SOME STUDIES ON THE LOW MOLECULAR WEIGHT RNA COMPONENTS OF MAMMALIAN CELLS

by

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Abbreviations

These are as laid down in the Instructions to Authors, of the Biochemical Journal, with the following additions:-

BSS	Balanced salt solution
SSC	Standard saline citrate (0.15M-NaCl, 0.015M sodium citrate, pH 7.0)
SDS	Sodium dodecyl sulphate
RNase	Ribonuclease
BHK-21	BHK-21/C13, Baby hamster kidney cells, clone 13
PPLO	Pleuropneumonia-like organisms.
pre-tRNA	Transfer RNA precursor RNA.

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INTRODUCTION

INTRODUCTION

1. General

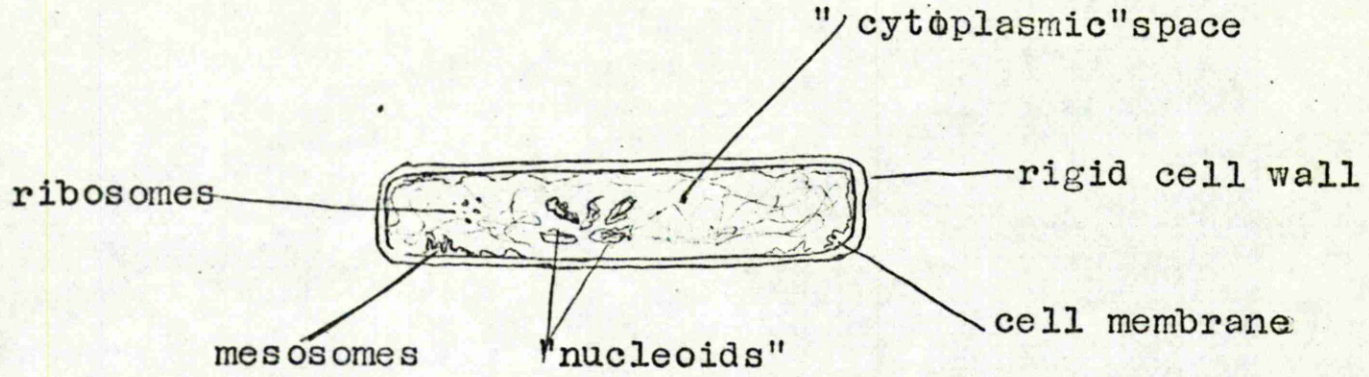
1.1. The Unilateral Nature of biological systems

Three major systems of thought have stressed the unilateral nature of biological systems; Schleiden & Schwann (1838) emphasised that all living matter is composed of units called cells, each with individual yet common characteristics; Darwin (1858) proposed a theory of evolution whereby the diversity of living forms could have developed by the modification of a common ancestor; Mendel (1866) provided by his laws of heredity, a general mechanism by which combination and resegregation of genetic units of organisms within any species could give rise to the transmission of characteristics inherited from evolutionary development. All living matter is therefore composed of cellular units which are themselves the smallest units of living material capable of an independent existence and which possess varying degrees of individuality. The recent advent of electron microscopy has delineated two distinct cell types; the prokaryotic system and a more complex eukaryotic system. The prokaryotes lack the nuclear organisation of the eukaryote and show an absence of such subcellular organelles as mitochondria, golgi apparatus and endoplasmic reticulum although the functions carried out by these organells in

Schematic diagram of a prokaryotic and a eukaryotic cell.

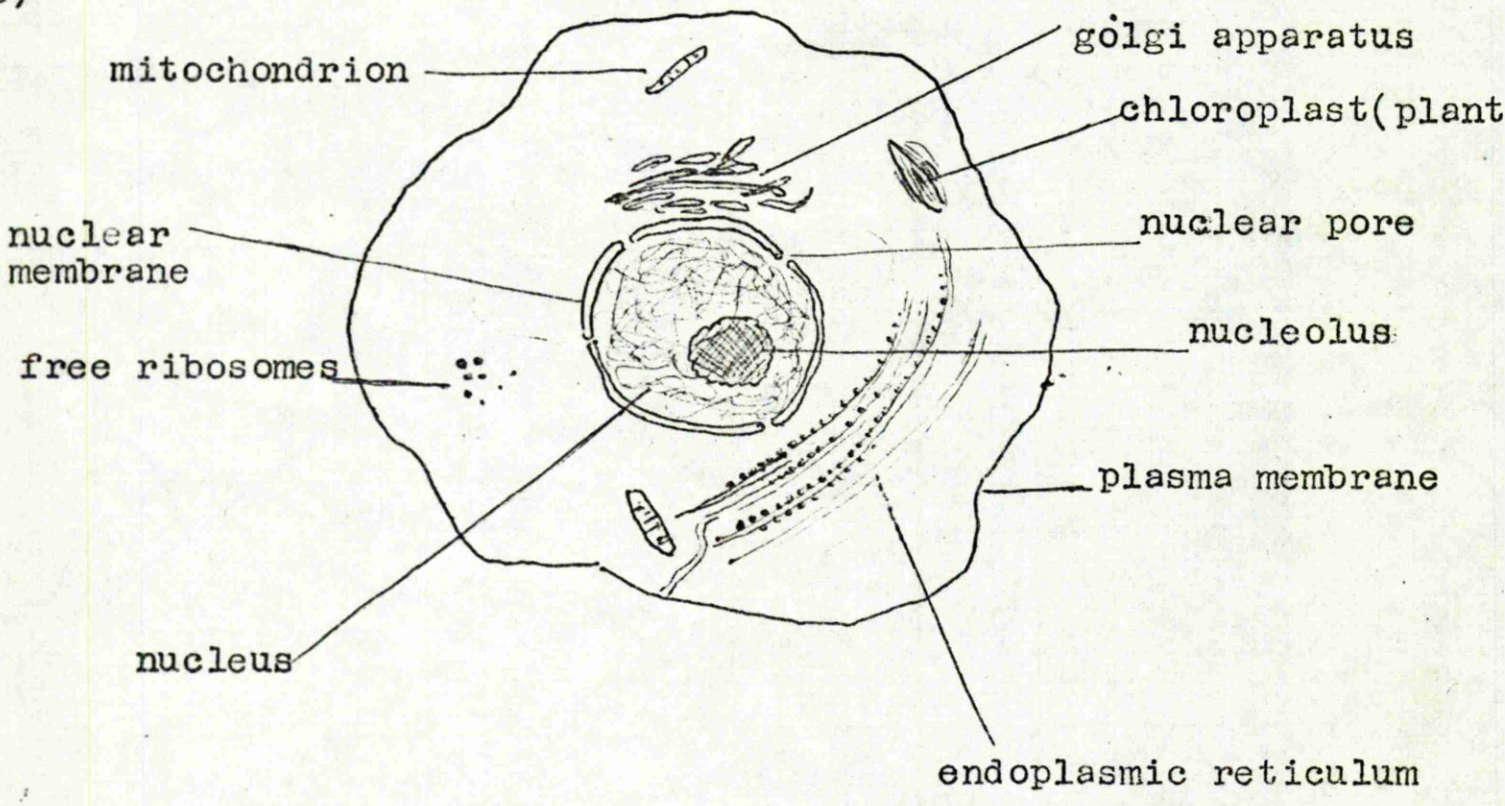
Fig I.1

(a)



The prokaryotic cell

(b)



The eukaryotic cell

eukaryotes appear to be performed in prokaryotes by involutio of the plasma membrane (mesosomes) and this absence may there fore simply reflect the evolutionary process by which eukaryotes have arisen. Although a degree of individuality is conferred to the cellular units by these distinguishing features, it is yet true that there exists a great deal of similarity in the nature of their basic "machinery of life", for they appear to store and duplicate their genetic information in the same manner and to express the information encoded therein by identical or similar mechanisms. For reference both types of cell system are shown diagramatically in Fig.1.1 (a) and (b).

1.2. The macromolecular components of life processes

Organisms owe their existence to a process of information transfer from one generation to another. Biochemical investigations of the molecular "make-up" of the machinery of living processes have revealed that proteins and nucleic acids contribute approximately 90% of the dry weight of most cells and that the nucleic acid fraction can be further subdivided into two distinct but related parts, namely DNA (desoxyribonucleic acid) and RNA (ribonucleic acid). Although rapid advances concerning the chemical nature and histological location of the nucleic acids were made, it was not until about 1940 that the identification of DNA as

a transforming factor in bacteria suggested its role as the carrier of genetic information (Avery, McLeod and McCarty, 1944). The intensive chemical and physical studies on the structure of DNA culminated in perhaps one of the most striking proposals of molecular biology; the double helical structure of DNA (Watson & Crick, 1953). This structure immediately offered an explanation of the mode of replication of DNA and its suitability as the genetic material. Early cytochemical observations on cells actively engaged in the synthesis of proteins for secretion (Caspersson & Brachet, 195(had suggested a role for RNA in protein synthesis, and in vit studies confirmed this suspicion (Spiegelman, 1957), finally leading to the proposal by Crick, (1958) of the "central dogma of molecular biology". This proposal suggested a flow of information, in the form of RNA molecules, from the genome to the sites of protein synthesis where they directed the assembly of amino acids to form proteins. Confirmation of this hypothesis was obtained in 1961 by the isolation of a "messenger RNA" template in prokaryotic cells (Jacob & Monod, 1961). However investigations over the last decade have indicated that despite this most of the RNA present in eukaryotic and prokaryotic cells has no template function in protein synthesis.

2. The RNA of Prokaryotic & Eukaryotic cells

2.1. The Diversity of RNA Species

Eukaryotic and prokaryotic cells contain a wide variety of RNA molecules of varying size and chain length whose molecular weights range from 2.5×10^4 to 4×10^6 daltons and greater, and considerable effort has been invested in the elucidation not only of the mechanisms whereby these molecules are synthesised but also how they participate in the synthesis of proteins in these cells. These studies have indicated that although many RNA molecules arise as a result of direct transcription, certain species whose existence is transitory, may represent intermediates in a sequence of maturation events leading to mature, well characterised RNA molecules found in both eukaryotic and prokaryotic cells.

2.2. Ribsomal RNA

Ribosomes, ribonucleoprotein particles found ubiquitously in prokaryotic and eukaryotic cells, and which consist of approximately 50% RNA and 50% protein, are integral components of the protein synthesising machinery in both types of cells. They consist of two subunits, one large and one small. There is now a considerable body of experimental evidence pointing to the existence of two classes of ribosomes in living cells, one form, with a sedimentation coefficient of approximately 70s and composed of 50s and 30s subunits

is present in prokaryotes and in some eukaryotic organelles (mitochondria and chloroplasts) and another, with a sedimentation coefficient of approximately 80s, and composed of 60s and 40s subunits, is found exclusively in eukaryotic cells. Ribosomes from prokaryotic and eukaryotic cells are characterised by their possession of three types of RNA species (Osawa, 1968), two high molecular weight species, one pertaining to the large subunit and one to the small subunit, and a species of low molecular weight, called 5sRNA on the basis of its sedimentation behaviour, which is associated with the larger ribosomal subunit. In addition the ribosomes of many eukaryotic cells have a "7s" RNA species attached by hydrogen bonding to the high molecular weight RNA species of the larger subunit (Pene, Knight & Darnell, 1968).

2.2.1. Biogenesis of Ribosomal RNA

The biogenesis of ribosomal RNA (rRNA) has been perhaps the most widely studied area of RNA metabolism in eukaryotic cells. The processes leading to the formation of mature high molecular weight rRNA species in both eukaryotic and prokaryotic cells involve transcription followed by secondary modification of the nascent molecules.

2.2.1.1 High molecular weight ribosomal RNA in eukaryotic cells

Early cytochemical and autoradiographic evidence for the nucleolar location of rRNA cistrons in eukaryotic cells was

confirmed by the observations of Perry,(Perry,1962,1964) that low doses of the drug actinomycin D, while inhibiting the appearance of rRNA in the cytoplasm, blocked the synthesis of nucleolar RNA as judged by autoradiography. The more recent studies of nucleolar function in ribosome formation have largely been as a result of improved cell fractionation techniques(Penman,Smith & Holtzman,1966) but direct evidence as to the location of rRNA genes comes from the demonstration that anucleolate mutants of the clawed toad, Xenopus laevis, which lack the ability to form nucleoli also lack the ability to synthesise rRNA(Brown & Gurdon,1964). The biochemical synthesis of rRNA was first examined in Hela and L cells by Perry(Perry,1964) and in most eukaryotic cells the two species of high molecular weight rRNA appear to be derived, by a non-conservative maturation process,from a single unique precursor molecule containing the sequences of both the larger and small high molecular weight rRNA species together with some non-ribosomal sequences destined to be degraded. When Hela cells are exposed to isotopically labelled RNA precursors, such as [^{3}H]-uridine, for varying lengths of time, radioactivity first appears intwo classes of rapidly sedimenting RNA species associated with the nuclei. The first of these, characterise by its rapid turnover and heterogeneous distribution on sucros gradients (20s-80s) is called "heterogeneous nuclear RNA"

(Scherrer,Latham & Darnell,1963, Attardi, Parnas, Hwang & Attardi, 1966, Warner, Girard, Latham & Darnell,1966(a), Warner, Soiero, Birnboim, & Darnell,1966(b)) and will be discussed later. The second class of rapidly labelled RNA, on sucrose gradients sediments in a homogeneous fashion with a sedimentation coefficient of approximately 45s,(Scherrer et al,1963, Scherrer & Darnell, 1962) and is confined to the nucleolus. As the time of exposure to isotope increases, radioactivity appears progressively in a 32s RNA molecule confined to the nucleus and in an 18s RNA molecule which is rapidly transported to the cytoplasm, and finally in a 28s RNA molecule which appears firstly in the nucleoplasm and later in the cytoplasm (Penman,1966, Penman et al,1966). When cultures of Hela cells are exposed to isotope for a short time and all further RNA synthesis then blocked by treatment of the cultures with actinomycin D, the radioactivit first associated with the 45s RNA molecule disappears from it and appears progressively in RNA species sedimenting at 32s and 18s(Girard, Penman & Darnell,1964). These kinetic and pulse-chase data together with the observation that the base composition of 45s RNA is distinct from that of the cellular DNA and similar to that of rRNA (Scherrer et al, 1963, Soiero, Birnboim & Darnell,1966) point strongly to its role as a rRNA precursor molecule. Polyacrylamide gel electrophoresi

of Hela cell nucleolar RNA (Weinberg, Loening, Willems & Penman, 1967) revealed in addition to 45s, 32s, 28s and 18s RNA, minor components corresponding to 41s, 36s, 24s and 20s. After poliovirus infection of these cells, which was shown to interfere with normal nucleolar processing (Weinberg et al, 1967), these minor species were found to accumulate. Elegant analyses of these components, after poliovirus infection of Hela cells, by Weinberg & Penman (1970) has led to the scheme of maturation of 45s rRNA precursor represented in Fig 1.2. The accuracy of this maturation scheme has been confirmed by nucleotide composition analyses (Wagner, Penman & Ingram, 1967, Willems, Wagner, Laing & Penman, 1968, Amaldi & Attardi, 1968), by hybridisation data (Attardi, 1969) and recently by finger-printing of RNase T_1 digests of intermediates in the maturation process (Salim, Williamson & Maden, 1970).

Methylation of ribosomal RNA and content of non-ribosomal sequences

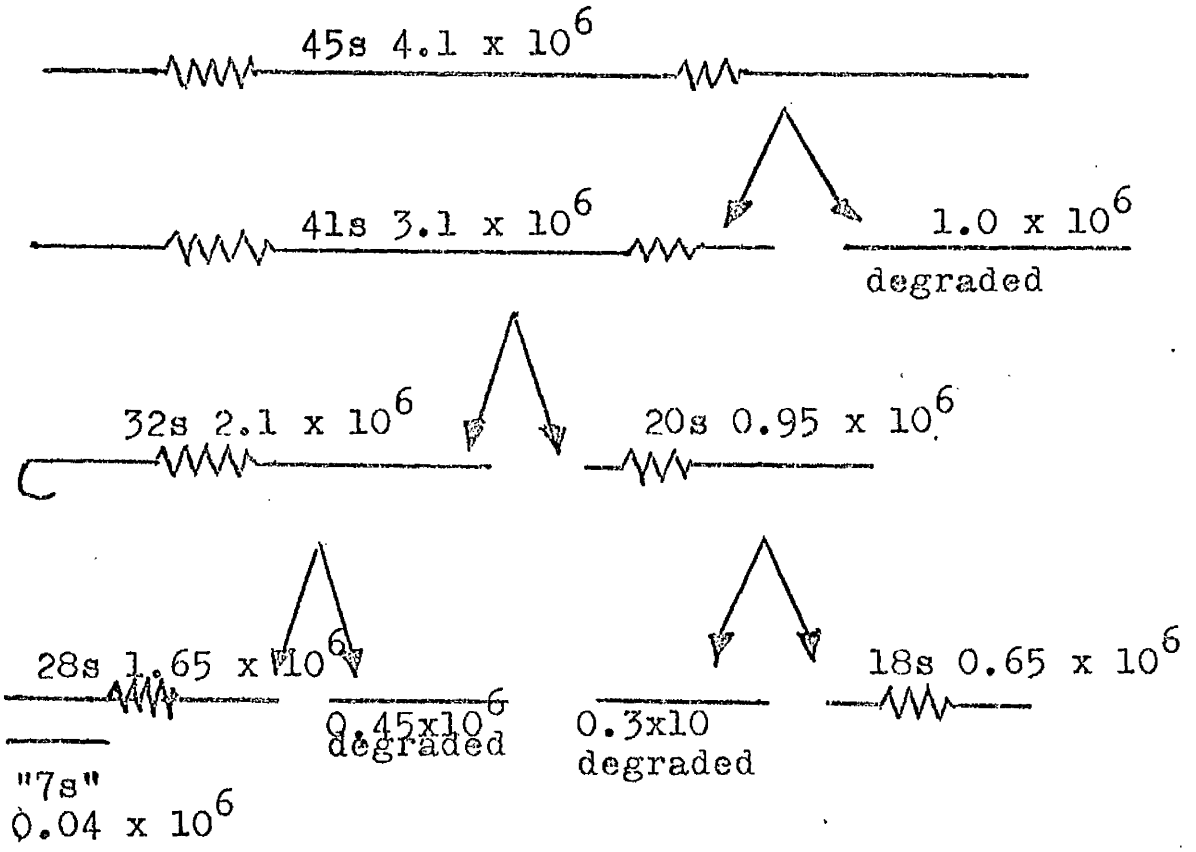
The ribosomal RNA from different organisms displays not only size differences but also a wide range of base compositio (Maden, 1970) but a characteristic feature of rRNA in both eukaryotic and prokaryotic cells is its high content of methylated nucleotides (Fellner & Sanger, 1968, Fellner, 1969, Wagner et al, 1967). In the prokaryotes the majority of

these methyl groups are present on the various bases with a few existing as 2'-O-methylribose (Fellner, 1969) but in eukaryotes at least 80% of the methyl groups occur as 2'-O-methylribose (Wagner et al, 1967, Brown & Attardi, 1965, Burdon, 1967, Lane & Tamaoki, 1969). The chemical effects of 2'-O-methylation are to render the adjacent phosphodiester bond resistant to alkali and nuclease treatment and it is therefore tempting to suggest that such methylation serves a protective function in vivo. Methylation of rRNA occurs at the level of the 45s precursor (Greenberg & Penman, 1966, Burdon, 1967) and is an early event in the synthesis of this molecule since treatment of cultures with actinomycin D for 5 min prior to the addition of methyl label prevents the subsequent incorporation of methyl groups (Burdon, 1967, Zimmerman & Holley, 1967). Only one methyl group is added subsequent to the synthesis of the 45s RNA molecule (Zimmerman, 1968).

Base composition studies (Ameldi & Attardi, 1968) indicated that 45s and 32s RNA possessed a significantly higher (G C) content than 28s or 18s RNA and it has been observed that in the multistep maturation of 45s rRNA precursor molecules (r-preRNA) to the mature 28s and 18s rRNA there is a progressive increase in the relative level of methylation (Weinberg et al, 1967, Weinberg & Penman, 1970). It is

Scheme of the maturation of ribosomal RNA in mammalian cells.

Fig.I.2



Scheme reproduced from Weinberg & Penman (1970).

therefore apparent that 45s rRNA contains (G+C) rich non-ribosomal sequences which in the course of maturation are lost and probably degraded. Various workers have shown that these non-ribosomal sequences contribute as much as 45% - 50% of the polynucleotide sequences of the 45s RNA molecule (Amaldi & Attardi, 1968, Jeanteur, Amaldi & Attardi 1968, Attardi, 1969).

The ribosomal RNA transcription unit in eukaryotes

Much of the above data concerning the maturation events leading to mature cytoplasmic rRNA species has been gained from studies with Hela cells but RNA possessing properties of a rRNA precursor has been identified in a wide variety of mammalian cells, where the process of maturation appears to be identical (Yoshikawa-Fukada Fukada & Kawade, 1965, Burdon, 1965, 1967, Muramatsu & Busch, 1967, Torelli, Henry & Weissman, 1968). r-preRNAs have also been examined in plants (Leaver & Key, 1970, Rogers, Loening & Fraser, 1970) and in lower eukaryotes (Rae, 1970, Greenberg, 1969, Edstrom & Daneholt, 1967, Hughes & Kafatos, 1970). The maturation sequences in these lower organisms are similar to that of the higher organisms although the molecular weights of the transcriptional units and the intermediates vary as does the content of non-ribosomal sequences, (Perry, Cheng, Freed, Greenberg, Kelley &

Table I.1 Species variation in the rRNA transcription unit

Species origin	Mol. Wt x 10^{-6}		% of transcription unit not conserved
	gene prod.	rRNA	
Mouse	4.19	2.35	44
Potoroo (Marsupial)	4.19	2.35	44
Chicken	3.92	2.24	43
Iguana	2.74	2.13	22
Frog	2.76	2.19	21
Trout	2.70	2.20	19
Drosophila	2.85	2.05	28
Tobacco	2.76	1.95	29

Data in this table was taken from Perry et al(1970).

Tartoff, 1970). In higher organisms the primary transcription unit (45s r-preRNA) has a molecular weight of approximately 4.1 x 10^6 daltons and in plants and lower organisms of 2.7 - 2.8 x 10^6 daltons, reflecting the higher content of non-ribosomal sequences in higher organisms. Similar observations are made for the other intermediates of the maturation processes. rRNA processing at various evolutionary levels is reflected in the data presented in Table I.

2.2.1.2. High molecular weight ribosomal RNA in prokaryotic cells

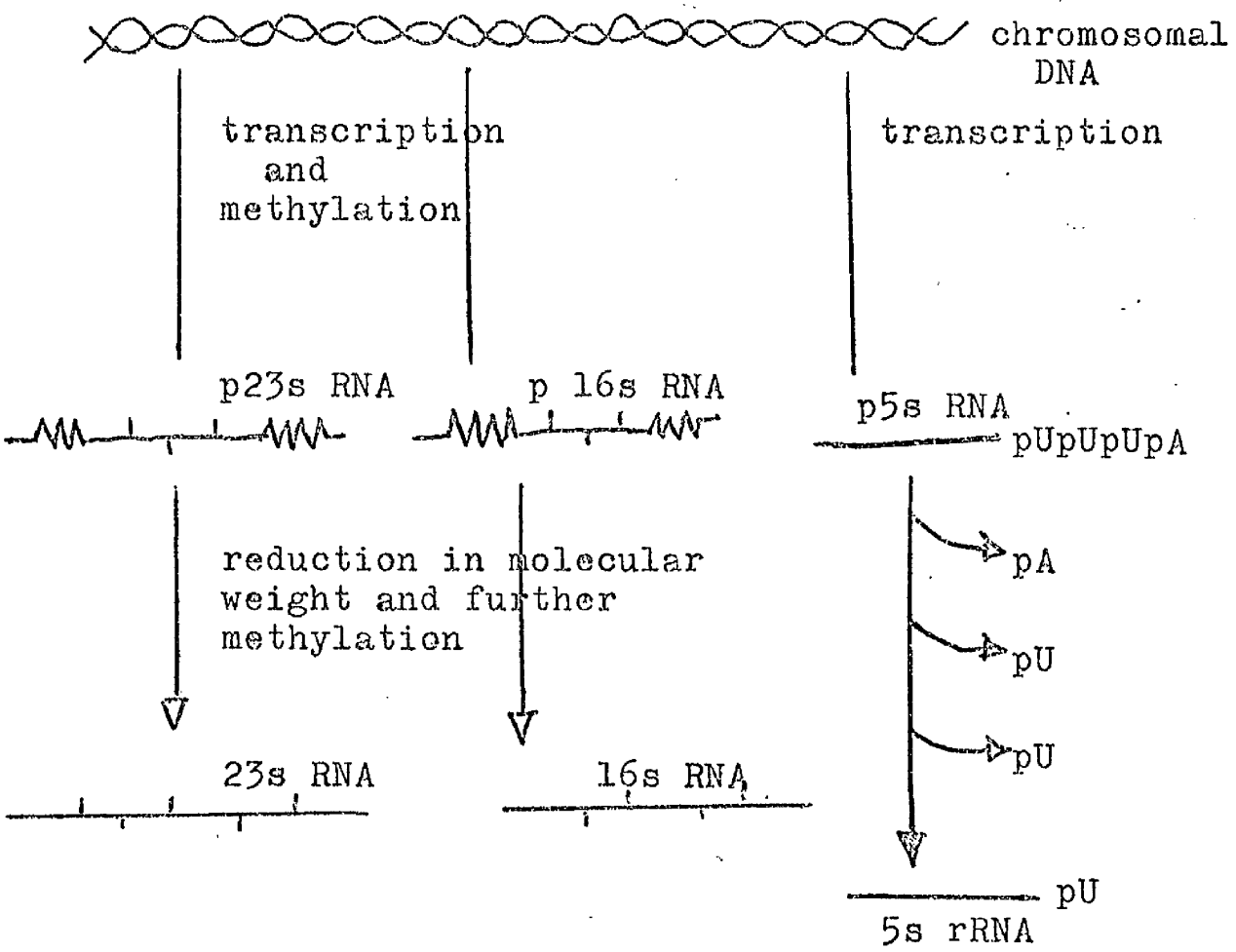
The rRNA components of prokaryotic cells have been identified as single polynucleotide chains (Stanley & Bock, 1965) of sedimentation values 23s (from the larger 50s subunit) and 16s (from the smaller 30s subunit) by Kurland (1960). These molecules show little variation in base composition even when isolated from a variety of different species (Midgely, 1962). In addition to the 4 major bases these RNA molecules, like their eukaryotic counterparts contain methylated nucleotides and the minor nucleoside Ψ (pseudouridine), but 80% - 90% of the methyl groups are present on the bases and only a few occur as 2'-O-methylribose (Fellner & Sanger, 1968) in contrast to the situation in eukaryotes. In an exponentially growing culture of E.coli equal numbers of 16s and 23s RNA chains

are synthesised per unit time (Adesnik & Levinthal, 1969) and since there exists the same number of gene copies for 23s and 16s RNA (Smith Dubnau, Morell & Marmur, 1968, Yanofski & Speigelman, 1962) Mangiarotti, Apirion, Schlessinger & Silengo (1968) have suggested that in E.coli a 23s RNA molecule may be assembled from two halves of similar size. This possibility is given further support by the observations of Fellner & Sanger (1968) that the major methylated nucleotides occur twice in E.coli 23s RNA. Although in bacteria there is no entity corresponding to the ribosomal transcription unit of eukaryotes, recent evidence (Adesnik & Levinthal, 1969) suggest that the two species of rRNA are each transcribed as slightly larger though separate molecules which are then processed to the mature rRNA species. The existence of macromolecular precursors to bacterial rRNA was suggested by the accumulation, in cells in which protein synthesis had been inhibited by chloramphenicol, of abnormal rRNA of higher sedimentation coefficient and contained in ribonucleoprotein particles referred to as CM particles (Dubin & Elkort, 1964, McConkey & Dubin, 1965, Sypherd & Fenster, 1967). Much of this accumulated RNA was incorporated into mature ribosomal subunits when the block on protein synthesis was removed (Osawa, 1965; Nomura & Hosokawa, 1965, Nakada, Anderson & Magasanik,

1964). Such CM particles sedimented in sucrose gradients at 22s, 26s, 30s and 40s and were thought to be intermediates in ribosome assembly since the 23s and 26s particles contained undermethylated 17s RNA and the 30s and 40s particles contained undermethylated 23s RNA. Furthermore "labelled" RNA extracted from such particles competed in hybridisation experiments with mature 23s and 16s RNA of the ribosomes and the particles also contained ribosomal proteins (Mangiarotti et al, 1968). Recently Lewandowski & Brownstein (1969) have identified, in a mutant of E.coli a 43s ribonucleoprotein particle posessing some ribosomal protein and 23s RNA and which appears to be a precursor stage in 50s ribosomal subunit assembly. Polyacrylamide gel electrophoresis of pulse labelled RNA from B.subtilis, by Hecht & Woese (1968) has demonstrated slower migrating species to 23s and 16s RNA and the kinetics of labelling of these components compared to that of mature 16s and 23s RNA is consistent with a precursor role for these slower migrating species. Similar experiments by Adesnik & Levinthal (1969) in E.coli confirm this and suggest one or possibly two precursors to 16s RNA with one detectable precursor to 23s RNA, each of lower electrophoretic mobility than the corresponding mature species. Furthermore these precursors have the same electrophoretic mobility as

The maturation of prokaryotic rRNAs

Fig.I.3



-non-ribosomal sequences

- methyl groups

p23s, precursor to 23s rRNA
p16s, precursor to 16s rRNA
p5s, precursor to 5s RNA.

Modified from Smillie (1970).

RNA extracted from CM particles or from particles which accumulate in methionine starved cells (Adesnik & Levinthal, 1969). It therefore appears that in the maturation events leading to mature 16s and 23s RNA molecules in prokaryotic cells, non-ribosomal sequences are removed and secondary modifications of methylation and minor nucleoside conversion occur. This is diagramatically represented in Fig 1.3. The concept of maturation of rRNA is therefore not restriced to eukaryotic cells and indeed Pace, Peterson & Pace (1970) have suggested, on the basis of kinetic data, that all stable RNA species in E.coli arise as a result of post-transcriptional modification.

2.2.2. Low Molecular Weight Ribosomal RNA

2.2.2.1. 5s RNA

The major ribosomal subunits of both eukaryotic and prokaryotic cells possess a low molecular weight rRNA component designated as 5s RNA by virtue of its sedimentation characteristics. This RNA molecule, which shows little sequence variation from different sources, is 120 nucleotides long (Brownlee, Sanger & Barrell, 1968, Forget & Weissman, 1967), is devoid of methylated nucleosides and Ψ (Comb & Catz, 1964, Galibert, Larsen, Lelong & Boiron, 1965, Rosset & Monier, 1964) and possesses a base composition rich in (G+C), (64%) (Rosset & Monier, 1964) but distinct from that of high

molecular weight rRNA.

(a) Synthesis of 5s RNA eukaryotes

The 45s ribosomal precursor molecule in eukaryotes contains 40% of its polynucleotide sequence as (G+C) rich, unmethylated non-ribosomal sequences which are lost during maturation. Since 5s RNA, a (G+C) rich, unmethylated low molecular weight rRNA species, could be found associated with nascent ribosomes in the nucleus and with the mature ribosomes in the cytoplasm (Knight & Darnell, 1967) it was originally proposed that this molecule might be derived from the 45s rRNA precursor molecule but a comparison of the kinetics of labelling of 5s RNA and 45s RNA indicated a large pool of 5s RNA molecules in the nucleus of Hela cells (Knight & Darnell, 1967). When low doses of actinomycin D are administered, 45s RNA synthesis is blocked and there is little inhibition of nuclear RNA synthesis (Perry, 1962, Penman, Vesco & Penman, 1968) but under these conditions 5s RNA synthesis is uninhibited (Burdon, Martin & Lal, 1967, Perry & Kelley, 1968, Smillie, 1970). The anucleolate mutants of Xenopus laevis, which lack all the DNA complementary to rRNA possess the DNA complementary to 5s RNA (Brown & Weber, 1968) and furthermore the DNA complementary to 5s RNA displays a density in CsCl density gradients distinct from that complementary to rRNA (Brown & Weber, 1968). Also during oogenesis in Xenopus laevis oocytes the genes for rRNA

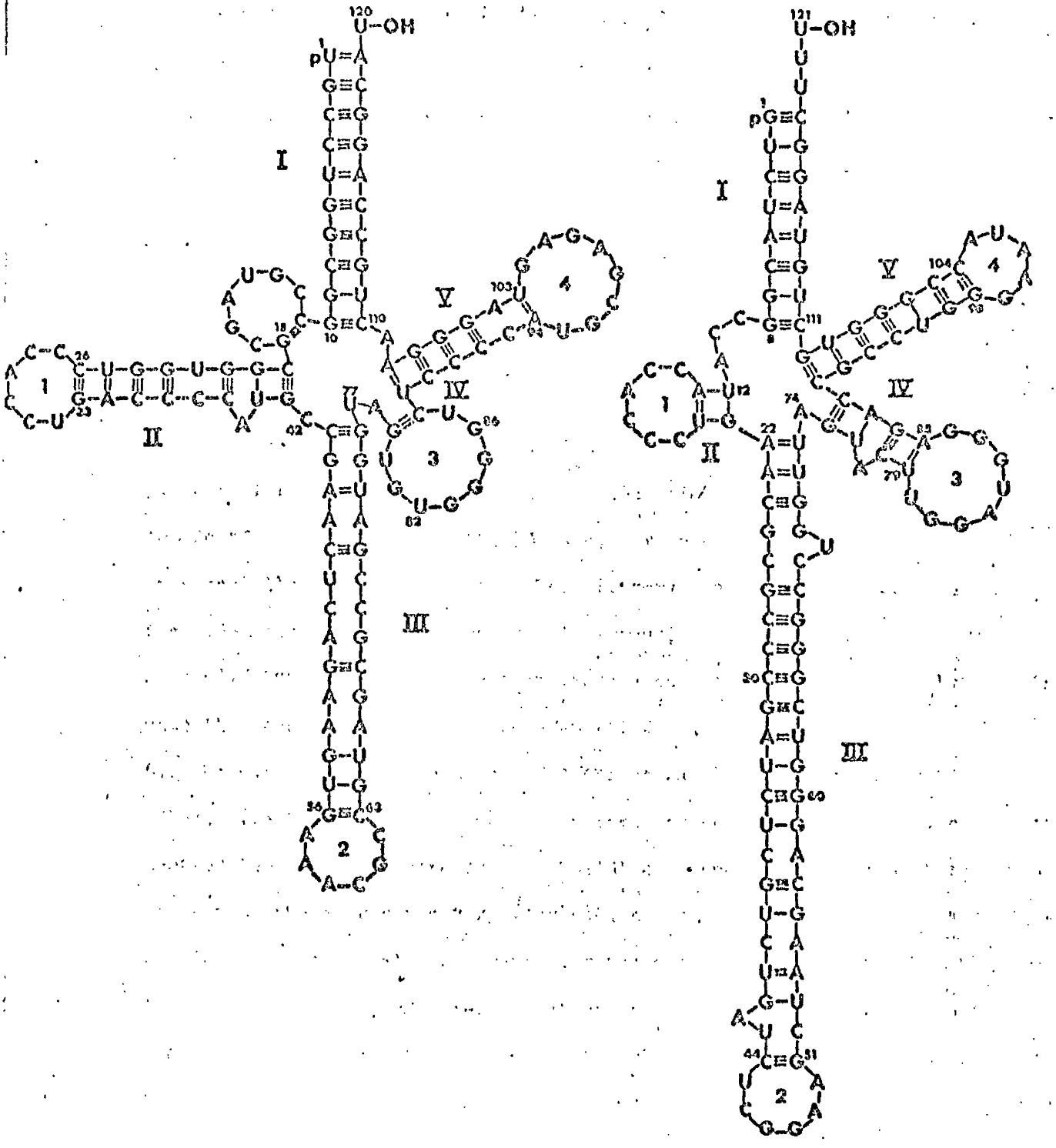
but not 5s RNA are amplified (Brown & Dawid,1968). Together these data indicate that in eukaryotes 5s RNA originates independently of the 45s r-pre-RNA and from an extranucleolar site and indeed the majority of the genes for 5s RNA in Hela cells are located on chromosomes distinct from those bearing the nucleolar organisers and the genes for 28s and 18s rRNA (Attardi & Amaldi,1970).

(b) Synthesis of 5s RNA in prokaryotes

It has been estimated that an equal number of 5s, 16s and 23s RNA molecules are synthesised in E.coli in a given time (Galibert, Lelong, Larsen & Boiron, 1967) and since there are twice as many gene loci for 16s and 23s RNA as for 5s RNA in the B.subtilis chromosome and since these loci are separated from that of 5s RNA by the genes for tRNA (Smith, et al,1968), a common precursor for 16s, 23s and 5s RNA is unlikely. Precursor molecules for 5s RNA have been suggested (Hecht, Bleyman & Woese, 1968), for some 5s RNA does appear in the absence of transcription and a pool of 5s RNA molecules is suggested by kinetics of labelling experiments. Recently Doolittle and Pace(1970) using rifampicin, (which inhibits initiation of transcription by RNA polymerase,(Wehrli, Knusel, Schmid & Staehelin, 1968) have shown that in E.coli, 5s RNA is derived from a transcription product 15-30 times the size of 5s RNA itself. Adesnik & Levinthal, (1969) also studied

the synthesis of 5s RNA in E.coli and showed that the kinetics of synthesis were essentially first order, therefore suggesting the absence of a pool of 5s precursor molecules in E.coli. These observations were supported by pulse-chase experiments using actinomycin D, but when cells were treated with chloramphenicol or starved for the amino acid methionine there accumulated 5s RNA-like molecules of lower electrophoretic mobility than 5s RNA itself. These species were not derived from the precursors to the larger rRNAs, and on restoration of methionine to the culture, or reversal of the block on protein synthesis, they matured to molecules with an electrophoretic mobility identical to that of 5s RNA from the cytoplasmic ribosomes. When protein synthesis in E.coli is inhibited by chloramphenicol, puromycin or amino acid starvation, several 5s RNA-like molecules (CM 5s RNA) accumulate (Adesnik & Levinthal, 1969, Forget & Jordan, 1970, Faunteun & Monier, 1970, Jordan, Forget & Monier, 1971). Partial sequence studies of such CM 5s RNAs reveal that they are homologous to 5s RNA but at the 5' end there are either one, two or three extra nucleotides. Normal 5s RNA has pUG at the 5' end while CM 5s RNAs have either pUUG, pUUUG, or pAUUUG (Forget & Jordan, 1970, Jordan et al, 1971). These abnormal molecules are suggested to be intermediates in the maturation pathway leading to 5s RNA and they have also been found in exponentially

Fig. I.4



Hypothetical structures for the 5s RNAs of E.coli and KB cells.
Taken from I. D. Raacke (1968).

growing E.coli cells (Jordan et al, 1970). CM 5s RNA is found free in the cytoplasm and not bound to the 50s subunits but Forget & Varichio (1970) have identified 5s RNA in the 43s ribonucleoprotein precursor particle (see above) which suggests that 5s RNA is inserted into the ribosomal structure at or close to the 43s precursor stage (Reynier & Monier, 1968) and is then rapidly reduced in size, becoming more tightly bound to the 50s subunit. The function of 5s RNA is still obscure but Siddiqui & Hosokawa (1969) have found that 50s subunits lacking 5s RNA can bind 30s subunits but their capacity to subsequently bind tRNA is considerably impaired. It is therefore unlikely that the function of 5s RNA is simply one of binding the ribosomal subunits together as was originally proposed by Rosset & Monier, (1964). Its function in binding tRNA and its ribosome binding ability are accommodated in the two dimensional cloverleaf structure proposed for 5s RNA by Raake (1968) and shown in Fig. 1.4

2.2.2.2. "7s" RNA

The major ribosomal subunits of eukaryotic cells have, in addition to 5s RNA, a low molecular weight RNA component first described by Pene, Knight & Darnell,(1968) and referred to as "7s" RNA. This RNA has been reported in a wide variety of eukaryotes (Pene et al,1968, Sy & M^{c}Carty, 1969, King & Gould, 1970, Burdon & Clarson, 1969), is 130-150 nucleotides in length,

contains no methylated nucleosides and is tightly hydrogen bonded to the 28s RNA component of the 60s ribosomal subunit (Pene et al, 1968). For this reason Weinberg & Penman (1969) who further characterised it as 5.7s RNA have referred to it as 28sA RNA (28s associated). Unlike 5s RNA, 28sA RNA appears to be derived from the 45s r-pre-RNA molecule and is associated in molar amounts not only with cytoplasmic 28s RNA but also with nucleoplasmic 28s RNA obtained from 50s ribosomal precursor particles (Pene et al, 1968). It in fact appears to be generated at the 32s to 28s cleavage point in 45s rRNA maturation. This RNA species appears to be absent from prokaryotic ribosomes but Goldstein & Harewood (1969) have isolated, from 105,000g supernatants of E.coli cytoplasm, an RNA species of identical size and base composition ($\sim$55% (G+C)) but have not characterised its synthesis or function. The sequence of this "6s" RNA has recently been established by Brownlee (1971) who has shown that this RNA possesses the same 5' terminal as some of the 5sRNA precursor molecules but that "6s" RNA is a stable molecule and a functional relationship between "6s" RNA and 5s precursor molecules is therefore unlikely. The relationship of E.coli "6s" RNA with the RNAs of higher organisms is unclear but it is however unlikely to be related to the low molecular weight rRNA called 7s RNA

for it is not found on ribosomes, nor is it released from ribosomes by treatment with hot-phenol-sodium dodecylsulphate (Brownlee,1971). Its role is not at present understood.

2.3 Transfer RNA

The expression of information, encoded in the genome and transmitted by the mechanisms embodied in the "central dogma of molecular biology" (Crick, 1958) requires a process whereby the genetic information "written" in the four letter alphabet of the nucleic acids is translated by an "adaptor mechanism" to the twenty letter alphabet of the proteins. Such an adaptor function in the process of protein synthesis is fulfilled by the low molecular weight RNA component referred to as transfer RNA (tRNA). This RNA species, first described by Hoagland, Zamecnik & Stevenson (1957), has a sedimentation coefficient of approximately 4s, a molecular weight of about 25,000 daltons and is found ubiquitously in all living systems where it constitutes 10% of the cytoplasmic RNA (about 10^8 molecules per eukaryotic cell).

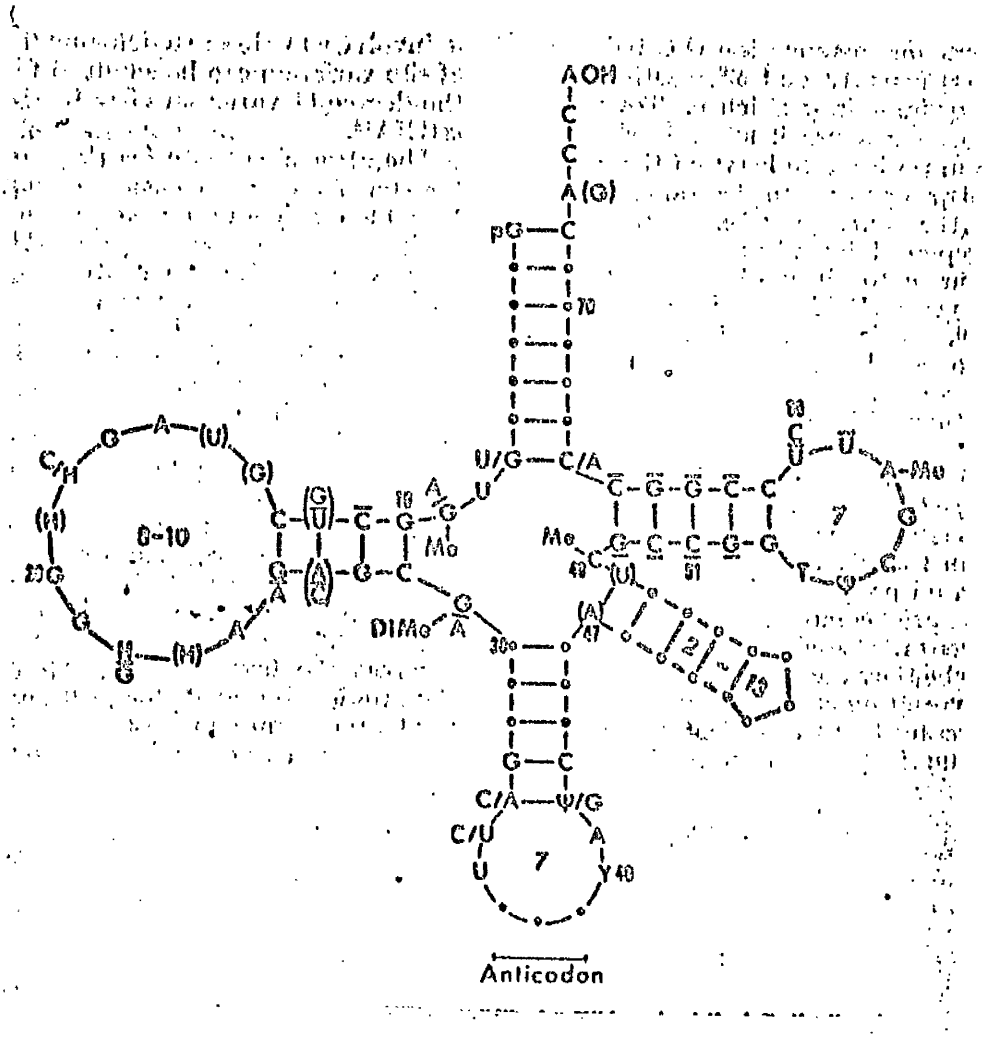
2.3.1 Structure of tRNA

The heterogeneous class of low molecular weight RNA molecules collectively referred to as transfer RNA consists of a mixture of single polynucleotide chains ranging in size from 75 to 85 nucleotides in length (Phillips, 1969). Despite the relatively small size of the nucleotide chains, tRNA has a

complicated secondary and tertiary structure and contains a high proportion of atypical bases such as methylated nucleosides, dihydrouracil, thiouracil and pseudouridine. The first complete nucleotide sequence of a tRNA molecule was published in 1965 by Holley (Holley et al, 1965) but since then many more sequences have been determined for a variety of tRNA species from yeast, bacterial and animal sources (Zachau, Dutting & Feldman, 1966, RajBhandary et al, 1967, Dube, Marcker, Clark & Cory, 1968, Cory, Marcker, Dube & Clark, 1968, Staehelin, Rogg, Baguley, Ginsberg & Wehrli, 1968). All tRNA sequences at present known can be arranged in the cloverleaf conformation (Fig.1.5) proposed by Holley (Holley et al, 1965). This two dimensional conformation allows the formation of a maximum number of hydrogen bonds between the Watson-Crick base pairs A-U and G-C (Crick, 1966). In this model three loops of unpaired, non-hydrogen bonded regions are joined by short, base paired helical arms to a stem formed by the pairing of seven bases near each end of the molecule. At the 3' end of the stem and not involved in base pairing is the $CpCpA_{OH}$ end group common to all transfer RNA molecules and essential to their function in amino acid transfer. Adjacent to the stem are located the pentanucleotide loop and the dihydrouracil loop. The pentanucleotide loop is so called because of the occurrence within its sequence of the sequence GpTpUpCpG common to almost

Generalised secondary configuration of a tRNA molecule.

Fig. I.5



Generalised stucture of tRNA. The dihydro U loop is at the left, the pentanucleotide loop at the right. The discrepancy in nucleotides in the dihydro-U loop reflects the fact that in none of the known structures do all nucleotides appear together. Nucleotides designated by a capital letter appear always in this position. Those marked with a bar and a capital letter appear most often although they might be exchanged for any other nucleotide. Nucleotides in parenthesis can, but most do not, occur in this position. Nucleotides indicated by the dots are variant. Methyl groups have been included in three positions where they are found.

Reproduced from Phillips (1969).

all tRNAs (Sanger, Brownlee & Barrell, 1965, Zamir, Holley & Marquissee, 1965) and suggested to be involved in the non amino acid specific binding site of tRNA to the larger ribosomal subunit (Zamir et al,1965; Ofengand & Henes, 1969). Between the pentanucleotide loop and the dihydrouracil loop is the anticodon arm of the molecule and this contains a loop of seven nucleotides, three of which form the anticodon and pair in a specific manner with the corresponding code triplet of the mRNA molecule. The anticodon loop in many tRNAs also contains, on the 3' side of the anticodon sequence, an alkylated purine (Fuller & Hodgson, 1967) which in tRNAs from most sources is 2'-methylthio-N^6-(Δ^2-isopentenyl)-adenosine, (Hall, 1970) and on the 5' side of the anticodon sequence, a pair of pyrimidine bases. The function of these and other modified nucleosides will be discussed later. Between the pentanucleotide loop and the anticodon arm of the tRNA molecule is located an "extra" arm of variable length, being in smaller tRNAs almost non existant, consisting of only a few nucleotides, and in the larger tRNAs ranging in size up to 13 nucleotides in a base paired formation (Phillips, 1969). The sequences of 14 tRNA molecules from various sources were compared by Phillips (1969) who found that as much as 50% of the polynucleotide sequences were invariant in tRNAs and suggested that a common structure exists for

all tRNAs, determined largely by these invariant sequences. This is supported by X-ray crystallographic data of Blake, Fresco & Langridge (1970) who have found that crystals containing multiple species of tRNA give single crystal X-ray diffraction patterns and indicates a marked level of similarity in the tertiary structure of tRNAs. Several models for the tertiary structure of tRNA have been proposed (Cantor, Jaskunas & Tinoco, 1966, Lake & Beeman, 1968, Cramer, Doepner, Von der Haar, Schlimme & Seidel, 1968, Fuller & Hodgson, 1967, Doctor, Fuller & Webb, 1969, Froholm & Olsen, 1969, Melcher, 1969, Abraham, 1971) and the general model emerging from these various proposals is one of a hairpin structure with the dihydrouracil and the pentanucleotide loop folded up and the amino acid arm protruding (see Cramer, 1971). It is however apparent that a detailed model of the tertiary structure of tRNA must await the results of X-ray diffraction studies with suitable crystals of tRNA.

2.3.2. Biogenesis of tRNA

In addition to its key role in the synthesis of proteins, tRNA has been suggested, by a number of workers, to be of key importance in the regulation of cell metabolism at both a transcriptional and a translational level (Ames & Martin, 1964, Stent, 1964, Taylor, Buck, Granger & Holland, 1967, Sueoka & Kano-Sueoka, 1970). It is apparent

therefore that qualitative or quantitative differences in the available species of tRNA could alter the rate or quality of translation of genetic information and thus influence profoundly cell differentiation and function. Consequently it is therefore of utmost importance to understand the molecular processes involved in the biogenesis of such a key molecule.

There is now a considerable amount of experimental evidence that, like the RNA of the ribosomes, tRNA molecules are derived in both eukaryotic and prokaryotic cells by a sequence of maturation events involving secondary modification of an original gene transcription product. The existence of an unstable, rapidly labelled, methyl deficient low molecular weight precursor to tRNA has recently been demonstrated in a number of mammalian systems (Burdon & Lal, 1967, Lal & Burdon, 1967, Perry & Kelley, 1968, Bernhardt & Darnell, 1969, Moshowitz & Darnell, 1969, Kay & Cooper, 1969, Smillie, 1970), in insects, (Sirlin & Loening, 1968, Egyhazi, Daneholt, Edstrom, Lambert & Ringborg, 1969) and most recently in E.coli (Altman, 1971).

(1) tRNA biogenesis in eukaryotes

The chromosomal non nucleolar origin of tRNA is suggested by the studies with anucleolate mutants of the clawed toad Xenopus laevis (Brown & Gurdon, 1964), by autoradiography

(Woods & Zubay, 1968) by hybridisation experiments (Ritossa, Atwood & Spiegelman, 1966) and by actinomycin D inhibition experiments (Perry & Kelley, 1968). Exposure of eukaryotic cells for varying lengths of time to [^{3}H]-uridine and L-[^{14}C-methyl]-methionine indicated that although the radioactivity was initially confined to the nucleus, at short time periods there appeared in the cytoplasm a class of polydisperse low molecular weight methylated RNA possessing features compatible with tRNA (Burdon, et al, 1967, Lal & Burdon, 1967, Darnell, 1968). The kinetics of labelling of this class of RNA molecules was consistent with its role as a precursor of tRNA (Burdon et al, 1967) although they appear to be deficient in methyl groups in comparison to tRNA itself. Pulse-chase experiments utilising actinomycin D have indicated that these RNA species are indeed precursors to tRNA, for radioactivity, initially present in this polydisperse RNA fraction after short time pulses, was shown to subsequently and quantitatively mature to species of RNA possessing characteristics of tRNA (Burdon et al, 1967, Bernhardt & Darnell, 1969, Kay & Cooper, 1969). Furthermore since these RNA species (referred to as "pre-tRNA" eluted in Sephadex G100 columns and migrated in gels of poly-acrylamide in a position intermediate between that of 5s RNA and tRNA it was suggested that they possessed a greater

molecular length than tRNA itself (Burdon et al, 1967, Bernhardt & Darnell, 1969). Chromatographic examination, on columns of Sephadex G100, of "pre-tRNA" under conditions where contributions of secondary structure were eliminated, (Boedtker, 1967, 1968) revealed this to be that case and the "pre-tRNA" was longer than tRNA by an average of about 30 nucleotides (Burdon & Clason, 1969). Precursor molecules to tRNA appear in the cytoplasm of eukaryotic cells within minutes of their transcription from nuclear genes (Burdon et al, 1967, Lal & Burdon, 1967, Burdon & Clason, 1969) and then undergo certain maturation processes in the cytoplasm, during which the extra sequences are removed and methylation and minor nucleoside alterations occur and are completed (Burdon et al, 1967, Bernhardt & Darnell, 1969). In agreement with this finding it was demonstrated that the bulk of the tRNA-methylase enzymes are located in the cytoplasmic fraction (Burdon et al, 1967). Investigations of the intracellular location of tRNA precursors showed that although "pre-tRNA" molecules were found in the soluble portion of the cytoplasm rather than associated with mitochondrial of microsomal components, there was no obvious intimate association of the precursor molecules with any soluble cell sap proteins (Burdon & Clason, 1969), nor was there any indication of their accumulation in the nucleus (Burdon & Clason, 1969, Weinberg & Penman, 1969).

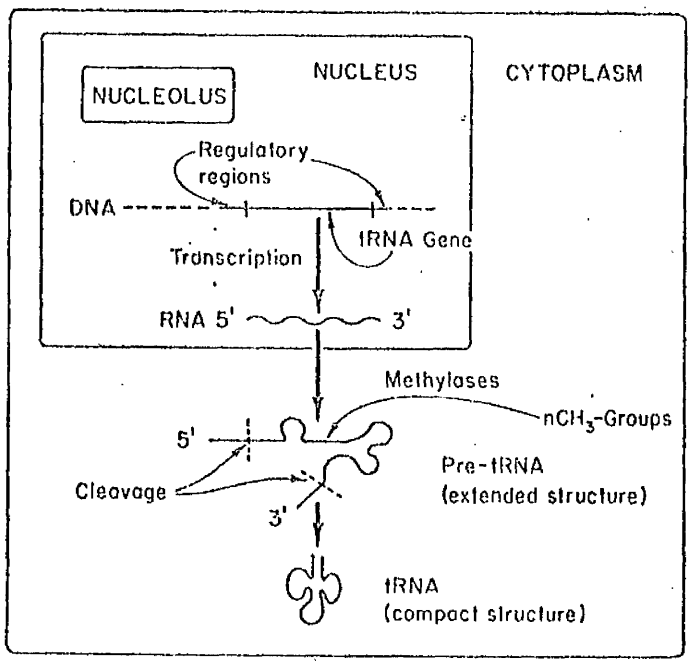
However, Egyhazi et al, (1969) by isolation of nuclei by a microsurgical procedure rather than by an aqueous cell fractionation technique, have indicated that tRNA precursors may be found in insect nuclei. This may reflect a species difference in the nuclear location of tRNA cistrons for Sirlin & Loening (1968) and Sirlin, Jacob & Birnstiel (1966) have indicated the possible nucleolar location of tRNA cistrons in insect cells. The nature and location of the "extra" sequences in "pre-tRNA" have been investigated by Smillie, (1970) who found that in the "pre-tRNA" of hamster fibroblasts (BHK-21/C13) the "extra" sequences were rich in pyrimidines and most were located at the 5' end of the molecule although some were possibly at the 3' end. It was further observed that "pre-tRNA" possessed different 3' and 5' terminal sequences from the mature tRNA. In addition Smillie & Burdon, (1970) have prepared from these hamster fibroblasts a cell-free enzyme preparation capable of at least partially trimming "pre-tRNA" to a tRNA-like material, apparently by an exonuclease action, for no oligonucleotides were detectably released. The 4s product of this "trimming enzyme" preparation is still however deficient in methylated nucleotides and pseudouridine and presumably represents a biologically non functional molecule since it has been reported that methylated nucleosides are essential to the interaction of

tRNAs with the appropriate amino acid activating enzymes (Shugart, Chastain, Novelli & Stulberg, 1968). It would therefore appear that methylation is not a prerequisite for tRNA maturation. At present it is uncertain whether the extra sequences in tRNA precursors are identical for all tRNA species and possibly represent a common mechanism for the control of their transcription or whether heterogeneity in these sequences exists.

