthesis

Since the RNA chain consists of arrangements of only four nucleotides, some form of code is necessary to transcribe the information contained in the sequence of bases in the polynucleotide chain into sequences of the twenty or so amino acids in the protein chain. Such a code in which a group of three bases codes one amino acid has been described (Crick, Barnett, Brenner & Watts-Tobin, 1961) and extended (Nirenberg & Matthaei, 1961; Lengyel, Speyer & Ochoa, 1961; Speyer, Lengyel, Basilio & Ochoa, 1962; Wahba et al., 1963; Jukes, 1963) so that it is now considered to be universal (Crick, 1963). Stachelin, Wettstein, Oura and Noll (1964) have provided direct and unequivocal experimental evidence that the coding ratio is three.

The amino acid to be incorporated into protein is first activated by ATP (Hoagland, Zamecnik & Stephenson, 1957) and transferred to a specific transfer RNA (sRNA) molecule by a specific enzyme (Hoagland, Stephenson, Scott, Hecht and Zamecnik, 1958; Berg & Ofengand, 1958). After this step, the role of sRNA in the synthesis of proteins is that of translator from the nucleotide code to the amino acid code, the "adaptor" suggested by Crick (1958).

It was considered for many years that the ribosomes were the site of protein synthesis, but this concept has now been extended. When T2 bacteriophage specific RNA is attached to host cell ribosomes (Brenner, Jacob & Meselson, 1961), heavy ribosomes are observed (Risebrough, Tissières & Watson, 1962). Similar structures have been

observed in rabbit reticulocytes and in rat liver and have been given the name of polyribosomes or ergosomes (Warner, Knopf & Rich, 1963; Wettstein, Staehelin & Noll, 1963). Ribosome monomers are thought to attach to one end of a messenger RNA strand and then move along it as the polypeptide chain lengthens (see Gilbert, 1963). The number of ribosomes which can attach at any one time depends on the length of the messenger RNA strand.

5.5. The appearance of bacteriophage precursors and assembly of mature bacteriophage

The DNA of T-even bacteriophages is unique in containing 5-hydroxymethylcytosine (HMC) in place of cytosine (Wyatt & Cohen, 1953) so that synthesis of bacteriophage DNA can readily be followed. In the first few minutes after infection a class of new proteins is synthesised. These proteins differ from the host cell proteins and the bacteriophage coat protein and are made up of a range of enzymes, including deoxycytidine-5'-phosphate hydroxymethylase (Flaks & Cohen, 1957, 1958), hydroxymethyldeoxycytidine-5'-phosphate kinase, deoxycytidine triphosphatase and an enzyme involved in the direct glucosylation of HMC-DNA (Kornberg, Zimmerman, Kornberg & Josse, 1959), which are necessary for the synthesis of bacteriophage DNA. About halfway through the eclipse phase, that is, six minutes after infection, the synthesis of bacteriophage DNA begins, but mature virus particles do not appear until more than fifty bacteriophage equivalents of DNA have been produced (Hershey, Dixon & Chase, 1956; Vidaver and Kozloff, 1957). Bacteriophage protein first appears in the

infected cell about nine minutes after infection, that is, just after the appearance of DNA and just before the production of infective particles (Maaløe & Symonds, 1953).

Following assembly of the mature bacteriophage, the host cell is lysed by an enzyme under the control of the bacteriophage genome and the bacteriophage particles are liberated into the environment (Streisinger, Mukai, Dreyer, Miller & Harrar, 1961).

It is apparent that the bacteriophage components are synthesised separately and then withdrawn randomly from the pools for assembly of the mature virus. This concept is supported by experiments on phenotypic mixing. If E. coli B is infected simultaneously with bacteriophage T2 and T4 and the progeny isolated, it can be shown that about half the particles which possess the protein tail structure (phenotype) of bacteriophage T2 display the genetic constitution (genotype) of bacteriophage T4 (Novick & Szilard, 1951; Delbrück & Bailey, 1946).

3.6. Application of the bacteriophage model to problems of infection by animal viruses

The well defined conditions employed in the study of bacterial viruses were largely responsible for the rapid progress made in this field. Similar studies on the replication of animal viruses have now become much more feasible through the development of tissue culture techniques and it now seems clear that an eclipse phase is a general characteristic of the multiplication of animal viruses. Moreover, the methods which have proved so successful in studying bacteriophage replication, such as one-step

growth experiments (Ellis & Delbrück, 1939) and the agar overlay technique for producing discrete plaques on cell monolayers (Dulbecco, 1952), have been applied to investigating animal viruses. As a result, the mechanisms of replication of animal viruses are beginning to be elucidated.

4. The biosynthesis of deoxyribonucleic acid

Investigation of DNA biosynthesis *in vitro* demonstrated that an enzyme preparation (DNA nucleotidyltransferase) from *E. coli* would incorporate deoxyadenosine triphosphate, deoxyguenosine triphosphate, deoxycytidine triphosphate and thymidine triphosphate into polydeoxyribonucleotide material, liberating four equivalents of inorganic pyrophosphate (Fig. 5) (Lehman, Bessman, Simms & Kornberg, 1958; Bessman, Lehman, Simms & Kornberg, 1958; Adler, Lehman, Bessman, Simms & Kornberg, 1958). The purified enzyme required the presence of deoxyribonucleoside triphosphates and Mg^{2+} ions. A similar enzyme has been described in extracts of mammalian cells (Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959; Davidson, Smellie, Keir & McArdle, 1958; Smellie, Keir & Davidson, 1959; Smellie *et al.*, 1960).

The Watson-Crick structure of DNA provides a model whereby the conservation of the genetic message contained in the sequence of bases in the DNA chain can be envisaged in the polymerisation reaction. If the twin strands of the primer molecule separate to give the two complementary strands, then, by forming adenine-thymine and guanine-cytosine base pairs, two new complementary strands can be produced by the

FIGURE 5

The mechanism of DNA biosynthesis.

(After Lehman, I. R., Bessman, M. J., Simms, E. S. & Kornberg, A. (1958). J. biol. Chem., 233, 163).

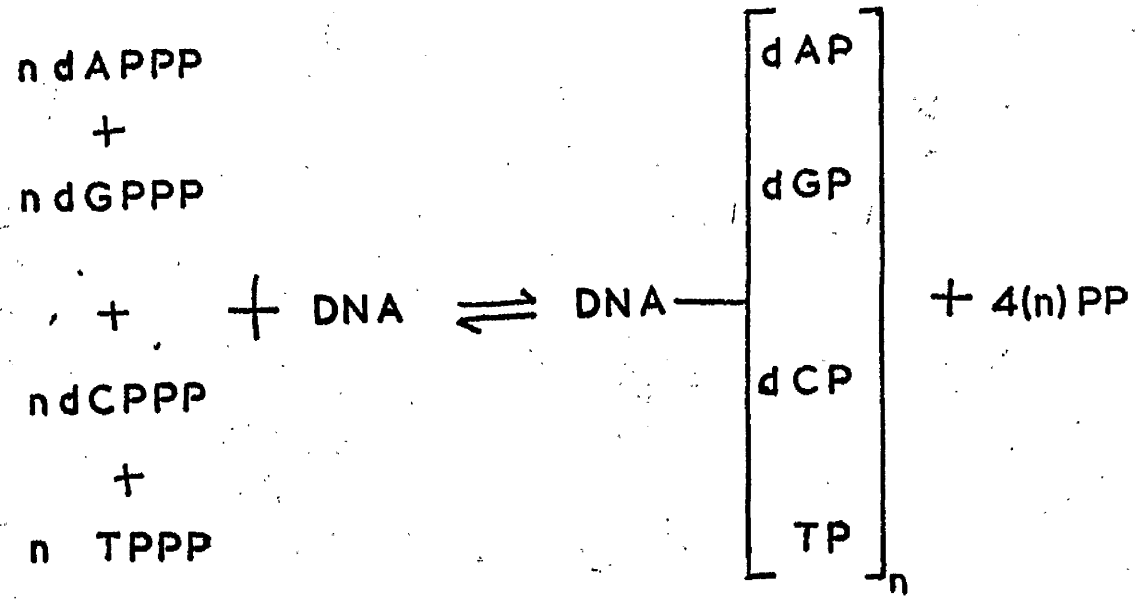


FIGURE 5.

polymerising enzyme. This mechanism of DNA replication is termed "semi-conservative" replication. The formation of complementary strands by a semi-conservative mechanism has been demonstrated with E. coli DNA nucleotidyltransferase and dAT copolymer as primer (Weke & Baldwin, 1962).

Evidence that this mechanism operates in vivo comes from the work of Meselson and Stahl (1958). E. coli cells were grown in a medium heavily labelled with nitrogen-15 and transferred to a medium containing $^{14}NH_4Cl$. Samples of cells were withdrawn at intervals, the DNA extracted and examined by sedimentation equilibrium ultracentrifugation. DNA isolated from cells immediately prior to their transfer to unlabelled medium formed one single band corresponding to the ^{15}N-labelled DNA in the caesium chloride density gradient. Thereafter, molecules containing half this amount of ^{15}N began to appear until after one generation time, only half-labelled molecules were present. After two generations, half-labelled molecules and unlabelled molecules were present in equal amount.

These experiments clearly demonstrate that the parent DNA molecule is split into two units which remain intact, each daughter molecule receiving one parental subunit. The possibility that the two units are associated end-to-end instead of side-to-side has been excluded by investigating sonic fragments of ^{13}C-^{15}N-DNA (Rolfe, 1962).

Replication of the genome of bacteriophage λ occurs in a semi-conservative manner (Meselson & Weigle, 1961; Kellenberger, Zichichi & Weigle, 1961).

5. The biosynthesis of ribonucleic acid

1. In vitro synthesis

1. General

It is difficult to differentiate precisely between the types of RNA synthesis described below and some degree of overlap is unavoidable. The evidence from *in vitro* systems serves to indicate the potentialities of the different systems but provides relatively little indication of the interrelationships between them.

5.1.2. The formation of terminal sequences

The pioneering studies on the formation of polyribonucleotide chains were performed with soluble enzyme preparations from rat liver homogenates. It was first observed by Heidelberger, Harbers, Liebman, Takagi and Potter (1956) that ^{32}P-labelled AMP residues could be incorporated into RNA by a cytoplasmic fraction from rat liver. Alkaline hydrolysis of the product, which allows the ^{32}P originally attached to the 5'-hydroxyl group of the adenosine residue to be recovered on the 2'(3')-hydroxyl groups of the adjacent nucleoside residues, revealed that most of the radioactivity was associated with 2'(3')-CMP. These experiments demonstrated that most of the ^{32}P-AMP residues were incorporated adjacent to CMP units in the polyribonucleotide chain.

The first attempts to purify the enzyme involved precipitation at pH 5.2 of a fraction from the 105,000 g. supernatant material of rat liver homogenate. Incubation of this fraction with ^{14}C-ATP indicated that 90 per cent of the AMP residues were located in terminal positions of the

polyribonucleotide chain since hydrolysis with alkali yielded principally adenosine (Zamecnik, Stephenson, Scott & Hoagland, 1957; Paterson & LePage, 1957; Edmonds & Abrams, 1957). Herbert (1958) showed that, in general, the enzymes which incorporate AMP residues from ATP into non-terminal positions are associated with the particulate fractions of the cell whereas those responsible for terminal addition to a specific type of RNA are present in the soluble portions of the tissue homogenate. The enzyme system that incorporated AMP residues will also incorporate CMP units into both terminal and non-terminal positions of RNA, but when both ATP and CTP are present, most of the CMP residues are incorporated in non-terminal positions (Hecht, Stephenson & Zamecnik, 1958; Hecht, Zamecnik, Stephenson & Scott, 1958; Herbert, 1958). Both AMP and CMP residues are incorporated into RNA by this system with the liberation of inorganic pyrophosphate, and the terminal groups of the soluble RNA of rat liver can be cleaved with pyrophosphate to produce ATP. A similar pyrophosphorolysis of RNA giving rise to ATP has been demonstrated in extracts of embryonic chicken liver (Chung, 1958; Chung & Mehler, 1958). Further investigation has revealed that the RNA of these fractions accept the nucleotide residues in a specific manner to give the terminal sequence pCpCpA (Hecht, Stephenson & Zamecnik, 1959; Canellakis, 1957; Canellakis & Herbert, 1960a, 1961; Herbert & Canellakis, 1961; Preiss, Dieckmann & Berg, 1961; Furth, Hurwitz, Krug & Alexander, 1961). GMP and UMP, as well as CMP and AMP, can be linked to the ends of pre-existing RNA chains by these enzyme systems but to a much smaller degree (Hecht, Zamecnik,

Stephenson & Scott, 1958; Harbers & Heidelberger, 1959; Canellakis & Herbert, 1960b). The RNA taking part in these reactions is the low molecular weight, soluble RNA and it has been established that this RNA with the terminal sequence pCpCpA accepts amino acids from the amino acid-activating enzyme complex.

5.1.3. RNA-dependent synthesis of ribonucleic acid

Many enzymes which exhibit a requirement for a polyribonucleotide primer catalyse the addition of nucleotide units to the end of the primer chain and, as a rule, only one or two types of nucleotide are involved in these reactions.

An enzyme from Micrococcus lysodeikticus has been described by Nakamoto and Weiss (1962) which catalyses the incorporation of ribonucleotides from ribonucleoside triphosphates into RNA in the presence of tobacco mosaic virus RNA. This enzyme is also stimulated by polycytidylic acid, poly CA, poly AUC and poly CU primers, the products being polyguanylic acid, poly GU, poly UAG and poly GA respectively. The formation of polyriboadenylate chains by an enzyme from E. coli has been described by Moldave (1960). A similar enzyme has been purified 300-400-fold from E. coli by August, Ortiz and Hurwitz (1962). This enzyme catalyses the RNA-dependent incorporation of ribonucleotides producing principally poly A and also an exchange of ^{32}P-pyrophosphate with ribonucleoside triphosphates.

In animal tissues a number of similar systems has been reported.

An enzyme has been described in calf thymus that incorporates CMP

residues from CTP into the terminal position of thymus RNA in the sequence pCpA (Hurwitz & Bresler, 1961), and for which the primer requirement is highly specific. The system is primed to some extent by E. coli sRNA suggesting that the enzyme system is a fragment of that responsible for forming the pCpCpA sequence. Extracts of calf thymus nuclei catalyse the synthesis of poly A and appear to depend on a poly A primer (Edmonds & Abrams, 1960a,b, 1962). Polymerisation of ATP has also been described by an enzyme from chorioallantoic membrane which uses either poly A+ poly U or microsomal RNA as primer (Venkataraman & Mahler, 1963). In preparations of nuclear ribosomes from Lendschutz ascites-tumour cells, ATP uptake is stimulated by poly A while UTP uptake is stimulated by poly U (Burdon, 1963). Similarly, an enzyme from the 105,000 g. supernatant fraction of rat liver incorporates UMP residues into poly U and is stimulated by poly U, poly C and rat liver sRNA. A similar Mn^{2+}-dependent enzyme from rat liver incorporates AMP residues into polyriboadenylate chains (Klemperer, 1963a,b). Enzyme systems have been described in rat liver microsomes (Klemperer & Kammen, 1962) pigeon liver microsomes (Straus & Goldwasser, 1961) and Ehrlich ascites-tumour cells (Burdon & Smellie, 1961a,b; 1962) which incorporate UMP residues into RNA and which require the addition of RNA primers. The requirement for RNA has been most clearly demonstrated by Burdon and Smellie who find that RNA prepared from the cytoplasm, mainly microsomal RNA, of the ascites cells promotes the reaction but RNA derived from the cell nuclei is a much more potent primer.

5.1.4. DNA-dependent synthesis of ribonucleic acid

A particulate enzyme named RNA-nucleotidyltransferase (RNA polymerase) has been demonstrated in rat liver by Weiss and Gladstone (1959; Weiss, 1960). This enzyme requires the presence of all four ribonucleoside triphosphates and primer DNA for the incorporation of any one ribonucleotide into RNA. Enzymes with similar properties have been described by several authors (Weiss & Nakamoto, 1961; Hurwitz, Bresler & Diringer, 1960; Furth, Hurwitz & Goldmann, 1961a,b; Stevens, 1960, 1961; Ochoa, Burma, Krüger & Weill, 1961; Burdon & Smellie, 1962; Abrams, Edmonds & Biswas, 1962) showing, in general, an absolute requirement for DNA.

The DNA used to prime the reaction determines both the over-all base composition and the base sequence of the RNA formed. Thus, dAT copolymer primes the synthesis of a polyribonucleotide composed of alternating units of AMP and UMP (Furth, Hurwitz & Goldmann, 1961a,b) and polyriboadenylate is formed in a reaction primed by polydeoxyribothymidylate (Hurwitz, Furth, Anders, Ortiz & August, 1961). When single-stranded DNA from the bacteriophage ØX 174 is used as primer, the proportion of bases in the RNA synthesised approximates to that of a complementary copy of the DNA (Chamberlin & Berg, 1962; Hurwitz, Furth, Anders & Evans, 1962). Determination of nearest neighbour base pairs of RNA produced with DNA primers further confirms this hypothesis (Weiss & Nakamoto, 1961; Hurwitz, Furth, Anders & Evans, 1962).

Further evidence of complementary sequence of bases in the RNA product of the RNA polymerase reaction has been obtained from studies on

the formation of DNA-RNA hybrids. Polyribonucleotide single chains can interact to produce two- and three-stranded helices (Davies & Rich, 1958; Rich, Davies, Crick & Watson, 1961) and, similarly, hybrid helices of polyribonucleotide and polydeoxyribonucleotide chains can be produced (Rich, 1960; Schildkraut, Marmur, Fresco & Doty, 1961). It is also possible to anneal naturally-occurring DNA and RNA provided the strands possess a certain degree of homology (Hall & Spiegelman, 1961). Complexes can be formed between the RNA product and the template DNA (Geiduschek, Nakamoto & Weiss, 1961).

RNA polymerase can utilise either single- or double-stranded DNA as primer. When double-stranded ØX 174 DNA is used, both strands serve equally well as primer since equivalent amounts of AMP and UMP and of CMP and GMP are incorporated (Hurwitz, Furth, Anders & Evans, 1962; Chamberlin & Berg, 1962).

Double-stranded DNA maintains its structural and functional integrity after acting in the RNA polymerase reaction. Native transforming factor DNA from B. subtilis loses no transforming activity after acting as primer in the polymerase reaction (Furth, Hurwitz & Anders, 1962) and the sedimentation characteristics of bacteriophage T2 DNA remain unchanged after recovery from an RNA polymerase reaction mixture (Geiduschek, Nakamoto & Weiss, 1961).

Krakow and Ochoa (1963) have observed that purified preparations of RNA polymerase from Azotobacter vinelandii, which normally utilise DNA as primer for the synthesis of RNA, are also stimulated by polyribonucleotides

such as poly A, poly U and poly C. This evidence suggests that a single enzyme may catalyse both DNA- and RNA-dependent incorporation of ribonucleotides into RNA.

5.2. In vivo synthesis of ribonucleic acid

1. General

Great progress has been made of recent years in elucidating the molecular and functional heterogeneity of cellular ribonucleic acid. The question is one of such complexity that it is convenient to consider each functional species of RNA separately to examine the mechanisms whereby RNA is synthesised in the cell.

An important recent development in the study of the biosynthesis of RNA has been the use of metabolic inhibitors and antimetabolites. For example, one of the most useful inhibitors has been meractinomycin (actinomycin D). This is a peptide-containing antibiotic produced by species of Streptomyces (Vining & Waksman, 1954) which binds with DNA to form a complex (Kirk, 1960; Rauen, Kersten & Kersten, 1960; Müller, 1962; Kersten & Kersten, 1962) and selectively inhibits the synthesis of RNA but not of DNA or protein (Reich, Franklin, Shatkin & Tatum, 1961, 1962; Hurwitz, Furth, Malamy & Alexander, 1962). An important part has also been played by recent technical developments, such as sucrose density gradients (McQuillen, Roberts & Britten, 1959), methylated serum albumin column techniques (Mandell & Hershey, 1960) and hybridisation methods (Hall & Spiegelman, 1961), in elucidating this problem.

5.2.2. Messenger RNA synthesis

The observation of messenger RNA by Volkin and Astrachan (1956) in bacteriophage-infected E. coli has been extended to uninfected cells. In normal E. coli a metabolically labile fraction of RNA, related in its base composition to the DNA of the cell, has been described by Gros et al. (1961a). The molecules of messenger RNA appear to be very heterogeneous in size (Ishihara, Mizuno, Takai, Otaka & Osawa, 1962; Monier, Naono, Hayes, Hayes & Gros, 1962). A similar fraction of messenger RNA has been reported in other micro-organisms (Yeas & Vincent, 1960; Gros et al., 1961b).

It now appears beyond question that messenger RNA is built on a DNA template and the interesting problem arises as to whether the information contained in the DNA molecule is transcribed from one strand or from both. In vitro experiments indicate that both strands can be copied (Geiduschek, Moohr & Weiss, 1962) and that useful messenger RNA is produced from a native DNA primer (Chamberlin & Berg, 1962; Wood & Berg, 1962). In the intact cell, however, the indications are that only one strand is active as a source of messenger RNA.

When the two strands of pneumococcal transforming DNA are separated at pH 12, it can be shown that one strand expresses its genetic information more rapidly than the other (Guild & Robison, 1963). It appears, therefore, that effective messenger RNA is copied from only one of the strands of the DNA, either of which may however serve to transform the cell.

In other experiments, the component polydeoxyribonucleotide strands of the DNA of the lysogenic bacteriophage c, which parasitises Bacillus megatherium, are sufficiently different to allow their separation into light and heavy strands (Cordes, Epstein & Marmur, 1961). The bacteriophage-specific RNA purified after a pulse label during virus infection fails to interact by hybridisation with the light strand but does interact with the complementary heavy strand (Tocchini-Valentini et al., 1963). Both light and heavy strands can serve as templates for the in vitro synthesis of RNA by RNA polymerase. The ribonucleic acids so produced can anneal by complementary base pairing with their respective DNA primers.

When the DNA from bacteriophage SP 8 (virulent for B. subtilis Marburg) is denatured and centrifuged in a caesium chloride density gradient, a light and a heavy band of DNA can be demonstrated. Both strands can serve as templates for E. coli RNA polymerase in vitro (Marmur & Greenspan, 1963). The light strand differs in base composition from the heavy strand, the latter being relatively rich in pyrimidines and the former rich in purines. When the bacteriophage-specific RNA is isolated from the infected host, it will only form hybrids with the heavy strand of DNA (Hayashi, Hayashi & Spiegelman, 1963). Further light has been cast on the origin of messenger RNA in other experiments by the same authors when it was shown that messenger RNA induced by infecting E. coli with bacteriophage ϕX 174 does not hybridise with DNA from the mature bacteriophage particle, but does hybridise with the

double-stranded replicative form of ØX 174. The implication here is that the single-stranded DNA from the mature bacteriophage must first direct the synthesis of a complementary DNA strand which acts as the template from which a meaningful message can be transcribed to RNA.

By examining the growth of rII mutants of bacteriophage T4 on E. coli in the presence of 5-fluorouracil, Champe and Benzer (1962) found that only one strand of the DNA of the rII locus specified messenger RNA for that region. Discrepancies in the base composition of the RNA formed after infection of E. coli with bacteriophage T4 suggest that it is not complementary to both strands of the DNA (Bautz & Hall, 1962).

Either single or double-stranded DNA will act as primer for the RNA polymerase and it might well be that double-stranded DNA is the real primer and that only one of the strands is transcribed. In certain systems, complementary RNA which is functional in amino acid incorporation is produced from double-stranded templates (Wood & Berg, 1962).

If, in fact, messenger RNA is transcribed from only one of the strands of the DNA, a function must be afforded the other. It might conceivably fulfil a mechanical role in providing support as the message is read off the other strand. Alternatively, one DNA strand may carry information for messenger RNA and the other that for ribosomal or sRNA, or, yet again, one strand may transcribe information for protein synthesis while the other is responsible for the production of regulator substances. It is also possible that one of the strands is a replicating strand while the other transcribes information to RNA.

Zubay (1962) has described a scheme for coding messenger RNA on double-stranded DNA in which a DNA base pair codes for one nucleotide in the RNA chain with the intermediate formation of a triple-stranded helix. This mechanism, however, is rendered less likely by the evidence outlined above.

The participation of a DNA-RNA stable complex of the type reported in E. coli and Neurospora crassa (Spiegelman, Hall & Storck, 1961; Schulman & Bonner, 1962), as a possible intermediate in information transfer is thought to be unlikely (Geiduschek, Nakamoto & Weiss, 1961) since transcription probably involves only small unwound areas or loops in the DNA template.

When E. coli is infected by bacteriophage, bacteriophage-directed proteins appear as they are required to produce specific bacteriophage precursors. Thus, there appears to be a sequential production of bacteriophage-specific proteins (Kornberg et al., 1959; Dirksen, Wiberg, Koerner & Buchanan, 1960; Wiberg, Dirksen, Epstein, Luria & Buchanan, 1962; Luria, 1962), and evidence that the genome is indeed transcribed in a non-random, ordered manner has been provided (Kano-Sueoka & Spiegelman, 1962).

A mechanism for such a sequential and oriented process has been suggested by Jacob and Monod (1961) who proposed the concept of transcription from a linear array of structural genes, each of which functions by producing a specific polyribonucleotide transcript of its own particular sequence. The transcription can only be initiated at certain points or

segments called the operators and where the transcription of several adjacent genes is dependent on a single operator, the coordinated structure constitutes an operon. Genes with a function other than transcription are designated regulator genes, able to build RNA transcripts, called repressors, which can associate reversibly with a homologous operator and block transcription of the whole operon.

The production of messenger RNA in bacterial systems, therefore, is well supported by experimental evidence to form a nicely coordinated picture. The same cannot, unfortunately, be said for mammalian systems.

The presence of messenger RNA in mammalian systems is inferred principally from the appearance of rapidly-labelled RNA fractions after exposure to labelled precursor, as for example, in rat liver (Hiatt, 1962), rabbit blood cells (Marks, Willson, Kruh & Gros, 1962), human amnion cells (Cheng, 1961) and HeLa cells (Scherrer & Darnell, 1962). In addition, RNA fractions have been described in Ehrlich ascites-tumour cells (Georgiev & Mantieva, 1962) and in calf thymus nuclei (Sibatani, de Kloet, Allfrey & Mirsky, 1962) which are rapidly labelled and which resemble the cellular DNA in base composition.

Up to this point, the proof of the messenger function of this species of RNA has been considered on the basis of its relationship with the primer DNA molecule. It is now necessary to examine the status of messenger RNA with respect to the transcription of information into a specific protein.

A large body of evidence has arisen from bacterial systems where the

observation was first made that an RNA fraction synthesised by crude extracts of E. coli stimulated amino acid incorporation into protein (Tissieres & Hopkins, 1961). Addition of a purified RNA polymerase plus T2 bacteriophage DNA to a soluble protein ribosome system from E. coli increased incorporation of labelled amino acids up to twenty-fold (Wood & Berg, 1962). Similar results have been obtained with preparations from Salmonella typhimurium (Ning, Stevens & Loper, 1962). A more conclusive demonstration was provided by the formation of β-galactosidase in a cell-free system from E. coli with purified RNA polymerase in the presence of both DNA from the cells induced to form β-galactosidase and the inducer (Eisenstadt, Kameyama & Novelli, 1962). Similar specific demonstrations have been published providing further evidence of a specific messenger function (Nisman, Fukuhara, Demailly & Genin, 1962; Oishi, Takahashi & Maruo, 1962; Rueckert, Zillig & Doerfler, 1962). The messenger RNA fractions also link to ribosomes at the site of protein synthesis to form the aggregates known as polyribosomes (Risebrough et al., 1962).

It has been shown that bacteriophage-induced RNA turns over rapidly and that in cell-free systems, bacteriophage-induced RNA is rapidly degraded to 5'-ribonucleotides by polynucleotide phosphorylase with the production of 5'-deoxyribonucleotides in the presence of ATP and $NADH_2$ (Cohen, Barner & Lichtenstein, 1961). However, in E. coli, calculations show that a triplet of messenger RNA bases must be used about six times in protein synthesis which indicates that there is no obligatory destruction

of messenger RNA after it specifies the synthesis of a molecule of protein (Woese, Naono, Soffer & Gros, 1963). Furthermore, breakdown of messenger RNA may depend neither on ribonuclease or polynucleotide phosphorylase in E. coli supernatant fractions but on a phosphodiesterase which removes nucleoside 5'-monophosphates sequentially from the end of the chain (Spahr & Schlessinger, 1963).

Confirmatory evidence for the model proposed by Jacob and Monod (1961) has come from the work on enzyme induction in E. coli where it was found that the regulation of induction occurs at the level of the gene and, more particularly, on the transcription reaction leading to the formation of the corresponding messenger RNA (Attardi, Naono, Gros, Buttin & Jacob, 1963; Hayashi, Spiegelman, Franklin & Laurin, 1963). In animal systems, the suggestion has been put forward that in the Jacob-Monod system of genetic regulation of protein synthesis, histones can be equated with the operator genes. The regulator gene could control the binding and release of structural genes by controlling the production of histones (Bartalos, 1963). Studies on isolated nuclei from calf thymus have indicated that histones inhibit the synthesis of messenger RNA and that when histone is removed by tryptic digestion, new messenger RNA molecules are formed (Allfrey, Littau & Mirsky, 1963).

There is so far little evidence of an RNA species from animal cells that acts as a messenger for the synthesis of a specific protein. However, an RNA fraction from rat liver that stimulates the uptake of leucine into a non-specific protein has been demonstrated (Brawerman,

Gold & Hisenstadt, 1963). The ribosome fraction of rabbit reticulocytes contains aggregates produced by several ribosomes producing protein simultaneously on one strand of messenger RNA (Gierer, 1963).

5.2.3. The synthesis of ribosomal RNA

The origin of ribosomal RNA has been the subject of considerable interest in recent years. In E. coli, there are two sequential precursors of the ribosome called the eosome and the neosome (McCarthy & Britten, 1962; Britten & McCarthy, 1962; McCarthy, Britten & Roberts, 1962; Britten, McCarthy & Roberts, 1962), both of these fractions having a much higher RNA to protein ratio than E. coli ribosomes. The RNA of the eosome fraction has properties similar to messenger RNA and since the base ratios of the RNA of this fraction are intermediate between those of DNA and ribosomal RNA, it has been considered to be a mixture of messenger RNA and ribosomal precursor (Kitazume, Ices & Vincent, 1962); the RNA of the eosomes has been separated into two such fractions (Bolton & McCarthy, 1962).

In E. coli, ribosomal RNA consists of two main components of sedimentation coefficients 23S and 16-17S (Littauer & Eisenberg, 1959; Kurland, 1960; Aronson & McCarthy, 1961; Green & Hall, 1961; Boedtker, Möller & Klemperer, 1962; Huxley & Zubay, 1960). The two ribonucleic acids appear to be derived from the 50S and 30S subunits that make up the ribosomes (70S) (Tissières & Watson, 1958; Tissières, Watson, Schlessinger & Hollingworth, 1959; McQuillen et al., 1959). The 23S component of ribosomal RNA probably consists of long, continuous

polynucleotide chains rather than dissociable fragments (Spirin & Milman, 1960; Spirin, 1961). Annealing experiments reveal that the base sequence of ribosomal RNA is complementary to regions of E. coli DNA which suggests that DNA may act as template in the synthesis of ribosomal RNA. The fact that the base composition of ribosomal RNA shows no tendency to correlate with that of homologous DNA (Woese, 1961) does not exclude DNA-directed synthesis since the segment of DNA involved might be so small as to constitute a statistically inadequate sample of the overall base composition (Yankofsky & Spiegelman, 1962a). The amount of RNA complexed per unit of DNA at saturation suggests a number of repeating or identical sequences which are probably side-by-side in the DNA chain (Yankofsky & Spiegelman, 1962b). Annealing and double-labelling experiments have shown that the 16S and 23S ribosomal ribonucleic acids of B. megatherium are complementary to different sites in the homologous DNA and that the two ribosomal RNA molecules in E. coli are derived from separate loci on the DNA template (Yankofsky & Spiegelman, 1963). This had been previously suspected since the nucleotide sequences of the RNA from the 30S and 50S E. coli ribosomes are so different as to rule out a common origin (Aronson, 1962).

Turning now to animal systems; it is well known that the ribonucleic acids in different parts of the cell have different base compositions (Edström, 1960; Edström, Grampp & Schor, 1961), but, as we have seen, this does not exclude a common origin for all these species.

Autoradiographic experiments have shown that radioactive precursors

of RNA are incorporated into RNA first in the nucleus then later in the cytoplasm (Amano & Leblond, 1960; Feinendegen, Bond, Shreeve & Painter, 1960; Zalokar, 1960; Rho & Bonner, 1961; Sirlin, 1960). Isolated nuclei can synthesise RNA in vitro (Allfrey & Mirsky, 1957; Rho, Birnstiel, Chipchase & Bonner, 1961) whereas the ability of enucleated cytoplasm to do this is greatly impaired (Prescott, 1959). Evidence is available that a substantial portion of cytoplasmic RNA is synthesised in the nucleus and then migrates to the cytoplasm (Prescott, 1960; Sirlin, 1960). When HeLa cells are treated with concentrations of actinomycin D which strongly inhibit the synthesis of nucleolar and cytoplasmic RNA, the synthesis of RNA in the extra-nucleolar part of the nucleus is not inhibited. These results suggest that nucleolar and extranucleolar RNAs are synthesised independently of each other (Perry, 1963). Interference with nucleolar RNA synthesis greatly modifies cytoplasmic RNA synthesis (Perry, Hell & Errera, 1961) suggesting that it is a precursor of a major part of ribosomal RNA (Perry, 1960). The above autoradiographic studies have been linked to studies on the sedimentation behaviour in sucrose gradients of the RNA extracted from L strain fibroblasts with phenol and sodium dodecylsulphate (Perry, 1962). The results of these experiments suggest that extra-nucleolar RNA consists of a 4S component, probably mostly transfer RNA, which migrates to the cytoplasm and a fast-sedimenting component which is labile and which was identified with messenger RNA. Nucleolar RNA was present as a heterogeneous, fast-sedimenting component which was an obligatory precursor of the ribosomal components of cytoplasmic RNA

(Perry, 1963).

On the other hand, the RNA extracted from HeLa cell nuclei and from two cytoplasmic fractions was studied after a short exposure to radioactive RNA precursor. The rapidly-labelled nuclear RNA was not transferred to the microsomes and was probably being degraded in the nucleus (Harris, Fisher, Rodgers, Spencer & Watts, 1963). Analyses of the base composition of nuclear and cytoplasmic ribonucleic acids revealed no RNA having a base composition similar to that of DNA. In the presence of actinomycin D, very little of the rapidly-labelled nuclear RNA was transferred to the cytoplasm in a stable form (Harris, 1963).

In other experiments, sedimentation analysis of RNA from HeLa cells shows that rapidly labelled RNA sediments faster than ribosomal RNA (Scherrer & Darnell, 1962). This rapidly labelled fraction is confined to the nucleus and its formation is inhibited by low concentrations of actinomycin D (Tamaoki & Mueller, 1962). It has been suggested that the sequence of steps in ribosomal RNA synthesis involves firstly an actinomycin-sensitive incorporation of nucleotides into a polymeric precursor A which matures by a puromycin-sensitive process to give precursor B which is in turn converted by an actinomycin-insensitive process to 28S and 18S ribosomal RNA (Tamaoki & Mueller, 1963). This obviously is very similar to the eosome/neosome concept discussed earlier.

A rather similar situation has been described in nuclear RNA from rat liver where four fractions (over 40S, 33S, 19S and 6S) have been found, the 6S component having physical properties distinct from cytoplasmic

sRNA (Sporn & Dingman, 1963).

A great deal of new evidence has accumulated that supports the concept of transfer of RNA to the cytoplasm from the nucleus. In "pulse"-labelled HeLa cells it has been found that cytoplasmic RNA is probably formed from precursors synthesised in the nucleus and nucleolus and that messenger RNA constitutes an important fraction of the RNA radioactivity transferred from the nucleus to the cytoplasm (Srinivasan, Miller-Faures, Brunfaut & Errera, 1963). Then again, when HeLa cells are exposed to ^{14}C-uridine, labelling first appears in a 45S fraction, then in a 35S fraction and ultimately accumulates in the 28S, 16S and 4S fractions of the RNA. When actinomycin D is added after 30 min. exposure to the radioactive precursor, radioactivity from the 45S fraction is transferred sequentially to the 35S fraction then to the 28S and 16S fractions so that the 45S and 35S fractions appear to be precursors of ribosomal RNA (Scherrer, Latham & Darnell, 1963). The 45S fraction from these cells stimulates a protein-synthesising system from E. coli and forms hybrids with HeLa cell DNA. A similar ribosomal RNA precursor has been described in mouse ascites-tumour cells (Harel, Harel, Lacour, Boor & Imbenotte, 1963).

Other studies suggest that nuclear ribosomal RNA in HeLa cells is probably the immediate precursor of microsomal RNA (Singh, Koppelman & Evans, 1963), the nuclear ribosomal RNA either being synthesised on a DNA template or on an RNA molecule which has itself been built on a DNA template.

It is of great interest that a step in the synthesis of ribosomal RNA might be RNA-dependent. In L5 cells, the uptake of ^{3}H-uridine is a function of exposure to radioactive precursor and actinomycin D. The synthesis of a major nuclear RNA component and a ribosomal component are resistant to actinomycin D and therefore probably RNA primed, while the rapidly labelled, actinomycin D sensitive nuclear RNA never becomes associated with cytoplasmic ribosomes (Paul & Struthers, 1963).

It appears therefore that some of the contradictory results are a function firstly of the different exposures to labelled precursors and actinomycin and secondly to the uncertainties in the kinetics of incorporation of labelled precursors into RNA due to the variability of pool size and whether the concept of "pulse" labelling in intact mammalian cells is valid.

Most workers appear to hold the view that all RNA synthesis is DNA dependent since actinomycin D inhibits virtually all RNA synthesis (Franklin, 1965; Levinthal, Keynan & Higa, 1962; Aes, Reich & Valenju, 1963). Similarly, virtually all RNA synthesis in lampbrush chromosomes of isolated oocytes of the newt is DNA dependent (Izawa, Allfrey & Mirsky, 1963a,b). This limitation would confine all RNA synthesis to the nucleus because of the DNA requirement and therefore by this hypothesis any cytoplasmic RNA must originate in the nucleus.

5.2.4. The biosynthesis of sRNA

sRNA amounts to about 10-20 per cent of the total cellular RNA and its molecular weight ranges from 25,000 to 30,000, being a continuous chain

of 80-100 linked nucleotide residues (Tissières, 1959; Brown & Zubay, 1960; Cox & Littauer, 1960; Allen, Glassman, Cordes & Schweet, 1960; Singer & Cantoni, 1960).

Certain bacterial strains continue to synthesise RNA when deprived of an essential amino acid (Borek, Ryan & Rockenbach, 1955; Borek, Rockenbach & Ryan, 1956) this being due to a transferable genetic locus called the RC locus (relaxed control as opposed to stringent control where RNA is not synthesised in the absence of the required amino acid) (Stent & Brenner, 1961). The control of this locus extends both to ribosomal and sRNA, pointing to a common step in the formation of ribosomal and sRNA, possibly a DNA template mechanism. Base sequences in sRNA have, in fact, been shown to be complementary to those in the homologous DNA (Giacomoni & Spiegelman, 1962; Goodman & Rich, 1962).

Among the species of RNA, sRNA is unique in containing methylated bases (Dunn, Smith & Spahr, 1960). However, RNA polymerase lacks specificity in differentiating between methylated bases and normal bases so that ribothymidylate is not distinguished from uridylate when being incorporated into RNA (Kahan & Hurwitz, 1962). The distribution of methylated bases in sRNA is not random and the sequence of bases and content of methylated bases is related to the amino acid accepting capacity of the sRNA (Cantoni, Richards & Tanaka, 1963; Madison, Everett, Holley & Zamir, 1963). Methylation of bases of sRNA occurs at the polynucleotide level and not in the mononucleotides prior to their incorporation (Gold, Hurwitz & Anders, 1963; Srinivasan & Borek, 1963). It appears, however,

that the methylated bases are not involved in the amino acid acceptor function of the sRNA (Stern, 1963).

6. Objectives

The purpose of this work is to investigate certain aspects of the biosynthesis of ribonucleic acid in mammalian tumour cells and, more particularly, in mammalian tumour cells infected with encephalomyocarditis virus. The problem was investigated from two points of view. Firstly, the enzymes which catalyse the synthesis of polynucleotides in the tumour cell were examined, then the changes which occur after virus infection were determined. In the second approach, the ribonucleic acid components of the uninfected tumour cell were examined and the changes which occur after virus infection investigated. Efforts were made to elucidate the manner in which the viral RNA was replicated.

In the light of the results obtained, the mechanisms of animal virus replication are discussed.

