THE MATURATION OF LOW MOLECULAR WEIGHT RNA IN MAMMALIAN CELLS

by

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ABBREVIATIONS.

These are as laid down in the Biochemical Journal Instructions to Authors (revised, 1969) with the following additions :-

BSS	Balanced salt solution
SSC	Standard saline citrate (0.15M - NaCl, 0.015M - sodium citrate, pH 7.0)
MAK	Methylated albumin kieselguhr
PPLO	Pleuropneumonia - like organisms
RNase	Ribonuclease
sRNA	soluble RNA
C13	BHK21/C13 i.e. Baby hamster kidney cells, clone C13.
pre-tRNA,	RNA eluting from Sephadex G - 100 in a position between 5s ribosomal RNA and transfer RNA.
A_{260nm}	Extinction at 260nm
Esch. coli	Escherichia coli

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

I. General Introduction

The smallest unit of living matter, which is capable of an independent existence, is the cell. Protein and nucleic acids contribute as much as 90% of the dry weight of most cells. The proteins form a very complex group of giant polymers built from only twenty basic units, the amino acids, but despite the relatively small number of basic units, thousands of different proteins are present in a living cell. These molecules are capable of forming the complexity of the cell from relatively simple materials. Therefore in examining living systems, it seems reasonable that some attention should be paid to the synthesis of such important molecules. It was by considering this problem that the function of the other major constituent of cells became apparent. The nucleic acid fraction can be divided into two parts, DNA and RNA. Contained within the structure of DNA is the information required to direct the synthesis of the complex protein molecules. The most conclusive direct evidence in support of this belief is derived from studies of bacterial transformation which was first observed by Griffith (1928). For example, several different types of pneumococci exist each possessing a different capsular polysaccharide and in 1944,

Avery, MacLeod and McCarty showed that cells of one strain of pneumococcus could synthesise the characteristic polysaccharide of a second strain if it were grown in the presence of DNA purified from that second strain. However, it is now known that DNA is not directly involved in the formation of proteins; in vitro work has shown that it is RNA not DNA which plays a vital role in the protein synthesising system (Spiegelman, 1957). This requirement for RNA during protein synthesis was first explained by Crick (1958) who suggested that the information contained in the DNA molecule was transcribed or copied to give an informational RNA molecule which then directed the synthesis of proteins. However, over the last ten years it has become apparent that most of the RNA does not act as a template in protein synthesis. There are in fact several types of RNA to be found in living cells and they range in molecular weight from 25,000 to 4×10^6 and larger. Considerable effort has been invested in elucidating not only the mechanisms whereby these RNA species are manufactured in prokaryotic and eukaryotic cells but also how they participate in the sequence of events leading to the synthesis of new proteins. Some of this

work will now be discussed.

II. The RNA of prokaryotes

The RNA of prokaryotic cells can be divided into three types, ribosomal, messenger and transfer. These will be described in the following sections:

a) Ribosomal RNA

Bacteria contain numerous small particles known as ribosomes (Schachman, Pardee and Stanier, 1952). When cells are exposed to amino acids containing the isotope ^{35}S, the radioactivity is first found associated with these ribosomes and later migrates to the cell sap where it is found incorporated in proteins (McQuillen, Roberts and Britten, 1959). This indicates that the ribosomes are involved in protein synthesis.

The ribosomal particles have a diameter of 0.015μ and ultracentrifugal analysis gives a sedimentation coefficient of 70s (Schachman et al, 1952). These particles consist of about 60% RNA and 40% protein (Spirin and Gavrilova, 1969); they account for more than 80% of the total RNA in the cell. Under conditions of low magnesium concentration, the ribosomes dissociate into two sub-units

(Tissières, Watson, Schlessinger and Hollingworth, 1959). These sub-units have sedimentation coefficients of 50s and 30s and, like the complete ribosomes, they are composed of about 60% RNA, the remaining 40% being protein (Kurland, 1960; Spirin and Gavrilova, 1969). It has been estimated that as many as 60 different proteins are present in the ribosomal particle of Esch. coli and that at least 20 of these occur on the 30s sub-unit (Spirin and Gavrilova, 1969). This small sub-unit also contains one large RNA molecule with a molecular weight of about 0.55×10^6 while the 50s sub-unit possesses an RNA of molecular weight 1.1×10^6 (Kurland, 1960; Stanley and Bock, 1965). These RNA molecules have a sedimentation coefficient of 16s and 23s respectively (Kurland, 1960). In addition to the large RNA molecules, there occurs a low molecular weight ribosomal RNA which is tightly bound to the 50s sub-unit (Aubert, Monier, Reynier and Scott, 1967; Comb and Zehavi-Willner, 1967) and has a sedimentation coefficient of 5s (Rosset and Monier, 1963). The two large ribosomal RNAs (rRNAs) have similar base compositions and show very little variation when isolated from different species of bacteria (Midgley, 1962). Although they are of so similar composition, it has been

shown that the sequences of nucleotides in these RNA molecules are not identical (Sanger, Brownlee and Barrell, 1965; Oishi and Sueoka, 1965). In addition to the four major nucleosides, rRNA contains several others in minor amounts. The principle group of these minor nucleosides is made up of the methylated derivatives of the major nucleosides. Pseudouridine (5-ribosyl-uridine) is also present. The content of these minor nucleosides varies between the 16s and 23s rRNAs (Dubin and Günalp, 1967).

The 5s RNA, on the other hand, contains neither pseudouridine nor methylated nucleosides (Rosset and Monier, 1963; Brownlee, Sanger and Barrell, 1967). It has a different base composition from 16s and 23s rRNA and a much higher content of G + C:-64% compared with 54% in the large rRNAs (Spirin and Gavrilova, 1969). In 1967, Brownlee, Sanger and Barrell described the complete sequence of 5s RNA from Esch. coli. Their work demonstrated that this RNA is composed of 120 nucleotides with no modified bases.

Since ribosomes carry out an important role in the process of protein synthesis, their own formation has attracted much attention. The next section deals with the production of the RNA components of the ribosome.