2.3.2. tRNA biogenesis in prokaryotes

A similar process of tRNA maturation in prokaryotes is suggested by the isolation of tyrosine suppressor tRNA precursor molecules from E.coli by Altman (1971). These molecules are rapidly labelled, short-lived intermediates in a process of maturation leading to mature suppressor tRNA. Pulse labelling experiments and analysis by two dimensional fingerprinting indicate that they are indeed precursors to tyrosine suppressor tRNA. It appears that they lack the 3' end group $CpCpA_{OH}$ but are longer than the mature species by some 30-40 nucleotides. Their existence has however so far only been detected in bacteriophage infected cells. In summary therfore, molecules of transfer RNA are probably produced by a series of maturation events from an original gene transcription product of tRNA cistrons. Nucleotide modifications occuring during this maturation process take

Fig.I.6



A scheme showing the possible steps involved in the biogenesis of mammalian cell tRNA and their intracellular location.

Reproduced from Burdon (1971a).

place upon an essentially unmodified precursor molecule and there is suggestive evidence that the common 3' $C_pC_pA_{OH}$ end group is added to the precursor molecule during maturation, for this sequence seems to be absent from the tRNA cistrons (Daniel, Sarid & Littauer, 1970) and there are enzymes in the cytoplasm which can bring about its addition (Canellakis & Herbert, 1960). The possibility that "pre-tRNA" itself is derived from a macromolecular precursor component in the nucleus of eukaryotic cells cannot at present be excluded. A scheme showing the possible steps involved in tRNA biogenesis is shown in Fig 1.6.

2.3.3. Content of modified nucleosides and their function

Transfer RNA is characterised by it high content of atypical bases and minor nucleosides and it is generally recognised that these modified nucleosides confer upon tRNA molecules properties which in many cases are essential to their biological function. Such minor nucleotides are found in definite locations within the primary sequences of the tRNAs and have recently been reviewed by Hall (1970(a)). The extent of modification varies greatly, ranging from a relatively simple replacement of functional group (OH with S in thiouridine e.g.), to the hypermodified nucleosides resulting from more complex alterations. Although the simpler modifications do not greatly alter the

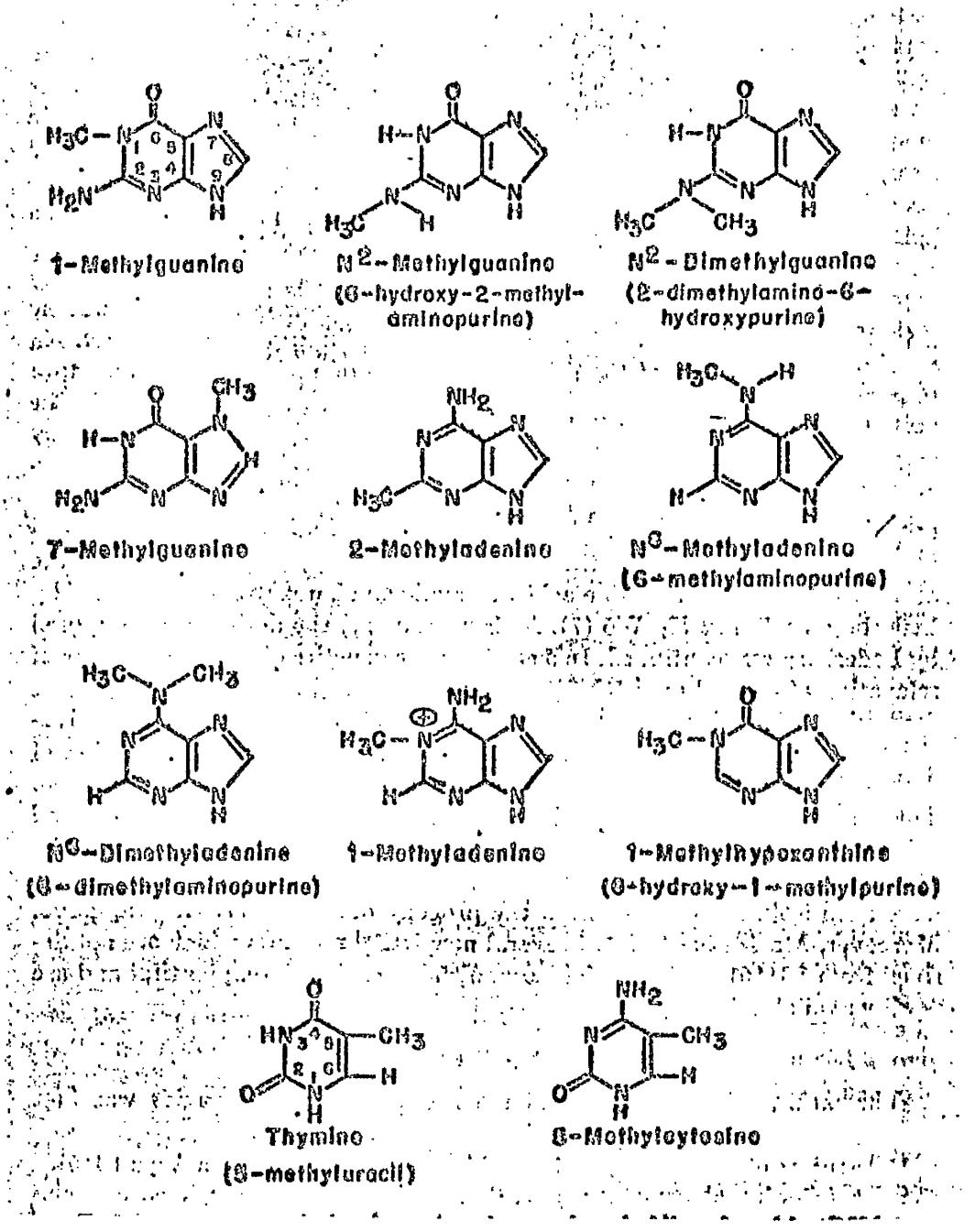
structure of the nucleoside affected, they can give rise to changes in the hydrogen bonding base stacking and covalent characteristics of the tRNA molecule and consequently affect its biological function (Hall, 1970(b)).

2.3.3.1 Hypermodified nucleosides

The anticodon loop of many tRNA molecules possesses, at the 3' end of the anticodon sequence, a hypermodified nucleoside which in most tRNAs is a modified $N^6(\Delta^2$-isopentenyl)-adenosine residue (Hall, 1970(b)). Nishimura, Yamada & Ishikura (1969) have demonstrated its presence in many tRNAs from E.coli and suggest that it is involved in the recognition of codons starting with uridine. It is apparently essential for correct codon recognition (Thiebe & Zachau, 1968) and Fuller & Hodgson (1967) suggest that since there are seven nucleotides in the anticodon loop, the alkylated purine at the 3' end of the anticodon sequence provides a punctuation mark" to ensure the correct alignment of the anticodon and mRNA sequence. Experimental evidence in support of this hypothesis is obtained from the work of Gefter & Russell (1969) and Thiebe & Zachau (1968) who indicate that the base modification is essential for ribosome binding and consequently for participation in protein synthesis. It is however apparently non essential for amino acyl synthetase recognition for not all tRNAs contain the base (Nishimura et al,

Structures of methylated nucleosides commonly found the RNA components of eukaryote and bacterial cells

Fig.I.7



Reproduced from Borek & Srinivasan (1966)

1969) and one synthetase recognises several serine tRNAs (Sundharadas, Datgi, Soll, Konigsberg & Lengyel, 1968) and several leucine tRNAs (Kan & Sueoka, 1971). The modification arises by an enzymic maturation event (Kline, Fattler & Hall, 1969) upon preformed tRNA precursors in the cytoplasm (Burdon, 1971(a)).

2.3.3.2 Methylated nucleosides

Methylation of nucleic acid bases provides the largest single group of molecular modifications and in tRNA the net methylation of unfractionated tRNA is between 2.5% and 7%, most of the methyl groups being present on the bases and only 10-20% of the total methyl groups occurring as 2'-O-methylribose. (Tamaoki & Lane, 1969). The major methylated derivatives commonly found in tRNA (Iwanami & Brown, 1968) are shown in Fig.1.7. Although there is a general heterogeneity in the base composition of tRNA from various sources, heterologous tRNA from yeast and rat liver have the same 6 oligonucleotide sequences surrounding 1-methyladenine and it is therefore possible that the methylated bases may be found in common locations in a number of tRNAs where a common function is required. The precise biological function of the methylated derivatives in tRNA is not understood but it is unlikely that they serve a mere protective role, for no detectable difference in nuclease sensitivity has been found in normal and methyl

deficient tRNAs (Borek & Srinivasan, 1966). Methyl group deficiency leads to restrictions in amino acid acceptance (Shugart et al, 1968, Peterkofsky, 1964), to miscoding effects (Revel & Littauer,1965,1966, Capra & Peterkofsky,1968) and the involvement of methylated derivatives in codon anticodon recognition is indicated by the presence of these bases in the anticodons of several bacterial tRNAs (Phillips,1969, Capra & Peterkofsky, 1968). Methyl derivatives are also suggested to be tertiary structure determinants in tRNA (Borek & Christman, 1968, Ludlum, Warner & Wahba,1964, Brahms & Sadron, 1966).

2.3.3.3 Thionucleotides, Inosine, Pseudouridine and Dihydrouracil.

tRNA from bacterial and animal sources contains thionucleotide derivatives (Elicieri,1970, Carbon, David,& Studier, 1968) which in bacteria appear to be principally 4-thiouracil, 2-thiocytosine and 5-methylaminomethyl-2-thiouracil. In addition inosine residues occur in a number of tRNA molecules and are implicated in codon recognition (Staehelin & Fol, 1969, Yoshida, Furiuchi, Kaziro & Ukita,1968) and all tRNAs so far examined have dihydrouracil residues in their base sequences (Phillips, 1969). Pseudouridine is a constituent of all tRNAs in the common pentanucleotide sequence TpCpΨpCpG and suggested to be involved in ribosome binding (Zamir et al, 1965 Ofengand & Henes, 1969), and in tertiary structure maintenance

(Millar, 1969, Yoshida et al, 1969). The precise function of these and other modified nucleosides however remains to be determined.

2.4. Messenger RNA

The DNA which comprises the hereditary material of both eukaryotic and prokaryotic cells can be divided into regions (genes) which code for proteins (structural genes), those which are transcribed into RNA but not translated into protein (eg. rRNA or tRNA cistrons) and those whose functional significance does not require that they are transcribed (operator, promotor or regulator genes). Growth and protein synthesis is accompanied, largely by the transcription of structural genes, by a flux of RNA molecules from the sites of transcription in the genome to the sites of translation in the cytoplasm. The mediator in this transfer of genetic information from DNA to protein is called "messenger RNA" (mRNA). The concept of messenger RNA was formally derived by Jacob & Monod (1961) investigating the synthesis of inducible enzymes in bacteria. The nature of the synthesis of these proteins, combined with the genetic evidence, suggested that information issuing from the genome was contained in rapidly labelled, short-lived unstable structures. Further investigations showed these molecules to possess a base composition reflecting that of

the cellular DNA (Astrachan & Fischer, 1961, Bolton & McCarthy, 1962, Midgely & McCarthy, 1962), to be associated rapidly with protein synthesising structures called polyribosomes. (Warner, Knopf & Rich, 1963, Penman, Scherrer, Becker & Darnell, 1963, Risenborough, Tissieres & Watson, 1962), to be active in the stimulation of amino acid incorporation (Willson & Gross, 1964, Bautz, 1963(a), Arnstein, Cox & Hunt, 1964, Kruh, Dreyfus & Schapiro, 1964, Kruh, Schapiro, Lareau & Dreyfus, 1964) and to rapidly hybridise to homologous DNA (Bautz, 1963(b)). Bacterial mRNAs were shown to possess extremely short half-lives of the order of a few minutes (Jacob & Monod, 1961, Britten & Roberts, 1960, Willson & Gross, 1964, Higa & Levinthal, 1964, Kepes, 1963, Hartwell & Magasanik, 1963) whilst eukaryotic cells displayed considerable diversity in the stabilities of their mRNAs, ranging from a few hours to several days and exhibiting both stable and unstable populations (Bloom, Goldberg & Green, 1965, Drysdale & Munro, 1965, Bekhor, Mohseni, Nimmi & Bavetta, 1965, Trakatellis, Axelrod & Montijar, 1964). Messenger RNA has been shown to contribute only a few per cent of the total RNA content of cells (Brenner, Jacob & Meselson, 1961, Gross, Hiatt, Gilbert, Risenborough & Watson, 1961) and as yet no detectable modification of its constituent bases

has been reported and Moore (1966) has confirmed that E.coli mRNA certainly contains no methylated nucleosides. Both in vitro and in vivo transcriptional studies have led to the conclusion that mRNA is the transcription product of only one strand of the DNA duplex. (Hayashi, Hayashi & Spiegelman, 1964, Geiduschek, Tochini-Valentini & Sarnat, 1964, Aloni & Attardi, 1971). Although the functional properties of mRNA in both eukaryotic and prokaryotic cells appear to be identical, the different structural organisation of these cell types allows possible differences in the mode of transport and utilisation of mRNAs and this point is worth discussion.

(1) Eukaryotic cells

In higher organisms, where the sites of protein synthesis are separated from the sites of mRNA synthesis not only by longer distances than in bacteria but also by a nuclear membrane, the risk of degradation of unprotected mRNA molecules before they become associated with ribosomes or polysomes is high. The mechanism of transfer of genetic information from the nucleus where mRNA is transcribed to the cytoplasm where it is translated is still obscure but the recent evidence suggests that, in eukaryotic cells, newly synthesised mRNA molecules are transferred to the cytoplasm not as naked strands of RNA but in the form of ribonucleoprotein

associates. Two different interpretations have been placed upon the nature of these ribonucleoproteins. One, based largely upon observations with mammalian cells, is that mRNA is transferred in association with the smaller ribosomal subunit. (Joklick & Becker, 1965, Kohler & Arends, 1968, Latham & Darnell, 1965, Henshaw, Revel & Hiatt, 1965, McConkey & Hopkins, 1965). The other, largely from studies with loach or sea urchin embryos, suggests that mRNA is associated with ribonucleoprotein particles distinct from ribosomal subunits (Spirin, Belitsna & Ajtkhozhin, 1964, Spirin & Nemer, 1965, Infante & Nemer, 1968, Samarina, Lukanidin, Molnar & Georgiev, 1968, Samarina, Krichevskaya & Georgiev, 1966). Such mRNA bearing particles have been observed in both cytoplasmic (Spirin, 1969), and nuclear fractions (Samarina et al, 1968, 1968, Cartouzou, Poiree & Lissitzky, 1969, Moule & Chauveau, 1968) of mammalian cells and have been termed "informosomes" by Spirin (1964). They are found both free in the cytoplasm or bound to polysomes (Spirin, 1969, Lee & Brawerman, 1971, Spohr, Granboulan, Morel & Scherrer, 1970) and have a heterogeneous distribution, but a constant RNA/protein ratio independent of both the size of the particle and the cell type from which it is isolated (Spirin, 1969). Informosome particles contain approximately

80% protein and have a buoyant density distribution in CsCl centred around a value of 1.40g/cm^3. In sucrose gradients the particles sediment with coefficients from 30s to 120s, each class of "informosomes" containing a characteristic size of mRNA. Samarina et al, (1968) have examined the structural organisation of nuclear informosomes in rat liver and have found them to contain specific globular proteins of the nucleus and referred to by them as "informofers". Similar observations on the "informosomes" released from rat liver nuclei, by incubation with ATP, have been made by Ishikawa, Kuroda, Ueki & Ogata (1969, 1970 (a)(b)) who suggest that the protein component of these d-RNA bearing particles is less basic than the basic proteins of ribosomes or histones. In L cells Perry & Kelley (1968) have identified ribonucleoproteins released from polyribosomes by EDTA treatment and which are similar, with respect to buoyant density distribution, RNA/protein ratio and mRNA content, to "informosomes" found free in the cytoplasm. The functions of such "informofers" or protein components of the ribonucleoprotein complexes have been suggested to be protective (McConkey & Hopkins, 1965), a ribosome recognition signal (Henshaw, 1968) or a means of control at the translational level (Spirin, 1966). Recently Lee & Brawerman, (1971) have

shown that informosome particles accumulating in amino acid starved cells are precursors of polysomal mRNA with a rate limiting step being the addition of a cytoplasmic protein for the initation of polysome formation. Bach & Johnson (1966) however proposed that mRNA enters polysomes at or near the nuclear membrane and Spirin (1969) has therefore suggested that polysome bound "informosomes" represent readily translatable messages whilst those found free in the cytoplasm are examples of messages which are temporarily masked by "informofers". Such a suggestion offers some explanation of the observations that at certain periods of embryonic development and cell differentiation the synthesised mRNA is translated not immediately but after a significant delay (Spirin, 1966, 1969) and indeed Cartouzou et al, (1969) found the "free" informosomes of sheep thyroid cytoplasm to be inactive in protein synthesis but found their constituent d-RNA to be active. The size heterogeneity of the "informosomes" (30s to 120s) and their included dRNA (8s to 30s) suggests the possibility of polycistronic messengers, in keeping with the observations from a variety of virus infected systems (Nathans, Natani, Schwartz & Zinder, 1962, Nathans, 1965, Ames & Martin, 1964) but the experiments of Kuff & Roberts (1967) suggest that, at least in mammalian cells, mRNA is monocistronic.

2. Prokaryotic cells

To date "informosome-like" particles have not been reported in prokaryotic cells and although the structural organisation of the eukaryotes is absent and the migratory distances from the sites of transcription to the sites of translation are shorter in bacterial cells, it is apparent that some mechanism for the protection of nascent mRNA molecules against nuclease degradation must also exist in bacterial cells In prokaryotic cells the genetic material exists in locations in the cytoplasm proximal to the sites of mRNA translation, and Stent (1967) has proposed a model whereby mRNA is removed from the template DNA by ribosomes. The *in vitro* experiments of Byrne, Levin, Bladen & Nirenberg (1964) and Bladen, Byrne, Levin & Nirenberg (1965) have demonstrated the attachment of ribosomal particles to mRNA whilst the RNA is still attached to the DNA template. The plausibility of this model as a scheme for mRNA transport is given further support by the observations of Shin & Moldave (1966) and Ravel, Herzberg, Becarevic & Gros (1968) that *in vitro* transcription by RNA polymerase is stimulated by the addition of ribosomes. In addition, ribosomes of prokaryotic cells have been shown to be subject to an obligatory cycle, being dissociated to subunits when released from mRNA chains and reassociating to 70s particles when

attached to the initiation site for protein synthesis on a mRNA molecule (Mangiarotti & Schlessinger, 1966, 1967, Kaempfer, Meselson & Raskas, 1968). Forchammer and Kjelgaard (1968) suggest that it is the 30s ribosomal subunit rather than the 70s ribosomes which may be involved in the transport of mRNA and their data, obtained with E.coli mutants deficient in 50s ribosomal subunits, are given further support by the observation that 30s subunits carry the site for mRNA attachment (Takanami & Okamoto, 1963, Raskas & Staehelin, 1967) and are involved in the initiation of protein synthesis (Nomura & Lowry, 1967, Nomura, Lowry & Guthrie, 1967). These results therefore indicate that, in prokaryotic cells, the means of mRNA transport is probably the ribosome itself or its constituent 30s subunit.

2.5. Nuclear RNA

Eukaryotic cells are characterised by the location of their genetic material within a defined organelle, the nucleus, and recent improvements in cell fractionation techniques have allowed the identification of distinct classes of RNA molecules restricted to the nucleus.

2.5.1. Heterogeneous Nuclear RNA (HnRNA)

When eukaryotic cells are exposed to radioactive RNA precursors for short time periods, radioactivity first

appears in two classes of high molecular weight RNA associated with the nucleus. The first, referred to as "r-pre-RNA" or ribosomal precursor RNA, whose metabolism has been discussed in section 2.2.1.1., is the product of ribosomal genes and has a sedimentation coefficient of 45s, with a molecular weight of 4-4.5 x 10^6 daltons (Scherrer et al, 1964, Weinberg & Penman, 1970). Coincident with or shortly after its synthesis, this molecule is methylated and becomes associated with nuclear proteins to form a ribonucleoprotein complex within which a process of maturation, involving specific enzyme cleavage of the RNA moiety, occurs and gives rise eventually to mature ribosomal subunits in the cytoplasm. The second class of rapidly labelled nuclear RNA, characterised by its rapid turnover and heterogeneous sedimentation in sucrose gradients, is referred to as heterogeneous nuclear RNA (HnRNA). This class of RNA molecules, which in short time pulses may comprise as much as 50% of the rapidly labelled cellular RNA (Warner et al, 1966(b), Yoshikawa-Fukada, Fukada & Kawade, 1965) has been identified in a number of mammalian cells (Warner et al, 1966(a)(b), Soiero et al, 1966, Willems, Musilova & Malt, 1969, Stevenin, Mandel & Jacob, 1969, Shearer & McCarthy, 1967) in insects (Edstrom & Daneholt, 1967, Sirlin & Loening, 1968)

in amphibians (Brown & Gurdon, 1966, Loening, 1969) and also in plants (Retjel & Planta, 1968) but as yet has not been found in bacteria. The free RNA itself has sedimentation coefficients ranging from about 30s to 100s (Warner et al, 1966, Soiero et al, 1966, Attarki et al, 1966, Scherrer et al, 1966) but is found in the nucleoplasm in the form of ribonucleoprotein particulates with sedimentation coefficients ranging up to 5000s (Penman, Vesco & Penman, 1968). This heterodispersity reflects a true range of high molecular weights in this class of RNA molecules of from about 2×10^6 to 10^7 daltons (Attardi et al, 1966) for these values are unaffected by treatments which disrupt non specific binding or aggregation (Lindberg & Darnell, 1970) and electron micrograph examinations of isolated heterogeneous nuclear RNA molecules reveal chains 7-8 microns in length and corresponding to molecular weights of $7-8 \times 10^6$ daltons (Scherrer, Marcaud, Zajdela, London & Gros, 1966). In addition to heterogeneity of size they show heterogeneity of base composition (Shearer & McCarthy, 1970) but their overall base composition reflects that of the cellular DNA with a (G C) content of 42-47% (Soiero et al, 1966, Attardi et al, 1966, Loening, 1969, Brown & Gurdon, 1966, Scherrer et al, 1966, Houssais & Attardi, 1966). HnRNA is synthesised

in the nucleus in a location distinct from the nucleolus (Soiero et al, 1966, Attardi et al, 1966) and is restricted to the nucleus (Shearer & McCarthy, 1967) where 80-90% of it turns over with a half-life of 30-60 mins, being degraded to acid soluble fragments without leaving the nucleus (Shearer & Marcaud, 1965, Scherrer et al, 1966, Burns, Fisher & Lowy, 1965, Soiero et al. 1968). Presaturation hybridisation experiments (Soiero & Darnell, 1969) reveal that in Hela cells, HnRNA is complementary to about 5% of the genome. These complementary sequences contain a fraction which rapidly hybridises to homologous DNA, possibly representing reiterated sequences or sequences containing a fair degree of sequence homology, and a fraction which is only partially hybridised even after "exhaustive" hybridisation (Pagoulatos & Darnell, 1970(a). The rapidly hybridising sequences appear to be equally distributed among the chromosomes (Pagoulatos & Darnell, 1970(a)). A comparison of HnRNA during the various periods of the cell growth cycle (Pagoulatos & Darnell, 1970(b)) reveals that HnRNA synthesis is not restricted to any phase of the cell cycle (G_1, S or G_2) and some molecules were equivalent at all times but small significant differences in the classes of HnRNA at various periods may exist but are undetectable by the presently available techniques.

2.5.1.1 Heterogeneous nuclear RNA - its relationship to messenger RNA.

From experiments with duck erythrocytes (Scherrer et al, 1966, Attardi et al, 1966), Hela and L cells (Soiero et al, 1968, Shearer & McCarthy, 1967) it is clear that the vast majority of HnRNA cannot represent messenger RNA sequences since approximately 90% of it is degraded to acid soluble fragments in the nucleus. However its characteristics of rapidity of labelling, metabolic instability and DNA-like base composition suggest the possibility that a minority of these large molecules might serve as precursors to cytoplasmic mRNA. Examination of cytoplasmic extracts obtained from cells labelled for short time periods reveals two classes of rapidly labelled heterogeneous RNA in the cytoplasm (Penman et al,1968, Warner et al,1966). The first class of RNA molecules is found associated with polysomes, is released from these by treatment with EDTA (Darnell, 1968), is assumed to represent mRNA and has a heterogeneous distribution on sucrose gradients with s values ranging from 8 - 30s. However a comparison of the kinetics of labelling of this class of RNA molecules with that of HnRNA suggests that no precursor product relationship exists between polysome associated RNA and HnRNA (Penman et al, 1968). The second class of cytoplasmic heterogeneous

RNA (10s-70s) cosediments with the polysomes but is not apparently associated with them or related to mRNA, and forms a contaminating fraction which may be an isolation artifact as a result of leakage of HnRNA from the nucleus. Competition hybridisation of polysome associated mRNA and HnRNA with homologous DNA reveals considerable sequence homology in the two fractions (Aaron & Giorgiev, 1967, Birnboim et al, 1967, Soiero & Darnell, 1970) and hybridisation experiments (Stevenin et al, 1969) have indicated that, in rat brain, 65% of the total dRNA is found on microsomes although no nuclear specific d-RNA species with sedimentation characteristics of microsomal RNA was detected. Similar observations have been made by Aaronson & Wilt, (1969) in sea urchin embryos, thus suggesting specific limited cleavage of nuclear d-RNA. The most convincing evidence for the possible precursor relationship of HnRNA to mRNA is provided by the experiments of Lindberg & Darnell, 1970) in SV 40 transformed cells. SV 40 transformed cells contain SV40 DNA integrated into the cellular genome, and produce virus specific RNA. HnRNA isolated from SV 40 transformed cells contains virus specific sequences in RNA molecules considerably larger than the presumed viral mRNA molecules isolated from cytoplasmic polysomes. Again these data suggest that mRNA

is derived from HnRNA by limited specific cleavage. The apparently wasteful synthesis of large amounts of RNA which is destroyed during maturation of nuclear RNA species subsequent to enzyme cleavage has precedent in the case of ribosome maturation as discussed in section 2.2.1.1.. Evidence from hybridisation experiments (Birnbiom et al, 1967, Soiero & Darnell, 1970) has confirmed the hypothesis of Brown & Gurdon (1966) that at least some sequences of HnRNA may be converted to stable mRNA species. Recently Niessing & Sekeris (1970) have identified an endonuclease activity, associated with the 50s - 80s d-RNA containing ribonucleoprotein particles of rat liver nuclei, which specifically cleaves high molecular weight DNA-like RNA (30s) to a size comparable with that of mRNA extracted from cytoplasmic polysomes (10-18s). This cleavage takes place within the 50-80s ribonucleoprotein complex. In parallel with these observations, Willems et al, (1969) have shown that after unilateral nephrectomy, compensatory growth of the remaining kidney is accompanied by a faster rate of processing of the HnRNA prior to an increase in cytoplasmic RNA. In contrast to these observations however, Penman, Rosbash & Penman (1970), on the basis of the differential response of HnRNA and mRNA synthesis to the adenosine analogue, cordycepin, suggest that HnRNA and

mRNA are transcribed by two distinct nuclear located RNA polymerases and that they therefore cannot be related. Other functions ascribed to HnRNA fall largely into the concept of regulation and will be discussed in section 3.

2.5.2 Chromosomal RNA

Chromosomal proteins obtained, by dissociation by salt of nucleohistone preparations, from pea bud, pea cotyledon, calf thymus, rat liver, rat ascites cells and chick embryos have been shown to contain a covalently associated special class of low molecular weight RNA molecules referred to as "chromosomal RNA" (Huang & Bonner, 1965, Bonner & Widholm, 1967, Shih & Bonner, 1969, Dahmus & McConnell, 1969). This class of RNA molecules has a homogeneous size distribution of about 3.3 to 3.8s, corresponding to a molecular length of about 40-50 nucleotides (Huang & Bonner, 1965). It possesses considerable sequence heterogeneity and hybridises to a relatively large portion of the genome (2-4%) and is characterised by its high content of dihydrouracil or dihydrothymidine (9 moles per cent). It is apparently distinct from tRNA or mRNA and studies of dissociation and reassociation of chromatin suggest that chromosomal RNA may play a key role in gene regulation by conferring specificity on the DNA-chromosomal protein interaction (Bonner & Huang, 1966, Bonner & Widholm, 1967, Bekhor,

Kung & Bonner, 1969, Huang & Huang, 1969). A recent reexamination of chromosomal RNA in calf thymus (Heyden & Zachau, 1971) however suggests that it is at least partially composed of fragments of transfer RNA linked by peptide bond to nucleohistone, but no competition hybridisation between fractions is demonstrated and these authors do not exclude the possibility that chromatin specific RNA does exist in calf thymus.

2.5.3 Low molecular weight monodisperse RNAs of the nucleus.

The development of acrylamide gel electrophoresis for the separation of RNA molecules (Loening, 1967) has revealed that in addition to the high molecular weight fractions discussed in sections 2.2.1.1 and 2.5.1., the nuclei of eukaryotic cells contain a class of low molecular weight (5-10s) monodisperse RNA molecules (Prestayko & Busch, 1968 (a)(b), Weinberg & Penman, 1968, 1969). These RNA molecules range in size from 100 to 200 nucleotides in length, have a high content of (G+C) (47-54%), appear to be restricted to the nucleus, are metabolically stable and most are highly methylated (Weinberg & Penman, 1968, 1969, Zapisek, Saponara & Enger, 1968). They are found in birds, mammals and amphibians (Rein & Penman, 1969) and are distributed between the nucleoplasmic and nucleolar fractions.

They are contained within ribonucleoprotein complexes ranging in size from 30s to 700s (Weinberg & Penman, 1969) and the nucleoplasmic species are less tightly bound to the chromatin than heterogeneous nuclear RNA. They are unrelated to HnRNA and are metabolically stable, many being as metabolically stable as the cytoplasmic ribosomes. They survive mitosis, during which they are found in particles whose size range from 30-180s, with different RNAs being distributed over this particle size range (Rein, 1971). In Hela cells their synthesis is strongly affected by drugs and inhibitors which inhibit nucleolar synthesis and does not appear to be coordinated with the synthesis of DNA. As yet their function is unknown.

2.6 Mitochondrial RNA (mit-RNA)

The mitochondria of eukaryotic cells are semiautonomous organelles possessing their own distinctive genetic material and capable of synthesising proteins by a system which resembles that of bacteria in its response to a variety of metabolic inhibitors (Roodyn & Wilkie, 1968). Mitochondrial DNAs from a variety of sources show a close distribution of buoyant densities in CsCl (Rabinowitz & Swift, 1970) and have been identified as closed circular, double stranded molecules of an overall length of about 5μ (Borst, Van Bruggen, Ruttenberg & Kroon, 1967) which therefore possess

a limited coding ability (Rabinowitz & Swift, 1970). Despite their partial dependence upon nuclear genes, mitochondria possess their own RNA polymerase (Kalf, 1964, Kroon, Saccone & Bottman, 1967). Mitochondrial DNA is transcribed into rRNA, tRNA and mRNA, the complete genome being expressed almost entirely by the transcription of the heavy strand" (Attardi & Attardi, 1971(a), (b), Aloni, Hatlen & Attardi, 1971). Mitochondrial RNAs are characterised by the sensitivity of their synthesis to inhibition by the drug ethidium bromide (Zylber, Vesco & Penman, 1969, Knight, 1969).

2.6.1 Mitochondrial ribosomal RNA (mit-r-RNA).

RNase sensitive, ribosome-like particles within the matrix areas of mitochondria were first observed by Andre & Marinozzi (1965) and Adams (1966). Ribosome particles have been isolated from a wide variety of mitochondrial sources and are characterised by their lower sedimentation velocity and their content of smaller rRNA species than those from cytoplasmic ribosomes (Kuntzel & Noll, 1967, Kuntzel, 1969, Edelman, Verma & Littauer, 1970, Wintersberger & Viehauser, 1968, Rifkin, Wood & Luck, 1967). Ribosomes from eukaryotic mitochondria appear to have a sedimentation coefficient of 55-60s and are composed of 40s and 30s

subunits (Swanson & Dawid, 1970, Brega & Vesco, 1971, Attardi & Ojala,1971, O'Brien & Kalf,1967). Associated with the larger 40s subunit is a 21s rRNA species and with the 30s subunit, a 12s rRNA species. The low molecular weight 5s RNA component found in cytoplasmic ribosomes appears to be absent from mitochondrial ribosomes (Lizzardi & Luck, 1971, Attardi & Attardi,1971(b)). The base composition of mit-r-RNA differs widely from that of the cytoplasmic species (Fauman, Rabinowitz & Getz, 1969, Vesco & Penman, 1969) being richer in (A+U) and mit-r-RNA has been shown to hybridise to about 10-12% of mitochondrial DNA, there being at least 4 gene copies for mit-r-RNA in Neurospora (Wood & Luck, 1969, Dawid, 1969) although Aloni & Attardi (1971) have found no such multiplicity in Hela cells. Mit-r-RNA does however hybridise with nuclear DNA (Wintersberger & Viehauser, 1968) but it is as yet unclear whether this represents partial sequence homology or true nuclear cistrons for mit-r-RNA. Polyribosome structures active in protein synthesis have recently been identified in Hela mitochondria (Brega & Vesco, 1971, Attardi & Ojala, 1971) and Kuntzel (1969) has shown that mitochondrial ribosomes possess protein components distinct from those of the cytoplasmic species. Mit-r-RNA appears in addition to be poorly methylated (Attardi & Attardi, 1971(b))

2.6.2 Mitochondrial tRNA (mit-tRNA).

Mitochondria of Neurospora, yeast and rat liver have been shown to contain aminoacyl tRNAs that differ from those of the cytoplasm (Epler, 1969, Epler & Barnett, 1967, Buck & Nass, 1968,1969(a)) and tRNA synthetases specific for mit-tRNA have been identified in Neurospora (Barnett, Brown & Epler, 1967). Reverse phase chromatography has revealed fifteen distinct separable species of mit-tRNA in Neurospora (Epler, 1969) and six in rat liver (Buck & Nass, 1969(a)) unable to be charged by the cytoplasmic synthetases. Polyacrylamide gel electrophoresis of cytoplasmic and mitochondrial tRNA from Hela cells (Knight & Sugiyama, 1969) revealed a class of mit-tRNA with different coding properties to cytoplasmic transfer RNA species. N-Formyl-methionine tRNA, the initiating tRNA in bacterial and eukaryotic protein synthesis (Smith & Marcker,1970, Brown & Smith, 1970) has been shown to exist in mitochondria of Hela cells (Galper & Darnell, 1969) and yeast and rat liver (Smith & Marcker, 1968). Hybridisation data (Nass & Buck, 1969, Casey, Fukuhara, Getz & Rabinowitz, 1969) reveal that most if not all mit-tRNAs are mit-DNA specified, and Aloni and Attardi, (1971) have shown the existence of 11 tRNA genes present in Hela mit-DNA, the bulk of these being present on the "heavy strand". That mit-tRNA biogenesis may involve a

similar maturation scheme to that proposed for cytoplasmic tRNA synthesis, is suggested by Lizzardi & Luck (1971) who have demonstrated the existence of a putative mit-tRNA precursor in Neurospora mitochondria.

2.6.3 Mitochondrial messenger RNA (mit-mRNA).

Most mitochondrial enzymes and many structural components are probably derived from nuclear genetic information and are synthesised on cytoplasmic ribosomes prior to assembly into mitochondrial structures, but polysome structures engaged in mitochondrial specific protein synthesis have been identified in Hela and Xenopus mitochondria (Attardi & Ojala, 1971, Brega & Vesco, 1971, Swanson & Dawid, 1970, Perlman & penman, 1970). Pulse labelling of yeast with RNA precursors rapidly labels an RNA fraction which hybridises efficiently with mit-DNA and is presumably messenger RNA (Rabinowitz & Swift, 1970). In addition RNA/DNA hybridisation studies indicate an RNA fraction, associated with the membrane bound polysomes of Hela cells, which is distinct from nuclear mRNA with respect to base composition and metabolic behaviour and which hybridises efficiently with mit-DNA (Attardi & Attardi, 1967,1968). Attardi suggests that this mRNA may provide information for the synthesis of cellular membrane proteins (Attardi & Attardi, 1968). However, there also exists the possibility that this mit-mRNA leaves the mitochondrion to

specify the synthesis of mitochondrial ribosomal proteins, for they appear to be synthesised in an extramitochondrial site insensitive to chloramphenicol and sensitive to cycloheximide (Kuntzel, 1968).

3. Regulation of RNA synthesis.

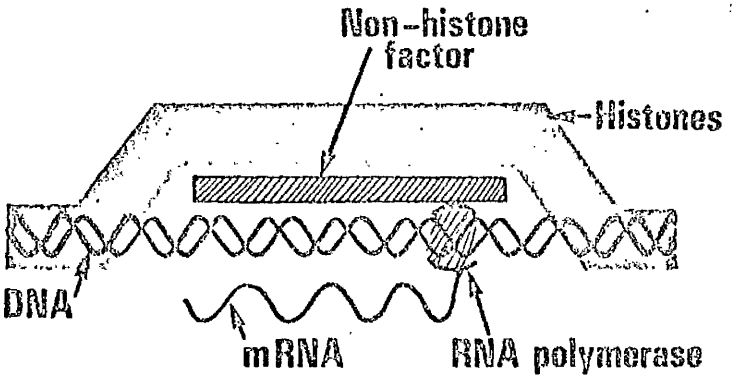
Despite the equivalence of genomes in the cells of higher organisms (Gurdon, 1962) the individuality expressed in the various differentiated states implies an ordered epression of genetic information. Since the immediate products of gene transcription are RNA molecules it is important to consider the processes whereby RNA synthesis is regulated:-

3.1 Gene masking

Some 80-90% of the DNA of the interphase nuclei appears to be, in some way, repressed and unavailable for transcription. (Allfrey, Littau & Mirsky, 1963, Bonner & Huang, 1963, Frenster, Allfrey & Mirsky, 1963, Paul & Gilmour, 1968). "Repressed" interphase DNA appears as condensed heterochromatin and "active" interphase DNA as extended euchromatin fibrils (Hsu, 1962, Frenster, Allfrey & Mirsky, 1963). In addition the euchromatin fibrils are deficient in the basic proteins, histones, (Frenster 1963) and contain the bulk of the rapidly labelled RNA of the nucleus (Frenster et al, 1963). Since histone molecules inhibit the synthesis of both RNA by RNA polymerase and DNA by DNA polymerase in vitro, (Huang & Bonner, 1962, Hnilica &

Billen, 1964) it is suggested that only a limited range of DNA sequences is available for transcription in chromatin, the majority being masked by the basic proteins (Allfrey & Mirsky, 1962, Paul & Gilmour, 1966(a)). RNAs extracted from several mammalian organs anneal with no more than 5-10% of the DNA from the same animal (Paul & Gilmour, 1968) and when chromatin preparations are used as templates, *in vitro*, similar results are obtained in contrast to the 40-50% annealing obtained with RNA transcribed from purified DNA (Paul & Gilmour, 1968). This restriction of chromatin is organ specific, some sequences being peculiar to particular organs and some sequences being common to various organs. Whe deproteinised chromatin is used as template it behaves exactly like DNA, and when dehistoned chromatin is the template there is at least partial restriction but when histones alone are combined with pure DNA, no RNA is transcribed *in vitro*. (Paul & Gilmour, 1968) From this data Paul & Gilmour conclude that histones can mask chromatin in a non specific manner, the specificity of histone-DNA interaction being mediated through the non-histone acidic proteins (Paul & Gilmour, 1969) allowing the unmasking of organ specific sequences.(see Fig. 1.8). There is now some evidence that non-histone proteins can catalyse the modification of chromosomal macromolecules (Burdon, 1971(b)).

Fig.I.8



A model of the possible arrangement of chromosomal proteins in the gene regulation system of mammalian cells as suggested from experimental findings.

Reproduced from Paul, Gilmour, & Thomou (1970).

Moreover in multicellular organisms cyclic AMP (cAMP) can be considered as a positive allosteric effector for the phosphorylation of proteins by the enzymic action of protein kinases and ATP (Tomkins & Martin, 1970). Langan (1968, 1969(a),(b)) has shown that after cAMP administration specific serine residues in liver F,histones are phosphorylated. The proposed role of histones is to regulate gene transcription and the phosphorylation of histones is proposed to uncover regions of the genome thus allowing transcription. In addition Burdon (1971(c)) has found that, in Krebs II cells, cAMP stimulates phosphorylation of histones in vivo and AMP promotes methylation of histones. The precise role of these hormone mediated modifications in gene regulation is however at present unclear.

3.2 Gene dosage

Rapid cell development in early embryogenesis demands an increasing capacity for protein synthesis which is met in amphibian oocytes by an increased synthesis of ribosomes achieved by the selective amplification of appropriate genetic material (Brown & Dawid, 1968). Interestingly however only the genes for 28s and 18s RNA are amplified (450 copies to greater than 1000 copies/haploid complement) and the coordinately expressed 5s genes are highly redundant, there being about 20,000 copies per haploid genome (Brown & Weber,

1968). Perhaps the high redundancy of 5s genes allows the synthesis of 5s RNA to keep pace with the increased synthesis of 28s and 18s RNA during oogenesis and embryogenesis. The rate at which 5s RNA synthesis proceeds in Xenopus appears to be regulated by the rate of synthesis of 28s and 18s RNA for no 5s RNA accumulates in anucleolate embryos unable to synthesise 28s and 18s RNA although such embryos do contain the genes for 5s RNA (Brown & Dawid, 1968). In Hela cells and BHK cells however this regulation system does not appear to operate for there is persistant synthesis of 5s RNA when rRNA synthesis is inhibited by low levels of actinomycin D (Perry & Kelley, 1968, Burdon et al, 1967, Smillie, 1970).

3.3 Regulation by RNA polymerase

Control of transcription mediated by RNA polymerase has been suggested by Brown & Dawid (1970), Roeder & Rutter (1970) and by Travers (1971) and has been most widely studied in bacterial cells. A single RNA polymerase species is thought to be responsible for the synthesis of all types of bacterial RNA (Geiduschek, 1969) and has been shown to possess the subunit composition $\alpha_1\beta\beta_2\sigma$ (Travers & Burgess, 1969, Burgess, 1969). The α subunit has a yet unknown function and the β subunits are involved in the binding to DNA (Travers, 1971) whilst the σ factor appears to be necessary for the accurate initiation of transcription and is reversibly dissociable from the

holoenzyme (Sugiura, Okamoto & Takanami, 1970). The σ factor acts catalytically in the initiation of RNA chains (Travers & Burgess, 1969) and is not required for chain elongation (Darlix, Sentenac, Ruet & Fromageot, 1969). It is released from the DNA-enzyme complex *in vitro* during or shortly after initiation and Pettijohn, Stettington & Kossman (1970) have suggested that σ release may occur *in vivo*. *In vitro* release of σ factor is mediated by the binding of single stranded polynucleotide chains to the holoenzyme and it appears that release occurs when a single stranded polynucleotide structure is generated such as the new RNA chain or by the "melting" of the DNA duplex which would occur shortly after initiation of transcription (Travers, 1971). It is possible to alter the initiation specificity by substituting one sigma-like factor for another (Travers, 1969). The flexibility of most reversible transcriptional transitions can only be provided by a control of initiation specificity and reversible dissociations of factors could feasibly provide such flexibility. The holoenzyme does not, however, appear to use bacterial DNA as a template efficiently (Travers & Burgess, 1970) and Travers (1971) has suggested that a further control by ϕ factors is superimposed upon σ factor action. These additional factors, of which several types are postulated, are themselves regulated by low molecular weight effectors

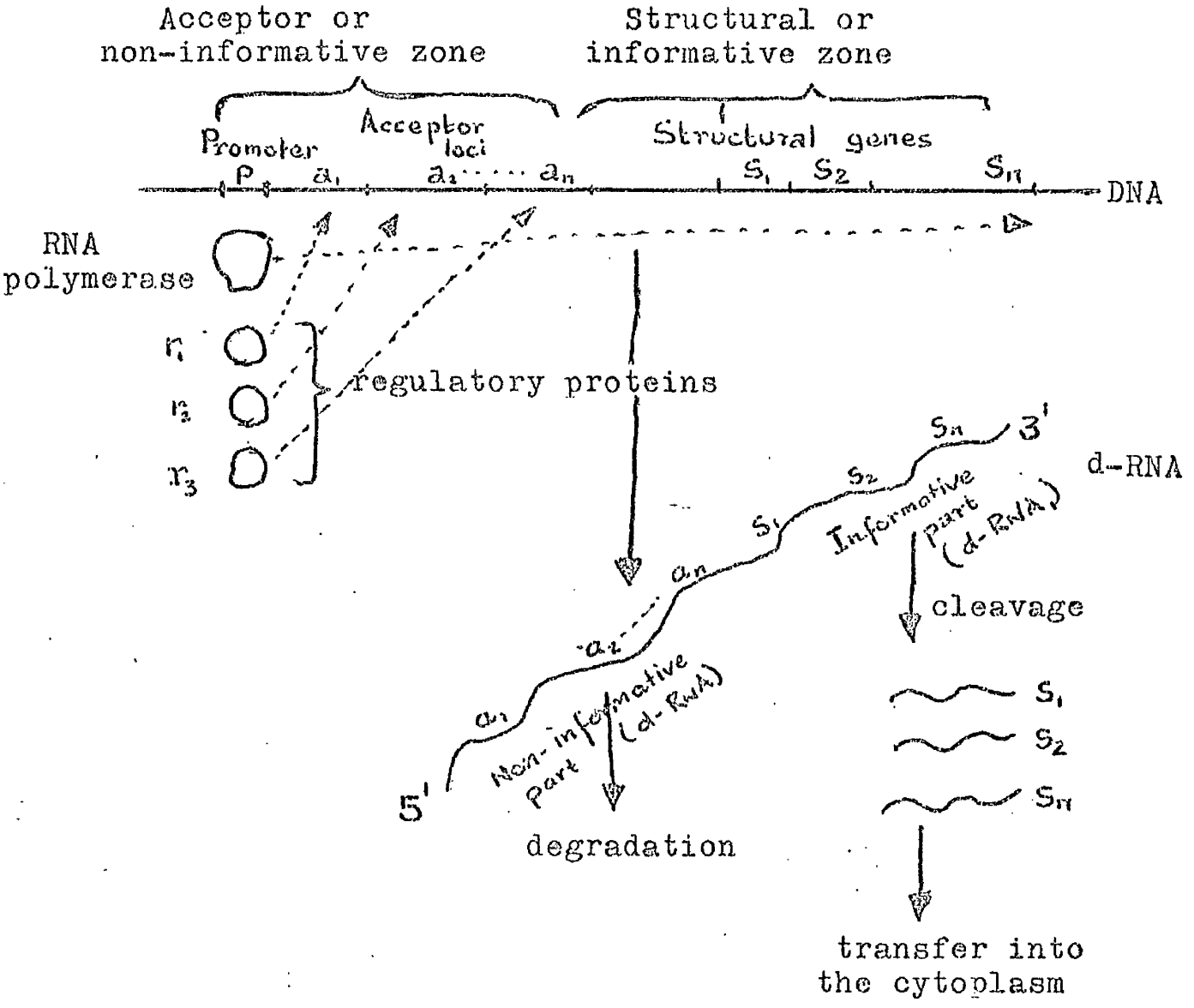
and interact with the holoenzyme to alter its initiation specificity. Examples of this type of modification have been identified for rRNA and tRNA cistrons (σr factors) (Travers, Kamen & Schlief, 1970). Fine control of such reversible transitions could be mediated by an other category of regulatory protein, examples of which are the λ and lac. repressors. Multiple forms of RNA polymerase have been identified in a number of eukaryotic cells (Roeder & Rutter, 1969, 1970) and changes in the proportion or activity of these have been reported following hormone administration (Tomkins & Martin, 1970). Brown & Dawid (1970) have suggested that multiple forms of RNA polymerase or its subunits could serve as positive control elements in higher cells, and visualise a model in which linked genes would be recognised by defined polymerase species by their content of the same initiation sequences. Thus regulation of transcription via RNA polymerase or its constituent subunits appears to be a feasible proposition for both eukaryotic and prokaryotic cells.

3.4 The structural organisation of gene regulation in higher cells.

Theories of gene regulation in higher cells are a direct extension of the Jacob & Monod theory of gene regulation in bacterial cells. Pace et al, (1970) have suggested that all stable RNAs of prokaryotic cells arise as a result of post-

"Operon structure" of gene regulation in eukaryotes

Fig.I.9



After Georgiev (1969)

transcriptional modifications and it is becoming increasingly apparent that, in eukaryotic cells also , RNA maturation features extensively in gene regulation (Burdon, 1971(a), Willems, Penman & Penman, 1969). A recent theory of Georgiev (1969) on the structural organisation of the "operon" in higher cells encompasses the role of such maturation phenomena. Georgiev envisages the "operon" to be composed of a non-informative region containing the promoter site, to which RNA polymerase binds, and separated from the informative region of structural genes by a linear array of acceptor loci or operators (see Fig. 1.9). These "operators " are recognised by various regulatory proteins functioning like bacterial repressors and preventing transcription by the prevention of the passage of RNA polymerase. The existence of several loci in any one "operon" ensures that an "operon" may be regulated by different factors such as hormones or other cellular effectors, and the multiplicity of some acceptor loci would then accommodate the expression of linked genes. Operons may be combined in groups of higher order and determine the synthesis of cell specific proteins. A coordinate expression of such linked genes mediated by the high multiplicity of acceptor loci in various operons easily accommodates our present impressions of cell differentiation. Expression of an operon therefore results in a large RNA

molecule containing informative and non-informative sequences and Georgiev suggests that such molecules represent the so called HnRNA which undergoes a maturation process during which the non-informative sequences are selectively degraded and the informative regions pass to the cytoplasm as mRNA. A similar but slightly more detailed model is proposed by Britten & Davidson,(1969) who suggest that interaction of effectors with"sensor genes" allows the expression, in the form of "activator RNA" molecules, of integrator genes" and that such nuclear confined "activator RNAs" complex with "receptor genes" linked to "producer genes" to determine the pattern of cellular gene expression. Some 20-40% of the mammalian DNA base sequence is represented by highly repeated sequences scattered throughout the genome (Britten & Kohne, 1968) and Georgiev (1969) suggests that these repeated sequences represent non-informative regions of "operons". The concept of the regulatory action of histones and certain non-histone proteins in higher cells has been expounded for some time and has been discussed in section 3.1, but Georgiev, Ananieva & Kozlov (1966) have reported that transcription of repeated DNA sequences is specifically inhibited by F, histones and therefore suggest that this represents a prevention of transcription by the binding of histones to acceptor loci of operons".

3.5 Regulation of RNA synthesis in relation to growth.

RNA synthesis in relation to the periods of rapid cell development occurring during oogenesis and embryogenesis has been discussed in section 3.1, but in less active cells, such as adult liver or kidney, there is still extensive incorporation of RNA precursors into rRNA (Muramatsu, Hodnett, & Busch, 1966) and Quincey & Wilson (1969) have estimated that rRNA genes in rat liver are transcribed twice a minute, 4s genes once a minute and 5s genes once every two minutes. Again there exists a high degree of redundancy of 5s genes and since the half-life of an adult liver ribosome is aproximately 4-6 days (Loeb, Howell & Tomkins, 1965) it is apparent that some post-transcriptional control of ribosome production must exist. Such a system could be explained by the "wastage phenomenon" reported in resting human lymphocytes by Cooper (1969) which proposes that some of the newly synthesised rRNA is immediately degraded, a fixed level surviving, and this survival level controls the rate of rRNA synthesis. Similar observations of a wastage phenomenon in lens tissue have been reported by Papaconstantinou & Julka, (1968). RNA synthesis appears to be continous throughout interphase with a slightly higher rate in G_1 phase than in the G_2 phase of the cell cycle (Enger & Tobey, 1969) but it is reduced greatly during mitosis (Johnson & Holland, 1965)

possibly due to the condensation of the chromatin template. The synthesis of 45s r-pre-RNA and its processing to 28s and 18s RNA appears to occur in the S, G_1 and G_2 phases of the cell cycle to an equal extent (Scharff & Robbins, 1965).

Transfer RNA is a stable component of the cell and has a half-life in rat liver estimated at approximately 90h (Bloebel & Potter, 1968) yet it is estimated that 11,000 tRNA molecules are synthesised per minute in rat liver (Quincey & Wilson, 1969). tRNA synthesis is accelerated when resting human lymphocytes are stimulated to grow and divide by treatment with phytohaemagluttinin (Kay & Cooper, 1969) perhaps in response to an increased demand for protein synthesis. Starvation of cultures of Hela or BHK-21 cells for the essential amino acid, methionine, promotes a retardation in pre-tRNA maturation (Smillie, 1970, Bernhardt & Darnell, 1969) as does infection of BHK cells with pseudorabies virus (Shepherd, 1969) possibly not as a result of inhibition of protein synthesis but as a result of methyl group deficiency, for pre-tRNA is synthesised and processed at a normal rate in valine starved cells. (Smillie, 1970) Regulation of tRNA synthesis might therefore be mediated through a control of maturation of pre-tRNA molecules.

Inhibition of protein synthesis by amino acid starvation appears also to affect the regulation of rRNA synthesis

(Maden, 1971(a), Shields & Korner, 1969). When cultures are starved for methionine, ribosome formation continues at a much reduced rate (Maden, Vaughan, Warner & Darnell, 1969, Maden & Vaughan, 1968) and there is a deficiency of ribosomal proteins (Vaughan, Soiero, Warner & Darnell, 1967, Maden & Vaughan, 1968). The intranucleolar content of RNA however remains constant (Maden, 1971) and during lysine or valine starvation similar effects are observed but, in addition, there exists a ribosomal subunit imbalance indicating abortive synthesis (Maden, 1969). Similar effects are obtained for a variety of other amino acids (Maden, 1971(b)). During complete inhibition of protein synthesis by cycloheximide (Willems, Penman & Penman, 1969, Craig & Perry, 1970) the nucleolus still produces mature ribosomes at a highly reduced rate, the rate of 45s RNA synthesis declines but the intranucleolar content remains constant. After prolonged inhibition the export of ribosomes is however greatly reduced. Inhibition of protein synthesis by puromycin does not appear to impair 45s rRNA synthesis nor its cleavage to 32s and 18s rRNA, but no RNA leaves the nucleolus (Soiero, Vaughan & Darnell, 1968). Together these data suggest that rRNA production in eukaryotes is regulated by the actual level of protein synthesis and by a mechanism distinct from that in E. coli where the control mediated by the cytoplasmic product of a particular genetic

locus, the RC locus, (Edlin & Broda, 1968) but Willems *et al*, (1969) have suggested that rRNA synthesis may be controlled by the intranucleolar level of 32s rRNA.

Aims of the present work.

Polyacrylamide gel electrophoresis of extracts of mammalian cell cytoplasm has revealed the presence of a number of hitherto undetected low molecular weight RNA species. The aim of the present study was to investigate the nature of these RNA species in the cytoplasmic fraction of normal, malignant and virus transformed animal cells. It was proposed to characterise these molecules with respect to their mode of synthesis; their relationship to one another and to the other RNA species present in these sub-cellular fractions; to investigate their content of methylated nucleosides and their precise location within the cell. With a view to investigating their possible function within the cell it was also proposed to examine the association of these RNA molecules with subcellular particles and their possible release from them; their pattern of synthesis in relation to the cell division cycle and the effects of a variety of metabolic inhibitors upon their synthesis and subsequent metabolism.

MATERIALS & METHODS

MATERIALS

(A) BIOLOGICAL

Tissue Culture Material

BHK-21/C13 cells and the transformed cell lines BHK-21/SR8/V1 and BHK-21/PyY were a continuous line of hamster fibroblasts after McPherson and Stoker,(1962).

Brain heart infusion broth and Sabouraud medium, utilised in the analyses of cultures for contamination by bacteria and yeast, were obtained from Oxoid Ltd., London. Difco Laboratories, Detroit 1, Michigan, U.S.A., provided trypsin, agar, PPLO broth for production of PPLO agar plates, and Difco/Bacto tryptose phosphate broth. Calf serum and penicillin/streptomycin were purchased from Flow Laboratories Inc., Irvine, Scotland.

Composition of Media and Standard Solutions

(a) Eagles medium(Glasgow modification), Busby, House and McDonald, (1964) containing 100µg/ml streptomycin, 100 units /ml penicillin, 0.2µg/ml of an antimycotic agent, n-butyl, p-hydroxy benzoate and 0.002%(w/v) phenol red was the growth medium used for propagation of the cells.

EC_{10} medium consisted of Eagles medium supplemented with 10%(v/v) calf serum.