EXPERIMENTAL

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

1. The growth of Krebs II ascites tumour cells

The parent strain of Krebs II ascites tumour cells, originally supplied by Dr. F. Kingsley Sanders of the Medical Research Council Virus Research Unit, Carshalton, England, was propagated by serial transplantation in albino mice from a pure Porton strain maintained as a departmental colony.

Tumour for transplantation was withdrawn aseptically by syringe from mice inoculated seven days previously. 0.2 ml. of this cell suspension was inoculated intraperitoneally into young mice and was allowed to grow for seven days when about 4-5 ml. of tumour suspension was present in each mouse.

To minimise changes in the cell line over a large number of generations, a stock of frozen cells was maintained as a standard preparation. After every fifth passage, serial transplantation was discontinued and mice were inoculated with tumour cells from stock. Ascitic fluid was mixed with aqueous glycerol (final concentration, 6 per cent (v/v)), and 4 ml. samples dispensed into ampoules which were then flame sealed and set on ice for one hour. The ampoules were then transferred to a bath of ethanol at 0°. The temperature of the ethanol was lowered over a period of ten minutes to -15° by careful addition of solid CO_2, and further cooled to -25° over a period of 15 min. As soon as the ampoules had reached -25°, they were rapidly cooled to -70° and stored at -80°.

When required for inoculation, the cells were thawed rapidly at 40° and injected as above.

2. Virus techniques

1. General

The following solutions were used throughout the work to be described.

Phosphate-buffered saline: three stock solutions were prepared. Solution A contained NaCl (40 g.), KCl (1.0g), Na_2HPO_4 (5.75 g., or equivalent of hydrated salt), KH_2PO_4 (1.0 g.) and water (4 l.). Solution B contained $MgCl_2$, $6H_2O$ (0.5 g.) in water (500 ml.). Solution C contained $CaCl_2$ (0.5 g.) in water (500 ml.). Each solution was autoclaved and stored at 0°. For use, solutions A, B and C were mixed in the proportions 40:5:5. This solution is referred to throughout as PBS.

Earle's medium: four stock solutions were prepared. Solution A contained NaCl (68 g.), KCl (4 g.), $MgSO_4$, $7H_2O$ (2 g.) and water (400 ml.). Solution B contained NaH_2PO_4, $2H_2O$ (1.58 g.), glucose (20 g.) and water (400 ml.). Solution C contained $CaCl_2$ (2.0 g.) in water (200 ml.). Solution D contained $NaHCO_3$ (4.4 g.) in water (100 ml.). Solutions A, B and C were autoclaved and stored at 0° and solution D was filtered free of bacteria then stored at 0°. For use, solutions A (4.0 ml.), B (4.0 ml.) and C (2.0 ml.) were mixed and diluted to 100 ml. with sterile water; 5 ml. of this mixture was discarded and replaced by 5 ml. of

solution D, previously saturated with CO_2. Benzylpenicillin (100 i.u. per ml.) and streptomycin-$CaCl_2$ (60 µg. per ml.) were added.

All glassware used in experiments on virus replication was washed using the method of Martin, Malco, Sved and Work (1961). Calgon (Albright and Wilson, Ltd., England; 20 g.) was mixed with water (2,270 ml.) and sodium metasilicate (180 g.) and after 24 hr. the mixture was filtered. Glassware was cleaned in a neutral detergent and soaked overnight in diluted silicate solution (1 part to 99 of water). The solution was heated to boiling and the glassware was removed, rinsed in tap water, soaked in 0.1N-HCl (30 min.), rinsed in distilled water and steam-sterilised. Pipettes were cleaned in H_2SO_4-HNO_3 mixture and treated as above.

2.2. Propagation of virus

Mice were killed by cervical dislocation, swabbed with absolute alcohol and the skin resected from the abdomen. Tumour was withdrawn with a sterile syringe and transferred aseptically to a chilled universal container which contained either heparin (100 units) or 5 ml. ice-cold, sterile PBS to prevent clotting. Any sample of cells which was contaminated with erythrocytes was rejected. The cells were washed aseptically with ice-cold PBS and centrifuged at 600 g for 5 min. to remove plasma. Washing was repeated three times and the cells were finally suspended in PBS at a concentration of 1×10^8 per ml.

10 ml. of this suspension was transferred to a sterile bottle and 10 ml. of Earle's solution was added. Encephalomyocarditis (EMC) virus,

originally supplied by Dr. F. K. Sanders, was then added (about 3-5 plaque-forming units per cell) and allowed to adsorb at room temperature for 30 min. 80 ml. of Earle's solution at 37° was then added. The bottle was transferred to a shaking water bath at 37° and allowed to stand for 15 min., after which shaking was begun. After 18-20 hr., the cells were centrifuged down at 750 g for 10 min. and the supernatant fluid, containing the virus, was harvested. To prepare very high titre virus, this supernatant fluid was centrifuged at 105,000 g in the Spinco Model L preparative ultracentrifuge and the virus pellet suspended in a small volume of PBS.

2.3. Methods of virus assay

1. Haemagglutination assay

4.5 g. glucose was dissolved in 100 ml. distilled water and mixed with 100 ml. double-strength PBS. 95 ml. of this solution was taken and 5 ml. of 1 per cent gelatin solution in PBS was added. 1 ml. of sheep blood in Alsever's solution (Burrough's Wellcome & Co., Beckenham, England) was centrifuged at 750 g for 10 min. and the packed cells were washed three times in glucose-PBS by re-centrifuging at 750 g for 5 min. The cells were then diluted to 0.1 per cent in glucose-PBS-gelatin.

0.2 ml. aliquots of glucose-PBS-gelatin were pipetted out in a serology tray. 0.2 ml. of virus suspension was added to the first cup, mixed and 0.2 ml. transferred to the second cup. This was repeated until a dilution of about 2^{20} had been obtained. One volume (0.2 ml.) of 0.1 per cent sheep red blood cell suspension was added to each cup and the tray shaken

gently to mix the contents. Ten cups containing no virus were prepared to act as controls. After 12-15 hr., the haemagglutination titre was recorded.

2.3.2. Plaque Assay

Bacto-agar (Difco Laboratories, Detroit, Mich., U.S.A.) was washed by decantation with a large volume of water which was then removed by washing three times in acetone. The agar was dried in open trays and 3.0 g. batches were dissolved in 100 ml. amounts of water by autoclaving at 10 lb. per sq.in. for 10 min.

One volume of melted agar at 44° was added to one volume of double-strength Earle's solution at 44°. 3 ml. of this mixture was measured into small petri dishes and allowed to set. Virus was diluted in tenfold steps in phosphate-buffered gelatin (0.2 g. gelatin dissolved in 25 ml. boiling water, mixed with 4.5 g. glucose and made up to 100 ml. with water. This solution was mixed with an equal volume of double-strength PBS just before use). 0.1 ml. of each dilution of virus was added to 0.5 ml. of a suspension of Krebs II ascites tumour cells (1 x 10^8 per ml.) in sterile test-tubes and warmed to 44°. 3 ml. of Earle's-agar was added to the infected cells then poured to cover the agar base layer and allowed to set. The dishes were incubated for three days in an incubator gassed with 5 per cent CO_2 in air at 37°, then stained with 1/10,000 neutral red in PBS for one hour. The stain was poured off and the plaques were counted.

2.3.3. Cell death assay

This method was used in conjunction with the haemagglutination assay to assess the virus titre, as an alternative to plaque assay. A sample of infected cells was taken about 16 hr. after the addition of virus, diluted one hundred-fold, then stained with 0.2 per cent trypan blue in PBS. The number of dead (stained) and live (unstained) cells was counted in a Neubauer haemocytometer.

2.4. Purification of the virus

Virus could be freed from contaminating cellular debris by passage through a column of calcium phosphate. Calcium phosphate was prepared by mixing dropwise equal volumes of 0.5M-$CaCl_2$ and 0.5M-Na_2HPO_4 with vigorous stirring. The supernatant fluid was decanted off and the calcium phosphate washed extensively with 0.1M-phosphate buffer, pH 7.5. A slurry of this material was packed to give a column 4" by 1". Bromocresol purple dye was added to the column as marker and washed just below the surface of the calcium phosphate with a small volume of 0.1M-phosphate buffer. The narrow band of dye was then washed a little distance down the column by adding 5 ml. 0.1M-phosphate buffer and just before the meniscus reached the top of the column, 2 ml. of virus suspension was added. The column was washed with 0.1M-phosphate buffer until the indicator dye came off the column. The virus was then eluted with 0.5M-phosphate buffer, the eluate being collected in 5 ml. amounts. These fractions were scanned at 280 mμ for protein and assayed for haemagglutination titre.

3. The preparation of enzyme fractions

1. From whole Krebs II ascites tumour cells

Krebs II ascites tumour cells were harvested from mice and washed as before in PBS until free of red blood cells and plasma. The cells were then packed by centrifugation at 750 g for 5 min. at 0°, suspended either in 5 vol. ice-cold distilled water or 0.01M-tris-HCl buffer, pH 7.5, in 0.001M-$MgCl_2$, and submitted to sonic vibration (50W; 20Kc) for 30-60 sec. Complete disruption of cells and nuclei by this treatment was confirmed microscopically after staining with crystal violet (1 per cent in 0.1M-citric acid). The suspension was centrifuged at 10,000 g for 15 min. at 0°, the supernatant fraction removed and its protein concentration adjusted to 1.5-2.5 mg. per ml. with 0.01M-tris-HCl buffer, pH 7.5. Enzymes from virus-infected cells were prepared in a similar manner. This crude enzyme fraction represents only a selected portion of the total protein of the cell and more complete fractionations of cellular components were performed.

3.2. Cell fractionation in sucrose solutions

Washed Krebs II ascites tumour cells were disrupted osmotically by the method of Smellie, Keir and Davidson (1959). The cells were suspended in 10 vol. ice-cold distilled water and gently homogenised in a pre-cooled Potter-type homogeniser (Potter and Elvehjem, 1936). Disruption of cells, but not of nuclei, was confirmed microscopically using 1 per cent crystal violet in 0.1M-citric acid. To the suspension was added one-half volume of 0.75M-sucrose, pH 8.0, in 0.003M-$MgCl_2$ to

give a final concentration of 0.25M-sucrose in 0.001M-$MgCl_2$. This mixture was centrifuged at 750 g for 20 min. at 0°, giving well-packed nuclear material and a cytoplasmic supernatant fraction (A) of non-staining particulate material which was decanted off. The nuclear material was suspended in 0.25M-sucrose in 0.001M-$MgCl_2$ and re-centrifuged as above. The supernatant fraction was discarded and the nuclei washed once more. The packed nuclei were re-suspended in water and lyophilised, then re-suspended in 0.01M-tris-HCl buffer, pH 7.5, as required.

An alternative method of freeing nuclear material from cytoplasmic material was employed. The disrupted cells in 0.25M-sucrose in 0.001M-$MgCl_2$ was layered on top of 70 per cent (w/v) sucrose in 0.001M-$MgCl_2$ and centrifuged at 35,000 g for 15 min. at 0°. The nuclear material sedimented through the sucrose while the cytoplasmic material remained on top.

The cytoplasmic supernatant fraction (A) was centrifuged at 10,000 g for 20 min. at 0° to give a pellet and a supernatant fraction. The pellet was suspended in water then lyophilised for storage. The supernatant fraction was centrifuged in the Spinco Model L preparative ultracentrifuge at 105,000 g for 60 min. at 0°. The pellet fraction so obtained was suspended in water and lyophilised; the supernatant fraction was dialysed against 0.001M-tris-HCl buffer, pH 7.5, to remove sucrose and then lyophilised.

3.3. Cell fractionation using Tween 80

Infected and uninfected cells were mainly fractionated by a method

derived from that of Fisher and Harris (1962).

Krebs II ascites tumour cells were suspended in 5 vol. of ice-cold 0.01M-tris-HCl buffer, pH 7.5, in 0.1 per cent (v/v) Tween 80 (L. Light & Co., Colnbrook, England) and either stirred gently in a Nelco blendor or homogenised in a chilled Sireica homogeniser (Sireica, New York, U.S.A.). This process disrupted the cells but left the nuclei intact.

The nuclei were centrifuged down at 600 g. for 5 min. at 0° and the supernatant material was removed with a pasteur pipette. The nuclei were suspended in ice-cold PBS and re-centrifuged to remove the bulk of the contaminating cytoplasmic material, which was decanted off. The nuclei were then subjected to sonic vibration (50W; 20Kc) for 15-30 sec. to give a nuclear enzyme.

The original supernatant material was re-centrifuged at 600 g. and any sediment was discarded. It was then centrifuged at 10,000 g. for 15 min. at 0°. The sediment was washed with PBS then suspended in tris-Tween by brief exposure to sonic vibration. The 10,000 g. supernatant fraction was centrifuged at 105,000 g. for 2 hr. giving a pellet, which was suspended in tris-Tween by brief exposure to sonic vibration, and a soluble supernatant fraction.

For incorporation experiments, the protein content of each of the fractions just described was adjusted to about 1.5-3.0 mg. per ml.

3.4. The preparation of polynucleotide phosphorylase

The method employed was that described by Steiner and Beers (1961).

20 g. of spray-dried Micrococcus lysodeiktieus cells (Calbiochem,

Los Angeles, Calif., U.S.A.) were suspended in 200 ml. 0.5 per cent (w/v) NaCl, brought to pH 8.0 with 1.0N-NaOH, at 37°. 50 mg. crystalline lysozyme (Armour Pharmaceutical Co., Ltd., Sussex, England) in 2.0 ml. water was added and thoroughly mixed with the cell suspension, which was stirred occasionally as lysis occurred. During lysis, the colour of the cell suspension darkened and the consistency thickened slightly. After about 10 min., the suspension became lumpy and it was stirred until a liquid phase began to separate. This stage was accompanied by a failure of the cell debris to adhere to the sides of the vessel and by the appearance of a glistening texture to the lumpy material. 100 ml. saturated ammonium sulphate at 0° was added to the lysed cells, mixed and the preparation was centrifuged at 20,000 g for 30 min. at 0°.

An equal volume of saturated ammonium sulphate was added to the clear, yellowish-brown supernatant fluid to precipitate polynucleotide phosphorylase. The precipitated material was collected by centrifugation at 20,000 g for 30 min. at 0° then dissolved in 20 ml. 0.1M-tris-HCl buffer, pH 8.0. This material was dialysed against distilled water for 8 hr. then lyophilised.

The lyophilised material was dissolved in 0.1M-tris-HCl buffer, pH 8.0, at a concentration of 1.0 g. per 100 ml. Solid ammonium sulphate was added to the solution to bring the concentration to 45 per cent of saturation. The precipitated material was centrifuged down at 20,000 g for 30 min. at 0° and discarded. The concentration of ammonium sulphate in the supernatant fluid was brought to 57 per cent of saturation by adding

solid ammonium sulphate. The precipitate was collected as before and dissolved in a small volume of 0.1M-tris-HCl buffer, pH 8.0. This solution of polynucleotide phosphorylase was then dialysed for 6 hr. against 0.1M-tris-HCl buffer, pH 8.0.

The enzyme preparation was further purified by adding 2 g. Norite A charcoal per 100 ml. of enzyme in tris. The suspension was allowed to stand on ice for 30 min. and the charcoal was removed by centrifugation at 20,000 g for 10 min. at 0°.

This enzyme preparation was employed in the phosphorolysis of ^{32}P- and ^{3}H-labelled RNA from infected cells and EMC virus.

4. Assay of enzymes

1. Nucleotidyltransferase assays

1. Incubation conditions

DNA nucleotidyltransferase was assayed as described by Keir, Binnie and Smellie (1962), DNA-dependent RNA nucleotidyltransferase by a modification of the method of Weiss and Nakamoto (1961) and RNA-dependent RNA nucleotidyltransferase by a modification of the method of Burdon and Smellie (1961b).

Incubations were performed in 15 ml. round-bottomed centrifuge tubes sealed with "parafilm" to prevent evaporation. The volume of the incubation mixtures was between 0.2 and 0.5 ml. and the tubes were incubated with shaking at 37°. After incubation, the tubes were plunged into a freezing-mixture of solid CO_2 in ethanol and stored at -20° until required.

4.1.2. Analytical procedures

(a) Disc method

This method was modified from that of Bollum (1959) and was used for the assay of DNA nucleotidyltransferase, DNA-dependent RNA nucleotidyltransferase and RNA-dependent RNA nucleotidyltransferase.

0.05 ml. portions of the incubation mixtures were pipetted on to numbered discs of Whatman No. 1 filter paper, 1" in diameter. The discs were dropped into a beaker containing ice-cold 5 per cent (w/v) trichloroacetic acid (TCA; 15 ml. per disc), and allowed to stand for 15 min., the beaker being swirled gently at intervals. The TCA was decanted off, replaced with an equal volume of ice-cold acid and allowed to stand as before. This procedure was repeated a total of four times. The discs were washed twice with cold 95 per cent (v/v) ethanol (15 ml. per disc), once with ether and dried on stainless steel planchettes for assay of radioactivity.

4.1.2. (b) An alternative method for the assay of DNA-dependent- and RNA-dependent RNA nucleotidyltransferases

This method was modified from that of Weiss (1960). To the frozen incubation mixtures was added 3.0 ml. of ice-cold 5 per cent (w/v) TCA along with 0.5 ml. of carrier yeast RNA (2 mg. per ml.). After thawing, the mixture was allowed to stand for 15 min. and was then centrifuged at 750 g. at 0° for 5 min. The acid-insoluble precipitate was washed thoroughly with three further 3 ml. portions of TCA in the same manner, then twice with ethanol-ether (3:1). The sediment was extracted twice

with 2.0 ml. of 10 per cent (w/v) NaCl, pH 8.0, and 0.5 ml. of carrier RNA (2 mg. per ml.) in a boiling water bath for 30 min. The combined extracts were precipitated with two volumes of ice-cold ethanol and centrifuged at 750 g for 15-30 min. The precipitate was redissolved in 5.0 ml. water and 0.5 ml. samples dried on planchettes for assay of radioactivity.

On occasion, high background samples were observed in the RNA-dependent reaction using this method. These could be eliminated by a minor modification suggested by Baltimore and Franklin (1963), which was adopted in part. The frozen incubation mixtures were thawed in 5 per cent (w/v) TCA with carrier RNA (1 mg.) and centrifuged down as before. The sediment was then dissolved in 0.2 ml. 0.5M-NaOH then immediately precipitated with 4.0 ml. ice-cold TCA. This was repeated four times after which the precipitate was dried in ethanol-ether as before. The residue was dissolved in 1.0 ml. concentrated formic acid and 0.5 ml. dried for assay of radioactivity.

4.2. Ribonuclease assay

The method used to determine the sensitivity of radioactive viral ribonucleic acids to ribonuclease was based on the descriptions given by Fillem, Colter and Kuhn (1962) and Shortman (1961), but since purified commercial bovine pancreatic ribonuclease was used, p-chloromercuribenzoate was omitted from the reaction mixture. Incubation was performed in a 25 ml. conical flask at 37° with shaking in a total volume of 3.15 ml. 0.5 ml. samples were withdrawn at intervals and added to 1.0 ml. acid-ethanol

(1N-HCl in 76 per cent (v/v) aqueous ethanol) plus 0.5 ml. carrier RNA (2 mg. per ml.). The mixture was centrifuged at 750 g for 10 min. to sediment the precipitated material and the supernatant fraction was assayed for radioactivity.

4.3. Phosphorolysis by polynucleotide phosphorylase

The method used in this work to determine the phosphorolysis of radioactive ribonucleic acids by polynucleotide phosphorylase, has been described by Steiner and Beers (1961). Incubation was carried out in 50 ml. conical flasks at 37° with shaking, in a volume of 14.0 ml. The reaction was stopped by adding 1.0 ml. aliquots of the reaction mixture to 0.5 ml. 5 per cent (v/v) perchloric acid and 0.5 ml. carrier RNA (2 mg. per ml.). After standing for 10-20 min. at room temperature, the mixture was centrifuged at 750 g. for 10 min. and the supernatant fluid assayed for radioactivity.

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

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

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

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

alcohol were added and the solution concentrated *in vacuo* to an oil at 40°. A second portion of anhydrous pyridine was added and the solution again concentrated to an oil. Then 5 ml. of anhydrous pyridine and 2.1 g. (2.3 ml.) dicyclohexylcarbodiimide (DCC) were added and the reaction set aside overnight at room temperature in a well-stoppered flask.

Water (5 ml.) was added to stop the reaction and the resulting mixture heated in a boiling water bath for 30 min. The mixture was concentrated to dryness *in vacuo* and 10 ml. water and 10 ml. saturated barium hydroxide were added to the residue. After 5 min. at room temperature, the solution was adjusted to pH 7.5 with glacial acetic acid and filtered to remove dicyclohexylurea (DCU) and barium phosphate. Two volumes of ethanol were added to precipitate the barium 2-cyanoethyl-phosphate, which was collected after one hour at 0° by centrifugation. The crystals were redissolved in water (5 ml.) by adding a minimum volume of glacial acetic acid, neutralised with barium hydroxide, centrifuged to remove a trace of insoluble material and recrystallised by adding 10 ml. of ethanol. The product was collected by centrifugation in a pre-weighed tube, washed with ethanol, acetone and finally ether. The yield at this step was usually about 60 per cent. The product was dissolved in water (plus acetic acid to a total volume of about 20 ml.) and passed through a Dowex-50-H^+ column (3 cm. by 2 cm.). The effluent was taken to dryness and dissolved in anhydrous pyridine to which isopropylidene uridine had been added in the ratio 1m-mole isopropylidene uridine per 0.5m-mole cyanoethylphosphate.

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

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

After 20 hr. at room temperature, the well-stoppered flask was opened and 10 ml. water was added. After 1 hr., the mixture was concentrated to dryness *in vacuo*. The residue was hydrolysed for 90 min. in 10 per cent acetic acid (40 ml.; v/v) at 100° to remove the isopropylidene groups and cleave phosphoamide bonds. The acetic acid was then removed by evaporating the solution to dryness with the last traces being removed by a second evaporation after adding 10 ml. water. The residue was heated with 40 ml. 2N-ammonium hydroxide (to remove cyanoethyl groups) at 60° for 90 min. and the ammonia removed by concentrating the mixture to dryness. 10 ml. water was added to the residue and the insoluble dicyclohexylurea removed by filtration under reduced pressure. The precipitate was washed with a small volume of water and a sample of the combined filtrates was taken for paper chromatography of the reaction products. The precipitate was then washed very thoroughly with 100-200 ml. water. Chromatography was performed in one dimension on sheets of Whatman No. 1 chromatography paper, using UMP, uridine and isopropylidene-uridine as markers. The chromatogram was developed in the system iso-butyric acid-ammonia-water (see Section 14.1) for 18 hr. as a descending

chromatogram. The sheet was then dried and scanned for ultraviolet-absorbing spots using a Hanovia chromatolite.

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

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

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

1.2 ml. tri-n-butylamine, 6 ml. pyridine, 0.2 ml. 85 per cent (v/v) phosphoric acid and 3.0 g. DCC.

The mixture was allowed to stand at room temperature for 48 hr. At the end of the reaction, a thick precipitate of DCU had formed. About 2 volumes of water were added to precipitate DCU from unreacted DCC. The flask was shaken and left for 1 hr. at 0°. The DCU was filtered off under reduced pressure and washed with water. The eluate was extracted with four 50 ml. portions of ether to remove pyridine and the ether washes were extracted with two 10 ml. portions of water, these water washes being added to the main aqueous phase.

The combined aqueous phases were concentrated at 40° *in vacuo*. The material was applied to a Dowex-50-Na^+ column (4 cm. by 10 cm.) to remove tri-n-butylamine and the uridine derivatives were eluted by washing with

water until the extinction of the eluate at 260 mμ was less than 0.05. The total extinction of the eluate at 260 mμ was determined and the amount of uridine derivatives calculated. The effluent was diluted to one litre and adsorbed on to a Dowex-1-Cl$^-$ column (2 cm. by 20 cm.). The column was washed with water to remove ultraviolet-absorbing material, the total extinction being recorded.

Gradient elution from the Dowex-1-Cl$^-$ column was then carried out with 1.5 litres of 0.01N-HCl in the mixing vessel and 2 litres of 0.5M-LiCl in 0.01N-HCl in the reservoir. The eluate was collected in 20-30 ml. fractions which were scanned automatically for radioactivity. The fractions containing (^{32}P)UTP were pooled and the volume was reduced *in vacuo* at 25°, the yield having been determined. LiCl, HCl and inorganic phosphate were removed on active charcoal, which was prepared in the following manner. A column of charcoal was washed with ethanol-ammonia (70 per cent (v/v) aqueous ethanol containing 10 ml. concentrated ammonia per litre) until the absorbancy at 260 mμ fell to less than 0.1. The column was then washed with water, 0.01M-$NaHCO_3$ and then with about 5 column volumes of 1N-HCl. The charcoal was suspended in water, fine particles were decanted off and washing was continued until neutral. The column was then re-packed.

The mixture containing (^{32}P)UTP was adsorbed on the charcoal column which was washed with water until chloride ions were completely eluted. Washing was continued with small volumes of 0.01M-$NaHCO_3$ to remove inorganic ortho- and pyro-phosphate until ultraviolet-absorbing material

began to be eluted. The column was allowed to drain and was washed with 2 column volumes of water. (^{32}P)UTP was eluted with about 1 litre of ethanol-ammonia and was concentrated *in vacuo* at 25°. The material was re-dissolved in water and passed through a small Dowex-50-Na^+ column then checked for purity by chromatography in the ammonium isobutyrate solvent (see Section 14.1). The chromatogram was tested for the presence of phosphate. 1 g. of ammonium molybdate was dissolved in 8.0 ml. water. 5.0 ml. 72 per cent (v/v) perchloric acid and 3.0 ml. concentrated HCl were added and made up to 100 ml. with acetone. The chromatogram was dipped in this mixture, dried and exposed to ultraviolet light for 30 min. Inorganic phosphate gives a yellow spot on dipping; inorganic and ester phosphate give blue spots after exposure to ultraviolet light.

6. The preparation of DNA

DNA was prepared from Krebs II and Landschutz ascites tumour cells by the method of Kay, Simmons and Dounce (1952).

About 50 x 10^8 cells were harvested and washed as described in Section 2.2, then disrupted osmotically by adding ten volumes of water followed by homogenisation in a Potter homogeniser. The resulting mixture was centrifuged at 750 *g* for 15 min. at 0°. The supernatant fluid was discarded and the residue was homogenised in 0.9 per cent (w/v) NaCl in 0.01M-sodium citrate and made up to 200 ml. with the same solution. The resulting mixture was centrifuged at 900 *g* for 10 min. and the supernatant fluid was discarded; this was repeated three times. The sediment finally

obtained was suspended in 0.9 per cent (w/v) NaCl, homogenised in a Potter homogeniser and made up to 800 ml. with the same solution. 27 ml. 0.5 per cent (w/v) sodium dodecyl sulphate in 45 per cent (v/v) aqueous ethanol were added and the mixture was stirred for 30 min. Solid NaCl was added to give a 1.0M-solution and stirring was continued until all the NaCl had dissolved. The mixture was centrifuged at 20,000 g. for 30 min. and the supernatant fluid decanted off. Two volumes of absolute ethanol were added to the supernatant fluid to precipitate the DNA which was collected on a glass rod, washed three times with ethanol, three times with acetone and allowed to dry. The DNA thus obtained was dissolved in 300 ml. water at room temperature with stirring. 27 ml. sodium dodecyl-sulphate in ethanol was added and the mixture was stirred for 30 min. before 20-25 g. NaCl was added. After the NaCl had dissolved, the mixture was centrifuged at 20,000 g. for 30 min. and the DNA was precipitated from the supernatant fluid with ethanol, washed and dried as before. The DNA was dissolved in 200 ml. water, 1.8 g. NaCl was added and the mixture was centrifuged as before. 10 g. NaCl was dissolved in the supernatant material, two volumes of ethanol added and the DNA was collected as before. The DNA was then dissolved at a concentration of 2 mg. per ml. in distilled water.

7. The preparation of bentonite

It has been reported that bentonite binds to and inhibits ribonucleases (Brownhill, Jones and Stacey, 1959) and also stabilises and protects TMV-RNA

(Fraenkel-Conrat, Singer and Tsugita, 1961). Bentonite was therefore used in the preparation of RNA from Krebs II ascites tumour cells by the method to be described (Section 8).

Bentonite suspensions were prepared from commercial bentonite (The British Drug Houses Ltd., Poole, England). 2 g. bentonite was suspended in 40 ml. water and centrifuged at 750 g. for 15 min. The sediment was discarded and the supernatant material was centrifuged at 8,700 g. for 20 min. The sediment so obtained was re-suspended in 0.1M-EDTA, pH 7.0, and stored in this solution for 48 hr. at room temperature. The material was centrifuged once more at 750 g. and 8,700 g. The 8,700 g. sediment was suspended in 0.01M sodium acetate, pH 6.0, centrifuged at 8,700 g. and the sediment taken up in the acetate buffer at a concentration of 2-6 per cent (w/v).

8. The preparation of RNA

1. Non-radioactive RNA

1-2 ml. washed, packed Krebs II ascites tumour cells (approximately 4×10^8 cells per ml.) were suspended in 0.8 ml. 2.5 per cent (w/v) bentonite in 0.01M-sodium acetate buffer, pH 6.0, 1.6 ml. 0.5 per cent (w/v) aqueous sodium dodecyl sulphate and 8.0 ml. of 0.01M-sodium acetate buffer, pH 5.2. The mixture was suspended with a pasteur pipette. After the cells had disrupted, 8.0 ml. 90 per cent (v/v) aqueous phenol was added. It was found that these reagents had to be added in the sequence described and as rapidly as possible to avoid degradation of RNA.

The mixture was shaken for 10-15 min. in a mechanical shaker at room temperature. The resulting emulsion was centrifuged at 10,000 g. for 5 min. The aqueous phase was removed with a pasteur pipette and transferred to a chilled 50 ml. centrifuge tube which contained 0.1 ml. 2.5 per cent bentonite (w/v) suspension. The phenol-interphase residue was re-extracted with 4.0 ml. 0.01M-sodium acetate buffer by shaking for 10 min. as before. After centrifugation, the aqueous phase was combined with the first. The combined aqueous extracts were shaken for 10 min. with an equal volume of 90 per cent (w/v) aqueous phenol and centrifuged at 10,000 g. for 10 min. The aqueous phase was removed and made 2 per cent (w/v) with respect to sodium acetate. The RNA was precipitated with two volumes of ice-cold ethanol. The precipitate was collected by centrifugation at 760 g. for 15-30 min. at 0° and dissolved in 10 ml. buffer A (0.01M-tris-HCl buffer, pH 7.5, in 0.001 M-$MgCl_2$). 10-20 µg. bovine pancreatic deoxyribonuclease (crystalline; Sigma) was added to the solution which was incubated for 15 min. at 37°. The mixture was cooled on ice, the RNA precipitated with 2 volumes of ice-cold ethanol and, after collection by centrifugation, was dissolved in 5-10 ml. buffer B (0.01M-sodium acetate buffer, pH 5.2/0.05M-NaCl/0.001M-$MgCl_2$). The solution of RNA was then dialysed against two changes of 7 litres of this buffer for 18 hr. The dialysed solution was centrifuged at 10,000 g. for 10 min. to remove insoluble material and the RNA was precipitated with ethanol as before. The final RNA precipitate was dissolved in a small volume of buffer B, the mixture was extracted four times with an equal

volume of ether and the last traces of ether were removed with a stream of nitrogen.

8.2. Preparation of (^{32}P)- and (^{3}H)RNA

Krebs II ascites tumour cells were harvested aseptically and suspended in 50-100 ml. sterile Earle's medium (1 x 10^7 cells per ml.) by the method described in Section 2.2. The bottles were transferred to a shaking water incubator at 37°. ^{3}H-uridine or ^{32}P-orthophosphate was added to a final concentration of 5 μcuries per ml. and 20 μcuries per ml. respectively and shaking was commenced.

At various time intervals thereafter, samples of the cell suspension were removed and poured on to crushed, frozen PBS to stop the incorporation of labelled precursors into RNA. The cells were centrifuged down at 750 g. for 5 min. at 0°, the supernatant fluid was discarded and the cells were re-suspended in ice-cold PBS with a pasteur pipette. The suspension was centrifuged as before and the cells were washed twice more in ice-cold PBS.

RNA was extracted from the packed cells by the method described in Section 8.1; dialysis of the extracted RNA was performed three times, instead of twice as indicated in Section 8.1, to ensure removal of any residual radioactive precursor of RNA. The contents of the dialysis vessel were stirred by means of a magnetic stirrer to remove unincorporated precursors of RNA more efficiently.

In other experiments, the labelled cells were fractionated as described in Section 5.3 and RNA was extracted from these fractions in the manner

described above.

8.3. The preparation of labelled RNA in the presence of actinomycin D

Krebs II ascites tumour cells were suspended in Earle's solution as already described (Section 8.2). Varying amounts of actinomycin D (dissolved in water) were added to the cell suspension which was swirled and allowed to stand at room temperature for 45 min. Labelled precursors of RNA were added and RNA was prepared by the method described in Section 8.2.

8.4. The preparation of labelled RNA from virus-infected cells

(a) In the absence of actinomycin D

Encephalomyocarditis virus was used to infect Krebs II ascites tumour cells by the procedure described in Section 2.2 and was allowed to adsorb for 20 min. at room temperature and 15 min. at 37°. 60 min. after the addition of virus, the cells were diluted in Earle's solution at 37° and radioactive RNA precursors were added to the infected culture and to a non-infected control culture. RNA was prepared as in Section 8.2.

8.4. (b) In the presence of actinomycin D

Actinomycin D was added to 10 ml. of a suspension of washed Krebs II ascites tumour cells (0.5×10^8 cells per ml.) in each of two sterile bottles and allowed to stand at room temperature for 45 min. EMC virus was added to one bottle, the other acting as an uninfected control, and allowed to adsorb as before. One hour after infection, the cells were diluted ten-fold in Earle's solution and labelled as in Section 8.2. Labelled RNA was prepared from whole cells and from cell fractions by the

method described in Section 8.2.

9. Ultracentrifugal studies of the nucleic acids

1. In sucrose density gradients

Sedimentation analyses of RNA preparations were performed by centrifugation in linear sucrose density gradients.

The gradients were originally prepared by layering 0.5 ml. 20 per cent (w/v) sucrose in buffer B (see Section 8.1) at the bottom of a 5 ml. cellulose centrifuge tube then 0.5 ml. of solutions of decreasing sucrose concentration, until 5 per cent (w/v) sucrose was added to give a total volume of 4.5 ml. The tubes were set aside on ice for 4-5 hr. before use.

Alternatively, a mixing device (Bock and Ling, 1954) was used to deliver 4.5 ml. of a solution of sucrose in buffer B linearly graded from 20 per cent (w/v) to 5 per cent (w/v) in a 5 ml. cellulose centrifuge tube.

0.2-0.5 ml. RNA in buffer B, containing about 0.5 mg. of RNA (10-20 optical density units), was layered on the sucrose gradients and the tubes were centrifuged in the swinging bucket rotor (SW 39) of a Spinco Model L preparative ultracentrifuge for varying periods of time and at varying speeds as indicated in the legends to the appropriate figures.

Between 30 and 55 fractions were collected from each tube by puncturing the base with a hypodermic needle and allowing the contents to drip out into the test tubes. Since the drop size varied because of the changing sucrose concentrations, the volume of the fractions was not constant.

0.1 ml. of each fraction was diluted to 3.0 ml. with water and the ultraviolet absorption of the fractions was measured (Section 13). With radioactive RNA, 1 ml. samples of the diluted fractions were assayed for radioactivity (Section 12).

In those experiments where a particular fraction of the RNA was isolated, the drops were collected directly in test tubes containing 3.0 ml. water so that no material would be lost. Where the amount of RNA was small, 0.8-1.0 ml. amounts of water were used in each tube instead of 3 ml.

The required fractions were pooled, 0.05 ml. 2.5 per cent (w/v) bentonite was added and the material was dialysed against water overnight to remove sucrose. The solution was then lyophilised and the RNA dissolved in a small volume of the appropriate buffer solution.

The sedimentation pattern of DNA preparations was obtained by the same methods that were used for RNA.

9.2. Analytical ultracentrifuge studies

Solutions of RNA in buffer B were centrifuged in the Spinco Model E analytical ultracentrifuge equipped both with Schlieren and ultraviolet optical systems to determine the sedimentation coefficients of the various components.

10. Annealing experiments

1. Annealing of DNA and RNA

The procedure for annealing RNA and DNA mixtures was that described

by Hall and Spiegelman (1961).

The rapidly labelled, rapidly-sedimenting peak of RNA from Krebs II ascites tumour cells which had been exposed to radioactive precursors of RNA for a short time, was isolated as described in Section 9.1. The pooled fractions from the sucrose gradient were dialysed against 0.015M-NaCl in 0.001M-sodium citrate, pH 7.8, and concentrated ten-fold by lyophilisation.

The resulting solution was heated to 100° for 5 min. and rapidly cooled in an attempt to disrupt the tertiary structure of the RNA chain. It was then mixed with Krebs II, Landschutz or calf thymus DNA (dissolved in 0.15M-NaCl in 0.01M-sodium citrate, pH 7.8), which had been denatured by heating to 100° for 10 min. then cooling rapidly. The final concentration of DNA in the mixture was 100 µg. per ml. The mixture was heated to 57-60° for two hours in an insulated water bath then allowed to cool slowly overnight to 25-30°.

10.2. Analysis of the annealed material on caesium chloride gradients

Sedimentation analyses of DNA- RNA mixtures in CsCl density gradients were carried out by the method of Meselson, Stahl and Vinograd (1957).

Caesium chloride (AnalaR) was obtained from Hopkins and Williams Ltd., and was freed from material absorbing at 260 mµ by passage in dilute solution through a charcoal column. The dilute solution was then concentrated in vacuo until crystals began to appear. A minimum volume of water was added to dissolve the crystals and the density of the solution was determined using an Abbe refractometer. An amount of the DNA-RNA

mixture sufficient to dilute the CsCl solution to a density of 1.73 g. per ml. was added, giving a total volume of 3.0-4.0 ml. This was transferred to a 5 ml. cellulose centrifuge tube and the remainder of the tube was filled by carefully adding 1-2 ml. of a light silicone oil which formed a layer on top of the CsCl mixture and prevented the tube from collapsing during the centrifugation.

Ultracentrifugation was performed in the SW 39 rotor of the Spinco Model L preparative ultracentrifuge at 35,000 rev./min. at 24° for 48 hr. Fractions were collected as in Section 9.1 and were assayed for ultraviolet absorption and radioactivity.

10.3. Annealing of RNA with RNA.

(a) Virus RNA

Two samples of RNA from virus infected Krebs II ascites tumour cells, one labelled with ^{3}H and the other with ^{32}P, were prepared as described in Section 8.4.(b). Each RNA preparation was centrifuged in a 5-20 per cent (w/v) sucrose gradient (Section 9.1). The peaks of radioactivity which had sedimentation constants of about 35-40s were isolated and dialysed against 0.015M-NaCl in 0.001M-sodium citrate, pH 7.8, to remove sucrose. The fractions were then concentrated ten times by lyophilisation.

The ^{3}H-labelled peak was heated at 100° for 10 min. then placed in an insulated water bath at 60° for 2 hr. and allowed to cool slowly to 25-30° overnight.

The ^{32}P-labelled peak was heated at 100° for 10 min. and cooled

rapidly on ice.

The two samples were then mixed, layered on 5-20 per cent (w/v) sucrose gradients and centrifuged for various times. The fractions collected were assayed for radioactivity.

The converse experiment was also performed. The ^{32}P-labelled peak was heated and cooled slowly and the ^{3}H-labelled peak heated and rapidly cooled. The mixed samples were analysed on sucrose gradients in the same manner.

(b) "40s" Krebs II RNA

To serve as a control for the previous experiment, the rapidly labelled, rapidly sedimenting peaks ("40s") from Krebs II ascites tumour cells exposed to either ^{3}H-uridine or ^{32}P-orthophosphate for a short time were isolated (Sections 8.2 and 9.1). These RNA fractions were then treated in a manner identical to the corresponding fractions described in Section 10.3.(a).

11. The preparation of polyribosomes

1. From uninfected cells

The method was that used by Warner, Knopf and Rich (1963).

Krebs II ascites tumour cells were suspended in Earle's solution at a concentration of 1 x 10^{7} cells per ml. and transferred to a shaking water bath at 37°. After varying times, 0.5 μcuries of ^{14}C-valine were added and the mixture was incubated for 2 min. Incorporation was stopped by pouring on to crushed, frozen PBS. The cells were centrifuged down

at 750 g for 5 min. at 0° then washed twice in ice-cold PBS.