Recently polyacrylamide gel electrophoresis has been introduced as a method for fractionating RNA (Loening, 1967). This technique gives a much higher degree of resolution than ultracentrifugation and using it, Hecht and Woese (1968) and Adesnik and Levinthal (1969) have observed RNA species of slightly slower electrophoretic mobility than 16s and 23s rRNA. Studies of the kinetics of labelling carried out by these workers indicate that these may be precursors to the rRNA molecules. When Esch. coli are grown in the presence of chloramphenicol, small ribonucleoprotein particles (CM particles) accumulate (Nomura and Watson, 1959). The RNA molecules contained in them have slightly higher sedimentation coefficients than 23s and 16s rRNAs (Dubin and Elkort, 1964). After the removal of the chloramphenicol, the RNA species originally present in the CM particles can be detected in mature ribosomes (Osawa, 1965) suggesting that they are rRNA precursors. Adesnik and Levinthal (1969) confirmed this when they showed that RNA from CM particles has the same electrophoretic mobility on polyacrylamide gels as the rRNA precursors which they studied. Thus the precursor RNAs have lower electrophoretic mobilities and higher sedimentation coefficients than mature rRNA. Weinberg, Loening, Willems and Penman (1967) suggested that if lower

electrophoretic mobility is due to a more open configuration and not a higher molecular weight, then the molecule should also have a lower sedimentation coefficient. Since this is not the case, it follows that the precursors must have slightly higher molecular weights than mature rRNA. Therefore the precursors must contain non-ribosomal sequences which are removed during the maturation process, yielding 23s and 16s rRNA. The RNA contained in the CM particles has been shown to be undermethylated compared to the mature rRNAs. (Hayashi, Osawa and Miura, 1966; Dubin and Günalp, 1967). This means that the methylated nucleotides are formed after the completion of the polynucleotide chain and not by insertion of methylated nucleotides during the transcription process. Indeed Srinivasan, Nofal and Sussman (1964) have isolated several enzymes capable of transferring methyl groups from methionine to undermethylated rRNA molecules.

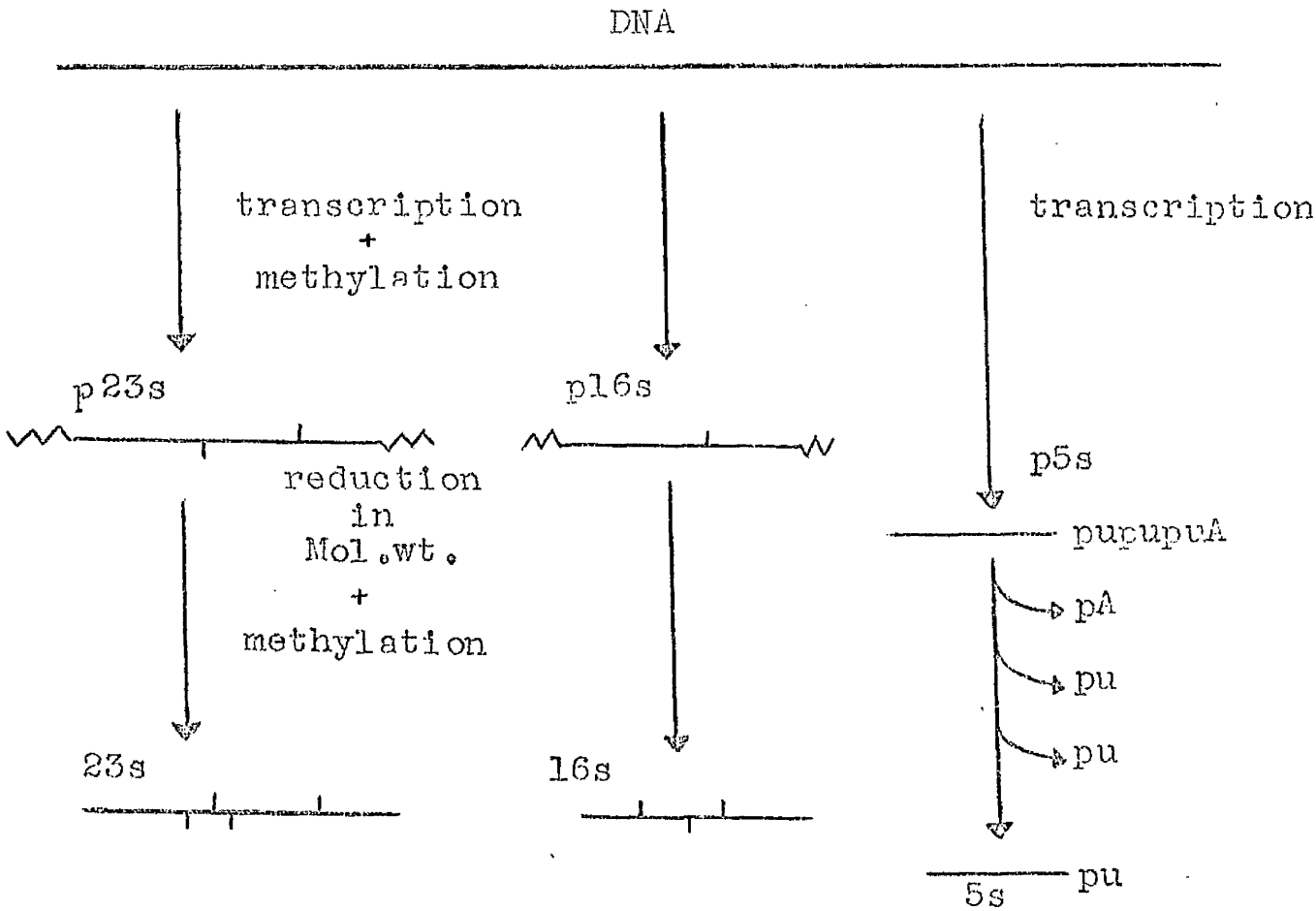
5s RNA synthesis in bacteria has been studied by several groups (Hecht, Bleyman and Woese, 1968; Smith, Dubnau, Morell and Marmur, 1968; Morell, Smith, Dabnau and Marmur, 1967; Forget and Jordan, 1970). Hecht et al (1968) carried out studies of the kinetics of incorporation of RNA precursors into 5s RNA of Bacillus subtilis. Their work indicates the presence of a precursor which accumulates during growth of the cells with chloramphenicol. Since this treatment

also prevents the maturation of the 16s and 23s precursors, these authors postulated that 5s RNA is derived from the fragments released during the processing of the precursors of the large rRNAs. However, Smith et al (1968) reported that Bacillus subtilis contains 10 cistrons for 23s and 16s RNA and only 3 or 4 for 5s. Also the gene mapping studies of Morell et al (1967) showed that the 5s genes appear to be separated from those of the other rRNAs by the genes for transfer RNA (tRNA). Recently Forget and Jordan (1970), using Esch. coli, have reported sequencing studies of 5s RNA formed in the presence of chloramphenicol. They found evidence for three 5s RNA molecules which differed from normal Esch. coli 5s RNA by having one, two or three extra nucleotides at the 5' end of the molecule. These extra sequences could also be found in a small proportion of 5s RNA from logarithmically growing cells. The authors therefore suggest that these three different 5s RNA molecules represent stages in the processing of the 5s RNA precursor.

Thus current work suggests that, although 23s, 16s and 5s RNA are found in equimolar amounts in ribosomes, they are synthesised separately. Each is produced by the modification of a precursor molecule. Extra sequences are removed and, in the case of the large rRNAs, methyl groups must be added before the mature RNA molecules are formed.

Fig. I.1.