ETC_{10} medium consisted of Eagles medium supplemented with 10%(v/v) calf serum and 10%(v/v) tryptose phosphate broth.

Tryptose Phosphate Broth was a 2.95%(w/v) solution of Difco Bacto tryptose phosphate broth in distilled water.

Buffered Saline Solution (BSS) consisted of 0.116M NaCl.5.4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$ and 0.002%(w/v) phenol red. This mixture was adjusted to pH7.0 by the addition of 8.4%(w/v) $NaHCO_3$.

Phosphate Buffered Saline (PBS) consisted of 0.17M NaCl, 3.4mM KCl, 10mM Na_2HPO_4 and 2.4M KH_2PO_4 and was adjusted to pH7.4.(Dulbecco & Vogt. (1954).

Versene was 0.6M disodium EDTA in PBS solution A. to which was added 0.002%(w/v) phenol red.

Trypsin/versene was a 0.05%(w/v) solution of trypsin in versene.

1x SSC was a solution of 0.15M NaCl and 15mM trisodium citrate adjusted to pH7.0 with HCl.

2x SSC was a solution of 0.3M NaCl and 30mM trisodium citrate adjusted to pH7.0 with HCl.

(B) CHEMICAL

(a) General

Sodium deoxycholate, disodium EDTA, and bentonite powder were obtained from BDH-Biochemicals Ltd., Poole, Dorset. Trizma base, glycine, adenine, thymidine and L-amino acids were purchased from Sigma London Chemical Co. Ltd., London, S.W.6. Tween 80 and sodium dodecyl sulphate were from

Koch-Light Laboratories Ltd., Colnbrook, Bucks, England

Esch. coli(strain B.) soluble RNA was purchased from Calbiochem Ltd., Los Angeles, California, U.S.A.

CsCl(Analar grade) was obtained from Hopkins & Williams Ltd., Chadwell Heath, Essex.

(b) Drugs and Inhibitors

Actinomycin D was obtained from Calbiochem Ltd., London and also from Merck, Sharpe and Dohme Inc., Rahway, New Jersey, U.S.A. Ethidium bromide was the generous gift of Dr. J.D.Pitts of this department and α-Amanitin was purchased from C.H.Boehringer Sohn, Abtielung Chemikalein, 6507, Ingelheim am Rhein, Germany. A generous gift of 1mg of this drug from Dr. J.Szilagyi, Institute of Virology, University of Glasgow is also gratefully acknowledged. Puromycin dihydrochloride and Cordycepin were obtained from Sigma London Chemical Co., London S.W.6. Toyocamycin was a generous gift from Dr. Acs. Institute for Muscle Disease, New York.

(c) Enzymes

Electrophoretically purified RNase and DNase were obtained from Sigma London Chemical Co., Ltd., London, S.W.6. Pronase grade B was purchased from Calbiochem Ltd., Los Angeles, California, U.S.A. Pronase was used at a final concentration of 100μg/ml and was auto-digested at 37^{o}C for 2h prior to use to remove any contaminating nucleases.

(d) Acrylamide gel materials

Acrylamide was obtained from Eastman Organic Chemicals Ltd., Rochester, New York, U.S.A. Methylene bisacrylamide and ammonium persulphate were purchased from BDH-Biochemicals Ltd., Poole, Dorset. N,N,N',N',-tetramethylethylenediamine (TEMED) was provided by Koch-Light Laboratories, Colnbrook, Bucks.

(e) Autoradiographic materials

Nuclear Research emulsion(type L4) and ID19 developer fluid were provided by Ilford Ltd., Ilford, Essex. Amfix fixative solution was purchased from May and Baker Ltd., Dagenham, Essex. Haematoxylin/Eosin, obtained from BDH-Biochemicals Ltd., Poole, Dorset, was used as an 0.01%(w/v) solution in distilled water. Giemsa stain was a suspension of 1.5%(w/v) Giemsa in glycerol heated to 56°C for 90 min, then diluted with an equal volume of methanol.(Dancie, 1956).

(f) Chromatographic materials

Sephadex G100 was obtained from Pharmacia Ltd., Uppsala, Sweden. Whatman No.1 filter discs and 3MM filter paper were purchased from H. Reeve-Angel & Co. Ltd., London, England.

(g) Radioisotopes

[8-^{3}H-] -guanosine (4.5-5.0 Ci/mmol), [G-^{3}H]- uridine (4.25 Ci/mmol) [G-^{3}H]-uridine (Ci/mmol), [2-^{14}C] uridine (62mCi/mmol)

L-[Me-^{3}H]-methionine (8.3 Ci/mmol), L-[Me-^{14}C]methionine (56.8mCi/mmol), [6-^{3}H]-thymidine (26.Ci/mmol), [6-^{3}H]-thymidine (17.4Ci/mmol) and[^{32}P]-orthophosphate. (37-78Ci/mg P) were purchased from the Radiochemical Centre, Amersham, Bucks, England.

(h) Chemicals for scintillation spectrometry

Hyamine hydroxide, 1M in methanol and naphthalene were purchased from Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh. Koch-Light Laboratories, Colnbrook, Bucks, supplied 2.5 diphenyl oxazole and dioxan. BDH-Biochemicals Ltd., provided 2-methoxyethanol and 30%(w/v) hydrogen peroxide. Cellulose acetate filters, 0.45μ pore size (HAWP02500) were obtained from Millipore (U.K.) Ltd., Wembley, Middlesex. Membrane filters, 2.5cm diameter 0.45μ pore size were also obtained from Sartorius-Membranfilter GMBH, 34, Gottingen, West Germany.

(i) Fluids for scintillation spectrometry

Toluene based scintillation fluid was prepared by dissolving 5g 2,5 diphenyl oxazole in 1 litre of Analar toluene at room temperature and storing this at room temperature. Dioxan based scintillation fluid was constituted by 7g of 2,5 diphenyl oxazole dissolved with 100g naphthalene in 1 litre of scintillation grade dioxan. A scintillant consisting of 4 parts 2-methoxyethanol to 6 parts toluene

based scintillator was routinely used for the assay of $^{3}H/^{14}C$ in acrylamide gel samples (methods section 5b).

Miscellaneous

All other chemicals were, wherever possible, Analar grade or its equivalent. Beckman Spinco Ltd., Palo Alto, California, U.S.A., supplied cellulose nitrate tubes ($\frac{1}{2}$" x 2", 1" x 3" and 9/16" x 3$\frac{3}{4}$"). Macaloid powder was obtained from Texas Lead Co., Houston, Texas, U.S.A.

Minimum essential medium (Glasgow modification)

AMINO ACIDS	mg/l.
L-Arginine HCl	42.1
L-Cystine	24.0
L-Glutamine	292.0
L-Histidine HCl	19.2
L-Isoleucine	52.5
L-Leucine	52.5
L-Lysine HCl	73.1
L-Methionine	14.9
L-Phenylalanine	33.0
L-Threonine	47.6
L-Tryptophan	8.2
L-Tyrosine	36.2
L-Valine	46.9

VITAMINS	mg/l.
D-Calcium pantothenate	2.0
Choline Chloride	2.0
Folic Acid	2.0
i-Inositol	4.0
Nicotinamide	2.0
Pyridoxal HCl	2.0
Riboflavin	0.2
Thiamin HCl	2.0

INORGANIC SALTS AND OTHER COMPONENTS

$CaCl_2 . 2H_2O$	264.0
Dextrose	4500.0
$Fe(NO_3)_3 . 9H_2O$	0.1
KCl	400.0
$MgSO_4 . 7H_2O$	200.0
NaCl	6400.0
$NaH_2PO_4 . 2H_2O$	140.0
Phenol Red	17.0
$NaHCO_3$	2750.0

10^7 units Penecillin / litre

100 µg/ml. Streptomycin S.

METHODS

A. BIOLOGICAL

1) Cell Culture Systems

BHK-21/C13 cells, BHK-21/SR8/V1, and BHK-21/PyY cells were routinely cultured as monolayers in rotating 80oz winchester bottles essentially according to the method of House and Wildy(1965). The cells were seeded initially at a concentration of 2×10^7 cells per bottle in 180ml ETC_{10} or EC_{10}, the buffering capacity of which was maintained by an atmosphere of 5%(v/v) CO_2 in air, and the culture maintained at 37°C for 2 - 3 days, at which time the cell density had reached approximately 4×10^8 to 10^9 cells per bottle. The cells were then removed from the glass by treatment with trypsin/versene, suspended in ETC_{10} and dispensed, in appropriate concentrations in 100ml ETC_{10}, into 80oz winchester bottles, or in 5ml amounts into plastic 60mm petri dishes, for experimental procedures. Although the ETC_{10} (Glasgow modification) (Busby, House and McDonald, 1964) contained antibiotics as detailed in the opposite table, the cultures were routinely examined for contamination by the following methods.

Bacterial contamination was monitored by blood agar plates and brain/heart infusion broths, while contamination by yeast and fungi was checked by incubation in Sabourauds

Growth characteristics of BHK-21/C13 cells.

Fig. II.1

(a) Growth.

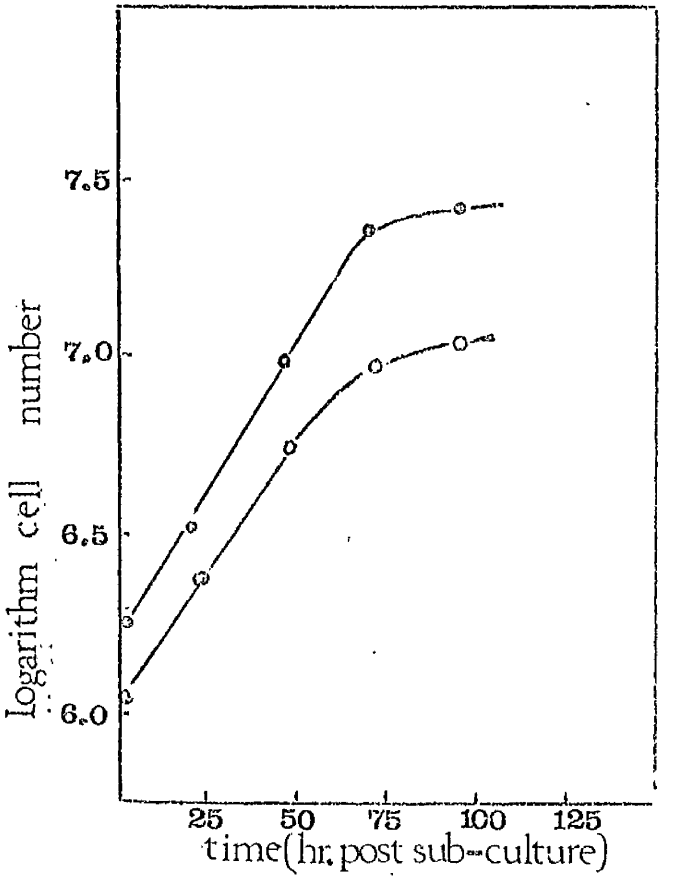
Cultures of BHK-21/C13 cells containing either 1.5×10^6 cells in 10ml EC_{10}, or 2×10^6 cells in 10ml of ETC_{10} were grown in 4oz. bottles at 37°C. After appropriate times the medium was removed from duplicate cultures and the cell monolayers bathed with 5ml of trypsin solution. The cells were then removed from the glass in 10ml of EC_{10} and an aliquot of this suspension counted in a haemocytometer slide. The cell counts were estimated as the average of three fields per culture.

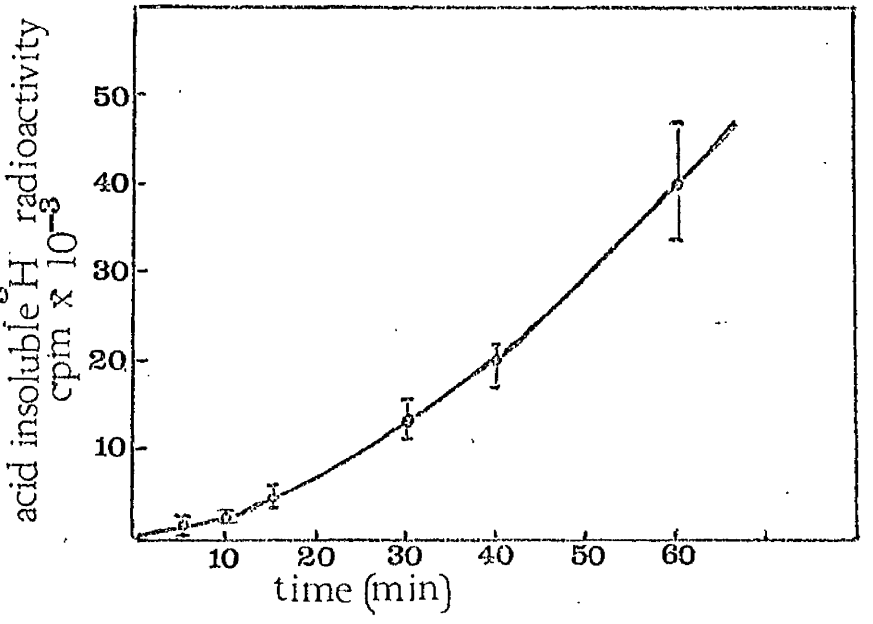
Growth of cells in EC_{10} (—o—)
Growth of cells in ETC_{10} (—o—)

(b) Isotope incorporation.

Cultures of BHK-21/C13 cells containing 2.5×10^5 cells in 3.0ml of ETC_{10} in 60mm petri dishes were grown at 37°C for 12h. Each dish was then inoculated with 5μCi of [^{3}H]-uridine (1.52Ci/mmol) and the cultures exposed to the radioisotope for appropriate times. After this exposure time each dish was washed three times with ice cold BSS, three times with ice cold 5%(w/v) trichloracetic acid and the cell monolayer then dissolved in 1.5 ml of 0.3N KOH. The resultant solution was then evaporated to dryness and the residue treated with 0.5ml of hyamine hydroxide at 60°C for 30min, before adding 10ml of a toluene based scintillant and estimating the incorporated radioactivity by liquid scintillation spectrometry.

Acid insoluble radioactivity cpm (—o—)


Logarithm cell number
7.5
7.0
6.5
6.0
25
50
75
100
125
time(hr. post sub-culture)


acid insoluble H^3 radioactivity
cpm x 10^{-3}
50
40
30
20
10
10
20
30
40
50
60
time (min)

medium. The cultures were also routinely examined for contamination by pleuropneumonia-like-organisms (PPLO) by the method of Fogh and Fogh (1964) or by growth of the cultures on PPLO agar. Samples of the cell suspension were taken up in a sterile pasteur pipette and inoculated into the solidified agar by puncturing the surface approximately 10 times with the pasteur pipette, thus allowing the cells to spread out in the agar in a number of different locations and facilitating their location for microscopic examination. The PPLO assay plates were incubated in an atmosphere of 5%(w/v) CO_2 in nitrogen in an anaerobic Leyden jar for 7 days at 37°C. After this incubation period the plates were examined by microscopy for the appearance of characteristic fried egg colonies of PPLO infected cultures. As a check on the efficiency of the method a culture of BHK-21/C13 cells known to be infected with known strains of PPLO was assayed simultaneously. All glassware and pipettes used in tissue culture methods were steeped in chloros or CMS overnight, washed thoroughly, rinsed in distilled water and sterilised by autoclaving at 15p.s.i. for 45 mins.

2) Harvesting of cells from experimental cultures

Cells were harvested from experimental cultures by one of the following procedures:-

(a) After exposure of the cell monolayer to isotopically labelled RNA precursors for the appropriate time period, the growth medium was removed and the cell sheet washed with two successive 25ml portions of ice cold BSS. The cells were then removed from the glass by treatment with a 10ml volume of trypsin/versene, suspended in 25ml ice cold ETC_{10} and collected by centrifugation at 800g for 2.5 min at $4^{o}C$.

(b) The "labelled" cell monolayer was washed twice with 25ml portions of ice cold BSS and the cells removed from the glass by scraping with a rubber "windscreen wiper" into 25ml ice cold BSS. The cells were pelleted by centrifugation at 800g for 2.5 min and washed with two 10ml volumes of ice cold BSS.

3) Cell Synchronisation Procedures

Cell cultures were synchronised by the inhibition of DNA synthesis by one of the following techniques:-

(a) Aminopterin Induced Synchrony:- Cultures were grown for approximately 18h in rotating 80oz winchester bottles as previously described. Aminopterin (200nM), adenine (0.7mM) and glycine (0.3mM) were added and after approximately 12h the block imposed on DNA synthesis was released by the addition of thymidine (0.3mM).

The cultures were then exposed to radioactive RNA precursors for the appropriate time and the cells harvested as described

above.

(b) Thymidine Induced Synchrony:- Cultures grown as described above were synchronised by treatment with 5mM thymidine for 10 - 12h at 37°C. The block imposed on DNA synthesis by this treatment was reversed by washing the cell sheet with three 50ml portions of prewarmed ETC_{10} and then adding 100ml prewarmed ETC_{10} for continued growth. The cultures were then exposed to isotopically-labelled RNA precursors and the cells harvested as previously described.

4) Assay of Synchrony

At the same time as the above synchronisation treatments were performed, smaller cultures (10^5 cells/60mm petri dish) of the same batch of cells were blocked with aminopterin or thymidine as detailed above. However after release from the block, 5μCi [^{3}H]-thymidine (17.4 Ci/mmol) were added to each culture for 1h periods, at various times after release, in order to follow the rates of DNA synthesis as judged by the incorporation of thymidine. Each monolayer culture so labelled was washed three times with 3ml ice cold BSS, and DNA extracted and assayed by one of the following procedures:-

(a) The washed cell monolayer was further washed with three portions of ice cold 5%(w/v) trichloracetic acid and

dissolved in 1.0ml of 0.3NKOH and the resulting solution evaporated to dryness. The vials were then allowed to cool. 4.75ml of toluene based scintillation fluid together with 0.25ml of hyamine hydroxide were added and the radioactivity estimated by liquid scintillation spectrometry.

(b) The washed cell monolayer was lysed by exposure to 3ml of BSS containing 1%(w/v) SDS for 5 min at room temperature. The lysate was quantitatively removed, made 1M with respect to sodium perchlorate and shaken vigorously with an equal volume of chloroform-isoamyl alcohol (24:1, by vol). The emulsion was separated by centrifugation at 10,000g, the aqueous phase removed, made 5%(w/v) with respect to trichloracetic acid and acid precipitable radioactivity collected on membrane filters and, after drying, radioactivity assayed by liquid scintillation spectrometry. The efficiency of the synchronisation procedure was frequently checked by autoradiography.

5) Autoradiography

Autoradiography of synchronised cell populations was carried out as follows:-

The "labelled" cell monolayer was washed twice with 3ml portions of ice cold BSS, three times with 3ml volumes of ice cold 5%(w/v) trichloracetic acid and once with a 3ml

volume of absolute ethanol. The dishes were then dried at 37°C, 1.0ml nuclear track emulsion (type L4) spread evenly over each dish in a very thin layer and the dishes exposed for 7 days. After this exposure time the dishes were developed in ID19 developer for 2 min, followed by 2 min in a 1 in 5 dilution of Amfix. Dishes were then immediately rinsed in running tap water and stained in 0.1%(w/v) ammonaical Haematoxylin/Eosin or in Giemsa stain. Microscopic examination showed background grains to be a minimum and labelled nuclei were counted over an average of 3 fields per dish.

(B) BIOCHEMICAL

1) Purification of bentonite

Bentonite was purified essentially according to the method of Fraenkel-Conrat, Singer and Tsugita (1961). Approximately 60g bentonite powder was suspended in 400ml distilled water by homogenisation and centrifuged at 800g for 15 min. The supernatant fraction was recentrifuged at 8500 rev/min for 20 min and the sediment from this was then resuspended in 0.1M disodium EDTA(pH7.0) for 48h at 25°C. The EDTA suspension was then centrifuged differentially and the 8500 rev/min sediment suspended in 0.01M sodium acetate (pH 6.0) and again centrifuged at 8500g for 20 min. The sediment from this was then taken up in 0.01M sodium

acetate (pH 6.0) to a concentration of 1.5%(w/v) as determined by dry weight estimation. The solutions were then sterilised by autoclaving at 15p.s.i. for 30 min.

2) Purification of Macaloid

Macaloid powder was suspended in distilled water to a concentration of 1.0%(w/v) and the solution sonicated in an MSE sonibath for 3 to 4 min. This solution was then dialysed against 100 vol distilled water at 4°C overnight. The supernatant dialysate was removed and sterilised by autoclaving at 15p.s.i. for 30 min. This stock solution was sonicated for 3 - 4 min just prior to use and was used at a final concentration of 0.001%(w/v) in RNA or subcellular preparation techniques.

3) Preparation of Subcellular Fractions

(a) Nuclei:- Nuclei were prepared from cell pellets by one of the following techniques:-

(1) Nuclei were prepared by a modification of the method of Fisher and Harris (1962). The cell pellet obtained from cell cultures as previously described was suspended in 5ml of 1.0%(w/v) Tween 80 and homogenised in a glass/teflon Potter type homogeniser. The resultant homogenate was centrifuged at 2,000 rev/min for 5 min, the pellet resuspended in 5ml 1.0%(w/v) Tween 80 and homogenised again in ice. The homogenate was again centrifuged at

2,000 rev/min and the pellet suspended in 5ml of 1x SSC, layered over 3 volumes 0.33M Sucrose in 0.5M ammonium acetate (pH 5.1) and centrifuged at ?,500 rev/min for 15 min at 4°C. This process was repeated until nuclei free of contaminating cytoplasmic tabs were obtained as judged by phase contrast microscopy.

(?) Nuclei were prepared according to the method of Penman et al(1967). Briefly, the cells are suspended and washed in hypotonic RSB buffer (0.001M-NaCl, 0.003-$MgCl_2$, 0.01M-Tris, pH7.4) and allowed to swell in this buffer in ice for 10 min. The cells are then ruptured by homogenisation in a glass/teflon homogeniser and the crude nuclei are deposited by centrifugation at ?,500 rev/min. The nuclei are washed once more in RSB buffer and then resuspended in RSB buffer and treated with a detergent mixture of 10%(v/v) Tween 80/10%(w/v) sodium deoxycholate (?:1, by vol) and the nuclei, which now have their outer nuclear membrane removed, are collected by centrifugation.

(b) Nucleoli and Nucleoplasm

These fractions were prepared according to the method of Penman et al(1967). Detergent cleaned nuclei, prepared as described above, were resuspended in 1.0ml of HSB buffer (0.5M NaCl, 0.05M-$MgCl_2$, 0.001M-Tris, pH 7.4) and treated with 100µg/ml electrophoretically purified DNase

at room temperature for 2 min to digest the DNA of the chromatin. The digested nuclei were then layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in HSB buffer and centrifuged for 20 min at 22,000 rev/min, in an SW25.1 rotor of a Beckman model L ultracentrifuge at 4°C, to yield a nucleolar pellet and the nucleoplasmic contents spread throughout the gradient. The gradient was decanted, made 0.5%(w/v) with respect to sodium dodecyl sulphate, and precipitated with two volumes of absolute ethanol to yield a pellet of nucleoplasmic material which was suspended in 0.05M-ammonium acetate for extraction of the nucleoplasmic RNA. The nucleolar pellet was similarly suspended in 0.05M-ammonium acetate.

(c) Cytoplasmic Components

The cells, after incubation with appropriate additions, were washed and harvested as described in section A2, suspended in 3ml of RSB buffer and collected by centrifugation. The cell pellet was resuspended in 3ml of RSB buffer containing 0.5%(w/v) bentonite and allowed to stand for 10 min in ice. The suspension of swollen cells was then homogenised for 1.5 min in a glass/teflon Potter type homogeniser or in a hand homogeniser and the resultant homogenate centrifuged at 2,500 rev/min to deposit the crude nuclei and any unbroken cells.

The supernatant fraction was retained and the pellet was resuspended in 1.0ml of RSB buffer by vigorous pipetting and repelleted by centrifugation. The supernatant from this step was retained and combined with the previous one to yield a cytoplasmic fraction.

This cytoplasmic fraction was then centrifuged at 12,000g for 15 min in an MSE 18 centrifuge to yield a fraction rich in mitochondria. This sediment, after resuspension in 0.05M ammonium acetate was further purified by layering it over a linear gradient of 0.8M - 1.2M sucrose in NET buffer (0.001M-NaCl, 0.001M disodium EDTA, 0.01M-Tris, pH 7.4) and centrifuging at 25,000 rev/min for 30 min in an SW 25,1 rotor in a model L ultracentrifuge to give a purified mitochondrial pellet (Penman et al 1969), which was then resuspended in 0.05M ammonium acetate for extraction of the RNA. The supernatant from the 12,000g spin was further centrifuged at 140,000g for 90 min to sediment microsomal material and to yield a supernatant fraction referred to as cell sap (Burdon & Clason 1969). The pellet from this treatment was resuspended in 0.05M ammonium acetate for extraction of RNA and referred to as the microsomal fraction.

(d) Polysomes

Polysomes were prepared essentially according to the method

of Penman et al (1967).

When polysome preparations were required these were prepared from cytoplasmic fractions, derived from labelled cell pellets as described in Methods section 3(c) but in the presence of 0.001% Macaloid rather than bentonite as it was found that bentonite interfered with the harvesting of CsCl gradients, when such preparations were examined by CsCl gradient analysis. The cytoplasmic fraction was treated with 0.12 vol of 10%(w/v) sodium deoxycholate and layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in TKM buffer and centrifuged at 25,000 rev/min for 3h at 4°C in an SW25.1 rotor of a Beckman model L ultracentrifuge. The gradient was harvested in 1.0ml fractions, the extinction being automatically monitored and recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. Frequently a pellet of polysome material was obtained by layering the deoxycholate treated cytoplasmic fraction over 2.0ml of 2M sucrose in TKM buffer, overlayered with 1.0ml 0.5M sucrose in TKM buffer and centrifuging at 40,000 rev/min at 4°C in an SW50 rotor of a model L ultracentrifuge.

(e) Ribosomes

Ribosomes were prepared (according to the method of Pene

et al 1968) from cytoplasmic fractions, derived from labelled cell pellets in the presence of 0.001%(w/v) Macaloid as described above, by the following procedure:- The cytoplasmic fraction was treated with 0.05 vol 10%(w/v) Tween 80, then with 0.05 vol 10%(w/v) sodium deoxycholate and 0.08 vol 1M $MgCl_2$ and allowed to stand in ice for 45 - 60 min. After this time the ribosomes were pelleted by centrifugation at 10,000 rev/min for 10 min and the pellet rinsed once with ?ml RSB buffer. The ribosomal pellet was then resuspended in 0.05M disodium EDTA by hand homogenisation in a glass/teflon homogeniser and centrifuged through 15%(w/v) to 30%(w/v) gradients of sucrose in disodium EDTA to display the ribosomal subunits.

4) RNA Isolation

(a) Glassware and solutions

All glassware used in the preparation of RNA was either flamed immediately before use or autoclaved at 15p.s.i. for 30 min, and all solutions and media were sterilised by autoclaving to remove contaminating nucleases. Visking tubing for dialysis was boiled in 0.10%(w/v) sodium dodecyl sulphate and disodium EDTA for 15 min then rinsed with sterile dialysis medium.

After incubation of cells with appropriate additions,

washing and harvesting as described, RNA was isolated from intact cells or from isolated cellular components. The following techniques were employed:-

(a) Cold Phenol technique

This was either applied directly to intact cells or to isolated subcellular fractions and was essentially the method described by Burdon et al (1967). To a suspension of intact cells or subcellular components in 3ml of 0.05M ammonium acetate (pH5.1) was added 0.5ml of 1.5%(w/v) bentonite (purified as described in Methods section B1,) together with 3ml 80%(w/v) phenol equilibrated with the same buffer. The mixture was shaken thoroughly for 2 min and the resultant emulsion separated by centrifugation at 10,000g for 10 min. The aqueous phase was then added to a further 3ml 80%(w/v) phenol and 0.5ml of 1.5%(w/v) bentonite and re-extracted as above. The aqueous phase from this was then precipitated with 2 vol absolute ethanol, deposited by centrifugation at 2,500 rev/min and then resuspended in 0.5ml of 0.05M ammonium acetate (pH 5.1). This solution was then centrifuged at 18,000 rev/min in an MSE 18 high speed centrifuge for 5 min to remove the bentonite and the supernatant added to 2 volumes of absolute ethanol. RNA was collected after 18h at -20°C by centrifugation at 10,000g for 10 min

at 4°C. The RNA pellet was drained, dissolved in 1.0ml 0.05M ammonium acetate (pH 5.1) and reprecipitated from 2 vol. absolute ethanol at -20°C.

(b) Hot Phenol-sodium dodecyl sulphate technique

This was carried out essentially as described by Burdon and Clason (1969):-

Intact cells or subcellular fractions (mitochondria, nuclei, nucleoli and nucleoplasm) in 0.05M ammonium acetate (pH 5.1) were made 0.1%(w/v) with respect to sodium dodecyl sulphate, 0.5%(w/v) with respect to bentonite (purified as in section B1) and shaken at 60°C for 5 min with an equal volume of 80%(w/v) phenol equilibrated with the same buffer. The resultant emulsion was separated by centrifugation at 10,000g for 10 min in an MSE 18 high speed centrifuge at 4°C. The aqueous phase was then re-extracted with a further portion of phenol/sodium dodecyl sulphate in the presence of 0.5%(w/v) bentonite. The aqueous phase from this extraction was precipitated with 2 vol absolute ethanol at -20°C for 18 h and the precipitate collected by centrifugation at 2,500 rev/min for 10 min at 4°C. The pellet was drained, dissolved in 1.0ml 0.05M ammonium acetate buffer (pH 5.1) and centrifuged at 18,000g for 5 min to deposit the bentonite and the supernatant decanted into 2 vol absolute ethanol. RNA was collected after 18h by centrifugation

at 10,000g for 10 min. The RNA pellet was drained, dissolved in 1.0ml 0.05M ammonium acetate buffer and reprecipitated from 2 vol absolute ethanol at -20°C.

On occasions when the time of exposure to isotopically-labelled RNA precursors was short (of the order of 5 min or less) the growth medium was quickly decanted from the culture and 10ml of ice cold phenol-ammonium acetate-bentonite extraction mixture added directly to the cell monolayer in the 80oz winchester bottle or Roux flask. The bottle was then shaken vigorously for 2 min and the emulsion removed by pipette for further extraction as described above.

RNA samples isolated by the above procedures were suspended in either (a) 1.0ml of 0.05M ammonium acetate (pH 5.1) for gel filtration studies or sucrose gradient analyses, or (b) in 0.5ml 0.05M ammonium acetate (pH 5.1) containing 10%(w/v) sucrose and bromophenol blue (0.1%(w/v)) for examination by polyacrylamide gel electrophoresis, or (c) stored as an alcoholic slurry in 2 vol absolute ethanol containing 0.01%(w/v) sodium dodecylsulphate.

5) RNA Fractionation

(a) Gel Filtration Studies

These were carried out on long columns of Sephadex G100 at 4°C essentially according to the procedure of Galibert, Larsen, Lelong and Boiron (1965) with 0.05M ammonium acetate

(pH 5.1) as the eluting buffer. This procedure affords a separation of high molecular weight RNA, which is excluded from the gel matrix, from the lower molecular weight 5s and 4s components as indicated in fig.III-14, 3ml fractions of the eluate were collected in tubes containing 2 drops of 1.0%(w/v) sodium dodecylsulphate, and each fraction was examined for extinction at 260nm. Sodium dodecylsulphate was added to the tubes to minimise the risk of degradation of the RNA by contaminating nucleases.(Burdon et al(1967). Fractions were made 5%(w/v) with respect to trichloracetic acid and the acid insoluble radioactivity collected on membrane filters and assayed by liquid scintillation spectrometry. (section 7).

(b) Electrophoretic Analyses using Gels of Polyacrylamide

High molecular weight RNA was examined by electrophoresis on gels containing 2.5%(w/v) acrylamide and low molecular weight material was examined in gels containing 7.5%(w/v) acrylamide in a vertical tube system by one of the following procedures:-

(1) This was a system similar to that described by Loening (1967). The following stock solutions were prepared:- (A) 20%(w/v) Cyanogum 41 in distilled water, (B) 8ml of N,N,N',N',-tetramethylethylenediamine in 250ml of 0.32M-Tris-0.26M sodium acetate 0.016M disodium EDTA buffer

(pH 7.8), (C) 10%(w/v) ammonium persulphate in distilled water. These solutions were mixed as follows to provide a 7.5%(w/v) gel; 2 parts (A): 1 part (B):0.15 parts (C): 2.45 parts distilled water, and 2.0ml portions of this mixture were pipetted into 5mm x 120mm glass tubes. The surface of the gel was overlayed with a small volume of water and the gels polymerised in approximately 3 min. Electrophoresis of 100µl samples of RNA preparations dissolved in 0.05M ammonium acetate buffer containing 10%(w/v) sucrose and bromophenol blue was then carried out at room temperature at 5ma per gel in 0.04M-Tris-0.033M-sodium acetate-2mM disodium EDTA (pH 7.8) buffer. The gels were run in this system until the bromophenol blue marker dye, added with the RNA sample, was approximately 1cm from the end of the gel. The gels were then removed, stained in 0.1%(w/v) toluidine blue in distilled water (McIndoe and Munro, (1967) for 30 min and after destaining in distilled water overnight, were scanned for optical density using a Vitatron universal densitometer fitted with a U1 red absorption filter. After scanning, the radioactive gels were frozen in powdered Drikold, fractionated into 1mm segments and these treated for assay of radioactivity as described in section 5,3b.

(2) This was a modification of the method of Ornstein and Davis (1964).

The following stock solutions were prepared from acrylamide and methylene bisacrylamide purified as described below:- (A) 30%(w/v) acrylamide, 0.8%(w/v) methylene bisacrylamide in distilled water, (B) 0.46ml N,N,N',N',-tetramethylethylene-diamine in 100ml 0.3M-Tris/HCl(pH 8.2), (C) 10%(w/v) ammonium persulphate in distilled water. 7.5% gels were prepared by mixing these solutions as follows:- 1 part (A) 2 parts (B) 0.25 parts (C) 0.75 parts distilled water. The gels were cast and run at 5ma per gel, stained, destained and scanned as described in (1) above. Radioactive gels were treated as described and assayed as in section 5,3b.

(3) This was the system described by Loening (1967).

(a) Recrystallisation of the monomers

Since many impurities found in commercial preparations of acrylamide and methylene bisacrylamide are UV absorbing materials and would therefore interfere with the UV scanning of acrylamide gels, the monomers were first purified as described below.

Approximately 150g acrylamide was dissolved in 2l of AR chloroform at 60°C and the solution was filtered hot. Crystals were recovered by filtration in a chilled filter funnel after 18h at -20°C. The crystals were washed briefly with cold chloroform and then dried in an oven at 37°C.

Bisacrylamide was dissolved in acetone (approximately 10g/l. at 50°C and filtered hot. The solution was then allowed to cool slowly to -20°C and the crystals were recovered and washed with acetone by filtration.

(b) Preparation of gels

For the preparation of 2.5% gels a stock solution containing 15%(w/v) acrylamide, 0.8%(w/v) methylene bisacrylamide, purified as described above was prepared. For the preparation of 7.5% gels a stock solution containing 30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide was used. Suitable volumes of these solutions were mixed with appropriate quantities of 0.36M-Tris-0.3M-NaH_2PO_4-10mM disodium EDTA (pH 7.8) and water to give the desired concentration of acrylamide. 33µl of N,N,N',N',-tetramethylethylenediamine and 0.33ml of 10%(w/v) ammonium persulphate were added per gram of acrylamide present. The solution was then mixed and 2.8ml aliquots dispensed into 50mm x 120mm glass or plexiglass tubes. The gels were overlayed with a small volume of water and allowed to polymerise at room temperature. The 2.5% gels polymerised in 10 to 15 min, while the 7.5% gels only required about 1 min to polymerise. The gels were then pre-electrophoresed at 5ma per tube for 30 min in 0.036M-Tris-0.03M-NaH_2PO_4-1mM disodium EDTA (pH 7.8). 100µl to 200µl samples of RNA dissolved in the appropriate

buffer (section 4b) were then layered on top of the gels and electrophoresed at 5ma per gel for a suitable time in this buffer system at room temperature. After electrophoresis, the gels were removed and scanned for extinction at 260nm in the linear transport system of a Gilford recording spectrophotometer. Radioactive gels were frozen, sliced into 1mm segments, and radioactivity assayed by the procedures described below.

(b) Assay of radioactivity in polyacrylamide gel slices

[^{32}P]-labelled gel slices were dried on 2.5cm diameter Whatman No. 1 filter discs and radioactivity assayed by low background gas flow counting or by liquid scintillation spectrometry in a toluene based scintillator. [^{3}H] and [^{14}C]-labelled samples were assayed for radioactivity by one of the following procedures.

(1) The gel slices were dried in scintillation vials by incubating at 60°C for 2h 0.3ml of 30%(w/v) hydrogen peroxide was then added per vial, the vials capped and incubated at 60°C for 18h. After this period the gel slices had been totally dissolved and 10ml of scintillator consisting of 4 parts 2-methoxyethanol to 6 parts toluene based scintillant was added to each vial. The radioactivity was assayed by liquid scintillation spectrometry. This is a modification of the method of Tishler and Epstein(1968).

(2) [^{3}H] and [^{14}C]-labelled gel slices were immersed in 0.3ml of 0.880 ammonia and incubated at 37°C for 18h. To each dried sample was added 0.5ml distilled water and the gel slices allowed to swell by incubation at 37°C for 1 h. 10ml of a scintillant consisting of 4 parts 2-methoxy-ethanol to 6 parts toluene based scintillator was then added to each vial and radioactivity assayed by liquid scintillation spectrometry.

(c) Sucrose Gradient Analyses

0.3ml of the appropriate RNA sample was layered on top of a linear gradient of 5%(w/v) to 20%(w/v) sucrose in 0.05M ammonium acetate (pH 5.1) -0.01%(w/v) sodium dodecyl-sulphate prepared according to the method of Britten and Roberts (1960). After centrifugation for 3¾h at 40,000 rev/min in an SW50 rotor or for 5h at 39,000 rev/min in an SW40 rotor of a Beckman model L2 65B ultracentrifuge at 4°C, fractions of an appropriate volume were collected in tubes containing 2 drops of 1%(w/v) sodium dodecyl-sulphate. SW40 gradients were harvested by passage of the sample through the flow cell of a Gilford recording spectrophotometer where the extinction at 260nm was automatically recorded. SW50 gradients were harvested in 2 drop fractions by puncturing the base of the centrifuge tube. Each fraction so collected was then examined for extinction at 260nm; trichloracetic acid was added to a

final concentration of 5%(w/v) and the acid insoluble radioactivity was collected on membrane filters and assaye d by liquid scintillation spectrometry in a toluene based scintillator.

(6) CsCl centrifugation

(a) Equilibrium centrifugation

Samples to be analysed were made 4%(w/v) with respect to formaldehyde (Spirin, Belitsna & Lerman, 1965) in an ice bath and after 2h were mixed with sufficient CsCl solution to yield a final density of 1.48g/cm^3. The CsCl solution was made by dissolving 10g of CsCl in 6.4ml TKM buffer, followed by the addition of 0.05ml of 1M $MgCl_2$ and 0.51ml of 40%(w/v) formaldehyde. The gradients were centrifuged at 40,000 rev/min at 4^{o}C in an SW65 rotor of a Beckman model L2 65B or at 36,000 rev/min in an SW40 rotor of a Griffen Christ ultracentrifuge for 24h to 48h. Centrifugation gradients were harvested in 3 drop fractions and every fifth tube examined for density determination using an Abbe refractometer. Each fraction was then made 5%(w/v) with respect to trichloracetic acid, acid insoluble radioactivity collected on membrane filters and assayed by liquid scintillation spectrometry.(section 7).

(b) On preformed gradients of CsCl

Samples to be analysed were fixed in 8% glutaraldehyde, previously neutralised with 1M NaOH, and the fixed particles

layered on top of a preformed linear gradient of 30%(w/v) to 80%(w/v) CsCl in RSB buffer (section 3(a) ?) and centrifuged for 12h to 18h as described in (a) above. The gradients were then harvested and analysed as described above.

(7) Procedures for the estimation of radioactivity in fractions from gel filtration studies and centrifugation gradients.

Aqueous samples, arising from the analyses of RNA by gel filtration or centrifugation in density gradients, were made 5%(w/v) with respect to trichloracetic acid and acid insoluble radioactivity collected on membrane filters. The filters were washed with an appropriate volume of 5%(w/v) trichloracetic acid and, after drying, radioactivity was assayed by liquid scintillation spectrometry in 10ml of a toluene based scintillant.

(8) Determination of base composition of RNA samples

(1) [$^{32}P_i$]-labelled RNA was isolated from monolayer cultures of BHK-21/C13 cells grown in 100ml ETC_{10} containing 500uCi [$^{32}P_i$]- orthophosphate, for periods of 24 to 48h. Such [$^{32}P_i$]-labelled RNA preparations were fractionated on 2.5% and 7.5% polyacrylamide gels as described in section 5b, the gels frozen, sliced, and radioactivity assayed as described in section 7. Gel slices corresponding to distinct RNA species, as judged by optical density, were pooled and RNA eluted by the following procedure.

(2) Elution of RNA from polyacrylamide gel slices

This was carried out according to the method of Sirbasku and Buchanan, (1970). Dried gel slices were further fragmented by the use of scissors and the fragments immersed in 2.0ml of 2x SSC containing 0.1%(w/v) sodium dodecyl sulphate. This system was then incubated at 37°C for 48h and the gel fragments separated from the liquid, which now contained at least 70% of the radioactivity, by centrifugation at 2,500 rev/min. The RNA solution was then dialysed against 100 vol. 2x SSC to remove the sodium dodecyl sulphate and evaporated to dryness.

(3) Hydrolysis of RNA samples

RNA samples, eluted from polyacrylamide gel slices as described above were suspended in 100µl of 0.3M KOH and incubated at 37°C for 18h. 1mg Esch. coli sRNA was similarly treated and after incubation at 37°C the RNA samples were neutralised by the addition of an equimolar amount of perchloric acid, and the $KClO_4$ precipitate removed by centrifugation at 2,500 rev/min.

(4) High voltage electrophoresis of RNA hydrolysates

50µl to 100µl of an alkaline hydrolysate of BHK-21/C13 RNA together with 10µl of a similar hydrolysate of Esch. coli sRNA, prepared as described above and adjusted to pH 3.5 with perchloric acid, was applied as a 2cm streak to a

46cm x 57cm Whatman 3MM paper. This was subsequently moistened with pyridine-acetate-EDTA buffer, (pH 3.5) (16ml pyridine, 17-ml glacial acetic acid, 18.5g disodium EDTA water to 5,000ml.) Electrophoresis was carried out at 300 volts for 30 min, to remove salt, then at 3,000 volts for 3h. The dried chromatograms were then exposed to steam in an autoclave for 15 min at atmospheric pressure, which effectively removed the ultraviolet absorbing solvent, and the nucleotides were located under ultraviolet light. The spots were cut from the paper and sectioned into scintillation vials, 0.5ml hyamine hydroxide was then added to each vial and the vials incubated at 37°C for 30 min. 10ml of toluene based scintillator was then added to each vial and radioactivity assayed by liquid scintillation spectrometry. Electrophoresis by this method is as according to Sebring & Salzman (1964).

RESULTS

III. RESULTS

III.1 Low molecular weight RNA content of different fractions of mammalian cells.

The improved resolution of low molecular weight RNA species afforded by polyacrylamide gel electrophoresis (Loening, 1967) has enabled the identification of a multiplicity of hitherto unidentified RNA species in a wide variety of both eukaryotes and prokaryotes. The optical density profiles of RNA extracted from the nuclear and cytoplasmic fractions of BHK-21/C13 cells (BHK-21 cells), after electrophoresis in gels of polyacrylamide, are shown in Fig. III.1. The units of optical density are expressed in an arbitrary scale proportional to the extinction at 260nm and suggest the relative amounts of each RNA species present in the cells. These species are composed of RNA in that they are labelled with RNA precursors such as [^{3}H]-uridine and [^{32}P]-orthophosphate, are resistant to degradation by DNase and pronase and are degraded by treatment with RNase or alkali. Cytoplasmic RNA was extracted from 10^{8} BHK-21 cells by the "cold phenol" technique described in Methods (section 4(a)) and nuclear RNA was extracted from the detergent cleaned nuclei of approximately 5×10^{8} cells by the "hot-phenol-SDS" technique (Methods section 4(b)). The RNA extracted from intact cells by the "cold phenol" procedure shows an identical optical density profile, after

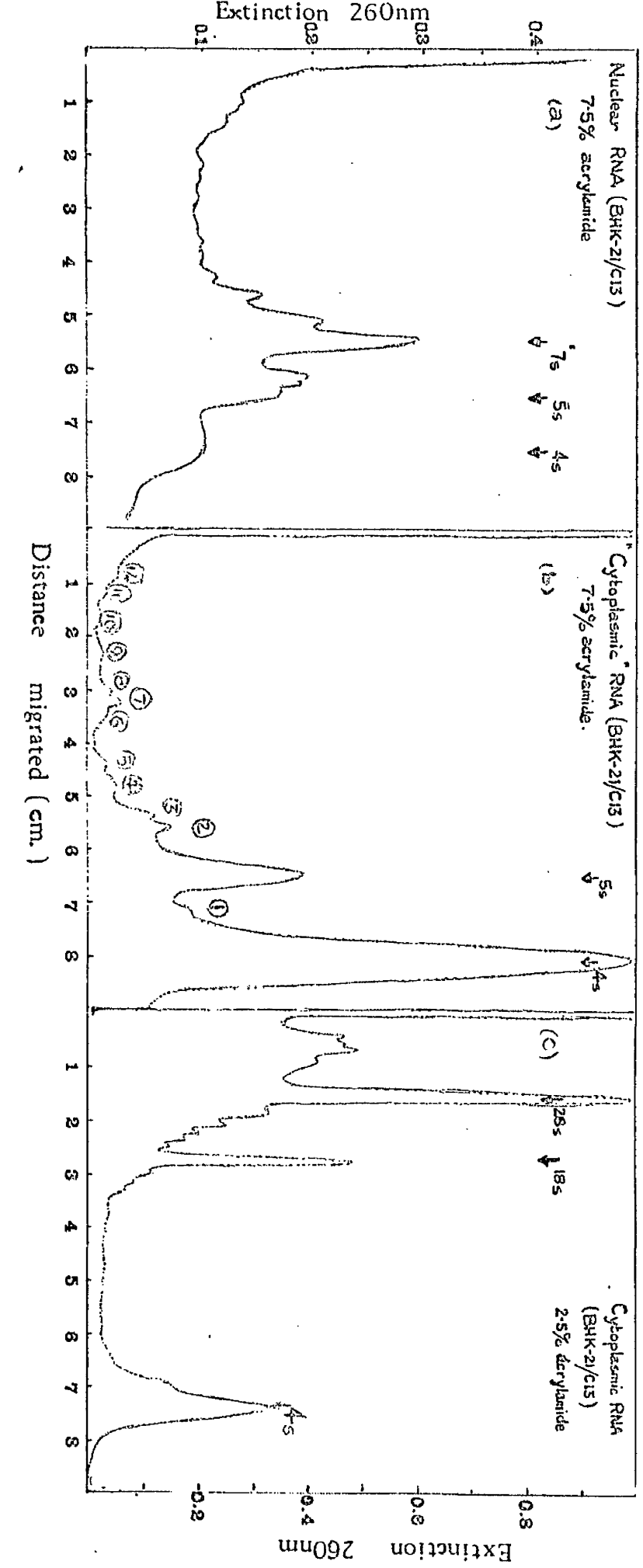
Optical density profiles of RNA components of BHK-21/C13 cells after electrophoresis in gels of polyacrylamide.

Fig. III.1

Cytoplasmic (cold phenol) or nuclear RNA was prepared from 10^8 or 5×10^8 BHK-21/C13 cells and analysed by acrylamide gel electrophoresis as described in Methods (section 5(b)). Following electrophoresis, the optical density of each gel was measured using either a Vitatron Universal densitometer fitted with a U1 red filter, for gels stained with toluidine blue, or the linear transport attachment of a Gilford recording spectrophotometer for unstained gels. 7.5% gels, for the analysis of low molecular weight RNA components of the nucleus or cytoplasm, were electrophoresed for approximately $5\frac{1}{2}$h and 2.5% gels, for the examination of high molecular weight RNA species were similarly electrophoresed for $2\frac{3}{4}$h. The ordinates are expressed in arbitrary units proportional to the extinction at 260nm.

(a) low molecular weight RNA from the nuclei.
(b) low molecular weight RNA from the cytoplasm.
(c) high molecular weight RNA from the cytoplasm.

The ordinates on the left hand scale refer to diagrams (a) & (b) and those on the right to diagram (c)


Extinction 260nm
Nuclear RNA (BHK-21/C13)
7·5% acrylamide
(a)
7s
5s
4s
"Cytoplasmic" RNA (BHK-21/C13)
7·5% acrylamide.
(b)
5s
4s
(c)
28s
18s
4s
Cytoplasmic RNA
(BHK-21/C13)
2·5% acrylamide
Distance migrated (cm.)
Extinction 260nm

electrophoresis in gels of polyacrylamide, as that obtained from examination of RNA extracted by this procedure from cytoplasm prepared by the hypotonic rupture of BHK-21 cells in RSB buffer (cf. Methods section 3(c)). High molecular weight RNA was analysed by electrophoresis in 2.5% polyacrylamide gels and low molecular weight RNA components (defined as RNA molecules with an average chain length not exceeding 500 nucleotides) were examined by electrophoresis in 7.5% polyacrylamide gels from which the high molecular weight RNAs are excluded.(Methods sect. 5(b)). Similar profiles to those shown in diagrams (b) and (c) were obtained as a result of electrophoresis of RNAs prepared from the cytoplasm of a number of mammalian tissue culture cell lines, (Hela, L929, BHK-21/C13, BHK-21/SR8/V1, BHK-21/PyY) and Krebs II mouse ascites tumour cells. Fig.III.1 presents the relative proportions of the various RNA species present in mammalian cells but a more accurate measure of the relative molar amounts of these RNA species present in the cytoplasm of BHK-21 cells is presented in Table III.1 The amounts listed there represent the estimated steady-state number of molecules per cell as computed from the optical density tracings and the estimated molecular weights of the RNA species. These molecular weight estimates, also shown in Table III.1, were made assuming a logarithmic relationship between migration

Table III.1

ecies of A	REM†	Estimated Mol. Wt.	Estimated no. of nucleotides	Estimated molecules per cell.
RNA(tRNA)	80	2.8×10^4 *	80*	5.5×10^7
	72	3.5×10^4	100	4.4×10^6
RNA	64	3.9×10^4	115	1.0×10^7
	56	4.5×10^4	130	2.2×10^6
	54	5.0×10^4	145	1.8×10^6
	48	5.6×10^4	160	7.7×10^5
	43	6.3×10^4	180	4.8×10^5
	35	7.2×10^4	205	2.5×10^5
	32	7.9×10^4	225	2.9×10^5
	29	8.9×10^4	250	2.4×10^5
	18	1.1×10^5	315	8.1×10^4
	15	1.25×10^5	350	1.0×10^5

ayhoff & Eck (1968) and represents an average value for ammalian tRNA

elative electrophoretic mobility expressed in mm travelled rom origin of the gel.

species of RNA represented in Table III.1 are enumerated as Fig. III.1(b). The molecular weights shown are estimated uming a logarithmic relationship between electrophoretic ility and molecular weight (Bishop et al,1967) adopting the wn molecular weights of 4s and 5s RNA as standards. From average molecular weight of one nucleotide (computed as daltons), the molecular lengths of the various RNA chains e computed assuming no contributions to electrophoretic ility by conformational differences.

rates in polyacrylamide gels and molecular weights (Bishop, Claybrook & Spiegelman, 1967). The relative migration rates are quite reproducible from one gel to another but the estimation of molecular weights is empirical and may be slightly inaccurate, a more accurate determination must await precise biochemical analysis of these RNA species. A comparison of Figs. III.1(a) and (b) reveals that the nuclei of mammalian cells contain relatively low levels of the 4s RNA (tRNA) components which contribute approximately 80% of the low molecular weight RNA of the cytoplasm. It is also apparent that such nuclei contain a large amount of 5s RNA molecules, in accord with the results found in Hela cells by Knight & Darnell (1967) and Weinberg & Penman (1969). It should also be noted that a number of nuclear RNA species display electrophoretic mobilities in polyacrylamide gels comparable to the species found in the cytoplasm. The most prominent of the nuclear RNA species, that referred to as "7s RNA" is apparently absent from the cytoplasmic preparation shown in Fig. III.1(b). This however is a facet of the RNA extraction procedure, for this RNA species can also be found in the cytoplasm if the isolated subcellular fraction is extracted with the hot-phenol-SDS technique (Burdon & Clason, 1969). It is a component of the larger ribosomal subunit and remains associated with the 28s RNA component during "cold

phenol" extraction but is melted free of the 28s rRNA upon heating the preparation to the temperatures used in the "hot-phenol-SDS" extraction procedure (Pene et al, 1968). This RNA fraction exhibits both a sedimentation velocity and an electrophoretic mobility of approximately 5.5s (Weinberg & Penman, 1969) and has thus been referred to by these authors as "28s-associated RNA".

III.2 Synthesis of low molecular weight cytoplasmic RNA. Previous reports indicate that the study of the labelling behaviour of low molecular weight nuclear RNA components during short labelling periods is made difficult by the presence of large amounts of co-sedimenting unstable nuclear heterogeneous RNA of low molecular weight (Weinberg & Penman, 1969). No similar heterogeneous material appears to exist in the cytoplasm however and it is therefore possible, using the selective extraction of cytoplasmic RNA species by the "cold phenol" technique (Burdon & Clason, 1969, Harris 1963), to investigate the synthesis of the low molecular weight RNA components of the cytoplasm. The results of such an experiment are shown in Fig. III.2 (a) and (b). Identical cultures of BHK-21 cells were exposed to [^{3}H]-guanosine for periods of 5min, 10min, 20min, and 60min and the "cold phenol" RNA examined by electrophoresis on gels of polyacrylamide. Fig. III.2(a) shows the electrophoretograms of cytoplasmic

Labelling kinetics of low molecular weight RNA components of BHK-21/C13 cell cytoplasm.

Fig. III.2

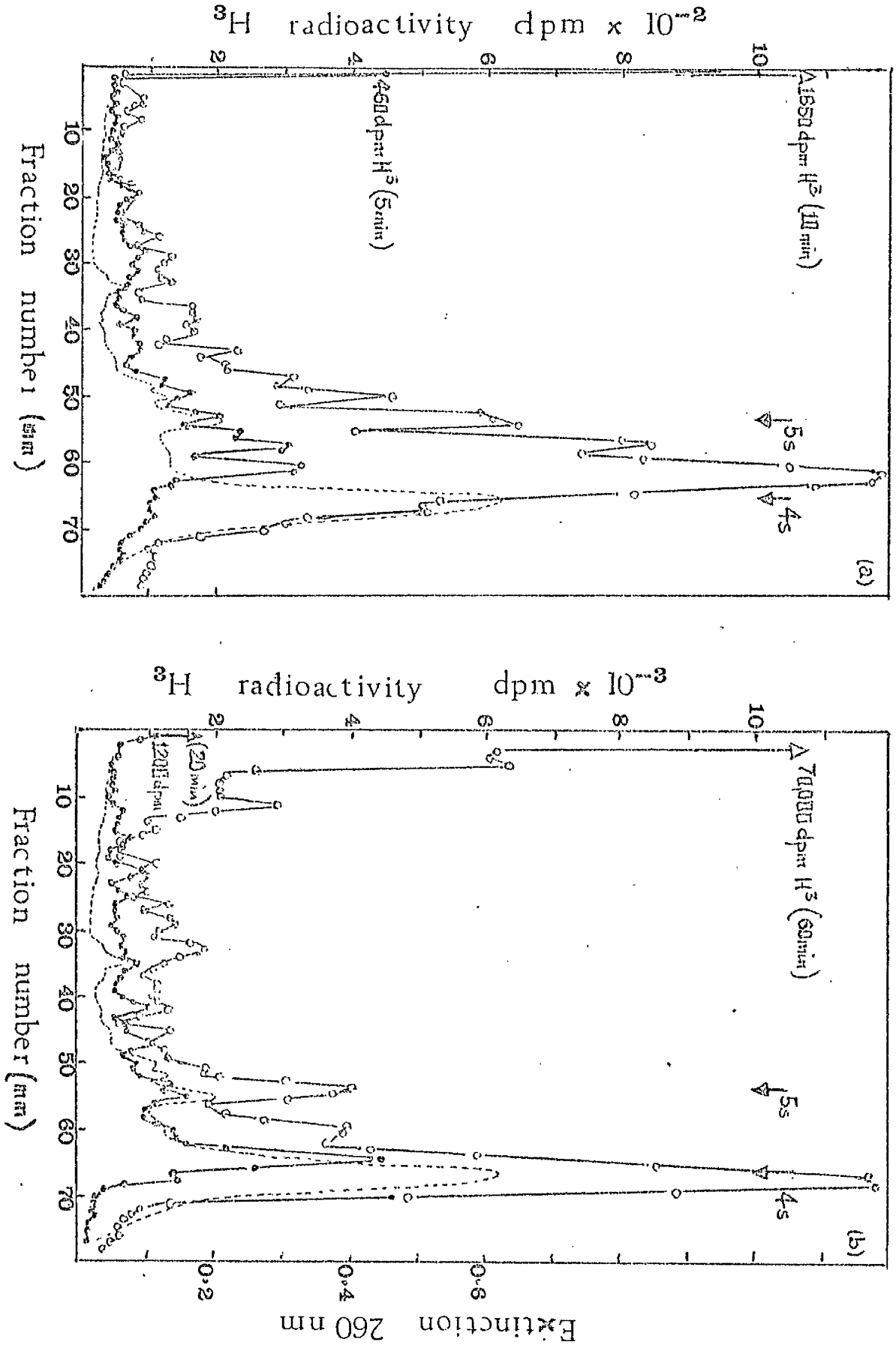
Profiles of the distribution of radioactivity in low molecular weight RNA components of BHK-21/C13 cytoplasm after electrophoresis in gels of polyacrylamide (Methods section 5(b)).