The cells were disrupted in two volumes of 0.001M-$MgCl_2$. It was observed that the longer the cells were incubated in Earle's solution, the more difficult they were to disrupt. Two volumes of 0.01M-tris-HCl buffer, pH 7.5, in 0.001M-$MgCl_2$ in 0.01M-NaCl (TMS) were added and the mixture was centrifuged at 10,000 g for 15 min. at 0°. 0.4 ml. of the supernatant material was layered on top of a 15-30 per cent (w/v in TMS) sucrose density gradient and centrifuged in the SW 39 rotor for 2 hr. at 27,000 rev./min. at 0°. The gradient was analysed as in Section 9.1.

The 10,000 g supernatant fraction was also made 0.5 per cent (w/v) with respect to sodium dodecyl sulphate and warmed to 37° for 3-5 min. (see Stoehelin et al., 1964). This material was layered on top of a 5-20 per cent (w/v in buffer B; section 8.1) sucrose density gradient and centrifuged for 12 hr. at 20,000 rev./min. at 0°. The gradient was analysed as in Section 9.1.

11.2. From virus-infected cells

Cells were infected and were labelled, along with uninfected control cells, with ^{3}H-uridine as in Section 8.4.(b). Six hours after adding virus, 0.05 ml. of an aqueous solution of ^{14}C-valine (50 μcuries per ml.) was added both to infected and uninfected cultures. The mixtures were incubated for 5 min. then the cells were disrupted and analysed as in Section 11.1.

12. Isotope determination

^{3}H- and ^{14}C-labelled samples were assayed in a two-channel Packard Tricarb liquid scintillation spectrometer or a three-channel Nuclear Chicago model 725 liquid scintillation spectrometer. 1.0 ml. samples of aqueous solution were mixed with 8.0 ml. of a scintillator consisting of "Scinstant" NE 572 (Nuclear Enterprises, Edinburgh, Scotland) dissolved in scintillation grade dioxane. The variation in quenching encountered was not sufficient to distort the qualitative patterns obtained, but corrections could easily be made for this using the channel ratio data from the Nuclear Chicago spectrometer. When samples containing CsCl were counted, they had to be diluted so that the components of the scintillator did not precipitate out of solution.

The data obtained in the simultaneous counting of ^{3}H and ^{32}P in the Packard Tricarb spectrometer were obtained in the form of simultaneous equations which were solved on a Sirius computer (Western Regional Hospital Board, Regional Physics Department, Glasgow). Counting of ^{3}H and ^{14}C simultaneously was carried out in the Nuclear Chicago scintillation counter.

^{32}P-labelled samples in solution were usually dried on stainless steel planchettes and counted in a Nuclear Chicago gas flow counter fitted with a thin window. No correction for self-absorption was necessary.

Strips from paper chromatograms were scanned for radioactivity in a Nuclear Chicago Actigraph coupled to a chart recorder which moved synchronously with the paper strip.

13. Spectrophotometric analysis

Analyses of ultraviolet absorption of the large number of tubes obtained from sucrose density gradients were performed on a recording Beckman DB spectrophotometer. Where the volume of the RNA fractions was only 1.0 ml., the optical density was determined using micro-cells in a Unicam SP 500 spectrophotometer fitted with micro-cell attachment.

14. Analysis of ribonucleotides

1. Chromatographic analysis

The acid-soluble fractions obtained after treatment of reaction mixtures with TCA (Section 4.1.2.(b)) were neutralised and applied to sheets of Whatman No. 1 chromatography paper. Descending chromatograms were developed in the ammonium isobutyrate system, consisting of 100 ml. isobutyric acid, 55.8 ml. water and 4.2 ml. ammonia, for 16 hr. The paper was dried, cut into strips 2.5 cm. broad and scanned for radioactivity in a Nuclear Chicago Actigraph.

The acid washed, insoluble material obtained in Section 4.1.(b) was hydrolysed in 0.3N-KOH for 18 hr. at 37°, neutralised with 7N-perchloric acid and set aside on ice for 1-2 hr. The precipitate of $KClO_4$ was centrifuged down at 750 g. for 15 min. and 0.2 ml. of the supernatant material applied as a spot on a sheet of Whatman No. 1 paper as before. After development in the ammonium isobutyrate system, the spots were cut out and eluted with water. The eluted fractions were assayed for radioactivity.

14.2. Electrophoretic separation of ribonucleotides

The acid washed, insoluble material described in Section 14.1 was hydrolysed in KOH, neutralised as before and applied as a narrow band on a strip of 3MM Whatman chromatography paper 72 cm. long. The paper was moistened in 0.5M-citrate buffer, pH 3.5 (66 ml. 0.5M citric acid and 22 ml. 0.5M trisodium citrate made up to 2200 ml.) and 720 volts (10mA) was applied across the strip for 16 hr. The strip was dried, the nucleotides located as before and eluted for assay of radioactivity.

15. Estimation procedures

1. Protein estimation

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

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

To 1 ml. protein solution, 5 ml. reagent C was added and after 10 min., 0.5 ml. reagent E was rapidly added. The mixture was allowed to stand for at least 30 min. and was read in a Unicam SP 600 spectrophotometer at 500 and 750 mμ against a reagent blank. A standard solution of bovine serum albumin was used to calibrate the assay.

15.2. Estimation of DNA

DNA was estimated by the Ceriotti procedure (Ceriotti, 1952, 1955). 1 ml. 0.04 per cent indole in distilled water and 1 ml. concentrated HCl were added to 2 ml. DNA solution (containing from 2.5-15 µg. of DNA per ml.). The tube was then immersed in a boiling-water bath for 10 min. and then cooled in running water. The cooled solution was extracted with 4.0 ml. chloroform three times and the chloroform layer discarded each time. The tubes were centrifuged at 600 g. for 5 min. to ensure complete removal of chloroform. The yellow colour was read at 490 mµ in a Unicam SP 600 spectrophotometer against a reagent blank.

15.3. Estimation of pentose

Pentose was determined by the orcinol method (Kerr and Seraidarian, 1945). 3.0 ml. pentose-containing solution was treated with 3.0 ml. orcinol reagent (60 mg. orcinol dissolved in 10.0 ml. 0.02 per cent $FeCl_3$, $6H_2O$ in concentrated HCl) and the mixture was heated in a boiling water bath for 30 min. The colour developed was estimated at 665 mµ in a Unicam SP 600 spectrophotometer. A standard ribose solution containing 1-10 µg. ribose per ml. was used for calibration.

16. Materials

dATP, dGTP, dCTP, ATP, GTP and CTP were purchased from Pabst Laboratories, the Sigma Chemical Company or the British Drug Houses Ltd. Actinomycin D was a gift from Merck, Sharp and Dohme, Inc. ^{3}H-uridine, carrier-free ^{32}P-orthophosphate and ^{32}P-cyanoethylphosphate were obtained

from the Radiochemical Centre, Amersham, England. Crystalline pancreatic ribonuclease and deoxyribonuclease were purchased from the Sigma Chemical Company.

RESULTS

R E S U L T S

1. The incorporation of (^{32}P)UMP into RNA

1. Preliminary considerations

Many factors affect the extent of synthesis of polynucleotide material by enzymes present in cellular extracts. One of the most important of these is the breakdown of the newly synthesised polynucleotide by nucleases which are invariably present in crude enzyme fractions. In studies on the synthesis of RNA, this effect can be reduced, or even eliminated by the addition of bentonite to the reaction mixtures to inhibit ribonuclease. In the preliminary studies on RNA synthesis, bentonite was therefore added to tubes containing the appropriate RNA precursors, buffer and essential ions before incubation was carried out. It was found that in these circumstances, the standard methods used to assay the amount of (^{32}P)UMP incorporated into RNA produced considerable variation in duplicate assays, this being most noticeable when the disc method of assay was employed. It was found that this variation was caused by the use of a gross excess of bentonite (0.05 ml. 2 per cent (w/v) bentonite suspension per assay) which absorbed radioactive precursors from the incubation medium. The bentonite was not removed by the subsequent washing procedure and it was necessary, therefore, to determine the amount of bentonite which would give low background counting rates and still inhibit ribonuclease activity. This was found to be in the range of 0.01-0.05 ml. of 0.02 per cent (w/v) suspension of bentonite

per assay.

In evaluating the disc method of assay (experimental, section 4.1.2.(a)), it was observed that the most satisfactory results were obtained with soluble enzyme fractions. When particulate enzyme preparations were used, it proved to be essential to avoid overloading the disc with an excessive amount of the incubation mixture, otherwise satisfactory removal of adsorbed (^{32}P)UTP was not achieved. In the alternative method for the assay of DNA-dependent and RNA-dependent RNA nucleotidyltransferases (experimental, section 4.1.2.(b)), it was necessary to suspend the contents of the tubes in a manner sufficiently vigorous to ensure the breakdown of any large associations of material. This was achieved by the use of a Vortex Mixer (Scientific Industries, England).

It is important to note that when crude enzyme systems are used, it is very difficult to obtain results which duplicate precisely from experiment to experiment. However, all the experiments were repeated many times with consistent results.

1.2. DNA nucleotidyltransferase, DNA-dependent RNA nucleotidyltransferase and RNA-dependent RNA nucleotidyltransferase in uninfected Krebs II ascites tumour cells

1. General

The procedure for the assay of DNA nucleotidyltransferase in extracts of Landschutz ascites tumour cells employed by Keir, Binnie and Smellie (1962) gave satisfactory levels of activity in extracts of Krebs II ascites tumour cells.

The results of Burdon and Smellie (1961a, b, 1962) pointed to the existence in Landschutz ascites tumour cells of RNA-dependent and DNA-dependent systems for the synthesis of RNA in vitro. These observations were confirmed using crude enzyme preparations from Krebs II ascites tumour cells and experiments were therefore carried out to distinguish these two activities in preparations of sonically disrupted Krebs II cells.

Table 1 demonstrates that the reaction primed by DNA was favoured more at pH 7.5 and in the presence of Mn^{2+} ions and 2-mercaptoethanol. The reaction primed by RNA was favoured more at pH 9.5 and when Mn^{2+} ions and 2-mercaptoethanol were absent. With either the DNA-dependent or RNA-dependent systems, absence of the appropriate primer led to greatly diminished incorporation.

The effect of added Mn^{2+} ions and 2-mercaptoethanol on the incorporation of (^{32}P)UMP residues into RNA in both RNA-dependent and DNA-dependent systems is shown in more detail (Table 2). The results indicate that Mn^{2+} ions stimulated the activity of the DNA-dependent enzyme to the same extent as a combination of Mn^{2+} ions and 2-mercaptoethanol. The RNA-dependent reaction was inhibited by Mn^{2+} ions alone and to a greater degree by Mn^{2+} ions plus mercaptoethanol. Inhibition by the Mn^{2+} ions may in part have been caused by the precipitation of Mn^{2+} salts at pH values higher than 7.5.

In order to obtain further evidence for the distinction between RNA-dependent and DNA-dependent RNA nucleotidyltransferase activities, experiments

TABLE 1

The influence of pH, primer and $MnCl_2$ plus 2-mercaptoethanol on the DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 μmoles tris-HCl buffer of the appropriate pH; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2 x 10^7 counts/min./μmole); 50 μg. DNA or RNA and 0.2 ml. enzyme (2 mg. protein per ml.) in a total volume of 0.4 ml. Where indicated, 1 μmole each of $MnCl_2$ and 2-mercaptoethanol was included. Incubations were carried out at 37° for 20 min.

TABLE 1.

Primer	pH	$MnCl_2$ + 2-mercaptoethanol	μμmoles (^{32}P)UMP incorporated per mg. protein
DNA	7.5	+	216
DNA	7.5	−	84
DNA	9.5	+	25
DNA	9.5	−	76
NIL	7.5	+	54
NIL	9.5	−	67
RNA	9.5	+	32
RNA	9.5	−	198
RNA	7.5	+	46
RNA	7.5	−	54

TABLE 2

The effect of Mn^{2+} ions and 2-mercaptoethanol on the DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

The reaction mixture for the assay of DNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 7.5; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2 x 10^7 counts/min./μmole); 50 μg. DNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

The reaction mixture for the assay of RNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 9.5; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2 x 10^7 counts/min./μmole); 50 μg. RNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Where indicated, 1 μmole of $MnCl_2$, 1 μmole of 2-mercaptoethanol or 1 μmole of $MnCl_2$ plus 1 μmole of 2-mercaptoethanol were included. Incubations were carried out at 37° for 20 min.

TABLE 2.

System	Additions	μμmoles (^{32}P)UMP incorporated per mg. protein
DNA'	0	86
DNA'	$MnCl_2$	194
DNA'	2-mercaptoethanol	108
DNA'	$MnCl_2$ + 2-mercaptoethanol	198
RNA'	0	180
RNA'	$MnCl_2$	94
RNA'	2-mercaptoethanol	146
RNA'	$MnCl_2$ + 2-mercaptoethanol	74

were performed in which the crude enzyme system was subjected to digestion with pancreatic ribonuclease or deoxyribonuclease to remove endogenous primer. 2 ml. portions of the enzyme system from sonically disrupted ascites tumour cells were digested for 20 min. at 37° with concentrations of ribonuclease ranging from 0.1 - 10 µg. per ml. or with concentrations of deoxyribonuclease ranging from 20-200 µg. per ml. Thereafter, 0.05ml. 0.1 per cent (w/v) bentonite suspension was added to inhibit ribonuclease and the mixture was centrifuged at 750 g for 10 min. at 0°. The supernatant fluid was then assayed for DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities in the absence of added primers. The results of the experiments with ribonuclease (Fig. 6) demonstrate that at concentrations of ribonuclease greater than 0.1 µg. per ml. the RNA-dependent RNA nucleotidyltransferase was substantially inhibited whereas the DNA-dependent RNA nucleotidyltransferase was only slightly inhibited at concentrations of ribonuclease exceeding 1.0 µg. per ml. No inhibition, but in fact a slight stimulation of the RNA-dependent system (Fig. 7) was observed at concentrations of deoxyribonuclease up to 200 µg. per ml., although about 20 per cent inhibition of the DNA-dependent system was found at this concentration of deoxyribonuclease.

Actinomycin D has been shown (Introduction, section 5.2.1) to bind with double-stranded DNA and thus to inhibit DNA-dependent synthesis of RNA. Experiments were therefore performed to determine the effect of actinomycin D on the DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

FIGURE 6

The effect of removal of endogenous RNA with ribonuclease on the unprimed DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

The reaction mixture for the assay of DNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 7.5; 2 μmoles $MgCl_2$; 1 μmole $MnCl_2$; 1 μmole 2-mercaptoethanol; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole) and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

The reaction mixture for the assay of RNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 9.5; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole) and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Incubations were carried out at 37° for 20 min.

—○— DNA-dependent reaction.

—●— RNA-dependent reaction.

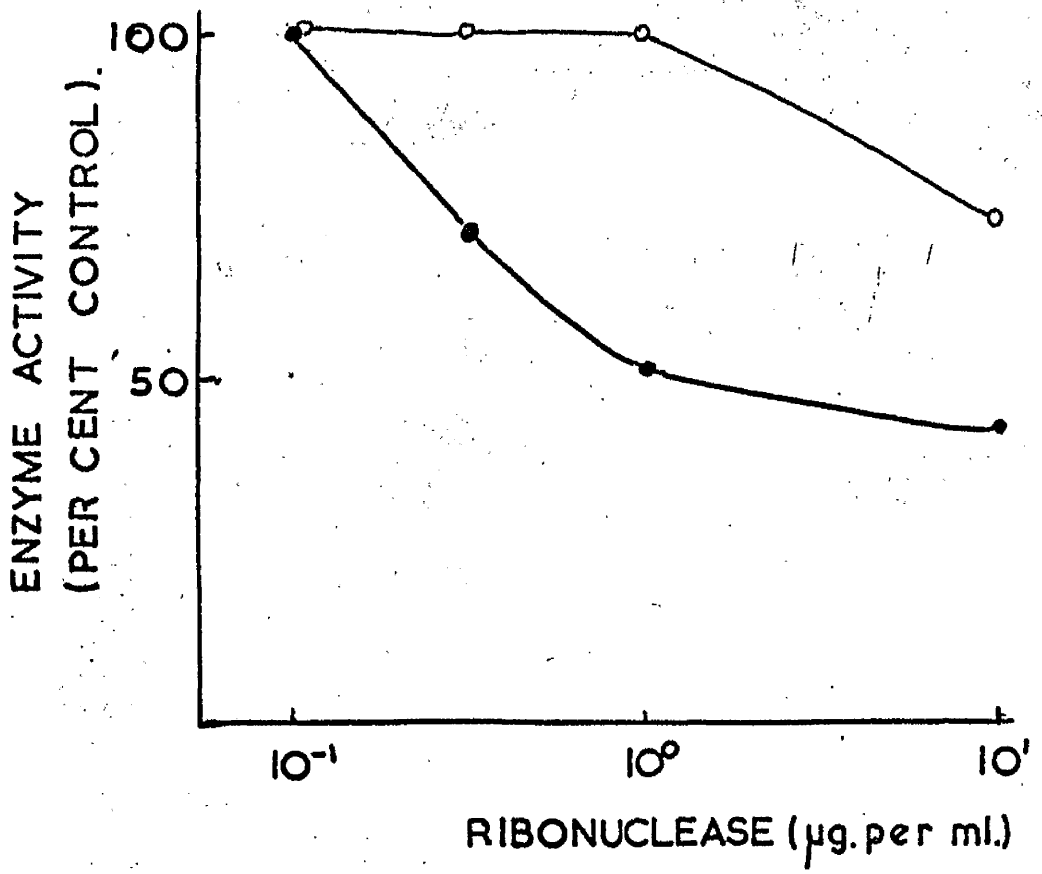

ENZYME ACTIVITY
(PER CENT CONTROL).
100
50
10^{-1}
10^{0}
10^{1}
RIBONUCLEASE (µg. per ml.)

FIGURE 6.

FIGURE 7

The effect of removal of endogenous DNA with deoxyribonuclease on the unprimed DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

The incubation mixtures are described in Figure 6.

—○— DNA-dependent reaction.

—●— RNA-dependent reaction.

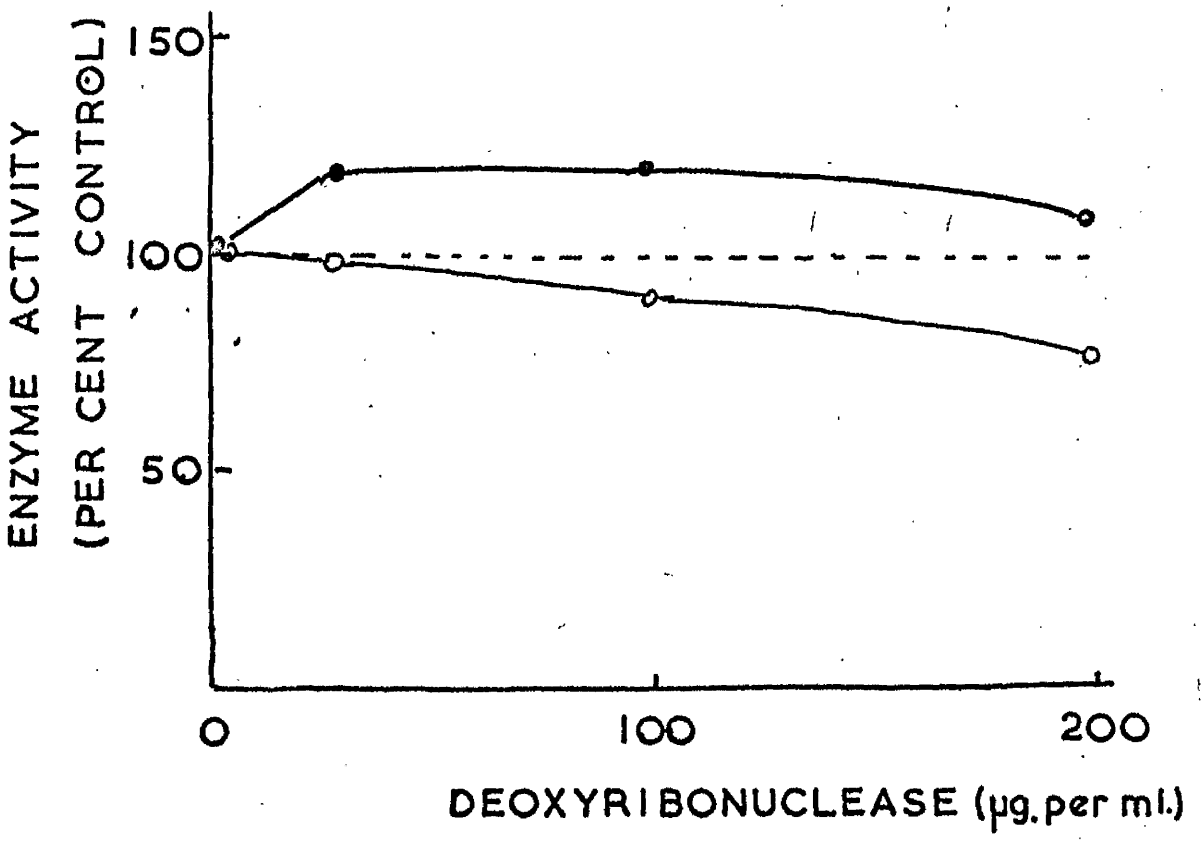

ENZYME ACTIVITY
(PER CENT CONTROL)
150
100
50
0
100
200
DEOXYRIBONUCLEASE (µg. per ml.)

FIGURE 7.

Table 3 shows that while the DNA-dependent reaction was inhibited by actinomycin D, the RNA-dependent reaction was stimulated to an appreciable extent.

When crude enzyme preparations are employed as a source of enzyme for studies on RNA nucleotidyltransferases, it seems possible that the incorporation of (^{32}P)UMP residues is due in part to a DNA-primed and in part to an RNA-primed reaction. Nevertheless, two systems for synthesising polyribonucleotide material, one depending on DNA and the other on RNA, appear to be present in the uninfected Krebs II ascites tumour cell.

1.2.2. Characteristics of the DNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells

The DNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells was at a maximum at pH 7.5 under the conditions of incorporation employed (Fig. 8) and the amount of Mg^{2+} ions became limiting below 2 µmole per assay (Fig. 9). The reaction was stimulated to the greatest extent by 0.5-1.0 µmole each of Mn^{2+} ions and 2-mercaptoethanol per assay (Fig. 10).

Table 4 presents the results of a series of experiments on the characteristics of the DNA-dependent reaction. It is clear that the incorporation of (^{32}P)UMP residues was highly dependent on the presence of Mg^{2+} ions and native DNA, which was a more effective primer than heat denatured DNA. No specificity was observed using Krebs II, Landschutz,

TABLE 3

The effect of actinomycin D on the DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities of extracts of Krebs II ascites tumour cells.

The reaction mixture for the assay of DNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 7.5; 2 μmoles $MgCl_2$; 1 μmole $MnCl_2$; 1 μmole 2-mercaptoethanol; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. DNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

The reaction mixture for the assay of RNA-dependent RNA nucleotidyltransferase activity contained: 50 μmoles tris-HCl buffer, pH 9.5; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. RNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Where indicated, 10 μg. actinomycin D was added.

Incubations were carried out at 37° for 20 min.

TABLE 3.

Assay System	Actinomycin D	counts per min incorporated per mg. protein
RNA'	—	291
RNA'	+	360
DNA'	—	375
DNA'	+	270

FIGURE 8

The effect of pH on the DNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 μmoles tris-HCl buffer of the appropriate pH; 2 μmoles $MgCl_2$; 1 μmole $MnCl_2$; 1 μmole 2-mercaptoethanol; 0.2 μmoles each of ATP, GTP, CTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. DNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Incubations were carried out at 37° for 20 min.

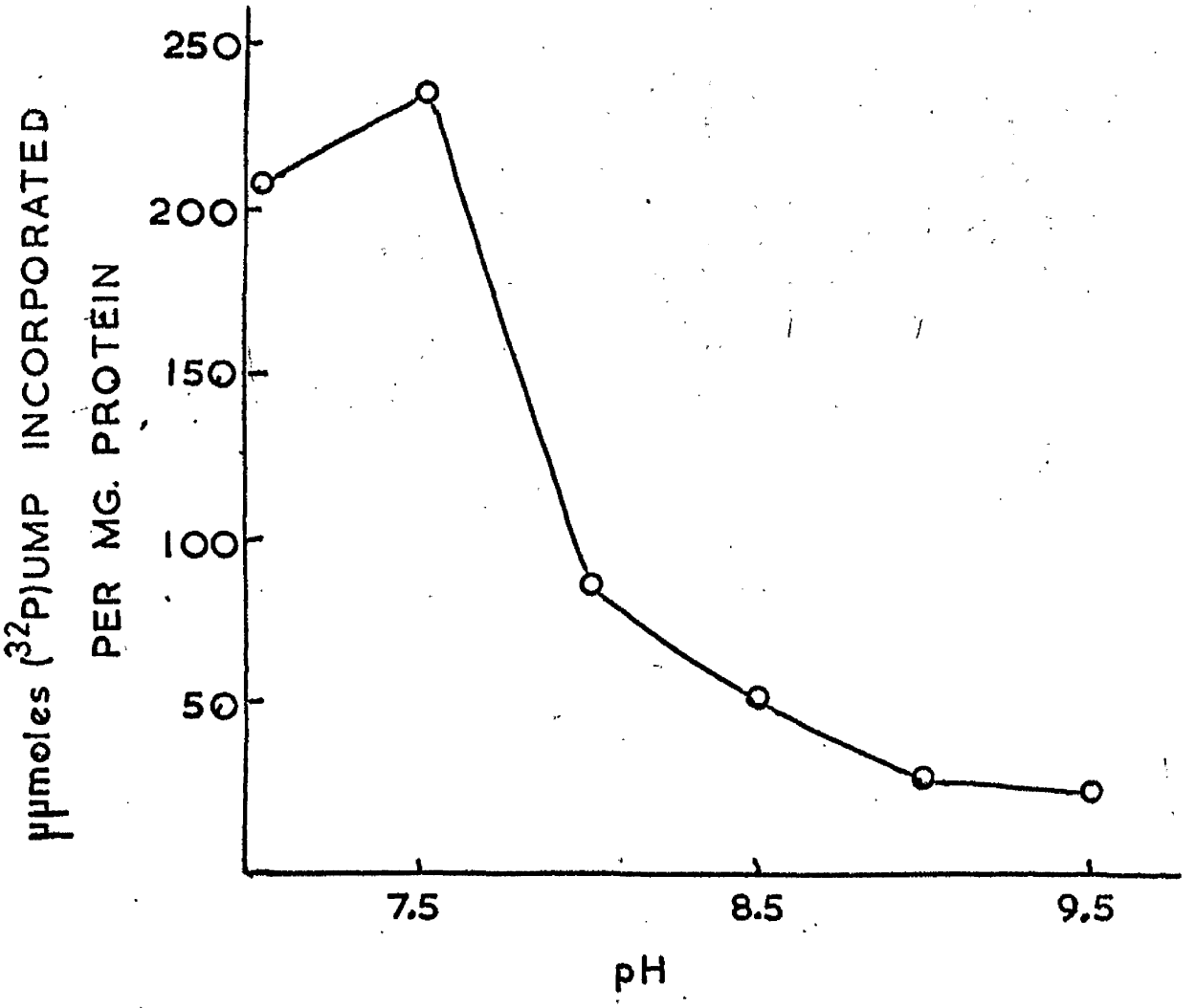

μμmoles (^{32}P)UMP INCORPORATED
PER MG. PROTEIN
250
200
150
100
50
7.5
8.5
9.5
pH

FIGURE 8

FIGURE 9

The effect of Mg^{2+} ions on the DNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 µmoles tris-HCl buffer, pH 7.5; 1 µmole $MnCl_2$; 1 µmole 2-mercaptoethanol; 0.2 µmoles each of ATP, GTP, CTP and (α-^{32}P)UTP (2×10^7 counts/min./µmole); 50 µg. of DNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

The appropriate amount of $MgCl_2$ was included.

Incubations were carried out at 37° for 20 min.

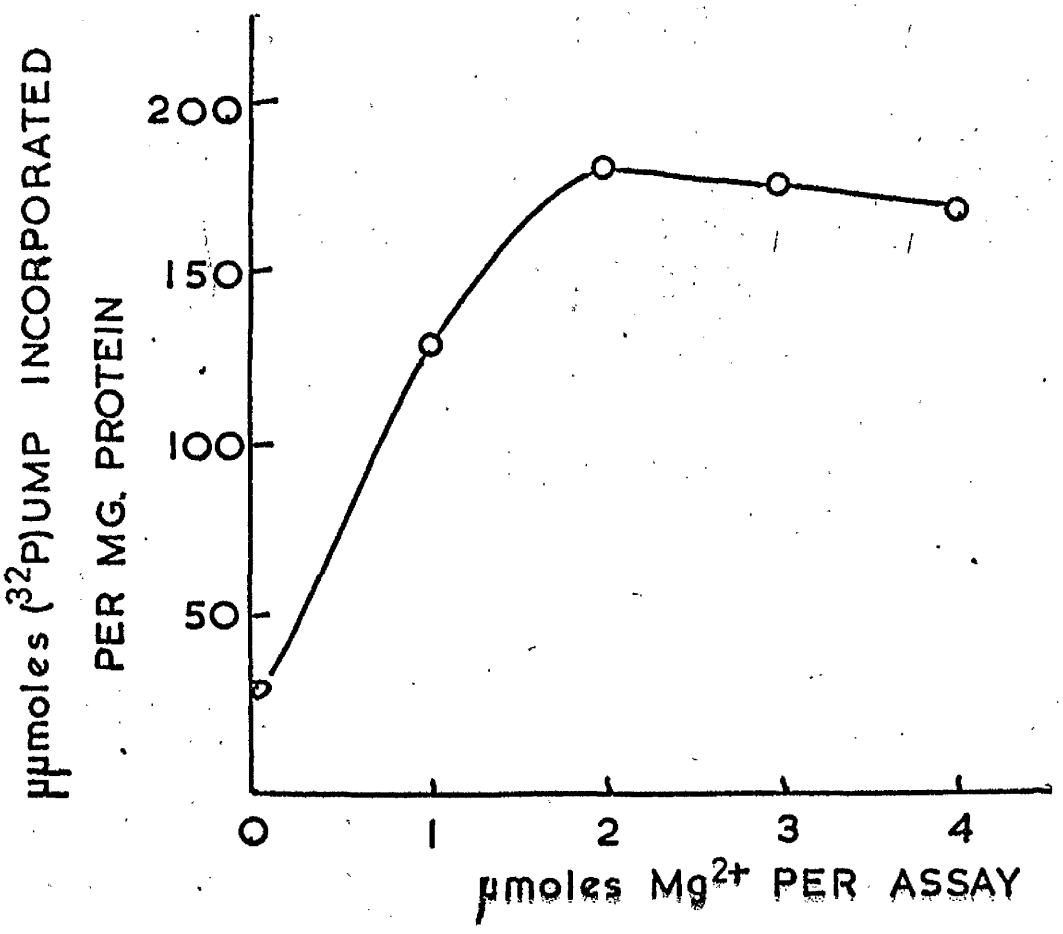

μμmoles (^{32}P)UMP INCORPORATED
PER MG. PROTEIN
200
150
100
50
0
1
2
3
4
μmoles Mg^{2+} PER ASSAY

FIGURE 9.

FIGURE 10

The effect of Mn^{2+} ions plus 2-mercaptoethanol on the DNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 μmoles tris-HCl buffer, pH 7.5; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. DNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

The appropriate amounts of $MnCl_2$ plus 2-mercaptoethanol was included.

Incubations were carried out at 37° for 20 min.

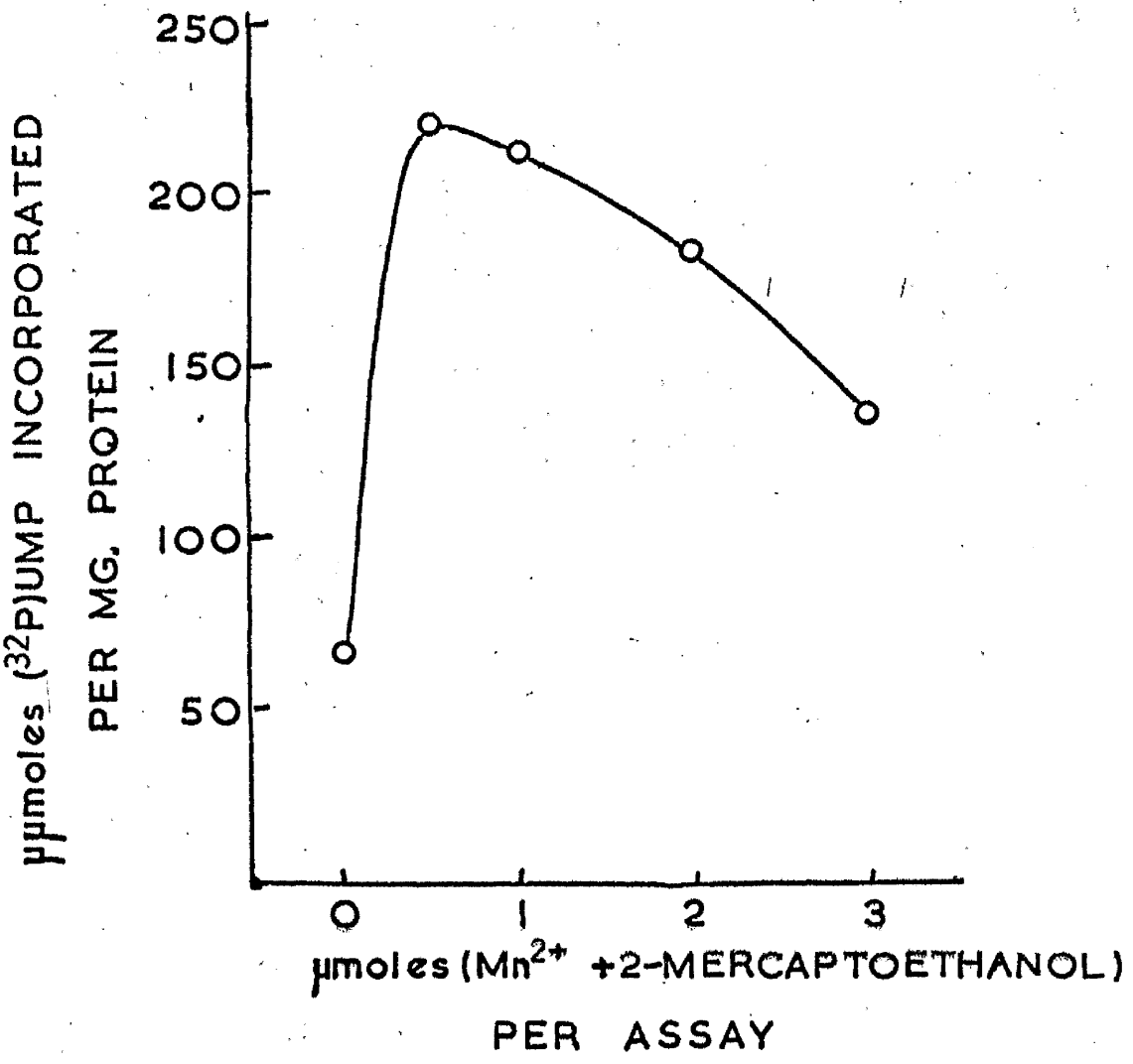

250
200
150
100
50
μμmoles (^{32}P)UMP INCORPORATED
PER MG. PROTEIN
0
1
2
3
μmoles (Mn^{2+} +2-MERCAPTOETHANOL)
PER ASSAY

FIGURE 10.

TABLE 4

The effect of omitting components of the incubation mixture in the assay of DNA-dependent RNA nucleotidyltransferase activity in extracts of Krebs II ascites tumour cells.

The complete reaction mixture for the assay of DNA-dependent RNA nucleotidyltransferase is described in Table 3.

TABLE 4.

Incubation Mixture	Enzyme activity (per cent).
COMPLETE	100
MINUS $MgCl_2$	20
MINUS DNA	32
Heat denatured DNA substituted for native DNA.	61
MINUS ATP	14
MINUS GTP	28
MINUS CTP	20
MINUS ATP, CTP and GTP	35

E. coli or calf thymus DNA. The omission of any one or all three of the ribonucleoside 5'-triphosphates led to a sharp reduction in the extent of the reaction. This suggested that polyribonucleotide chains containing all four ribonucleoside 5'-monophosphate residues were synthesised and this was confirmed by the data in Table 5, where analysis of the products of alkaline hydrolysis of the RNA indicated considerable randomisation of ^{32}P among the four nucleotides.

The time course of the incorporation of (^{32}P)UMP residues into RNA in the presence and absence of bentonite is shown in Fig. 11. In the absence of bentonite, incorporation of (^{32}P)UMP showed a maximum at about 30 min. Thereafter, the amount of (^{32}P)UMP present in the RNA declined. In the presence of bentonite, however, maximum incorporation was reached at about 30 min., after which the level remained steady up to about 3 hr. This difference can probably be accounted for in terms of stabilisation of the product since, after about 30 min., all the (^{32}P)UMP had been hydrolysed to the mono- and diphosphate. These assays may therefore be carried out in the absence of bentonite for incubation times not exceeding 30 min., or for longer times in the presence of bentonite. Since bentonite probably inhibits ribonuclease by adsorption, the possibility that pre-treatment of the crude enzyme fraction with bentonite might effect a purification of the nucleotidyltransferase activity was considered. The increases in specific activity after such treatment were small, however, and the method was not pursued.

In other experiments, the intracellular distribution of the DNA-

TABLE 5

The distribution of radioactivity in the ribonucleoside 5'(2')-monophosphates obtained on alkaline hydrolysis of RNA synthesised by the DNA-dependent RNA nucleotidyltransferase of Krebs II ascites tumour cells.

TABLE 5.

RIBONUCLEOTIDE	PER CENT TOTAL RADIOACTIVITY
CYTIDINE 3'(2')-PHOSPHATE	40
URIDINE 3'(2')-PHOSPHATE	21
ADENOSINE 3'(2')-PHOSPHATE	9
GUANOSINE 3'(2')-PHOSPHATE	30

FIGURE 11

The time course of the incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by the DNA-dependent RNA nucleotidyltransferase from extracts of Krebs II ascites tumour cells and the effect of bentonite on the reaction.

The reaction mixture for the assay of DNA-dependent RNA nucleotidyltransferase is described in Table 3.

In examining the effect of bentonite, 0.05 ml. of 0.02 per cent (w/v) bentonite suspension in 0.01M-sodium acetate buffer, pH 6.0, was included in the reaction mixture.

—o— plus bentonite.

—●— no bentonite.

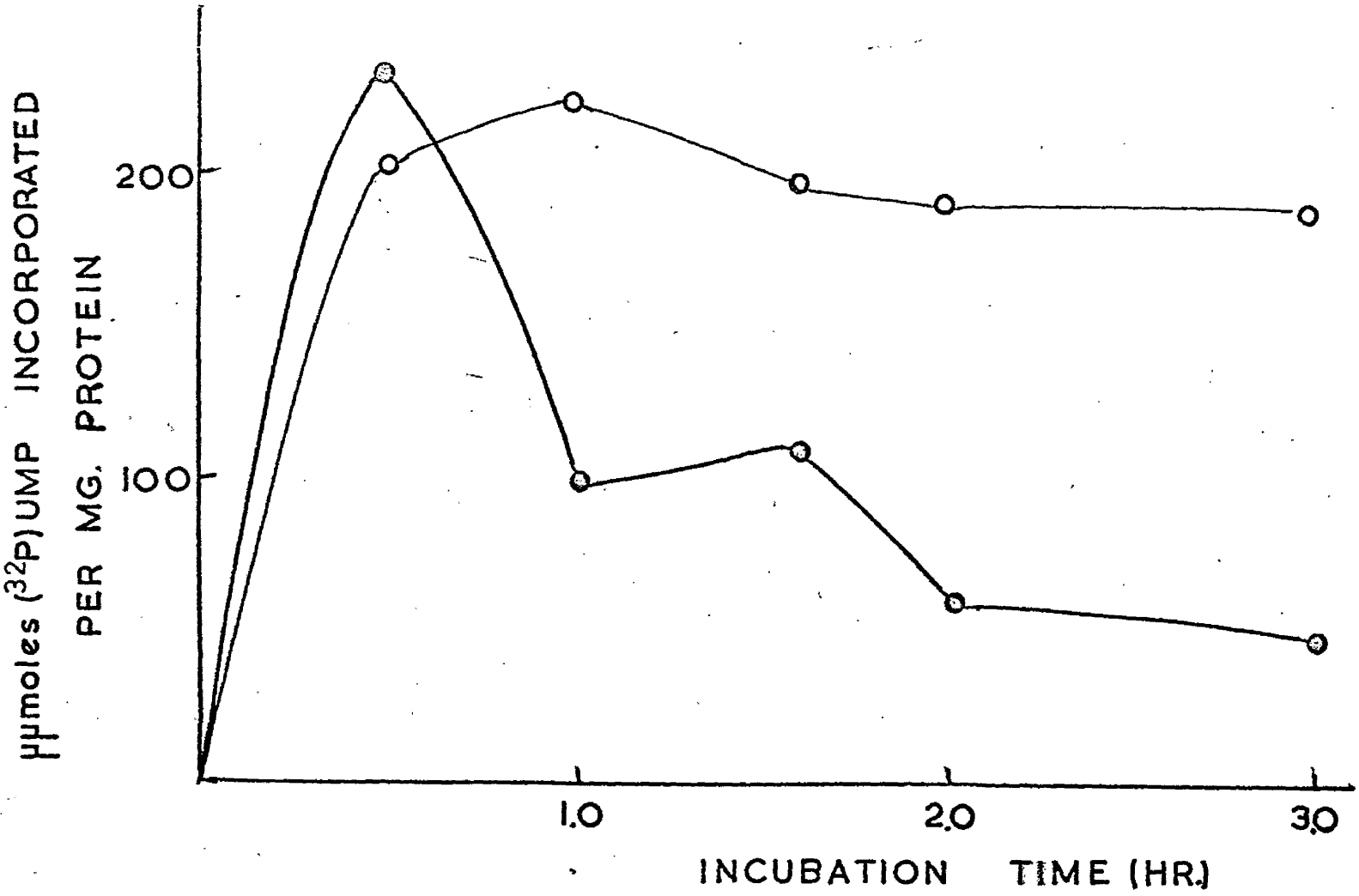

μμmoles (^{32}P)UMP INCORPORATED PER MG. PROTEIN
200
100
1.0
2.0
3.0
INCUBATION TIME (HR.)