Synthesis of Prokaryotic ribosomal RNAs.



~~~ - non-ribosomal material.

⊥⊤ - methyl groups.

Mol.wt. - molecular weight.

p23s, p16s, p5s. - precursors to 23s, 16s and 5sRNA.
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b) Messenger RNA

The concept of messenger RNA (mRNA) as a distinct species was first put forward by Jacob and Monod (1961). It is now generally accepted that mRNA is a copy of the DNA sequence and contains the information for protein synthesis in the form of a series of codons or nucleotide triplets. 64 of these can be formed using the 4 major bases, however, as only 20 amino acids are found in proteins, the code is degenerate and several codons represent each amino acid.

In rapidly growing cells many different proteins and therefore many mRNA must be produced. This fact together with the short half life of bacterial mRNAs (Jacob and Monod, 1961; Britten and Roberts, 1960; Astrachan and Fisher, 1961; Willson and Gros, 1964) hinders studies of their structure and synthesis. In spite of this, Moore (1966) has succeeded in proving that mRNA of Esch. coli contains no methylated nucleosides. However, whether or not mRNA molecules are synthesised with extra sequences, as is accepted for rRNA synthesis, is not yet known.

c) Transfer RNA

A third species of RNA is involved in the synthesis of proteins - transfer RNA. Hoagland, Zamecnik and Stephenson (1957) first

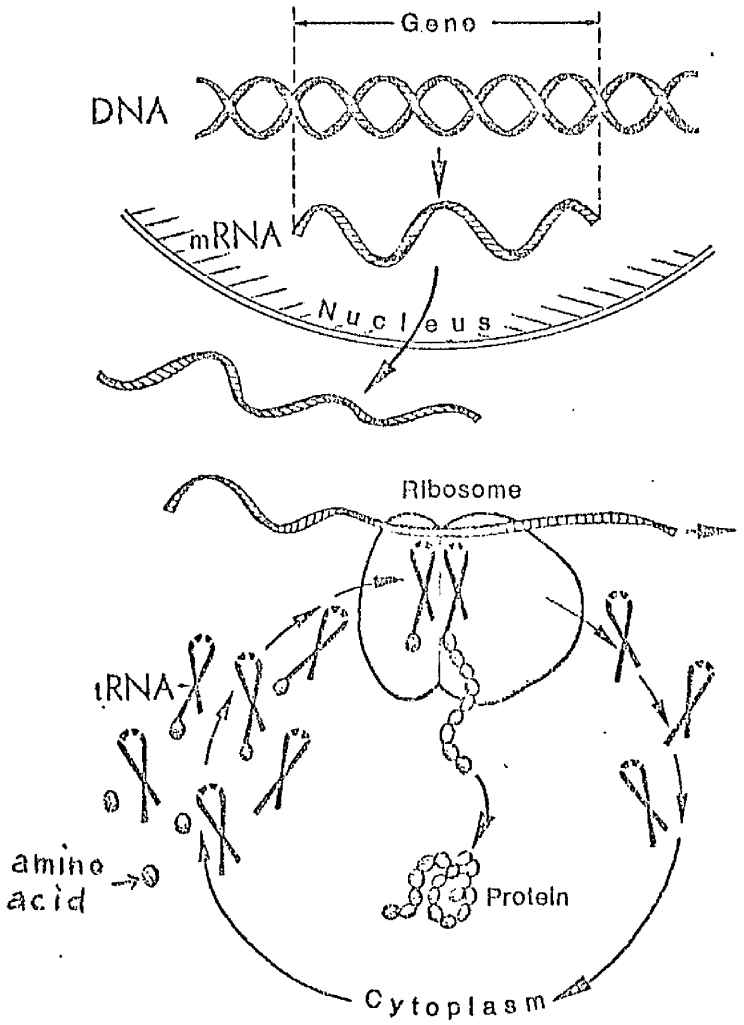
discovered this RNA when they observed that, prior to their incorporation into proteins, amino acids became attached to a low molecular weight RNA when incubated with ATP and a cell-free extract of rat liver. Although this new class of RNA was first discovered in mammalian cells, much work has also been done with bacteria and yeast to determine its structure and functions.

Transfer RNA (tRNA) has a sedimentation coefficient of 4s and a molecular weight of about 25,000. It consists of a single polynucleotide chain of 75-85 units. Despite its small size, tRNA takes part in several interactions vital to the synthesis of proteins. For example the activation of amino acids followed by their transfer to ribosomes engaged in protein synthesis requires the presence of tRNA molecules.

The discovery of tRNA occurred because of its ability to bind amino acids. This reaction takes place in two stages both of which are under the direction of one of a group of enzymes called aminoacyl-tRNA synthetases (amino acid-tRNA ligases, E.C.6.1.1.). At least one of these enzymes is specific for each of the 20 amino acids found in proteins. The first step in the reaction involves the activation of the carboxyl group of the amino acid. This interacts with ATP to form aminoacyl adenylate which

Fig. I.2

General scheme of protein biosynthesis.



Reproduced from Spirin & Gavrilova, (1969)

then reacts with a tRNA molecule to form aminoacyl-tRNA. The synthetase shows specificity for both the amino acid and the tRNA and therefore combines the amino acid with a particular tRNA. The importance of this dual specificity was demonstrated by Chapeville et al (1962) who showed that cysteine could be reduced to alanine with Raney Nickel. These workers found that, as this reaction did not destroy the link between the amino acid and the tRNA, they could prepare alanine attached to the tRNA specific for cysteine. When this altered aminoacyl-tRNA complex is used in a cell-free protein synthesising system, alanine is incorporated in place of cysteine thus proving that the fidelity of translation rests mainly in the specificity of the aminoacyl-tRNA synthetases.

The incorporation of amino acids into protein involves several specific interactions between tRNA and other macromolecules. The tRNA loaded with its amino acid must bind to a ribosome but only after recognition of a specific codon in the mRNA. Before the tRNA can be released from the ribosome, its amino acid must be incorporated into the growing peptide chain by the formation of two peptide bonds.

Considerable effort has been devoted to the determination of the structure of tRNA. It is hoped to decide which features are important in each of the wide variety of reactions carried out by these molecules.

The tRNAs specific for different amino acids can be separated by several methods (see Weiss and Kelmers, 1967, and references therein); they have different base compositions and sequences but all possess the same pCpCpA group at the 3' end of the molecule. It can be shown that amino acid acceptance is dependent on the presence of these three terminal nucleotides since they can be removed and replaced by the action of the enzyme adenylate pyrophosphorylase. Indeed, Zachau, Acs and Lipman (1958) discovered that leucine is attached to the 3' hydroxyl of the terminal A in the aminoacyl-tRNA complex. When they repeated this work, Marcker and Sanger (1964) discovered that two compounds are attached to the ribose of the terminal A in Esch. coli methionyl-tRNA. One is methionine, as expected, but some of the tRNA molecules appear to carry formylmethionine residues instead. In fact 2 species of tRNA are specific for methionine. Both accept methionine but only one of the methionyl-tRNAs can be formylated (Kellogg, Doctor, Loebel and Nirenberg, 1966). Several workers have shown that this formylmethionyl -tRNA is involved in the initiation of the polypeptide chain during protein synthesis (Clark and Marcker, 1966; Capecchi, 1966; Adams and Capecchi, 1966; Webster, Engelhardt and Zinder, 1966). The formyl

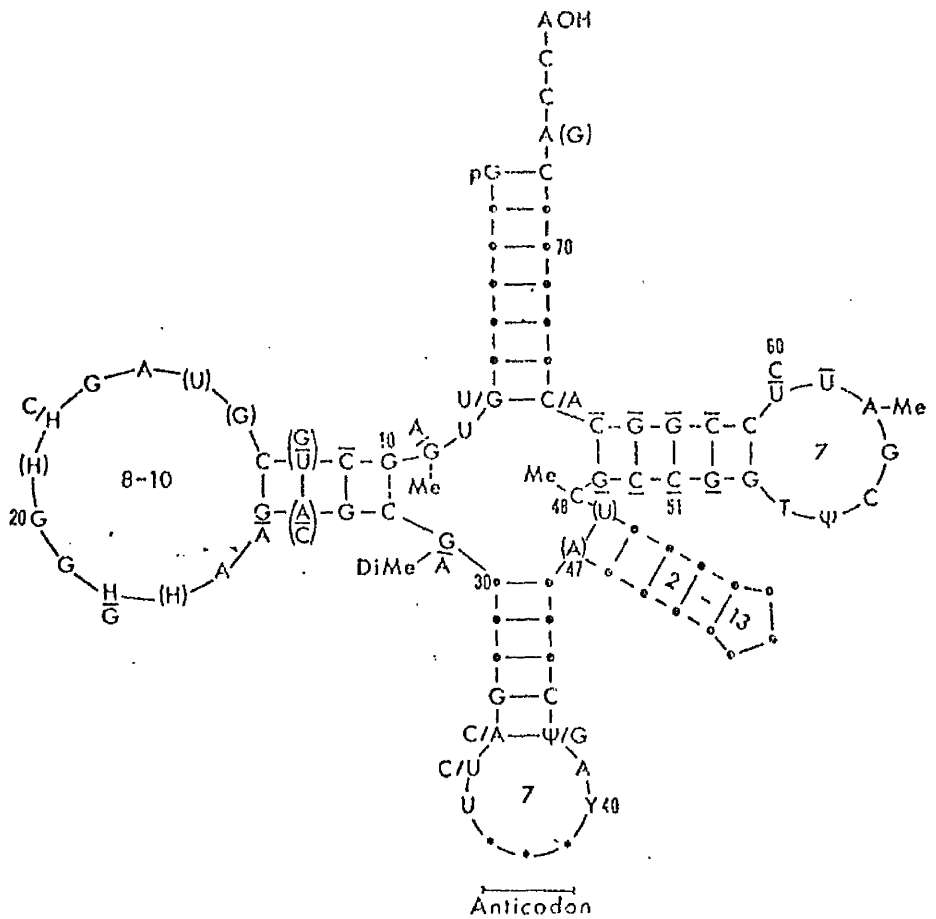
group is removed before completion of the protein (Adams, 1968).