Cultures of BHK-21/C13 cells(30×10^6 in 100ml EC_{10}) were grown for 18h at 37°C in rotating 80oz. winchester bottles. At this time there was added to each culture 100μCi [^{3}H]-guanosine (6Ci/mmol) and the cultures exposed to isotopically labelled precursor for the appropriate time period. The cells were then harvested, washed with ice cold BSS, and RNA extracted by the "cold phenol" procedure (Methods section 4(a)) and examined by polyacrylamide gel electrophoresis as described in Methods section 5(b).

(a) Extinction 260nm (------), [^{3}H]-radioactivity after a 5min exposure to isotope (—o—), [^{3}H]-radioactivity after a 10min exposure to isotope (—o—).

(b) Extinction 260nm (----), [^{3}H]-radioactivity dpm after a 20min exposure to isotope(—o—), [^{3}H]radioactivity dpm after a 60min exposure to isotope (—o—).

The arrows in Figs.III.2(a) and (b) indicate the optical density peaks corresponding to the RNA components referred to as 4s (tRNA) and 5s RNA. On the left hand edge of each profile is recorded the [^{3}H]-radioactivity in RNA species excluded from the gel matrix and which did not enter the gel.


^{3}H radioactivity dpm x 10^{-2}
A 1650 dpm H^3 (10 min)
450 dpm H^3 (5 min)
5s
4s
(a)
Fraction number (5 m)
^{3}H radioactivity dpm x 10^{-3}
A 7000 dpm H^3 (60 min)
A (20 min)
1200 dpm
5s
4s
(b)
Fraction number (5 m)
Extinction 260 nm

RNA species extracted from cells labelled for 5min and 10 min, and Fig.III.2(b) the electrophoretograms of these RNA species from cells exposed to isotope for 20min and 60min. It is apparent that after short time exposures, (5min) the bulk of the radioactively labelled RNA migrates in a somewhat heterogeneous fashion in positions intermediate between the ribosomal 5s RNA and the 4s RNA optical density peaks, but that the radioactivity, in fractions with a lower electrophoretic mobility than 5s RNA, corresponds fairly well with the optical density profile. When the labelling period is increased to 10min, much of the radioactivity still migrates in positions intermediate between 4s and 5s RNA but there is now a distinct fraction of the radioactivity associated with both the 4s and 5s RNA peaks and also with those optical density peaks showing a lower electrophoretic mobility than 5s RNA. After a 20min or 60min exposure of the cells to radioactivity (Fig. III.2(b)) the distribution of radioactivity follows much more closely the optical density profile but the radioactive material corresponding to 4s RNA does not quite correspond exactly with the optical density marker at 20min but does so after 60 min. From the profiles of distribution of radioactivity shown in Figs. III.2(a) and (b) it is apparent that the species of RNA shown in the optical density profile are distinct RNA species which seem to bear no obvious

precursor-product relationship one to another. The bulk of the radioactivity which at short time periods migrates in positions intermediate between 5s and 4s RNA, and which contributes an ever decreasing proportion of the total radioactivity as the time of exposure to isotope is increased, displays the characteristics of the tRNA precursor molecules previously described in mammalian cell cytoplasm after short time pulses of radioactivity (Burdon & Clason, 1969, Bernhardt & Darnell, 1969; Smillie, 1970). This is the only cytoplasmic low molecular weight RNA component which appears to function as a precursor molecule. If the kinetics of labelling of each individual RNA species present in the optical density profile is plotted as the incorporation of radioactivity versus the time of exposure to isotope, it is found that the synthesis of these species proceeds, after a short lag of approximately 5min, and which may represent the time for equilibration of isotope with the cellular nucleotide pools, in a fairly linear fashion, following the same pattern as the incorporation of $[^3H]$-guanosine into total acid insoluble material (see Fig. 11.1(b)). These observations may therefore indicate that these low molecular weight RNA components of the cytoplasm may be derived not as a result of direct transcription but as the cleavage product from some larger RNA molecules, or indicate the possible existence of a pool

of these molecules located in the nucleus.

III.3 Comparison of long and short exposures to isotope.

Exposure of cell cultures to isotopically labelled RNA precursors for periods of one generation time or longer labels predominantly the stable RNA components of the cell. Since the low molecular weight RNA components displayed in Fig.III.2 (a) and (b) appear to be fairly extensively labelled in short time periods such as 30min or one hour, an indication of their metabolic stability may be gained by the comparison of the profiles of distribution of radioactivity in these RNA species after short and long exposures to radioisotopes. Therefore, petri-dish cultures of BHK-21 cells were grown in the presence of $[^{32}P]$-orthophosphate for periods of 24 to 48 hours, the "cold phenol" RNA extracted (Methods section 4(a)) and examined by electrophoresis in polyacrylamide gels as before. The distribution profiles of such "long labelled" RNA is shown in Fig.III.3. This profile is distinctly different from that obtained with the shorter pulse times shown in Fig.III.2(a) and (b) and indicates that many, if not all, of the low molecular weight cytoplasmic RNA species, with a lower electrophoretic mobility than 5s RNA, are metabolically unstable. In addition it can be seen that both 5s RNA and tRNA are extensively labelled and presumably therefore represent stable components. Fig.III.3 shows the profile of cytoplasmic RNAs

Distribution of radioactivity in RNA components of BHK-21 cytoplasm after long exposure of cultures to isotopically labelled RNA precursors.

Fig. III.3

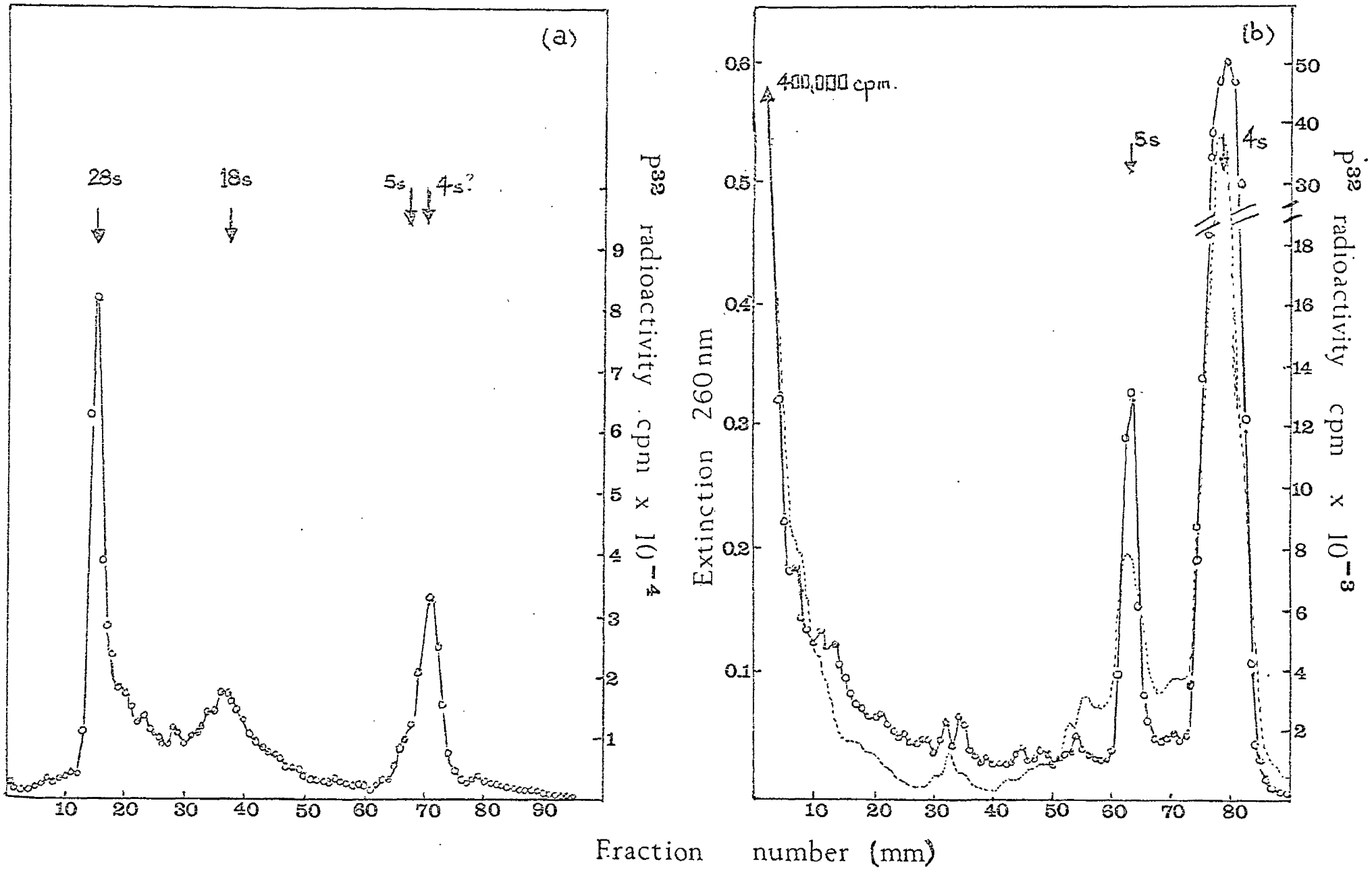
Cultures of BHK-21/C13 cells (3.0×10^6 cells/90mm petri dish) were grown for 48h in 100ml ETC_{10} containing 400μCi[^{32}P]-orthophosphate (60Ci/mg P) at 37°C in an atmosphere of air containing 5%(v/v) CO_2. The radioactive growth medium was then removed, the cell monolayer washed twice with 10ml portions of ice cold BSS and the cells removed from the dish by treatment with trypsin/versene. The collected cell pellet was then extracted with "cold phenol and the cytoplasmic RNA isolated as described in Methods section 4(a). The RNA, after dissolution in the appropriate buffer (electrophoresis buffer Methods section 5(b)), was examined by electrophoresis in gels of polyacrylamide. (Methods section 5(b)). The gels were scanned for optical density using the linear attachment of a Gilford recording spectrophotometer, frozen, sliced into 1mm segments and radioactivity assayed by gas flow counting as described in Methods section .

(a) High molecular weight RNA components examined by electrophoresis in 2.5% polyacrylamide gels.

[^{32}P]-radioactivity cpm (—∘—).

(b) Low molecular weight RNA components examined by electrophoresis in 7.5% polyacrylamide gels.

Extinction 260nm (----), [^{32}P]-radioactivity cpm (—∘—).


(a)
28s
18s
5s
4s?
P32 radioactivity cpm x 10−4
10
20
30
40
50
60
70
80
90
(b)
400,000 cpm.
5s
4s
Extinction 260 nm
P32 radioactivity cpm x 10−3
0.6
0.5
0.4
0.3
0.2
0.1
Fraction number (mm)

extracted from cells labelled for 48 hours but a similar, although quantitatively different, profile of distribution of radioactivity was obtained with RNA extracted from cells exposed to isotope for 24 hours.

III.4 Methylation of low molecular weight cytoplasmic RNA.

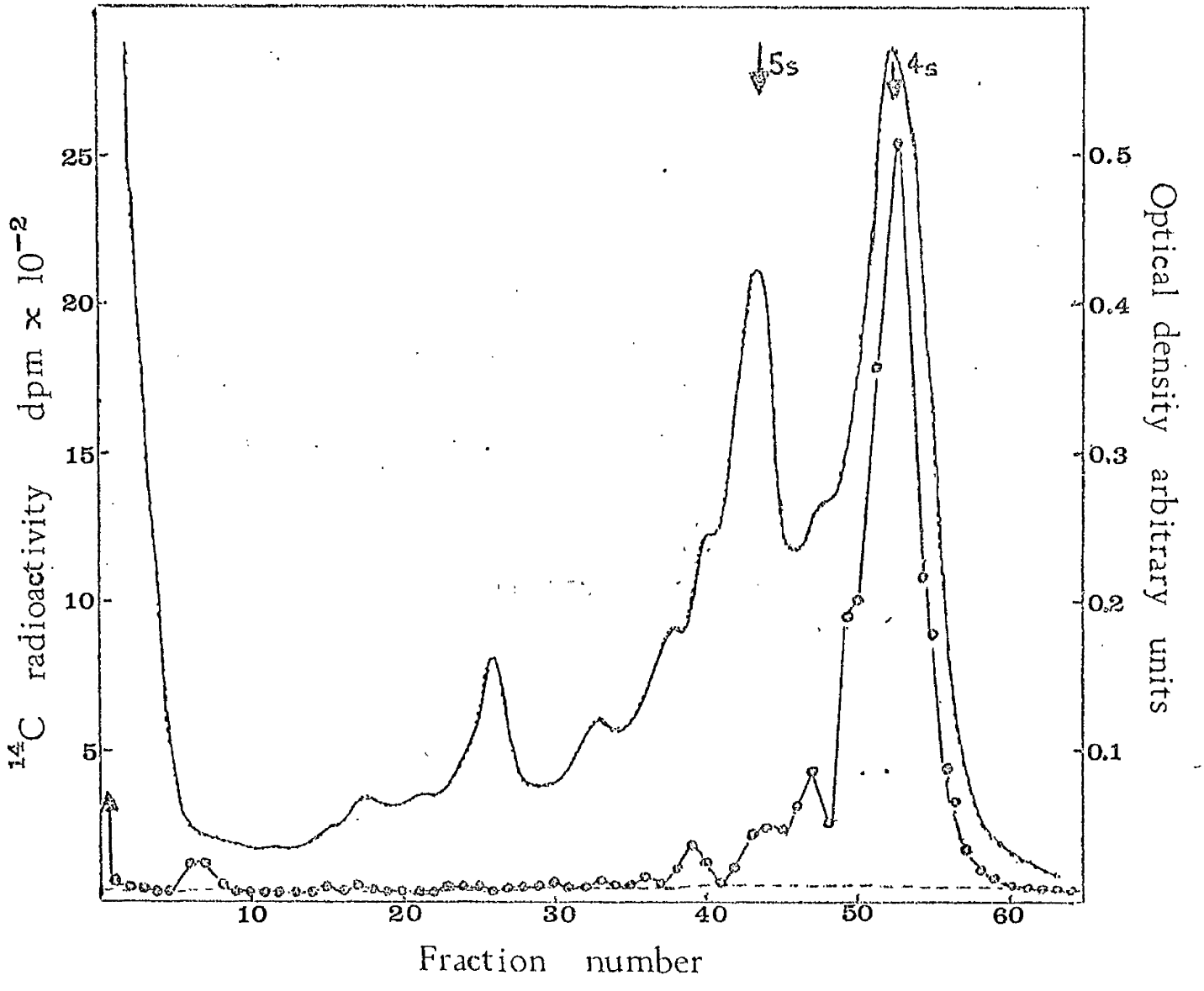
Three classes of RNA are presently known to contain methylated nucleosides; tRNA, rRNA and its nuclear precursors, and the low molecular weight RNAs of the eukaryotic nucleus. It is assumed that, as in E.coli, methyl groups in eukaryotic RNA molecules arise directly from L-methionine via the methyl-donor S-adenosylmethionine (Mandel & Borek, 1961, 1963, Fleissner & Borek, 1962). Should the low molecular weight RNA components of the cytoplasm examined in Fig.III,2(a) and (b) be the degradation products of either rRNA, rRNA-precursors, precursors to tRNA, or leakage to the cytoplasm of low molecular weight components of the nucleus, it might be expected that they would be methylated. It was therefore decided to examine the low molecular weight cytoplasmic RNA species for their content of methylated nucleosides. The measurement of the extent of methylation implies the measurement of methyl groups which have been joined to the RNA molecule subsequent to its transcription from the DNA template. Care must therefore be taken to prevent radioactivit from labelled methyl groups from being incorporated into

Methylation of low molecular weight RNA components found in cytoplasmic fraction of BHK-21/C13 cells.

Fig. III.4

A culture of BHK-21/C13 cells (30 x 10^6 cells in 100ml ETC_{10}) were grown for 18h at 37°C. The growth medium was then removed and replaced with 100ml prewarmed ETC_{10} medium containing 20μCi L-methionine (methyl-^{14}C) (56.8mCi/mmol) and the incubation continued for a further 18h. After this time the culture was harvested, the cells washed and pelleted as described in Methods section 2(b)., and cytoplasmic RNA isolated from the cell pellet as described in Methods section 4(a). The RNA was dissolved in electrophoresis buffer and examined by electrophoresis in gels of polyacrylamide and the gels then stained with toluidine blue and scanned for optical density as described in Methods section (5b). The scanned gels were frozen, sliced into 1mm segments and the segments assayed for radioactivity by liquid scintillation spectrometry. (Methods section 5(b)).

Optical density, arbitrary units (——), [^{14}C]-radioactivity dpm, (—o—).
Background level of radioactivity (- - - - -).


5s
4s
25
20
15
10
5
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
^{14}C radioactivity dpm x 10^{-2}
Optical density arbitrary units
Fraction number

moeities other than the methyl groups attached to the ribose or base moeities of the constituent nucleosides of the RNA molecule. In particular the incorporation of radioactivity from methyl groups into the purine ring skeletons via the one carbon pool will confuse any examination, either quantitative or qualitative, of the degree of methylation of RNA. In this experiment the entrance of label into the purine biosynthetic pathways was suppressed by the addition to the growth medium of 20mM-sodium formate (Winocour, Kay & Stoller, 1965). Such an addition has been shown to have no effect upon the growth rate of BHK-21 cells and presumably therefore upon the rate and extent of RNA synthesis (Low, 1970). A culture of BHK-21 cells was therefore grown overnight in the presence of L-[methyl-^{14}C]-methionine and the cytoplasmic RNA isolated by the "cold phenol" technique as before. In order to ensure that the incorporated methyl radioactivity was present on the base or ribose moeities of any apparently methylated RNA species and not as an aminoacyl ester, this cytoplasmic RNA preparation was incubated at 37 in 0.05M-tris/HCl buffer (pH 10) for 30min (Svensson, Boman, Eriksson & Kjellin, 1963) prior to its examination by polyacrylamide gel electrophoresis. The results of such an examination of low molecular weight cytoplasmic RNAs are shown in Fig. III.4 and indicate that the only methylated species present in the cytoplasm of BHK-21

Base compositions of the low molecular weight RNA Components of BHK-21/C13 cytoplasm.

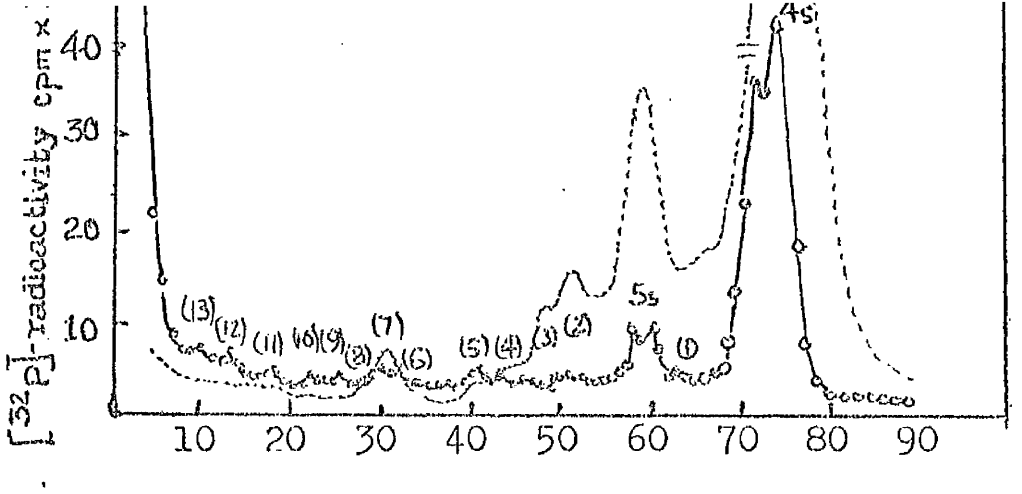
Fig.III.5

A culture of BHK-21/C13 cells (10^7 cells in 10ml ETC_{10}) grown for 18h at 37°C in petri dishes, was exposed to [^{32}P]-orthophosphate (70Ci/mg P) (500μCi per dish) for a further 48h. After this time, the radioactive medium was decanted, the cell monolayers washed with two 10ml portions of ice cold BSS and the cells removed from the dishes with the aid of a rubber policeman. "Cold phenol" cytoplasmic RNA was then isolated from the harvested cell pellet (Methods section 4(a)) and after dissolution in the appropriate buffer (Methods section 4(b)) was mixed with unlabelled cold phenol cytoplasmic RNA(marker RNA) and electrophoresed in both a 2.5% and a 7.5% polyacrylamide gel. The gels were scanned for optical density, frozen, sliced into 1mm segments and the fractions assayed for radioactivity as described in Methods section 5(b).

Extinction 260nm (----), [^{32}P]-radioactivity cpm (—•—).

Table III.2

Appropriate fractions of the gels and corresponding to the low molecular weight RNA components as enumerated in Fig.III.5 were further fragmented and the RNA eluted from them by the method of Sirbasku & Buchanan (1970). Nucleotide composition analyses were then performed on each RNA species, enumerated in Fig.III.5, as described in Methods section B.8. Ribosomal RNA species (28s and 18s) obtained from the 2.5% polyacrylamide gel electrophoretic analysis, were similarly treated.



Fraction number

Table III.2 Nucleotide composition of low molecular weight cytoplasmic RNA components of BHK-21/C13 cells

RNA species	Base (moles per 100 moles)				fractions
	Adenine,	Guanosine,	Cytosine,	Uridine	
4s RNA	18.8	34.6	35.4	11.2	72-75
5s RNA	27.8	26.9	31.2	14.1	58-61
1	24.2	30.3	33.3	12.2	63-65
2	23.7	28.6	32.2	15.5	51-53
3	24.4	22.9	31.4	21.3	48-50
4	20.9	28.4	36.0	14.8	43-45
6	23.9	18.1	43.3	14.6	32-34
7	23.9	16.2	22.1	37.8	30-32
8	35.5	11.7	37.8	11.7	27-29
9	36.8	17.1	30.6	15.5	24-26
10	22.9	20.4	36.4	20.3	22-23
11	23.0	21.5	36.1	19.4	17-19
12	21.6	14.4	48.9	15.0	12-14
13	25.0	19.2	34.6	21.2	9 -11
28s RNA	21.2	28.1	39.7	11.0	-
18s RNA	23.4	28.9	37.4	10.3	-

cells are tRNA and possibly the RNA species referred to in Table III.1 as component (1). It is also possible that a minor component which co-electrophoreses with 5s RNA is methylated, for a similar methylated minor"5s" species has been described in the low molecular weight RNA of Hela cell nuclei (Weinberg & Penman, 1969). The apparent absence of extensive "methylation" of the ribosomal 5s RNA however shows the efficiency of the sodium formate dilution of the one carbon pool and indicates no incorporation of methyl groups into purine ring skeletons. The pattern of methylation shown in Fig III.4 therefore probably represents true methylation of the base or ribose mocities of the RNAs and indicates the absence of methyl ation in the RNA species with lower electrophoretic mobilities than 5s RNA (referred to as species (?) - (10) in Table III.1 and Fig III.5.).

III.5 Base composition of the cytoplasmic RNA.

The base compositions of the small molecular weight cytoplasmic RNA components have been examined by the $[^{32}P]$-orthophosphate equilibrium labelling technique, (Volkin & Astrachan, 1956). Petri-dish cultures of BHK-21 cells were grown for 48 hours in phosphate depleted medium containing carrier-free $[^{32}P]$-orthophosphate. The cytoplasmic RNA was then isolated by the "cold phenol" technique (Methods section 4(a)) and the RNA examined by polyacrylamide gel electrophoresis as before.

The profile of distribution of radioactivity in the small molecular weight RNA components is indicated in Fig III.5. Fractions of the gel corresponding to the RNA species enumerated as (1)-(13), tRNA and 5s RNA were taken, the RNA eluted and the base compositions determined by the procedure described in the Methods section. The results of these analyses are listed in Table III.2. The base compositions of the various species of RNA vary significantly and are consistent with the possibility that each species represents a unique type of RNA. The RNA species referred to in Table III.1 as (2)-(10) are seen to be relatively (G+C) rich but to be significantly different in base composition from the ribosomal RNA species, 28s and 18s RNA, whose base composition is shown for comparison.

III.6 Intracellular location of cytoplasmic low molecular weight RNA molecules.

The experiments reported in Fig III.2(a) and (b) have indicated that the small molecular weight RNAs found in the cytoplasm appear rapidly in this fraction in BHK-21 cells. Unlike the precursor tRNA molecules which form the bulk of the radioactively labelled cytoplasmic RNA in short time pulses, the species (2) to (10) do not appear to be the substrates for the cytoplasmic methylases. However the precise location of these low molecular weight RNA components have not been determined. In order to do this, cultures of BHK-21 cells were exposed to [^{14}C]-uridine

Intracellular location of low molecular weight RNA components of BHK-21/C13 cytoplasm.

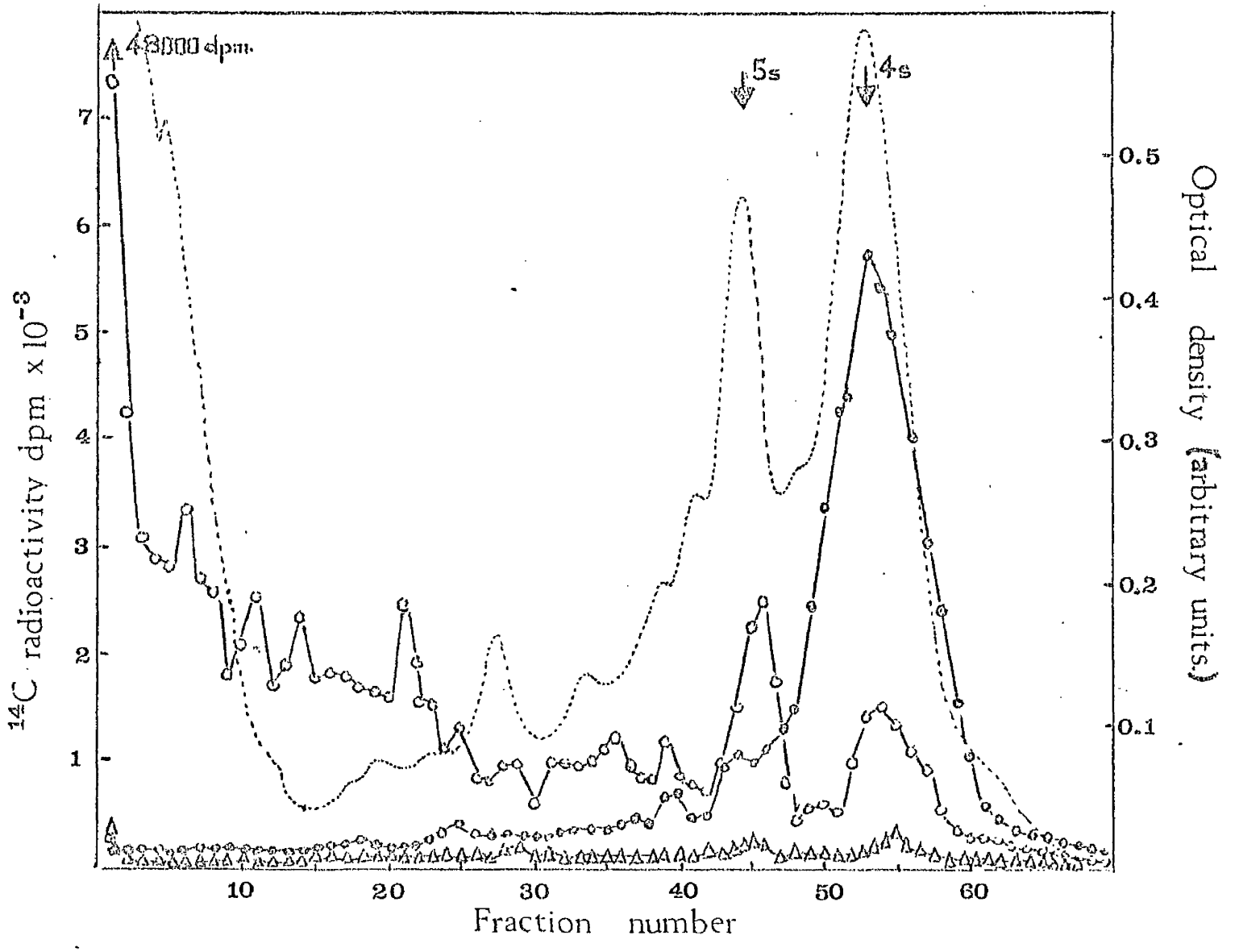
Fig. III.6

30×10^6 BHK-21/C13 cells were grown for 18h in 150ml EC_{10} at 37°C and after this time the growth medium was removed and replaced with 100ml EC_{10} containing 5μCi[^{14}C]-uridine (62mCi/mmol). Growth was continued for a further 24h when the cells were removed from the glass by trypsin treatment. The cells were then washed with a 25ml portion of ice cold BSS and collected by centrifugation at 2,500 rev/min for 2min. Subcellular fractions were prepared from the cell pellet as described in Methods section 3 , and RNA extracted from the isolated subcellular fractions by the appropriate method as described in Methods section 4 . The extracted RNA was then fractionated by polyacrylamide gel electrophoresis in 7.5% gels as described in Methods section 5(b). The gels were then sliced and examined for radioactivity as described previously.

Optical density, arbitrary units (-----), [^{14}C]-radioactivity dpm in, :-

(a) Mitochondrial fraction (—Δ—Δ—)
(b) Cell sap (—□—○—)
(c) Microsomal material (—○—○—).

The figures recorded in the top left hand corner of the profile represents the [^{14}C]-radioactivity in RNA species of high molecular weight which do not enter the gel matrix.


43000 dpm.
5s
4s
7
6
5
4
3
2
1
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
^{14}C radioactivity dpm x 10^{-3}
Optical density (arbitrary units.)
Fraction number

for 24 hours and the subcellular components isolated from the harvested cells as described in the Methods section (B.3). [^{14}C]-labelled RNA was then isolated from the various subcellular fractions and examined by electrophoresis in 7.5% polyacrylamide gels. The results of these examinations are shown in Fig III.6 which is a composite electrophoretogram of the distributions of radioactivity among the various low molecular weight RNA components isolated from the subcellular fractions. It is clear from Fig.III.6 that the only species of low molecular weight RNA found free in the "cell sap" is the 4s RNA (tRNA) component although a small amount of 5s RNA was detected free in this fraction. The bulk of the low molecular weight cytoplasmic RNA species are seen to be associated with the microsomal fraction and there appears to be no detectable association of these species with either the mitochondria or the cell sap. The major microsomal associated low molecular weight RNA species is, as expected, 5s RNA with some 4s or tRNA also being present.

III.7 The effects of actinomycin D on RNA synthesis in BHK-21 cells.

Although a comparison of the distributions of radioactivity in the low molecular weight cytoplasmic RNA components after a short and long exposure to isotope (Figs.III.2(b) and III.3) reveals the possible metabolic instability of these components, a clearer indication of their stability may be obtained under

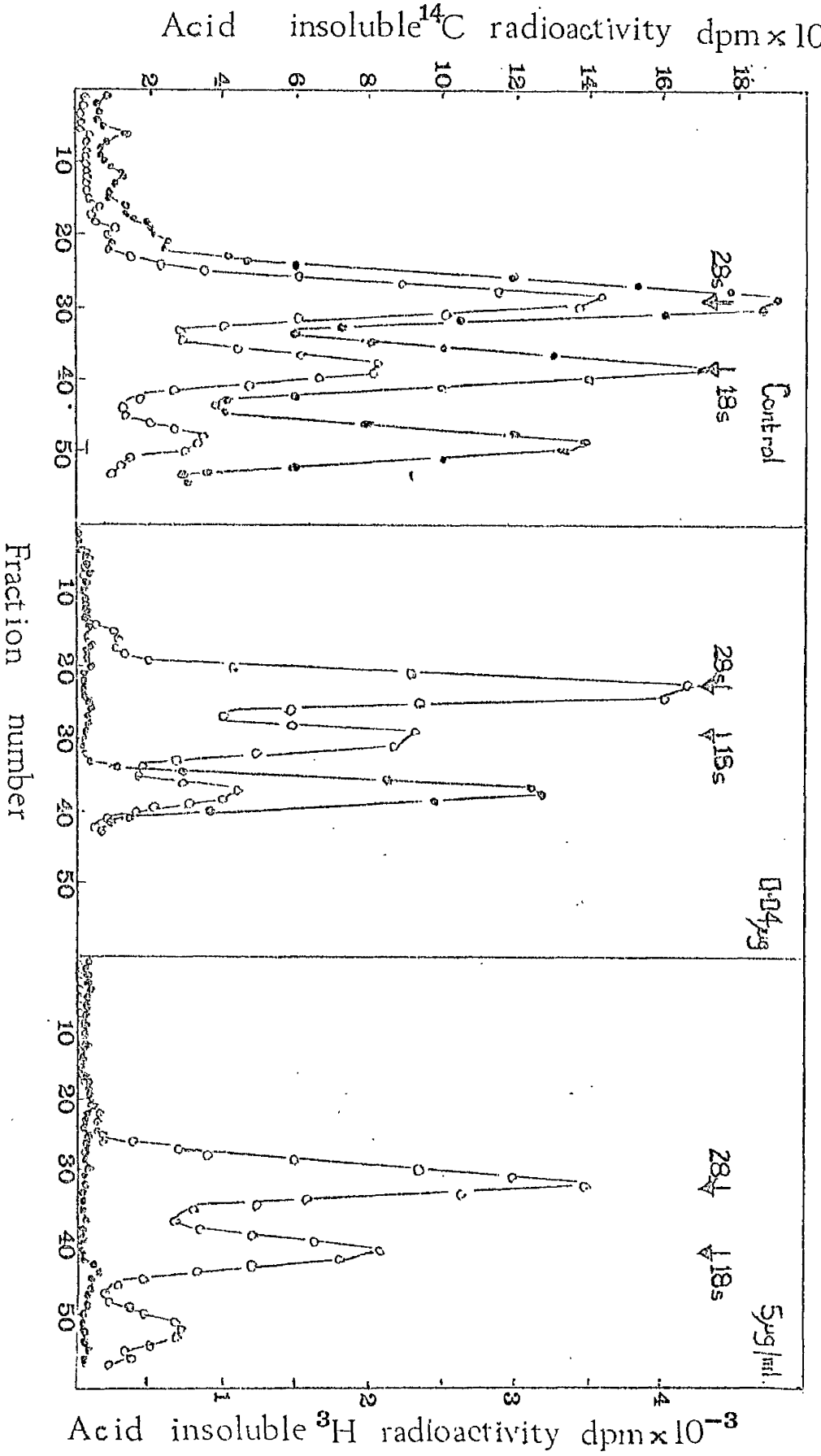
conditions when the rate of decay of the incorporated label can be examined without the complication of continued incorporation of isotope. Such a situation is attainable in pulse-chase experiments utilising actinomycin D. Perry(1962) has reported that low concentrations of the drug, eg. 0.04µg/ml, can selectively inhibit the synthesis of ribosomal RNA and a total inhibition of RNA synthesis is attainable with higher concentration of the drug. In order to determine the drug concentrations essential to achieve these situations in BHK-21 cells, the experiments reported in Figs.III.7 and III.8 were performed. Cultures of BHK-21 cells were preincubated for 30min in the presence or absence of either 0.04µg/ml or 5µg/ml actinomycin D and then exposed to [^{3}H]-uridine for either 15min or 60min. Total cell RNA was isolated by the "hot-phenol-SDS" procedure from cells exposed to isotope for 15min and "cold phenol" cytoplasmic RNA was prepared from cultures which had been exposed to isotope for 60min. The extracted [^{3}H]-labelled RNA was mixed with 50µl of a [^{14}C]-labelled RNA preparation (obtained by cold phenol extraction of BHK-21 cells grown for 24h in the presence of [^{14}C]-uridine) and analysed by sucrose density gradient centrifugation (Methods section B.5c). The radioactivity profiles obtained from these analyses are shown in Figs.III.7 and III.8. Fig.III.7 shows the distribution profiles of radioactivity in cytoplasmic RNA species isolated from control

Effects of actinomycin D on the synthesis of cytoplasmic RNA components of BHK-21/C13 cells.

Fig. III.7

3 Roux bottles of BHK-21/C13 cells (15×10^6 in 50ml ETC_{10}) were grown for 18h at 37°C and actinomycin D was then added to two of the bottles to give a final concentration of either 0.04µg/ml or 5µg/ml and incubation continued for 30 min. 50µCi [^{3}H]-uridine (8Ci/mmol) were then added to each bottle and the incubation of the cultures continued for a further 60 min in the presence of the drug. The radioactive medium was then decanted, the cell monolayer washed with a 50ml portion of ice cold BSS and the cells harvested by trypsinisation. From the collected cell pellets RNA was extracted by the "cold phenol" technique (Methods section 4(a)) and, after dissolution in the appropriate buffer (Methods section 4(b)) was layered together with 50µl of a [^{14}C]-labelled marker RNA over a linear gradient of 5%(w/v) to 20%(w/v) sucrose in 0.05M ammonium acetate (pH 5.1). The gradients were centrifuged at 39,000 rev/min in an SW40 rotor for 5h as described in Methods section 5(c), harvested in 8 drop fractions and the fractions assayed for radioactivity as described in Methods section 7.

[^{14}C]-acid insoluble radioactivity dpm (—•—), [^{3}H]-acid insoluble radioactivity dpm (—○—).


Acid insoluble ^{14}C radioactivity dpm × 10^{-3}
Fraction number
Acid insoluble ^{3}H radioactivity dpm × 10^{-3}
28s
18s
Control
28s
15s
0.04μg
28s
18s
5μg/ml.

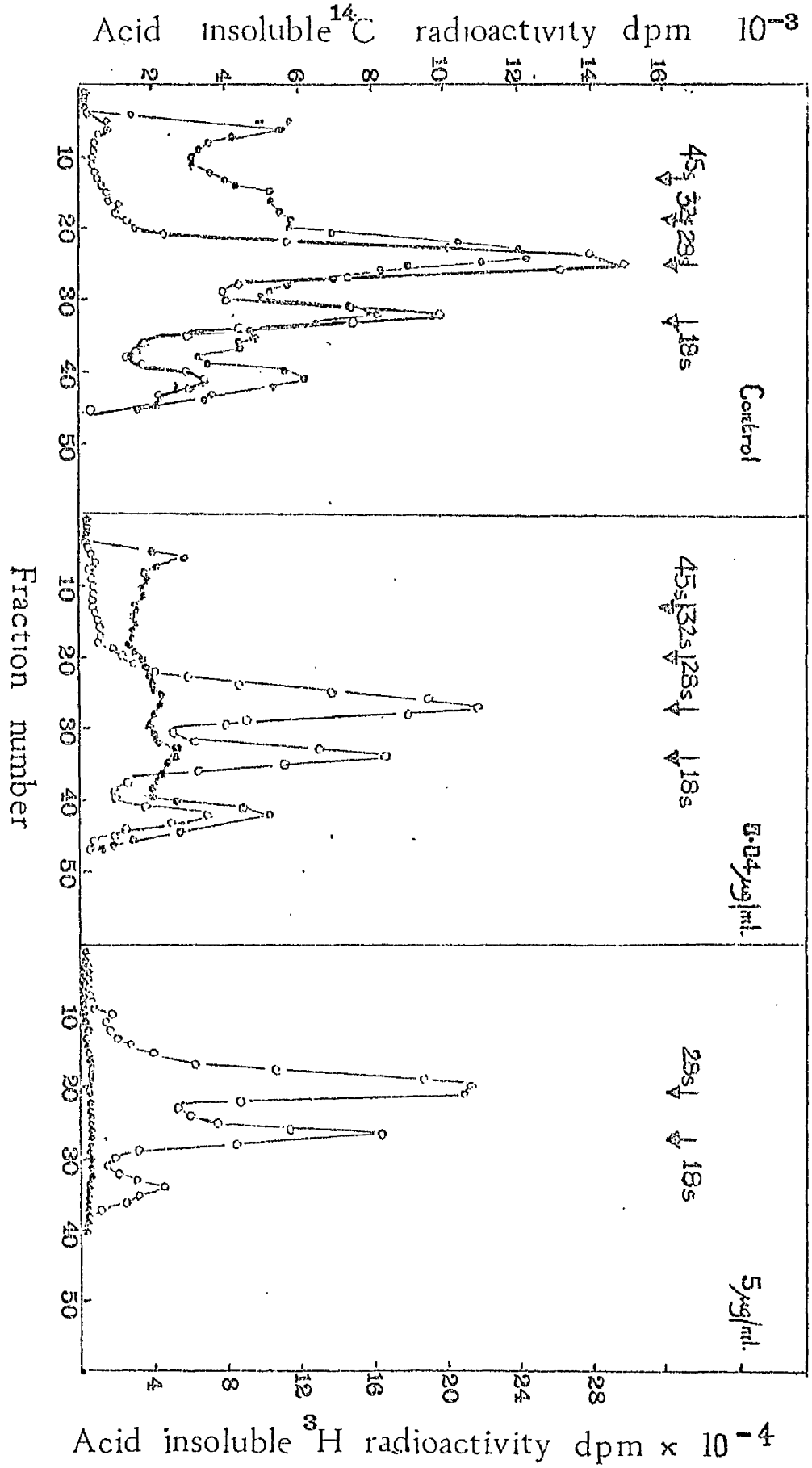
Effects of actinomycin D on the synthesis of total cell RNA in BHK-21/C13 cells.

Fig.III.8

3 petri dish cultures of BHK-21/C13 cells (10^6 cells/dish in 10ml ETC_{10}) were grown for 18h at 37°C and actinomycin D was then added to two of the cultures to give a final concentration of either 0.04µg/ml or 5µg/ml and the incubation continued for 30min. 50µCi [^{3}H]-uridine (8Ci/mmol) were then added to each dish and incubation of the cultures continued for a further 15min. The radioactive medium was decanted, the cell monolayers washed with two 10ml portions of ice cold BSS and the cells removed from the plates with the aid of a rubber policeman. The collected cell pellets were extracted by the "hot-phenol-SDS" technique (Methods section 4(b)) and the [^{3}H]-labelled RNA mixed with 50µl of a [^{14}C]-labelled marker RNA in an appropriate buffer.(methods section 4(b)). The RNA preparations were then layered over linear gradients of 5% (w/v) to 20% (w/v) sucrose in 0.05M ammonium acetate (pH 5.1) and the gradients centrifuged at 39,000 rev/min in an SW40 rotor for 5h as described in Methods section 5(c). The gradients were harvested in 8 drop fractions and the fractions assayed for radioactivity as described in section 7 of Methods.

[^{14}C]-acid insoluble radioactivity dpm (—∘—)

[^{3}H]-acid insoluble radioactivity dpm (—•—)


Acid insoluble ^{14}C radioactivity dpm 10^{-3}
2
4
6
8
10
12
14
16
45s
32s
28s
18s
Control
45s
32s
28s
18s
0.04 μg/ml.
28s
18s
5 μg/ml.
10
20
30
40
50
Fraction number
4
8
12
16
20
24
28
Acid insoluble ^{3}H radioactivity dpm x 10^{-4}

cells, cells treated with 0.04μg/ml actinomycin D and cells treated with 5μg/ml actinomycin D. It is apparent that the levels of the drug of 0.04μg/ml inhibit the appearance in the cytoplasm of labelled ribosomal RNA components and that a concentration of the drug of 5μg/ml causes a total inhibition of the incorporation of label into cytoplasmic RNA species. The results obtained with total cell RNA using the same drug concentrations, and shown in Fig.III.8, indicate that low levels of actinomycin D in BHK-21 cells do in fact inhibit the synthesis of ribosomal RNA but that at a concentration of 5μg/ml it causes a total inhibition of RNA synthesis in these hamster cells as it is reported to do in Hela cells.

III.9 Stability of low molecular weight cytoplasmic RNA to actinomycin D chase.

The experiments reported in Fig III.7 and III.8 have indicated that concentrations of the drug, actinomycin D, of 5μg/ml are sufficient to totally inhibit the synthesis of RNA in BHK-21 cells and pulse chase experiments utilising this concentration of the drug were therefore designed to determine the metabolic stability of the low molecular weight cytoplasmic RNA species. The results of the experiments reported in Fig III.2(a) and (b) have indicated that these RNA species appear in the cytoplasm of BHK-21 cells as discernible species in pulse times as short as 15 to 20 minutes. Cultures of BHK-21 cells were therefore

Stability of low molecular weight RNA components of BHK-21 cells to actinomycin D chase.

Fig. III.9

Cultures of BHK-21/C13 cells (5×10^6 cells in 50ml ETC_{10}) were grown for 24h at 37°C. Each culture was then exposed to 100µCi [^{3}H]-guanosine (3.5Ci/mmol) for 15min, then the radioactive medium decanted and the cell monolayer washed twice with 50ml portions of prewarmed ETC_{10}. One culture was harvested, and to the remaining culture bottles was added 40ml of prewarmed growth medium containing 5µg/ml actinomycin D and the incubations continued for the appropriate periods of time. The cultures were harvested as described in Methods section A2 and from the cell pellets "cold phenol"cytoplasmic RNA was isolated as described in Methods section 4(a). After dissolution in the appropriate buffer (Methods section 4(b)) 200µl samples of the RNA preparations were examined by electrophoresis in gels of polyacrylamide(Methods section 5) and after electrophoresis the gels were scanned for optical density, frozen, sliced into 1mm segments and the segments assayed for radioactivity as described in Methods section 5 subsection 3(b).

Extinction 260nm (-----)

^{3}H radioactivity cpm (---o---)

(a) 15 min pulse, (b) 15min pulse followed by 10 min chase procedure, (c) 15 min pulse followed by 30 min chase procedure, (d) 15 min pulse followed by 60 min chase procedure (e) 15 min pulse followed by 75 min chase procedure.

The distance migrated by 5s and 4s RNA under these conditions is indicated by the shaded box (▨).

Control
(a)
10 min "chase"
(b)
30 min "chase"
(c)
60 min "chase"
(d)
75 min "chase"
(e)
5s
4s
Extinction 260 mm

exposed to [^{3}H]-guanosine for 15 min, the radioactive medium removed fromthe cultures and replaced with prewarmed growth medium containing 5µg/ml actinomycin D, and the incubations continued for various lengths of time. "Cold phenol" cytoplasmic RNA was then isolated from all the cultures and examined by electrophoresis in 7.5% polyacrylamide gels. The results of these chase experiments are shown in the radioactivity profiles of Fig.III.9. For comparison, the optical density profile obtained by the electrophoresis of unlabelled "cold phenol" cytoplasmic RNA in an identical polyacrylamide gel is included in each of Figs. III.9(a) - (e). The distribution of radioactivity in the control gel after 15min exposure to isotope is similar to that obtained in the 10 min pulse represented in Fig. III.? (a) and shows that the bulk of the radioactivity migrates in a heterogeneous fashion in positions intermediate between 5s RNA and 4s RNA and presumably represents "pre-tRNA". However, prominent radioactivity peaks representing the cytoplasmic RNA components referred to in Fig. III.1 as (2) to (10) are visible at this time and a distinct fraction of the radioactivity migrates coincident with the 5s and 4s RNA peaks. After a 10 min "chase", the prominent peak of species (7) present in the control culture is considerably reduced and the material migrating between the 5s and 4s optical density peaks is apparently less heterogeneous. After 30 min "chase", almost

all of the heterogeneous material, previously migrating intermediate between 5s and 4s RNA, has disappeared and a prominent 4s RNA and 5s RNA peak have emerged, but the distinct peaks of radioactivity corresponding to the RNA species referred to in Fig III.1(b) as (2) -(10) can be seen to be little changed from the situation found after a 10min "chase". By 60 min the heterogeneous "pre-tRNA" has quantitatively been converted to 4s RNA components and once again there is little discernible change in the levels of radioactivity present in the RNA species (2) -(10) with a lower electrophoretic mobility than 5s RNA. A similar picture is obtained after 75 min of "chase" but the peak of "5s " RNA is reduced. The significance of this observation is obscure but it is apparent that the RNA species (2)-(10), although metabolically relatively unstable as suggested by the results of experiments shown in Figs. III.2 (b) and III.3, are measurably stable for periods greater than 75 minutes, but none the less apparently have a life span of less than 30 hours.

III.10 Metabolic stability of ribosomal RNA in BHK-21 cells.

To investigate this problem further the stability of ribosomal RNA was examined. Ribosomes are recognised as stable components of mammalian cells, exhibiting half-lives of approximately 4 - 6 days in the adult rat liver cell (Loeb et al, 1965). The results of kinetics of labelling and pulse chase experiments have indicated that many of the low molecular weight cytoplasmic

RNA species in BHK-21 cells are metabolically unstable by comparison. In order that some relative estimate of their metabolic stability might be obtained, it was decided to compare the stability of cytoplasmic low molecular weight RNAs with the known stable components of the cell, the ribosomes. An estimate of the metabolic stability of ribosomes in BHK-21 cells was therefore required. Since ribosomal RNA is methylated and since it has been reported that [methyl-^{3}H]-methionine incorporation can be effectively suppressed within five to ten minutes of the beginning of the "chase period" (Weinberg & Penman, 1969) cultures of BHK-21 cells were therefore labelled with L-[methyl-^{3}H]-methionine in the presence of 20mM sodium formate to prevent methyl group incorporation into the purine ring skeletons. After a 2h. exposure of the cells to [methyl-^{3}H]-methionine, the radioactive medium was removed, the cell monolayers washed several times with prewarmed non-radioactive growth medium and "chased" for various periods of time up to a maximum of 72h. "Cold phenol" RNA was then extracted from each culture, mixed with an aliquot of [^{14}C]-labelled cytoplasmic RNA, and examined by sucrose density gradient centrifugation (Methods section 5(c)). The profiles of the distribution of radioactivity in the ribosomal RNA species are shown in Fig III.10 (a) - (d). Fig III.10 (e) shows the total incorporated radioactivity in each of the ribosomal RNA

Metabolic stability of ribosomes in BHK-21/C13 cells.

Fig. III.10

Cultures of BHK-21/C13 cells (12 x 10^6 cells/Roux bottle) were grown in 20ml ETC_{10} containing 20mM sodium formate for 18h at 37°C. The cultures were then exposed to 100µCi L-(methyl-^{3}H)-methionine (8.3Ci/mmol) for a period of two hours. After this period the growth medium was removed and the monolayers washed three times with 50ml portions of prewarmed ETC and fresh prewarmed ETC_{10} growth medium then added to the cultures. The cultures were then "chased" for various lengths of time with this non-radioactive medium and harvested at appropriate times after the initiation of the "chase" conditions. RNA was then isolated from the cell pellets by the "cold phenol" method described in Methods section 4(a). The RNA, dissolved in a suitable buffer (Methods section 4(b)), together with 50µl of a [^{14}C]-labelled marker RNA preparation, was then examined by sucrose density gradient centrifugation in an SW40 rotor as described in Methods section 5(c). The gradients were harvested and the fractions examined for radioactivity as detailed in Methods section 7.

[^{3}H]-acid insoluble radioactivity dpm (—o—)

[^{14}C]-acid insoluble radioactivity dpm (—c—)

(a) 2 hour pulse control cells, (b) 2 hour pulse then 12h in chase conditions, (c) 2 hour pulse then 48h in chase conditions (d) 2 hour pulse then 72h in chase conditions, (e) decay rates of radioactivity present in ribosomal RNA components.

Acid insoluble ^{14}C radioactivity dpm 10^{-3}
(a)
2 hour pulse
28s
18s
(b)
12 hour chase
28s
18s
(c)
48 hour chase
(d)
72 hour chase
Fraction number
Acid insoluble ^{3}H radioactivity dpm $\times 10^{-2}$
(e)
28s
18s
Hours of 'chase'

species, 28s RNA and 18s RNA at various times after the initiation of the chase period. These data are computed from the radioactivity profiles shown in Fig III.10 (a)-(d) and indicate that, in BHK-21 cells, under the conditions of the experiment, rapid suppression of [methyl-^{3}H]-methionine incorporation is not attained. However these data indicate that in BHK-21 cells, grown under the conditions of the experiment, the average half-life of ribosomal RNA is approximately 142 hours and that ribosomes can therefore be considered as relatively stable components of BHK-21 cells,

III.11 Metabolic stability of low molecular weight cytoplasmic RNAs in BHK-21 cells

The results of experiments reported in Fig III.10 have indicated that ribosomal RNA is a relatively stable component of BHK-21 cells and is therefore a valuable parameter in gaining an estimate of the metabolic stability of the low molecular weight RNA components of these cells. However these low molecular weight RNA components appear to be devoid of methyl groups (see Fig III.4) and therefore [methyl-^{3}H]-methionine, whose incorporation can be more readily and more effectively suppressed, within a short period of the initiation of the chase, than the incorporation of [^{3}H]-guanosine, was an unsuitable labelled precursor for a study of the low molecular weight RNAs. Cultures of BHK-21 cells were therefore

exposed to [^{3}H]-guanosine for a period of two hours prior to subjection to identical chase conditions as in the experiment reported in section III,10. The data presented in Fig III.11 (a) - (e) indicate that radioactivity from [^{3}H]-guanosine cannot be rapidly diluted from the precursor pools and consequently still continues to be incorporated into the RNA components of the cell subsequent to the initiation of chase conditions. "Cold phenol" RNA isolated from cells labelled for two hours with [^{3}H]-guanosine and subjected to metabolic chase for various periods of time was examined by electrophoresis as 7.5% polyacrylamide gels and by sucrose density gradient centrifugation. Fig III.11 (a) - (e), indicates the distribution profiles of radioactivity in cytoplasmic RNA components of BHK-21 cells, at various times subsequent to "chase" as examined by sucrose density gradient centrifugation. These profiles indicated that [^{3}H]-guanosine has continued to be incorporated subsequent to the initiation of the chase and that there occurs a linear decay in the total radioactivity present in the ribosomal RNA species (see Fig III.11 (e)). It is also apparent that by 72 hours exposure to the "chase conditions" the radioactivity present in each of the RNA fractions separated by sucrose gradient centrifugation is approaching that originally present after the 2 hour exposure to [^{3}H]-guanosine. It should also be noted from Fig III.11 (e)

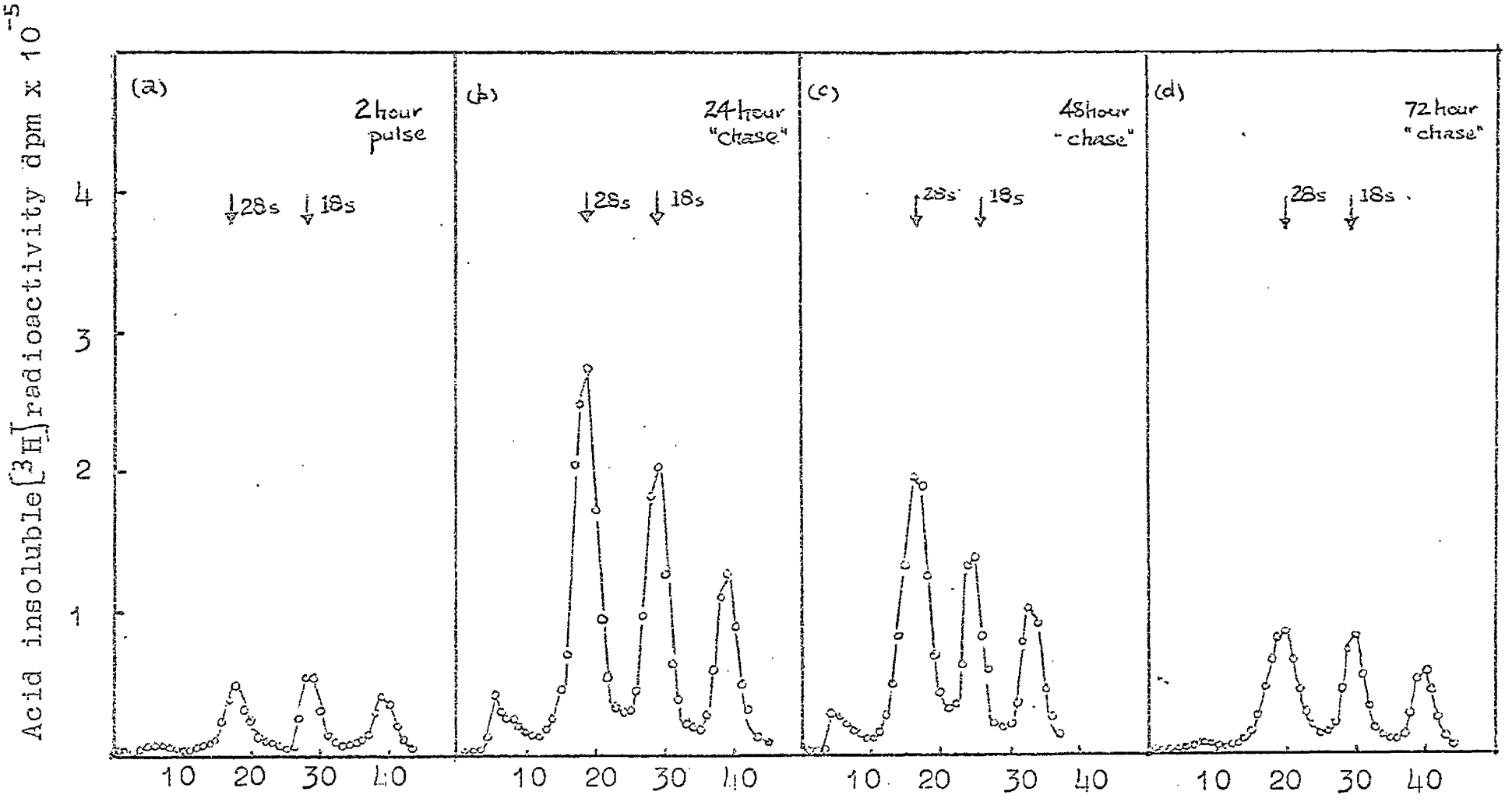
Metabolic stability of low molecular weight RNA components of BHK-21/C13 cytoplasm.