FIGURE II.

dependent RNA nucleotidyltransferase was examined. The results (Fig. 12) showed that the activity was almost exclusively located in the nuclear preparations. Since the DNA-dependent activity was observed in fractions of sonically disrupted cells, the enzyme must have been liberated from the nucleus by such treatment. Attempts to fractionate the DNA-dependent RNA nucleotidyltransferase from nuclear fractions were not successful and were therefore abandoned. The particulate material in the nuclear enzyme was rendered soluble by the addition of 0.02 ml. saturated ammonium sulphate, pH 7.5, to the incubation mixture, but this caused inhibition of the reaction under the conditions employed.

1.2.3. Characteristics of the RNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells

The RNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells was a maximum between pH 8.5 and 10 (Fig. 13) and the effect of added Mg^{2+} ions was indistinguishable from the effect of Mg^{2+} ions on the DNA-dependent RNA nucleotidyltransferase (see Fig. 9). The reaction was inhibited by about 50 per cent in the presence of 1 μmole $MnCl_2$ (Fig. 14) and by about 70 per cent in the presence of 2 μmoles $MnCl_2$.

The time course of the incorporation of (^{32}P)UMP residues into RNA in the presence and absence of bentonite is shown in Fig. 15. As in the case of DNA-dependent RNA nucleotidyltransferase (see Fig. 11), bentonite prevented the decline in the amount of (^{32}P)UMP present in

FIGURE 12

The time course of the incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by the DNA-dependent RNA nucleotidyltransferase of nuclear and cytoplasmic fractions of Krebs II ascites tumour cells.

The incubation mixture for the assay of DNA-dependent RNA nucleotidyltransferase is described in Table 3. In addition, 0.03 ml. of 0.02 per cent (w/v) bentonite suspension in 0.01M-sodium acetate buffer, pH 6.0 was included in the reaction mixture.

—●— cytoplasmic fractions.

—○— nuclear fractions.

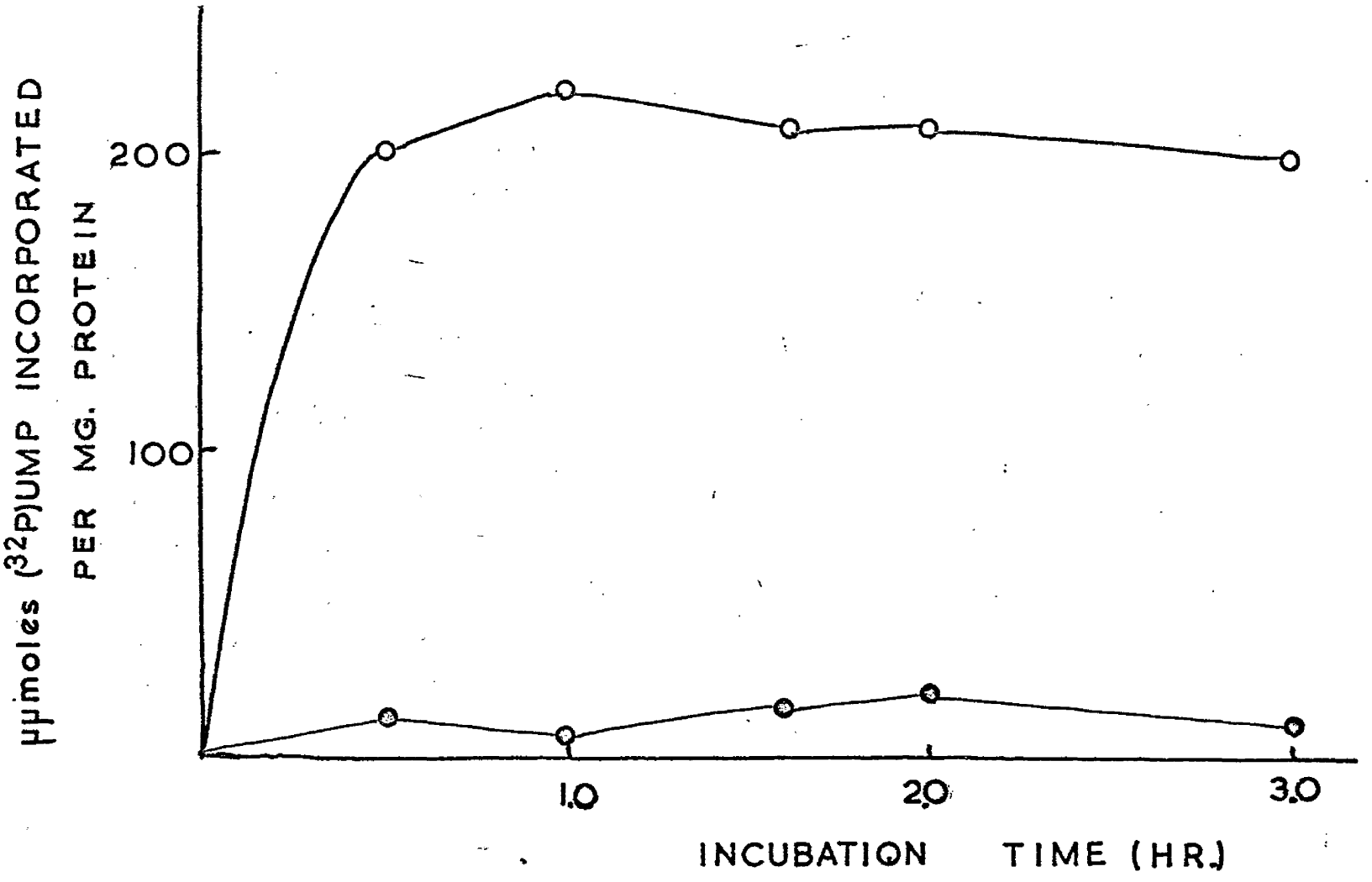

μμmoles (^{32}P)UMP INCORPORATED PER MG. PROTEIN
200
100
1.0
2.0
3.0
INCUBATION TIME (HR.)

FIGURE 12.

FIGURE 13

The effect of pH on the RNA-dependent RNA nucleotidyltransferase activity of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 μmoles tris-HCl buffer of the appropriate pH; 2 μmoles $MgCl_2$; 0.2 μmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. RNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Incubations were carried out at 37° for 20 min.

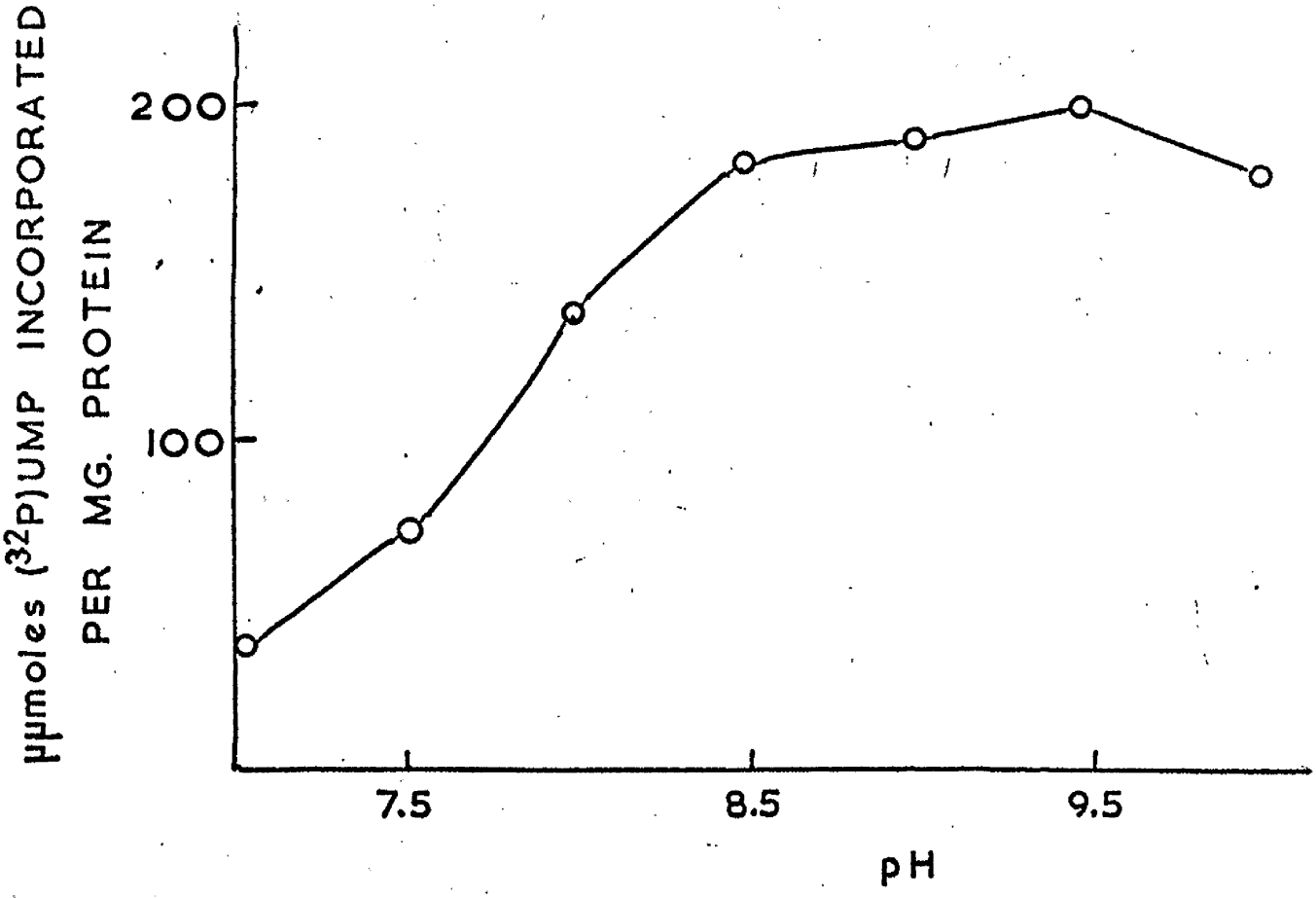

μμmoles (^{32}P)UMP INCORPORATED PER MG. PROTEIN
200
100
7.5
8.5
9.5
pH

FIGURE 13.

FIGURE 14

The effect of Mn^{2+} ions on the RNA-dependent RNA nucleotidyl-transferase activity of extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 µmoles tris-HCl buffer pH 9.5; 2 µmoles $MgCl_2$; 0.2 µmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2 x 10^7 counts/min./µmole); 50 µg. RNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml. The appropriate amount of $MnCl_2$ was included.

Incubations were carried out at 37° for 20 min.

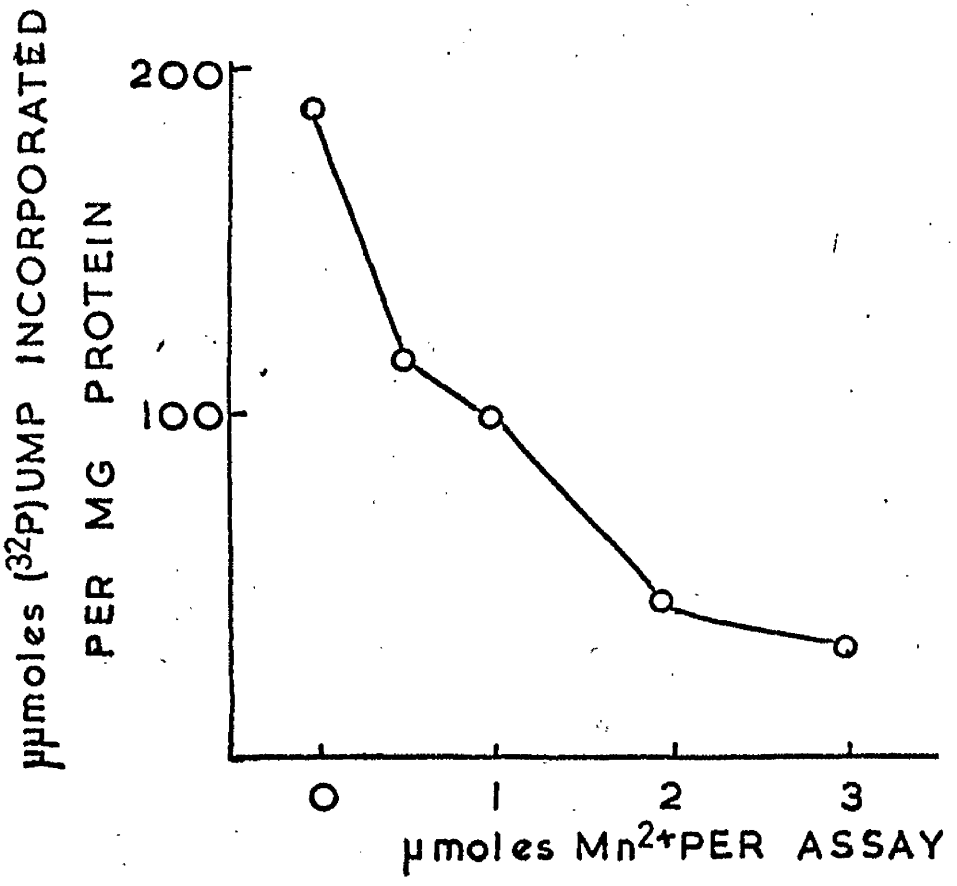

μμmoles (^{32}P)UMP INCORPORATED PER MG PROTEIN
200
100
0
1
2
3
μmoles Mn^{2+} PER ASSAY

FIGURE 14

FIGURE 16

The time course of the incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by the RNA-dependent RNA nucleotidyltransferase from extracts of Krebs II ascites tumour cells and the effect of bentonite on the reaction.

The reaction mixture for the assay of RNA-dependent RNA nucleotidyltransferase is described in Table 5.

In examining the effect of bentonite, 0.05 ml. of 0.02 per cent (w/v) bentonite suspension in 0.01M-sodium acetate buffer, pH 6.0, was included in the reaction mixture.

—o— plus bentonite.

—●— no bentonite.

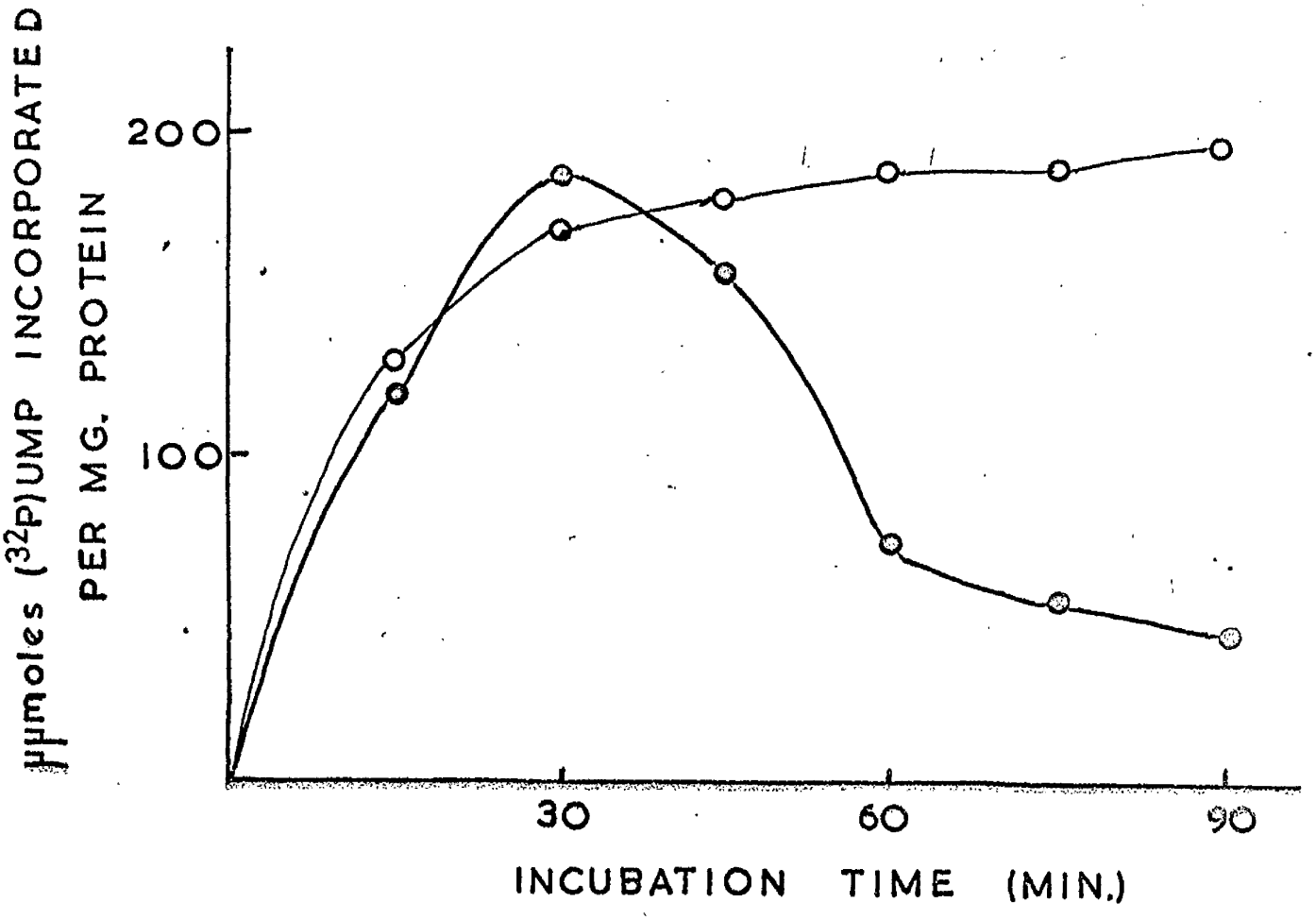

μμmoles (^{32}P)UMP INCORPORATED PER MG. PROTEIN
200
100
30
60
90
INCUBATION TIME (MIN.)

FIGURE 15.

RNA after 30 min. Krebs II ascites tumour cell RNA, yeast RNA and EMC virus RNA all served as primers for the RNA-dependent incorporation of (^{32}P)UMP residues into RNA.

Table 6 shows the influence of ribonucleoside 5'-triphosphates on the activity of RNA-dependent RNA nucleotidyltransferase. The effect was strikingly different from that observed for the activity of DNA-dependent RNA nucleotidyltransferase (see Table 4). In the RNA-dependent reaction, the addition of any one or all three ribonucleoside 5'-triphosphates markedly diminished incorporation. This suggested that polynucleotide chains containing mainly (^{32}P)UMP residues were synthesised and this was supported by the data in Table 7, where analysis of the products of alkaline hydrolysis of the RNA indicated that radioactivity was associated principally with UMP. Nevertheless, the data indicated some randomisation of ^{32}P from (^{32}P)UMP and some synthesis of polyribonucleotide containing all four ribonucleosides may have proceeded simultaneously with the formation of polyuridylic acid.

In other experiments, the intracellular distribution of the RNA-dependent RNA nucleotidyltransferase was examined. The results (Fig. 16) showed that the activity was principally located in the 105,000 g sediment, with least activity in the 10,000 g sediment. Addition of RNA stimulated the 105,000 g supernatant fraction to the greatest extent. This may in part have been due to amination of (^{32}P)UTP to (^{32}P)CTP followed by the formation of -pGpCpA sequences on exposed ends of the primer chain by enzymes in this soluble fraction (see Introduction, section 5.1.2).

TABLE 6

The effect of adding ribonucleoside 5'-triphosphates on the activity of RNA-dependent RNA nucleotidyltransferase in extracts of Krebs II ascites tumour cells.

The reaction mixture contained: 50 μmoles tris-HCl buffer, pH 9.5; 2 μmoles $MgCl_2$; 0.2 μmoles (α-^{32}P)UTP (2×10^7 counts/min./μmole); 50 μg. RNA and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml.

Where indicated, 0.2 μmoles of ATP, GTP or CTP were included.

Incubations were carried out at 37° for 20 min.

TABLE 6.

Nucleoside Triphosphates Present	Counts/min incorporated per mg. protein
(α-^{32}P)UTP	100
(α-^{32}P)UTP + ATP	48
(α-^{32}P)UTP + GTP	42
(α-^{32}P)UTP + CTP	27
(α-^{32}P)UTP + ATP + GTP + CTP	40

TABLE 7

The distribution of radioactivity in the ribonucleoside 3'(2')-monophosphates obtained on alkaline hydrolysis of RNA synthesised by the RNA-dependent RNA nucleotidyltransferase of Krebs II ascites tumour cells.

TABLE 7.

RIBONUCLEOTIDE	PER CENT TOTAL RADIOACTIVITY
CYTIDINE 3'(2')-PHOSPHATE	8
URIDINE 3'(2')-PHOSPHATE	69
ADENOSINE 3'(2')-PHOSPHATE	9
GUANOSINE 3'(2')-PHOSPHATE	14

FIGURE 16

The incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by enzymes in subcellular components of Krebs II ascites tumour cells and the effect of added RNA.

The reaction mixture contained: 50 µmoles tris-HCl buffer, pH 9.5; 2 µmoles $MgCl_2$; 0.2 µmoles each of ATP, CTP, GTP and (α-^{32}P)UTP (2×10^7 counts/min./µmole) and 0.2 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.4 ml. 50 µg. RNA was included where indicated.

Incubations were carried out at 37° for 20 min.

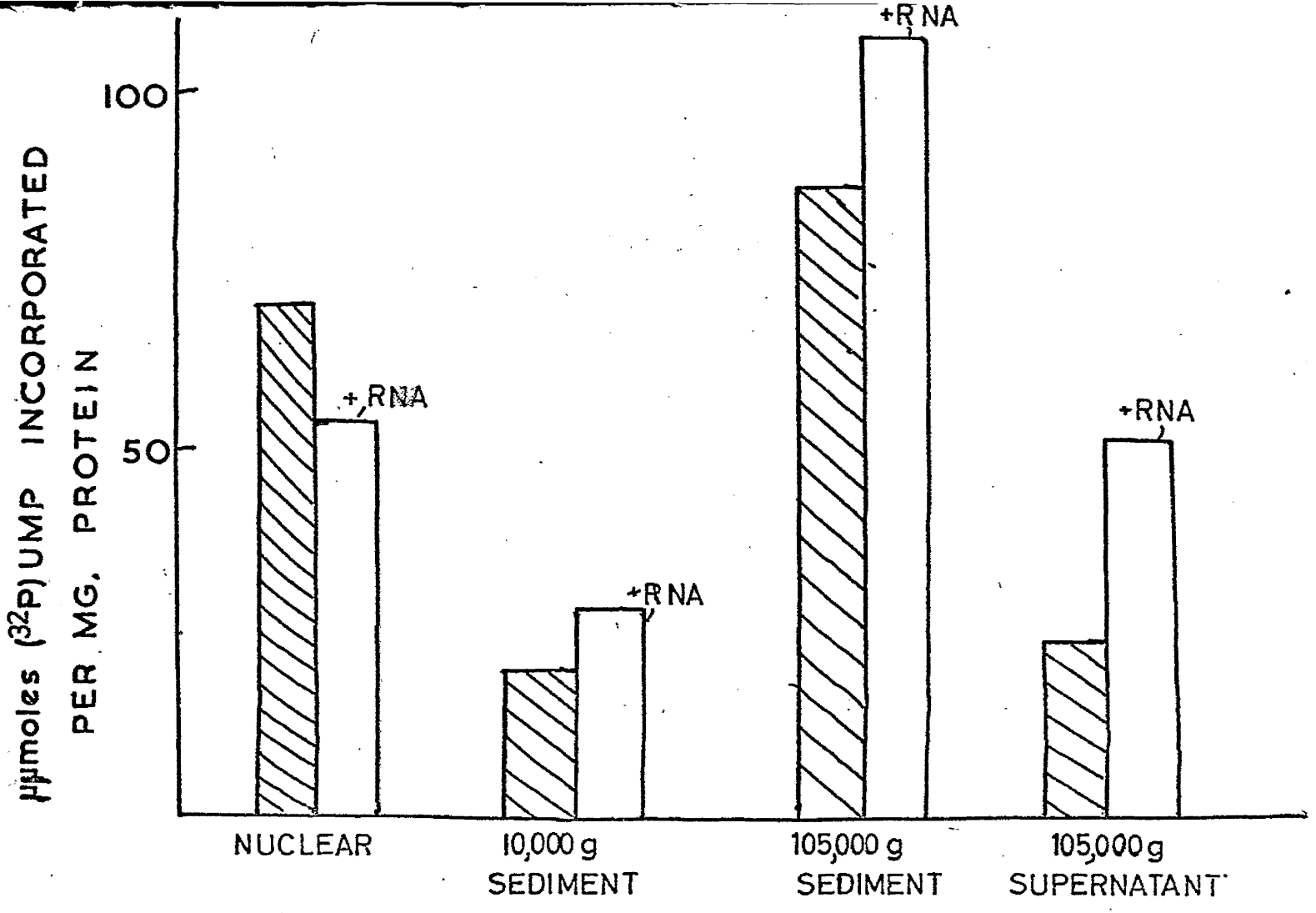

μμmoles (^{32}P) UMP INCORPORATED PER MG. PROTEIN
100
50
+RNA
+RNA
+RNA
+RNA
NUCLEAR
10,000 g SEDIMENT
105,000 g SEDIMENT
105,000 g SUPERNATANT

FIGURE 16

The other enzyme fractions were also stimulated, with the exception of the nuclear enzyme which was inhibited. The activity in the nuclear fraction may therefore have been due in part to some residual DNA-dependent RNA nucleotidyltransferase activity under the conditions employed. This conclusion was supported by the results shown in Table 6. Whereas in the nucleoside 5'(2')-monophosphates obtained on alkaline hydrolysis of RNA synthesised by non-nuclear enzymes (B, C and D), radioactivity was associated principally with UMP, in those obtained from the RNA synthesised by the nuclear enzyme, radioactivity was more evenly distributed among the four nucleotides.

In conclusion, therefore, the number of polynucleotide chains which contained all four ribonucleotides was small compared to the number of chains containing only UMP residues, which were synthesised under the conditions of RNA-dependent RNA nucleotidyltransferase.

1.3. The incorporation of (^{32}P)UMP into RNA and of (^{32}P)TMP into DNA by enzymes from Krebs II ascites tumour cells infected with encephalomyocarditis (EMC) virus

The DNA nucleotidyltransferase and DNA-dependent RNA nucleotidyltransferase activities of crude extracts of Krebs II ascites tumour cells were not appreciably altered after infection of the cells with EMC virus (Fig. 17 and Fig. 18). It was repeatedly observed, however, that after virus infection the level of RNA-dependent RNA nucleotidyltransferase activity increased and the result of one such experiment is shown in Fig. 19. The experiments were performed on more than ten occasions and

TABLE 8

The distribution of radioactivity in the ribonucleoside 3'(2')-monophosphates obtained on alkaline hydrolysis of RNA synthesised by enzymes in the subcellular components of Krebs II ascites tumour cells. The enzymes were assayed under the conditions for RNA-dependent RNA nucleotidyltransferase described in Table 3.

TABLE 8.

ENZYME SOURCE	PER CENT RADIOACTIVITY IN			
	CMP	GMP	AMP	UMP
A. NUCLEAR	22	14	17	47
B. 10,000 g SEDIMENT	4	4	10	82
C. 105,000 g SEDIMENT	9	4	7	78
D. 105,000 g SUPERNATANT FRACTION	2	10	8	80

FIGURE 17

The DNA nucleotidyltransferase activities in extracts of uninfected Krebs II ascites tumour cells and in extracts of Krebs II ascites tumour cells infected with EMC virus.

The reaction mixture contained: 10 μmoles tris-HCl buffer, pH 7.5; 9 μmoles KCl; 0.08 μmoles EDTA; 0.5 μmoles KH_2PO_4-K_2HPO_4 buffer, pH 7.5; 1 μmole $MgCl_2$; 50 μm-moles each of dATP, dCTP, dGTP and (α-^{32}P)TTP (2×10^7 counts/min./μmole); 50 μg. heat denatured DNA and 0.15 ml. of enzyme (1.5 mg. protein per ml.) in a total volume of 0.25 ml.

Incubations were carried out at 37° for 45 min.

—●— infected.

—○— uninfected.

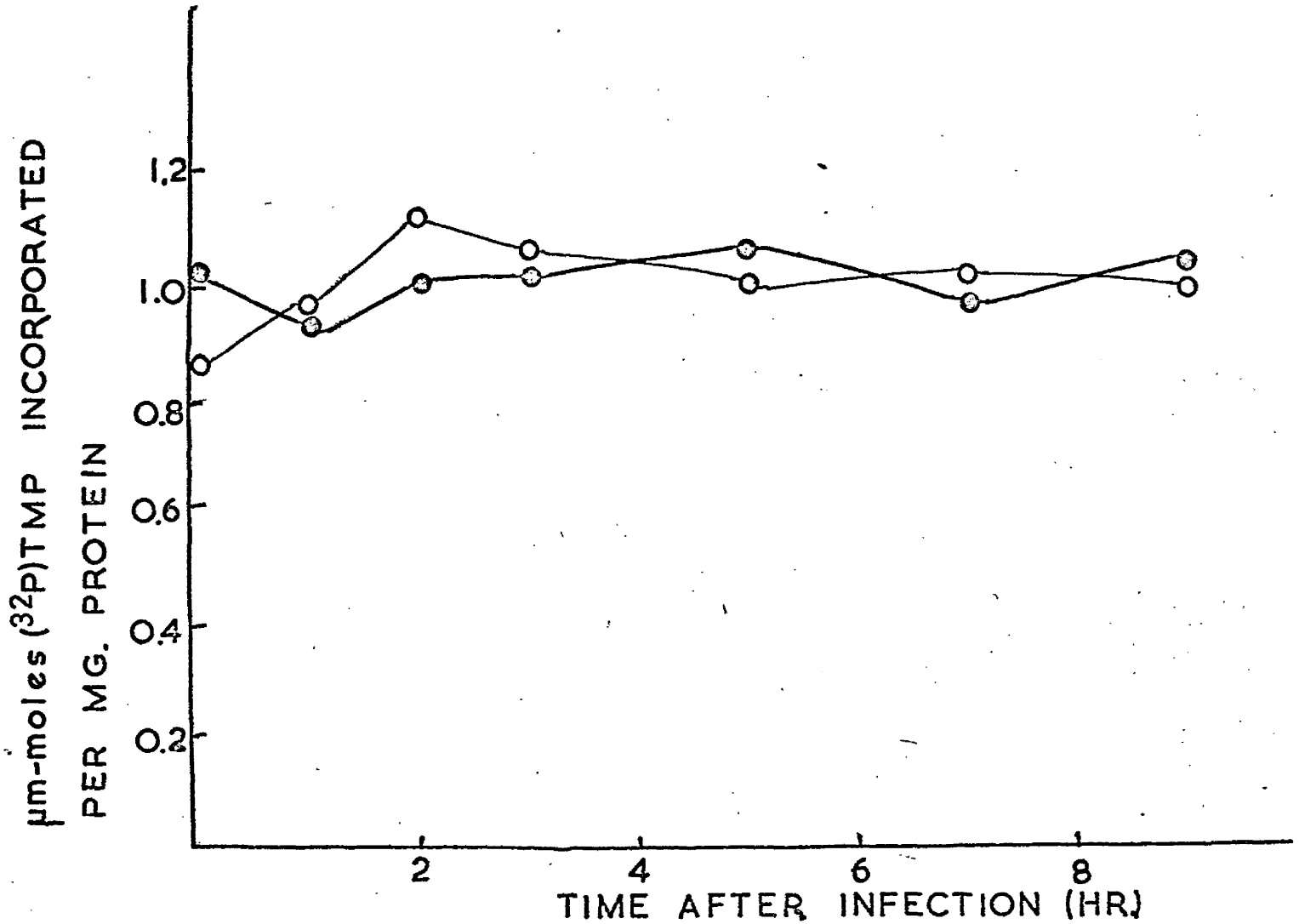

μm-moles (^{32}P)TMP INCORPORATED PER MG. PROTEIN
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
8
TIME AFTER INFECTION (HR.)

FIGURE 17.

FIGURE 18

The DNA-dependent RNA nucleotidyltransferase activities in extracts of Krebs II ascites tumour cells and in extracts of Krebs II ascites tumour cells infected with EMC virus.

The incubation mixture for the assay of DNA-dependent RNA nucleotidyltransferase is described in Table 3.

—●— infected.

—○— uninfected.

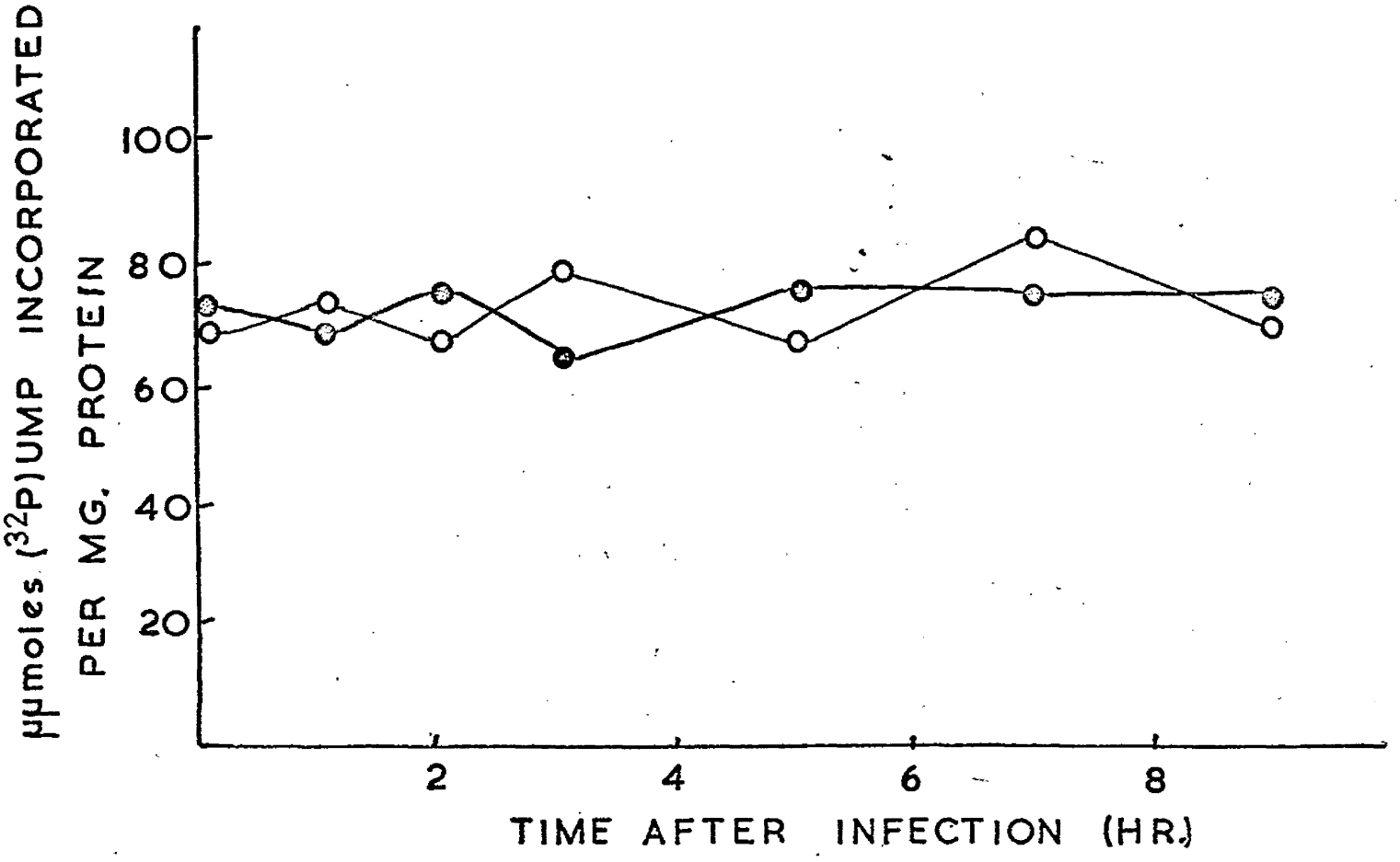
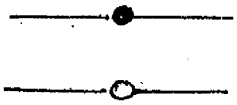

μμmoles (^{32}P)UMP INCORPORATED
PER MG. PROTEIN
100
80
60
40
20
2
4
6
8
TIME AFTER INFECTION (HR.)

FIGURE 18.

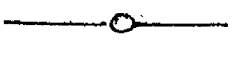
FIGURE 19

The RNA-dependent RNA nucleotidyltransferase activities in extracts of Krebs II ascites tumour cells and in Krebs II ascites tumour cells infected with EMC virus.

The incubation mixture for the assay of RNA-dependent RNA nucleotidyl-transferase is described in Table 3.



infected.



uninfected.

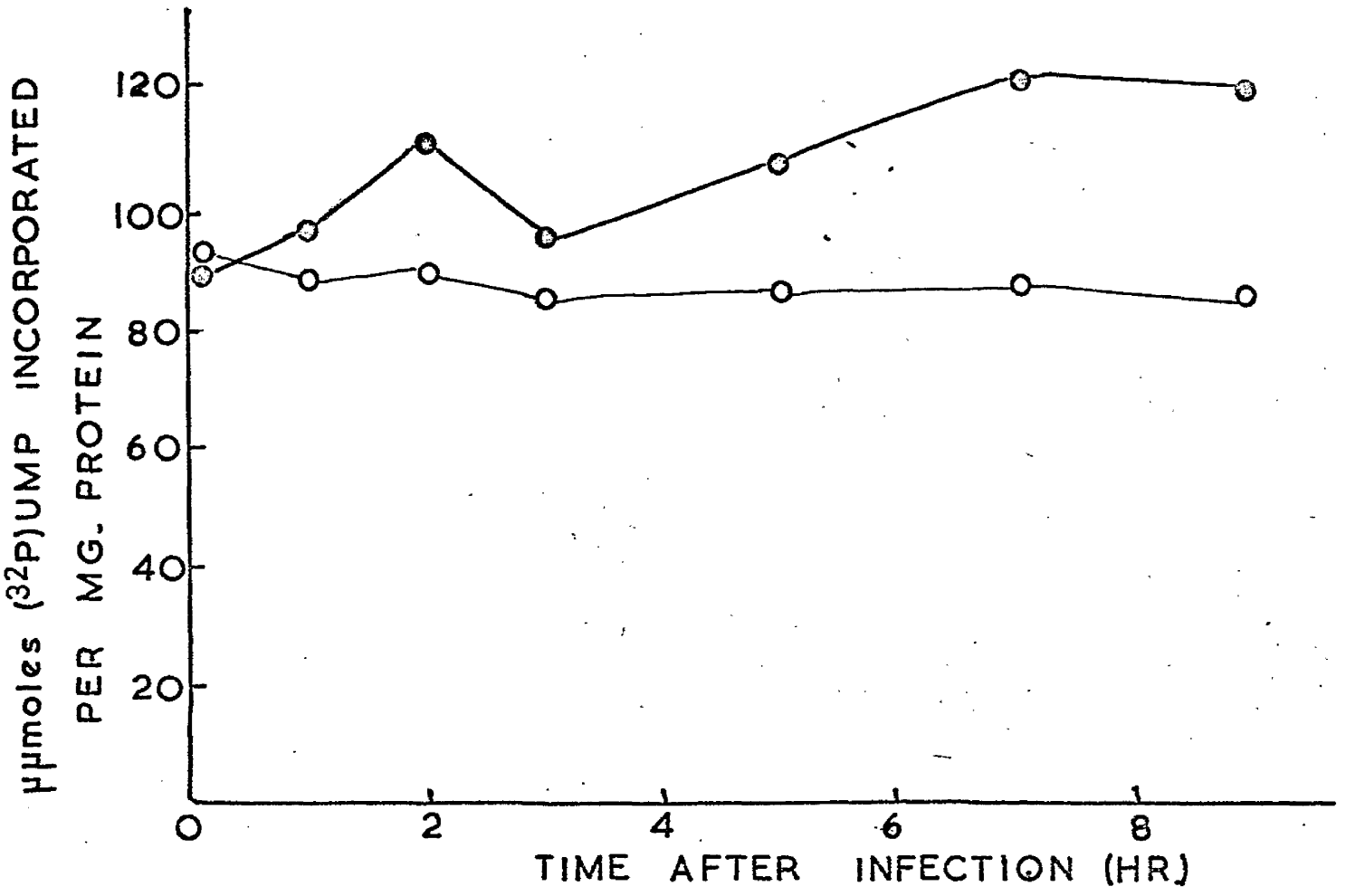

μμmoles (^{32}P)UMP INCORPORATED
PER MG. PROTEIN
120
100
80
60
40
20
0
2
4
6
8
TIME AFTER INFECTION (HR)

FIGURE 19.

a summary of the results obtained is shown in Fig. 20.