In 1965, Holley et al published the sequence of yeast alanine tRNA. This was the first determination of the primary structure of an RNA molecule. Since then many more sequences have been determined for various tRNAs from bacterial, yeast and animal sources. Each tRNA molecule has a unique sequence. Philipps (1969) has compared the sequences of 14 tRNA molecules and finds that about 50% of the nucleotides are similar for all 14. He suggests that these similar sequences are involved in interactions which are common to all tRNA molecules, for example the maintainance of a common tertiary structure or interactions with the ribosomes. The remaining variable nucleotides may be involved in recognition of the appropriate synthetase; they may however play a more neutral role in the structure, being quite compatible with it but not required for the function of the molecule.

All the tRNAs of known primary structure can be arranged in the cloverleaf formation (Fig.I.3) proposed by Holley et al (1965). This conformation allows the formation of the maximum number of hydrogen bonds between the bases in the pairs A and U, G and C. As the name suggests, three loops of non-paired nucleotides are joined by short

Fig I.3.

Generalized structure of tRNA.



Generalized structure 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 by 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 dots are variant. Methyl groups have been included in three positions, where they are found.

Reproduced from Philipps (1969).

base-paired arms to a stem formed by pairing seven bases near each end of the tRNA molecule. The pCpCpA end group is of course attached to the stem of the cloverleaf but is not involved in the base pairing. Adjacent to the stem are the pentanucleotide and dihydrouridine loops and between these lies the loop containing the anticodon. The pentanucleotide loop, which in fact contains seven nucleotides, is so called because in it occurs the five nucleotide sequence GpTpΨpCpGp common to almost all tRNA molecules (Sanger, Brownlee and Barrell, 1965). The anticodon loop also contains seven nucleotides, three of which form the anticodon and interact with mRNA. A variable structure, termed the extra arm, occurs between these loops. In small tRNA molecules, it is almost non-existent consisting of only two nucleotides, but it ranges in size up to 13 nucleotides in a base-paired formation in the largest tRNAs. The dihydrouridine loop varies in size from 8 to 12 nucleotides; the arm of this loop is also variable, 3 or 4 base pairs occurring in this portion. As its name implies, the dihydrouridine loop generally contains the modified nucleoside dihydrouridine. Since only the extra arm and the dihydrouridine loop show variation in size from one tRNA to another, it seems likely that these features may be involved in the tRNA-specific interaction with the synthetases.

The cloverleaf structure is of course only a two-dimensional model. A three-dimensional model would be much more useful in determining the features on which the interactions of tRNA with the other components of the protein synthesising system are dependent. Various suggestions have been made regarding folding the cloverleaf to form a compact structure for tRNA (Lake and Beeman, 1968; Doctor, Fuller & Webb, 1969; Ninio, Favre and Yaniv, 1969; Cramer, Doepner, v.d. Haar, Schlimme and Seidel, 1968) but it is likely that the final answer will have to await the results of X-ray diffraction studies of tRNA crystals which are currently in progress in several laboratories. So far Blake, Fresco & Langridge (1970) have reported initial studies with cocrystals containing at least 7 different tRNA species. The existence of these mixed crystals indicates the existence of a similar tertiary structure for these tRNA molecules.

tRNA contains many minor nucleosides but these are present in much higher amounts than in rRNA. The minor nucleosides can be considered as modifications of the 4 major ribonucleosides; to date about 35 of these have been identified in RNA (Hall, 1970). Because of their great variety, it is most convenient to consider them as several

related groups. Most modifications consist of relatively simple alterations of the basic nucleoside, for example methylation of the base or ribose or replacement of a hydroxyl group with sulphur. Pseudouridine (ψ) is an example of altered bonding between the base and sugar residues. More complex modifications are also possible resulting in "hypermodified" nucleosides. These are defined as having a relatively large side chain for example an isopentenyl group, a functional group such as hydroxyl and a location next to the 3' end of the anticodon (Hall, 1970). Since these "hypermodified" nucleosides occur next to the anticodon sequence in most tRNA molecules, they might be expected to play some part in recognition of the codon. Fuller and Hodgson (1967) suggest that, since there are 7 nucleosides in the anticodon loop, the modified nucleoside provides a 'punctuation mark' to ensure that the correct 3 interact with the mRNA. Expermimental evidence in support of this suggestion comes from the work of Gefter and Russell (1969) who isolated 3 species of suppressor tyrosine tRNA from Esch. coli infected with a defective transducing phage. These tRNA molecules differed only in the extent of modification of the nucleoside adjacent to the 3' end of the anticodon. All 3 forms could be charged with tyrosine and 2 of these, which contained A modified to

different extents, could support protein synthesis in vitro. The third form contained an unmodified A; it was unable to bind the ribosomes and therefore could not support protein synthesis.

As least 4 different thionucleosides have been identified in Esch. coli tRNA (Lipsett, 1965; Carbon, Hung and Jones, 1965; Schleich and Goldstein, 1965; Peterkofsky and Lipsett, 1965; Lipsett and Doctor, 1967; Goehler and Doi, 1968). The major one is 4-thiouridine but 2-thiocytidine and 5-methyl-aminomethyl-2-thiouridine have also been isolated (Carbon, David and Studier, 1968). So far the importance of these modifications to the functioning of tRNA has not been determined although they may be involved in maintaining the active configuration of the molecules (Goehler and Doi, 1968).