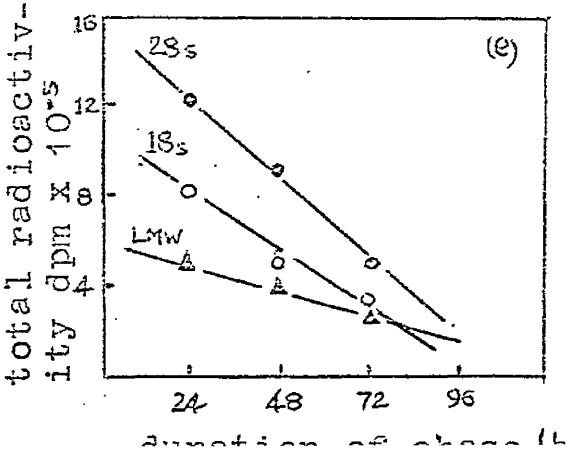
Fig. III.11

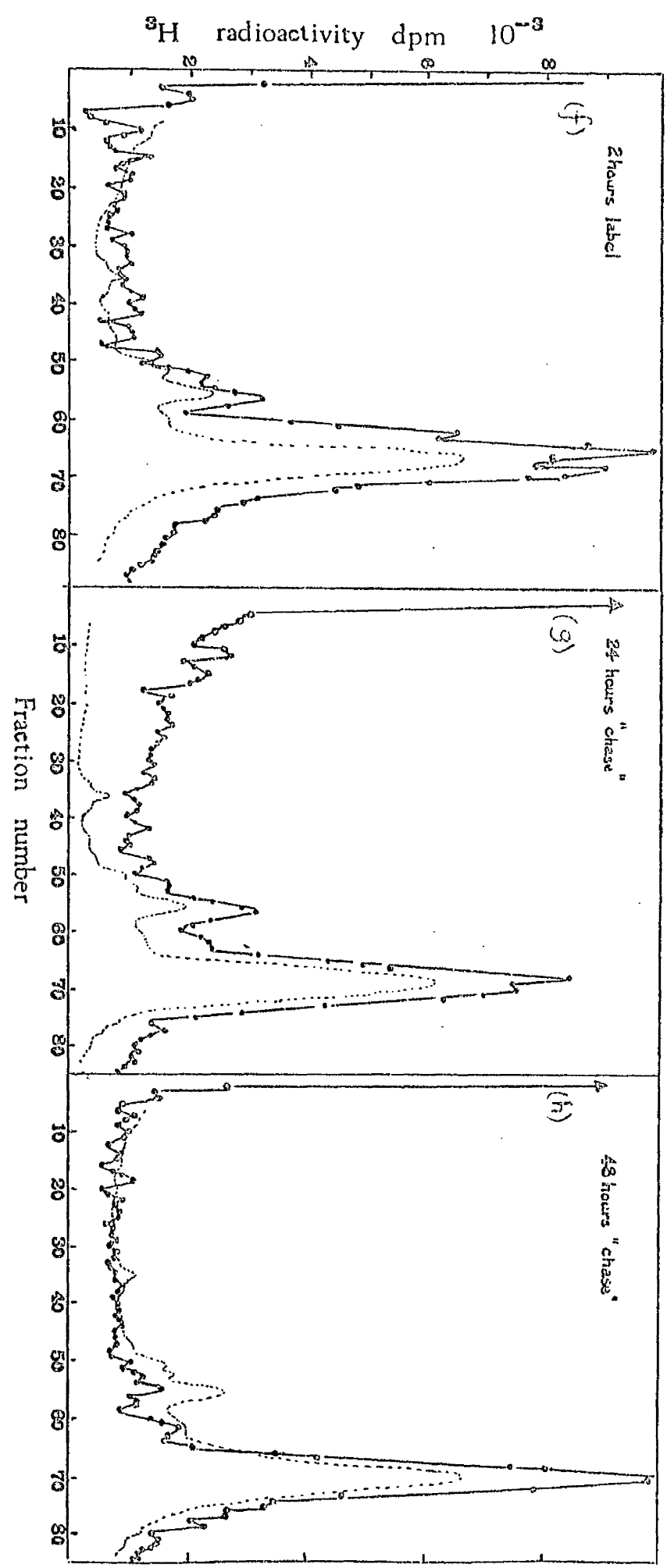
Cultures of BHK-21/C13 cells (10^7 cells in 50ml ETC_{10}) were grown for 18h at 37°C before exposure to 100μCi [^{3}H]-guanosine (6Ci/mmol) for 2 hours. After this time the radioactive medium was decanted and the cell monolayers washed three times with 50ml portions of prewarmed ETC_{10}. One culture was harvested at this point and to the remaining cultures 50ml of prewarmed growth medium was added, the incubation continued for various periods of "chase" and the cells harvested at 24 hour intervals. RNA was extracted from the cell pellets by the "cold phenol" technique described in Methods section 4(a). The extracted RNA was examined by centrifugation on linear 5%(w/v) to 20%(w/v) sucrose gradients (Figs.III.11(a)-(d)) or by polyacrylamide gel electrophoresis (Figs.III.11(f)-(h)). Recovery of the cells and the effects of varying precursor pool size were determined by the sucrose gradient analysis and each acrylamide gel was normalised to an equivalent label in the cytoplasmic ribosomal RNA. Fig.III.11(e) indicates the total incorporated [^{3}H]-radioactivity present in each of the RNA fractions separated by sucrose gradient centrifugation, at various times during the "chase" period.

Extinction 260nm (----), from unlabelled marker RNA electrophoresed simultaneously, [^{3}H]-radioactivity dpm (—o—)

(a) 2 hour pulse control cells, (b) 2hour pulse then 24h "chase" (c) 2 hour pulse then 48 hour chase, (d) 2 hour pulse then 72h chase, (f) as (a) but electrophoresis in polyacrylamide gels (g) as (b) but electrophoresis, (h) as (c) but electrophoresis.


Acid insoluble [3H] radioactivity dpm x 10-5
(a)
2 hour pulse
(b)
24 hour "chase"
(c)
48 hour "chase"
(d)
72 hour "chase"
28s
18s
1
2
3
4
10
20
30
40
Fraction number


total radioactivity dpm x 10-5
(e)
28s
18s
LMW
4
8
12
16
24
48
72
96
duration of chase (h)

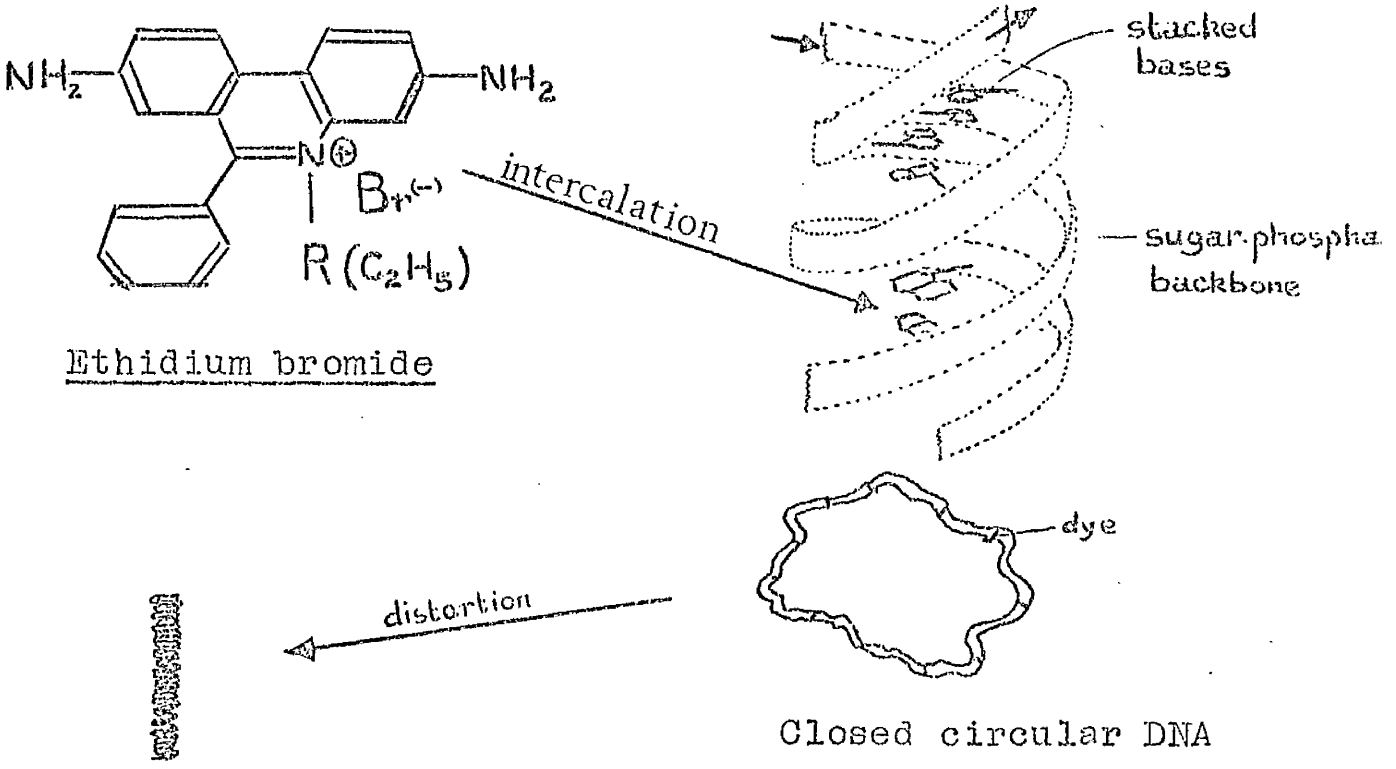

^3H radioactivity dpm 10^{-3}
2
4
8
8
(f)
2 hours label
(g)
24 hours "chase"
(h)
48 hours "chase"
10
20
30
40
50
60
70
80
Fraction number

that the decay rates for 28s and 18s RNA components are approximately equal. The significance of this decay of ribosomal RNA species will be discussed later. Figs III.11 (f) - (h) show the distribution profiles of radioactivity present in cytoplasmic low molecular weight RNA species after appropriate "chase periods" as examined by polyacrylamide gel electrophoresis. Each profile shown in Figs III.11 (f) - (h) was normalised to a constant level of cytoplasmic ribosomal RNA. The radioactivity present in each gel fraction was corrected by dividing by the ratio of radioactivity present in the ribosomal RNA at that chase time to that present in ribosomal RNA after the initial 2 hour pulse of [^{3}H]-guanosine, and therefore the stability of the low molecular weight RNAs measured here is a stability measured relative to that of the cytoplasmic ribosomal RNA. From a consideration of the profiles shown in Figs III.11 (f) - (h) it is apparent that the low molecular weight cytoplasmic RNA species referred to in Fig III.1 as (?)-(10) are not as stable as ribosomes and do not have a total life span of more than 24 hours and confirms the earlier results suggested by a comparison of Figs III.2 (b) and III.3

III.12 Effect of Ethidium bromide and low levels of actinomycin D on the synthesis of low molecular weight cytoplasmic RNA in BHK-21 cells.

The studies so far pursued have indicated that the relatively

Schematic diagram of the proposed mechanism of action of Ethidium bromide on mitochondrial DNA



Ethidium bromide

Closed circular DNA

Twisted tightly supercoiled circular DNA

unstable, (G+C) rich, unmethylated low molecular weight RNAs of mammalian cell cytoplasm are located principally in the microsomal fraction of the cells. Attardi and Attardi (1967) have reported a membrane bound RNA fraction in Hela cells, with similar properties but with a (G+C) content of 43% and which is relatively stable and hybridises efficiently with mit-DNA. It is therefore presumed to represent mit-DNA specified RNA sequences. In addition, Knight (1969) and Zylber, Vesco & Penman (1969) have indicated that the synthesis of mit-RNA is particularly sensitive to the drug, ethidium bromide. Experiments were therefore designed to determine whether the low molecular weight RNA fraction associated with the microsomes of BHK-21 cells represented species of RNA transcribed from mit-DNA. Except where noted, the cultures used in these experiments were treated with low concentrations of actinomycin D (0.04μg/ml) which has been shown to inhibit ribosomal RNA synthesis selectively (see Figs III.7 and III.8). This therefore permits the study of non-ribosomal RNA fractions in the cell without newly synthesised high molecular weight rRNA complicating the situation. In addition where ethidium bromide alone was omitted from the culture medium these low actinomycin D incubation conditions allow the discrimination between ribosomal and non-ribosomal RNAs. Various concentrations ethidium bromide were added to identical cultures of BHK-21

cells and after 30 min the cultures were exposed to [^{3}H]-guanosine for a continued incubation period of two hours. The cultures were then harvested, "cold phenol" RNA isolated and analysed by 7.5% polyacrylamide gel electrophoresis. The distribution profiles of the [^{3}H]-labelled RNA after electrophoresis are shown in Figs. III.12(a)-(f). For identification of the major optical density peaks, the optical density profile obtained as a result of co-electrophoresis of unlabelled "cold phenol" marker RNA is shown superimposed upon the radioactivity profile of Fig.III.12(a). It is apparent that in control cells [^{3}H]-radioactivity can be found associated with all the optical density peaks shown in Fig.III.12(a) and that those RNA species, tentatively identified as (10) -(13) are found at high specific activity near the origin of the gel. Fig. III.12(b) indicates that the synthesis of these "origin species" is sensitive to low levels of actinomycin D and that they appear to be of nucleolar origin. In addition it is evident from Fig.III.12(b) that the synthesis of those RNA species described as species (1) -(9) is insensitive to these low levels of actinomycin D as is the synthesis of 4s and 5s RNAs. This therefore indicates the possible nucleoplasmic location of their corresponding cistrons and their non-ribosomal nature in accord with the previously described base compositions of these RNA species. The distribution profiles

Effects of ethidium bromide on the synthesis of low molecular weight RNA in BHK-21/C13 cells.

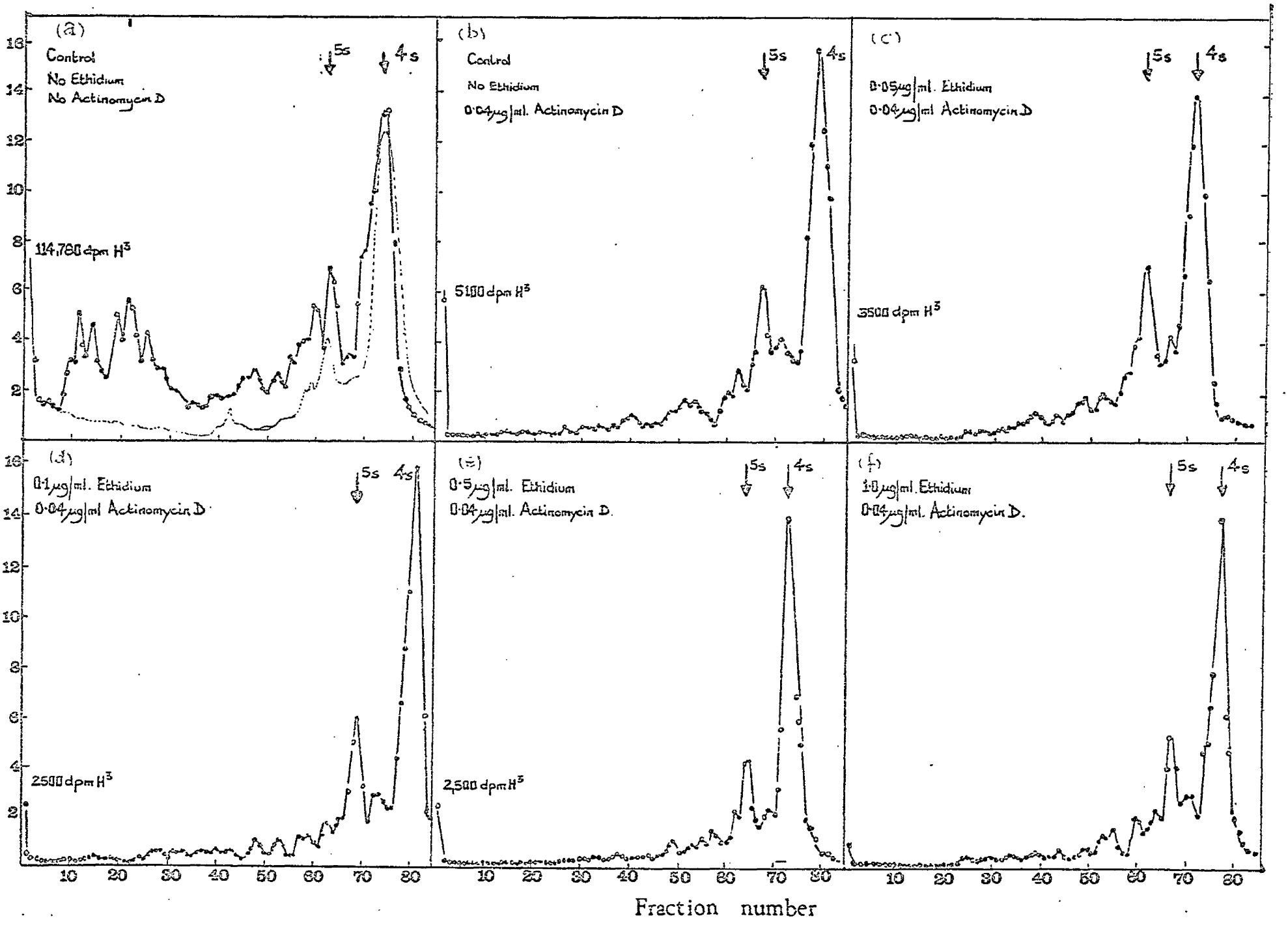
Fig. III.12

Cultures of BHK-21/C13 cells (5×10^7 cells in 100ml ETC_{10}) were grown for 18h at 37°C then treated with 0.04µg/ml of actinomycin D and appropriate concentrations of ethidium bromide for 30min. The cultures were then grown for 2h in the presence of 100µCi [^{3}H]-guanosine (3.5Ci/mmol) and after this period, the radioactive growth medium was removed, the cell monolayers washed with two 50ml portions of ice cold BSS and the cells harvested as described in Methods sect. A2. The cell pellets were extracted by the "cold phenol" procedure (Methods section 4(a)) and the isolated cytoplasmic RNA dissolved in an appropriate buffer(Methods section 4(b)). The RNA was then examined by polyacrylamide gel electrophoresis the gels scanned for optical density, frozen, sliced into 1mm segments and assayed for radioactivity as described in Methods section (5(b)).

Extinction 260nm (-----), [^{3}H]-radioactivity dpm (—•—).

Profiles from (a) control culture, no actinomycin D, no ethidium bromide, (b) control culture, no ethidium bromide, (c) in the presence of 0.05µg/ml ethidium bromide, (d) in the presence of 0.1µg/ml ethidium bromide, (e) in the presence of 0.5µg/ml ethidium bromide, (f) in the presence of 1.0µg/ml ethidium bromide.

The figures shown in the left hand edge of each profile,(a)-(f), indicate the [^{3}H]-radioactivity present in material of high molecular weight which does not enter the gel matrix but is retained at the origin.


(a)
Control
No Ethidium
No Actinomycin D
5s
4s
114,780 dpm H³
(b)
Control
No Ethidium
0·04μg/ml. Actinomycin D
5s
4s
5100 dpm H³
(c)
0·05μg/ml. Ethidium
0·04μg/ml Actinomycin D
5s
4s
3500 dpm H³
(d)
0·1μg/ml. Ethidium
0·04μg/ml Actinomycin D
5s
4s
2500 dpm H³
(e)
0·5μg/ml. Ethidium
0·04μg/ml. Actinomycin D.
5s
4s
2,500 dpm H³
(f)
1·0μg/ml. Ethidium
0·04μg/ml. Actinomycin D.
5s
4s
2
4
6
8
10
12
14
16
10
20
30
40
50
60
70
80
³H radioactivity dpm x 10⁻³
Fraction number

shown in Figs III.12 (c) - (f) further indicate that none of the low molecular weight RNA species insensitive to low levels of actinomycin D is sensitive to levels of ethidium bromide which have been shown to completely inhibit mit-RNA synthesis (Zylber et al, 1969). It must therefore be concluded that they are not transcribed from a mit-DNA template and cannot therefore be similar to the membrane bound RNA species of similar size in Hela cells described by Attardi & Attardi (1967).

III.13 The effect of ethidium bromide on the actinomycin D sensitive low molecular weight RNA fraction

The results of experiments reported in Fig III.12 have indicated that the RNA species tentatively referred to as (10) - (13), are sensitive to low levels of actinomycin D. Although this may possibly indicate the nucleolar location of their cistrons, the possibility still exists that they represent the transcription products of mit-DNA. The experiments reported in Fig III.12 using ethidium bromide were therefore repeated but with the omission from the medium of actinomycin D. The cultures were preincubated with various concentrations of ethidium bromide for 30 min, radioactive [^{3}H]-guanosine was then added to the cultures and incubation continued for two hours. The cultures were then harvested and cold phenol RNA isolated and examined by electrophoresis on 7.5% polyacrylamide gels. Figure III.13 shows the electrophoretograms of such [^{3}H]-labelled

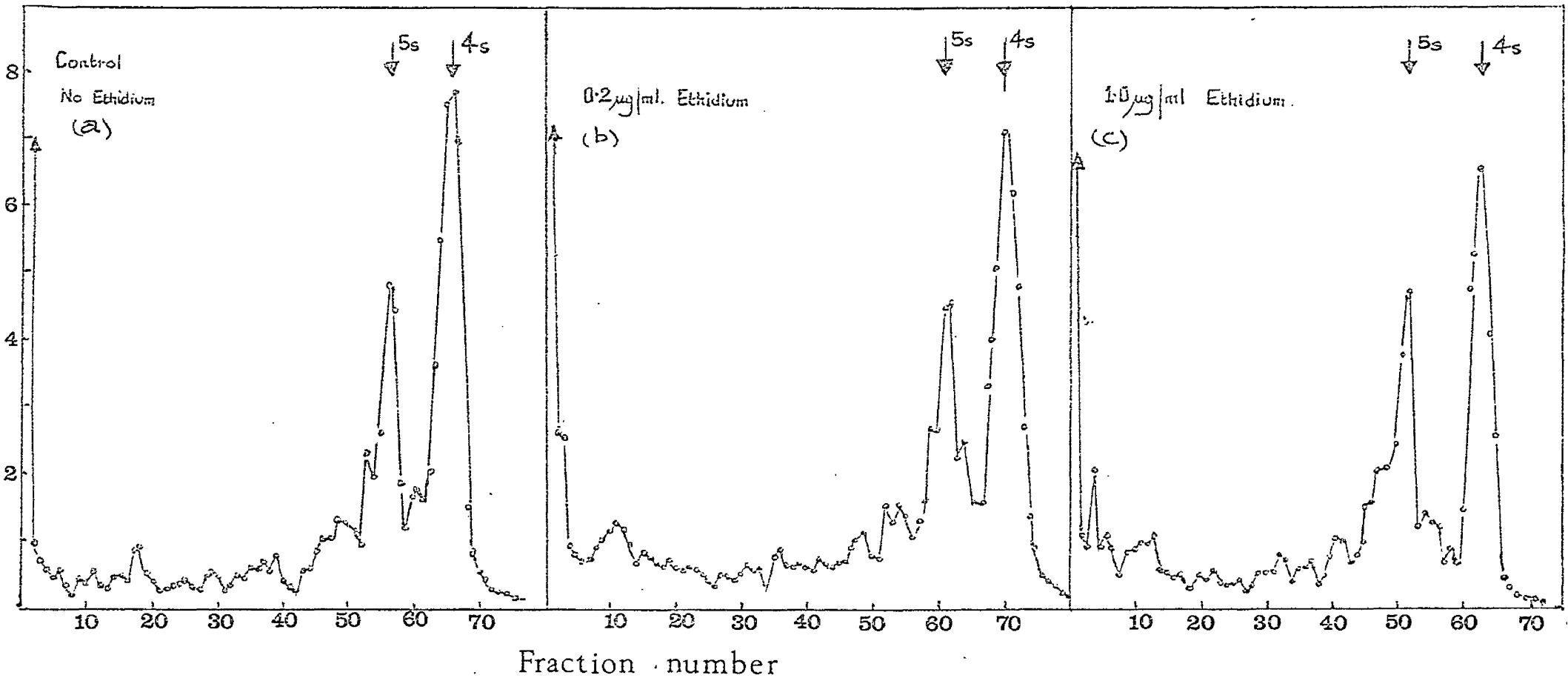
Effects of ethidium bromide on the actinomycin D sensitive RNA components of BHK-21/C13 cytoplasm.

Fig. III.13

Cultures of BHK-21/C13 cells (5×10^7 cells in 100ml ETC_{10}) were grown at 37°C for 18h, then treated for 30 min with various concentrations of ethidium bromide in the absence of actinomycin D, before being exposed to 100μCi [^{3}H]-guanosine (3.5Ci/mmol) for two hours. After this period the radioactive medium was decanted and the cell monolayers washed twice with 50ml portions of ice cold BSS. From the harvested cell pellets RNA was extracted by the "cold phenol" method described in Methods section 4(a), and examined by electrophoresis in gels of polyacrylamide (Methods section 5(b)) The gels were scanned for optical density, frozen, sliced into 1mm segments and assayed for radioactivity as described in Methods section 5.3(b).

^{3}H radioactivity dpm (—•—)

(a) no drug present, (b) 0.2μg/ml ethidium bromide, (c) 1.0μg/ml ethidium bromide


Control
No Ethidium
(a)
5s
4s
0·2 μg/ml. Ethidium
(b)
1·0 μg/ml Ethidium
(c)
8
6
4
2
10
20
30
40
50
60
70
Fraction number

RNA preparations after electrophoresis on 7.5% gels. It is apparent from Figs III.13 (a) - (c) that ethidium bromide alone causes no change in the distribution profiles of the cytoplasmic low molecular weight RNAs. It should therefore be concluded that the RNA species referred to as species (10) - (13) and previously described as "origin species" whose synthesis is sensitive to low levels of actinomycin D are not the products of mit-DNA but most probably derive from the transcription of nucleolar cistrons. Although variability in the extent of incorporation of labelled precursors into the low molecular weigh RNA components was experienced between experiments (cf. Fig III.12 (a) and III.13 (a)) the pattern of distribution was however constant and therefore reflects merely differences in the absolute amounts of the RNA species synthesised under growth conditions which although similar may vary within narrow limits.

III.14 The effect of ethidium bromide on "pre-tRNA" synthesis in BHK-21 cells

The kinetics of labelling of low molecular weight cytoplasmic RNA species in BHK-21 cells has previously revealed the existence of a heterogeneous class of RNA molecules migrating in positions intermediate between 5s and 4s RNA after short exposures to radioactive RNA precursors (see Fig III.2 (a)). These RNA species display the characteristics of precursor species to tRNA (see Fig III.9) and have been characterised in BHK-21 cells

by Smillie (1970). The intracellular location of these RNA molecules has been investigated in Krebs II ascites tumour cells (Burdon & Clason, 1969) and it was found that they exist in the cell sap in a free state rather than attached to microsomes or associated with the mitochondrial fraction. However, since in BHK-21 cells and Krebs II ascites cells these RNA molecules appear rapidly in the cytoplasm within a few minute of their transcription it was possible that they might be the products of cistrons present in mitochondrial DNA and be rapidly transported from these organelles into the cell sap. To rule out this possibility experiments were therefore designed to investigate this by using the selectivity of action of the drug ethidium bromide upon mitochondrial RNA synthesis. Cultures of BHK-21 cells were incubated for 30 min with various concentrations of ethidium bromide prior to the addition to the growth medium of [^{3}H]-guanosine. The cultures were then pulsed with isotope for 3 min in the presence of ethidium bromide. The cell monolayers were rapidly washed, the medium decanted and the "cold phenol" RNA extracted from the cells <u>in situ</u> by the direct addition of the extraction mixture to the culture flask. The extracted [^{3}H]-labelled RNA was then examined by Sephadex G100 chromatography and the RNA distribution profiles from these analyses are shown in Fig III.14. The optical density profiles shown in Figs III.14 (a) - (c)

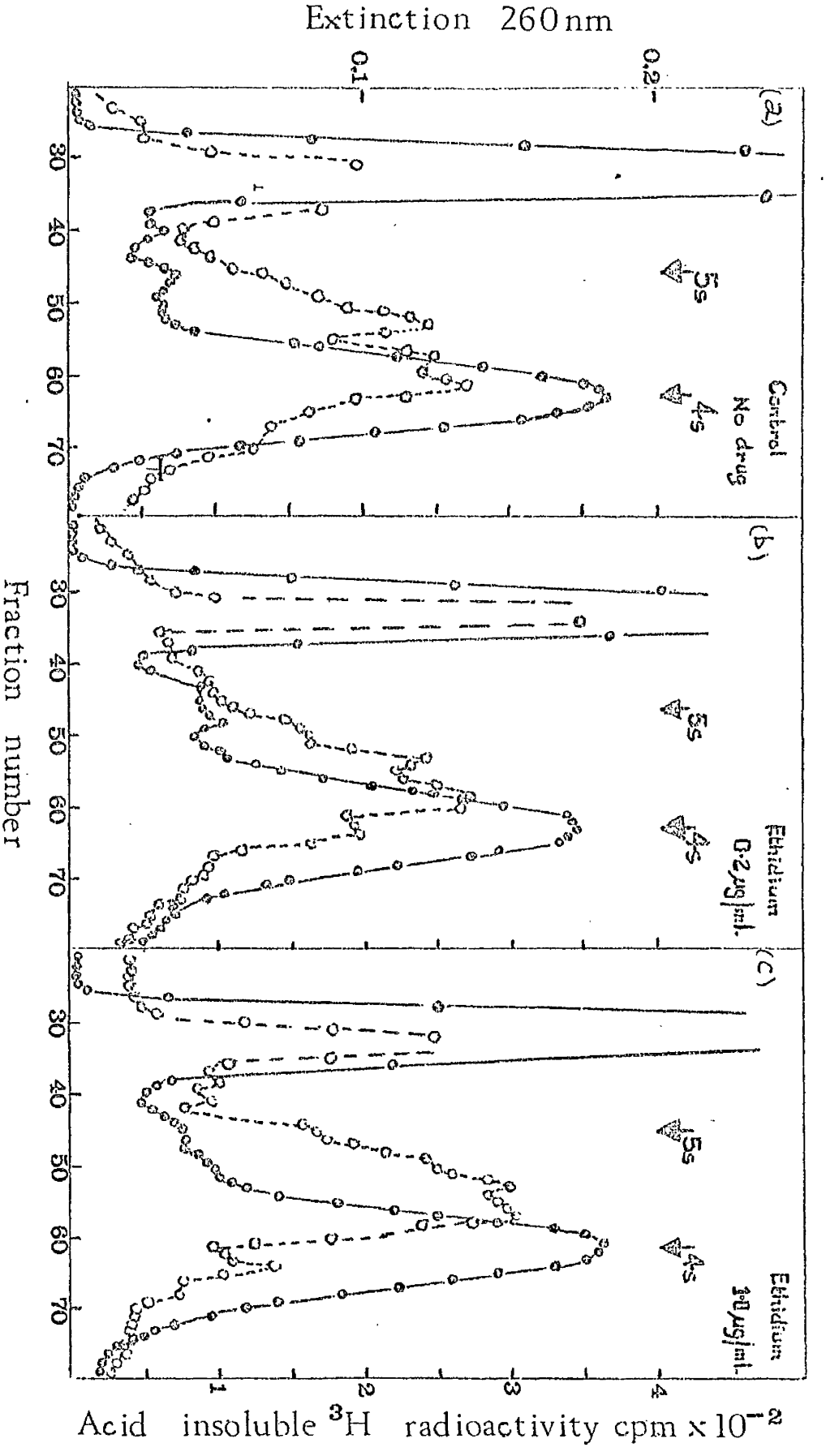
Effect of ethidium bromide on pre-tRNA synthesis in BHK-21, SR8/V1 and PyY cells.

Fig. III.14

30×10^6 cells of the appropriate cell line were grown for 18h in 100ml ETC_{10} at 37°C then treated with various concentrations of the drug, ethidium bromide, for 30 min. After this time 100µCi [^{3}H]-guanosine (5Ci/mmol) were added and the cells pulsed with this level of radioactivity for 3 min in the presence of the drug. The growth medium was then quickly decanted and the cell monolayer washed with a 50ml portion of ice cold BSS. The phenol/ammonium acetate extraction mixture (Methods section 4(a)) was then added directly to the cell monolayer in the 80oz. winchester bottle and the RNA extracted as described (Methods sect. 4(a)). The extracted [^{3}H]-labelled RNA was then examined by chromatography on a column of Sephadex G100, fractions collected and examined for extinction and radioactivity as described in Methods section 5(a).

(a) Pre-tRNA synthesis in the absence of the drug, (b) in the presence of 0.2µg/ml ethidium bromide, (c) in the presence of 1.0µg/ml ethidium bromide

Extinction 260nm (—○—), [^{3}H]-acid insoluble radioactivity cpm (—o—).


Extinction 260nm
0.1
0.2
(a)
Control
No drug
5s
4s
(b)
Ethidium
0.2μg/ml.
5s
4s
(c)
Ethidium
1.0μg/ml.
5s
4s
Fraction number
30
40
50
60
70
1
2
3
4
Acid insoluble ^{3}H radioactivity cpm $\times 10^{-2}$

indicate that using Sephadex G100 chromatography a separation of the low molecular weight 5s and 4s RNA components can be achieved from the high molecular weight rRNA components which are excluded from the gel matrix. It can be seen that after short time exposures to [^{3}H]-guanosine the bulk of the radioactively labelled low molecular weight cytoplasmic RNA elutes from Sephadex G100 columns in a heterogeneous fashion in positions intermediate between 5s and 4s RNA (Fig.III.14(a)). This distribution of radioactivity is not altered either qualitatively or quantitatively by the incubation of the cells with ethidium bromide (Figs.III.14(b)-(c)) and at concentrations known to inhibit mitochondrial RNA synthesis (Zylber et al, 1969). It is therefore apparent, as previously suspected, that the low molecular weight cytoplasmic tRNA precursor species are not of mitochondrial origin.

III.15 Polyribosome association of cytoplasmic low molecular weight RNA species.

The analysis of various subcellular fractions for the presence of low molecular weight cytoplasmic RNAs as reported in the experiments described in Fig III.6 have indicated the almost exclusive association of these RNA species with the membrane fraction of the cell. The question therefore arises, that since this fraction contains both the membrane bound and free polyribosomes of the cell, are these RNA species associated

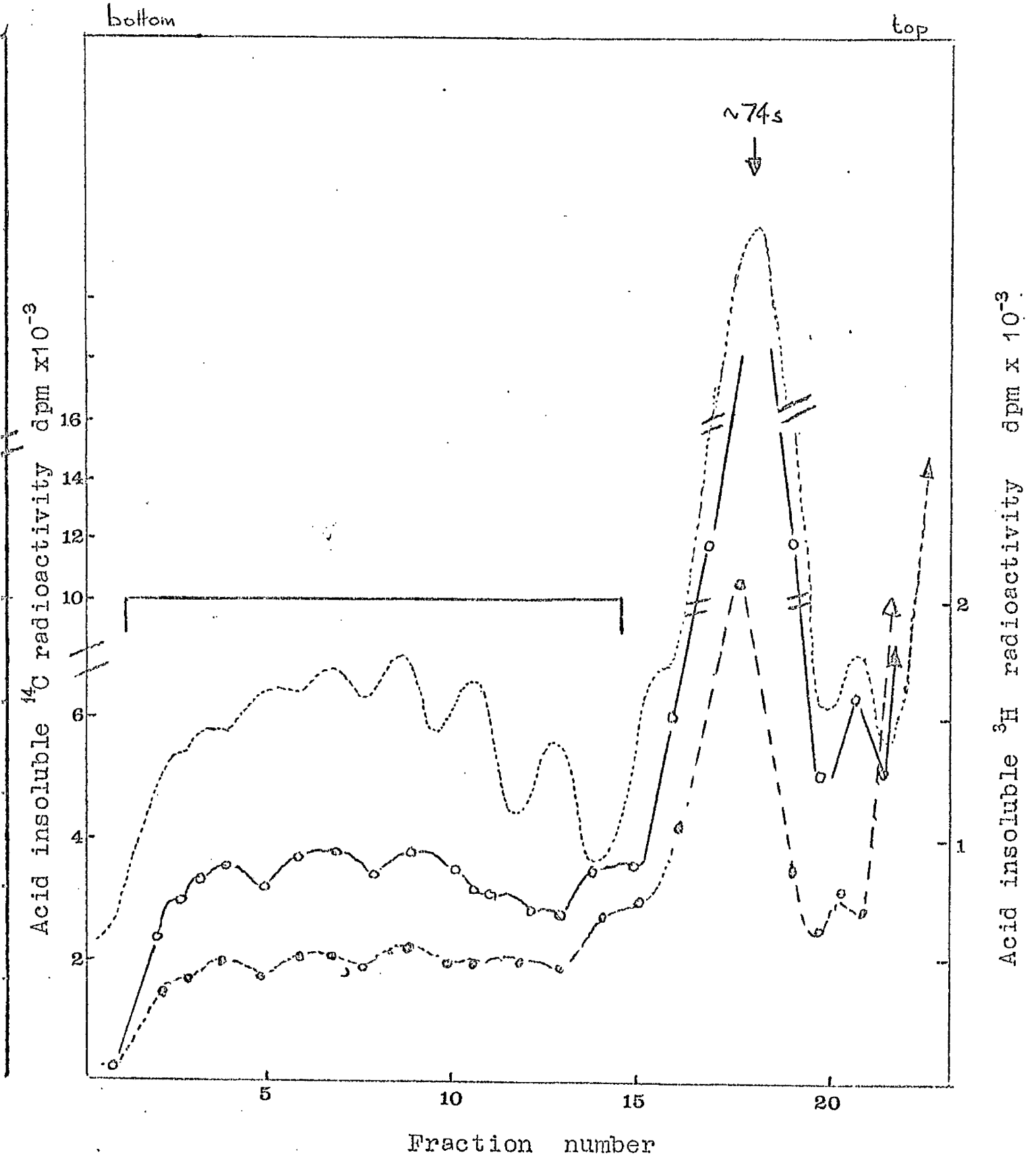
with the polyribosomes. In order to determine this, cultures of BHK-21 cells were grown overnight in the presence of [^{14}C]-uridine, to label the stable RNA components of the cell, then treated with 0.04µg/ml actinomycin D and labelled for one hour with [^{3}H]-uridine to detect the newly synthesised actinomycin D - resistant low molecular weight RNA components. The cells were then harvested and a cytoplasmic fraction isolated (Methods section 3.3). From this a total polyribosome preparation was obtained by the treatment of the cytoplasmic fraction with sodium deoxyocholate to release the membrane bound polysomes, and analysed by sucrose density gradient centrifugation to display the polyribosomes. The optical density profile and distribution of radioactivity in the polyribosomes after such analyses is shown in Fig.III.15.1. The cytoplasm of BHK-21 cells apparently contains a significant population of monoribosom (74s units) but the bulk of the optical density profile is composed of faster sedimenting, heavier polyribosomes. This pattern was obtained reproducibly when the extraction procedure described in Methods section was followed and indicates that no apparent severe degradation has taken place. The distribution of radioactivity is seen to follow quite precisely the optical density profile and is consistant with its being either ribosomal or mRNA. The [^{3}H]-radioactivity representing newly synthesised, rapidly labelled RNA components

Association of low molecular weight cytoplasmic RNA species with polyribosomes in BHK-21/C13 cells.

Fig. III.15.1

50×10^6 BHK-21/C13 cells, grown for 18h in 100ml ETC_{10} containing 10µCi[^{14}C]-uridine (62mCi/mmol) were treated with 0.04µg/ml actinomycin D, then exposed to 100µCi[^{3}H]-uridine (8Ci/mmol) for 60 min. After this time the cells were harvested and from the cell pellet a cytoplasmic fraction was prepared as described in Methods section 3(b). This cytoplasmic fraction was treated with sodium deoxycholate and centrifuged, through a linear 15%(w/v) to 30%(w/v) sucrose gradient for 3h, to display the polyribosomes as described in section 5 of Methods. The gradient was harvested and the fractions examined for radioactivity and extinction as described therein.

Extinction 260nm (----), [^{3}H] acid insoluble radioactivity cpm (—o—), [^{14}C]-acid insoluble radioactivity cpm (—e—).


bottom
top
~74s
Acid insoluble ^{14}C radioactivity dpm x10^{-3}
Acid insoluble ^{3}H radioactivity dpm x 10^{-3}
16
14
12
10
6
4
2
2
1
5
10
15
20
Fraction number

was however administered in the presence of levels of actinomycin D sufficient to totally inhibit the synthesis of high molecular weight ribosomal RNA and could therefore represent mRNA or some actinomycin D resistant polysome associated RNA species. Similar cultures of BHK-21 cells were next grown overnight in the presence of [^{32}P]-orthophosphate to label the stable ribosomal components and were then pulsed with [^{3}H]-uridine in the presence of low levels of actinomycin D as before. From the harvested cell pellets a cytoplasmic fraction was obtained as previously described and from this polyribosomes were either displayed as in Fig III.15.1, or pelleted and collected as described in Methods (section B3(d)). Fractions corresponding to the polysome material as indicated by the bracket in Fig III.15.1 were pooled, made 0.5% (w/v) with respect to sodium dodecyl sulphate and the RNA precipitated with two volumes of ethanol and examined by sucrose density gradient centrifugation. The distribution profiles of radioactivity and optical density of RNA obtained from such a preparation are shown in Fig III.15.2 (a) and indicate a heterogeneous sedimentation pattern of [^{3}H]-labelled RNA components sedimenting between 6 to 35s. There does however appear to be [^{3}H]-radioactivity associated with the 28s and 18s rRNA components and this may indicate only a partial inhibition of ribosomal RNA synthesis on this occasion. This was confirmed by the results depicted in Fig III.15,2 (b).

Association of low molecular weight RNA components of the cytoplasm with polyribosomes in BHK-21/C13 cells.

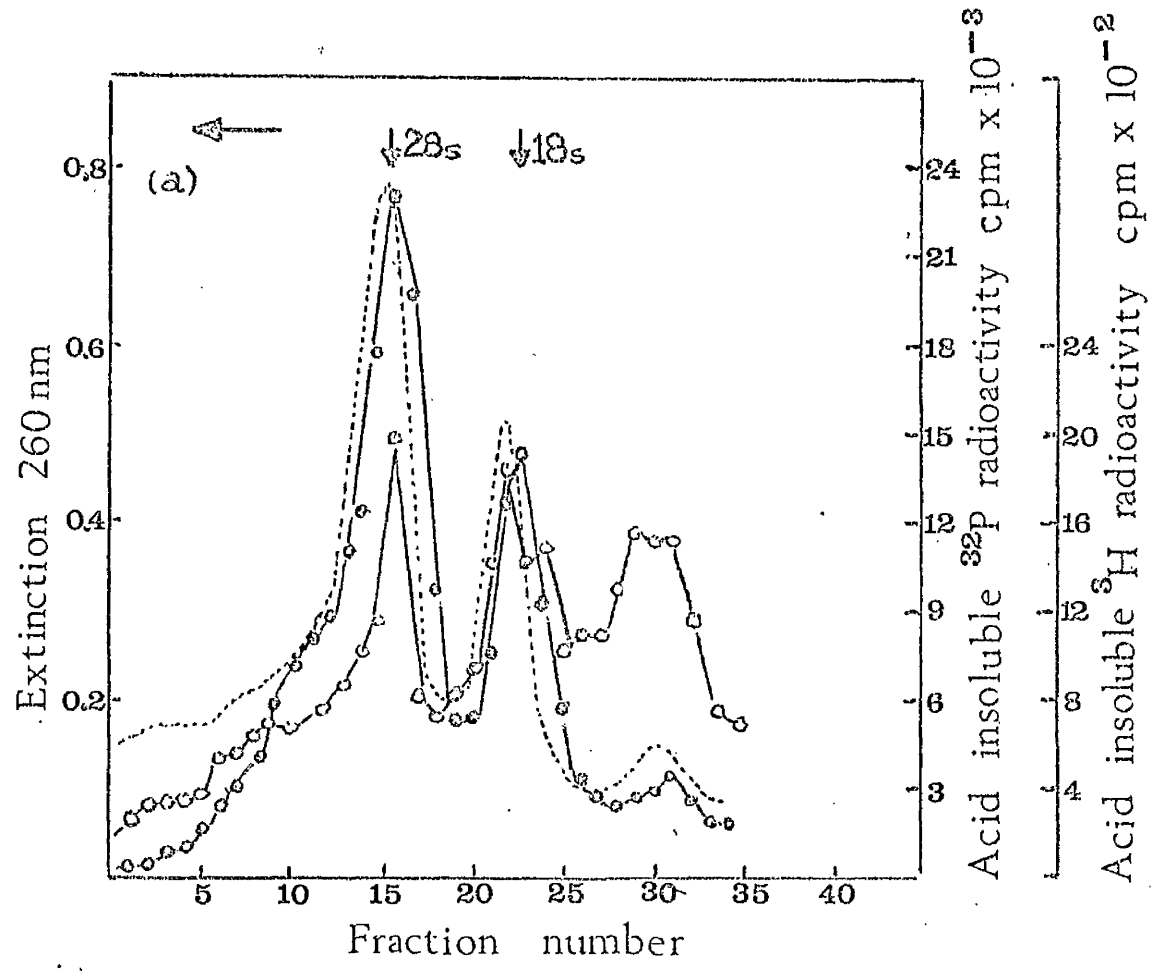
Fig. III.15.2

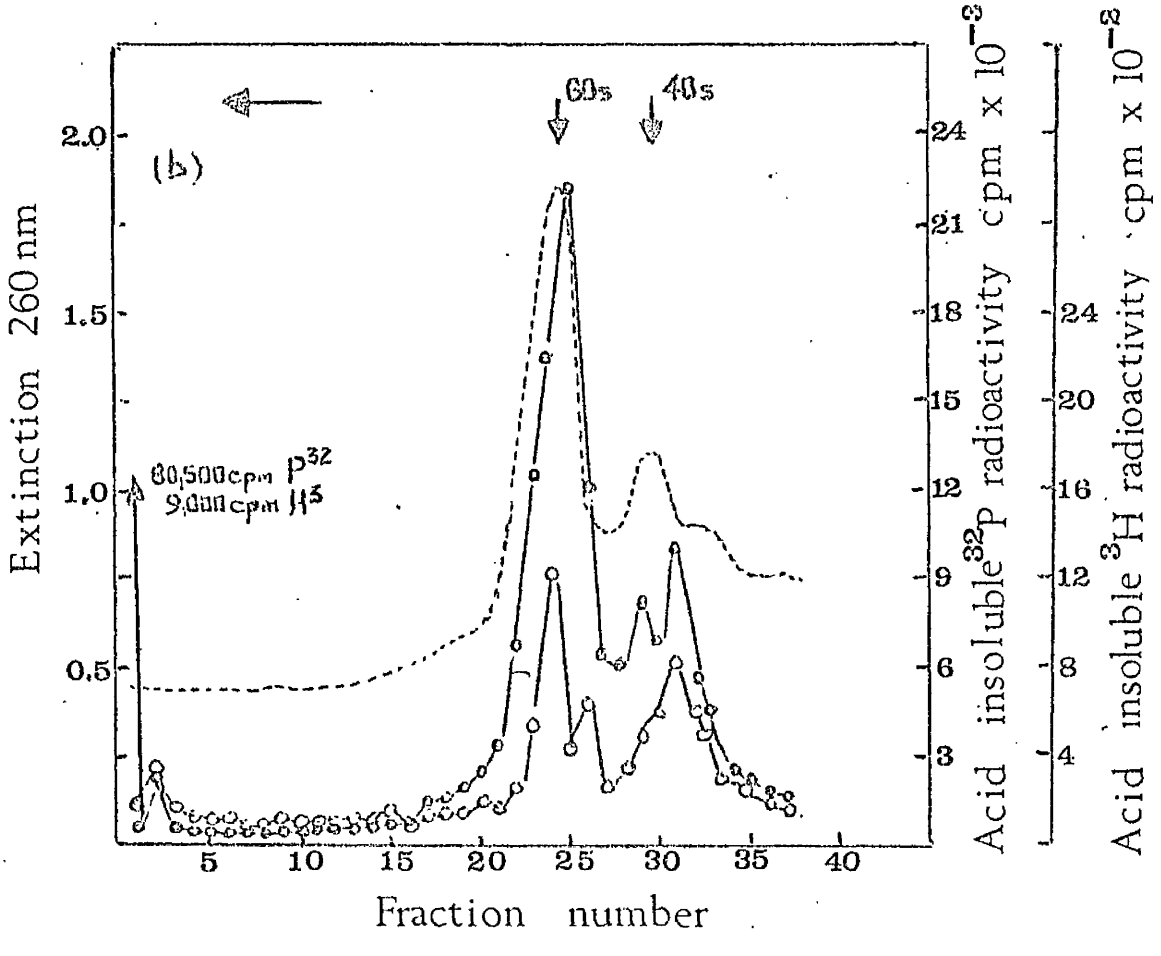
(a) 80 x 10^6 BHK-21/C13 cells, grown for 18h in 100ml ETC_{10} containing 25μCi [^{32}P]-orthophosphate (51.9Ci/mg P) were treated with 0.04μg/ml actinomycin D for 30min, then exposed to 100μCi [^{3}H]-guanosine (5Ci/mmol) for 60min at 37 C. From the harvested cells a deoxycholate treated cytoplasmic fraction was obtained and the polysomes displayed on a 15%(w/v) to 30%(w/v) sucrose gradiemt as in Fig.III.15.1. Fractions corresponding to the polysomal material as indicated by the bracket in Fig.III.15.1 were pooled, made 0.5%(w/v) with respect to SDS and precipitated with 2vol absolute ethanol. The RNA pellet, collected from this treatment by centrifugation at 2500 rev/min, was dissolved in 1.0ml TKM buffer (pH7.4) and layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in TKM buffer. The gradient was centrifuged at 24,500rev/min in an SW25.1 rotor of a Beckman model L2 65B for 15h at 4 C. The gradient was collected in 1.0ml fractions, the extinction being automatically monitored and recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. The fractions so collected were examined for radioactivity as described in Methods section 7.

(b) From a similar culture to (a) above, labelled to the same extent, a polyribosome pellet was obtained as described in Methods section 3(d). The polysome pellet was resuspended in 1.0ml 0.05M disodium EDTA (pH7.5) and centrifuged through a linear gradient of 15%(w/v) to 30%(w/v) sucrose in disodium EDTA (pH7.5) in an SW25.1 rotor of a Beckman model L ultra-centrifuge at 21,000 rev/min for 14½h. The gradient was The gradient was harvested in 1.0ml fractions and aliquots of the fractions examined for radioactivity as described above.

Extinction 260nm (----), [^{3}H] acid insoluble radioactivity cpm (—o—), [^{32}P]-radioactivity cpm (—•—)

The arrows shown in both Figs III.15.2(a) and (b) indicate the direction of sedimentation.


(a)
28s
18s
Extinction 260 nm
Acid insoluble ^{32}P radioactivity cpm x 10^{-3}
Acid insoluble ^{3}H radioactivity cpm x 10^{-2}
Fraction number


(b)
60s
40s
80,500cpm P32
9,000cpm H3
Extinction 260 nm
Acid insoluble ^{32}P radioactivity cpm x 10^{-3}
Acid insoluble ^{3}H radioactivity cpm x 10^{-2}
Fraction number

A preparation of pelleted ribosomes was resuspended in 0.05M disodium EDTA to dissociate the polyribosomes to ribosomal subunits (Gros et al, 1961) by the release of mRNA chains and the chelation of Mg^{++} ions. This EDTA suspension was then examined by sucrose density gradient centrifugation which revealed that dissociation to the subunits has taken place but that there was however still some [^{3}H]-radioactivity associated with the separated ribosomal subunits. Furthermore, appropriate fractions of the gradients depicted in Figs III.15,2 (a) and (b) and corresponding to either total polyribosomes, 60s subunits or 40s subunits were pooled, treated with SDS and after ethanol precipitation, the RNA was examined by polyacrylamide gel electrophoresis. The electrophoretograms of these RNA preparations are presented in Fig III.15.3 (a) - (c) respectively. It is apparent from Fig III.15.3 (a) that all the low molecular weight RNA species, tentatively referred to as species (1)-(10) can be found together with 5s and 4s RNA on the polyribosomes of BHK-21 cells. They are however absent from the EDTA isolated 60s subunits as indicated by Fig III.15.3 (b) but may be present on the 40s subunits although the 40s subunits, as obtained from the gradient depicted in Fig.III.15.2 (b), are visibly contaminated by a low molecular weight shoulder which may contain low molecular weight RNA species released by EDTA treatment of polysomes.

Association of low molecular weight RNA components of the cytoplasm with polyribosomes in BHK-21/C13 cells.

Fig.III.15.3

(a) Fractions 10-35 from the sucrose gradient analysis of polysomal RNA, indicated in Fig.III.15.2(a), were pooled, made 0.5%(w/v) with respect to SDS and precipitated with 2vol absolute ethanol at -20°C for 18h. The precipitated RNA was collected by centrifugation at 2500rev/min for 10min and dissolved in an appropriate buffer(Methods section 4(b)) in a volume of 100µl. This RNA sample was then examined by electrophoresis on gels of polyacrylamide as described in Methods section 5(b).(5ma/gel for 5½h). The gels were then stained, scanned for optical density, frozen, sliced into 1mm segments and assayed for radioactivity as described in Methods section 5(b).