The observation of increased RNA-dependent RNA nucleotidyltransferase after virus infection was extended to cell fractions (Fig. 21), but at this stage of the investigation, no serious attempt was made to determine the site of specific virus-induced changes within the cell and the "nuclei" prepared, for example, were seriously contaminated with cytoplasmic debris. In Fig. 21, the upper and lower limits of RNA-dependent RNA nucleotidyltransferase activity in control cells and virus-infected cells are indicated for a series of experiments. A general increase in activity after virus infection was evident and the increase in the "nuclear" fraction appeared to be greater because of the declining activity of the controls. The increase in activity was resistant to actinomycin D.

Once appreciable changes of this type had been established, the location of changes within the cell was examined by careful fractionation of cell constituents, special care being taken to avoid contamination of the nuclear fraction. Table 9 shows that the only clear-cut change occurs in the material sedimenting at 10,000 g, which would be expected to contain mitochondria, heavy microsomes and aggregated material. Table 10 shows that after infection, radioactivity in the ribonucleotides obtained after alkaline hydrolysis of the RNA synthesised by enzymes in the fraction sedimenting at 10,000 g is randomised to a greater degree, which suggests that a new enzyme has become associated with this fraction. The distribution of radioactivity among the nucleotides from the RNA synthesised by the other cell fractions did not change appreciably after

FIGURE 20

A summary of the changes which occur in the activities of DNA nucleotidyltransferase, DNA-dependent RNA nucleotidyltransferase and RNA-dependent RNA nucleotidyltransferase in Krebs II ascites tumour cells after infection with EMC virus.

(a) DNA nucleotidyltransferase.

(b) DNA-dependent RNA nucleotidyltransferase.

(c) RNA-dependent RNA nucleotidyltransferase.

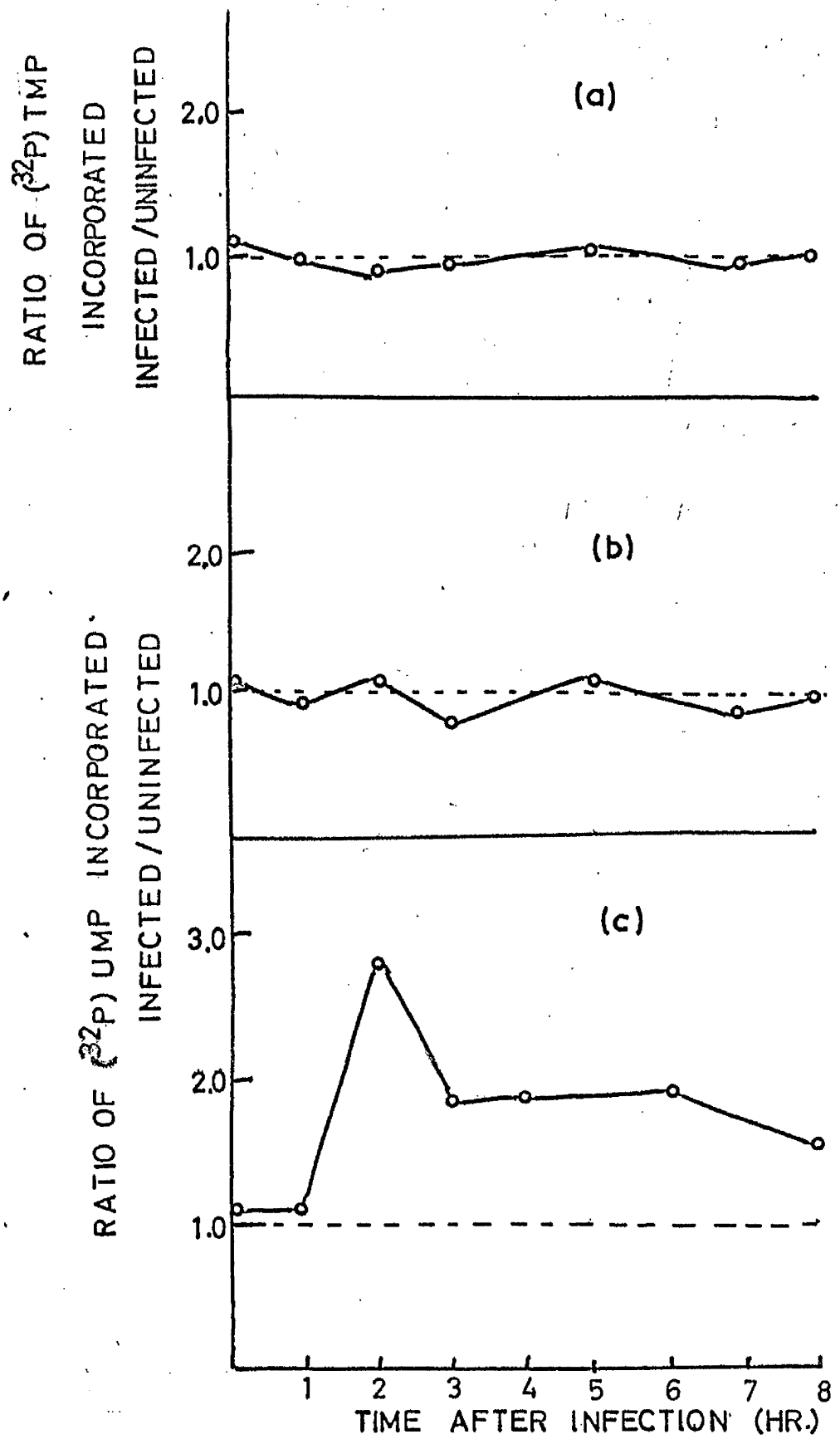

RATIO OF (^{32}P) TMP INCORPORATED INFECTED/UNINFECTED
(a)
2.0
1.0
RATIO OF (^{32}P) UMP INCORPORATED INFECTED/UNINFECTED
(b)
2.0
1.0
(c)
3.0
2.0
1.0
1 2 3 4 5 6 7 8
TIME AFTER INFECTION (HR.)

FIGURE 20.

FIGURE 21

The RNA-dependent RNA nucleotidyltransferase activity of whole cells, "nuclei" and cytoplasm in Krebs II ascites tumour cells and in Krebs II ascites tumour cells infected with EMC virus.

The incubation mixture for the assay of RNA-dependent RNA nucleotidyltransferase is shown in Table 5.

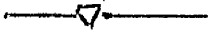
The upper and lower limits of a series of experiments are shown in each case.

(a) Whole-cell disruptate.

(b) "Nuclear" enzymes.

(c) Cytoplasmic enzymes.

Enclosing the results with enzyme from EMC virus infected cells.



Enclosing the results with enzymes from uninfected cells.

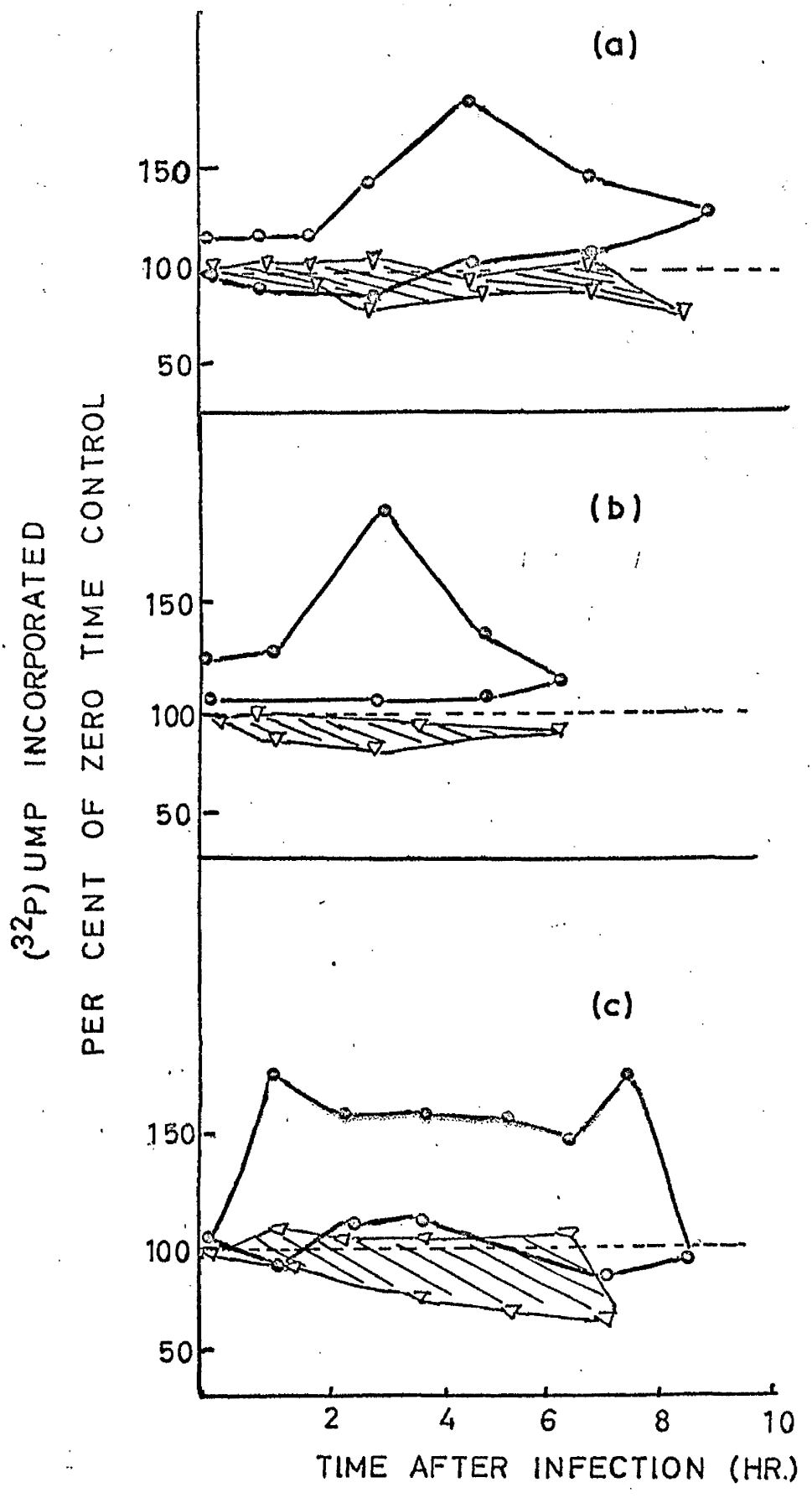

(a)
(b)
(c)
150
100
50
150
100
50
150
100
50
2
4
6
8
10
TIME AFTER INFECTION (HR.)
PER CENT OF ZERO TIME CONTROL
(^{32}P) UMP INCORPORATED

FIGURE 21.

TABLE 9

The incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by an RNA-dependent reaction catalysed by enzymes from Krebs II ascites tumour cells and from Krebs II ascites tumour cells infected with EMC virus.

The enzyme fractions were prepared 5.5 hr. after infection of the Krebs II cells both from virus-infected and uninfected cells.

The incubation mixture for the assay of RNA-dependent RNA nucleotidyltransferase is described in Table 3.

The results in this Table are a mean of three experiments.

TABLE 9

Enzyme Fraction	Ratio of (^{32}P)UMP incorporated Infected / Control
NUCLEAR	1·34
10,000 g SEDIMENT	4.48
105,000 g SEDIMENT	0.98
105,000 g SUPERNATANT FRACTION	0.97

TABLE 10

The distribution of radioactivity in the ribonucleoside 3'(2')-monophosphates obtained on alkaline hydrolysis of RNA synthesised by enzymes in the 10,000 *g* sediment fraction of Krebs II ascites tumour cells and in Krebs II ascites tumour cells infected with EMC virus.

The enzymes were assayed under the conditions for RNA-dependent RNA nucleotidyltransferase described in Table 3.

TABLE 10.

Source of 10,000g sediment	Per cent radioactivity in			
	CMP	GMP	AMP	UMP
UNINFECTED CELLS	4	4	10	82
INFECTED CELLS	11	7	18	64

infection. There was no appreciable change in the DNA-dependent RNA nucleotidyltransferase activity of these cell fractions after infection (Table 11).

The conclusion drawn from these experiments was that the increase in RNA-dependent RNA nucleotidyltransferase activity after infection of Krebs II ascites tumour cells with EMC virus was due to an enzyme, possibly a new enzyme, which occurred in the cytoplasmic material sedimenting at 10,000 g or which, in the process of cell fractionation, had been eluted from another site in the cell and became associated with the 10,000 g fraction.

2. The analysis of RNA from uninfected Krebs II ascites tumour cells and from Krebs II ascites tumour cells infected with encephalomyocarditis virus

When RNA was isolated from Krebs II ascites tumour cells by the method of Kirby (1956), it was found to be degraded and of low molecular weight when examined in the analytical ultracentrifuge. In consequence, the method described in the experimental section was evolved (Section 8.1). Sodium dodecylsulphate was added to extract all the RNA from the cells (see Perry, 1963) and bentonite was added to ensure that the RNA was not degraded. The extracted RNA was analysed to determine the RNA and DNA content by means of the orcinol and Ceriotti reactions (Experimental, sections 15.2 and 15.3). From the results of these analyses, together with the spectral properties and sensitivity to nucleases, the material was found to contain not less than 95 per cent RNA.

TABLE 11

The incorporation of (^{32}P)UMP residues from (α-^{32}P)UTP into RNA by a DNA-dependent reaction catalysed by enzymes from Krebs II ascites tumour cells and from Krebs II ascites tumour cells infected with EMC virus.

The enzyme fractions were prepared 3.5 hr. after infection of the Krebs II cells both from virus-infected and uninfected cells.

The incubation mixture for the assay of DNA-dependent RNA nucleotidyltransferase is described in Table 5.

The results are a mean of three experiments.

TABLE II

Enzyme Fraction	Ratio of (^{32}P)UMP incorporated Infected / Control
NUCLEAR	1.02
10,000g SEDIMENT	1.11
105,000g SEDIMENT	0.99
105,000g SUPERNATANT FRACTION	0.94

The main object of the work was to fractionate the cellular RNA from infected and uninfected cells and to examine its components. The RNA was therefore subjected to centrifugation in sucrose density gradients as already described (Experimental, section 9.1).

In the first instance, it was necessary to determine the conditions of centrifugation which would give the best separation of the RNA components. When the RNA was layered on top of the sucrose gradient and centrifuged at 37,000 rev./min., the maximum speed of the Spinco SW 39 rotor, for 90 min., no separation was achieved (Fig. 22(a)), since all the RNA was located in one band near the top of the gradient. More prolonged periods of centrifugation at a lower speed, however, produced a satisfactory separation (Fig. 22(b)). Three peaks of material absorbing at 256 mμ could be demonstrated in the extracted RNA. The sedimentation constants of these peaks were calculated from their rates of sedimentation observed in the Spinco Model E Analytical Ultracentrifuge and shown to be 30s, 19s and 4s. The 30s and 19s peaks were derived from the ribosomal RNA and the 4s peak from transfer RNA (sRNA). The 30s peak represented the major component in all cases and, although the 30s and 19s components did not separate completely, the patterns obtained were satisfactory.

Krebs II ascites tumour cell DNA was also analysed in sucrose density gradients (Fig. 23(a) and 23(b)). The DNA preparation appeared to be heterogeneous and the sedimentation pattern in no way resembled that of RNA. It might have been expected that DNA, by virtue of its high molecular weight, would sediment rapidly in a sucrose gradient. However, DNA

FIGURE 22

Sedimentation analysis in sucrose density gradients of RNA from Krebs II ascites tumour cells.

(a) The sucrose gradients (5-20 per cent (w/v)) were centrifuged for 90 min. at 37,000 rev./min. at 0° in the SW 39 rotor of the Spinco Model L Preparative Ultracentrifuge.

(b) The sucrose gradients were centrifuged at 20,500 rev./min. for 12.5 hr. at 0° in the SW 39 rotor.

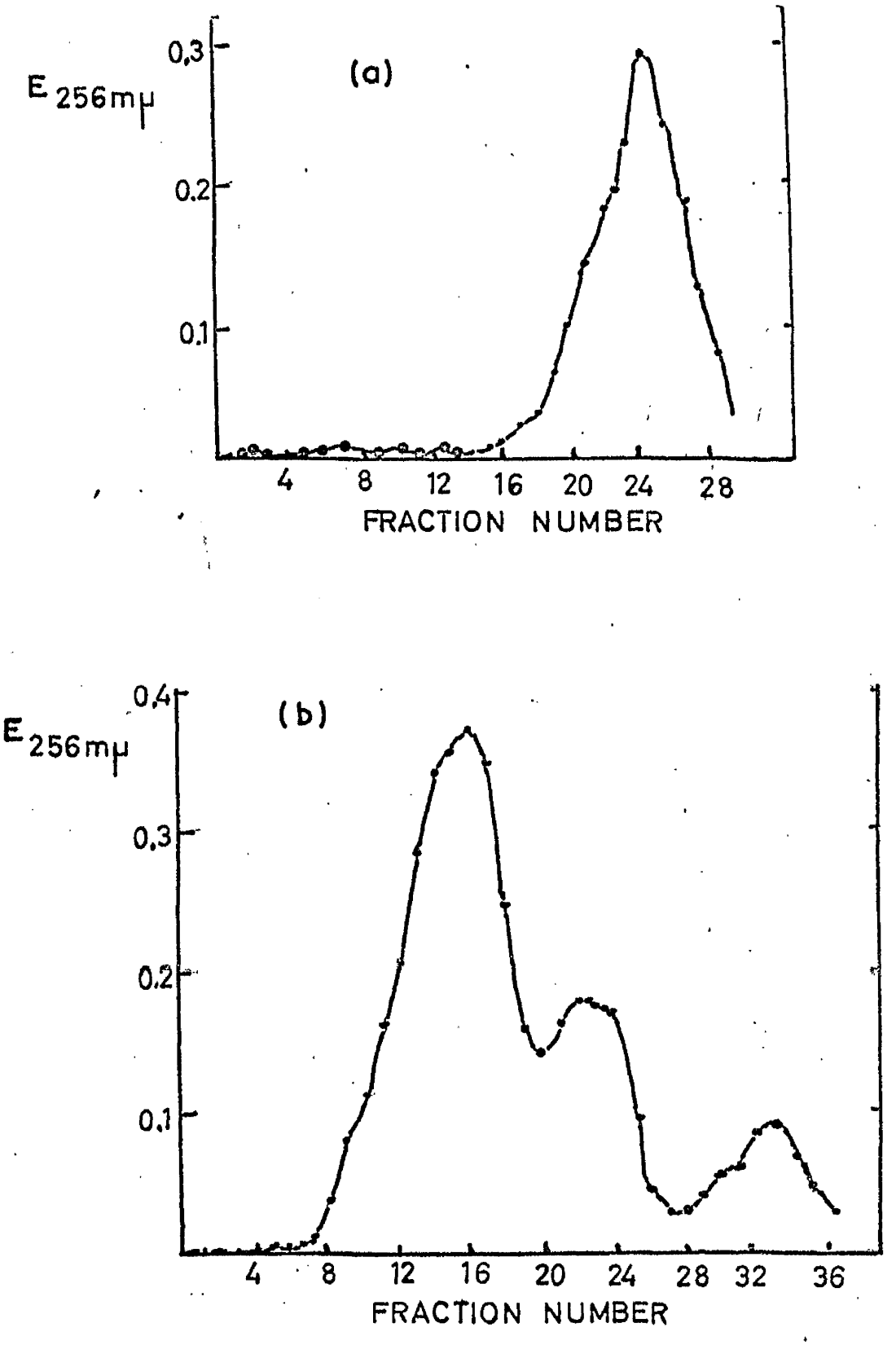

(a)
E 256mμ
0,3
0.2
0.1
4
8
12
16
20
24
28
FRACTION NUMBER
(b)
E 256mμ
0,4
0.3
0.2
0.1
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

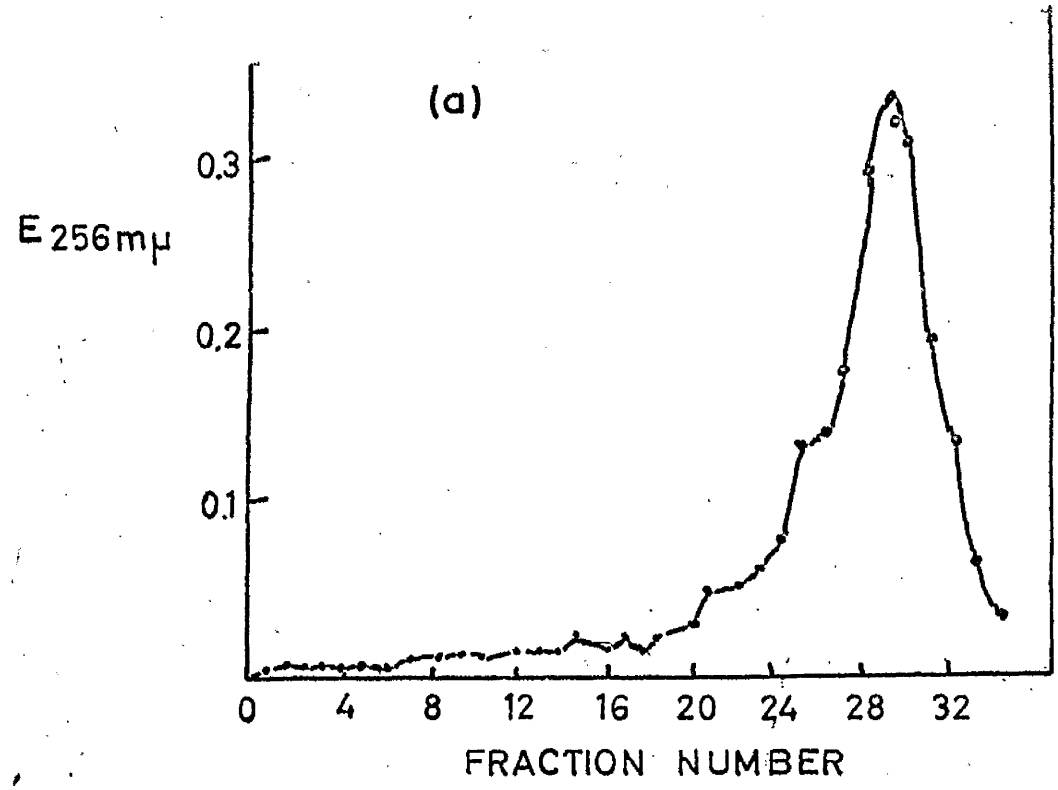
FIGURE 22.

FIGURE 25

Sedimentation analysis in sucrose density gradients of DNA from Krebs II ascites tumour cells.

(a) The sucrose gradients (5-20 per cent (w/v)) were centrifuged at 37,000 rev./min. for 90 min. at 0° in the SW 39 rotor of the Spinco Model L Preparative Ultracentrifuge.

(b) The sucrose gradients were centrifuged at 37,000 rev./min. for 6 hr. at 0° in the SW 39 rotor.


(a)
0.3
0.2
0.1
E 256 mμ
0
4
8
12
16
20
24
28
32
FRACTION NUMBER

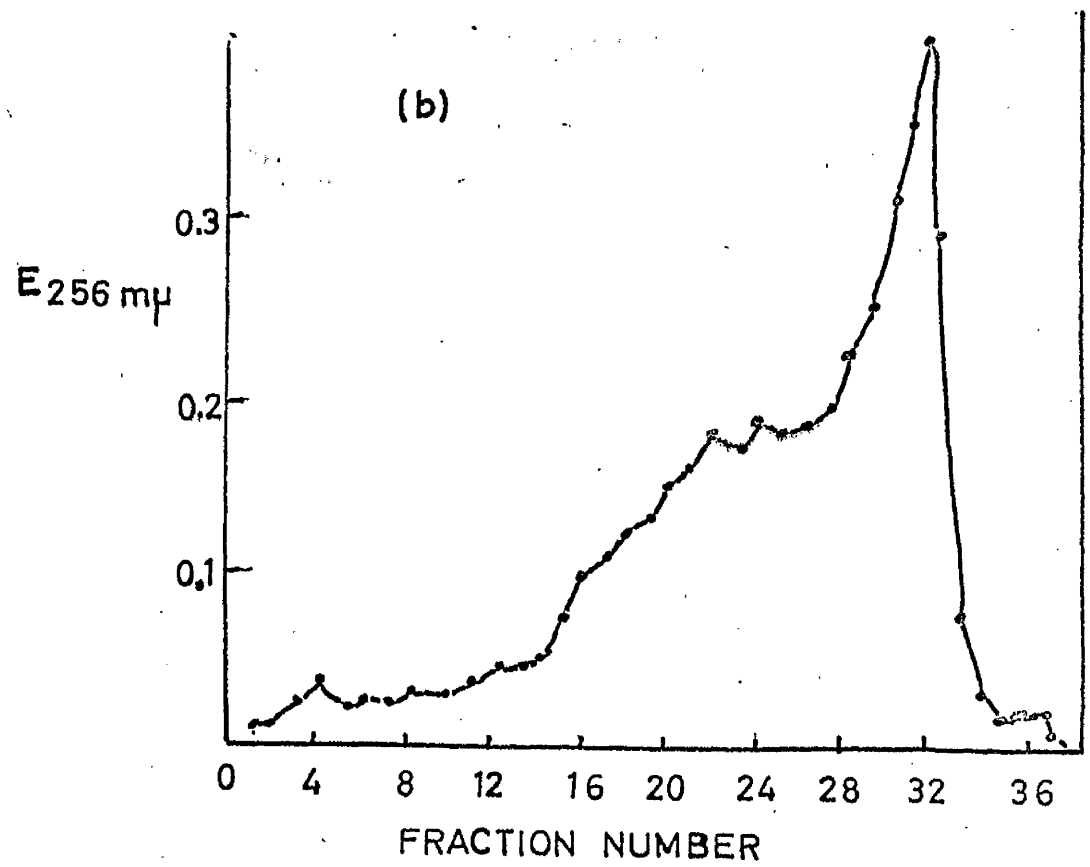

(b)
0.3
0.2
0.1
E 256 mμ
0
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

FIGURE 23.

possesses a much more rigid secondary structure than RNA, which can assume a compact, folded configuration. The DNA molecules encounter more resistance to sedimentation than the RNA and therefore tend to float on top of the sucrose gradient, while the RNA is able to migrate.

When a reproducible sedimentation pattern of RNA in sucrose gradients had been established, the cellular RNA was analysed in sucrose gradients after exposing the Krebs II ascites tumour cells to radioactive precursors of RNA. Thereafter, the distribution of radioactivity and ultraviolet-absorbing material was determined (Experimental, section 9.1). In this manner, the nature of newly-synthesised RNA and the changes occurring with time were determined. Bacterial cells can be pulse-labelled by brief exposure to a radioactive RNA precursor followed by addition of a large amount of non-radioactive precursor, which eliminates further incorporation of radioactivity by dilution. Mammalian cells, however, possess very large pools of nucleic acid precursors, which have interrelated pathways of metabolism. When a particular radioactive precursor of RNA is added, the radioactivity becomes distributed among the pools, so that when non-radioactive precursor is added, it may not prevent further incorporation of radioactivity as in the case of bacterial cells. Krebs II ascites tumour cells were therefore incubated continuously with a small amount of RNA precursor of high specific activity and no attempt was made to carry out conventional pulse labelling experiments.

When ^{3}H-uridine was used, a rapidly-sedimenting peak of radioactive RNA (40-45s) and a peak of radioactive 4s RNA appeared within about 20 min.

(Fig. 24). A radioactive 12s component was present in this instance, but it did not always appear and probably represented degradation of the labelled 40s component. After 50 min. exposure, (Fig. 24), a second component of the rapidly-sedimenting RNA appeared (33-35s) and incorporation began to appear in the 30s and 19s ribosomal components. Once again, the 12s component was present. The pattern of incorporation at 80 min. (Fig. 24) showed that the 30s and 19s components were labelled and some evidence remained of the two rapidly sedimenting components. From these experiments, it would appear that the first components to become labelled are the 40s and the 4s components. The 40s peak may act as a precursor for the 35s component and then for the 30s and 19s components of ribosomal RNA.

The corresponding experiment with ^{32}P-labelled orthophosphate is shown in Fig. 25. The most striking feature was that after 20 min. exposure, there was no evidence of a radioactive 40s component. This appeared only after 80 min. exposure, giving a pattern similar to that obtained after a 20 min. exposure to ^{3}H-uridine. In this instance, no 12s component was present, supporting the hypothesis that it represented a breakdown product of rapidly sedimenting, labelled RNA. After a 260 min. exposure (Fig. 25), the two rapidly-sedimenting components were present along with some degree of labelling of the ribosomal components. The difference in time between the patterns obtained with ^{3}H-uridine and ^{32}P-orthophosphate was probably caused by different rates of absorption of the two precursors and the large size of the phosphate pool relative

FIGURE 24

Sedimentation analysis in sucrose density gradients (5-20 per cent (v/v)) of RNA from Krebs II ascites tumour cells which have been exposed to ^{3}H-uridine (5 µcuries per ml. cell suspension) for 20 min., 50 min. and 80 min.

The sucrose gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E_{256} mµ.

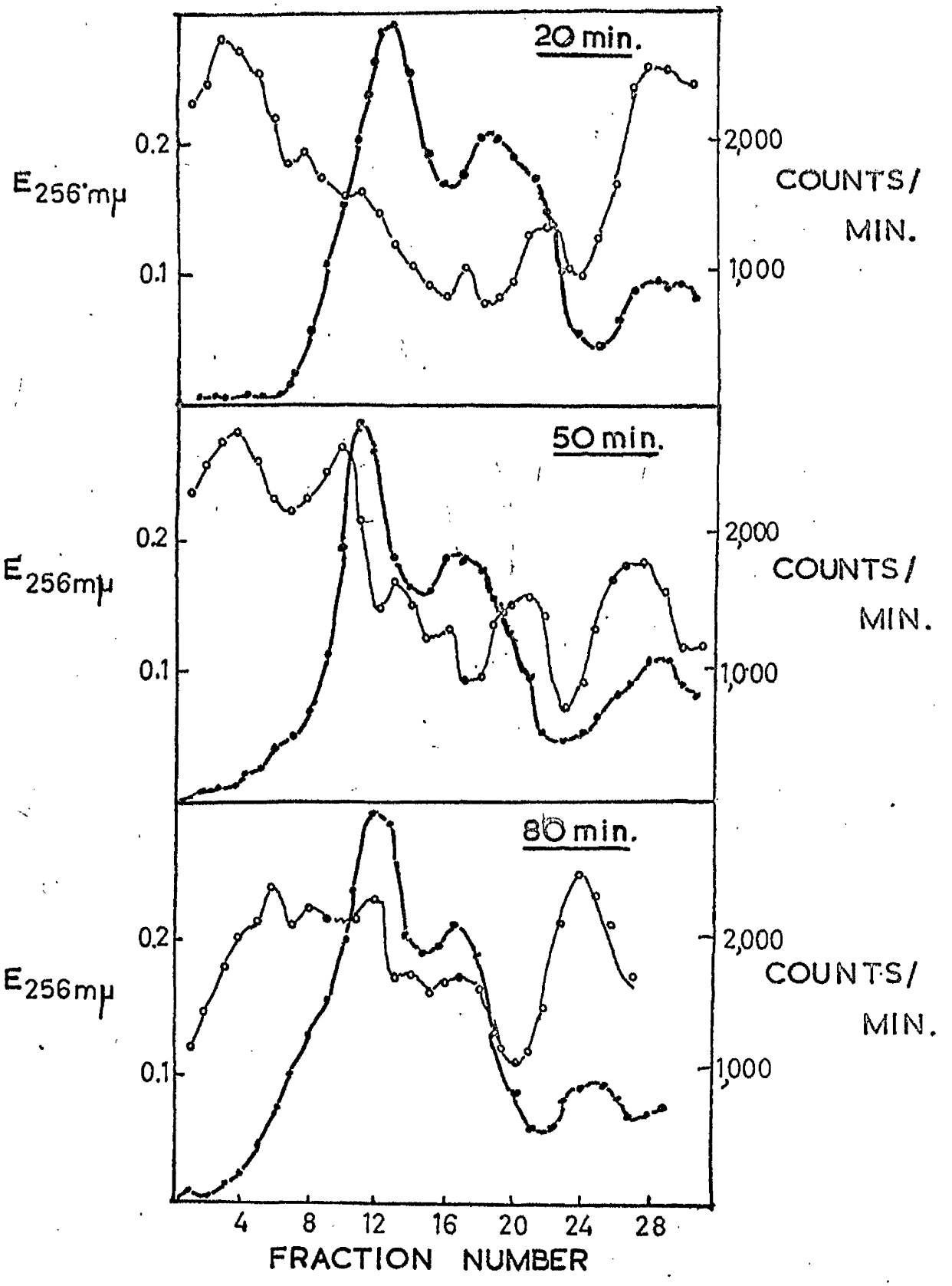

20 min.
50 min.
80 min.
$E_{256m\mu}$
0.2
0.1
2,000
1,000
COUNTS/
MIN.
4
8
12
16
20
24
28
FRACTION NUMBER

FIGURE 24.

FIGURE 25

Sedimentation analysis in sucrose density gradients (5-20 per cent (w/v)) of RNA from Krebs II ascites tumour cells which have been exposed to ^{32}P-orthophosphate (20 μcuries per ml. cell suspension) for 20 min., 90 min. and 260 min.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E_{256} mμ.

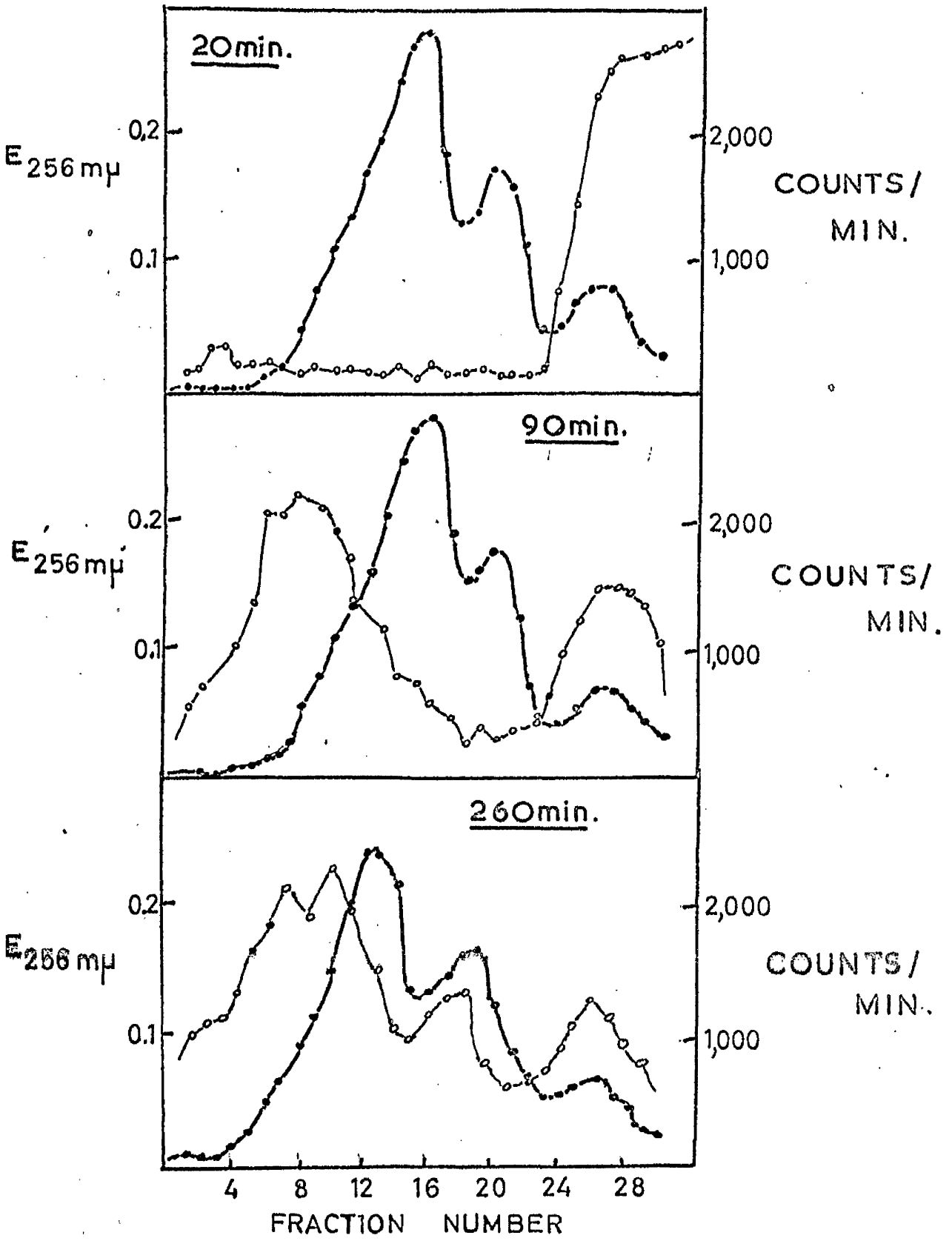

20min.
90min.
260min.
E 256 mμ
0.2
0.1
2,000
1,000
COUNTS/
MIN.
4
8
12
16
20
24
28
FRACTION NUMBER

FIGURE 25.

to that of the uridine nucleotides.

Further insight into the nature and sequence of RNA synthesis in Krebs II ascites tumour cells was obtained from experiments on cells exposed to varying amounts of actinomycin D. When a large amount of actinomycin D (10 µg. per ml. of cell suspension) was used, the pattern of ^{3}H-uridine incorporation was drastically altered (Fig. 26). Exposure to ^{3}H-uridine for either 20 or 150 min. gave rise to incorporation into the 4s component of RNA alone. This fraction was isolated, hydrolysed with alkali and the distribution of radioactivity amongst the nucleoside 3'(2')-monophosphates determined (Table 12). The radioactivity was located principally in cytidine monophosphate residues presumably derived from the ^{3}H-uridine by amination and probably represented non-DNA dependent terminal and sub-terminal addition to sRNA.

Exposure of the cells to a lower level of actinomycin D (2 µg. per ml. cell suspension) depressed the incorporation of ^{3}H-uridine to about 10 per cent of that in the controls (Fig. 27). At this low concentration of the antibiotic, the 40s component was either absent or present in very small amount after a short exposure to ^{3}H-uridine. Nevertheless, there was evidence of labelling of a component sedimenting more rapidly than the 30s component of ribosomal RNA and after longer incubation periods, radioactivity was distributed throughout all the components of RNA. The difference in pattern between cells exposed to different levels of actinomycin D is not easy to interpret, but may be a consequence of varying sensitivities of different systems for the synthesis of RNA or

FIGURE 26

Sedimentation analysis in sucrose density gradients (5-20 per cent (w/v)) of RNA from Krebs II ascites tumour cells which have been exposed to 10 µg. per ml. actinomycin D and thereafter to ^{3}H-uridine (5 µcuries per ml. cell suspension) for 20 min. and 150 min.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E_{256} mµ.