Pseudouridine was the first minor nucleoside to be discovered (Cohn, 1957; Davis and Allen, 1957); in tRNA it amounts to about 20% of the total uridine content (Dunn, 1959). Formylmethionine tRNA contains only one Ψ (Dube, Marcker, Clark and Cory, 1968) and this occurs in the pGpTpΨpCpG sequence. Siddiqui, Krauskopf and Ofengand, (1970) modified this Ψ without affecting any other nucleoside. They found that the modified tRNA had undergone an alteration of its tertiary

structure which presumably caused the loss of biological activity observed by these workers. This result suggests that Ψ, at least when present in the pentanucleotide loop, is involved in the maintenance of tRNA structure.

Methylation provides the largest single group of modifications. Studies of the function of methylated nucleotides have been greatly facilitated by the isolation of the relaxed control mutant Esch.coli K12 W-6 which, unlike normal bacteria, continues to synthesise RNA when the required amino acid methionine is withdrawn (Borek, Ryan and Rockenbach, 1955). However the RNA extracted after methionine starvation is undermethylated (Mandel and Borek, 1961a); it is thought to consist of some RNA devoid of methyl groups, plus normal RNA which had been synthesised prior to the removal of methionine. Initial work showed no distinct difference in the ability of tRNA to bind amino acids when it lacked methyl groups (Starr, 1963; Littauer, Muench, Berg, Gilbert & Spahr, 1963). However it is difficult to draw definite conclusions from this work as undermethylated tRNA preparations contain normally methylated tRNA. Also each tRNA species may be affected to a different degree by lack of methylated nucleosides. Thus differences in activity between normal and methyl-deficient tRNA

may be missed unless experiments are carried out with all 20 amino acids. Indeed the work of Peterkofsky (1964) and of Shugart, Chastain, Novelli and Stulberg (1968) indicate that the amino acid acceptance activity of tRNA is affected by the absence of methyl groups. Peterkofsky (1964) reported that although Esch. coli synthetases load amino acids equally well on to normal and methyl-deficient Esch. coli leucine tRNA, the synthetase isolated from yeast functions only with fully methylated Esch. coli tRNA. The later work of Shugart et al (1968) shows that when undermethylated tRNA is used, the loading of 4 amino acids onto the appropriate tRNA molecules is reduced. When the tRNA is methylated in vitro using tRNA methylase from Esch. coli, the activity of 2 of the tRNA species returns to normal.

Investigations have also been carried out to determine any effect that methylation may have on the transfer of amino acids from tRNA to the ribosomes. Revel and Littauer (1965) separated normal and methyl-deficient phenylalanyl-tRNA by chromatography on methylated albumin kieselguhr (MAK). They used these purified tRNAs to form polyphenylalanine under the direction of poly U, poly UC and poly UA - templates known to code for polyphenylalanine. It was found that methyl-deficient tRNA gave a greater response to poly UC and poly UA

than did normal tRNA (Revel and Littauer, 1966; Littauer, Revel and Stern, 1966). Since these templates contain codons for other amino acids as well as for phenylalanine, this result indicates that there is greater miscoding with methyl-deficient tRNA. Capra and Peterkofsky (1968) also noted differences in the coding properties of normal and undermethylated tRNA. These workers resolved leucine tRNA into four components using reverse phase chromatography. Two of the peaks were greatly increased by methionine starvation of the Esch. coli mutant. In vitro methylation caused almost complete reversion to the normal pattern. Also one of the peaks of tRNA, which originally responded to poly UC only, responded to poly UC and poly UG after in vitro methylation. In contrast to these results, Fleissner (1967) found no evidence of miscoding either when he used the complete complement of methyl-deficient tRNAs to form f_2 coat protein in vitro, or when he used purified phenylalanyl-tRNA to form polyphenylalanine. He did find, however, that the methyl-deficient phenylalanyl-tRNA was less efficient at binding to the ribosomes than was the normal tRNA. Obviously a final answer to the problem of the function of methylated nucleosides has not yet been obtained and further work will be required.

In summary, it appears that, although the detailed mechanism is not known, loss or alteration of the minor nucleosides can affect the amino acid acceptance, ribosome binding and coding properties of the tRNA molecule.

III. The RNA of Eukaryotes

By definition, eukaryotes have a nucleus bounded by a definite nuclear membrane. This nucleus contains at least one distinctive nucleolus. Another feature of eukaryotic cells is the presence in the cytoplasm of mitochondria and, in the case of plant cells, chloroplasts. From the 1920's new techniques in cytochemistry and cell fractionation have shown that the cellular DNA is confined to the nucleus while the RNA is found also in the cytoplasm. Recent studies indicate that less than 1% of the total DNA is present in the mitochondria (Luck and Reich, 1964); chloroplasts contain 2-4% of the cellular DNA (Brawerman and Eisenstadt, 1964).

It is possible to separate nuclei from the cytoplasm of cells and this allows a separate study to be made of the RNA present in these two fractions of the cell.

A. Nuclear RNA

When cells are exposed briefly to radioactive uridine, labelled

RNA molecules appear first in the nucleus, then in the cytoplasm. This is expected since, although over 80% of the total cell RNA is contained in the cytoplasm, virtually all the DNA, which is used as template in the transcription process, is present in the nucleus. Thus RNA must be synthesised in the nucleus and transported to the cytoplasm.

Attempts to analyse the nuclear RNA initially led to confusion until it was realised that two separate classes of RNA rapidly appeared after the introduction of labelled precursors (Yoshikawa, Fukada and Kawade, 1964; Yoshikawa.-Fukada, Fukada and Kawade, 1965).

a) Ribosomal Precursor RNA