Extinction 260nm (----), [^{3}H] radioactivity cpm (—o—), [^{32}P] radioactivity cpm (—•—)

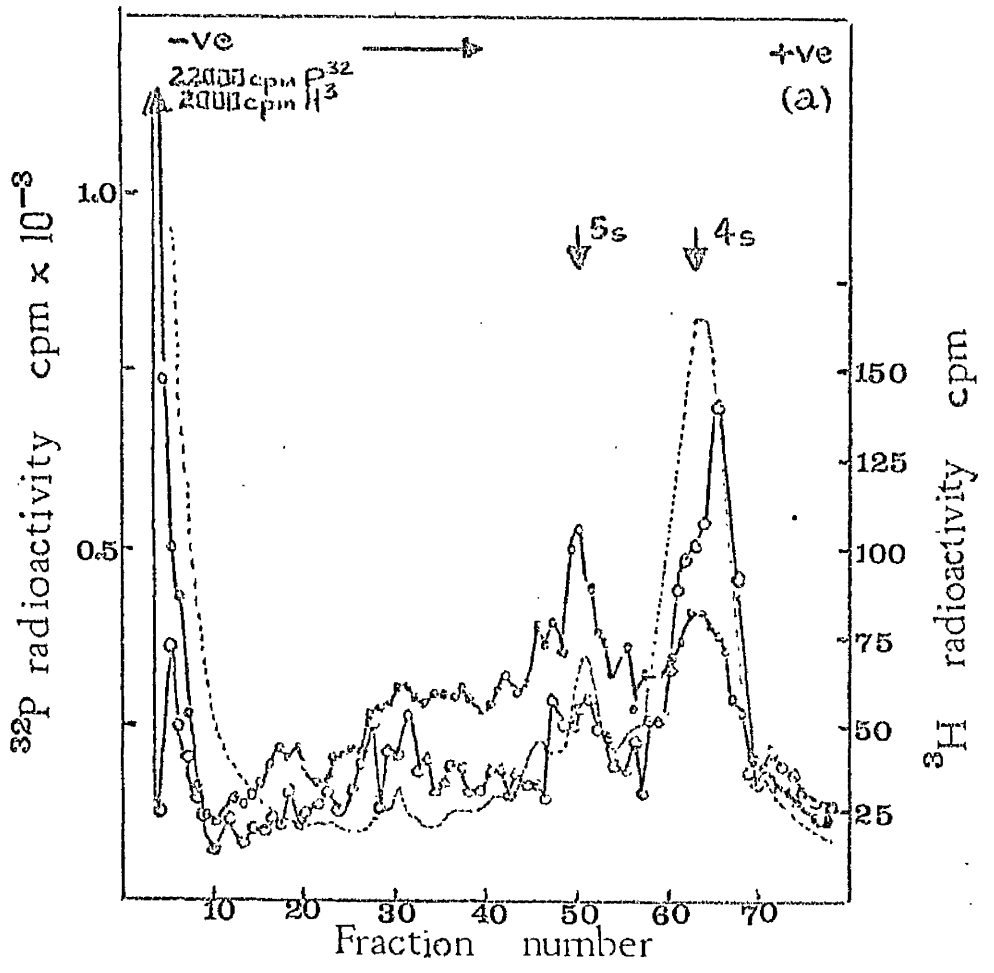
(b) Fractions 20-27 from the gradient displayed in Fig.III.15.2(b and corresponding to 60s ribosomal subunits were pooled, made 0.5%(w/v) with respect to SDS and precipitated with 2vol absolute ethanol at -20°C for 18h. The RNA obtained from this treatment was collected by centrifugation at 2500 rev/min dissolved in 100µl of the appropriate buffer (Methods section 4(b)) and examined by electrophoresis in gels of polyacrylamide. The gels were stained, scanned for optical density, frozen, sliced into 1mm segments and assayed for radioactivity as described in Methods section 5(b).

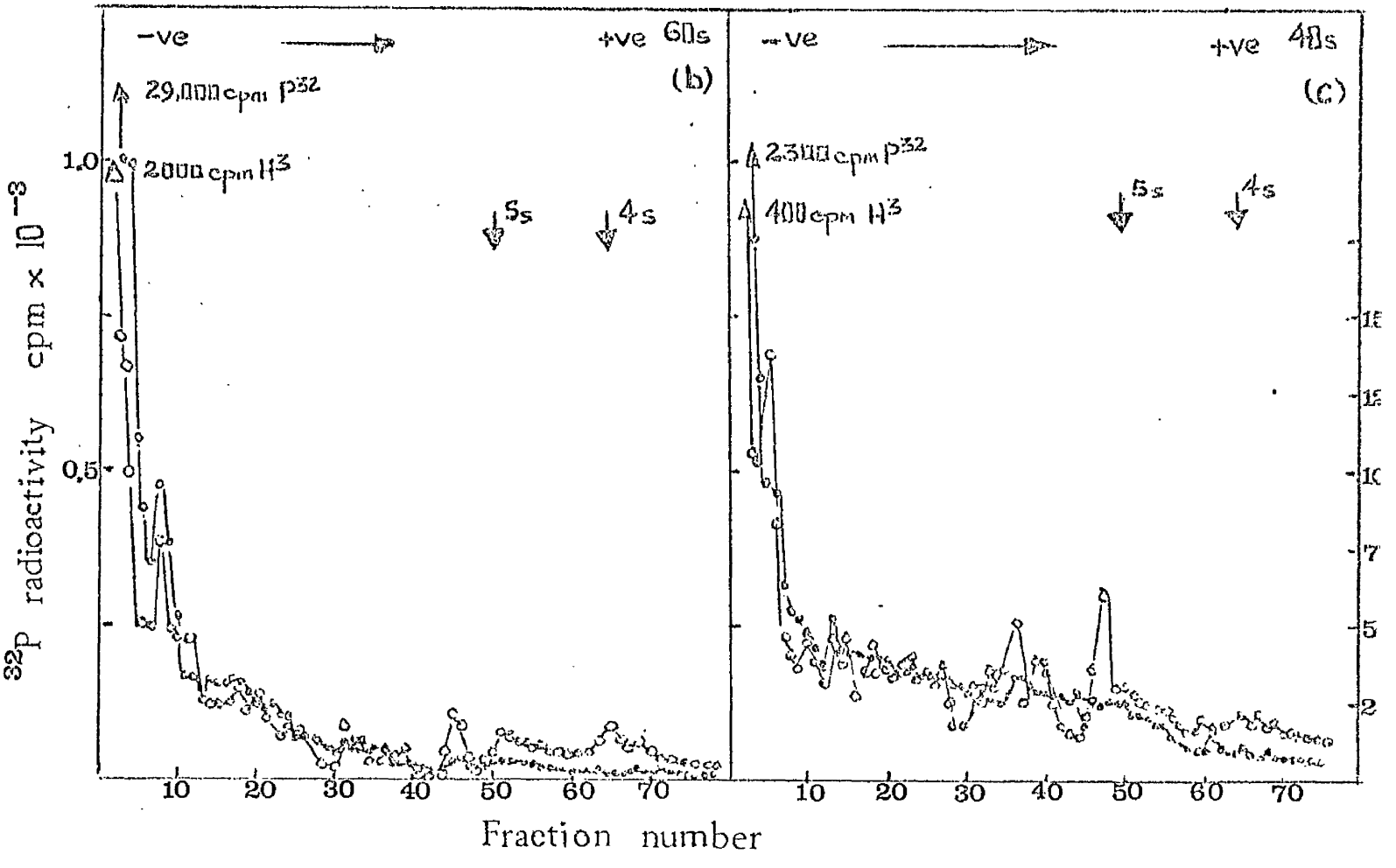
^{3}H radioactivity cpm (—o—), [^{32}P] radioactivity cpm (—•—)

(c) Fractions 28-36 from the gradient displayed in Fig. III.15 2(b) and corresponding to 40s ribosomal subunits, were pooled, made 0.5%(w/v) with respect to SDS and precipitated with 2vol absolute ethanol at -20°C for 18h. The RNA was collected as before and dissolved in 100µl of the appropriate buffer(Method section 4(b) and examined by polyacrylamide gel electrophoresis. as in (b) above.

[^{3}H]-radioactivity cpm (—o—), [^{32}P]-radioactivity cpm (—•—)

The direction of migration of the RNA species under the influence of the electromotive force is indicated by the solid black arrow in each of Figs. III.15.3(a)-(c).


-ve
+ve
(a)
22000 cpm P32
2000 cpm H3
5s
4s
1.0
0.5
32P radioactivity cpm x 10-3
3H radioactivity cpm
150
125
100
75
50
25
10
20
30
40
50
60
70
Fraction number


-ve
+ve 60s
(b)
29,000 cpm P32
2000 cpm H3
5s
4s
1.0
0.5
32P radioactivity cpm x 10-3
-ve
+ve 40s
(c)
2300 cpm P32
400 cpm H3
5s
4s
10
20
30
40
50
60
70
Fraction number

III.16 EDTA treatment and the release from polyribosomes of low molecular weight cytoplasmic RNAs

One of the presently accepted criteria for mRNA is that it should be present in the non-ribosomal RNA fraction released from polyribosomes by treatment with disodium EDTA (Gros et al, 1961). Since the low molecular weight RNA species found in the cytoplasm of BHK-21 cells and other mammalian cells show many of the characteristics representative of a class of non-ribosomal, polysome associated RNA species it was decided to investigate the possibility that they might represent relatively stable mammalian mRNA species. If this were so then they should be expected to be released from polyribosomes by EDTA treatment. Therefore cultures of BHK-21 cells were grown overnight in the presence of [^{14}C]-uridine then pulsed with [^{3}H]-guanosine in the presence of low levels of actinomycin D as with previous polyribosome preparations. The polyribosome pellets obtained from these cells were then treated with disodium EDTA as in the experiment reported in Fig III.15.2 (b). The results of sucrose density gradient analysis of this preparation are shown in Fig III.16 (a) which shows that the bulk of the [^{3}H]-radioactivity has been released from the polyribosomes and sediments in position between 6 and 35s with no [^{3}H]-radioactivity being associated with the [^{14}C]-labelled subunits derived from the polyribosomes. The [^{14}C]-

EDTA treatment and polyribosome associated low molecular weight RNA components of BHK-21/C13 cells.

Fig. III.16

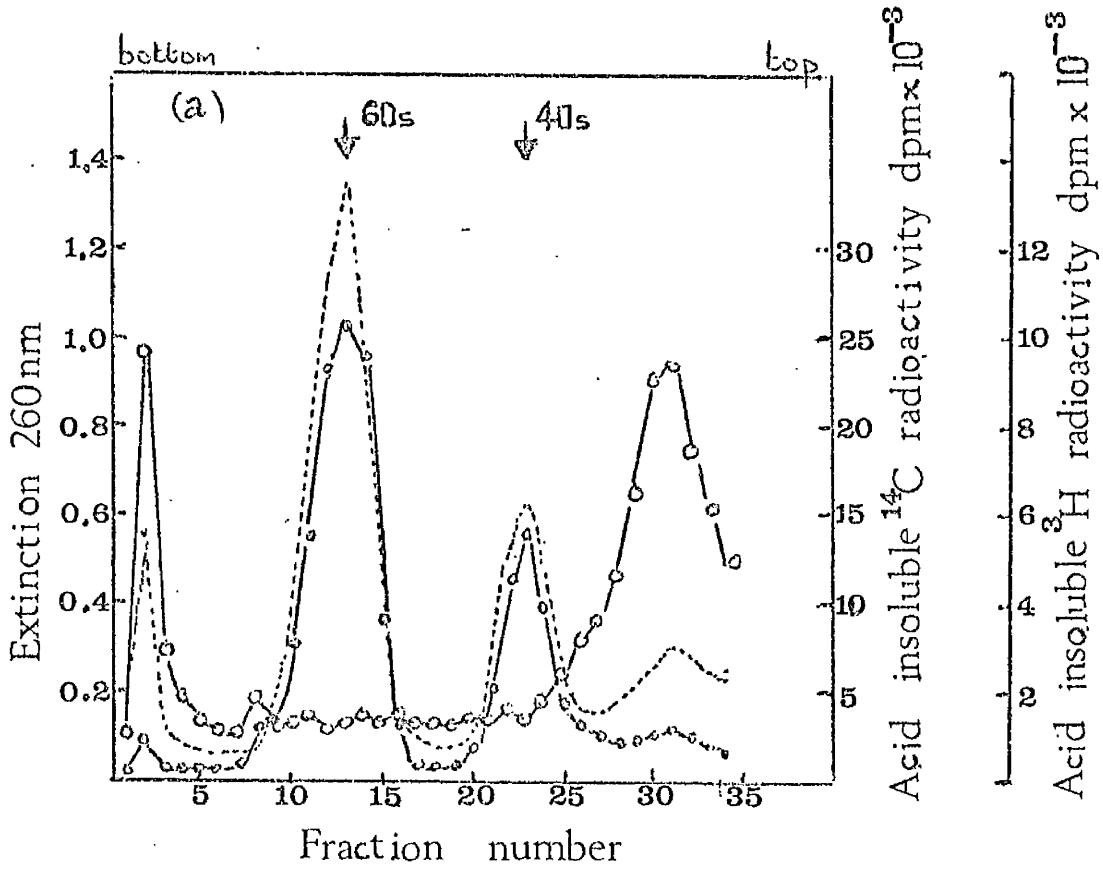
(a) 56 x 10^6 BHK-21/C13 cells were grown for 48h in 100ml ETC containing 50µCi [^{14}C]-uridine (62mCi/mmol) then treated with 0.04µg/ml actinomycin D for 30min before exposure to 250µCi [^{3}H]-guanosine (5Ci/mmol) for 60min. From the cell pellet a pellet of polysomes was obtained as described in Methods section 3(d). This pellet was then suspended in 1.0ml of 0.05M disodium EDTA by homogenisation and layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in 0.05M disodium EDTA (pH7.5) and centrifuged at 22,000 rev/min for 17h in an SW25.1 rotor of a Beckman L2 65B ultracentrifuge. The gradient was harvested in 1.0ml fractions, the extinction being automatically recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. An aliquot of each fraction was examined for radioactivity as described in section 7 of Methods.

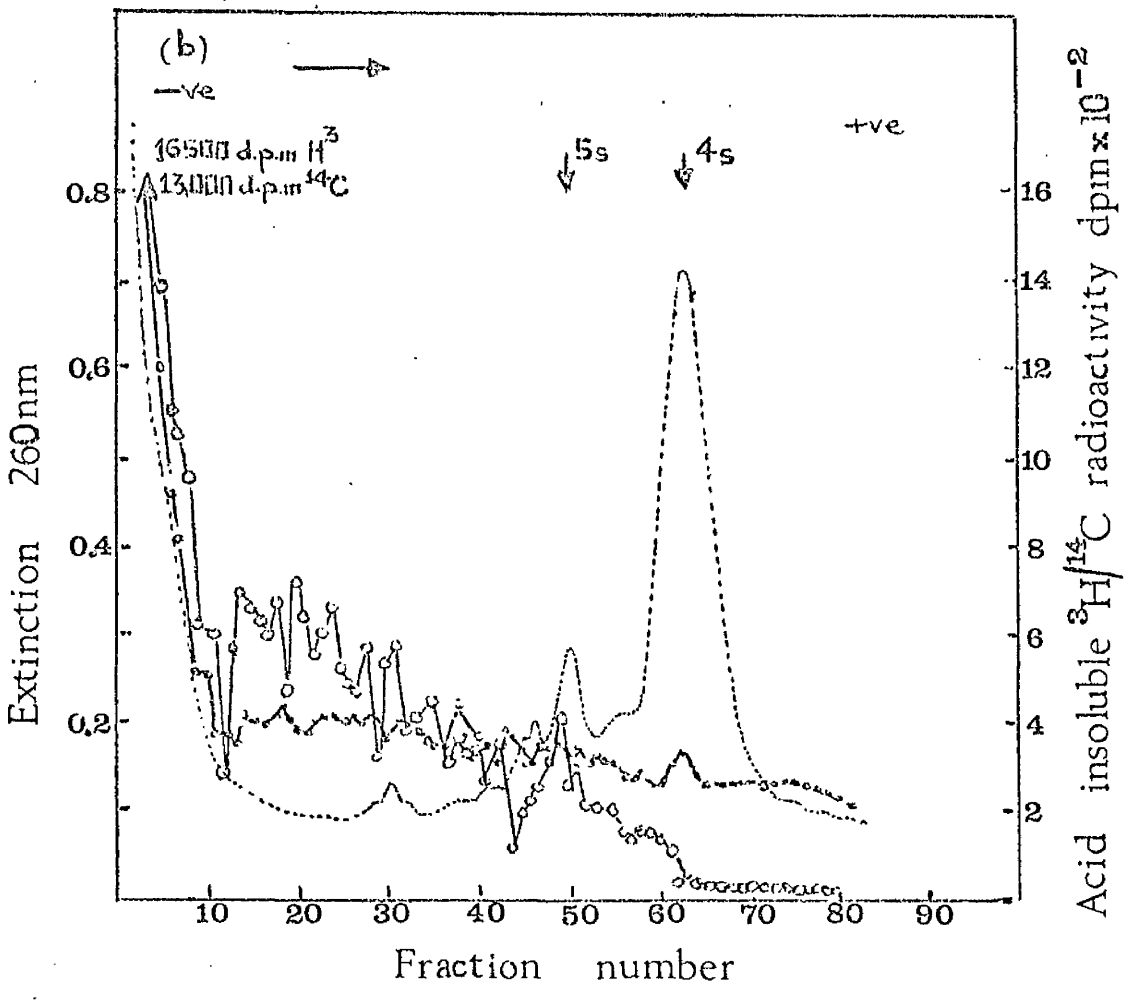
Extinction 260nm (---), [^{14}C] radioactivity dpm (—o—), [^{3}H]-radioactivity dpm (—o—)

(b) Fractions 26-33 from the gradient displayed in Fig.III.16(a) and corresponding to the EDTA released material, were pooled, made 0.5%(w/v) with respect to SDS and precipitated with 2vol absolute ethanol at -20°C overnight. The RNA was then dissolved in 200µl of an appropriate buffer(Methods section 4(b)) and examined by electrophoresis in 7.5% polyacrylamide gels. Electrophoresis was for 5h at 5ma per gel at room temperature.

Extinction 260nm (---) obtained as a result of electrophoresing unlabelled "cold phenol" RNA simultaneously. [^{14}C]-radioactivity dpm (—o—), [^{3}H] radioactivity dpm (—o—)

The direction of migration of the RNA species under the electromotive force is shown by the solid black arrow. The figures in the top left hand of Fig. III.16(b) refer to the radioactivity present in material of high molecular weight and excluded from the gel matrix.


bottom
top
(a)
60s
40s
Extinction 260nm
Acid insoluble ^{14}C radioactivity dpm x 10^{-3}
Acid insoluble ^{3}H radioactivity dpm x 10^{-3}
Fraction number


(b)
-ve
+ve
16500 d.p.m H^3
13000 d.p.m ^{14}C
5s
4s
Extinction 260nm
Acid insoluble $^{3}H/^{14}C$ radioactivity dpm x 10^{-2}
Fraction number

radioactivity follows precisely the optical density profile and therefore indicates that the sedimentation profile of the [^{3}H]-radioactivity could not be simply due to degradation but the true profile presented by the released RNA, for little [^{14}C]-radioactivity is associated with this released material. Fractions corresponding to the released material in Fig III. 16 (a) were pooled, treated with sodium dodecyl sulphate, ethanol precipitated and the RNA examined by polyacrylamide gel electrophoresis. The electrophoretogram of the released RNA, after analysis on 7.5% polyacrylamide gels is shown in Fig III.16 (b) from which it can be seen that the released material comprises a heterogeneous class of RNA molecules within which may be found the low molecular weight RNA species (1) - (10).

III.17 Nature of the EDTA-released polyribosome associated RNAs

Henshaw (1968) has shown that some 70% of the rapidly labelled RNA released from rat liver polyribosomes by EDTA treatment is in the form of ribonucleoprotein particles. To examine the question of whether the cytoplasmic polyribosome associated RNA species of BHK-21 cells were in the form of ribonucleoprotein particles or naked strands of RNA, the RNA or particles must be separated from the polyribosomes. This is accomplished by treating the polyribosome preparation with EDTA, a procedure shown by Gros et al, (1961) to separate the

ribosomes into their subunits and to release mRNA from bacterial polyribosomes. Cultures of BHK-21 cells were thereforegrown overnight in the presence of [^{32}P]-orthophosphate, pulsed for one hour in the presence of low levels of actinomycin D with [^{3}H]-guanosine, and polysome pellets obtained by centrifugation as before. A sucrose gradient analysis of an EDTA treated polysome preparation is shown in Fig.III.17(a). The [^{3}H]-labelled RNA has been separated from the [^{32}P]-labelled ribosomal subunits and the bulk of the released material sediments in the 6-40s region of the gradient with some material sedimenting between 40 and 60s. That a considerable portion of the released material is primarily in the form of free RNA strands is indicated by the results of CsCl gradient analysis shown in Fig.III.17(b) and (c). Polyribosome pellets from the cultures used in the experiments above were "fixed" with formaldehyde either without prior EDTA treatment or subsequent to EDTA treatment and were then analysed by CsCl gradient centrifugation as described in Methods section .

Formaldehyde fixation covalently cross-links macromolecules which are in close association, such as the RNA and protein of ribosomes, forming a structure which is then stable to exposure to the high salt conditions of CsCl gradients (Spirin et al, 1965). RNA which is associated with the ribosomes therefore becomes fixed to them and bands with them in CsCl gradients,

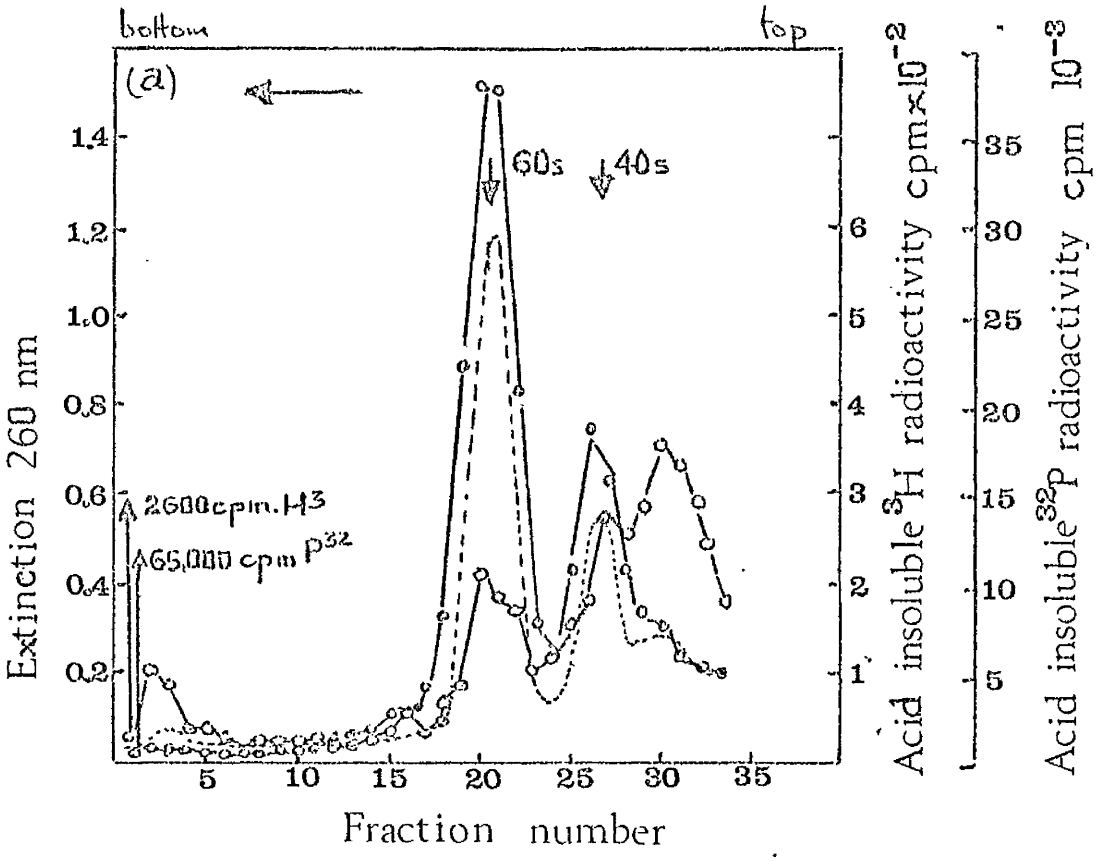
Fig.III.17

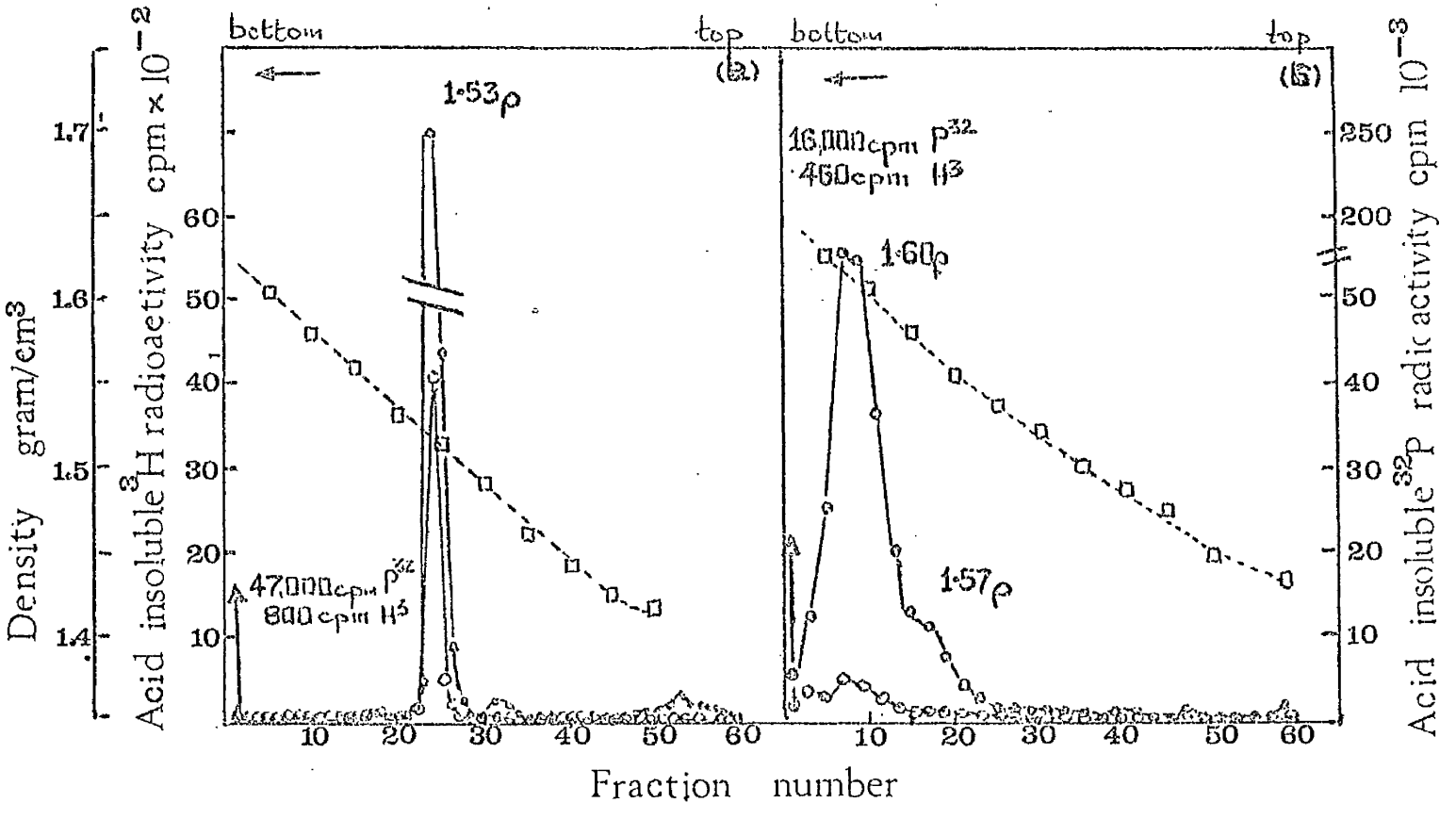
(a) 10^8 BHK-21/C13 cells, grown for 18h in 100ml ETC_{10} containing 50µCi [^{32}P] orthophosphate (67Ci/mg P) were treated with 0.04µg/ml actinomycin D for 30 min, then exposed to 100µCi [^{3}H]-guanosine (5Ci/mmol) for 60min at 37 C. From the cell pellet a cytoplasmic fraction was prepared as described in Methods section B.3(b), but in the presence of 0.01%(w/v) Macaloid. The cytoplasmic fraction was then layered over 2.5ml of 2M sucrose in TKM buffer, overlayed with 1.0ml of 0.5M sucrose in TKM buffer and a polysomal pellet was collected by centrifugation at 40,000rev/min in the SW40 rotor of a Griffen-Christ ultracentrifuge for 4h. The pellet was then resuspended in 1.0ml of 0.05M disodium EDTA(pH7.5) by hand homogenisation, layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in TKM buffer and centrifuged for 16h at 21,000 rev/min in an SW25.1 rotor of a Beckman model L ultracentrifuge The gradient was harvested in 1.0ml fractions, the extinction being automatically monitored and recorded by the passage of the sample through the flow cell of a Gilford recording spectrophotometer. An aliquot of each fraction was then analysed for radioactivity by the procedure described in Methods section 7.

Extinction 260nm (---), [^{32}P]-acid insoluble radioactivity cpm (—o—), [^{3}H]-acid insoluble radioactivity cpm (—o—).

(b) 10^8 BHK-21/C13 cells grown as in (a) above and the polysome pellet prepared as above. The pellet was resuspended in 1.0ml TKM buffer by homogenisation and the preparation made 4%(w/v) with respect to formaldehyde in an ice bath, and after 2h was mixed with sufficient CsCl solution to yield a final density of 1.48g/cm. This mixture was centrifuged at 36,000rev/min for 48h in an SW40 rotor and the gradients collected in 2 drop fractions. Density determinations were performed on every 5th fraction by Abbe refractrometry and the fractions were examined for radioactivity as described in Methods section 7.
[^{32}P]-radioactivity cpm (—o—), [^{3}H]-radioactivity cpm (—o—)
density g/cm (—□—)

(c) 10 BHK-21/C13 cells cultured as above and polysome pellet obtained as in (b) above but resuspended in 1.0ml disodium EDTA before being treated with formaldehyde, subjected to CsCl centrifugation and analysis as above
[^{32}P] radioactivity cpm (—o—), [^{3}H]-radioactivity cpm (—o—)
density g/cm (—□—).





The figures on the left hand extremities of the profiles represented above are the levels of radioactivity found in pelleted material at the bottom of the gradient tubes.

whilst unassociated RNA does not. Free RNA being of high buoyant density will, under the equilibrium conditions used here, form a pellet at the bottom of the CsCl gradient, and protein, being of relatively low buoyant density, forms a pellicle at the top of the gradient. Formaldehyde fixed ribonucleoprotein particles should therefore band at intermediate densities dependent upon the proportion of RNA to protein they contain. In Fig. III.17(b) the polyribosomes are seen to form a sharply sedimenting peak at a density of 1.53g/cm^3 and approximately 80% of the labelled RNA is present in the same region, indicating its association with polysomes. In Fig. III.17(c) is shown the CsCl density gradient profile of EDTA treated polyribosomes which shows a prominent [^{32}P]-radioactivity peak at a density of approximately 1.60g/cm^3 with a distinct shoulder at a density of 1.57g/cm^3. Little of the short time pulse [^{3}H]-radioactivity is found to be associated with either of the peaks of [^{32}P]-radioactivity at 1.60g/cm^3 or 1.57g/cm^3 but the bulk of the [^{3}H]-radioactivity is present as a pellet at the bottom of the gradient indicating that the released material is largely in the form of naked RNA strands rather than as ribonucleoprotein particles.

III.18 <u>The determination of the buoyant densities of ribosomal subunits in BHK-21 cells.</u>

In order to identify the peaks of [^{32}P]-radioactivity found, in

Determination of the buoyant density of BHK-21/C13 ribosomal subunits.

Fig.III.18

(a) 60 x 10^6 BHK-21/C13 cells were grown for 48h in 100ml ETC_{10} containing 10μCi[^{3}H]-guanosine (5Ci/mmol). From the cell pellet a ribosomal pellet was obtained as described in Methods section B.3(e). The ribosomal pellet was suspended in 1.0ml of 0.05M disodium EDTA (pH7.5) by hand homegenisation, layered over a linear gradient of 15%(w/v) to 30%(w/v) sucrose in 0.05M disodium EDTA(pH7.5), and centrifuged for 16h at 22,000 rev/min in a SW25.1 rotor of a Beckman L2 65B ultracentrifuge to display the ribosomal subunits. The gradients was collected in 1.0ml fractions, the extinction being automatically monitored and recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. An aliquot of each fraction was examined for radioactivity as described in Methods section B.7.

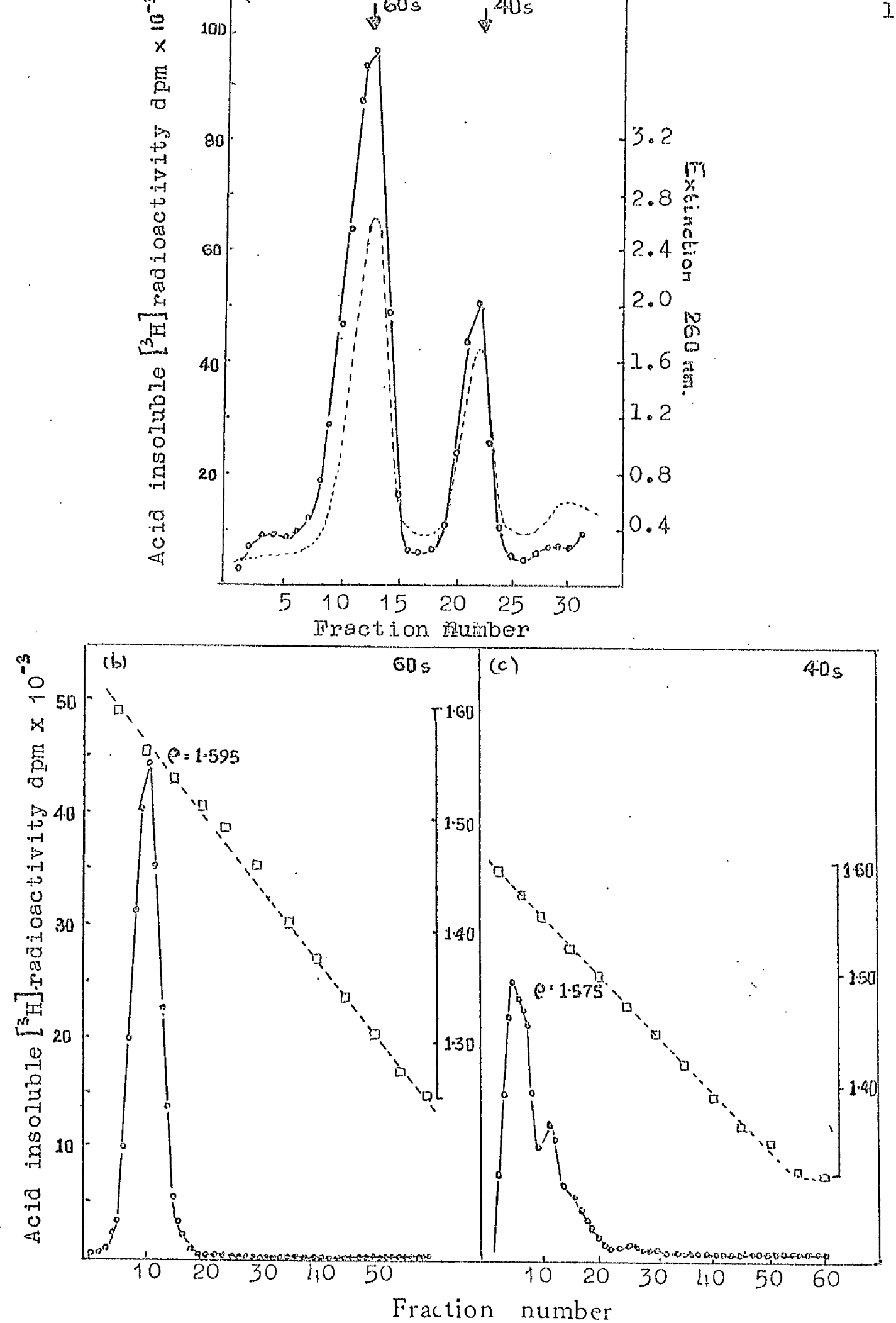
Extinction 260nm (----), [^{3}H] acid insoluble radioactivity dpm (—o—).

(b) The peak fraction (no. 12, Fig.III.18(a)) corresponding to 60s ribosomal subunits was made 8%(w/v) with respect to glutar aldehyde and subjected to CsCl density gradient centrifugation as described in Methods section B.6(b). The gradient was harvested in 3 drop fractions and density determinations were performed on every fifth fraction by Abbe refractometry. Each fraction was then analysed for radioactivity as described in section B.7 of Methods.

[^{3}H]-acid insoluble radioactivity dpm (—o—), density g/cm^3(—□—)

(c) The peak fraction (no. 22, Fig.III.18(a)) corresponding to 40s ribosomal subunits was made 8% with respect to glutaraldehyde and subjected to the procedure outlined in (b) above.

[^{3}H]-acid insoluble radioactivity dpm (—o—), density g/cm^3(-□-).


60s
40s
Acid insoluble [3H] radioactivity dpm × 10-3
Extinction 260 nm.
Fraction Number
(b)
60s
(c)
40s
Acid insoluble [3H]-radioactivity dpm x 10-3
e = 1·595
e = 1·575
Fraction number

the experiment described above, to have buoyant densities of 1.60g/cm^3 and 1.57g/cm^3 and to be derived from polyribosomes by EDTA treatment, the buoyant densities of BHK-21 ribosomal subunits were determined. A culture of BHK-21 cells was grown for 48 hours in the presence of [^{3}H]-guanosine to label predominantly the ribosomal RNA population, and a ribosomal pellet was prepared by Mg precipitation of a deoxycholate treated cytoplasmic fraction as described in Methods section. After EDTA treatment of the ribosomal pellet, ribosomal subunits were isolated by sucrose density gradient centrifugation as shown in Fig. III.18(a). Fractions corresponding to either "60s" or "40s" ribosomal subunits were then fixed with gluteraldehyde and analysed by CsCl gradient centrifugation (Baltimore & Huang, 1968). The results of these analyses are shown in Fig.III.18(b) and (c) and indicate that the "60s" ribosomal subunits have a buoyant density of approximately 1.595g/cm^3 and "40s" subunits have a buoyant density of 1.575g/cm^3. The [^{32}P]-radioactivity peaks shown in Fig.III.17(d) as possessing buoyant densities in CsCl of 1.60g/cm^3 and 1.575 g/cm^3 can therefore be identified as the "60s"and "40s" ribosomal subunits respectively. Therefore it can be stated that the bulk of the rapidly labelled, actinomycin D insensitive non-ribosomal RNA associated with the polyribosomes is released by EDTA as naked strands of RNA.

III.19 The kinetics of appearance in the cytoplasm of BHK-21 cells of newly synthesised ribosomal subunits.

The low molecular weight cytoplasmic RNA species which possess certain characteristics which resemble those of mRNA (Gros et al, 1961) for they can be isolated from polyribosomes by EDTA treatment and are rapidly labelled. In higher organisms mRNA synthesis is presumed to occur within the nucleus and the mRNA is then thought to be transported to the sites of protein synthesis, not as free RNA strands but in association with ribonucleoprotein particles. Two different interpretations have been placed upon the nature of these ribonucleoproteins. One suggests that they represent mRNA molecules attached to the smaller ribosomal subunit (Kohler & Arends, 1968, Joklik & Becker, 1965) and the other that they represent specific mRNA transporting particles distinct from ribosomes and called "informosomes" (Spirin, 1968). The "informosomes" model however suggests that, after EDTA treatment, mRNA should be released from polyribosomes in the form of ribonucleoprotein particles which will display intermediate and distinct buoyant densities in CsCl gradients. The results of experiments reported in Figs III.17(b) and (c) however have indicated that the material released by EDTA treatment of BHK-21 polyribosomes is released as free RNA. Experiments were therefore designed to investigate whether the low molecular weight

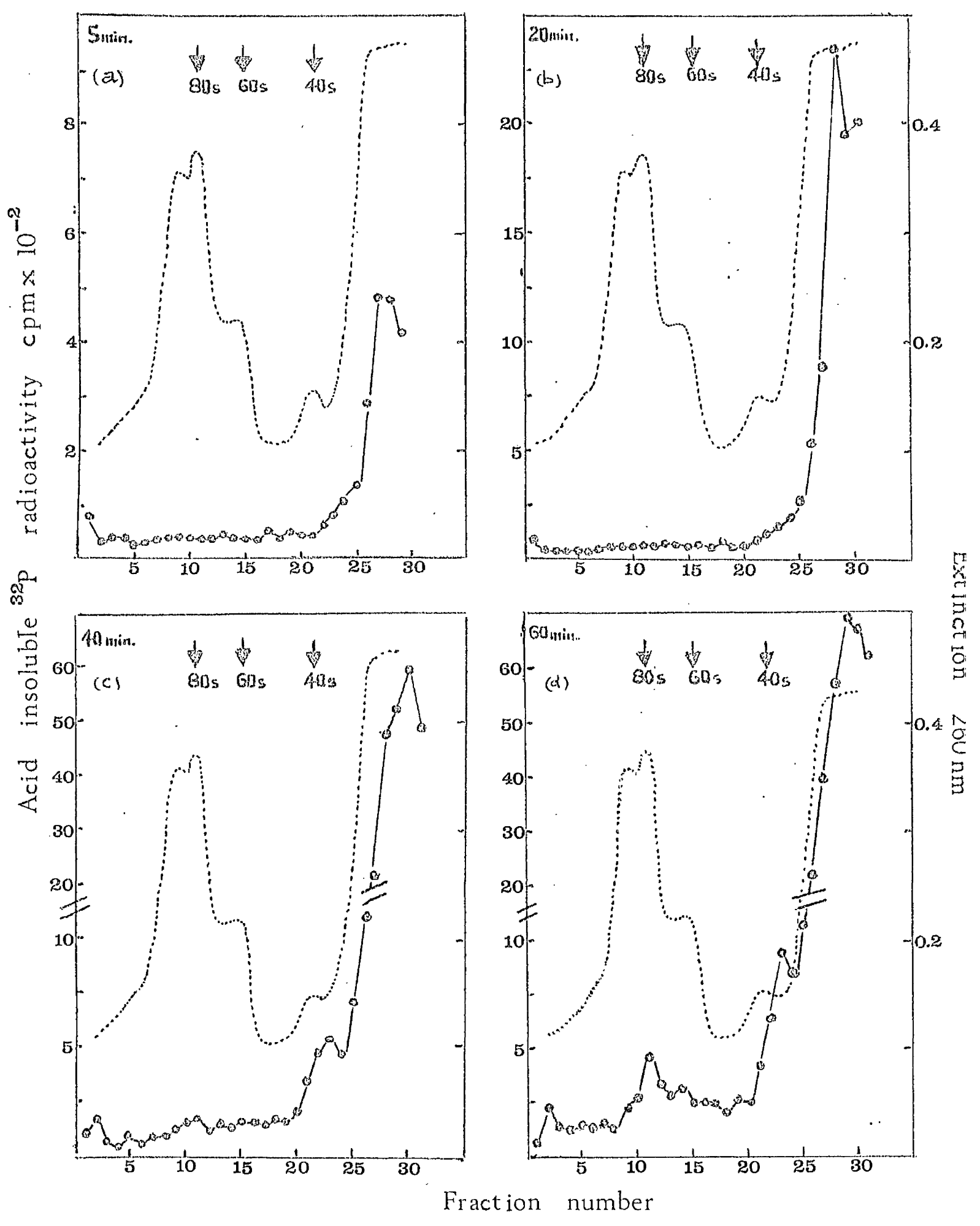
Kinetics of appearance of ribosomal subunits in the cytoplasm of BHK-21/C13 cells.

Fig. III.19

Cultures of BHK-21/C13 containing 50 x 10^6 cells were grown in suspension (10ml ETC_{10}) in 25ml spinner bottles at 37°C in the presence of 400μCi [^{32}P]orthophosphate (51.9Ci/mg P). The cultures were harvested at appropriate times after the addition of isotope and the cells collected by centrifugation at 2,500rev/min and washed with a 10ml portion of ice cold BSS. From the cell pellet a cytoplasmic fraction was derived as described in Methods section B.3(c), and subjected to centrifugation at 20,000g for 15min to remove the bulk of the polysomal material. The supernatant from this treatment was then layered over a linear gradient of 10%(w/v) to 30%(w/v) sucrose in TKM buffer and centrifuged for 6¾h at 22,500rev/min in an SW25.1 rotor of a Beckman model L2 65B ultracentrifuge The gradients were harvested in 1.0ml fractions, the extinction being automatically monitored and recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. An aliquot of each fraction was then examined for content of radioactivity as described in Methods section 7.

Extinction 260nm (-----), [^{32}P] acid insoluble radioactivity cpm (—•—),

(a) cytoplasm from cells pulsed for 5min, (b) pulsed for 20min (c) pulsed for 40min, (d) pulsed for 60min.


5min.
(a)
80s
60s
40s
20min.
(b)
40min.
(c)
60min.
(d)
Acid insoluble ^{32}P radioactivity cpm x 10^{-2}
Extinction 260 nm
Fraction number

cytoplasmic RNA species in BHK-21 cells might be transported to the cytoplasm in association with newly synthesised ribosomal subunits as proposed for mRNA by Joklick & Becker (1965). Cultures of BHK-21 cells were exposed to [^{32}P]-orthophorphate for short time periods and the cytoplasmic preparations obtained from these cells were analysed by sucrose density gradient centrifugation in order to display the monoribosomes and ribosomal subunits. The kinetics of appearance in the cytoplasm of BHK-21 cells, of newly synthesised [^{32}P]-labelled ribosomal subunits is shown in Figs III.19 (a)-(d) and it is apparent that newly synthesised "40s" ribosomal subunit first appear in the cytoplasm between 20 min and 40 min after the initiation of the labelling period and that 60s subunits appear later. After 60 min of labelling [^{32}P]-labelled monosomes (here referred to as 80s units) can be found in the cytoplasm. The results of kinetics of labelling experiments relating to the low molecular weight RNA species and reported in Figs III.2 (a) and (b) have shown that the low molecular weight polyribosome-associated cytoplasmic RNAs of BHK-21 cells can be found in the cytoplasm in as short a time as 10 min after the beginning of the labelling period. It therefore appears improbable that the newly synthesised 40s ribosomal subunits is the means of transport of these low molecular weight RNA species from the nucleus to the cytoplasm.

III.20 The effect of puromycin on the synthesis of low molecular weight cytoplasmic RNA in BHK-21 cells

The experiments described above (Fig III.19) have shown that newly synthesised "40s" ribosomal subunits are not the means of transport to the cytoplasm of low molecular weight polysome associated RNAs in BHK-21 cells. In puromycin treated Hela cells (Latham & Darnell, 1965) when the appearance of rRNA in the cytoplasm in inhibited, mRNA still continues to enter the cytoplasm attached to pre-existing ribosomal structures. The possibility therefore exists that, in BHK-21 cells, a situation similar to that present in puromycin treated Hela cells exists for mRNA transport from the nucleus to the polyribosomes. Cultures of BHK-21 cells were therefore grown for 30 min in the presence or absence of levels of puromycin (100 μg/ml) sufficient to cause a 98% inhibition of protein synthesis as judged by its effects upon the incorporation of [^{3}H]-leucine into acid-insoluble material. Cold phenol cytoplasmic RNA was isolated from these cells and mixed with an aliquot of a [^{14}C]-labelled cytoplasmic RNA preparation, prior to analysis by sucrose density gradient centrifugation. The results of such analysis are shown in Figs III.20 (a) and (b). These radioactivity distribution profiles indicate that levels of puromycin causing a total inhibition of protein synthesis in BHK-21 cells also cause

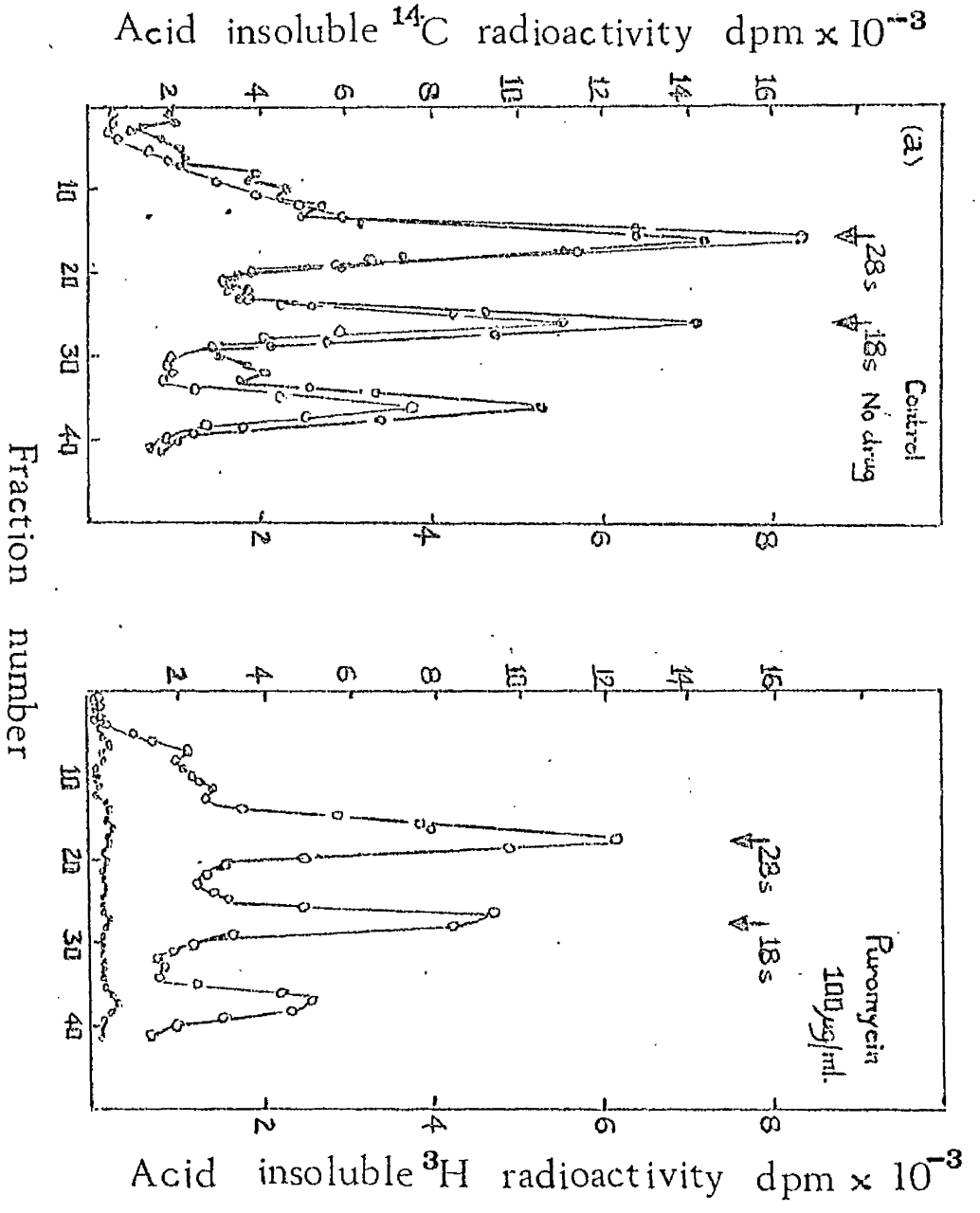
Effects of puromycin on the synthesis of low molecular weight RNA components of BHK-21/C13 cells.

Fig.III.20

Two cultures of BHK-21/C13 cells (10^7 cells in 50ml ETC_{10}) were grown for 18h at 37°C. To one of the cultures was then added 100µg/ml puromycin hydrochloride and the incubation continued for 15min. After this time 100µCi [^{3}H]-guanosine (5Ci/mmol) was added to each culture and the cells exposed to isotope, in the presence of absence of the drug, for 60min. The cultures were then harvested and RNA prepared from the cell pellet by the "cold phenol" method described in Methods section 4(a). The isolated RNA, dissolved in the appropriate buffer together with 50µl of a [^{14}C]-labelled marker RNA, was examined by sucrose density gradient centrifugation in a linear gradient of 5%(w/v) to 20%(w/v) sucrose in ammonium acetate buffer(pH5.1) as described in Methods section 5(c). The gradients were harvested as described, the extinction being automatically monitored and recorded by the passage of the sample through the flow cell of a Gilford recording spectrophotometer. Each fraction was then examined for radioactivity as described in Methods section B.7.

[^{14}C]-acid insoluble radioactivity dpm (—o—), [^{3}H]-acid insoluble radioactivity dpm (---o---)

(a) control cells, (b) puromycin treated cells.


Acid insoluble ^{14}C radioactivity dpm x 10^{-3}
(a)
Control
No drug
18s
28s
Puromycin
100 μg/ml.
18s
28s
Fraction number
Acid insoluble ^{3}H radioactivity dpm x 10^{-3}

an inhibition of the appearance of labelling of all types of cytoplasmic RNAs. Therefore unlike the mRNA species in Hela cells, the low molecular weight polyribosome associated RNA species of BHK-21 cells do not continue to appear in the cytoplasm after the inhibition of protein synthesis by puromycin. As found in Hela cells, however, it is apparent that puromycin inhibition of protein synthesis prevents the appearance in the cytoplasm of BHK-21 cells of newly synthesised ribosomes.

III.21 The effect of cordycepin on RNA synthesis in BHK-21 cells

Studies by Penman et al, (1970) have indicated that the 3' deoxyadenosine analogue, cordycepin, has a selective effect upon RNA synthesis in Hela cells in that the synthesis of rapidly labelled polyribosome associated RNA is inhibited by the drug whilst the synthesis of HnRNA is unimpaired. The effect of cordycepin upon the rapidly labelled polyribosome associated low molecular weight RNA species of BHK-21 cells was therefore studied. Cultures of BHK-21 cells were incubated in the presence or absence of various concentrations of the drug for a period of 30 min and were then labelled for 60 min with [^{3}H]-uridine. Cold phenol cytoplasmic RNA was isolated from the harvested cells. High molecular weight RNA was analysed by sucrose density gradient centrifugation and low molecular weight RNA was analysed by electrophoresis on 7.5%

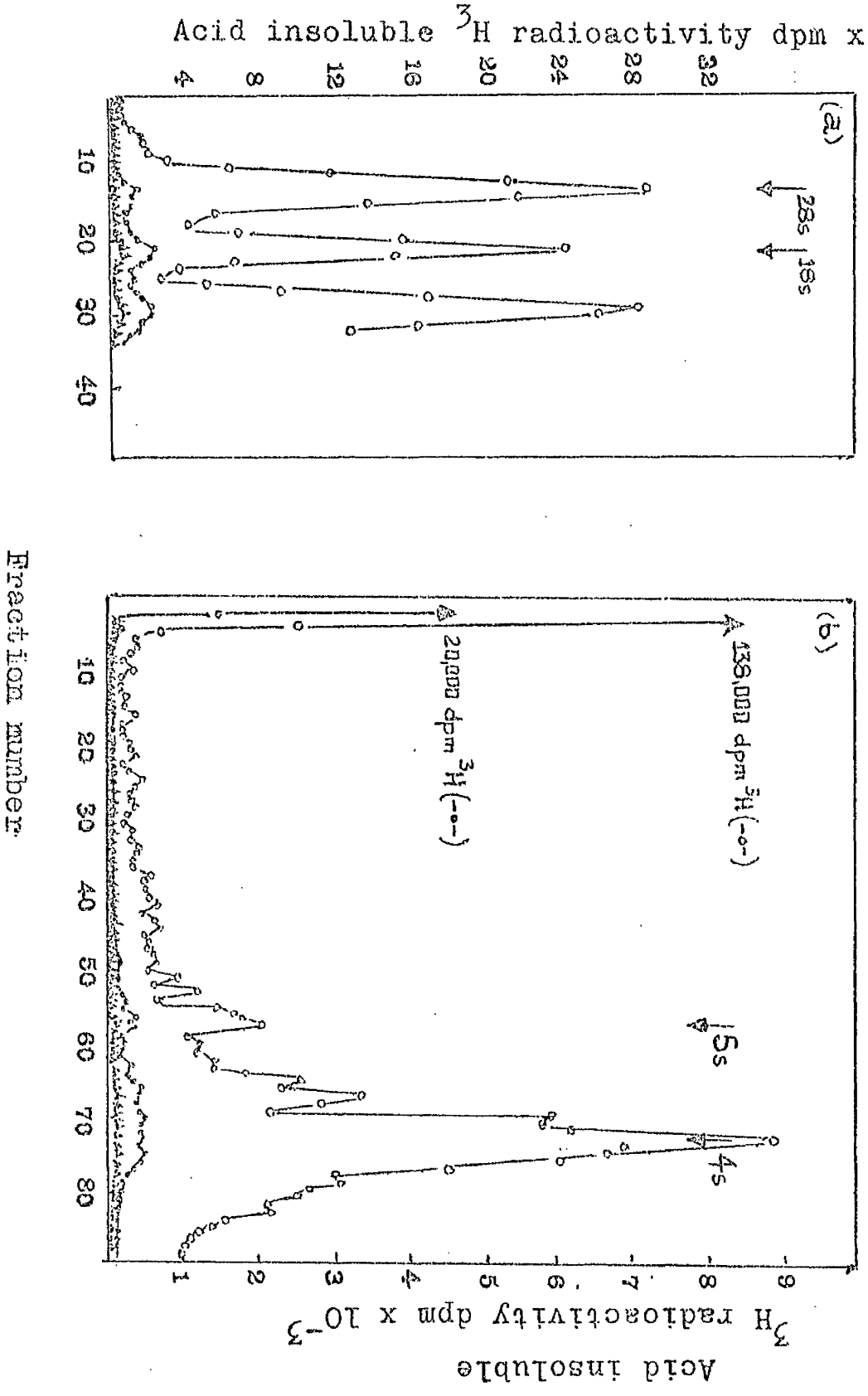
Effects of cordycepin on RNA synthesis in BHK-21/C13 cells

Fig.III.21

Roux flask cultures of BHK-21/C13 cells (10^7 cells in 50ml ETC_{10}) were grown for 18h at 37°C. To two of the cultures was added either 12.5µg/ml or 25µg/ml cordycepin and the cultures then incubated for 30min in the presence of the drug. After this preincubation period, 100µCi [^{3}H]-uridine (3.5Ci/mmol) was added to each culture and incubation continued for a further 60min. The cultures were then harvested and RNA extracted from the cell pellets by the cold phenol method (Methods section 4(a)). After dissolution in the appropriate buffer (Methods section 4(b)) samples of the appropriate RNA were either, layered over a linear sucrose gradient of 5%(w/v) to 20%(w/v) sucrose in 0.05M ammonium acetate (pH5.1) and centrifuged as described in Methods section 5(c), or examined by polyacrylamide gel electrophoresis as described in Methods section 5(b).