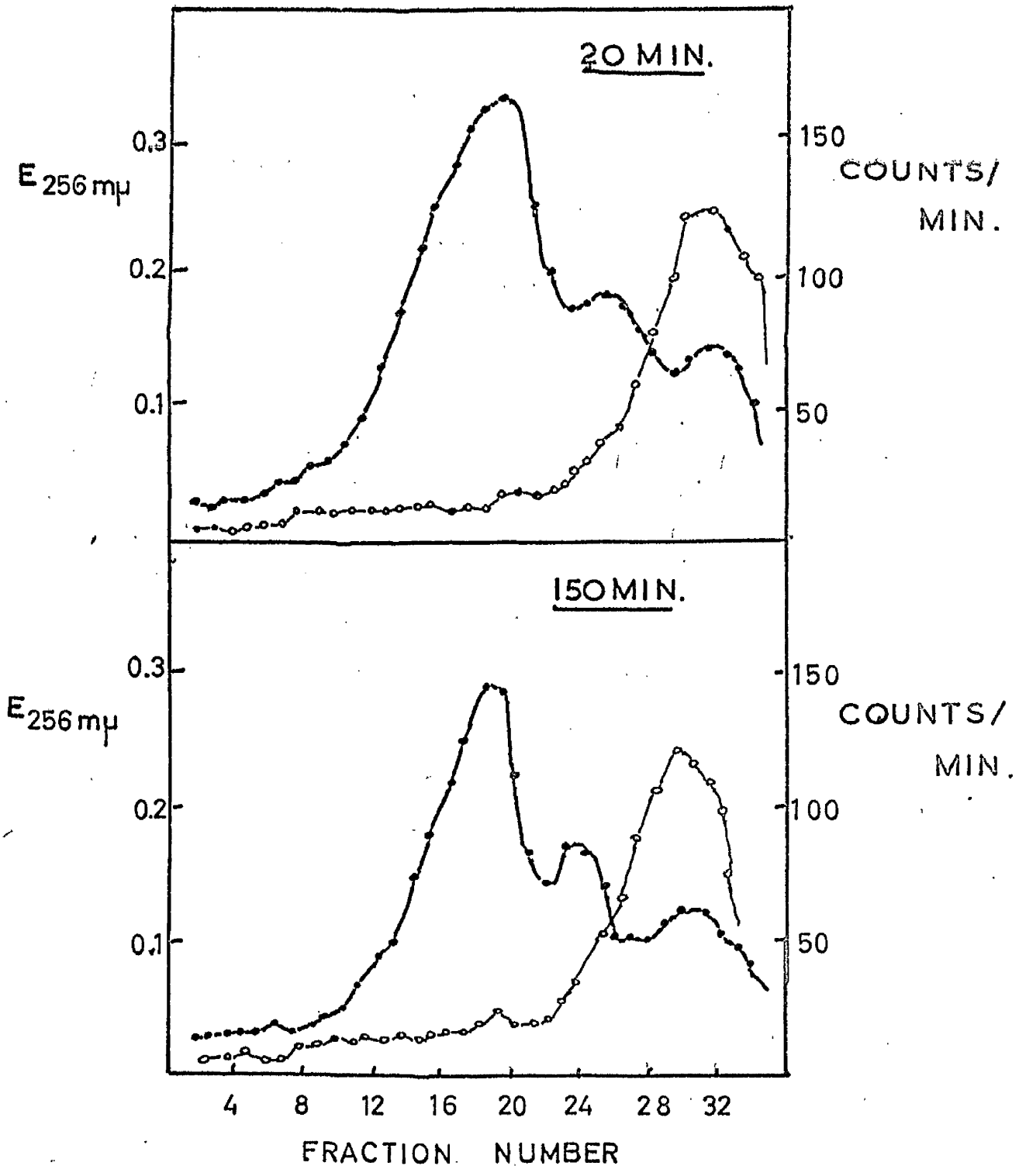

20 MIN.
150 MIN.
E 256 mμ
0.3
0.2
0.1
150
100
50
COUNTS/
MIN.
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 26.

TABLE 12

The distribution of radioactivity in the ribonucleoside 3'(2')-monophosphates obtained on alkaline hydrolysis of 4s RNA from Krebs II ascites tumour cells incubated with ^{3}H-uridine in the presence of actinomycin D.

TABLE 12.

Ribonucleotide	Per cent total radioactivity	
	20 min.	270 min.
CYTIDINE 3'(2')-PHOSPHATE	65	91
URIDINE 3'(2')-PHOSPHATE	21	9
ADENOSINE 3'(2')-PHOSPHATE	4	0
GUANOSINE 3'(2')-PHOSPHATE	10	0

FIGURE 27

Sedimentation analysis in sucrose density gradients (5-20 per cent (v/v)) of RNA from Krebs II ascites tumour cells in the presence of 2 µg. per ml. actinomycin D and exposed to ^{3}H-uridine (5 µcuries per ml. cell suspension) for 20 min., 50 min. and 80 min.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E256 mµ.

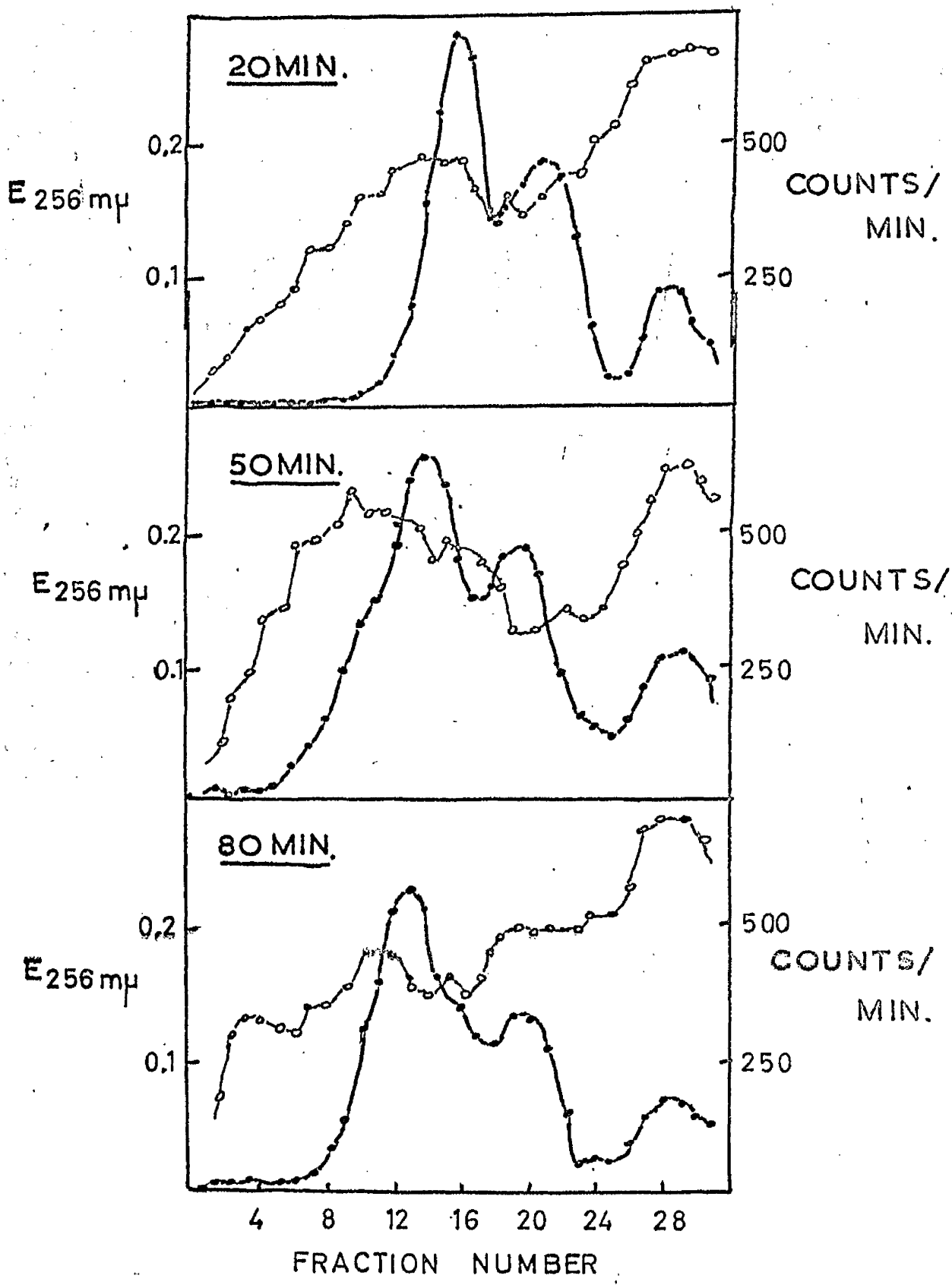

20MIN.
50MIN.
80 MIN.
E 256 mμ
0,2
0,1
0.2
0.1
500
250
COUNTS/
MIN.
4
8
12
16
20
24
28
FRACTION NUMBER

FIGURE 27

of the rate of absorption of actinomycin D into the cells.

Experiments were carried out to test whether the 40s component of RNA could form hybrids with DNA samples from a number of different sources. Fig. 23 shows that when the labelled 40s peak was annealed with the homologous DNA, radioactive material became associated with the DNA whereas with a heterologous DNA, no such association was evident. It may therefore be concluded that the 40s component contained regions of nucleotide sequence complementary to the Krebs II ascites tumour cell DNA but not to calf thymus or Landschutz ascites tumour DNA. Since the 40s RNA was also rapidly labelled, it fulfilled at least two criteria of messenger RNA. However, if ribosomal precursor RNA was also synthesised on DNA, as the results in Fig. 24 and Fig. 26 suggested, the above method would not distinguish between this species and messenger RNA. No experiments were performed on the nucleotide composition of this RNA since the total amount was too small to allow chemical determination of the bases to be performed, and since the alternative approach by way of ^{32}P distribution was open to many objections in this system.

The effect of virus infection on the distribution of RNA in sucrose density gradients is shown in Fig. 29. No marked qualitative changes could be observed between infected and uninfected cells incubated for 90 and 240 min. with ^{3}H-uridine (c.f. Fig. 24). When the Krebs II ascites tumour cells were treated with actinomycin D prior to infection, the distribution of radioactivity in RNA from infected and uninfected cells was similar up to about 4 hr., after which time a peak of radioactive

FIGURE 28

Sedimentation analysis in CsCl density gradients of DNA from Krebs II ascites tumour cells, calf thymus or Landschutz ascites tumour cells after annealing with ^{3}H-labelled RNA. The ^{3}H-labelled RNA was isolated from the rapidly-sedimenting material which appeared when Krebs II ascites tumour cells were exposed to ^{3}H-uridine (5 μcuries per ml. cell suspension) for 20 min.

The gradients were centrifuged at 35,000 rev./min. at 24° for 48 hr. in the SW 39 rotor of the Spinco Model L Preparative Ultracentrifuge.

(a) Krebs II DNA plus Krebs II "40s" RNA.

(b) Calf thymus DNA plus Krebs II "40s" RNA.

(c) Landschutz DNA plus Krebs II "40s" RNA.

—o— counts/min. (RNA).

—•— $E_{260\ m\mu}$ (DNA).

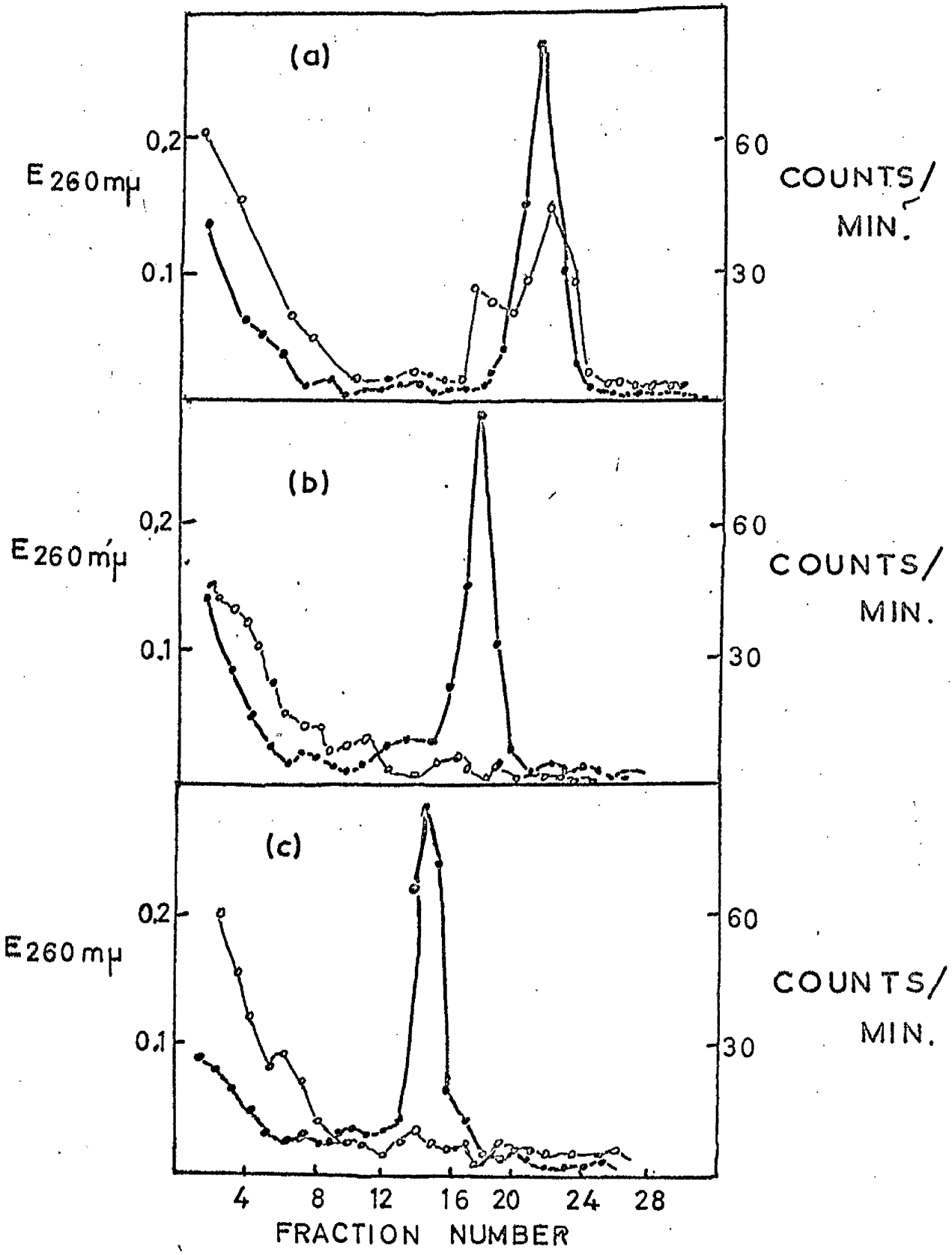

(a)
(b)
(c)
E 260 mμ
0.2
0.1
60
30
COUNTS/
MIN.
4
8
12
16
20
24
28
FRACTION NUMBER

FIGURE 28.

FIGURE 29

Sedimentation analysis in sucrose density gradients (5-20 per cent v/v)) of RNA from Krebs II ascites tumour cells infected with EMC virus and exposed to ^{3}H-uridine (5 μcuries per ml. cell suspension) during the intervals 60 min. - 90 min. and 60 min. - 240 min. after infection.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E_{258} mμ.

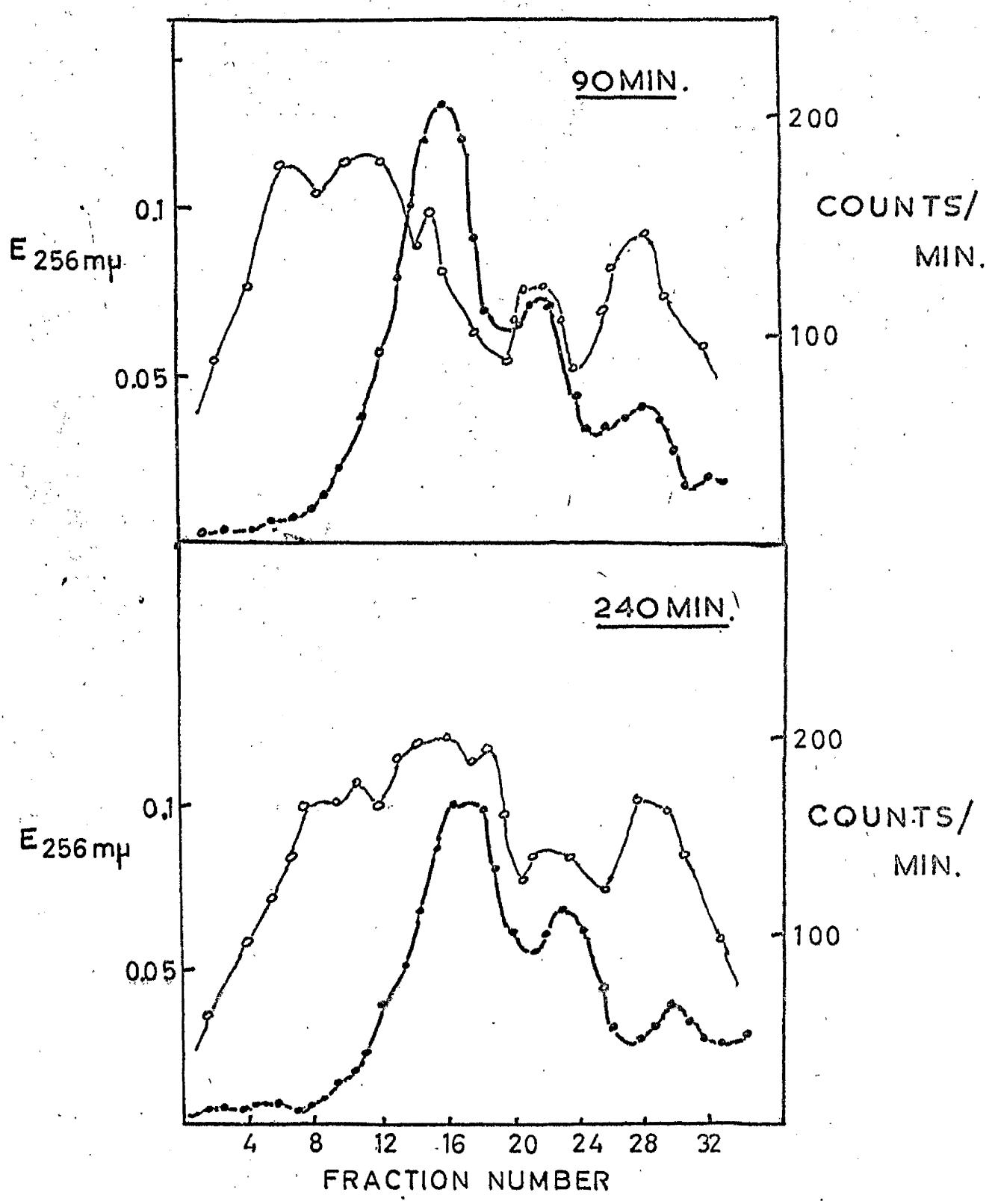

90MIN.
240MIN.
E 256mμ
0.1
0.05
200
100
COUNTS/
MIN.
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 29.

RNA (35-40s) began to appear in the RNA from infected cells (Fig. 30). This demonstrated that some RNA synthesis occurred in infected but not in uninfected cells in the presence of actinomycin D; this process was presumably therefore not primed by DNA. The development of this fraction with time is shown in Fig. 31.

To investigate whether there was any difference in the sedimentation behaviour of viral ribonucleic acids at different times in the replicative cycle, the radioactive ribonucleic acids (30-35s) in Fig. 31 were isolated and re-centrifuged in sucrose gradients against marker RNA prepared from Krebs II ascites tumour cells (Fig. 32). The patterns observed with these ribonucleic acids isolated at different stages during virus replication could be superimposed on the pattern obtained with RNA from mature virus particles. Slight shoulders were to be seen in the peaks of radioactivity which could have been due to some degree of association of the RNA molecules, but no major differences were evident.

Since the replication of a new viral RNA strand might have involved double-stranded intermediate, this was sought by examining the sensitivity of the different RNA preparations to phosphorolysis by polynucleotide phosphorylase. This enzyme readily attacks single-stranded synthetic polynucleotides, but multi-stranded polyribonucleotides are much more resistant. Preliminary experiments showed that the enzyme phosphorolysed complexes of poly A and poly U to only 30-50 per cent of the extent to which it attacked the individual homopolymers. Fig. 33 shows that the peaks isolated at various times after virus infection were all rapidly

FIGURE 30

Sedimentation analysis in sucrose density gradients (5-20 per cent (w/v)) of RNA from Krebs II ascites tumour cells infected with EMC virus and treated with 10 µg. per ml. actinomycin D and exposed to ^{3}H-uridine (5 µcuries per ml. cell suspension), 2 hr. and 4.5 hr. after infection.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E_{256} mµ.

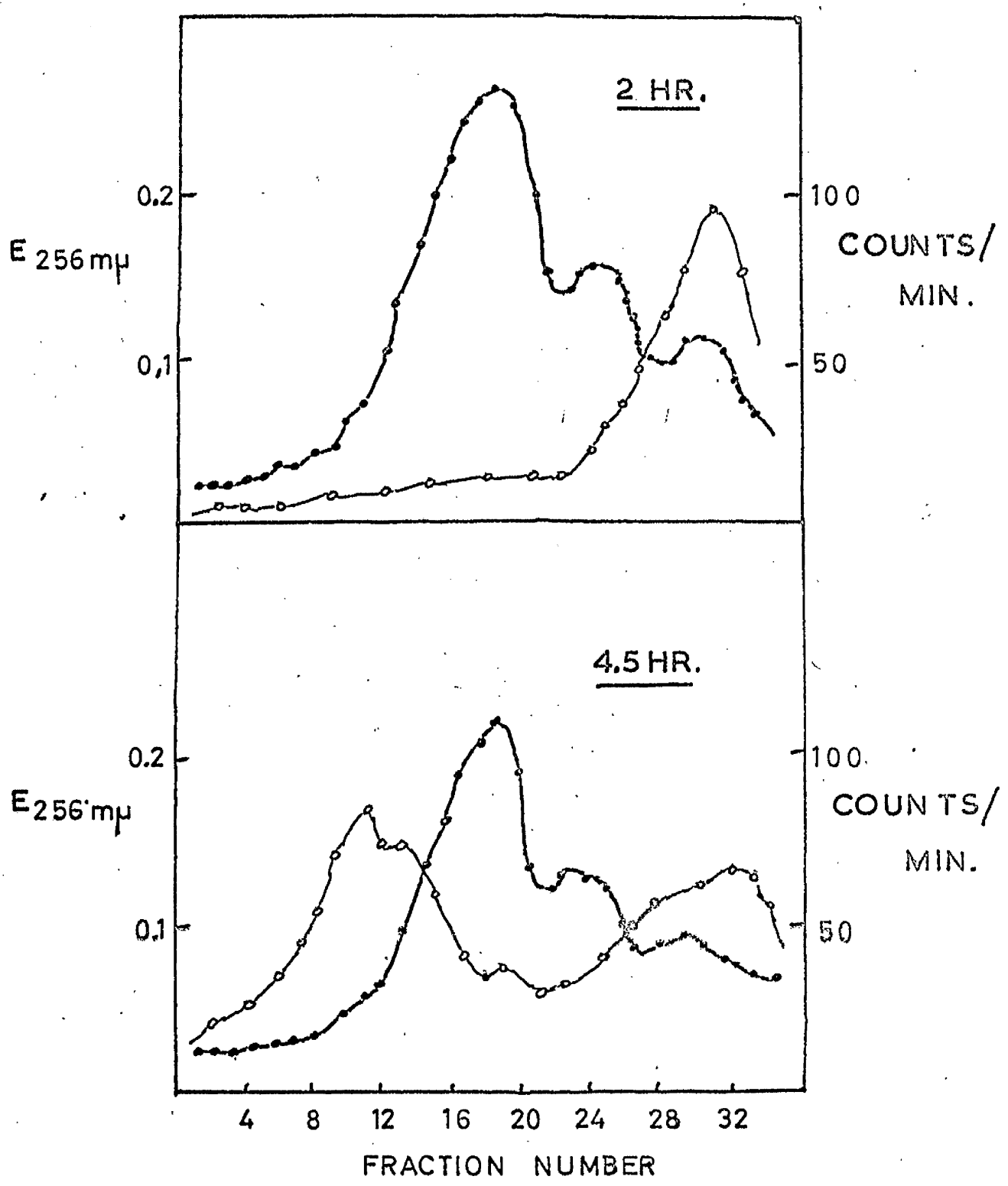

2 HR.
0.2
0,1
E 256 mμ
100
50
COUNTS/
MIN.
4.5 HR.
0.2
0.1
E 256 mμ
100
50
COUNTS/
MIN.
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 30.

FIGURE 31

Sedimentation analysis in sucrose density gradients (5-20 per cent (w/v)) of RNA from Krebs II ascites tumour cells infected with EMC virus and exposed to 10 µg. per ml. actinomycin D and ^{3}H-uridine (5 µcuries per ml. cell suspension), 4 hr., 6 hr. and 8 hr. after infection.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— E256 mµ.

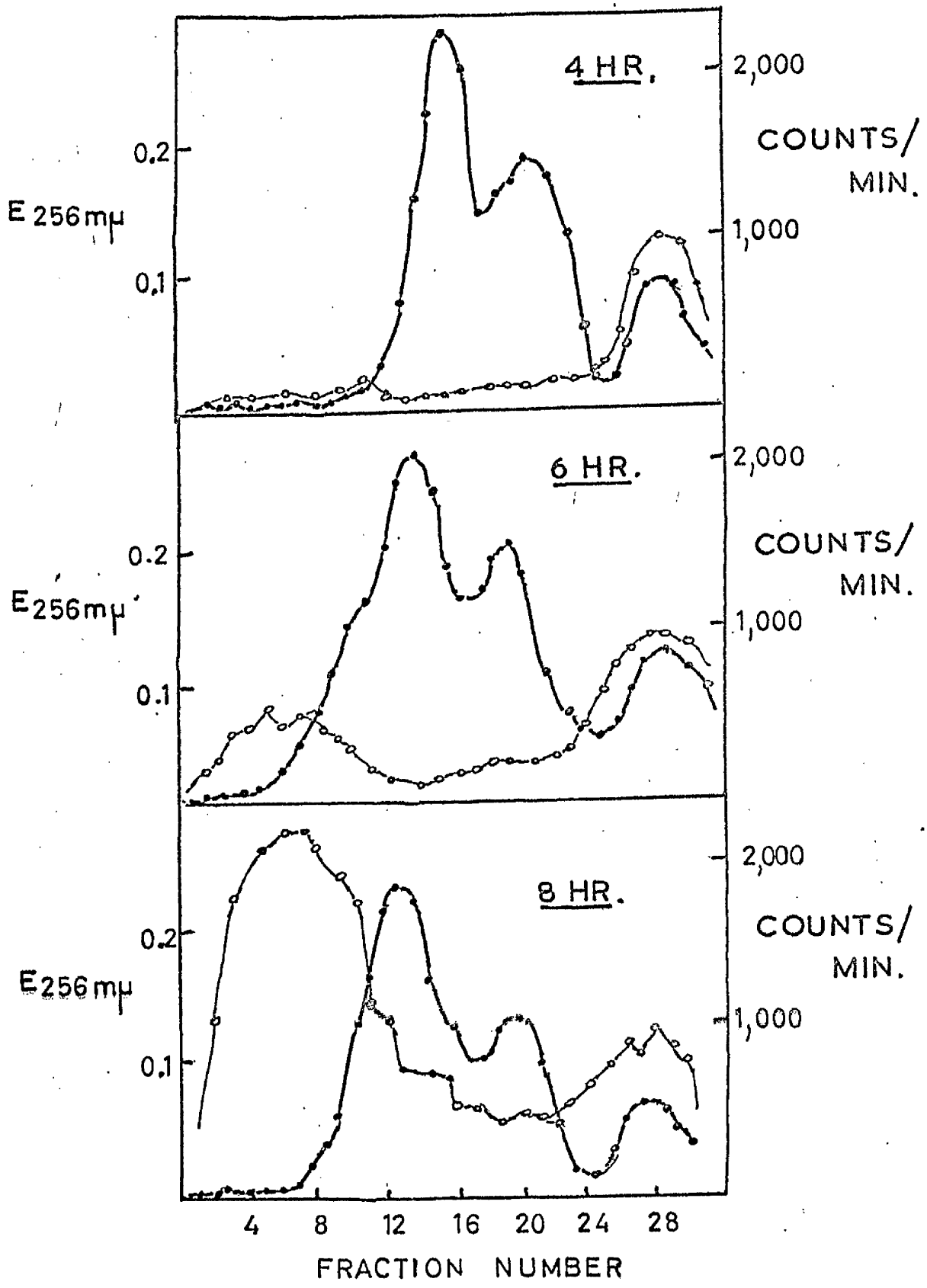

4 HR.
6 HR.
8 HR.
E 256mμ
0.2
0.1
2,000
1,000
COUNTS/
MIN.
4
8
12
16
20
24
28
FRACTION NUMBER

FIGURE 31.

FIGURE 32

The sedimentation behaviour in sucrose density gradients (5-20 per cent (w/v)) of ^{32}P-labelled RNA fractions isolated 6 hr., 8 hr. and 18 hr. after infection of actinomycin D-treated Krebs II ascites tumour cells with NWO virus, compared to a marker RNA fraction prepared from Krebs II ascites tumour cells.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

—o— counts/min.

—•— $E_{256\ m\mu}$.

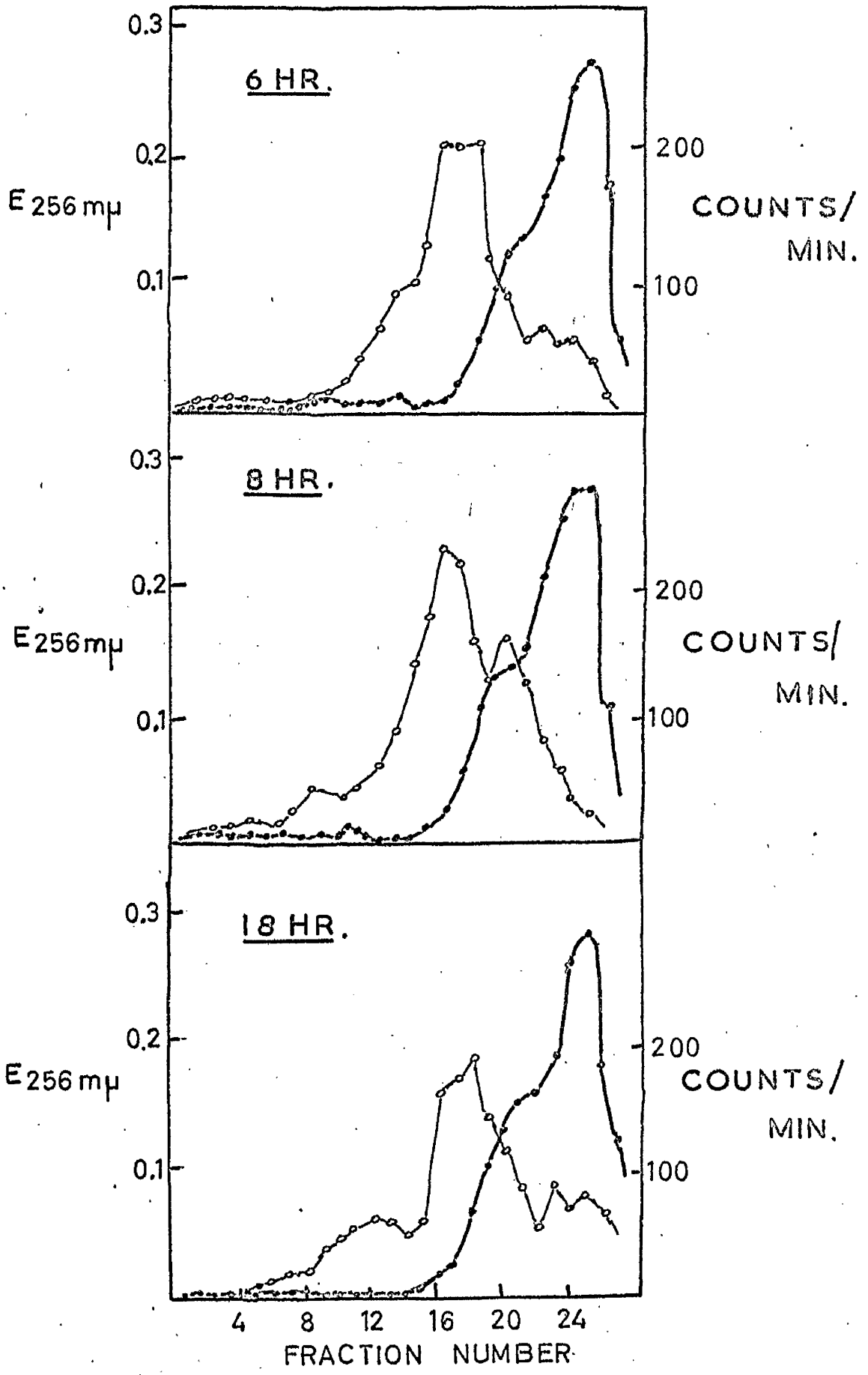

6 HR.
8 HR.
18 HR.
E 256 mμ
0.3
0.2
0.1
200
100
COUNTS/ MIN.
4
8
12
16
20
24
FRACTION NUMBER

FIGURE 32.

FIGURE 33

Phosphorolysis by polynucleotide phosphorylase of ^{3}H-labelled RNA isolated from mature EMC virus and from the rapidly-sedimenting RNA component of actinomycin D-treated Krebs II ascites tumour cells 6 hr. and 8 hr. after infection with EMC virus.

^{3}H-labelled RNA was incubated with 310 µmoles KCl; 3.1 µmoles $MgCl_2$; 31 µmoles K_2HPO_4; 154 µmoles tris-HCl buffer, pH 8.5, and 0.2 ml. polynucleotide phosphorylase in a total volume of 3.2 ml. at 37°.

0.5 ml. aliquots were removed at intervals, mixed with 1 mg. carrier yeast RNA and precipitated with 5 per cent (v/v) $HClO_4$. The supernatant fluid was diluted and samples taken for assay of radioactivity.

(a) 6 hr. RNA.

(b) 8 hr. RNA.

(c) Mature virus RNA.

—●— heated.

—○— native.

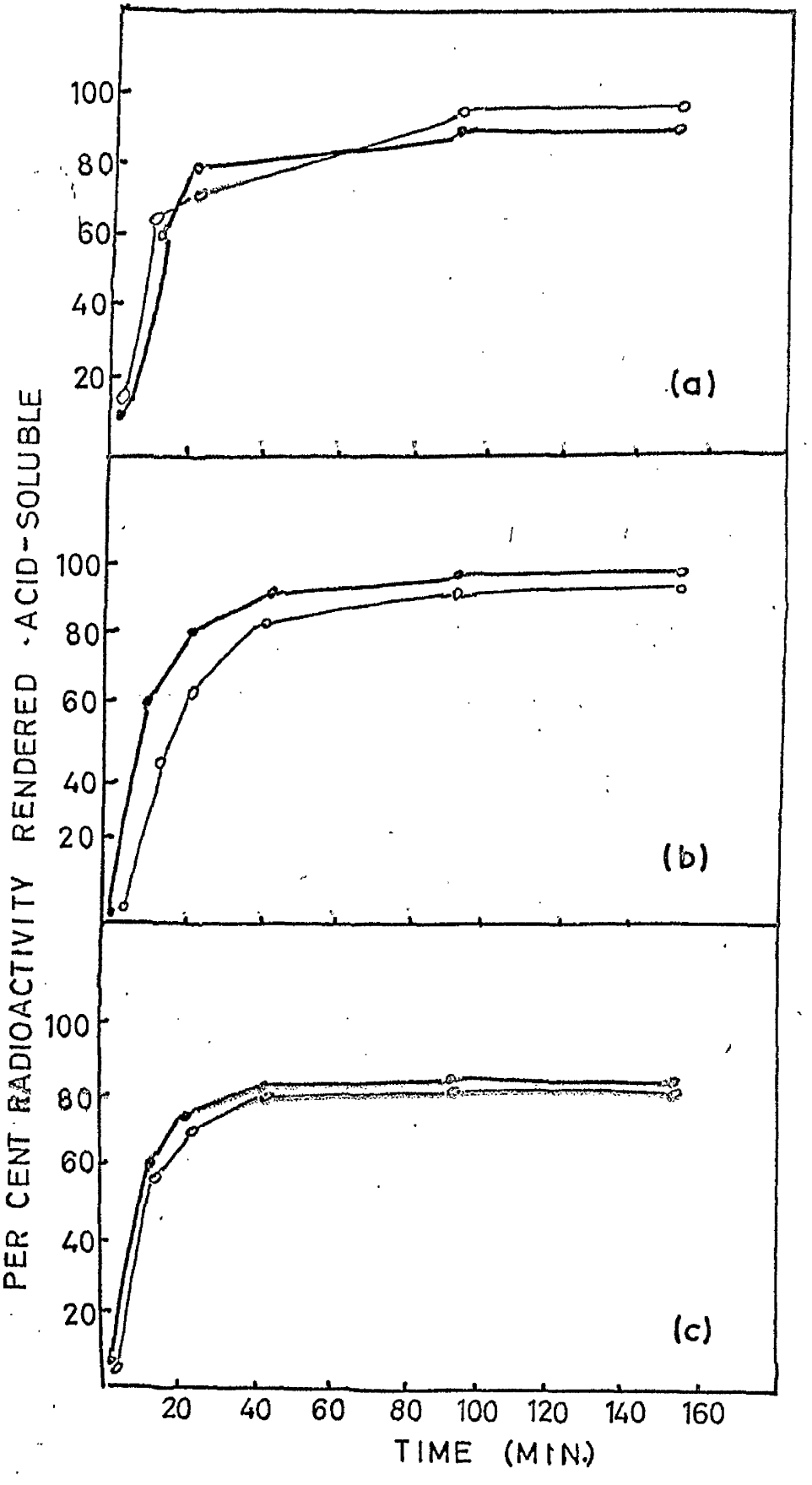

PER CENT RADIOACTIVITY RENDERED ACID-SOLUBLE
100
80
60
40
20
(a)
(b)
(c)
20
40
60
80
100
120
140
160
TIME (MIN.)

FIGURE 33.

and completely phosphorolysed, although in the case of mature virus RNA a fraction of the material remained intact. Denaturation of the RNA preparations by heating to 100° for 10 min. followed by rapid cooling had no effect on the rate or extent of the phosphorolysis. These results showed that no double-stranded component was present in the RNA synthesised in the presence of actinomycin D, or that such material was only transient in the infected cell and was present in quantities too small to be detected by the methods employed. Alternatively, the procedure used to extract RNA could have caused splitting of any double-stranded material to single strands. In this case, strands of RNA complementary in base sequence to each other might have been present. The possibility existed, therefore, that these strands could be annealed in a manner similar to annealing of DNA and RNA, and distinguished by sedimentation analysis and resistance to phosphorolysis.

Both ^{3}H-labelled and ^{32}P-labelled rapidly sedimenting ribonucleic acids were prepared from actinomycin D treated Krebs II ascites tumour cells 6 hr. after infection with EMC virus. The ^{3}H-labelled RNA was heated and slowly cooled and centrifuged in a sucrose density gradient along with ^{32}P-labelled RNA which had been denatured by heating and rapid cooling on ice (Fig. 34a). The reverse experiment in which the ^{32}P-labelled RNA was heated and slowly cooled and centrifuged in a sucrose density gradient with ^{3}H-labelled RNA, which had been denatured by heating and rapid cooling on ice, was also performed (Fig. 34b). The results showed that in both experiments, the slowly-cooled RNA sedimented slightly

FIGURE 34

Sedimentation analysis in sucrose density gradients (5-20 per cent (v/v)) of ^{32}P-labelled and ^{3}H-labelled rapidly-sedimenting RNA prepared 6 hr. after infecting actinomycin D-treated Krebs II ascites tumour cells with RNC virus.

(a) The ^{3}H-labelled RNA was heated then slowly cooled and the ^{32}P-labelled RNA was denatured by heating and rapid cooling on ice.

(b) The ^{32}P-labelled RNA was heated then slowly cooled and the ^{3}H-labelled RNA was denatured by heating and cooling rapidly on ice.

The sucrose gradients were centrifuged at 25,000 rev./min. for 12 hr. at 0° in the SW 39 rotor of the Spinco Model L Preparative Ultracentrifuge.

—●— ^{3}H-RNA.

—○— ^{32}P-RNA.