When eukaryotes are treated with radioactive RNA precursors for a short time, the immediately labelled RNA sediments much faster than ribosomal RNAs. Scherrer, Latham and Darnell (1963) obtained a distinct peak at 45s for Hela cells and a similar RNA was observed by Brown and Gurdon (1964) working with Xenopus laevis embryos. The work of both groups indicated that this high molecular weight RNA was a precursor of rRNA for when RNA synthesis was inhibited by the addition of actinomycin D, the 45s RNA appeared to be converted to rRNA (Scherrer et al, 1963). Also Brown and Gurdon (1964) noted that

the production of this high molecular weight RNA was greatly reduced in a certain mutant of Xenopus laevis which could not synthesise new ribosomes. These authors further noted that while cells of the normal wild-type Xenopus laevis contained two nucleoli per nucleus, the mutant was completely enucleolate. That this high molecular weight RNA was confined to the nucleolus was confirmed by Penman, Smith and Holtzman (1966) when they devised a technique for isolating nucleoli from Hela cells.

The combination of these facts suggests that the 45s RNA of the nucleolus is one of the first precursors of cytoplasmic ribosomal RNA. Penman and Attardi with their coworkers have used Hela cells to study the conversion of 45s RNA to the rRNAs, which in mammalian cells have sedimentation coefficients of 18s and 28s.

When the time of exposure to the RNA precursor is increased, label appears simultaneously as 32s RNA in the nucleolus and 18s rRNA in the cytoplasm. The $28s_{RNA}$ appears in the cytoplasm at a later time (Penman, 1966; Penman et al 1966). This sequential appearance of radioactivity in the RNA molecules led to the suggestion that the 45s precursor is split to form 32s and 18s RNA which is immediately

transported to the cytoplasm. The 32s RNA is subsequently converted to 28s RNA in the nucleolus and migrates into the cytoplasm more slowly than 18s RNA.

Since sedimentation coefficients depend partly on molecular weight and partly on the conformation of the molecule, some speculation arose as to whether or not nucleotide sequences were removed during the conversion of 45s to 18s and 28s RNA. Three different approaches have been made to this problem and each indicates that some material is indeed removed from the precursor RNA during the formation of rRNAs.

Jeanteur, Amaldi and Attardi (1968) studied the oligonucleotides released by pancreatic ribonuclease digestion of the various RNA species. The oligonucleotides obtained by this treatment are determined by the primary sequence of the RNA molecule. Small differences in sequence, even only one or two nucleotides, may be observed by the appearance of a new oligonucleotide (Sanger, Brownlee and Barrell, 1965). Such a "finger printing" method had previously proved useful in comparing proteins. Using this technique, Jeanteur et al (1968) found that up to 50% of the 45s RNA and 30% of the 32s RNA consisted of non-ribosomal sequences. These figures were later confirmed by Jeanteur and Attardi

(1969) using the technique of DNA-RNA hybridisation.

Further evidence for the non-conservative processing of 45s to 28s and 18s RNA is derived from studies of the methylation of rRNA and its precursors. In 1965 Brown and Attardi discovered that 18s and 28s rRNA of Hela cells contained methylated nucleosides. The following year this was confirmed by Burdon (1966) using Krebs II ascites cells. Since then it has become generally accepted that mammalian rRNA, like that from bacterial sources, contains methyl groups; however in the former case almost 80% of the methyl groups in 28s and 18s rRNA are present as 2-O'-methylribose (Brown and Attardi, 1965). Methylation in this position renders the adjacent phosphodiester bond resistant to alkaline hydrolysis (Morisawa and Chargaff, 1963). Recent work has indicated that with one exception (Zimmerman, 1968), all the methyl groups found in 28s and 18s rRNA, amounting altogether to about 80 (Wagner, Penman and Ingram, 1967), are added very soon after the synthesis of the 45s RNA (Greenberg and Penman, 1966). Wagner et al (1967) have shown that none of these are removed during the subsequent processing. These authors found that the pattern of alkali resistant oligonucleotides derived from 45s RNA is qualitatively identical to an equimolar mixture of 28s and 18s

rRNA; 32s RNA contains all the methyl groups of 28s RNA. These facts allowed Weinberg, Loening, Willems and Penman (1967), to conclude that the processing of 45s RNA involved the loss of RNA. They grew cells in the presence of [^{14}C]-uridine to label the RNA and [^{3}H-methyl]-methionine as a precursor to the methyl groups. They isolated RNA and measured the methyl group to nucleotide ratio. It was found that 45s and 32s RNA had much lower ratios than 28s and 18s RNA. Therefore, since only one methyl group is added after the formation of 45s RNA (Zimmerman, 1968), this indicates a loss of unmethylated nucleotides.

The third piece of evidence supporting the belief that material is lost during the processing of 45s RNA is provided by Willems, Wagner, Laing and Penman (1968); Amaldi and Attardi, (1968) and Jeanteur et al (1968). These groups determined the base ratios of highly purified rRNA and its precursors and found that 45s and 32s RNA have a much higher G+C content than do 28s and 18s rRNA. Therefore the non-ribosomal material present in the precursor molecules must have a very high G+C content.

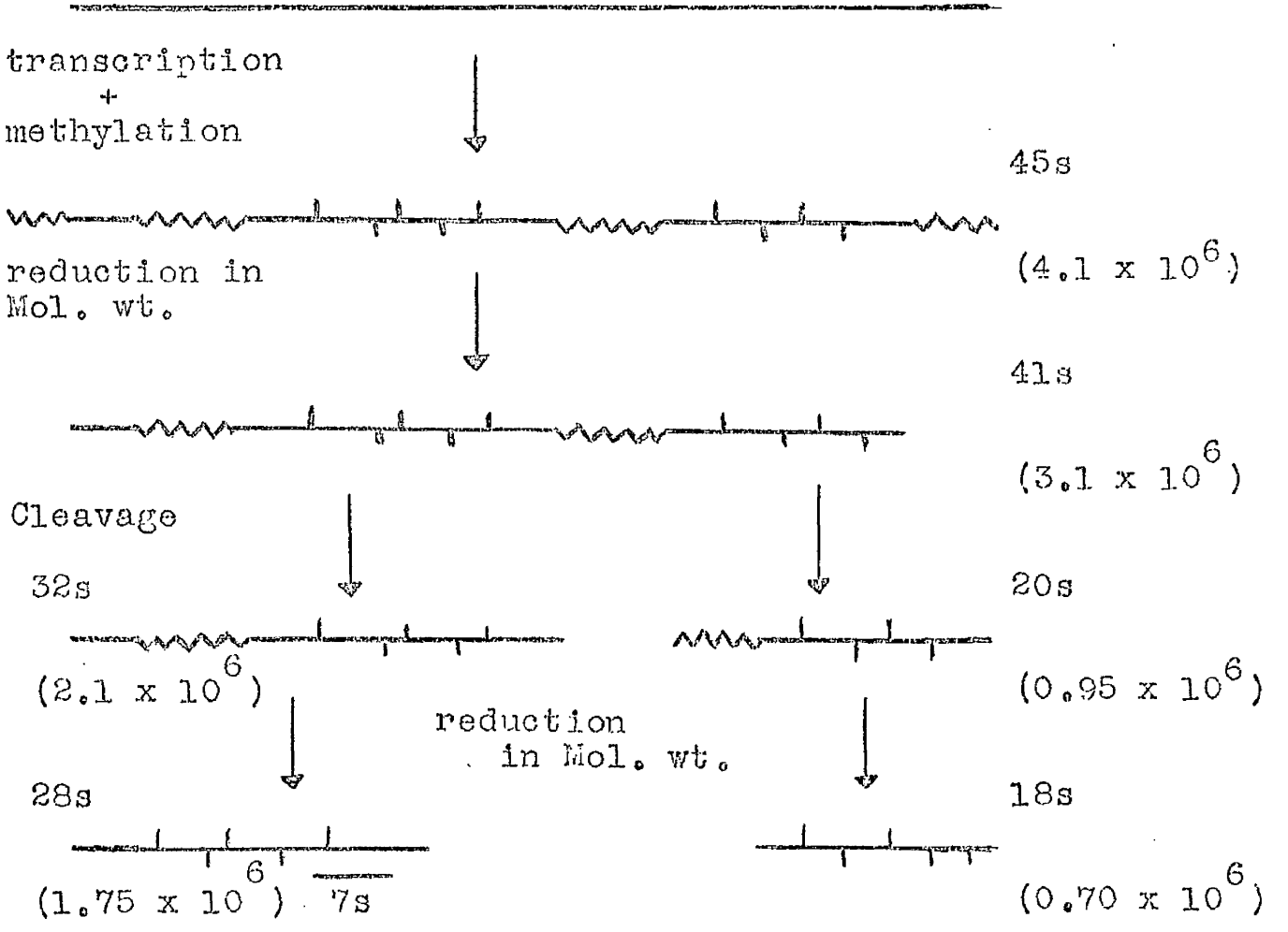
Summarizing all the above facts, it appears that 45s RNA contains 18s and 28s sequences but about 50% of its length is non-ribosomal.

32s RNA contains 28s sequences but not those of 18s; 30% of its sequence is lost in the conversion to 28s RNA. The RNA lost in the formation of rRNAs from their precursors has a very high G+C content and no methyl groups.