(a) Profiles of the distribution of radioactivity in the RNA components of BHK-21/C13 cytoplasm after centrifugation on 5%(w/v) to 20%(w/v) sucrose gradients, [^{3}H]-radioactivity in RNA from control cells (—o—), in RNA from cells treated with 12.5µg/ml cordycepin (—●—), in RNA from cells treated with 25µg/ml cordycepin (—▲—).

(b) Profiles of the distribution of radioactivity in the RNA components of BHK-21/C13 cytoplasm after electrophoresis in gels of polyacrylamide (7.5%). [^{3}H]-radioactivity in RNA from control cells (—o—), in RNA from cells treated with 12.5µg/ml cordycepin (—●—), in RNA from cells treated with 25µg/ml cordycepin (—▲—)


Acid insoluble ^{3}H radioactivity dpm x 10^3
4
8
12
16
20
24
28
32
(a)
23s
19s
10
20
30
40
(b)
138,000 dpm ^{3}H(-o-)
20,000 dpm ^{3}H(-•-)
15s
4s
Acid insoluble
^{3}H radioactivity dpm x 10^{-3}
1
2
3
4
5
6
7
8
9
10
20
30
40
50
60
70
80
Fraction number.

polyacrylamide gels. The profiles of the distribution of radioactivity in the RNA components of the cytoplasm of control and drug treated cells are shown in Fig III.21 (a) and (b) and indicate that levels of the drug of 12.5 µg/ml are sufficient to greatly inhibit the appearance of labelled RNA components in the cytoplasm of BHK-21 cells. A concentration of the drug of 25 µg/ml causes a total inhibition of labelling of the cytoplasmic RNA components. However Fig III.21 (b) indicate that both these drug concentrations totally inhibit the labelling of polyribosome associated low molecular weight RNA species in BHK-21 cells and that the synthesis of 4s and 5s RNA components is greatly impaired by drug treatment.

III.22 Relationship of the synthesis of low molecular weight cytoplasmic RNAs to the cell growth cycle in BHK-21 cells

Rapidly labelled polyribosome associated low molecular weight RNA components corresponding to histone mRNA species appear on Hela cell microsomes specifically during the S-phase of the cell growth cycle and their synthesis is linked to the replication of DNA (Brown, Scharff & Robbins, 1967, Gallwitz & Mueller, 1969). Since, in BHK-21 cells, the polyribosome associated low molecular weight RNA species appear to be stable for at least one generation time (14 - 16 hours in ETC_{10} growth medium, see Fig II.1(a)) it was decided to investigate the relationship of the synthesis of these low

molecular weight RNA species to the cell growth cycle and whether their synthesis was coordinated with the synthesis of the stable constituent of the nucleus, the DNA. Cultures of BHK-21 cells were therefore synchronised by the inhibition of DNA synthesis by treatment of the cultures with excess thymidine (Methods section A.3(b). At various times after release from the block the cultures were pulsed with [^{3}H]-guanosine for 60 min and cold phenol" RNA then isolated from the cells and examined by polyacrylamide gel electrophoresis. The distribution profiles of the radioactivity present in the various low molecular weight RNA components at selected times after release from the block are shown in Fig.III.22(a) and an analysis of the rates of synthesis of each RNA species at various times after release from the block is shown in Fig. III. 22(b). The rates of synthesis of most of the low molecular weight cytoplasmic RNA components during the S phase of the cell growth cycle appear to be independent of the rate of DNA synthesis and increase linearly in a fairly constant fashion throughout the period of examination but the changes in the rates of synthesis of the species referred to as (2) to (4) appear to show more pronounced changes than those of the other low molecular weight RNA species in that their rates of synthesis increase in a well defined manner over the late S to G_2 phase of the cell growth cycle and follows a similar pattern

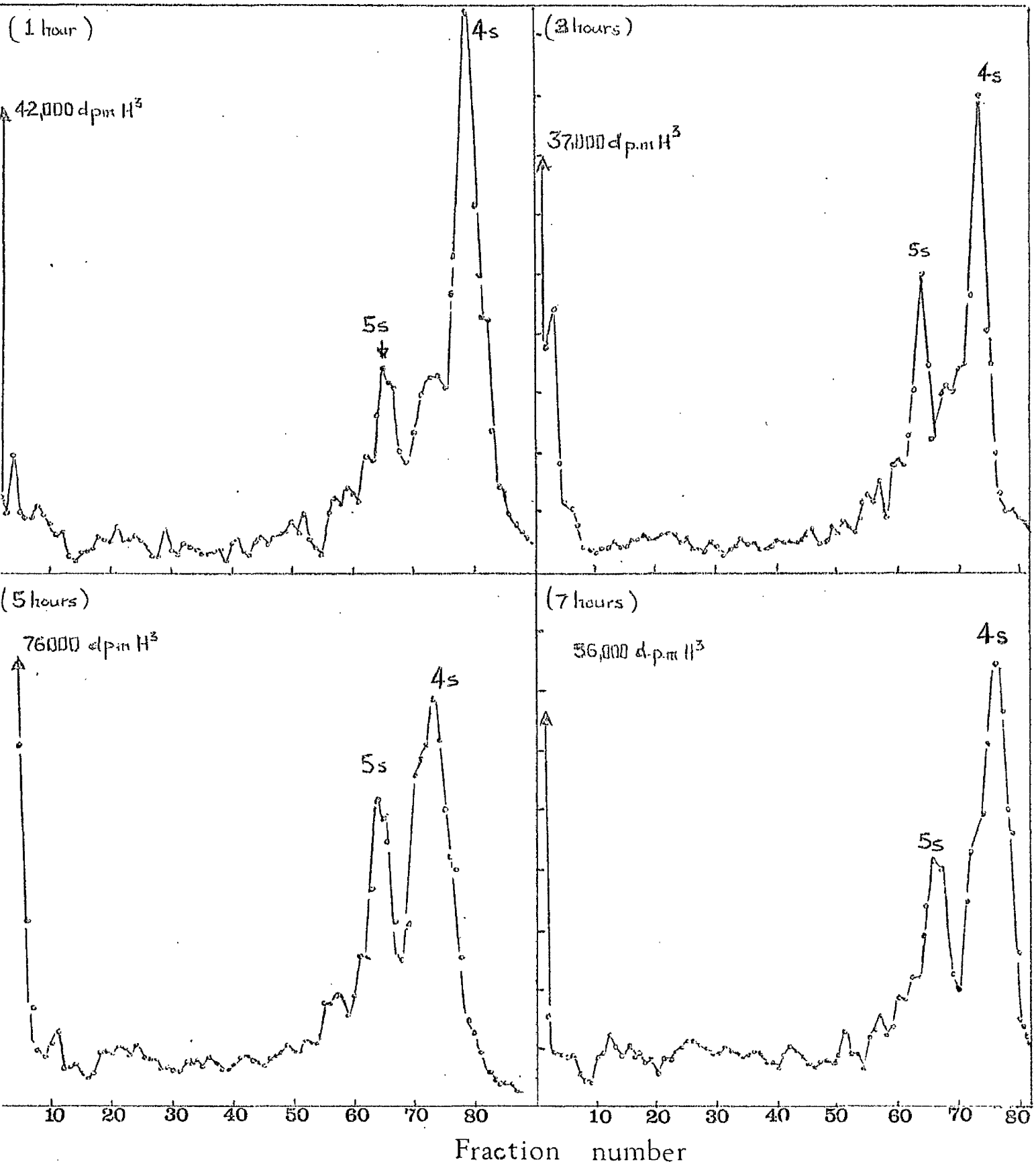
Synthesis of low molecular weight RNA components of BHK-21/C13 cytoplasm in relation to the cell growth cycle.

Fig.III.22(a)

Distribution profiles of radioactivity present in low molecular weight RNA components of BHK-21/C13 cytoplasm at various times after release from a thymidine block.

Monolayer cultures of BHK-21/C13 cells (5×10^7/80oz. winchester bottle) were grown for 18h in 100ml ETC_{10} at 37°C and synchrony induced by treatment of the cultures with 5mM thymidine as described in Methods section A.3(b), and the block reversed as described therein, by washing the cell monolayers with fresh prewarmed ETC_{10} growth medium. The cultures were then pulsed with 100µCi [^{3}H]-guanosine (5Ci/mmol) for 1h, in every hour after release, upto the 8th hour post release and the cells harvested, washed and collected as described in Methods section A2. Cold phenol RNA was isolated from each cell pellet and analysed by electrophoresis on 7.5% polyacrylamide gels. The profiles of the distribution of radioactivity in the low molecular weight RNA components at selected times after release from the block are shown in this figure. The gels were frozen, sliced into 1mm segments and assayed for radioactivity as described in Methods section 7.

^{3}H radioactivity dpm (—●—).


(1 hour)
42,000 d.p.m. H³
4s
5s
(2 hours)
37000 d.p.m. H³
4s
5s
(5 hours)
76000 d.p.m. H³
4s
5s
(7 hours)
36,000 d.p.m. H³
4s
5s
10 20 30 40 50 60 70 80
10 20 30 40 50 60 70 80
Fraction number

Synthesis of low molecular weight RNA components of BHK-21/C13 cytoplasm in relation to the cell growth cycle.

Fig.III.22(b)

The rates of synthesis of the various low molecular weight RNA components of BHK-21/C13 cytoplasm after release from a thymidine block.

Monolayer cultures of BHK-21/C13 cells (50 x 10^6/80oz. bottle) were grown for 18h in 100ml ETC_{10} at 37°C and synchrony induced by treatment of the cultures with 5mM thymidine as described in Methods section A3, and the block released, as described therein by washing the cell monolayers with fresh prewarmed ETC_{10} growth medium. The cultures were then pulsed with 100µCi [^{3}H]-guanosine (5Ci/mmol) for one hour, in every hour after release, up to the 8th hour after release, and the cells harvested, washed and collected as described in Methods section A2. From the cell pellets, "cold phenol" RNA was isolated (Methods section 4(a)) and analysed by electrophoresis on 7.5% polyacrylamide gels as described in Methods section 5(b).

At the same time smaller cultures of the same batch of cells (5 x 10^5 cells/60mm petri dish) were similarly treated with thymidine, but after release from the block, were pulsed with 5µCi [^{3}H]-thymidine (26Ci/mmol) and the rate of DNA synthesis measured, at various times after release from the block, by the incorporation of [^{3}H]-thymidine as described in Methods section A4. The same set of data regarding the different rates of [^{3}H]-thymidine incorporation at various times after release is presented in each section of Fig.III.22(b) (ie.(1) to (13)) to facilitate interpretation of the data regarding the rates of synthesis of various low molecular weight RNA components.

Incorporation of [^{3}H] radioactivity from [^{3}H]-thymidine (----)dpm. [^{3}H]-radioactivity from [^{3}H]-guanosine incorporated into low molecular weight RNA, dpm (—o—).

Section (14) shows similar data for ribosomal RNA synthesis obtained from 5%(w/v) to 20%(w/v) sucrose gradient analyses of RNA extracted from similar cultures treated with 100µCi [^{32}P]-orthophosphate (51.2Ci/mg P) after release from a thymidine block.

[3H] radioactivity dpm x 10^-3 (from [3H]-guanosine)
[32P]-radioactivity cpm
Ribosomal RNA
Time in hours after release from thymidine block

to that presented by the 18s and 28s ribosomal RNA species shown in Fig III.22 (b).14. The ribosomal RNA pattern shown in section 14 of Fig III.22 (b) is consistent with the transcription of the duplicated rDNA throughout the period of examination although it has been found that rDNA duplication occurs some 1½ to 3 hours after the initiation of the S phase (Amaldi, Giacomoni & Zito-Bignami, 1969) in chinese hamster cells.

III.23 The effect of toyocamycin on low molecular weight cytoplasmic RNA synthesis in BHK-21 cells

Earlier kinetics of labelling experiments reported in Fig III.2 (a) and (b) have suggested that the low molecular weight polyribosome associated species of the cytoplasm might possibly be derived not as a result of direct transcription but by the cleavage of some larger nuclear-located RNA molecules. Recently Tavitian, Uretsky & Acs, (1968) have reported that low concentrations of the adenosine analogue, toyocamycin, whilst allowing the production of 45s r-pre-RNA, prevents its maturation to cytoplasmic ribosomal RNAs. This is envisaged as occuring as a result of the incorporation of the analogue into the nucleolar precursors altering their tertiary structure and so inhibiting their cleavage to the mature species. It was therefore considered that since the polyribosome associated non-ribosomal RNA species of BHK-21 cytoplasm contained a

reasonable proportion of adenosine residues (see Table III.2), incorporation of the adenosine analogue into any putative precursor molecules for these species should possibly result in an inhibition of their appearance in the cytoplasm of these cells. Cultures of BHK-21 cells were therefore preincubated with various concentrations of toyocamycin (0.5μg/ml, 1.0μg/ml) prior to a 45 min incubation with ^{3}H -guanosine. Cytoplasmic RNA was then extracted by the cold phenol technique and analysed by sucrose density gradient centrifugation and electrophoresis in 7.5% polyacrylamide gels. The profiles of distribution of radioactivity in the RNA components of the cytoplasm as examined by sucrose density gradient centrifugation are shown in Fig.III.23(b). These profiles indicate that concentrations of the drug of either 0.5μg/ml or 1.0μg/ml totally inhibit the appearance in the cytoplasm of labelled 18s and 28s ribosomal RNA components in BHK-21 cells. Levels of the drug of 0.5μg/ml however suppress by 12.5% the incorporation of radioactivity into the low molecular weight RNA components displayed on sucrose gradients, and when the concentration of the drug of 1.0μg/ml is used the incorporation into this fraction is suppressed by about 50%. When these RNA preparations are examined by polyacrylamide gel electrophoresis it is apparent that both drug concentrations used totally suppress the appearance of radioactivity in the cytoplasmic

Effect of toyocamycin on cytoplasmic RNA synthesis in BHK-21 cells.

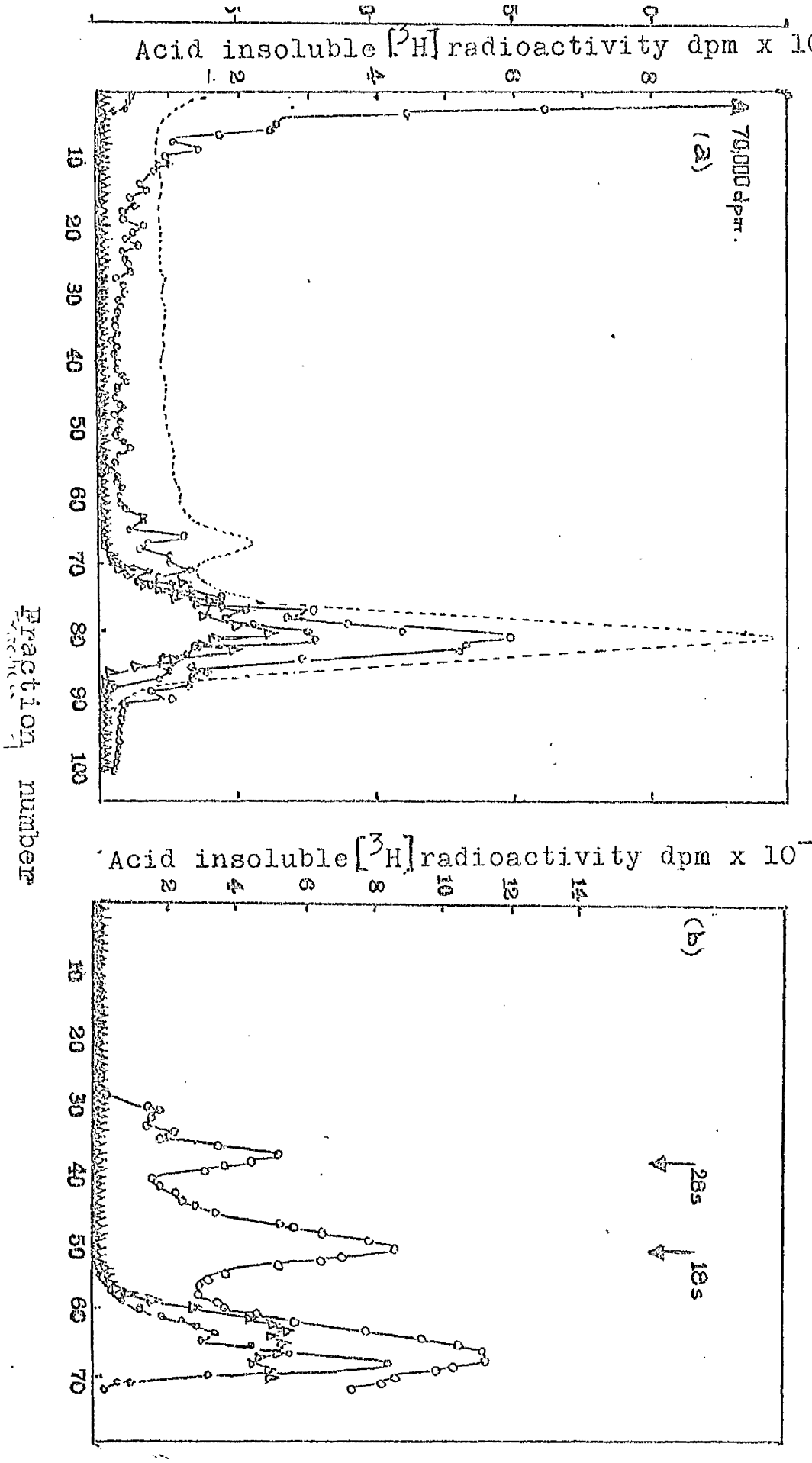
Fig.III.23

3 Roux bottle cultures of BHK-21/C13 cells (10^7 cells in 50ml ETC_{10}) were grown for 18h at 37°C. Toyocamycin was added to two of the cultures to a final concentration of either 0.5µg/ml or 1.0µg/ml and incubation continued for 30min. 100µCi of [^{3}H]-uridine (3.5Ci/mmol) were then added to each culture and the incubation continued for a further 45 min. After this time the radioactive growth medium was decanted, the cell monolayers washed twice with 50ml portions of ice cold BSS and the cells harvested by trypsinisation. "Cold phenol" cytoplasmic RNA was isolated from the cell pellets and after dissolution in the appropriate buffer(Methods section 4(b)),was examined by sucrose density gradient centrifugation in 5%(w/v) to 20%(w/v) sucrose gradients (Fig.III.23(b)) as described in Methods section 5(c), or by electrophoresis in gels of polyacrylamide (Fig.III.23(a)) as described in Methods section 5(b).

(a) Profile of the distribution of radioactivity in cytoplasmic RNA compoments of BHK-21/C13 cells after electrophoresis in 7.5% polyacrylamide gels.
[^{3}H]radioactivity in RNA from control cells (—o—), in RNA from cells treated with 0.5µg/ml toyocamycin (—•—), in RNA from cells treated with 1.0µg/ml toycamycin (—Δ—). [^{14}C]-radioactivity from coelectrophoresed marker RNA, dpm (– – – –).

(b) Profile of the distribution of radioactivity in cytoplasmic RNA components of BHK-21/C13 cells after centrifugation in 5%(w/v) to 20%(w/v) sucrose gradients.

[^{3}H]-radioactivity dpm in RNA from control cells (—o—), in RNA from cells treated with 0.5µg/ml toyocamycin (—•—), in RNA from cells treated with 1.0µg/ml toyocamycin (—Δ—).


Acid insoluble [3H] radioactivity dpm x 10-3
1 2 4 6 8
78,000 dpm.
(a)
10 20 30 40 50 60 70 80 90 100
Fraction number
Acid insoluble [3H] radioactivity dpm x 10-3
2 4 6 8 10 12 14
(b)
28s
18s
10 20 30 40 50 60 70

RNA species referred to as (2) to (10). In addition 5s RNA synthesis may also be inhibited by these relatively high concentrations and 4s RNA synthesis appears to be suppressed by about 50% in drug treated cultures.

III.24 The effects of α-amanitin on RNA synthesis in BHK-21 cells

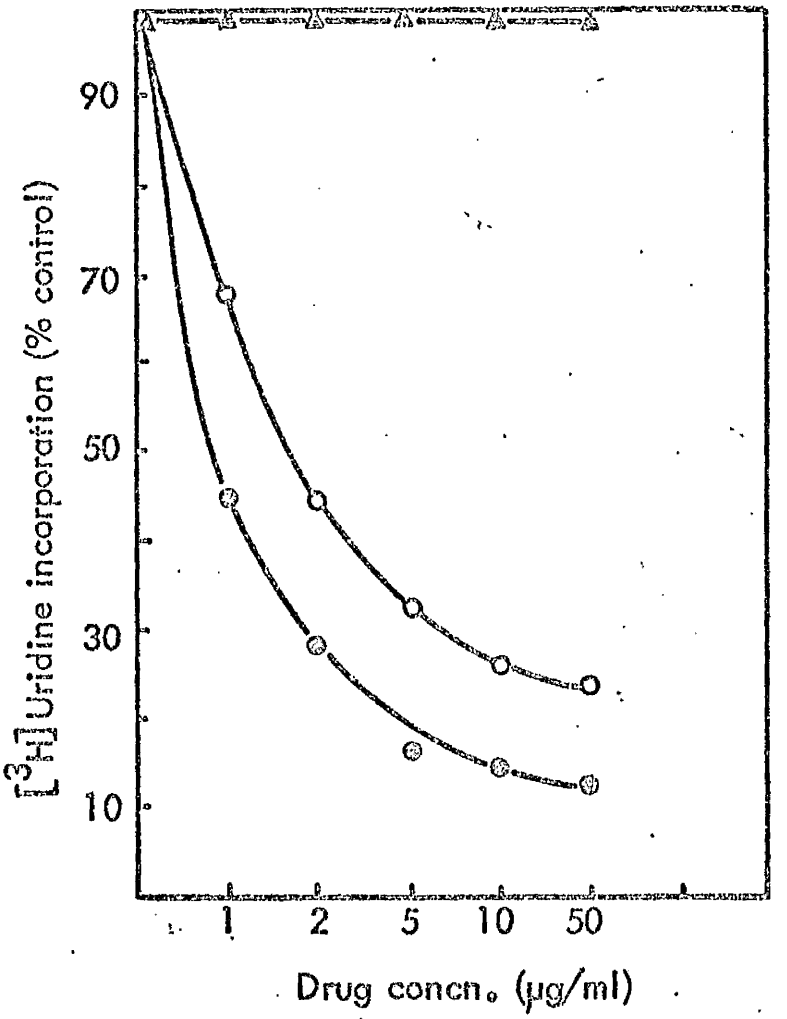
Experiments employing low levels of actinomycin D (see Fig. III. 12 (b)) have suggested the nucleoplasmic location of cistrons corresponding to some of the low molecular weight cytoplasmic RNAs but conclusive evidence in support of this hypothesis would only be obtained if some means of specifically inhibiting the synthesis of nucleoplasmic RNA was available. Soluble RNA polymerase activity extracted from various mammalian nuclei has exhibited two separate activities, one being associated with the nucleolus, the other extranucleolar (Roeder & Rutter, 1970, Jacob, Sajdel & Munro, 1970). In vitro polymerisation by the extranucleolar enzyme is inhibited by the drug α-amanitin, a slow acting toxic component of the toadstool, Amanita phalloides, whilst the antibiotic does not inhibit the nucleolar enzyme under similar conditions. Therefore a specific method for the inhibition of nucleoplasmic RNA synthesis apparently resided in the use of α-amanitin and the in vivo effects of α-amanitin upon RNA synthesis in BHK-21 cells were therefore investigated. Cultures of BHK-21 cells were preincubated for various times in the presence of various

concentrations of the drug before exposure to [^{3}H]-uridine for either 15 min or one hour. From each set of cultures the acid soluble radioactivity was obtained from the washed cell monolayers as described in the legend to Fig.24 (a). From the isolated subcellular fractions of similarly labelled cultures the total acid insoluble radioactivity present in nuclear RNA was estimated from cultures labelled for 15 min, and in cytoplasmic RNA from cultures exposed to isotope for 60 min. The results of these investigations are presented in Fig III.24 (a) and indicate that the incorporation of [^{3}H]-uridine into the RNA of the nuclear and cytoplasmic fractions of BHK-21 cells was reduced by a maximum of 70-80% by α-amanitin treatment. This effect was found to be independent of the duration of the preincubation period (5 min, 10 min or one hour) of the cells with the drug and therefore represents a true effect of the drug upon RNA synthesis rather than an expression of rapid cell death caused by drug treatment. Furthermore since the level of radioactivity present in the acid soluble fraction of both treated and untreated cultures is the same it is apparent that α-amanitin does not interfere with the uptake of the nucleoside. Since however the acid insoluble radioactivity in the various subcellular fractions falls with an increasing level of drug treatment there is therefore no apparent deficiency in drug uptake itself. The

Sensitivity of in vivo RNA synthesis in BHK-21/C13 cells to α-amanitin.

Fig.III.24

Cultures of BHK-21/C13 cells were grown for 18h in ETC_{10} and preincubated for an appropriate time with various concentrations of α-amanitin before exposure to 50μCi [^{3}H]-uridine (5Ci/mmol) for a suitable time period (15min or 60min). The growth medium was then removed and the cell monolayers washed with an appropriate volume of ice cold BSS. Acid soluble material was obtained from the washed cell monolayer (10^6 cells/60mm dish) by further washing with two 1ml portions of ice cold 5%(w/v) trichloracetic acid and the radioactivity in the combined fractions assayed by liquid scintillation spectrometry in 10ml of a dioxan based scintillant. (Methods section B7). (-△-). "Cold phenol" cytoplasmic RNA was extracted from 10^7 cells(-●-). and nuclear RNA (-○-) from 2 x 10^7 cells as described in Methods section 4(b). The RNA samples were examined for radioactivity by the procedure outlined in Methods section B7.


90
70
50
30
10
[3H] Uridine incorporation (% control)
1
2
5
10
50
Drug concn. (µg/ml)

shape of this in vivo dose response curve, with respect to RNA synthesis of components of the nucleus and cytoplasm of BHK-21 cells, is similar to that obtained with a soluble RNA polymerase activity isolated from rat liver, (Novello, Fiume & Stirpe, 1970). To investigate the nature of the α-amanitin sensitive RNA, cultures of BHK-21 cells were treated with α-amanitin as before and labelled for either 15 min or 60 min with [^{3}H]-uridine. Cold phenol RNA was isolated from cultures exposed to isotope for 60 min and nuclear RNA was isolated by the "hot phenol-SDS" technique from the detergent cleaned nuclei of cells labelled for 15 min. Cytoplasmic RNA was examined by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis and nuclear RNA was analysed only by sucrose density gradient examination. Profiles of the distribution of radioactivity in the RNA components of the cytoplasm from control and drug treated cultures after centrifugation in 5% - 20% (w/v) sucrose gradients are presented in Fig III.24 (b) and indicate a progressive reduction in the levels of incorporation of radioactivity in all species of cytoplasmic RNA as the level of drug treatment is increased. These profiles also reveal the unexpected inhibition of ribosomal RNA labelling. It should be noted also that some 10% of the total acid insoluble radioactivity is apparently resistant to α-amanitin

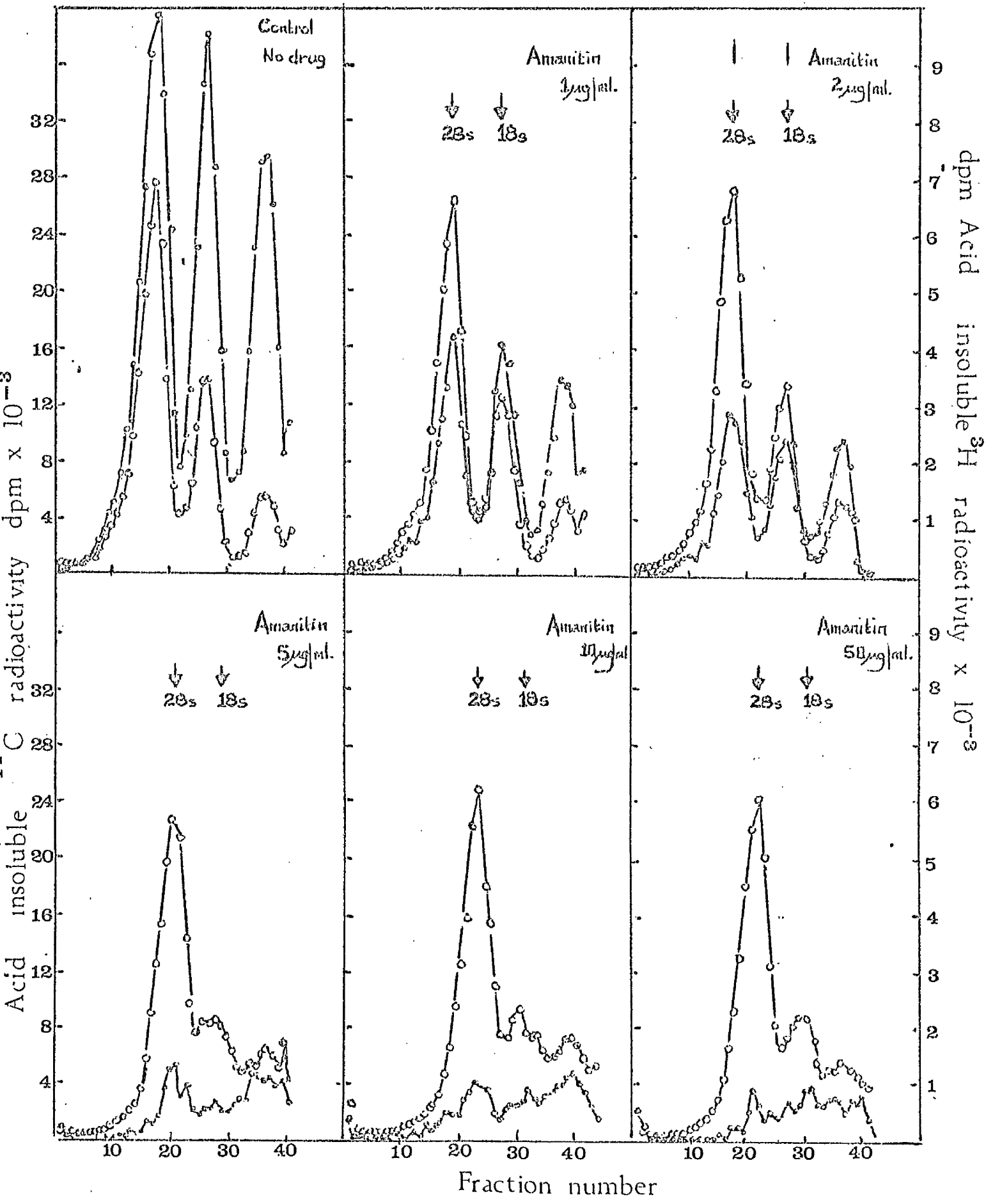
Effect of α-amanitin on the synthesis of RNA components of the cytoplasm of BHK-21/C13 cells.

Fig.III.24(b)

Profiles of the distribution of radioactivity in the RNA components of BHK-21/C13 cytoplasm synthesised in the presence of α-amanitin

BHK-21/C13 cells in 100ml ETC_{10} were grown to a density of 10^7 cells per culture and preincubated with various concentrations of α-amanitin for 30min before being exposed to 100μCi [^{3}H]-uridine (5Ci/mmol) for 60 min. After this time the cultures were washed, harvested and collected as described in Methods section A2. Cold phenol RNA was extracted from the cell pellets (Methods section 4(a)) and after dissolution in the appropriate buffer (Methods section 4(b)) was layered together with 50μl of [^{14}C]-labelled marker RNA over a linear gradient of 5%(w/v) to 20%(w/v) sucrose in ammonium acetate buffer (Methods section 5(c)) containing 0.1%(w/v) SDS. The gradients were centrifuged at 39,000rev/min for 5h in an SW40 rotor of a Beckman L2 65B ultracentrifuge, and were harvested in 8drop fractions, the extinction being automatically monitored and recorded by passage of the sample through the flow cell of a Gilford recording spectrophotometer. The fractions were then assayed for radioactivity as described in Methods section B7.

Acid insoluble [^{14}C] radioactivity dpm (—o—), acid insoluble [^{3}H]-radioactivity dpm (—o—).


Control
No drug
Amanitin
1μg/ml.
Amanitin
2μg/ml.
Amanitin
5μg/ml.
Amanitin
10μg/ml
Amanitin
50μg/ml.
28s
18s
Acid insoluble ^{14}C radioactivity dpm x 10^{-3}
dpm Acid insoluble ^{3}H radioactivity x 10^{-3}
Fraction number

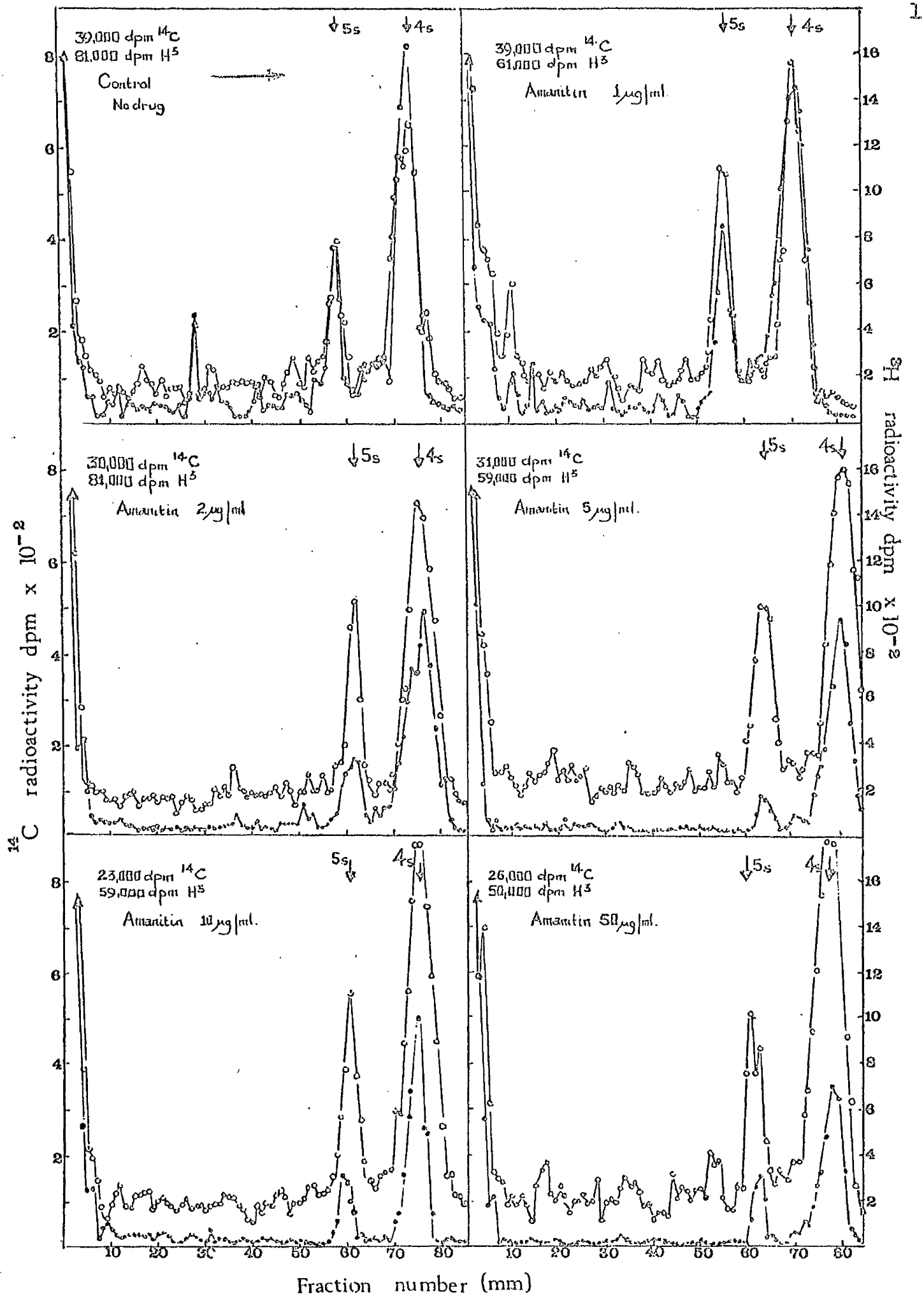
Effect of α-amanitin on the synthesis of RNA components of the cytoplasm of BHK-21/C13 cells.

Fig.III.24(c)

Profiles of the distribution of radioactivity in the low molecular weight RNA components of BHK-21/C13 cytoplasm synthesised in the presence of α-amanitin.

BHK-21/C13 cells in 100ml ETC_{10} were grown to a density of 10^7 cells/culture and preincubated with various concentrations of α-amanitin for 30min before being exposed to 100μCi [^{3}H]-uridine (5Ci/mmol) for 60 min. After this time the cultures were washed, harvested and collected as described in Methods section A2. Cold phenol RNA was extracted from the [^{3}H]-labelled cell pellets and after dissolution in the appropriate buffer (Methods section 4(b)) was mixed with 50μl of a [^{14}C]-labelled marker RNA preparation and examined by electrophoresis in 7.5% polyacrylamide gels.(Methods section 5(b)). Radioactive gels were then frozen, sliced into 1mm segments and the fractions assayed for radioactivity as described in Methods section 5(b). The direction of migration was from negative to positive as indicated by the arrow in the first of the figures shown in Fig.III.24(c) The figures quoted in the top left hand corner of each section of the figure represent the radioactivity present in material excluded from the gel and retained at the origin.

^{14}C radioactivity dpm (—o—), ^{3}H radioactivity dpm (—•—).


39,000 dpm 14C
81,000 dpm H3
Control
No drug
5s
4s
39,000 dpm 14C
61,000 dpm H3
Amanitin 1µg/ml.
30,000 dpm 14C
81,000 dpm H3
Amanitin 2µg/ml
31,000 dpm 14C
59,000 dpm H3
Amanitin 5µg/ml.
23,000 dpm 14C
59,000 dpm H3
Amanitin 10µg/ml.
26,000 dpm 14C
50,000 dpm H3
Amanitin 50µg/ml.
14C radioactivity dpm x 10-2
3H radioactivity dpm x 10-2
Fraction number (mm)

treatment and sediments in sucrose gradients with a rather heterogeneous distribution between 6 and 28s. The nature of this resistant material was examined by polyacrylamide gel electrophoresis as shown in Fig III.24 (c). These profiles of the distribution of radioactivity in the low molecular weight RNA species resolved by electrophoresis on 7.5% polyacrylamide gels reveal that at lower concentrations of α-amanitin the polyribosome associated cytoplasmic RNAs survive the drug treatment but that their synthesis is inhibited by concentrations of the drug above 2 μg/ml, above which the only detectably labelled RNA species are 5s and 4s species together with some larger higher molecular weight material which is excluded from the gels. Profiles of the distribution of radioactivity in the nuclear RNA components of BHK-21 cells, synthesised in the presence of α-amanitin are shown in Fig III.24 (d). Once again the same pattern of inhibition as occurred in the cytoplasm presents itself in the nuclear RNA species with a distinct inhibition of 45s and 32s r-pre-RNA labelling occuring with the increased drug concentration and a residual amount of incorporation in lower molecular weight components surviving at the highest drug concentrations used. These results of α-amanitin sensitivity of RNA synthesis in BHK-21 cells therefore tend to suggest that the *in vivo* effects of α-amanitin are quite different from the *in vitro* effects.

Effect of α-amanitin on the synthesis of RNA components of the nucleus in BHK-21/C13 cells.

Fig.III.24(d)

Profiles of the distribution of radioactivity in the RNA components of the nucleus of BHK-21/C13 cells synthesised in the presence of α-amanitin.

BHK-21/C13 cells in 50ml ETC_{10} were grown to a density of 2 x 10^7 cells per culture and preincubated for 30min with various concentrations of α-amanitin before being exposed to 100μCi [^{3}H]-uridine (5Ci/mmol) for 15min. Nuclei were then prepared from the harvested cells by the method of Penman et al,(1967) as described in Methods section B.3(a). RNA was extracted from the nuclei by the "hot phenol SDS" technique described in Methods section 4(b) and after dissolution in the appropriate buffer was layered, together with 50μl of a [^{14}C]-labelled marker RNA preparation, over a linear gradient of 5%(w/v) to 20%(w/v) sucrose in 0.05M ammonium acetate(pH 5.1) containing 0.1%(w/v) SDS. The gradients were centrifuged at 39,000rev/min in an SW40 rotor for 5h as described in Methods section 5(c), and were harvested in 8 drop fractions, the extinction being automatically monitored and recorded by the passage of the sample through the flow cell of a Gilford recording spectrophotometer. The fractions were then examined for radioactivity as detailed in Methods section B7.

[^{14}C]-acid insoluble radioactivity dpm (—o—), [^{3}H]-acid insoluble radioactivity dpm (---o---).

Acid insoluble ^{3}H radioactivity dpm x 10^{-3}

Fraction number

In addition if indeed α-amanitin, *in vivo*, interacts only with the extranucleolar polymerase, then these results suggest that ribosomal RNA synthesis *in vivo*; may be controlled by a product of the α-amanitin sensitive polymerase. A similar finding has been reported in rat liver nuclei by Jacφb, Muecke, Sajdel & Munro (1970).

III.25 Low molecular weight RNA components of the nucleus.

Several distinct species of low molecular weight RNAs from the nuclei and nucleoli of mammalian cells can be resolved by polyacrylamide gel electrophoresis (Burdon & Clason, 1969, Weinberg & Penman, 1968, Muramatsu, Hodnett & Busch, 1966). The results displayed in Fig.III.1(a) have shown that these low molecular weight RNA species also exist in the nuclei of BHK-21 cells. Labelling studies of Hela cells (Weinberg & Penman, 1968) show that the low molecular weight RNA of the nucleus is metabolically stable and partitioned between the nucleoplasm and nucleolus. Fig.III.25(a) shows the distribution profile of [^{32}P]-radioactivity in a number of low molecular weight RNA moeities (designated as 1 - 14) after labelling a randomly growing population of BHK-21 cells for 24 hours. Such a picture is similar to that obtained by Weinberg & Penman (1968) and the number of peaks was constant whatever labelling period greater than 1 hour was used, although the maximal level of label in each peak varied. Peak 14 of Fig.III.25(a) has

an electrophoretic mobility comparable with that of the cytoplasmic 4s RNA and as observed by Weinberg & Penman (1968), there was a double peak of radioactivity (designated as 12 and 12') with the electrophoretic mobility of 5s RNA. These low molecular weight nuclear species are composed of RNA in that they are resistant to degradation with DNase and pronase, are labile to alkali and are labelled with precursors such as [^{32}P]-orthophosphate of [^{3}H]-uridine. Their relative scarcity and metabolic stability are reflected by the fact that they label only slowly with [^{32}P]-orthophosphate or [^{3}H]-uridine and Weinberg & Penman (1968) have estimated them to be present to the extent of approximately 10^5 molecules per cell. Fig III.25 (b) shows the distribution profile of [^{3}H]-labelled RNA isolated from BHK-21 cell nucleoli after exposure to [^{3}H]-guanosine for 24 hours. A comparison of Figs III.25 (a) and (b) indicates that not all of the low molecular weight RNA species are present in both compartments of nucleus, for the components designated as 6, 7, and 11, do not appear to be present in the profile of Fig III.25 (b).

III.26 Methylation of low molecular weight nuclear RNA components of BHK-21 cells

Most if not all of the low molecular weight RNA components present in Hela cell nuclei are extensively methylated (Weinberg & Penman, 1968). In order to determine whether the low molecular weight nuclear RNA species in BHK-21 cells

Low molecular weight RNA components of the nucleus in BHK-21 hamster fibroblasts.

Fig.III.25

Distribution of radioactivity in the low molecular weight RNA components of the nucleus of BHK-21/C13 cells after electrophoresis in 7.5% polyacrylamide gels.

(a) A randomly growing population of BHK-21/C13 cells (5×10^7 cells/80oz. winchester bottle) was grown for 12h in 100ml Eagles -phosphate free minimal essential medium supplemented with 10% (w/v) dialysed calf serum. The growth medium was then decanted aseptically and 50ml fresh phosphate free EC growth medium and 500µCi [^{32}P]-orthophosphate (70Ci/mg P) added to the culture and incubation continued for 24h. Nuclei were then prepared from the harvested cell pellet by the Tween 80 method described in Methods section B.3(a), and [^{32}P]-labelled RNA extracted from the isolated nuclei by the hot-phenol-SDS method (Methods section 4(b)). After dissolution in the appropriate buffer [^{32}P] labelled RNA together with unlabelled marker RNA was examined by electrophoresis in polyacrylamide gels as described in Methods section 5(b). The gels were then frozen, sliced into 1mm segments and the fractions assayed for radioactivity as described in Methods section 5(b).

Optical density in arbitrary units (---), [^{32}P] radioactivity cpm (—o—)

(b) Distribution profile of the radioactivity present in low molecular weight RNA components of the nucleolus of BHK-21 cells after electrophoresis in 7.5% polyacrylamide gels.

A randomly growing population of BHK-21/C13 cells (5×10^7/80oz. winchester bottle) was grown for 24h in the presence of 25µCi [^{3}H]-guanosine (6Ci/mmol). Nucleoli were prepared as described in Methods section B.3(b) and RNA then extracted by the hot-phenol-SDS method. This RNA was then examined by polyacrylamide gel electrophoresis (Methods section 5(b)) and the gels assayed for radioactivity as described therein. The optical density profile was obtained as a result of electrophoresis of unlabelled nuclear RNA under the same conditions.

Optical density in arbitrary units (---), [^{3}H] radioactivity dpm (—•—).

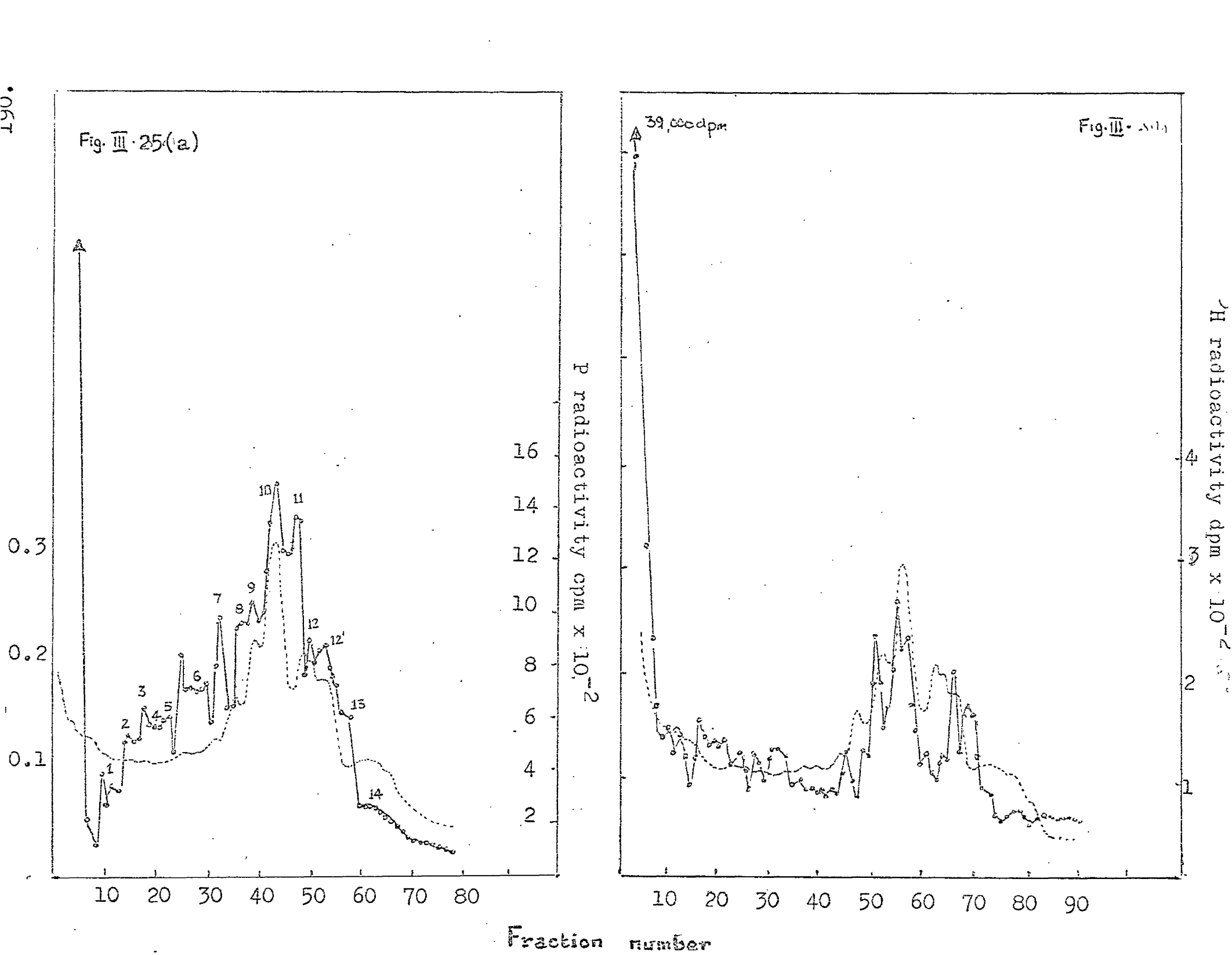


Fig. III·25(a)
39,000dpm
0.3
0.2
0.1
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
12'
13
14
10
20
30
40
50
60
70
80
90
P radioactivity cpm x 10⁻²
³H radioactivity dpm x 10⁻²
Fraction number

(enumerated as 1 - 14 in Fig III.25 (a)) contained methylated nucleosides, cultures of BHK-21 cells were grown overnight in medium containing 20mM sodium formate and [^{3}H -methyl]-methionine. The [^{3}H]-labelled nuclear RNA extracted from the Tween 80 purified nuclei was coelectrophoresed with [^{32}P]-labelled nuclear RNA from a similar culture exposed to [^{32}P]-orthophosphate for 24 hours. The electrophoretogram of such an examination is shown in Fig III.26 and indicates that the nuclear low molecular weight RNA species referred to as (1) - (6) are extensively methylated as is the 4s RNA-like species 14. The species referred to as (7) - (9) may be methylated to a low level but species 10 (the 7s RNA) and 12 and 12' (the 5s RNA) and also species (13) appear to be devoid of methyl groups.

III.27 <u>Synthesis of low molecular weight nuclear RNA in relation to the cell cycle in BHK-21 cells.</u>

Since many of the low molecular weight RNA species uniquely associated with the nuclei of mammalian cells are metabolically stable, the timing of their synthesis in synchronised cultures of BHK-21 cells was investigated to determine whether their synthesis was closely coordinated with that of the major stable constituent of the nucleus, the DNA. BHK-21 cells were therefore cultured as monolayers in phosphate-free Eagles minimal essential medium supplemented with dialysed

Methylation of low molecular weight RNA components of the nucleus of BHK-21/C13 cells.

Fig.III.26

A culture of BHK-21/C13 cells (5×10^7 cells in 100ml ETC_{10}) was grown overnight in medium containing 20mM sodium formate and 20μCi L-[^{3}H-methyl]-methionine (8.3Ci/mmol). The cell monolayer was then washed with a 50ml portion of ice cold BSS and the cells harvested by trypsinisation. From the cell pellet, nuclei were prepared by the Tween 80 method of Fisher & Harris (1962) and RNA extracted from these nuclei by the hot-phenol-SDS technique described in Methods section B.4(b). After dissolution in the appropriate buffer, the RNA was examined by electrophoresis in 7.5% polyacrylamide gels, and co-electrophoresed with [^{32}P]-labelled RNA from a culture exposed to 100μCi [^{32}P]-orthophosphate (70Ci/mg P) for 24h. The radioactive gel was frozen, sliced into 1mm segments, and the fractions assayed for radioactivity as described in Methods section 5(b).

Optical density, obtained as a result of electrophoresis of unlabelled marker RNA, in arbitrary units (——), [^{32}P] radioactivity cpm (– – – –), [^{3}H] radioactivity cpm (—o—)

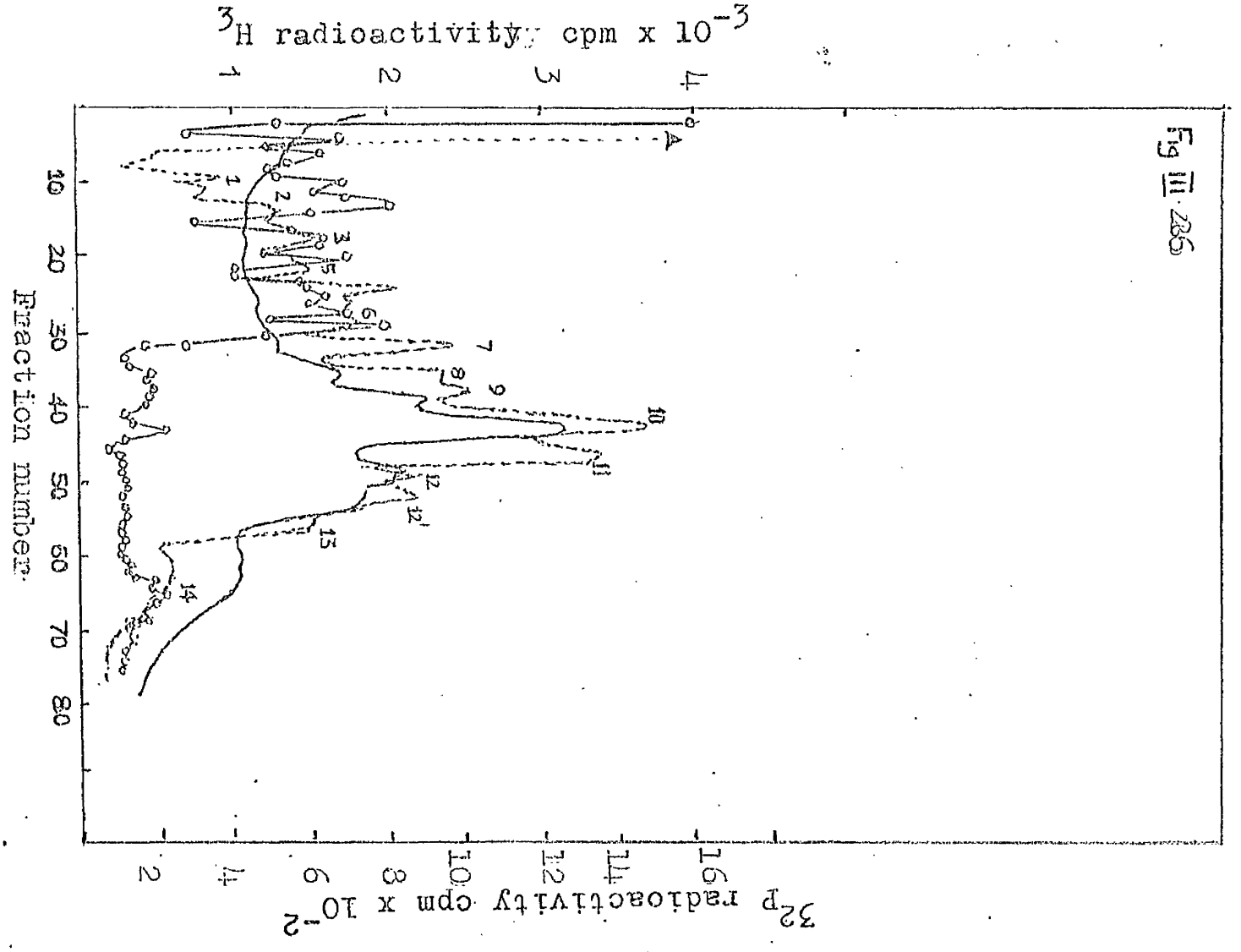

^{3}H radioactivity cpm x 10^{-3}
1
2
3
4
A
1
2
3
5
6
7
8
9
10
11
12
12
13
14
Fraction number
10
20
30
40
50
60
70
80
^{32}P radioactivity cpm x 10^{-2}
2
4
6
8
10
12
14
16

Fig III.28b

calf serum to deplete the cellular phosphate pools before labelling the cells with [^{32}P]-orthophosphate. DNA synthesis was arrested in each culture by the addition of aminopterin and after 12 hours the blockage imposed on DNA synthesis was released by the addition of thymidine. [^{32}P]-orthophosphate was added to each culture and incubation continued for various times. RNA was then isolated from the Tween 80 nuclei of these cells and electrophoresed through 7.5% gels of polyacrylamide. Because the amount of nuclear RNA present in the [^{32}P]-labelled samples was small, unlabelled nuclear RNA from BHK-21 cells was electrophoresed in identical conditions to act as a marker. At the same time as the incubation of the cultures with [^{32}P]-orthophosphate, smaller cultures of the same batch of cells were blocked with aminopterin and released from the block as described above. However, after release from the block the cultures were treated with [^{3}H]-thymidine and the rates of DNA synthesis measured by the incorporation of [^{3}H]-radioactivity at various times after release. Cells synchronised by this procedure show a peak of maximum DNA synthesis 3h after release from the blockage. The maximal amount of radioactivity corresponding to the low molecular weight RNA species enumerated in Fig III.25 (a) was then examined at each hour after release from the blockage. Fig III.27 (b) - (n)

Synthesis of low molecular weight RNA components of the nucleus of BHK-21/C13 cells in relation to the cell cycle.