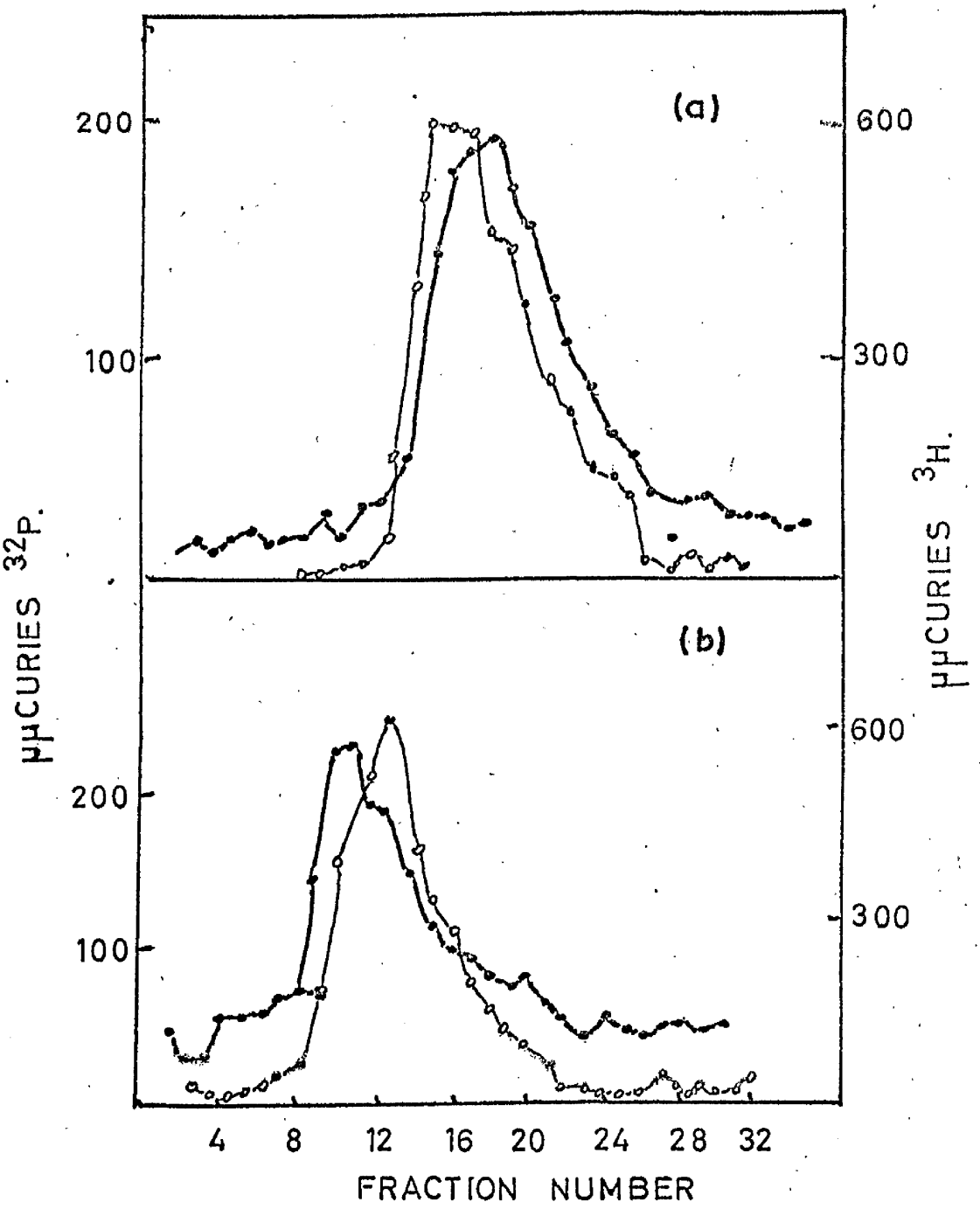

(a)
(b)
200
100
600
300
μμCURIES ^{32}P.
μμCURIES ^{3}H.
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 34.

more slowly than did the heat denatured RNA. Such a result would be expected if the RNA became more rigid by the formation of a double strand. As a control experiment, the rapidly-sedimenting (40s RNA) ^{3}H-labelled and ^{32}P-labelled ribonucleic acids were isolated from Krebs II ascites tumour cells exposed to ^{3}H-uridine for 20 min. or to ^{32}P-orthophosphate for 90 min. The ^{3}H-labelled RNA was heated then slowly cooled and centrifuged in a sucrose density gradient with the ^{32}P-labelled RNA which had been denatured by heating and rapid cooling on ice (Fig. 35a). The reverse experiment, in which the ^{32}P-labelled RNA was heated then slowly cooled and centrifuged in a sucrose density gradient with ^{3}H-labelled RNA which had been denatured by heating and rapid cooling on ice, was also carried out (Fig. 35b). The results showed no difference in sedimentation between the RNA subjected on the one hand to heating followed by rapid cooling and on the other to heating followed by slow cooling. This would indicate lack of double strand formation.

Although some slight differences were apparent between rapidly-sedimenting, labelled RNA, subjected to heating and either fast or slow cooling, from the virus infected cells, this alone did not provide sufficient grounds on which to postulate the presence of two complementary strands. The radioactive, heat-treated ribonucleic acids from the virus infected cells were therefore examined for resistance to hydrolysis by ribonuclease and phosphorolysis by polynucleotide phosphorylase. The results (Fig. 36) showed no detectable difference in the sensitivity of

FIGURE 35

Sedimentation analysis in sucrose density gradients (5-20 per cent (w/v)) of rapidly-sedimenting ^{3}H-labelled and ^{32}P-labelled RNA isolated from Krebs II ascites tumour cells exposed to ^{3}H-uridine (5 μcuries per ml. cell suspension) for 20 min. or to ^{32}P-orthophosphate (20 μcuries per ml. cell suspension) for 90 min.

(a) The ^{3}H-labelled RNA was heated then slowly cooled and the ^{32}P-labelled RNA was denatured by heating and cooling rapidly on ice.

(b) The ^{32}P-labelled RNA was heated then slowly cooled and the ^{3}H-labelled RNA was denatured by heating and rapid cooling on ice.

The sucrose gradients were centrifuged at 27,000 rev./min. for 6 hr. in the SW 39 rotor.

—●— ^{3}H-RNA.

—○— ^{32}P-RNA.

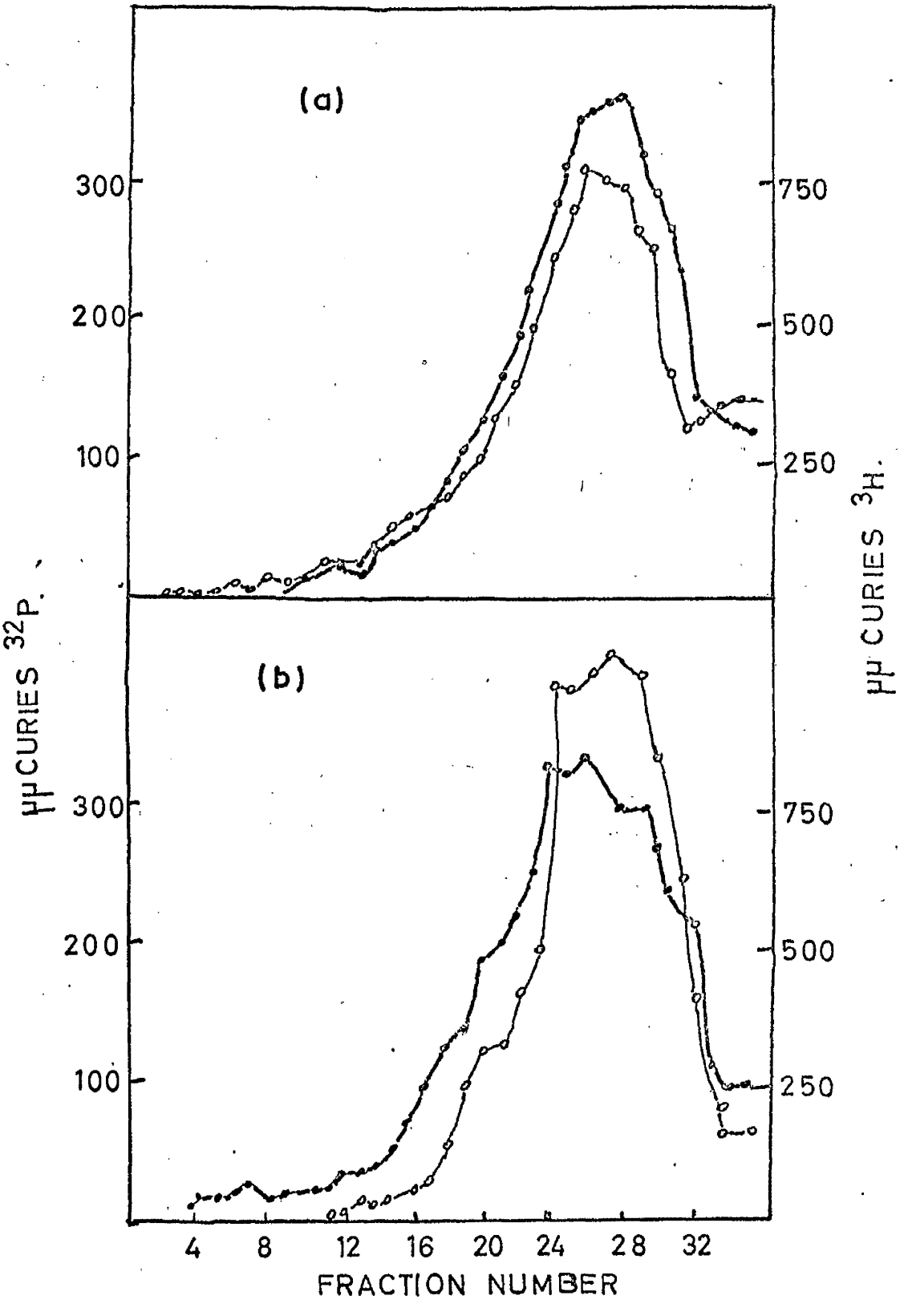

(a)
(b)
300
200
100
750
500
250
μμ CURIES ^{32}P.
μμ CURIES ^{3}H.
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 35.

FIGURE 36

Phosphorolysis by polynucleotide phosphorylase and by ribonuclease of the ^{32}P-labelled rapidly-sedimenting RNA prepared 6 hr. after infecting actinomycin D-treated Krebs II ascites tumour cells with EMC virus. The RNA was either denatured by heating followed by rapid cooling on ice or was heated then cooled very slowly.

Polynucleotide phosphorylase was assayed as described in Figure 34.

Ribonuclease activity was assayed by incubating the RNA preparations with 70 μmoles tris-HCl buffer, pH 8.0, and 0.02 μg. pancreatic ribonuclease in a total volume of 3.0 ml. at 37°, 0.5 ml. samples were withdrawn and precipitated with 1 ml. acid-ethanol (1N-HCl in 76 per cent (v/v) aqueous ethanol) in the presence of 1 mg. yeast RNA which acted as carrier.

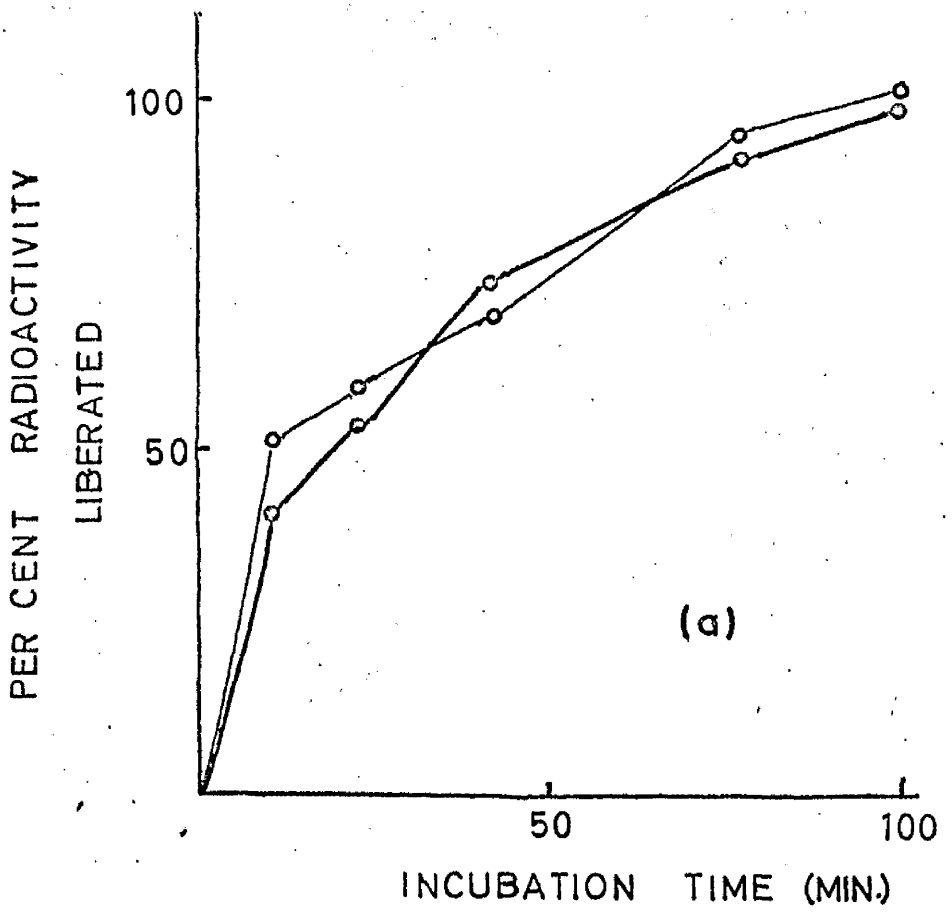
The supernatant fluid was diluted and assayed for radioactivity.

(a) Polynucleotide phosphorylase.

(b) Ribonuclease.

——●—— slowly cooled.

——○—— heat denatured.


PER CENT RADIOACTIVITY LIBERATED
100
50
(a)
50
100
INCUBATION TIME (MIN.)

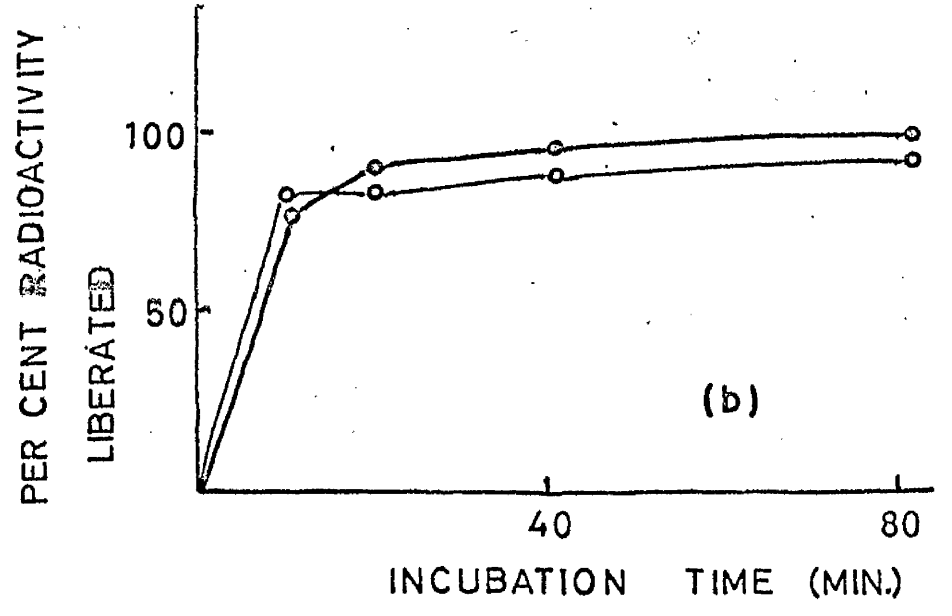

PER CENT RADIOACTIVITY LIBERATED
100
50
(b)
40
80
INCUBATION TIME (MIN.)

FIGURE 36.

the slowly and quickly cooled material. It would appear, therefore, either that no complementary strands were present in the fast-sedimenting, radioactive RNA synthesised in actinomycin D treated Krebs II ascites tumour cells infected with EMC virus, or that they were present in amounts too small to be detected by the methods employed.

The site of appearance of actinomycin D resistant RNA synthesis in EMC virus infected Krebs II ascites tumour cells in the presence of ^{3}H-uridine was examined by isolating various subcellular components at varying times after infection and analysing the ribonucleic acids extracted from them in sucrose density gradients. Fig. 37 shows the distribution in sucrose density gradients of RNA from nuclei, the 10,000 *g* supernatant fraction and 10,000 *g* sediment 2 hr. after infection. No rapidly sedimenting, labelled component was evident in any fraction. After 6 hr. (Fig. 38), however, although there was no evidence of a rapidly-sedimenting RNA component in the nuclei, such a component appeared in the 10,000 *g* sediment and to a lesser extent in the 10,000 *g* supernatant material. The peaks were associated with less-rapidly sedimenting material which could represent either breakdown of the RNA during cell fractionation or the presence of some other component involved in replication. It seemed possible therefore that the viral RNA was associated with the particulate fraction sedimenting at 10,000 *g* rather than with the cell nucleus.

Some preliminary studies were carried out, the final aim of which was to determine whether virus-induced polyribosomes appeared in the

FIGURE 37

Sedimentation analysis on sucrose density gradients (5-20 per cent (w/v) of ^{3}H-labelled RNA extracted from cell fractions of actinomycin D-treated Krebs II ascites tumour cells 2 hr. after infection with EMC virus.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

(a) Nuclei.

(b) 10,000 g sediment.

(c) 10,000 g supernatant fraction.

—o— counts/min.

—•— E_{256} mμ.

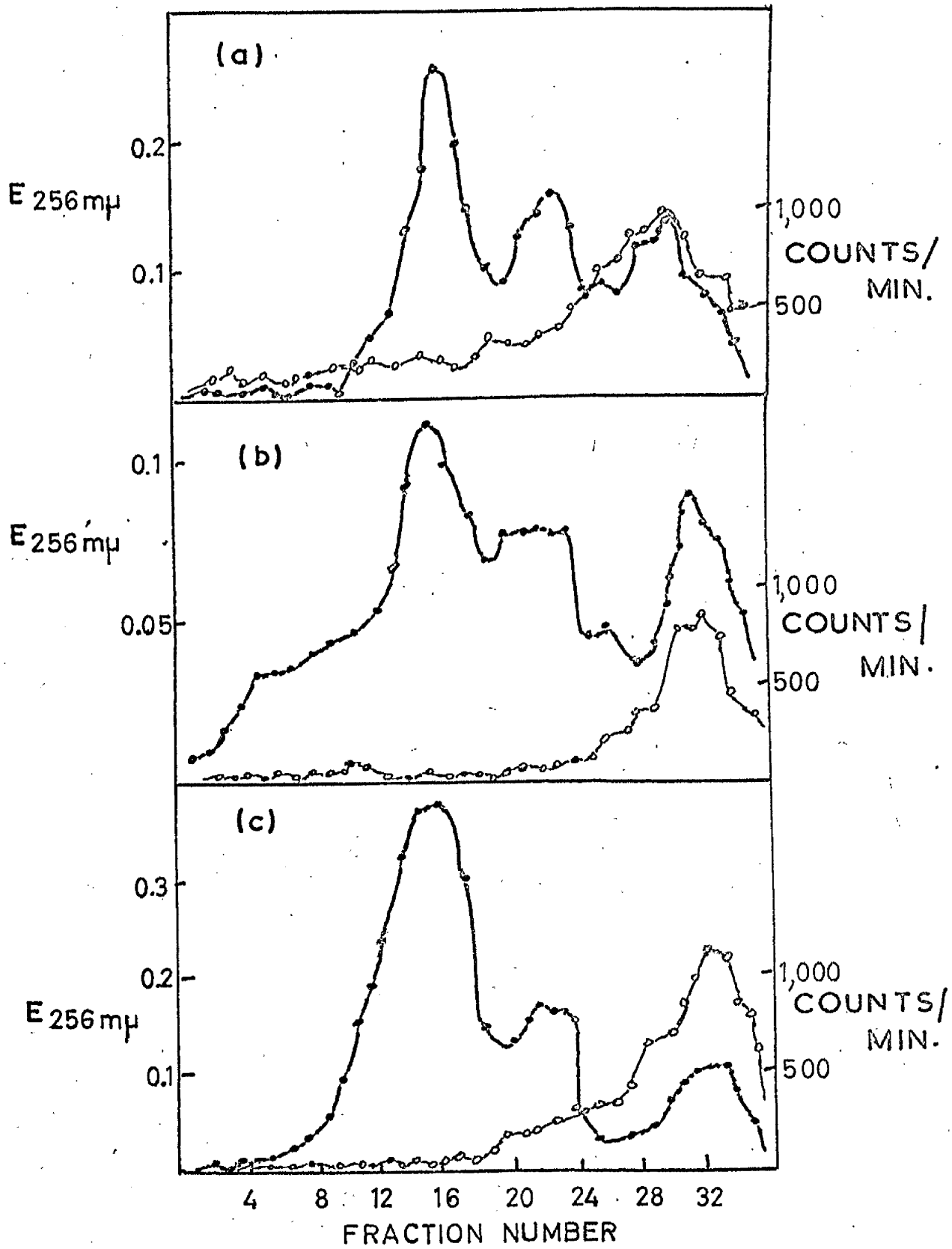

(a)
0.2
0.1
E 256 mμ
1,000
COUNTS/
MIN.
500
(b)
0.1
0.05
E 256 mμ
1,000
COUNTS/
MIN.
500
(c)
0.3
0.2
0.1
E 256 mμ
1,000
COUNTS/
MIN.
500
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 37.

FIGURE 38

Sedimentation analysis on sucrose density gradients (5-20 per cent (w/v)) of ^{3}H-labelled RNA extracted from cell fractions of actinomycin D-treated Krebs II ascites tumour cells 6 hr. after infection with EMC virus.

The gradients were centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

(a) Nuclei.

(b) 10,000 g sediment.

(c) 10,000 g supernatant fraction.

——o—— counts/min.

——•—— E_{256} mμ.

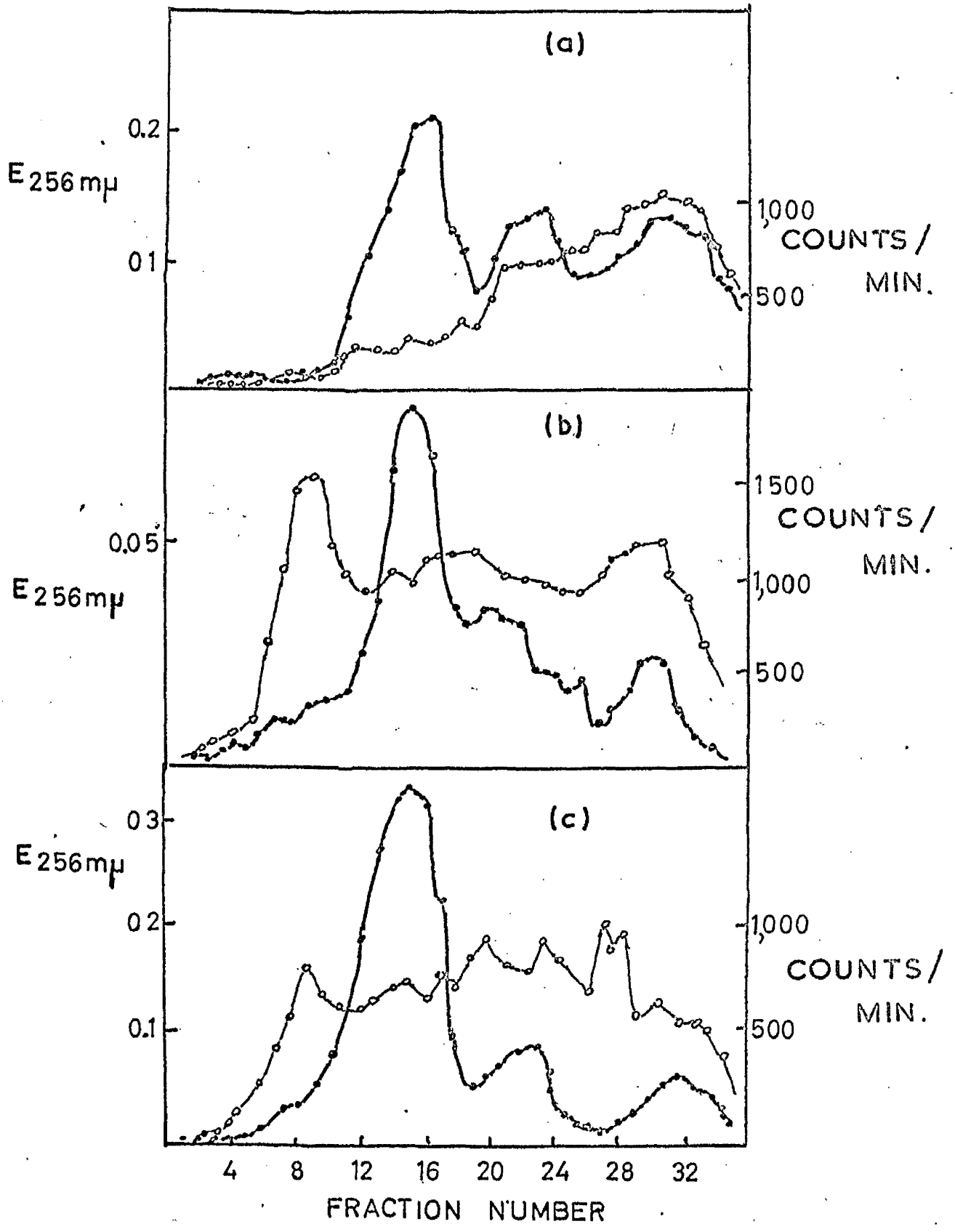

(a)
0.2
E 256 mμ
0.1
1,000
COUNTS /
MIN.
500
(b)
1500
0.05
E 256 mμ
COUNTS /
MIN.
1,000
500
(c)
0.3
E 256 mμ
0.2
0.1
1,000
COUNTS /
MIN.
500
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 38.

infected cells and, if so, to isolate the RNA from these and determine its nature.

In the system employed, the Krebs II ascites tumour cells were suspended in a maintenance medium and were not growing. The possibility therefore existed that the production of polyribosomes in the cells would not continue after the pool of cellular amino acids was exhausted. Evidence in favour of this is presented in Fig. 39. It appeared that while polyribosomes were present in cells immediately after they were harvested from the mice, they were not present after incubation of the cells in Earle's medium for 1 hr. The one main peak of optical density represented the 70s ribosome monomers. Presumably in this system, when EMC virus invades the Krebs II ascites tumour cell it must induce breakdown of cellular protein to provide amino acids for viral protein synthesis.

Fig. 40 shows the results of an experiment in which the 10,000 g supernatant fraction from Krebs II ascites tumour cells and also the same fraction treated with sodium dodecylsulphate were centrifuged in sucrose density gradients. It was clear that two peaks of ribosomal RNA were present (Fig. 40b) along with a large 4s peak due to low molecular weight material absorbing at 260 mμ in this fraction.

Similar experiments were attempted in actinomycin D treated Krebs II ascites tumour cells, 6 hr. after infection with EMC virus in the presence of ^{3}H-uridine, but an unexpected difficulty arose. After the cells had been incubated for 6 hr. in Earle's solution, they became virtually impossible to disrupt in the medium necessary to prepare polyribosomes. This factor has resisted all efforts to analyse polyribosomes in the infected cells.

FIGURE 39

Sedimentation analysis on sucrose density gradients (15-30 per cent w/v)) of ribosomal preparations from Krebs II ascites tumour cells.

(a) The Krebs II ascites tumour cells were suspended in Earle's medium, 1 µcurie ^{14}C-valine was added and the mixture was incubated for 2 min. at 37°. Thereafter ribosomes were prepared as described in the text.

(b) The Krebs II ascites tumour cells were incubated for 1 hr. at 37° in Earle's medium before 1 µcurie ^{14}C-valine was added. The mixture was incubated at 37° for 2 min. and ribosomes were prepared as described in the text.

The gradients were centrifuged at 28,000 rev./min. for 2 hr. in the SW 39 rotor of the Spinco Model L Preparative Ultracentrifuge.

—o— counts/min.

—•— E_{260} mµ.

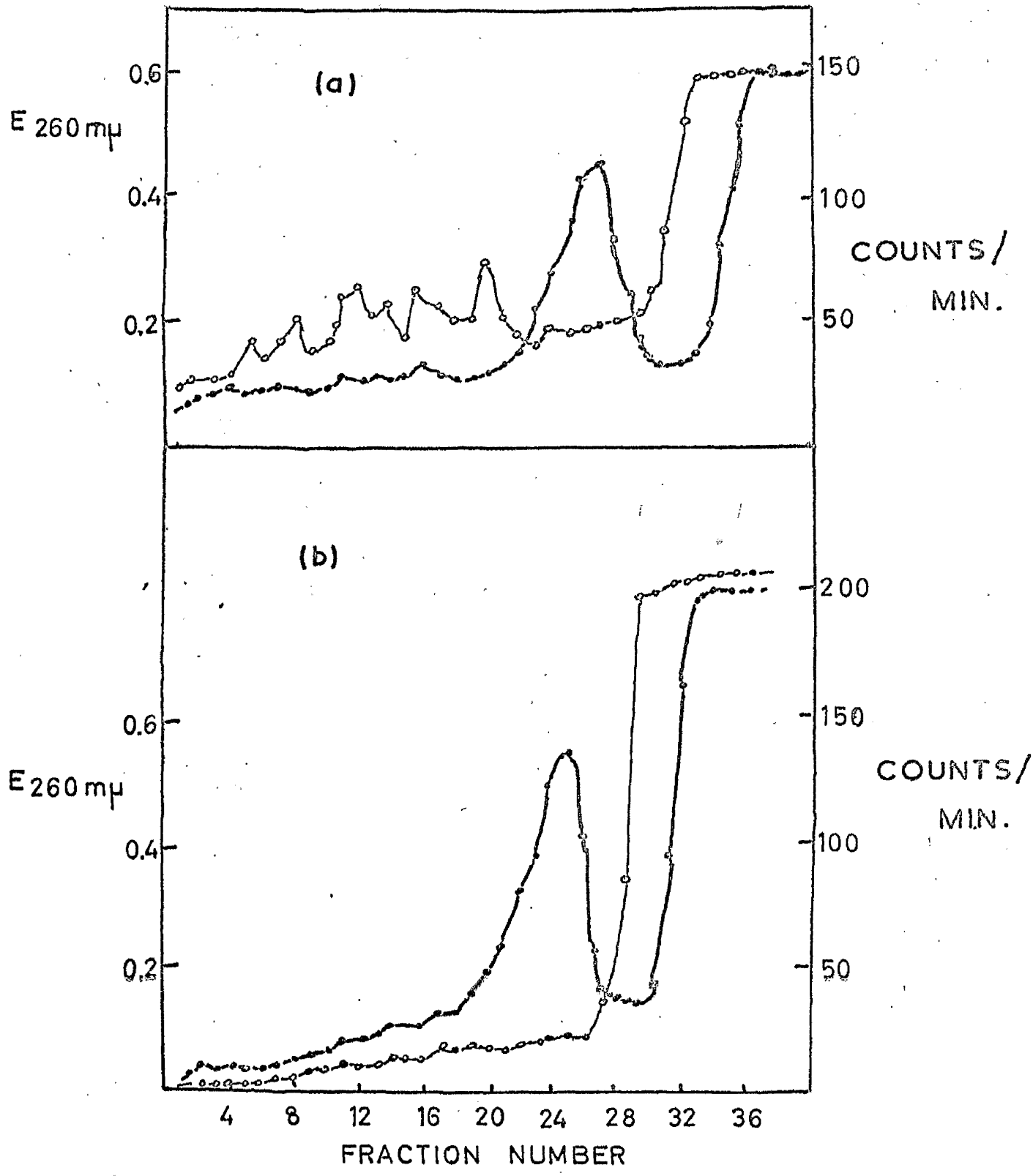

(a)
E 260 mμ
0.6
0.4
0.2
150
100
50
COUNTS/
MIN.
(b)
E 260 mμ
0.6
0.4
0.2
200
150
100
50
COUNTS/
MIN.
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

FIGURE 39.

FIGURE 40

Sedimentation analysis on sucrose density gradients of a 10,000 g supernatant fraction from Krebs II ascites tumour cells and of the RNA obtained from it by treatment with sodium dodecyl sulphate.

(a) The 10,000 g supernatant fraction was analysed on a 15-30 per cent (w/v) sucrose gradient which was centrifuged at 28,000 rev./min. for 2 hr. in the SW 39 rotor.

(b) The 10,000 g supernatant fraction was made 0.5 per cent with respect to sodium dodecyl sulphate, incubated at 37° for 3 min. then analysed on a 5-20 per cent(w/v) sucrose density gradient which was centrifuged at 20,500 rev./min. for 12.5 hr. in the SW 39 rotor.

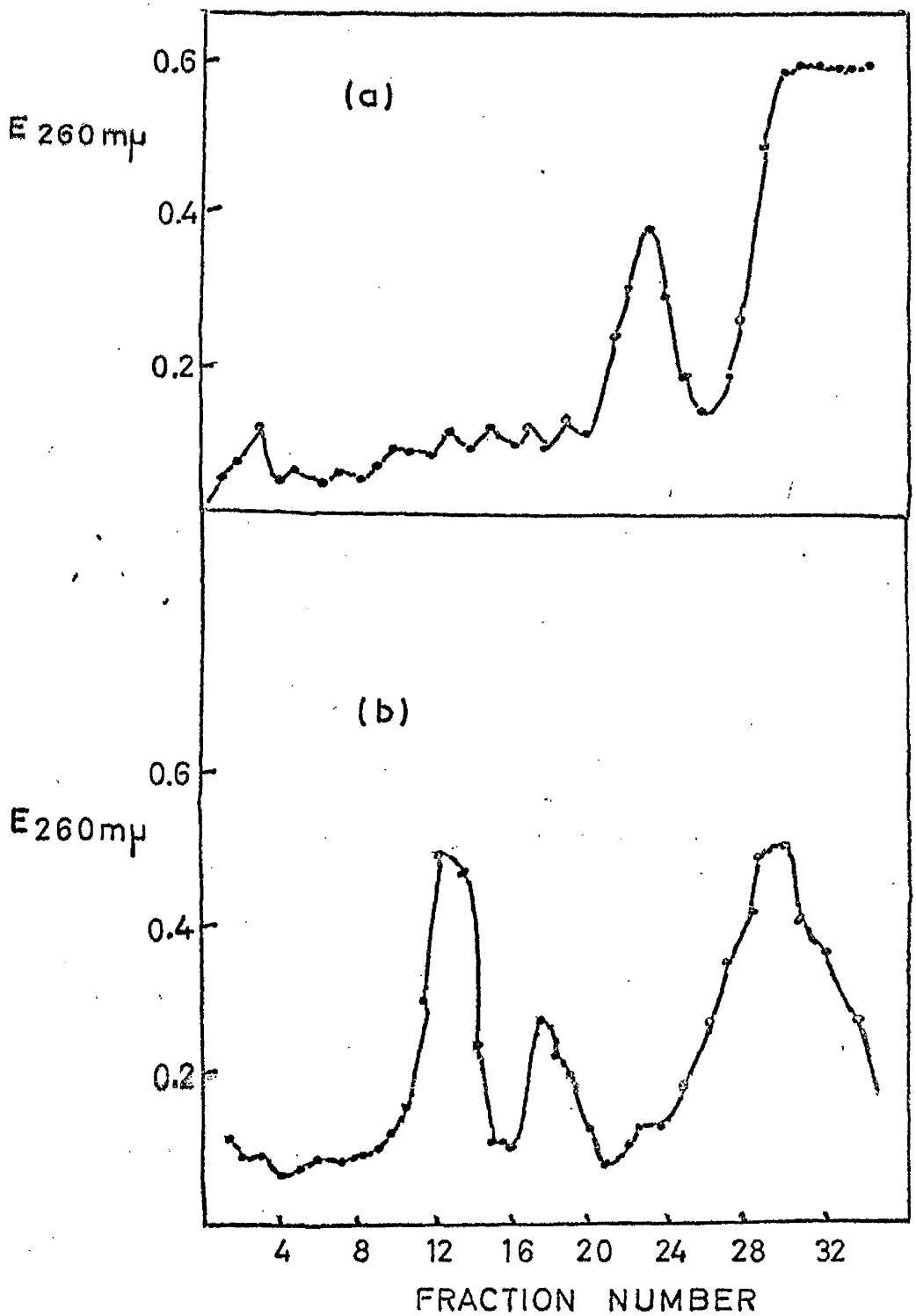

(a)
E 260 mμ
0.6
0.4
0.2
(b)
E 260 mμ
0.6
0.4
0.2
4
8
12
16
20
24
28
32
FRACTION NUMBER

FIGURE 40.

DISCUSSION

D I S C U S S I O N

1. The synthesis of RNA in uninfected Krebs II ascites tumour cells

Krebs II ascites tumour cells suspended in a buffered saline maintenance medium do not grow and undergo cell division. In some respects this is an artificial situation. Therefore, when the experimental results are compared with those obtained with actively growing cells, they must be interpreted with caution.

It is evident that two systems can be distinguished in extracts of Krebs II ascites tumour cells which incorporate (^{32}P)UMP residues into polyribonucleotide material. One is dependent upon DNA and the other is dependent on RNA as primer.

The DNA-dependent system is confined to the nucleus and is similar in its requirements to many other DNA-dependent systems which have been described. The results indicate that it catalyses the synthesis of polyribonucleotides containing all four ribonucleotide residues. This enzyme system probably represents that which catalyses the transcription of information from DNA to RNA in the nucleus of the intact cell.

The RNA-dependent activity is most evident in the cytoplasmic fractions and appears to catalyse synthesis mainly of homopolymer material. Whereas this activity was not diminished by actinomycin D, the DNA-dependent activity decreased markedly. Two possible interpretations can be made from these results.

Firstly, the two activities may represent the expression of the

potentiality of a single enzymic activity under different conditions. Thus Fox, Robinson, Haselkorn and Weiss (1964) have presented detailed evidence that both the DNA-directed and RNA-directed synthesis of RNA catalysed by extracts of M. lysodeikticus are due to the activity of a single enzyme. Similarly, Krakow and Ochoa (1963) observed RNA synthesis with both DNA and RNA primers catalysed by extracts of A. vinelandii and concluded that only a single enzyme was involved. These results cannot be related with those presented in this thesis, however, since the ionic requirements and pH optima they describe for the DNA-dependent and RNA-dependent activities are entirely different from those observed in Krebs II ascites tumour cells. Moreover, it is difficult to account for the DNA- and RNA-dependent reactions in mammalian cells in terms of one enzyme, since the activities are found in different parts of the cell.

The second possibility is that two enzymes are present. This implies that a distinct enzyme exists in the cell which catalyses the RNA-dependent synthesis of RNA, and evidence for this has been presented by Burdon and Smellie (1962); however, in the Krebs II cell, the amount of RNA produced in relation to the amount of homopolymer formed appears to be small. The role of such a homopolymer in the intact cell is obscure. These results do not exclude the existence in the cell of an enzyme catalysing the RNA-dependent synthesis of RNA, and the possible function of such an enzyme will be considered later.

The rapidly-sedimenting RNA that appears after a short exposure of Krebs II ascites tumour cells to radioactive precursors of RNA can be

annealed with homologous DNA but not with heterologous DNA. A section of the base sequences in the two chains appears therefore to be complementary. It would have been valuable to know the base composition of this RNA component and to relate it to the composition of the other nucleic acid components in the cell. Such an analysis was not undertaken since the RNA was not available in sufficient amount to allow the base composition to be determined by chemical methods. It would then have been necessary to label the RNA with ^{32}P-orthophosphate, hydrolyse it with alkali and determine the distribution of radioactivity amongst the nucleoside 3'(2')-monophosphates produced. This method presupposes that the proportion of radioactivity recovered in each of the four nucleotides accurately reflects the absolute proportions of these nucleotides in the polynucleotide chain. For this to occur, the specific activities of the precursor pools of ATP, CTP, GTP and UTP would require to be the same. For example, if the specific activity of the UTP pool were twice the specific activity of the ATP pool, and the number of UMP residues equalled the number of AMP residues, the total amount of radioactivity in the AMP and UMP would differ by a factor of two. One might therefore conclude, wrongly, that the amount of AMP in the RNA was only half the amount of UMP. When this approach is adopted, therefore, it is assumed that ^{32}P-orthophosphate is incorporated in such a fashion that the specific activities of the four nucleoside triphosphate pools are the same. This does not always appear to be a justifiable assumption since the rate of incorporation of ^{32}P-orthophosphate into the four pools

may be different and since the sizes of the pools certainly vary by considerable amounts. It was therefore felt that the use of this technique in the present experiments might give rise to results of questionable value.

Although the determination of nearest neighbour base frequencies is widely used to determine the nature of the product in enzyme assays, with intact cells the method appears to be open to criticism. This method requires the incorporation of a ^{32}P-labelled nucleoside 5'-monophosphate into RNA and analysis of the radioactive nucleoside monophosphates obtained on alkaline hydrolysis. In the intact cell, entry of nucleotides is limited by the permeability of the cell membrane and the radioactive nucleotide is subject to degradation within the cell. This would lead to randomisation of the radioactivity amongst the various nucleoside 5'-phosphates so rendering the method invalid. It therefore appeared that the base composition of the rapidly-sedimenting labelled RNA component, referred to above, could not be determined unequivocally by these methods.