The high resolution obtained with polyacrylamide gel electrophoresis has allowed the discovery of two further intermediates in rRNA synthesis (Weinberg et al 1967). These authors found that the proportion of these minor species could be increased by poliovirus infection of the Hela cells (Weinberg and Penman, 1970). From their mobility on polyacrylamide, Weinberg and Penman called them 41s and 20s RNA; studies of the kinetics of labelling indicated that these components are very short lived intermediates in the conversion of 45s to 28s and 18s rRNA. On comparing the methylation of the various RNA species, the 41s RNA was found to contain as many methyl groups as 45s and 20s as many as 18s rRNA. It therefore appears that 45s RNA is converted to 41s RNA with the loss of some non-ribosomal material; 41s RNA is then split to form 32s and 20s which is rapidly converted to 18s rRNA. 28s rRNA is derived from the 32s precursor, again with some loss of material. At the moment this sequence of events, summarized

Fig 1.4.

The synthesis of mammalian rRNA.



~~ - non-ribosomal material
⊣⊢ - methyl groups
Mol.wt. - molecular weight

Molecular weight values from Weinberg & Penman (1970).

in Fig I.4, seems to describe the synthesis of 28s and 18s rRNA.

b) High Molecular Weight Heterodisperse RNA

This is the second rapidly labelled species of RNA in the nucleus. Unlike 45s RNA, it is very heterodisperse ranging in sedimentation coefficient from less than 30s to 80s (Attardi, Parnas, Hwang and Attardi, 1966; Warner, Soeiro, Birnboim and Darnell, 1966). It has a base composition very similar to the DNA (Soeiro, Birnboim and Darnell, 1966) and is often referred to as DNA-like RNA. Another distinction between the two RNA species is the site of synthesis, the heterodisperse DNA-like RNA being produced in the nucleus but not in the nucleolus (Soeiro et al, 1966).

The function of this RNA species, which may constitute 30 to 50% of the rapidly labelled nuclear RNA (Yoshikawa-Fukada et al, 1965), is not at all clear. Since the DNA, assumed to contain all the information for the synthesis of proteins, is situated in the nucleus of the eukaryotic cell, while more than 95% of the cellular ribosomes are in the cytoplasm (Penman et al, 1966), the need for a Jacob and Monod type messenger RNA is obvious. The properties of such a messenger are that it should be produced in the nucleus and rapidly

labelled, assuming continual synthesis of proteins. It might also be expected to be heterodisperse owing to the many different proteins produced by the living cell and it must be transported to the cytoplasm and associate with ribosomes. While the first 3 points do apply to this DNA-like nuclear RNA, as regards transport to the cytoplasm, there is no evidence of the occurrence of such high molecular weight RNA outside the nucleus. However the cytoplasm does contain a rapidly labelled RNA fraction. It is heterodisperse with sedimentation coefficients ranging from 7s to 20s - the size expected for monocistronic messengers of the proteins found in living cells. Unlike the mRNA suggested by Jacob and Monod (1961) for bacterial cells, this species of RNA is reasonably stable (Staehelin, Wettstein and Noll, 1963; Revel and Hiatt, 1964). Thus the cytoplasmic messenger RNA appears to be much smaller than the nuclear rapidly labelled species. Also the latter type of RNA is very unstable with a half-life of about 30 min (Attardi et al., 1966) and one-third to two-thirds of the label incorporated into it never leaves the nucleus (Gvozdev and Tikhonov, 1964; Scherrer et al, 1963). A similar relationship exists between rRNA and 45s RNA. It is therefore possible that the rapidly labelled

nuclear RNA is a precursor to the cytoplasmic mRNA and is processed in a similar manner to the 45s RNA. However due to the heterogeneous nature of both the nuclear RNA and the cytoplasmic mRNA, such a hypothesis is very difficult to test; the final answer may have to await the introduction of new techniques for studying such high molecular weight RNA molecules.

c) Low Molecular Weight Monodisperse RNA

Recently a third class of RNA has been discovered in the nucleus. It consists of several species of low molecular weight RNA (Nakamura, Prestayko and Busch, 1968; Prestayko and Busch, 1968; Weinberg and Penman, 1968). They are highly methylated, mainly on the ribose (Weinberg and Penman, 1968; Zapisek, Saporana and Enger, 1969) and are present in cells from birds, mammals and amphibians (Rein and Penman, 1969; Weinberg and Penman, 1969). Unlike the other types of nuclear RNA which have lifetimes of 10 to 30 min, these RNA species are very stable; they all have lifetimes of a day and most survive for considerably longer (Weinberg and Penman, 1969). Prestayko and Busch (1968) and Clason and Burdon, (1969) have suggested that they may be involved in the regulation of gene expression. However further work will have to be done to confirm this.

These three types of RNA, 45s ribosomal precursor RNA, high molecular weight heterodisperse and low molecular weight monodisperse, are found exclusively in the nucleus and equivalent types of RNA have not so far been observed in prokaryotic cells. Species of RNA which do appear to be similar to those of bacterial cells are found predominantly in the cytoplasm of eukaryotic cells.

III.B. Cytoplasmic RNA

a) Ribosomal RNA

Eukaryotic cells, like prokaryotes, contain many ribosomes. These ribosomes, however, have higher sedimentation coefficients of about 80s (Spirin & Gavrilova, 1969). A few 70s ribosomes are also present but these are confined to the mitochondria and chloroplasts (Spirin & Gavrilova, 1969).

As in bacteria, the particles consist of 2 sub-units. The smaller one, with sedimentation coefficient of 40s, contains one molecule of 16-18s RNA and several proteins. The large 60s sub-unit contains one 28s RNA and a small 5s molecule together with about 30 proteins. Thus apart from the relative sizes of the molecules, there