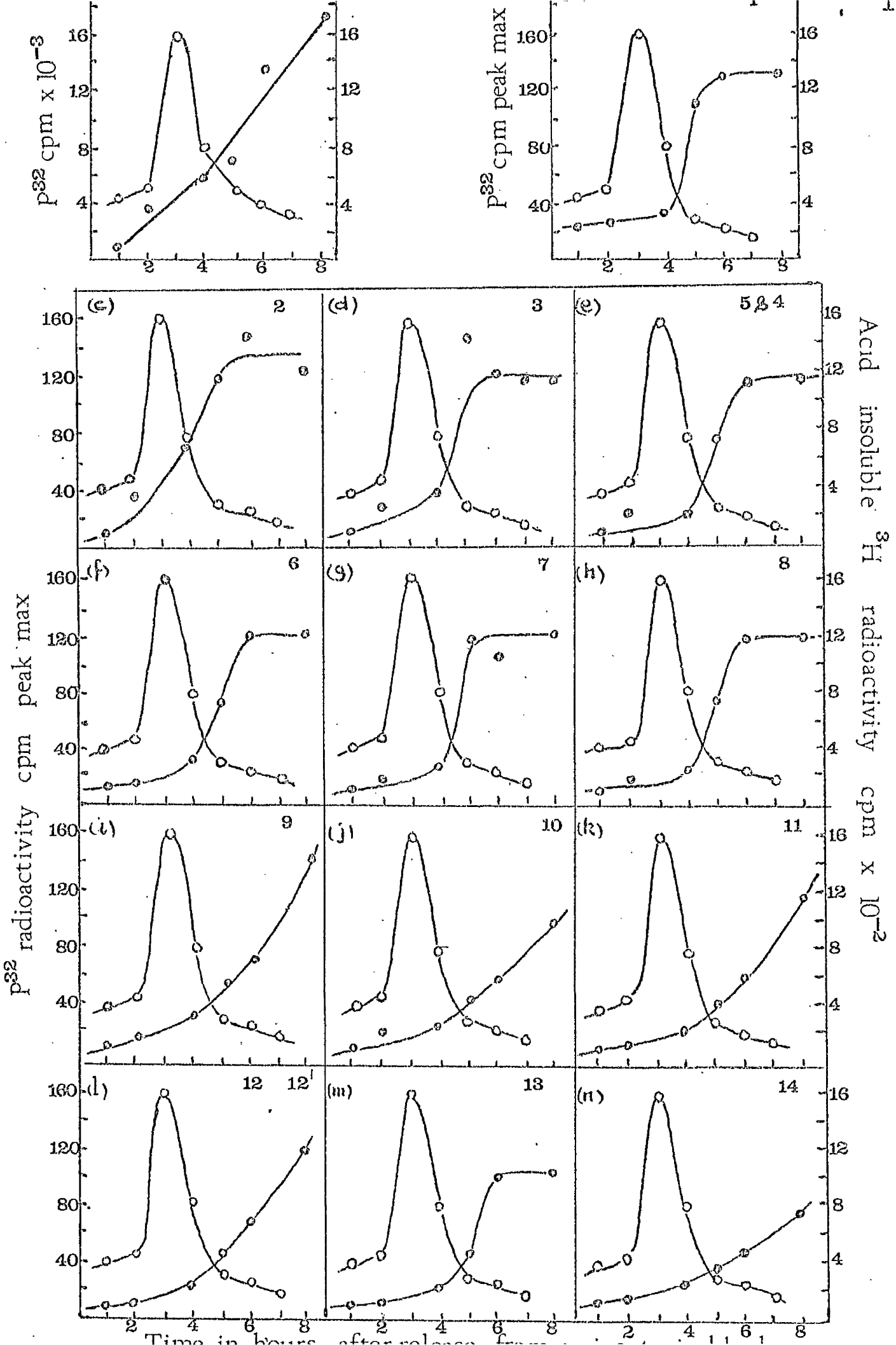
Fig.III.27

The time course of incorporation of ^{32}P into the low molecular weight RNA components of the nuclei of BHK-21/C13 cells enumerated in Fig.III.25(a), after release from aminopterin block.

Monolayer cultures of BHK-21/C13 cells (10^7 cells/80oz. bottle) were grown in 50ml phosphate free Eagles medium supplemented with 10%(v/v) calf serum(dialysed),for 24h. Synchrony was induced as described in Methods section A.3(a) and released by the addition of 0.3mM thymidine. 100μCi[^{32}P]-orthophosphate (67Ci/mg P) was added to each culture and the incubation continued for various lengths of time,after which[^{32}P]-labelled nuclear RNA was isolated and electrophoresed as detailed in Methods section 5(b). The maximal level of[^{32}P]-incorporation (—o—) into each of the RNA components indicated in Fig. III 25(a)(and enumerated as 1 to 14) is indicated in sections (b) to (n),after the cells had been exposed to ^{32}P continuously for various lengths of time after release from aminopterin block. Section (a) represents the total[^{32}P]-incorporation (—o—) into material excluded from the gel (ie high molecular weight ribosomal RNA(18s and larger) and DNA), under the same conditions.

At the same time smaller cultures of the same batch of cells were blocked with aminopterin and released from the block, as described in Methods section A.3(a). However after release from the block the dishes were treated with 5μCi[^3H]-thymidine (26Ci/mmol) and the rates of DNA synthesis measured by the incorporation of[^3H]-thymidine. The same set of data regarding the different rates of[^3H]-thymidine incorporation at various times after release from aminopterin blockage (maximum rate is found at 3h after release) is represented in each section of Fig.III.27 (ie (a) to (n)) to facilitate interpretation of the RNA labelling patterns.

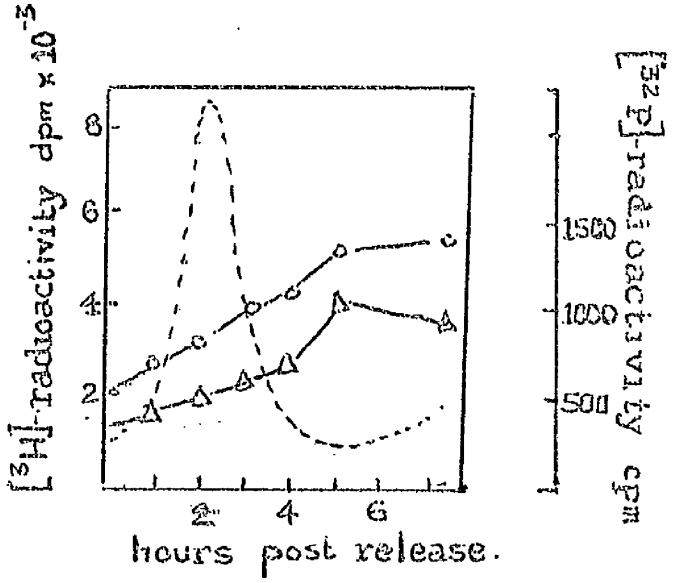
[^3H]-acid insoluble radioactivity cpm (—o—),[^{32}P]-radioactivity cpm (—•—).


P32 cpm x 10-3
P32 cpm peak max
(c) 2
(d) 3
(e) 5 & 4
(f) 6
(g) 7
(h) 8
(i) 9
(j) 10
(k) 11
(l) 12 12'
(m) 13
(n) 14
P32 radioactivity cpm peak max
Acid insoluble 3H radioactivity cpm x 10-2
Time in hours after release from

shows the time course of incorporation of [^{32}P]-radioactivity into these low molecular weight nuclear RNA components after release from the block. The same set of data regarding the different rates of thymidine incorporation at various times after release from the aminopterin blockage is presented in each section of Fig.III.27 to facilitate the interpretation of the RNA labelling patterns. It seems that certain of the low molecular weight RNA species are synthesised only at, or just after, the time of maximum DNA synthesis during the S phase of the cell cycle, namely components 1 - 8, and 13, whist other species 9, 10, 11, 12, 12' and 14 appear to be synthesised continually. To verify that aminopterin treatment had not interfered with the normal synthesis and processing of other RNA species, such as ribosomal RNA, which has been shown to be synthesised throughout the cell cycle in cells synchronised by mitotic selection (Robbins & Scharff, 1966), cells, cultured and blocked as before were labelled with [^{32}P]-orthophosphate for one hour at hourly intervals after the release from aminopterin blockage and cytoplasmic RNA analysed by sucrose gradient centrifugation. The amount of labelled 28s and 18s rRNA that appeared in the cytoplasm after each hourly pulse up to 8 hours after release from the aminopterin blockage is shown in Fig. III.28. This pattern of labelling is similar to the pattern obtained with

ls.

.III.28



bottles cultures of BHK-21 /C13 cells (10^7 cells in 50ml were grown for 12h at 37 C and synchrony induced by treat- with aminopterin as described in Methods section A.3(a). r release from the aminopterin block each culture was sed to 100μCi[^{32}P]-orthophosphate (67Ci/mg P) for one hour very hour after release up to the 8th hour post release. phenol cytoplasmic RNA was isolated from the harvested s and examined on linear 5%(w/v) to 20%(w/v) sucrose grad- s as described in Methods section 5(c). The gradients harvested in two drop fractions and the fractions assayed radioactivity as described in Methods section B7. included in Fig III.28 is the profile of the different s of DNA synthesis (---) at various times after release from aminopterin block and as obtained from the treatment of ler cultures of the same batch of cells with H -thymidine i/mmol).

acid insoluble radioactivity dpm (-----), [^{32}P] radioactivity 8s rRNA (—o—), in 18s rRNA (—Δ—).

the "thymidine synchronised" cells shown in Fig III.22 (b) 14 and indicates that no serious impairment of the biosynthesis and maturation of ribosomal RNA had occurred as a result of drug treatment of the cultures.

DISCUSSION

DISCUSSION

RNA fractionation techniques such as sucrose density gradient centrifugation, column chromatography on methylated albumin kieselguhr (MAK) or gel filtration in agarose or Sephadex columns have allowed only limited separations of the host of RNA species to be found in extracts of eukaryotic and prokaryotic cells, and have largely permitted a separation of high molecular weight RNA components from those of low molecular weight. Such techniques therefore afford a reasonable separation of the ribosomal RNA components (23-30s and 16-18s according to species) from the soluble or transfer RNA components of these cells. The fractionation of RNA by electrophoresis in supporting gels however provides the possibility of a more extensive and more precise separation of RNA species than is possible by the above mentioned techniques and Tsanev (1965) has achieved excellent separations of the ribosomal RNA components and various minor RNA species by electrophoresis in agar gels. The application of polyacrylamide gel electrophoresis which proved so successful in the separation of proteins (Frederick, 1964, Ornstein & Davis, 1964) and in which the gel pore size can be closely controlled, to the separation of RNA molecules (Loening, 1967), has enabled a resolution of RNA species which is greater and more detailed than can be achieved by sucrose density gradient analysis, gel filtration or MAK chromatography.

Electrophoresis in low concentration gels (2.2 - 2.6% acrylamide) gives a fractionation similar to that obtained by density gradient centrifugation and shows the two ribosomal RNA components, and a number of minor species (see Fig.III.1(c). In 7.5% gels 4s and 5s RNA are separated and ribosomal RNA is excluded but in addition a number of low molecular weight RNA species are resolved, (see Fig III.1(b)). Until recently, RNA was considered to belong to one of three categories; tRNA ribosomal RNA or messenger RNA. The development of more sophisticated analytical techniques such as polyacrylamide gel electrophoresis coupled with improved cell fractionation procedures (Penman et al, 1966) has permitted the detection of a number of hitherto unrecognised RNA species in both the nuclear and cytoplasmic fractions of mammalian cells. Among these RNA species are fractions of relatively low molecular weight. This report has described some of the characteristics of such small RNAs in the cytoplasm and nuclei of BHK-21 cells.

(1) Low molecular weight RNAs of the nucleus

The nucleus of mammalian cells contains, in addition to the nucleolar ribosomal precursor RNAs (Perry, 1962, Muramatsu et al, 1966, Weinberg et al, 1967), a class of unstable nucleoplasmic heterogeneous RNA of unknown function (Scherrer et al, 1966, Attardi et al, 1966, Penman et al, 1968). In addition recent work has indicated the existence of a new class

of low molecular weight RNA species in the nuclei of mammalian cells (Weinberg & Penman, 1968, Prestayko & Busch, 1968, Nakamura, Prestayko & Busch, 1968, Purdon & Clason, 1969). These RNA species were first observed by Knight & Darnell (1967) in acrylamide gel electrophoresis of Hela cell nuclear RNA. A detailed examination of these species in the nuclei of Hela cells has been reported by Weinberg & Penman (1968, 1969). These authors examined the low molecular weight RNA species of the nuclei with respect to base composition, methylation, occurrence in the nucleolar and nucleoplasmic fractions, relationship to cytoplasmic RNA species, metabolic stability and sensitivity to various inhibitors of RNA synthesis. In addition, the relationship of their synthesis to the cell cycle was examined in cells synchronised by a double thymidine block. Briefly, they describe nine low molecular weight RNA species amongst which can be found the 5s ribosomal RNA and the 28s associated low molecular weight RNA of the larger ribosomal subunit. Most of these RNA species are methylated and are metabolically as stable as ribosomes whilst others possess half-lives of approximately one generation time. These small nuclear RNAs are more weakly bound to the chromatin than the HnRNA as judged by ease of elution by salt, and appear to be restricted to the nucleus for they show no apparent relationship to the monodisperse metabolically unstable RNA

species found in the cytoplasmic supernatant. Their synthesis in Hela cells does not appear to be coordinated with the replication of DNA in the S phase and they appear to survive the mitosis of the cells (Rein, 1971). Furthermore, the synthesis of these low molecular weight RNA species in Hela cells is strongly affected by several of the inhibitors of nucleolar RNA synthesis and, in addition, Rein (1969) has reported that these low molecular weight nuclear RNA components are found in a wide variety of vertebrate cell lines (L.929, $3T_3$ mouse cells, WI38, human fibroblasts, Xenopus laevis, chick embryos). Similar low molecular weight RNA species have been reported in the nuclei of rat liver, (Peacock & Dingham, 1967) KB cells (Larsen, Galibert, Lelong & Boiron, 1967) and Novikoff hepatoma (Nakamura, Prestayko & Busch, 1968). This report describes similar species in the nuclei of BHK-21 cells and which range in size from 80 nucleotides (4s or tRNA) to approximately 350 nucleotides in length. Like the low molecular weight nuclear RNA species of Hela cells, most if not all of the low molecular weight RNA components of BHK--21 nuclei appear to contain methylated nucleosides (with the exception of "7s" or 28sA RNA and 5s RNA" and to be distributed in a similar fashion between the nucleoplasmic and nucleolar fractions of the organelle. Zapisek, Saponara & Enger, (1969) examined similar species in the chinese hamster ovary nuclei and have

shown them to possess distinct unique methylation patterns which indicate that they are not the degradation products of other types of methylated RNA. Weinberg and Penman (1969) noted a low level of 28sA RNA in the nuclei of Hela cells but in BHK-21 cells this is apparently the principal low molecular weight RNA component of the nucleus. Although this may suggest cytoplasmic contamination of the nuclear preparation, the low level of 4s-like RNA (referred to as species 14 in Fig III.25 (a)), as found in Hela cells, might argue against this possibility and the high levels of 28s-A RNA in BHK-21 nuclei may therefore reflect a true species difference in the relative content of the various low molecular weight RNA species in these cells. Evidence in support of such species differences in the relative distributions of low molecular weight nuclear RNA components may be reflected in the data of Prestayko & Busch (1968) who indicate high levels of the "4s-like" component in the chromatin fraction of Novikoff hepatomas, in contrast to the situation in Hela cells. In common with the findings for Hela cells, however, [^{32}P]-labelled BHK-21 low molecular weight nuclear RNA, after electrophoresis in gels of polyacrylamide, shows a double peak of radioactivity in a position of migration corresponding to that of 5s RNA from the cytoplasmic ribosomes. This bifid peak of radioactivity may represent the "native" and "denatured"

forms of 5s RNA, as suggested by Weinberg & Penman (1969) to be derived as a result of the hot phenol extraction procedure, or may indicate the presence, in addition to 5s RNA, of a minor, distinct low molecular weight RNA component, for under conditions when methyl group incorporation, into the purine ring skeletons, of RNA precursors is suppressed by 20mM sodium formate, a low level of radioactivity is found in the region of 5s RNA, an established unmethylated RNA species. A comparison of the electrophoretic mobilities in polyacrylamide gels of RNA species present in the nuclear and cytoplasmic fractions of BHK-21 cells, the distribution of methyl label radioactivity in these RNA species after exposure of the cells to [^{3}H -methyl]-methionine and their kinetics of synthesis in relation to the cell cycle, suggests that the low molecular weight RNA species of the BHK-21 cell nucleus are, as reported for similar RNA species in Hela cells (Weinberg & Penman, 1969), confined to this organelle and that they bear no apparent relationship to the low molecular weight RNA species of the cytoplasm with similar electrophoretic mobilities. At present however, the possible presence in the nuclear RNA fraction of those unmethylated cytoplasmic RNA species which, in polyacrylamide gels, possess electrophoretic mobilities closely similar to those of the low molecular weight methylated RNA species apparently confined to the nucleus, cannot be excluded.

It is apparent that their detection in the low molecular weight RNA fraction of the nucleus is made difficult by the presence of the comigrating methylated RNA species confined to this organelle. In addition the low molecular weight RNA species of the BHK-21 cell nucleus appear to be similar, with respect to the extent of their presence and their apparent stability, to the low molecular weight RNA species of the Hela cell nucleus, for their relative scarcity and metabolic stability are reflected in the observation that they are labelled only slowly with RNA precursors such as [^{32}P]-orthophosphate or [^{3}H]-uridine (see Fig III.25). Whilst transfer RNA molecules are present to a level of approximately 10^8 molecules per cell, these low molecular weight RNA species of the nucleus have been estimated each to be present to only approximately 10^5 molecules per cell (Weinberg & Penman, 1969, Prestayko & Busch, 1968). In Hela cells the synthesis of the metabolically stable low molecular weight RNA species of the nucleus is not apparently related to the synthesis of the DNA in the S phase of the cell growth cycle and they appear in fact to be synthesised at a fairly constant rate throughout the S and G_2 phases of the cell cycle. Similar investigations of the relationship to the cell cycle of the synthesis of low molecular weight RNA species in the nuclei of BHK-21 cells indicate however that, in contrast to the situation in Hela cells, certain of the low molecular weight

RNA species are probably synthesised only at, or just after, the time of DNA synthesis during the S phase of the cell cycle and early periods of G_2 phase, namely components 1-8 and 13 (see Fig III.25 (a)) whilst other species 9, 10, 11, 12 and 12' and 14 appear to be synthesised continually. The significance of these different patterns of synthesis during the cell growth cycle is at present obscure since no precise function can yet be ascribed to these low molecular weight nuclear RNA components. However, if some of these RNA species are only synthesised, as is suggested by the data presented in this thesis, at, or around the time of cellular DNA synthesis, ie. at the end of S phase or beginning of G_2 phase of the cell cycle, then they may represent RNA species involved, in some way, in the regulation of cellular gene expression as suggested by Prestayko & Busch (1968). Furthermore since these RNA species appear, on the whole, to be metabolically relatively stable, to contain methylated nucleosides and are not synthesised coordinately with the DNA in the S phase of the cell cycle, it is unlikely that they are related to the histone messenger RNA species reported in Hela cells by Gallwitz & Mueller, (1969). Since these low molecular weight RNA components are, in the main, extensively methylated and are confined to the nucleus, and since the evidence for nuclear protein synthesis is extremely circumstantial and quite suspect, it is unlikely

that these RNA species, although they are of the approximate size, represent mRNA species. The possibility also exists that they may be related to the chromatin RNA species reported by Bonner's group (Huang & Bonner, 1969, Dahmus & McConnell, 1969) and which are reported to confer the specificity upon the DNA-chromosomal protein interaction. However Weinberg & Penman (1969) reported the low molecular weight nuclear RNA species to be easily eluted from the chromatin by low salt conditions unlikely to remove the covalently attached "chromosomal RNA" of Bonner. These low molecular weight RNA species reported by Weinberg & Penman and here reported in BHK-21 cells, are therefore unlikely to be related to the "chromosomal RNA" reported by Bonner (Huang & Bonner, 1969). In addition, a recent reexamination of chromosomal RNA (Heyden & Zachau, 1971) suggests that it is at least partially composed of fragments of tRNA and Weinberg & Penman (1969) have shown these low molecular weight nuclear RNAs to possess base compositions distinct from that of tRNA. At present one can only speculate upon the probable function of these low molecular weight nuclear RNAs, but since they apparently survive mitosis it is possible that they perform a structural role in the determination of chromosome structure or they may be involved in chiasma formation during recombination events. However, it must be emphasised that there is only scant information at

present available regarding the molecular characteristics of these RNA molecules and that much investigation of the precise molecular structure of these RNA molecules requires to be made before a knowledge of their function will be obtained. The widths of the bands of migrating RNA in polyacrylamide gels suggests that each species of low molecular weight RNA (enumerated as 1-14 in Fig.III.25(a)) is homogeneous and represents a unique type of RNA molecule. In Novikoff hepatomas, Prestayko & Busch (1968) have shown that among the small molecular weight nuclear RNAs of hepatomas, only the 4s-like RNA component (species 14 in BHK-21 cells) contains amino acid acceptance activity and is therefore presumed to represeht tRNA molecules.

Although Egyhazi et al,(1969) have suggested that "pre-tRNA" molecules may be found in the nuclei of insect cells, no evidence could be found for their accumulation or transient association with the nuclei of BHK-21 cells, in keeping with the findings of Burdon et al,(1967) and Burdon & Clason,(1969) who have indicated that these molecules appear in the cytoplasm of mammalian cells within minutes of their transcription from nuclear genes.

(2) Low molecular weight RNA of the cytoplasm.

(1) Molecular characteristics.

Initial examination, by polyacrylamide gel electrophoresis of

RNA preparations from unlabelled Krebs II ascites tumour cells (Burdon & Clason,1969) suggested that, in addition to 5s and 4s RNA, the cytoplasm of mammalian cells also contained minor amounts of low molecular weight RNA components(defined as RNA molecules of an average chain length less than 500 nucleotides) possessing lower electrophoretic mobilities than 5s RNA but greater than that of the high molecular weight 18s RNA of the cytoplasmic ribosomes. Weinberg & Penman(1969) have referred to similar species in the cytoplasm of Hela cells and the present study has indicated their existence in a number of mammalian tissue culture cell lines (L929, BHK-21 and Hela cells). They also appear to be present in the cytoplasm of rat liver (Stevely,1971). However, these RNA species may be absent from the cytoplasm of insect cells for Egyhazi et al, (1969) have reported that the cytoplasm of the salivary gland of Chironomus tentans contains no low molecular weight RNA species besides the 4s to 5s RNA components. The low molecular weight RNA components of lower electrophoretic mobility than 5s RNA, and reported here to be constituents of several mammalian cell lines, may not therefore be a geheral feature of eukaryote cells but may be restricted to the vertebrates, although their presence in prokaryotic cells is suggested by the work of Adesnik & Levinthal (1970). In all the eukaryote cell types so far examined in this report, these RNA species show remarkably similar electrophoretic mobilities

in polyacrylamide gels and the homogeneity of each species is suggested by the band widths after electrophoresis, for these RNA species display bandwidths less diffuse than 4sRNA or 5s RNA. They are estimated to be between 80 and 350, nucleotides in length. Optical density profiles and methylation studies of the low molecular weight RNA components of BHK-21 cells indicate that the minor component of the cytoplasm referred to as species (1), which migrates in a position intermediate between 5s and 4s RNA, shows some of the characteristics of tRNA. A species of RNA with similar characteristics of electrophoretic mobility and methylation has been reported in the cytoplasm of E. coli and the mitochondria of Hela cells by Knight & Sugiyama (1969). This RNA species is referred to as tRNA B. and displays different amino acid acceptance patterns to the species found in Hela cytoplasm from which it is apparently absent. It is in part associated with the ribosomes both in Hela mitochondria and E. coli cytoplasm. The RNA species referred to in BHK-21 cytoplasm as component (1) is however unlikely to be related to the tRNA B of Hela cells mitochondria or E. coli. Since the "cold phenol" procedure for RNA extraction does not contain any detergents, which are necessary for the extraction of mitochondrial nucleic acids (Luck & Reich, 1964), it will not extract the nucleic acids of mitochondria and since the tRNA B species of Hela cells is present in mitochondria and absent

from the cytoplasm and the BHK-21 cell RNA component (1) is present in "cold phenol" extracts of intact cells, the two species are unlikely to be related. In addition experiments with the drug ethidium bromide, which specifically inhibits the synthesis of mit-DNA specified RNA species, (Zylber et al, 1969) indicate that in common with the other "cold phenol" RNA species of BHK-21 cells, component (1) is not specified by mit-DNA as are most, if not all, of the mit-tRNA species so far examined (Nass & Buck, 1969).

Methylation studies of RNA extracted from cells exposed to [^{3}H -methyl]-methionine have shown that the low molecular weight RNA components with an electrophoretic mobility lower than 5s RNA are devoid of methylated nucleosides. In addition estimates of the molecular weights of these RNA components suggest that they range from 25,000 to 110,000 and base composition data indicate them to have an average (G+C) content of approximately 54%. Since during the maturation of 45s r-pre-RNA to the 28s and 18s RNAs of the cytoplasmic ribosomes, (G+C) rich, unmethylated non ribosomal sequences, ranging in size from 0.3×10^6 to 1.0×10^6 daltons are lost and probably degraded (Weinberg & Penman, 1970), there exists the possibility that the low molecular weight RNA species of BHK-21 cytoplasm (referred to as species 1-10 in Fig III.1 (a)) are derived as the degradation products of these non-ribosomal sequences from

the 45s r-pre-RNA. However, when 45s r-pre-RNA synthesis is inhibited by low levels of actinomycin D (see Fig III.7) these low molecular weight RNA species continue to be synthesised, thus indicating that they cannot be the degradation products of either rRNA, or the non-ribosomal sequences contained within this precursor molecule. When rRNA synthesis in BHK-21 cells is inhibited by these low levels of actinomycin D, 28s and 18s rRNA synthesis is completely inhibited but there is persisten synthesis of 5s RNA molecules thus indicating that in BHK-21 cells, as in Hela, L929 and Krebs II cells (Darnell, 1968, Perry & Kelley, 1968, Burdon et al, 1967), 5s RNA is not derived from the same precursor molecule as the high molecular weight rRNAs and appears to be synthesised in an extranucleolar site. It is also evident therefore that the rate of 5s RNA synthesis in mammalian cells is independent of the rate of synthesis of 28s and 18s RNA, in contrast to the situation in amphibians (Brown & Dawid, 1968). Although it has been suggested (Brown & Dawid, 1968) that, in Xenopus embryos, 5s RNA genes are coordinately expressed with those for 28s and 18s RNA, BHK-21 cells they are evidently located in separate compartments of the nucleus and are not under any linked control mechanism with the genes for the high molecular weight ribosomal RNAs. The base compositions of low molecular weight cytoplasmic RNA components (see Table III.2) also argue against

the ribosomal origin of these RNA species and indicate that no apparent relationship exists between them, an observation which is supported by the results pulse labelling experiments. However, like the small molecular weight RNAs of the nucleus, the cytoplasmic components are rich in (G+C), but since they are devoid of methylated nucleotides and are approximately the same size as the nuclear low molecular weight RNA species, they are unlikely to be derived from them or related to them. Their presence in the nuclear RNA fraction cannot however be excluded for their detection in this fraction is made difficult by the comigrating methylated species of the nucleus.

(2) Intracellular location and origin of low molecular weight RNA species.

Although they do not appear to be ribosomal in origin, the low molecular weight RNA components of the cytoplasm do appear to be associated with the ribosomes, for intracellular location studies have revealed that, with the exception of tRNA, they are located principally on the microsomes, as originally found in Krebs II cells (Burdon & Clason, 1969) and appear in fact to be associated with the polyribosomes. Methyl deficient RNA species of similar size to the polyribosome associated low molecular weight RNAs of BHK-21 cytoplasm, but with a (G+C) content of approximately 44%, have been reported in Hela cells by Attardi & Attardi (1967,1968,1969). In Hela cells however these

membrane associated RNA molecules hybridise efficiently with mit-DNA and are therefore presumed to represent a possible mitochondrial messenger RNA species specified by mit-DNA. That the low molecular weight RNA species associated with the membrane fractions of BHK-21 cells are not of mitochondrial origin is however suggested by the fact that their synthesis is insensitive to inhibition by the drug ethidium bromide, which is reported to specifically inhibit mit-RNA synthesis (Zylber et al, 1969, Knight, 1969). These low molecular weight polyribosome associated RNA components of BHK-21 cytoplasm are therfore not apparently related to the mit-DNA specified membrane RNAs of Hela cells reported by Attardi & Attardi (1967, 1968, 1969). Low concentrations of actinomycin D have been used in determining the nucleolar origin of ribosomal RNA molecules, for these low levels are known to inhibit specifically the RNA synthesis of the nucleolus as judged from autoradiography and subcellular fractionation, and to leave uninhibited extranucleolar RNA synthesis When low levels of the drug were used in cultures of BHK-21 cells and the RNA from drug treated cultures examined by polyacrylamide gel electrophoresis, it was apparent that the bulk of the low molecular weight polyribosome associated RNA species were, at least on the above basis, the probable products of cistrons not located in the nucleolus. However some of the RNA species, namely species (10) to (13), <u>were</u> sensitive to the drug and

therfore maybe synthesised in the nucleolus. The significance of the nucleolar and nucleoplasmic origins of these polyribosome associated low molecular weight RNA components is at present obscure and presumably reflects the different functions possibly performed by the two classes of RNA molecules. In addition, since a number of these low molecular weight RNA species continue to be synthesised and to appear in the cytoplasm under conditions when nucleolar synthesis of RNA is inhibited by actinomycin D, it is apparent that their transport to the cytoplasm is by a mechanism which is independent of continuing nucleolar RNA synthesis. As a result of elegant experiments in which nucleolar activity (as judged by autoradiography) was inactivated by U.V. microbeam irradiation, Siddebottom & Harris (1969) have suggested that, in Hela cells, inactivation of the nucleolus alone inhibited the transport to the cytoplasm, not only of RNA synthesised at the nucleolar site but also of RNA made elsewhere in the nucleus. Since the polyribosome associated RNAs of BHK-21 cells continue to appear in the cytoplasm in the absence of nucleolar RNA synthetic activity, then either this system does not operate in BHK-21 cells or the low molecular weight cytoplasmic RNAs do not comprise part of the RNA fraction whose transport to the cytoplasm is, in Hela cells, governed by the synthetic activity of the nucleolus.

(3) Stability of low molecular weight RNA species.

Experiments employing levels of actinomycin D sufficient to suppress totally the incorporation of isotopically labelled precursors into RNA molecules have suggested that these low molecular weight RNA species of the cytoplasm have, in BHK-21 cells, a life span of some 24 to 30 hours. This is an additional feature which serves to differentiate these RNA species both from the membrane associated RNAs of Hela cells (Attardi & Attardi, 1969) which have short half-lives and from the small RNAs of the nucleus which are metabolically as stable as ribosomes (Weinberg & Penman, 1969). In order to compare the metabolic stability of these low molecular weight cytoplasmic RNA species of BHK-21 cells with that of the ribosomes of these cells, experiments were performed to measure the stability of the ribosomes in BHK-21 cells. Such studies employing [^{3}H-methyl]-methionine as the labelled precursor indicate the ribosomes of BHK-21 cells to have a half-life of approximately 142 hours as judged by the stability of the incorporated methyl groups. Since the low molecular weight polyribosome associated RNAs of the cytoplasm are devoid of methylated nucleosides, methyl-labelled methionine was an unsuitable precursor molecule to investigate the stability of these RNA species and [^{3}H]-uridine was therefore employed. These experiments suggested that the total life span of the low molecular weight RNA components was

of the order of 24 to 30 hours. In agreement with the findings of Harris (1963) it was observed that it was extremely difficult to quickly dilute the radioactivity in the nucleotide precursor pools of mammalian cells and the data from this experiment further suggested that there was considerable loss of radioactivity from ribosomal RNA species during the period of the chase. This therefore implied that, in contrast to the results obtained for the stability of ribosomal RNA species when [^{3}H-methyl]-methionine was employed as the labelled precursor, considerable turnover of rRNA was occurring and therefore suggests a further complication, ie. the existence of two classes of ribosomal RNA. One class of ribosomal RNA which is methylated and is relatively stable and a second class of unmethylated rRNA which is far less stable. This apparent heterogeneity of ribosomal RNA populations is supported by the work of Higashi et al, (1971) who report that the distributions of oligonucleotides in digests of rRNA from adult and embryonic rat tissues differ and suggest that rat tissues contain at least two species of ribosomal RNA. The possibility therefore arises that the low molecular weight RNA species of BHK-21 cytoplasm are derived from the degradation of these unmethylated unstable ribosomal components whose existence was inferred by the investigations of ribosomal RNA stability. However, since as previously stated these low molecular weight cytoplasmic RNA species are persistentl

synthesised when all ribosomal synthesis is inhibited by low levels of actinomycin D, they cannot be derived from the degradation of either the methylated or unmethylated classes of rRNA.

(4) Polyribosome association.

Intracellular location studies (see Fig.III.6) of the low molecular weight RNA species of BHK-21 cells indicate that these low molecular weight RNA species are located on the microsomes and are associated with the polyribosomes. Since they are unmethylated and have a total life span of 24 to 30 hours, they may, in BHK-21 cells, represent the stable mRNA fraction reported in $3T_3$ cells to be stable for periods greater than 6 hours (Cheevers & Sheinin, 1969) and reported in rat liver polysomes to possess half-lives of at least 80 hours (Wilson & Hoagland, 1967). The difference in the lifetimes of rat liver and BHK-21 polyribosome associated species can readily be explained by the differentiated state of the adult liver cell and the fact that BHK-21 cells are much more metabolically active than the adult liver cell and consequently would be expected to contain mRNA species possessing shorter half lives than those of the liver cell. Although the molecular weights of the cytoplasmic low molecular weight RNA species range from less than 36,000 to 110,000 daltons and lie in the 0.5 to 1.5 x 10^6 daltons range expected for mammalian mRNAs, they possess a base composition

rich in (G+C). However, like the mRNA of bacterial cells these RNA species in BHK-21 cells are released from the polyribosomes by EDTA treatment (Gros et al, 1961). The released material sediments in sucrose gradients with sedimentation coefficients of approximately 6 -35s and behaves as free RNA in CsCl gradient centrifugation in that the released material does not form a discrete sedimenting band within the density range 1.3g/cm^3 to 1.5g/cm^3. A number of workers have reported that, upon EDTA treatment of mammalian polyribosomes, the mRNA is released in the form of ribonucleoproteins sedimenting in CsCl with density distributions between 1.3 and 1.5g/cm^3 (Henshaw, 1968, Lee & Brawerman, 1970, Spohr et al, 1970, Cartouzou et al, 1969) and have equated these with the "informosomes" particles reported in loach embryos by Spirin (1969) and suggested to represent the "not immediately translatable" mRNA species observed during certain periods of early e embryogenesis. In the light of recent reports however some reservations must be made in interpretating such ribonucleoprotei particles as either these masked forms of mRNA or the state in which mRNA is apparently transported from the nucleus to the cytoplasm in eukaryotic cells. Chen, Schultz & Katchalski (1971) report that the ribonucleoprotein particles which are synthesised during early embryogenesis in wheat, and which sediment in the "informosome region" of CsCl gradients are not

of this nature but represemt a series of ribonucleoprotein particles with buoyant densities ranging from 1.42 to 1.54g/cm^3 and which appear to be intermediates in ribosome biosynthesis. These particles have been shown to contain labelled ribosomal RNA which matures in particles through this range of buoyant density before finally entering ribosomes sedimenting with a buoyant density of 1.565g/cm^3. These results therefore indicate clearly that "informosomes" in one species and precursor ribosomes in another may happen by chance to be characterised by the same buoyant density, and interpretation, upon the basis of data from CsCl density gradient centrifugation alone, of the nature of presumed ribonucleoprotein particles may be hazardous. In addition, if mRNA in polyribosomes is in the form of ribonucleoproteins, as is suggested by Henshaw (1968), then this is not apparently the case for all polyribosome associated RNA species which have been presumed to represent messenger RNA, for Lee & Brawerman (1971) have reported that, although the bulk of the material released from rat liver polyribosomes by EDTA treatment is in the form of ribonucleoproteins, a distinct portion exists apparently as free RNA strands. Furthermore Bloebel (1971) has reported that, if puromycin is used as the subunit dissociating reagent rather than EDTA, the material released from erythrocyte polyribosomes and possessing the characteristics of globin mRNA is in the form of free RNA strands since its

sedimentation behaviour in sucrose gradients is unaffected by subsequent treatment with sodium dodecyl sulphate. In addition the possibility that these polyribosome associates, found after EDTA treatment of polyribosomes, may be artifacts of the isolation procedure, is suggested by the work of Olsnes (1970) who reports that, dependent upon the method of preparation, polyribosomes from mammalian cells may be contaminated to a considerable extent with rapidly labelled d-RNA containing particles leaked out from the nuclei during homogenisation. Olsnes has further shown that this contaminating material cosediments with the polyribosomes in sucrose gradients and in CsCl gradients occupies regions of buoyant density similar to the "informosomes" or nuclear ribonucleoprotein particles containing RNA of DNA-like base composition. Therefore the fact that the low molecular weight polyribosome associated RNA species of BHK-21 cells are released from the polyribosomes, subsequent to EDTA treatment, in the form of free RNA strands rather than as ribonucleoproteins, is not sufficient evidence to indicate that these RNA components do not represent mRNA species. However, they are not transported to the cytoplasm via the newly synthesised ribosomal subunits as was originally proposed for the mechanism of transport of mRNA species from nucleus to cytoplasm by McConkey & Hopkins (1965). Indeed these low molecular weight RNA species can be found in the cytoplasm of these cells in as

short a time as two to three minutes after the initiation of the labelling period (Burdon & Clason,1969), in contrast to the 40 min lapse before labelled ribosomal subunits appear in the cytoplasm. Latham & Darnell (1965) have indicated that, in Hela cells, mRNA continues to enter the cytoplasm of cells treated with puromycin, although newly synthesised ribosomes do not and postulate that this transfer is mediated via existing ribosomal particles or by the attachment of mRNA to existing ribosomes at the nuclear envelope. In puromycin treated BHK-21 cells however the low molecular weight polyribosome associated RNA species do not appear in the cytoplasm nor does any cytoplasmic RNA species. This therefore suggests that, in BHK-21 cells, either no RNA is synthesised subsequent to puromycin treatment or that the newly synthesised RNA does not leave the nucleus, as was found for rRNA in puromycin treated Hela cells (Soiero, Vaughan & Darnell, 1968). Recently Penman, Rosbash & Penman (1970) have found that unlike the HnRNA of Hela cells, the mRNA synthesis in these cells is sensitive to the 3'deoxyadenosine analogue, cordycepin. This drug is reported to be incorporated into the growing chains of RNA molecules and, since it lacks the 3' hydroxyl group, to cause premature termination of RNA synthesis . Although in Hela cells this drug apparently acts specifically upon mRNA synthesis , in BHK-21 cells it apparently caused a total inhibition of the

appearance of labelled RNA species in the cytoplasm. Taken together these various observations tend to suggest that the low molecular weight polyribosome associated RNA components of BHK-21 cells do not represent mRNA species although they possess certain features, of rapidity of labelling, polyribosome association and release from polyribosomes by EDTA treatment, which are characteristics of molecules proposed to represent mRNA (Darnell, 1968). However since the experiments investigating polyribosome association and EDTA release were performed in cell cultures treated with low doses of actinomycin D, which are reported to be specific for nucleolar synthesis, it might be argued that such treatment could impair the synthesis of the ribonucleoproteins reported by other workers to represent mRNA species and which were apparently absent from the polyribosomes of BHK-21 cells. This is however unlikely since such ribonucleoproteins were found to be associated with the polyribosomes of actinomycin D treated Hela cells and to be released in this form subsequent to EDTA treatment of the polyribosomes (Spohr et al,1970). It has however been suggested earlier in this discussion that ribonucleoprotein forms of DNA-like RNA, purported to be mRNA, might be artifacts of the polyribosome isolation procedure (Olsnes, 1970) and that in many instances well characterised mRNA species are released from polyribosomes as free RNA strands (Bloebel, 1970). It may be, therefore,

that the material sedimenting to the bottom of the gradient when the EDTA-released RNA fraction of BHK-21 cell polyribosomes is examined by CsCl gradient centrifugation, contains not only the low molecular weight RNA species referred to as species (1) to (10) but also the mRNA species which in these cells appear to occur as free RNA strands. If however there is an absence, in these experiments, of mRNA species then this may, in part, be accounted for by the fact that, as observed in Hela cells impairment of the synthetic activity of the nucleolus, in this case by actinomycin D, interferes with the transport of certain classes of RNA from the nucleus to the cytoplasm (Siddebottom & Harris, 1969) and it is possible that mRNA species, under these conditions, may be trapped within the nucleus due to the impairment of nucleolar synthetic activity, whilst the low molecular weight polyribosome-associated RNA species are not. Recent investigations of mRNA in mammalian cells have indicated that these RNA molecules contain an almost monodisperse form of polyadenylic acid associated with them (Lim & Canellakis, 1970, Lee, Mendecki & Brawerman, 1971, Edmonds, Vaughan & Nakazato, 1971). This poly A core is approximately 80 - 150 nucleotides in length and lends to the mRNA, a tendency, depemdent on the pH and salt conditions, to enter the wrong phase during phenol extraction. This poly A core will therefore tend to give mRNA a high (A+U) content and therefore since the polyribosome

associated low molecular weight RNA components in BHK-21 cells have been shown to contain approximately 54% (G+C), this feature clearly distinguishes them from such mRNA molecules. However the unusual behaviour of poly A containing mRNA species with regard to their distribution in the various phases during phenol extraction may explain the apparent absence of these species in the polyribosome experiments in BHK-21 cells.

(5) The cell growth cycle and inhibitor studies.

The synthesis of the polyribosome associated low molecular weight RNA species does not appear to be coordinated with the synthesis of the DNA during the S phase of the cell growth cycle and these RNA species appear to be synthesised throughout the S and G_2 phases. The rate of synthesis of these molecules is however not constant but appears to increase continuously through the S and G_2 phases of the cell cycle. This is in accordance with the observations of RNA synthesis during the cell cycle in a number of cell types of higher organisms (Prescott & Bender, 1962, Monesi & Crippa, 1965, Scharff & Robbins, 1965) but Enger and Tobey (1969) hawe reported elevated rates of RNA synthesis only during the G_1 phase of thecell growth cycle in chinese hamster cells. The times of synthesis of the low molecular weight RNAs of the cytoplasm are therefore in contrast to the situation found in Hela cells for the microsomal associated histone mRNA molecules (Gallwitz & Mueller,1969, Borun, Scharff

& Robbins, 1967) for this RNA component is found on the microsomes only during the S phase of the cell cycle. It was not detected in synchronised populations of BHK-21 cells possibly due to the presence of the other low molecular weight polysome-associated RNA components. Although the synthesis of the polyribosome associated low molecular weight RNA species of BHK-21 cells appears to proceed continuously throughout the S and G_2 phase but to increase in rate through these phases of the cell cycle, a complicating factor which has not been taken into account in these experiments, is the possibility of a change in the size of the RNA precursor pools during the cell cycle, for this would affect the intracellular specific activity and thereby the apparent rate of incorporation of isotopically labelled RNA precursors. Such reservations upon the apparent increase in the rate of synthesis of these RNA species during these periods of the cell cycle must therefore be made.

Recently Niessing & Sekeris (1970)have indicated the specific cleavage of DNA-like RNAs, within the 80s precursor particles of the nucleus, to a size comparable with that of mRNA extracted from the cytoplasmic polyribosomes. In addition cleavage of the 45s r-pre-RNA molecule to the mature 28s and 18s RNA components of the cytoplasmic ribosomes has been shown to occur in a wide variety of mammalian and eukaryotic cells (Maden,1970). Pace, Peterson & Pace (1970) have suggested that

in prokaryotic cells much of the stable RNA arises as a result of post-transcriptional modifications of original gene transcription products. The results of experiments employing the adenosine analogue, toyocamycin, suggest that, in accordance with the results of kinetics of labelling experiments, the polyribosome associated RNA molecules of BHK-21 cells also might arise as the result of the specific cleavage of some larger precursor molecule(s). This drug, as is reported by Tavitian et al, (1968) inhibits the synthesis of 28s and 18s ribosomal RNAs in ammalian cells. The relatively poor response of tRNA synthesis to inhibitiom by the drug may possibly be explained by the fact that tRNA molecules are derived from precursor RNA molecules whose "extra" sequences are almost devoid of adenosine residues. (Smillie & Burdon, 1970).

Experiments utilising low doses of actinomycin D had suggested the nucleoplasmic location of cistrons corresponding to most of the polyribosome associated low molecular weight RNA species of BHK cytoplasm. Since α-amanitin, the toxic component of the toadstool, amanita phalloides, had been shown to inhibit in vitro the polymerisation of ribonucleotide monophosphates by the extranucleolar enzyme of mammalian cells whereas it did not inhibit the nucleolar enzyme under these conditions (Jacob, Sajdel & Munro, 1970), its effects upon the synthesis of the low molecular weight RNA components of BHK-21 cytoplasm

were investigated. The action of α-amanitin on RNA synthesis *in vivo* differs from its action *in vitro*. *In vivo* not only does α-amanitin inhibit the synthesis of RNA components known to be synthesised in the nucleoplasm (mRNA, tRNA and 5s RNA) but it apparently also inhibits the synthesis of rRNA components established to be of nucleolar origin. This suggests that in intact cells the function of the nucleolar polymerase responsible for the synthesis of rRNA components is regulated by some extranucleolar factor sensitive to α-amanitin. It is possible that α-amanitin prevents the synthesis of some species of RNA in the extranucleolar compartment which exerts a direct regulatory influence over ribosomal RNA formation or it may prevent the synthesis of some nucleoplasmic mRNA molecule which codes for a regulatory protein or indeed for the nucleolar polymerase itself. It is also possible that, *in vivo*, the toxin is metabolised to some product which does not possess the specificity of the native toxin towards the extranucleolar enzyme but instead interacts efficiently with both types of polymerase molecule. α-Amanitin apparently exerts its effects by binding directly to the RNA polymerase molecule (Jacob *et al*, 1970, Novello, Stirpe & Fiume, 1970) and has no direct effect upon protein synthesis (Fiume & Stirpe, 1966). It is therefore unlikely that α-amanitin inhibits the cytoplasmic synthesis of of nucleolar polymerase molecules. It is therefore apparent

that not only does nucleolar synthesis regulate the transport of RNA from the nucleus to the cytoplasm (Siddebottom & Harris, 1969), but that nucleolar synthesis is itself apparently regulated by the products of nucleoplasmic genes. Similar findings of an influence of nucleoplasm over nucleolar function has been reported in rat liver by Jacob, Muecke, Sajdel & Munro (1970).

Conclusions.

Although no definite conclusions as to the precise function of these low molecular weight RNA components of BHK-21 cells can be drawn from the data obtained in this study, it is apparent that there exists a class of unmethylated low molecular weight RNA molecules associated with the polyribosomes of mammalian cells and which are not representative of mRNA. In addition they do not appear to be related in any way with the synthesis of the DNA and do not appear to possess amino acid acceptance activity (as judged from amino acid labelling studies)[1]. One can at present only speculate upon the function of these low molecular weight RNA molecules but it is possible that they may represent adaptor or regulator molecules in the process of protein synthesis.

[1] (Hons. Project A.E. Clason, 1968. Univ. of Glasgow

SUMMARY

The increased resolution of RNA species afforded by polyacrylamide gel electrophoresis of extracts of eukaryotic cells has enabled the identification of a number of low molecular weight RNA components. The aim of this work was to investigate the identity and molecular characteristics of these various low molecular weight RNA species in the nuclei and cytoplasm of normal, malignant and virus "transformed" animal cells, to examine, if possible, their role within the cell and to define the molecular processes involved in their biogenesis.

(A) Low molecular weight RNAs of the nucleus

(1) Polyacrylamide gel electrophoresis of extracts of isolated mammalian nuclei indicate the existence of approximately 14 distinct low molecular weight RNA species with a size range of 80 to 350 nucleotides in length.

(2) Kinetics of labelling experiments indicate that these RNA species are stable and are present in the cell in low amounts for they are labelled only slowly with RNA precursors such as uridine or [^{32}P]-orthophosphate.

(3) Calculations based upon optical density tracings and estimated molecular weights suggest them each to be present to the extent of approximately 10^5 molecules per cell.

(4) Many of these RNA molecules are methylated either in their

base or sugar moieties and they are distributed between the nucleolar and nucleoplasmic fractions of the nucleus.

(5) Analysis of their patterns of synthesis in synchronised cell populations indicate that they display two distinct patterns of synthesis, for some appear to be synthesised only at, or just after, the time of maximum DNA synthesis during the S phase of the cell cycle whilst others appear to be synthesised continually.

(6) A comparison of the properties of these RNA species with those of the low molecular weight RNA components of the cytoplasm indicate that they are apparently confined to the nucleus and bear no obvious relationship to these cytoplasmic RNA species.

(B) Low molecular weight RNAs of the cytoplasm

(1) Electrophoresis of cytoplasmic preparations from mammalian tissue culture cell lines indicate the existence of approximately 13 low molecular weight RNA components ranging in size from 30 to 350 nucleotides in length. The bulk of these RNA components possess electrophoretic mobilities lower than that of the 5s RNA component of the cytoplasmic ribosomes.

(2) Kinetics of labelling experiments indicate that they are synthesised fairly rapidly in a linear fashion and show no precursor-product relationship one to another.

(3) Such kinetic studies also indicate that these RNA species

are fairly stable, observations which are confirmed by the results of pulse-chase experiments employing levels of actinomycin D sufficient to inhibit all further RNA synthesis.

(4) Investigations of the metabolic stability of these RNA components indicate them to possess total life spans of approximately 24 to 30 hours, to be less stable than tRNA or the RNA of the cytoplasmic ribosomes.

(5) Methylation studies in BHK-21/C13 and SR8/V1 cells, utilising both long and short exposures to methyl-labelled methionine, have indicated that most, if not all, of these species of RNA are devoid of methylated nucleotides and that the only methylated RNA species present in the cytoplasmic extracts is tRNA with the possibility of a minor RNA species migrating in positions intermediate between 5s RNA and tRNA.

(6) Studies of the intracellular location of these RNA species by the use of cell fractionation techniques have indicated that they are absent from the cell sap, are not associated with the mitochondrial fraction but are found in the microsomal fraction and appear in fact to be associated with the polysomes. Furthermore the only low molecular weight RNA species present in the cell sap is apparently tRNA and minor amounts of 5s RNA.

(7) Like the mRNA species of bacterial cells these low molecular weight RNA components are released from the polyribosome subsequent to treatment with EDTA and analysis in CsCl gradients

after fixation in formaldehyde have revealed however that they are released in the form of free RNA strands rather than as ribonucleoprotein associates.

(8) Experiments in BHK-21/C13 and the transformed cell lines (SR8/V1 and PyY) using ethidium bromide(which specifically inhibits mitochondrial RNA synthesis) have indicated that these RNA components are not of mitochondrial origin since they are synthesised in the presence of levels of the drug sufficient to totally inhibit mitochondrial RNA synthesis. Further this drug does not inhibit the synthesis of "pre-tRNA" in BHK-21/C13 or SR8/V1 cells.

(9) Base composition analysis of the polyribosome associated low molecular weight RNA components indicate them to possess high contents of (G+C), with an average value of 54%.

(10) Inhibitor studies using low levels of actinomycin D suggest them to be mainly of nucleoplasmic origin whilst experiments employing puromycin and cordycepin suggest that their function is probably not that of mRNA. Experiments utilising relatively low concentrations of the adenosine analogue, toyocamycin, raise the possibility that these low molecular weight RNA components could represent the cleavage products of some larger precursor molecule(s).

(11) Analysis of the synthesis of these low molecular weight cytoplasmic RNAs in thymidine synchronised populations of

BHK-21 cells has revealed that they are not synthesised coordinately with the DNA in the S phase of the cell cycle but are synthesised throughout S and G_2 phases with a rate which apparently increases through these phases of the cycle. (12) Additional experiments investigating the effects of α-amanitin on RNA synthesis in BHK-21 cells indicate that the *in vitro* and *in vivo* effects of the drug are different and suggest than an extranucleolar control of nucleolar function exists in mammalian cells.

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SUMMARY.

Some Studies on the Low Molecular Weight RNA Components of Mammalian Cells.

by

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Summary of a thesis presented for the degree of Doctor of Philosophy at the University of Glasgow, August, 1971.

Until recently, RNA was considered to belong to one of three categories; transfer RNA(tRNA), ribosomal RNA or messenger RNA. In addition however, the development of more sophisticated analytical techniques such as polyacrylamide gel electrophoresis, coupled with the improved cell fractionation procedures, has enabled the detection of hitherto unrecognised low molecular weight RNA species in both the nuclear and cytoplasmic fractions of eukaryote cells.

The aim of this work was to investigate the identity and molecular characteristics of these various low molecular weight RNA components in the nuclei and cytoplasm of normal, malignant and virus transformed animal cells and to define the molecular processes involved in their biogenesis.

(A) Low Molecular Weight RNAs of the Nucleus.

Polyacrylamide gel electrophoresis of extracts of isolated

mammalian nuclei indicate the existence of approximately fourteen distinct low molecular weight RNA species with a size range of 80-350 nucleotides in length. These RNA components are stable and appear each to be present to the extent of approximately 10^5 molecules per cell. Many of these RNA molecules are methylated either in the base or ribose moeities and they are distributed between the nucleolar and nucleoplasmic fractions of the nucleus. Analysis of their patterns of synthesis in synchronised cell populations indicate two distinct patterns of synthesis, for some appear to be synthesised only at, or just after, the time of maximum DNA synthesis during the S phase of the cell cycle whilst others appear to be synthesised continually. In addition these low molecular weight methylated RNA components appear to be confined to the nucleus for no methylated RNA components of similar size and base composition can be found in the cytoplasm.

(B) Low Molecular Weight RNAs of the Cytoplasm.

Electrophoresis of cytoplasmic preparations from mammalian tissue culture cell lines reveals the presence of approximatel thirteen low molecular weight RNA components with a size range of 80-350 nucleotides in length. The bulk of these RNA components possess lower electrophoretic mobilities than the 5s RNA component from the cytoplasmic ribosomes. Kinetic of labelling studies indicate them to be synthesised in a

fairly linear fashion and to show no precursor-product relationship one to another. They appear to be fairly stable, being less metabolically stable than tRNA or the RNA of the cytoplasmic ribosomes, but possessing total life spans of the order of approximately 24-30 hours. Unlike the low molecular weight RNA components of the nucleus they appear to be devoid of methylated nucleosides, the only methylated low molecular weight cytoplasmic RNA component being transfer RNA. Studies on the intracellular location of these RNA species indicate that they are absent from the cell sap, are not associated with the mitochondrial fraction but are associated principally with the microsomal fraction and appear in fact to be found on the polyribosomes. They are released from the polyribosomes subsequent to EDTA treatment and appear to be released in the form of free RNA strands rather than as ribonucleoprotein associates. Nucleotide composition analysis indicates these RNA species to possess an average (G+C) content of 54% and experiments employing ethidium bromide, which specifically inhibits mitochondrial RNA synthesis, indicate that they are not derived from the transcription of mitochondrial DNA. Inhibitor studies using low levels of actinomycin D suggest them to be of nucleoplasmic origin whilst experiments employing puromycin and cordycepin suggest that their function is not that of

mRNA. Experiments utilising the adenosine analogue, toyocamycin, raise the possibility that they could represent the cleavage products of some larger precursor molecule(s). Analyses of the synthesis of these low molecular weight RNA components of the cytoplasm in synchronised cell populations indicate that they are not synthesised coordinately with the DNA during the S phase of the cell cycle but are synthesised throughout the S and G_2 phases with a rate which apparently increases through these periods of the cell cycle. Additional experiments investigating the effects of the drug α-amanitin on RNA synthesis in mammalian cells indicate that the in vitro effects of the drug are different from the in vivo effects and suggest that an extranucleolar control of nucleolar function exists in mammalian cells.