It is relevant at this point to consider the relationship between the structure of DNA and the transcribed RNA. In the cell, the strands of DNA are responsible for the transcription of a very large number of separate base sequences, each with a unique coding function. The size of the transcribed RNA molecules will therefore be small compared with the length of the DNA chain. If the whole length of the DNA strand is transcribed into RNA, the base sequences in the RNA and DNA molecules will

be complementary. If, however, extensive areas of the DNA in the intact cell were repressed, only a portion of the DNA chain would be available for transcription and only certain segments of the DNA would be transcribed into RNA. In such circumstances, there is no reason to expect that the base composition of the transcribed RNA should bear any relationship to that of the complete DNA molecule.

From Fig. 24 and Fig. 25, it is evident that the rapidly-sedimenting, labelled RNA, obtained after brief exposure of Krebs II ascites tumour cells to radioactive precursors of RNA, resolves into a second rapidly-sedimenting component and radioactivity then becomes associated with ribosomal RNA. This suggests that the rapidly-sedimenting components act as precursors of ribosomal RNA, or that a messenger RNA has become associated with the ribosomal RNA. It should, of course, be remembered that the configurationsof the ribonucleic acids isolated with phenol are probably different from their configuration within the cell. Therefore, the association of radioactivity with the peaks of ribosomal RNA observed in sucrose density gradients may be due to factors other than those considered above.

It is evident from the literature that the origin of the various species of RNA in the intact cell is not yet established beyond question. The hypothesis supported by the bulk of the experimental evidence is that, in the normal cell, all species of RNA - messenger RNA, ribosomal RNA and sRNA - are synthesised on a DNA template. Newly-synthesised components have been described which resemble either DNA or ribosomal RNA in base

composition and they are thought to represent messenger RNA and ribosomal-precursor RNA respectively. In general, the synthesis of RNA, with the exception of some limited incorporation into sRNA, can be eliminated by actinomycin D. If this view is correct, the synthesis of cellular RNA must presumably be confined to the cell nucleus because of its dependence upon DNA and evidence in favour of this concept continues to accumulate.

Nevertheless, several pieces of evidence point to the existence of some extranuclear synthesis of RNA and it is by no means certain that all RNA synthesis takes place in the cell nucleus, or that DNA is in all cases the immediate template for all RNA synthesis. The present results demonstrate that virtually all synthesis of RNA in the Krebs II ascites tumour cell is susceptible to inhibition by actinomycin D. However, it is possible that the small amount of RNA synthesis evident at low concentrations of actinomycin D, may represent RNA synthesis which is not immediately dependent on DNA. The residual incorporation by Krebs II ascites tumour cells at low actinomycin D concentrations, which are, however, high enough to inhibit all incorporation in growing cell systems, might be a reflection of an RNA-dependent mechanism. On the other hand, it may only represent a remnant of the DNA-dependent system, which has escaped inhibition because of some factor controlling the permeability of the cell membrane. Similarly, the RNA-dependent enzymes that have been described may be a true indication of the existence in the cells of a mechanism of RNA synthesis that requires an RNA, rather than a DNA, template.

Extra-nuclear synthesis of RNA has been advocated principally by Harris (1965) who maintains that there is no significant transfer of newly-synthesised RNA from the nucleus to the cytoplasm. Although the results obtained by Harris have been interpreted in terms of the formation of pCpCpA sequences in the cytoplasm (see Franklin, 1965), the possible role of a cytoplasmic RNA-dependent synthesis of RNA must be considered. A template RNA molecule, arising in the first instance from DNA, could migrate to the cytoplasm and function in the production of RNA molecules. If this template were stable, the reaction it primed should be apparent after actinomycin D treatment, but if it were labile, the whole system would be sensitive to actinomycin D, since it depends in the first instance upon DNA-dependent synthesis of RNA.

2. The replication of animal viruses

1. General

The manner in which the virus parasite invades the host cell and compels it to produce new virus particles was, until recent years, a matter of conjecture. The problem was initially approached by examining the pathological changes induced in the cultured cell by infection with the virus. The investigation of cytopathological changes involved noting such effects as the distortion of the nuclear membrane and bubbling in the cytoplasm after virus infection and before virus release. However, the appearance of viral components could not be accurately observed since the methods lacked specificity. Some degree of specificity was achieved

by the use of fluorescent antibodies, but, on balance, the information provided by these techniques was rather imprecise.

A more fruitful approach has been the study of the metabolic changes within the infected host cells. The use of specific radioactive precursors of viral components has led to the establishment of a number of basic features of animal virus infection.

It is now generally established that shortly after an animal cell has been infected with virus, only a small fraction of the initial viral infectivity can be recovered. This is due not to the disappearance of viral infectivity, but to the separation of the viral nucleic acid from its protein capsomeres within the cell; the infectivity of the nucleic acid is only a small fraction of the infectivity of the intact virus. The functional role of the capsomeres appears to be fulfilled once the viral nucleic acid is introduced into the cell and their further fate is unknown. The entry of the virus particle into the host cell depends on its adsorption at specific attachment sites on the host cell. It appears, in some cases, that a pre-existing host cell enzyme begins the attack on the virus capsid and thereafter an uncoating enzyme is synthesised which completes the removal of the virus protein coat (Joklik, 1962). The virus host-range is limited by its inability to adsorb to certain cells. The nucleic acids, however, show a much more extensive host range, but this, too, is limited (Holland & Hoyer, 1962).

The expression of viral infectivity is determined by the manner in which the viral nucleic acid functions in the host cell. The conventional

sequence for the transfer of genetic information is DNA ⟶ RNA ⟶ protein (see Introduction). The nucleic acid of DNA-containing viruses replicates itself and also transcribes information to RNA, therefore functioning in a manner similar to cellular DNA. The manner in which the RNA of RNA-containing animal viruses transfers information for virus production is not quite so evident. It is the purpose of this section to extend the concepts of virus replication, already discussed in the case of bacteriophage replication, to the mechanism of replication of DNA- and RNA-containing animal viruses. The emphasis is placed on the RNA viruses and the results presented above will be discussed in the light of the current concepts of virus replication.

2.2. DNA-containing animal viruses

Infection of bacteria with virulent DNA-containing bacteriophages produces profound changes within the host cell. The invading viral DNA causes the production of lytic enzymes which destroy the host cell genome and completely disorganise the patterns of information transfer within the host. Viral DNA can then replicate in a semi-conservative manner and the transcription from DNA to RNA and protein can proceed by the mechanisms already described. Since the DNA of the bacteriophage øX 174 is single-stranded, the manner in which it replicates may shed some light on the replication of the single-stranded RNA of the RNA-containing animal viruses. During intracellular replication of øX 174, double-stranded DNA synthesis begins at an early phase and after an appreciable lag period, synthesis of single-stranded DNA begins (Sinsheimer,

Starman, Nagler & Guthrie, 1962). Both types of DNA have been isolated and observed in the form of closed rings which are resistant to exonuclease activity (Fiers & Sinsheimer, 1962; Burton & Sinsheimer, 1963). The synthesis of both protein and double-stranded DNA is required for the production of single-stranded DNA (Matsubara, Taketo & Takagi, 1963). By analogy, therefore, the production of a double-stranded RNA might be involved in the replication of RNA-containing viruses.

Within recent years, the mechanisms involved in the replication of DNA-containing animal viruses have been extensively studied. In particular, the replication of vaccinia virus has been the subject of intensive examination. Much of this work has been carried out in different strains of cells so that the results obtained by different groups must be compared with caution. However, with this limitation in mind, a reasonably full picture of the replication of vaccinia virus can be constructed.

Autoradiographic studies have revealed that this DNA-containing virus appears to replicate in the cytoplasm of the cells which it invades (Cairns, 1960; Reich et al., 1962; Dales, 1962). When the cell is infected with vaccinia virus, marked increases of the enzymes concerned with the biosynthesis of DNA occur during the time preceding the appearance of infectious virus (Kit, Piekarski & Dubbs, 1963; Shatkin & Salzman, 1963). In particular, striking increases have been observed in the DNA nucleotidyltransferase activity of HeLa cells and KB cells infected with vaccinia virus (Bach, 1963; Green, 1962; Magee, 1962). The uptake of ^{3}H-thymidine by the nuclei of mouse fibroblasts and rabbit kidney cells

is inhibited after infection with vaccinia virus (Kit, Dubbs & Hsu, 1963) and the deoxyribonuclease and ribonuclease activities of infected monkey kidney cells is increased (Allison & Sandelin, 1963).

In HeLa cells infected with vaccinia virus, viral DNA-directed synthesis of RNA occurs. Synthesis of this RNA is necessary throughout infection for the production of viral protein and infectious virus (Shatkin, 1965). Synthesis of vaccinia virus DNA is sensitive to actinomycin D although the replication of the host cell DNA is relatively resistant (Reich et al., 1962). This appears to be due to inhibition of the production of virus-specific RNA, which is responsible for the production of enzymes for viral DNA synthesis.

Polyribosomes, on which immunologically-specific protein is produced, have been observed in HeLa cells infected with vaccinia virus (Scharff, Shatkin & Levintow, 1963). Since the polyribosomes appear to change in size as infection proceeds, it seems possible that more than one species of virus-specific RNA is produced.

The antibiotics mitomycin C and porfiromycin inhibit the normal division and DNA synthesis of HeLa cells, but do not inhibit the production of vaccinia virus (Magee & Miller, 1962). Therefore, the synthesis of viral DNA can be dissociated from the synthesis of cellular DNA in the same manner as the synthesis of the RNA of RNA-containing viruses can be dissociated from cellular RNA by actinomycin D. In experiments with 5-fluorodeoxyuridine, which inhibits DNA synthesis by blocking the conversion of deoxyuridylic acid to TMP, it has been demonstrated that

the synthesis of viral DNA is complete when only a small fraction of the mature virus is formed (Salzman, 1960; Magee, Sheek & Burrous, 1960). This also demonstrates the absence of thymidylate stores in these cells and the unavailability of host cell DNA for virus production. The synthesis of viral DNA precedes the appearance of infectious virus and the virus protein is synthesised just prior to virus maturation (Salzman, Shatkin & Sebring, 1963).

The events which occur after infection with vaccinia virus, in so far as they are known, are therefore compatible with the conventional sequence of information transfer.

Not all DNA-containing animal viruses replicate in precisely the same manner as vaccinia virus. One major difference is that other viruses, such as the adenoviruses and herpes virus, replicate in the nucleus of the host cell (Green, 1962; Ginsberg & Dixon, 1959; Russell, 1962; Ben-Porat & Kaplan, 1962). Some features of the replication of these viruses, when viewed together, provide a more detailed picture of DNA virus replication.

Thus, in the early stages of infection, before the appearance of viral DNA, KB cells infected with Type 2 adenovirus increase in size due to the accumulation of protein, DNA and RNA (Green, 1962). Early RNA synthesis is required for the production of adenovirus DNA, antigen and infectious virus (Wilcox & Ginsberg, 1961, 1962). Studies on the changes occurring after infection with herpes virus reveal that in HeLa cells, DNA is broken down as a result of increased deoxyribonuclease activity (Wildy, Smith, Newton & Dendy, 1961; Keir & Gold, 1963). In HEp-2 cells,

duplication of viral DNA does not appear to occur until certain products of the eclipse phase attain a critical concentration (Roizmann, 1965). Increases have been observed in the DNA-synthesising enzymes in rabbit kidney cells infected with pseudorabies virus and in HEK 21 cells infected with herpes virus (Nohara & Kaplan, 1963; Keir & Gold, 1965). However, no marked changes have been observed in KB cells infected with adenovirus (Green, 1962).

In rabbit kidney cells infected with pseudorabies virus, a pool of viral DNA is formed from which molecules are withdrawn in a random manner to be incorporated into mature virus particles (Ben-Porat & Kaplan, 1963; Kaplan & Ben-Porat, 1965). This process is similar to the mechanism operating in bacteriophage replication.

Thus it would appear that the mechanisms of transfer of information in animal cells infected with DNA viruses follow those already established in normal cells and bacterial systems.

2.3. RNA-containing animal viruses

1. General

The isolation of infectious RNA from tobacco mosaic virus (TMV) and the production of mutants by treating TMV-RNA with nitrous acid have demonstrated that RNA, as well as DNA, can function as a carrier of genetic information. It is not readily apparent how the RNA of the RNA-containing animal viruses fits into the accepted schems of information transfer and to what extent the production of viral RNA is dependent on DNA.

The viruses which have been studied in greatest detail are the small RNA-containing animal viruses of approximately 25-30 mμ in diameter. A number of the Columbia SK group of viruses, encephalomyocarditis, Mengo and mouse encephalomyelitis viruses, have been investigated, but the most extensive work has probably been carried out on the enterovirus, poliomyelitis virus. These viruses consist of an RNA core surrounded by a protein shell; the molecular weights of the ribonucleic acids are of the order of 2 to 3 x 10^6 (Montagnier & Sanders, 1965a; Dales & Franklin, 1962; Hausen & Schäfer, 1961, 1962; Colter, Bird, Moyer & Brown, 1957). Poliovirus RNA, which is single-stranded (Holland, McLaren, Hoyer & Syverton, 1960; Henry & Youngner, 1963), is contained in a protein shell composed of 60 structurally identical subunits arranged with icosahedral symmetry (Horne & Nagington, 1959; Finch & Klug, 1959) so that the term spherical virus which is sometimes applied to this type of virus is not strictly accurate. The structures of these small RNA viruses are closely similar (Faulkner, Martin, Sved, Valentine & Work, 1961).

2.5.2. The rôle of DNA in viral RNA production

The biosynthesis of the RNA of these RNA viruses is a process fundamentally different from that of RNA biosynthesis in the normal cell. Thus, mitomycin, which destroys the host cell genome, does not affect Mengovirus growth under conditions in which DNA and RNA synthesis in mouse fibroblasts are no longer detectable (Reich & Franklin, 1961). Aminopterin, 5-flurodeoxyuridine, fluorouracil and bromouracil do not affect the synthesis

of poliovirus or of Newcastle disease virus (NDV) (Simon, 1961). Moreover, the growth of Mengovirus, poliovirus and NDV is resistant to actinomycin D (Reich et al., 1962; Darnell, 1962; Kingsbury, 1962). It therefore appears that the synthesis of the RNA of these viruses is independent of DNA. This is further supported by studies of the growth of the RNA-containing bacteriophage f2 and M12 (Cooper & Zinder, 1962; Hofschneider, 1963) and of TMV (Reddi & Anjaneyalu, 1963; Singer & Knight, 1963) which are independent of DNA.

It is clear from the results presented, that EMC virus can also grow in the presence of actinomycin D, confirming the observations that the synthesis of the RNA of certain RNA viruses is independent of DNA.

This situation, however, does not appear to be universal. Rous sarcoma virus appears to contain RNA but not DNA (Crawford & Crawford, 1961). Infection and virus production do not cause cell death (Temin & Rubin, 1959) and the virus can exist in a provirus state (Temin, 1962). Actinomycin D inhibits the production of Rous sarcoma virus so that the infection of cells may involve interaction of the virus with the host cell genome and may therefore be dependent upon DNA (Temin, 1963).

2.3.3. Alterations in host cell metabolism

One of the most important features of infection with the RNA-containing animal viruses is the manner in which the metabolism of the host cell is altered.

When Krebs II ascites tumour cells are infected with EMC virus, there

is no change in the total DNA, RNA and protein (Martin, Malec, Sved & Work, 1961) but the metabolism of RNA is profoundly altered (Martin & Work, 1961). The rate of RNA turnover increases during the period of rapid virus synthesis and this is accompanied by a small increase in the rate of protein turnover. There is a marked, progressive inhibition of orotic acid incorporation into nuclear RNA and also some net loss of RNA from the nucleus. The RNA of the mitochondrial fraction increases progressively during infection while that of the microsomal fraction increases to a lesser extent.

The effect of poliovirus infection on the synthetic activity of the host cell has yielded conflicting results. On the one hand, poliovirus infection was found to produce a great increase in HeLa cell RNA and protein synthesis (Maassab, Loh & Ackermann, 1957; Ackermann & Loh, 1960). On the other hand, no net change was reported in HeLa cell RNA, DNA and protein after poliovirus infection (Salzman & Sebring, 1961; Salzman, Lockart & Sebring, 1962; Darnell & Levintow, 1960). The conflicting results can be attributed to the fact that Ackermann et al. used cells supported in a maintenance medium whereas the latter workers used cells in the log phase of growth in a growth medium.

Poliovirus infection of HeLa cells causes progressive repression of cell-controlled RNA synthesis (Holland, 1963; Zimmerman, Hecter & Darnell, 1965) in a fashion analogous to the induced inhibition of host cell nucleic acid synthesis in E. coli infected with bacteriophage.

Autoradiographic studies of L cells infected with Mengo virus show

inhibition of normal nuclear RNA synthesis (Franklin & Rosner, 1962; Baltimore & Franklin, 1962). The decline in host cell nuclear RNA synthesis following infection can be prevented by puromycin (Franklin & Baltimore, 1962), which is a powerful inhibitor of protein synthesis (Yarmolinsky & de la Haba, 1959; Nathens & Lipmann, 1961), and seems to be caused by the elaboration of a viral-specific protein distinguishable from virus coat protein. There is progressive inhibition of the DNA-dependent RNA nucleotidyltransferase activity of extracts prepared from L cells at intervals after infection with Mengo virus (Baltimore & Franklin, 1962). A similar situation obtains in L cells infected with mouse encephalomyelitis virus where cellular protein, DNA and nuclear RNA synthesis is markedly depressed (Scholtissek, Rott, Hausen, Hausen & Schäfer, 1962). In L cells infected with reovirus, the synthesis of cellular DNA is specifically inhibited whereas the synthesis of RNA and protein is not (Gomatos & Tamm, 1963).

2.3.4. The messenger function of viral RNA

These changes in the host cell metabolism must be brought about directly or indirectly by the invading viral RNA and the mechanisms involved must be considered.

It is now reasonably well established that the viral RNA can act as its own messenger. Thus, when TMV-RNA is added to an E. coli protein-synthesising system, stimulation of the synthesis of a protein, which reacts with a TMV antiserum, is observed (Tsugita, Fraenkel-Conrat, Nirenberg & Matthaei, 1962). Similarly, f2 bacteriophage RNA, poliovirus RNA and turnip

yellow mosaic virus RNA can effect specific protein synthesis (Nathans, Notani, Schwartz & Zinder, 1962; Warner, Madden & Darnell, 1963; Darnell, 1962; Ofengand & Haselkorn, 1962). Although these RNA molecules function as messengers, they differ from the conventional concept of messenger RNA in that they do not appear to be unstable. Thus parental strands of MSϕ2 virus are completely conserved during all the replication and translation steps required to produce a full yield of mature virus particles and instability is not therefore a mandatory attribute of RNA molecules which serve as translatable messages for protein synthesis (Doi & Spiegelman, 1963). The RNA of certain animal viruses is similarly conserved (Laduron & Cocito, 1963).

Infection of HeLa cells with poliovirus leads to dissolution of the polyribosomes involved in the synthesis of cell protein and to the formation of larger, characteristic polyribosomes containing the pre-existing ribosomes of the cell (Penman, Scherrer, Becker & Darnell, 1963; Attardi & Smith, 1962). These polyribosomes are the site of production of immunologically identifiable protein (Scharff, Shatkin & Levintow, 1963). The RNA isolated from them has sedimentation coefficients of between 6 and 20s and is probably derived from the 45s precursor (Penman et al., 1963). This probably reflects the transcription of information for a number of different protein molecules. The inhibition of cellular RNA synthesis thus appears to be due to the synthesis of some virus-directed protein which is produced by the re-programming of ribosomes from which the host cell messenger RNA has been displaced by virus RNA (Kerr, Martin,

Hamilton & Work, 1962).

In order to produce these effects in the host cell and to direct the synthesis of new virus components, the viral RNA must carry information for more than one protein. An RNA molecule which can be translated into more than one protein has been termed polycistronic (Ohtaka & Spiegelman, 1965). Thus, Msϕ2 bacteriophage RNA is polycistronic and contains cistrons which code for at least 3 proteins. A control mechanism operates which determines the order and frequency of translation of each cistron into protein. Similarly, it is evident that the RNA of the small animal viruses codes for a number of proteins. An RNA molecule of molecular weight 2×10^6 contains about 6,000 nucleotides; this represents 2,000 coding triplets which could code for at least 10 proteins, assuming the average number of amino acid residues per protein to be of the order of 150-200. The RNA of the virus is therefore potentially able to produce the number of proteins required.

Probably the most important enzyme involved in virus infection is the RNA-dependent RNA nucleotidyltransferase responsible for the production of viral RNA.

Thus, E. coli cells infected with bacteriophage MS2, contain an enzyme for the synthesis of RNA which is not present, or is present in only small amount, in non-infected cells (Weissman, Simon & Ochoa, 1963). An RNA-dependent RNA nucleotidyltransferase has been isolated from E. coli cells infected with bacteriophage Msϕ2 which operates best with its own RNA as template (Haruna, Nozu, Ohtaka & Spiegelman, 1963). Similarly,

after infection of E. coli with bacteriophage f2, there is an increase in the actinomycin D and deoxyribonuclease resistant incorporation of CTP or GTP into RNA in a system that shows sensitivity to ribonuclease (Kay, Ortiz & August, 1963). In plant systems, an enzyme fraction has been observed in tobacco leaves infected with TMV which can produce infective RNA (Karasek & Schramm, 1962; Hudson, Kim, Smith & Wildman, 1963).

In animal cells, poliovirus induces the appearance of an RNA nucleotidyltransferase which is either absent, or present only in minute amounts, in uninfected cells (Baltimore, Eggers, Franklin & Tamm, 1963). Protein synthesis is necessary throughout the eclipse phase and the addition and removal of puromycin increases the eclipse period by a time equivalent to that during which the puromycin was present (Levintow, Thoren, Darnell & Hooper, 1962). The synthesis of poliovirus RNA can be inhibited by puromycin or high concentrations of p-fluorophenylalanine even after initiation of synthesis (Wecker, 1963; Scharff, Thoren, McElvain & Levintow, 1963). This inhibition may be due to the disappearance of functional RNA nucleotidyltransferase which must therefore be unstable (Eggers, Baltimore & Tamm, 1963; Eggers, Reich & Tamm, 1963). Lwoff (1962) has reported a critical thermosensitive event in the production of poliovirus RNA, which he has interpreted as follows. The viral RNA is considered to be liberated from the capsid and to act as a messenger by attaching to the ribosomes where it produces a protein called the polymerase monomer. The production of this protein is inhibited by

infra-optimal temperatures and by D_2O (deuterium, by replacing H, strengthens hydrogen bonds), but is not inhibited by supra-optimal temperatures or by urea (which split hydrogen bonds). The next reaction involves polymerisation of the monomer to give the functional polymerase. This reaction is inhibited by supra-optimal temperatures and urea and is favoured by D_2O; it can take place at infra-optimal temperatures. This step is considered to be the thermosensitive critical event, following which the synthesis of infective RNA occurs.

Mengovirus infection of L cells induces the formation of a new RNA nucleotidyltransferase (Baltimore & Franklin, 1963). The enzyme operates at a pH of between 7.5 and 9.5 and is reduced to below 50 per cent at pH 7.2. The reaction is completely dependent on Mg^{2+} ions, is inhibited by Mn^{2+} ions and is unaffected by deoxyribonuclease and actinomycin D. It is therefore similar in many respects to the enzyme described above in Krebs II ascites tumour cells infected with EMC virus. The enzyme in the Mengovirus system seems to appear rather later than in the EMC virus system.

2.5.5. The production of viral components

With small animal viruses, the production of virus RNA and virus protein generally appear to be separate events.

Thus in poliovirus infected HeLa cells, infective RNA appears before the production of mature virus particles (Holland, Mclaren, Hoyer & Syverton, 1960; Darnell, Levintow, Thoren & Hooper, 1961), but at no time does there appear to be a large amount of free viral RNA. After a lag

period of up to 4 hr., HeLa cells infected with poliovirus produce RNA which has base ratios approximating to those of poliovirus RNA, indicating the production of either viral RNA or virus-directed RNA or both (Holland, 1962; Darnell, 1962; Fenwick, 1963). This newly-formed RNA in actinomycin D treated, poliovirus infected HeLa cells has been shown to be viral RNA (Zimmerman, Heeter & Darnell, 1963). The synthesis of poliovirus RNA in L cells is dependent upon the continued synthesis of a specific protein (Scharff, Thoren, McElvain & Levintow, 1963). A similar situation has been described in HeLa cells infected with poliovirus, where the continued synthesis of a non-enzyme protein is required for the formation or preservation of infectious poliovirus RNA (Becker, 1963). Therefore, there appears to be an interdependence of protein and RNA synthesis in poliovirus replication.

There is strong evidence that poliovirus RNA is replicated in the cytoplasm of infected cells (Darnell, 1962) and the cytoplasmic site of formation of the capsid has been clearly established by electron microscopy (Horne & Nagington, 1959). Poliovirus RNA and protein are derived not from cell RNA and protein, but from the cellular pool of precursors (Darnell & Levintow, 1960; Salzman & Sebring, 1961). An intracellular particulate fraction has been observed in HeLa cells infected with poliovirus (Becker, Penman & Darnell, 1963). This material is apparently held together by lipid since it is disrupted by sodium deoxycholate, and it has been suggested by these authors that this represents the site of viral RNA and protein synthesis as well as of viral assembly.

Cytological studies on L cells infected with Mengo virus show degenerative changes in the nucleus and proliferative changes, associated with the production of viral subunits and new virus particles, within the cytoplasm (Dales & Franklin, 1962). However, this type of study can only locate the sites of accumulation of virus-specific products. Autoradiographic studies have revealed new RNA synthesis in the cytoplasm of L cells infected with Mengo virus (Franklin & Rosner, 1962). Furthermore, the location of the new RNA nucleotidyltransferase is in the post-mitochondrial and microsomal fractions (Baltimore & Franklin, 1963). Both viral RNA and protein are synthesised no later than 1 hr. and at least 30 min. before the appearance of completed virus (Krug & Franklin, 1964). The same workers failed to demonstrate any separation in time between the synthesis of viral RNA and cytoplasmic RNA as was described by Martin and Work (1961, 1962). They were unable, however, to determine whether protein or RNA was produced first.

Similar studies on the synthesis of mouse encephalomyelitis virus RNA and NDV-RNA have also led to the conclusion that these are synthesised in the cytoplasm (Hauson, 1964; Wheelock, 1963).

The course of events after infection of Krebs II ascites tumour cells with EMC virus has been carefully examined (Sanders, 1960; Martin & Work, 1962). Virus RNA is synthesised about 1 hr. before virus protein and the formation of complete virus follows almost immediately after the synthesis of virus protein. Much of the evidence on the site of production of EMC virus RNA appears to conflict with the results from the closely

related Mengo, MM and polioviruses; electron microscopy shows the earliest changes in the cell nucleus (Kerr, Martin, Hamilton & Work, 1962). The evidence suggests that infective EMC virus RNA is found in the nucleus shortly after infection and increases in amount until about 4.5 hr. after infection, when infectious RNA begins to be associated with the mitochondria (Bellet & Burness, 1965). Similarly, nuclei isolated from Krebs II cells infected with EMC virus, when incubated with ribonucleoside triphosphates and energy generating systems, can increase in infectivity (Zalta, Rosenewajg, Breugnon & Huppert, 1963), thus leading to the conclusion that the synthesis of EMC virus RNA normally takes place in the nucleus and can continue in the isolated nuclei. EMC virus RNA production begins about 3 hr. after infection (Martin & Work, 1962). These authors therefore state that the massive synthesis of RNA, observed in previous experiments (Martin & Work, 1961), that occurs in the cytoplasm of infected cells commencing about 4 hr. after infection, does not represent synthesis of viral RNA. However, the conditions employed in the infection of the cells in the two sets of experiments were not identical so that direct comparisons between the time of viral RNA and cytoplasmic RNA production may not be valid.

The results presented in this thesis show actinomycin D resistant increases of both RNA and RNA nucleotidyltransferase activity in cytoplasmic components of Krebs II ascites tumour cells infected with EMC virus. These results could be interpreted as indicating an extra-nuclear site of synthesis of EMC virus RNA. This interpretation must be accepted with

caution since it depends on the results of cell fractionation experiments, a technique which rarely yields unambiguous results.

Krug and Franklin (1964) point out the marked disagreement between their results on the location of Mengo virus RNA synthesis and the results of Bellet and Burness (1963), who advocate a nuclear site of MVO virus RNA replication. Indeed, since the two viruses are almost indistinguishable, it is not clear why their modes of replication should apparently be so different. Krug and Franklin (1964) indicate that the early increase in infective RNA noted by Bellet and Burness (1963) represents only a small fraction of the total RNA produced during infection. They also indicate that the decrease in the amount of infective EMC RNA obtained by Bellet and Burness (1963) 5 hr. after infection is probably due to the failure to extract infectious RNA from the virions at 0° under the conditions employed.

In their turn, Bellet and Burness (1963) suggest that cytoplasmic synthesis of RNA in L cells infected with Mengo virus cannot be due to viral RNA synthesis, because it occurs only after viral RNA synthesis is complete. This point, however, is refuted by Krug and Franklin (1964). Sanders (1962) suggested a possible reason for the apparent discrepancy and noted that if fingers of cytoplasmic material extend into the nucleus, the site of viral RNA production might appear to be nuclear, although, in fact, it was not. Alternatively, he suggested that the RNA might be synthesised in the nucleus then very rapidly transferred to the cytoplasm. Hausen (1964), using 30 sec. pulses of ^{3}H-uridine followed by analysis

of RNA, has been unable to detect any evidence for a nuclear phase in the production of ME virus RNA. Therefore an extremely fast transfer from nucleus to cytoplasm would be required.

In an earlier publication, Dulbecco (1962) suggested that, for a unitary view, the RNA viruses should be considered to multiply in the nucleus. This suggestion was made because no localised sites of virus production appear in the cytoplasm of L cells infected with Mengo virus; such a view favours the concept of synthesis of RNA in the nucleus followed by rapid transfer to the cytoplasm. However, the theory of EMC virus RNA replication propounded by Martin and Work (1962) suggests accumulation of RNA within the nucleus followed by a sudden change in the permeability of the nuclear membrane which results in the release of viral RNA into the cytoplasm. Dulbecco also based his suggestion on the study of phenotypic mixing of poliovirus, which demonstrated that the earliest recoverable progeny were already maximally mixed (Ledinko & Hirst, 1961).

Montagnier and Sanders (1963b) have recently demonstrated that an RNA component accumulates late in the infective cycle of EMC virus RNA replication and have suggested that this RNA is double-stranded. This material is also infective, but no conclusive evidence of its double-stranded nature has been presented. It is interesting to recall that Martin and Work (1962) stated that the cytoplasmic RNA synthesised late in the infection cycle is not viral RNA.

The results presented in this thesis provide no evidence for the presence of large amounts of either double-stranded RNA or of a strand

of RNA complementary to virus RNA. It is therefore necessary to examine further the possible manner in which the viral RNA strand might replicate.

Experiments on the production of poliovirus RNA (Darnell, 1962) and the results presented above, in conditions where the production of viral RNA is differentiated from the production of cellular RNA by actinomycin D, give no evidence for the appearance of a rapidly-labelled RNA analogous to T2 bacteriophage-specific RNA. These results, along with those discussed earlier, suggest that the viral RNA acts as messenger for the production of specific protein, one of the earliest of which is probably an RNA-dependent RNA nucleotidyltransferase. The nature of the primer for this enzyme is not yet clear, but there are a number of possible alternatives. The invading viral RNA strand could act as template for the synthesis of a complementary strand. Further replication could operate by means of a semi-conservative mechanism, involving a double-stranded intermediate. Such a mechanism would produce an equal number of RNA strands identical to the invading viral RNA and of strands complementary to them. Since there is no real evidence for a double-stranded intermediate in the production of infectious viral RNA, such material would have to be transient. It is unlikely that the code for transcribing information contained in the nucleotide sequence into protein is so degenerate that the complementary strand could constitute infectious virus RNA. This mechanism would therefore be wasteful.

A mechanism other than semi-conservative replication may operate, in which the invading viral RNA strand builds a complementary copy. This could then function as a template on which many strands of viral RNA

are synthesised. In this case, any double-stranded material involved as an intermediate would represent only a small proportion of the total RNA synthesised and might therefore fail to be detected by the methods employed.

In yet another mechanism, the active site of the nucleotidyl-transferase may be such that it can recognise each nucleotide residue and cause an identical chain to be synthesised with no base pairing taking place; that is, for an AMP residue on the viral RNA chain the enzyme would add an AMP residue to the strand being synthesised and so on.

In conclusion, the conflicting results obtained by different groups of workers may be a result of the different experimental systems which they employ. However, the simplest mechanism for the replication of RNA viruses appears to be one in which the viral RNA, acting as messenger, induces the synthesis of specific enzymes. These enzymes modify the metabolism of the host cell and catalyse the synthesis of the viral RNA, by a mechanism other than a semi-conservative one, in the cytoplasm. The newly-produced RNA will act as messenger for the production of capsid protein units which are then assembled into mature virus particles. It may not, however, be possible to integrate the synthesis of all RNA viruses into such a simple scheme.

8. Perspectives

The virus is a parasite and is absolutely dependent upon the host. It is not in the interest of the virus, therefore, that in the great

majority of cases, virus infection causes the death of the host cell, so that its means of support disappears. It is interesting to consider precisely the manner in which the small RNA-containing animal viruses kill the host cell. Cellular metabolism does not stop abruptly when the RNA from the invading particle enters. The viral RNA causes the progressive production of enzymes and proteins to prepare for the replication of new particles. Only about 5 per cent of the new viral RNA and protein produced are assembled into mature virus particles so that a very large excess of the viral components are formed. Because of the very small size of the virus compared to the cell, these components represent only a small percentage of the cell RNA and protein and the accumulation of these products probably does not of itself suffice to kill the cell. However, the viral RNA induces the production of proteins, which appear to suppress transcription from cellular DNA, cause actual damage to the host cell genome and thereby disorganise the host to such an extent that it dies.

The viruses, by killing the host cells, are therefore obliged to seek out new hosts to avoid extinction of the species. It would be more satisfactory for the virus to co-exist with the host cell without causing its death and integrate its metabolism with the host. The nearest approach of this concept of virus-host equilibrium is provided by the lysogenic bacteriophages of E. coli and the latent animal viruses such as herpes simplex virus. The conditions of symbiosis in these cases is not, however, ideal, since by suitable stimulation, the virus can begin to multiply in

the host and causes its death as before. Another situation akin to virus-host equilibrium is that situation in which the virus can interact with the host cell genome to effect a neoplastic transformation of the cell.

Some advance has been made in the chemotherapeutic control of virus infection (see Tamm & Eggers, 1963). The investigations have been carried out principally with cells in tissue culture, with notable exceptions, such as the control of herpes virus infection of the cornea with 5-iododeoxyuridine. However, the effects observed with cells in culture may be quite different from the situation in the intact animal. For example, whereas poliovirus appears to destroy its host in tissue culture, it apparently is almost nonpathogenic in those tissues where it replicates in the animal host. The one exception to this is the specific attack on the anterior horn cell in the animal host, to which all other effects of poliovirus infection are secondary.

In the whole animal the virus must contend with problems such as antibodies, phagocytes and the purely mechanical barrier of cell membranes outwith its host range. The establishment of the virus in the animal host remains a mystery and its elucidation awaits the extension of tissue culture techniques to the complex, dynamic interplay within the intact organism.

SUMMARY

S U M M A R Y

1. Experiments were performed to distinguish between the DNA-dependent and RNA-dependent incorporation of (^{32}P)UMP into RNA catalysed by enzymes in crude extracts of Krebs II ascites tumour cells. The DNA-dependent system showed optimal activity at pH values in the region of 7.5 in the presence of Mn^{2+} ions and 2-mercaptoethanol, and the RNA-dependent reaction showed optimal activity at pH values of about 9.5 and was inhibited by Mn^{2+} ions and 2-mercaptoethanol. Moreover, the DNA-dependent system was sensitive to treatment with actinomycin D and deoxyribonuclease, but was resistant to ribonuclease while the RNA-dependent system was resistant to actinomycin D and deoxyribonuclease, but was sensitive to ribonuclease.

2. The characteristics of the DNA-dependent incorporation of (^{32}P)UMP into RNA were determined. The activity was absolutely dependent on Mg^{2+} ions and native DNA was a more effective primer for the reaction than DNA which had been denatured by heat. The reaction showed no specificity with respect to primer DNA from a number of different sources. The reaction was stimulated in the presence of ATP, GTP and CTP and the removal of any one or all three of these markedly reduced incorporation of (^{32}P)UMP into RNA. Analysis of the product of the reaction showed that UMP residues were incorporated adjacent to AMP, GMP and CMP residues, thus suggesting synthesis of polyribonucleotides containing each of the four ribonucleotides.

3. Cell fractionation experiments showed that the DNA-dependent enzyme was located almost exclusively in nuclear preparations.

4. The characteristics of the RNA-dependent incorporation of (^{32}P)UMP

into RNA were also examined. The activity was at a maximum over a wide range between pH 8.5 and 10, was absolutely dependent on Mg^{2+} ions and was inhibited by Mn^{2+} ions. The reaction showed no specificity with respect to primer RNA from a number of different sources. Addition of ribonucleoside 5'-triphosphates to the system inhibited incorporation of (^{32}P)UMP into polyribonucleotides and analysis of the product demonstrated that the predominant reaction was the formation of polyuridylic acid, but there were also indications of the simultaneous formation of polyribonucleotides containing all four ribonucleotide residues.

5. Cell fractionation experiments showed that the enzymes catalysing the RNA-dependent incorporation of (^{32}P)UMP into RNA were located principally in fractions derived from the cytoplasm. While the activity of the cytoplasmic RNA-dependent reaction was stimulated by added RNA, the activity of nuclear fractions assayed under the same conditions was reduced by adding RNA.

6. No changes were observed in the activities of DNA nucleotidyltransferase or DNA-dependent RNA nucleotidyltransferase in extracts of Krebs II ascites tumour cells following infection with EMC virus.

7. The activity of the system catalysing the RNA-dependent incorporation of (^{32}P)UMP into polyribonucleotide was increased following infection with EMC virus. This increase was associated with cytoplasmic rather than nuclear enzymes and analysis of the reaction products suggested the presence of a new enzyme.

8. A method was developed for the extraction of all the RNA of Krebs II ascites tumour cells and Krebs II ascites tumour cells infected with EMC virus in an undegraded form.

9. Conditions for sedimentation analysis of RNA in sucrose density gradients were determined. Under the conditions employed, RNA extracted from Krebs II ascites tumour cells was resolved into three ultraviolet-absorbing components; the ribosomal RNA had sedimentation coefficients of 30s and 19s, while the sRNA had a sedimentation coefficient of 4s.

10. Brief exposure of Krebs II ascites tumour cells to small amounts of ^{3}H-uridine of high specific activity gave rise to a rapidly-sedimenting, radioactive component (sedimentation coefficient about 40s) and to radioactivity associated with the sRNA. On more prolonged exposure, the radioactivity associated with the ribosomal RNA components increased. Similar patterns were observed in experiments with ^{32}P-orthophosphate, but the appearance of radioactive components was somewhat later than in experiments with ^{3}H-uridine.

11. The rapidly-sedimenting, radioactive peak obtained after a brief exposure of Krebs II ascites tumour cells to radioactive precursors of RNA was isolated and it was demonstrated that it would anneal to homologous, but not to heterologous, DNA.

12. Exposure of Krebs II ascites tumour cells to high concentrations of actinomycin D eliminated the incorporation of ^{3}H-uridine or ^{32}P-orthophosphate into all RNA except sRNA. When lower concentrations of the antibiotic were employed, incubation of the cells with ^{3}H-uridine or ^{32}P-orthophosphate

led to limited incorporation into the rapidly-sedimenting components and ribosomal RNA in addition to sRNA.

13. Infection of Krebs II ascites tumour cells with EMC virus produced no marked qualitative effects on the sedimentation pattern in sucrose density gradients of the RNA extracted after exposure of the cells to radioactive precursors of RNA.

14. Analysis of the RNA extracted from actinomycin D treated Krebs II ascites tumour cells infected with EMC virus showed that a rapidly-sedimenting, radioactive component appeared about 4 hr. after infection. No such fraction could be demonstrated in uninfected control cells.

15. Fractionation of the subcellular components of Krebs II ascites tumour cells infected with EMC virus followed by extraction and analysis of the RNA showed that the RNA synthesised in the presence of actinomycin D was associated with cytoplasmic components of the cell.

16. No evidence was obtained for the occurrence of either a double-stranded RNA or of detectable amounts of an RNA complementary in base composition to viral RNA during the replication cycle.

17. Preliminary experiments performed to examine polyribosomes in Krebs II ascites tumour cells showed that after the cells were incubated in a buffered salt solution (Earle's medium), polyribosomes could no longer be detected. RNA could be prepared from the ribosomal fraction by treatment with sodium dodecyl sulphate. Difficulties in disruption of the cells after incubation in Earle's medium prevented the analysis of polyribosomes after infection with EMC virus.

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