appeared to be little difference between eukaryotes and prokaryotes as regards RNA content of the ribosomes. However recently Pene, Knight and Darnell, (1968) discovered that a small RNA molecule is released from the 28s RNA by heating to 60°C. Further study revealed that this RNA is tightly hydrogen - bonded to the 28s RNA and that it has a chain length of 150 nucleotides. Like 5s RNA it contains no methylated nucleosides. By calculation from its mobility on polyacrylamide gel electrophoresis, Pene et al (1968) suggested that it should be called 7s RNA; more precise measurement of its sedimentation coefficient showed that it is in fact 5.7s (Weinberg and Penman, 1968) and these authors therefore suggested that this new ribosomal RNA be referred to as 28s-associated RNA (28sA RNA). The occurrence of 28sA RNA has been studied in various cell types (Pene et al, 1968; Sy and McCarty, 1970); it is found in several eukaryotic cells but not in Esch. coli. Of course many more investigations will have to be made before it can be said that this is a definite difference between prokaryotic and eukaryotic ribosomes. It would be of interest to test for the presence of this new RNA in the 70s ribosomes of the mitochondria.

Eukaryotic ribosomes therefore contain 28s, 18s, 28sA and 5s RNA molecules in a one-to-one basis. The synthesis of the two large rRNAs

has already been discussed in the section on nuclear ribosomal precursor RNA; the formation of the low molecular weight rRNA molecules remains to be considered.

It has been discovered that up to 50% of the 45s RNA is not converted to 28s and 18s rRNA. The portion lost has a high G+C content and contains no methylated nucleosides. Neither 28sA nor 5s RNA contain methyl groups (Pene et al, 1968; Forget and Weissman, 1967). Also, unlike tRNA, they are found in close association with the larger ribosomal sub-unit; they cannot be removed without disruption of the particle (Pene et al, 1968; Knight and Darnell, 1967). It therefore seems reasonable to suppose that they may be synthesised from the same precursor molecule as the other two ribosomal RNAs. Pene et al, (1968) reported a very detailed study of 28sA RNA. This new species of RNA showed kinetics of labelling with radioactive ribonucleosides identical to that of 28s rRNA suggesting that it is derived from the same precursor molecule. Since 28s RNA has been shown to have several precursors, Pene and his colleagues isolated each one using conditions known not to release 28sA RNA from 28s rRNA. They found that the small RNA molecule could not be released subsequently from any of the ribosomal

precursor molecules or from 18s RNA. It is only found associated with 28s rRNA either in the cytoplasm or in the nucleus. The 28s RNA found in the nucleus is believed to be newly synthesised from the 32s precursor RNA. Pene et al therefore decided that 28sA RNA is formed from the 32s molecule at the same time as 28s rRNA (see Fig I.4).

Regarding the synthesis of 5s RNA, Knight and Darnell (1967) found that it is present in cytoplasmic and nuclear ribosomal particles in a one-to-one basis with 28s rRNA. However comparison of the kinetics of labelling of the two species of RNA indicated the existence of a pool of 5s RNA. Extraction of the nuclear RNA confirmed that 20% of the total cellular 5s RNA exists in the nucleus while only 1-2% of the 28s RNA occurs there. This means that either 5s RNA is not synthesised from the 45s precursor or else more than one of the small RNA molecules is derived from at least some of the 45s molecules. Brown and Weber (1968) also examined the problem of 5s RNA synthesis when they studied the enucleolate mutant of Xenopus laevis. Using the technique of DNA-RNA hybridisation, they found that although the genes for ribosomal RNA are completely absent, DNA complementary to 5s RNA is still present in the same amount as in wild type cells. However no 5s RNA is synthesised in the mutant cells. These authors also estimated the

relative numbers of 28s, 18s and 5s genes and found a vast excess of 5s genes. (Brown and Weber, 1968). It therefore seems that 5s RNA is synthesised independently of 28s, 18s and 28sA RNA.

b) Messenger RNA

The existence of a cytoplasmic RNA with the properties expected of a messenger molecule has already been mentioned in the section on high molecular weight nuclear RNA. In contrast to the bacterial system, mRNA appears to be relatively stable in eukaryotes, with lifetimes varying from a few hours to several weeks (London, Shemin and Rittenberg, 1950; Revel and Hiatt, 1964; Pitot, Peraino, Lamar and Kennan, 1965). Once again the heterogeneity of mRNA makes detailed studies difficult. However, in certain systems this problem is greatly reduced, for example, using rabbit reticulocytes, several groups have isolated an RNA with sedimentation coefficient of 8-9s; this is exactly the size expected of an RNA coding for the polypeptide chains of globin (Burny, Huez, Marbaix and Chantrenne, 1969; Laycock and Hunt, 1969). In fact the latter group used this 8-9s RNA to stimulate protein synthesis in a cell-free system from Esch. coli. The product produced gave a pattern of polypeptides, after tryptic digestion,

identical to that obtained with normal rabbit globin. Further work with this natural mRNA may help to answer some of the problems of protein synthesis.

c) Transfer RNA

Like eukaryotic rRNA, tRNA shows some slight variations from the picture obtained with prokaryotic cells. For example, there is only one report, for yeast tRNA, of sulphur-containing nucleosides (Baczynskyj, Biemann and Hall, 1968), a modification well documented for bacterial tRNAs.

An interesting point has arisen regarding formylmethionine tRNA. This tRNA has a sequence different from methionine tRNA (Cory, Marcker, Dube & Clark, 1968) and is required in bacteria for chain initiation during protein synthesis. Until recently this specific tRNA had not been observed in eukaryotes and appeared not to be required. However it has been reported that mitochondria and chloroplasts, in addition to possessing a small quantity of DNA and ribosomes, also contain tRNA (Barnett and Brown, 1966 and 1967). Formylmethionine tRNA has been found among these non-cytoplasmic tRNAs (Smith & Marcker, 1968; Schwartz, Meyer, Eisenstadt and Brawerman, 1967) where it presumably functions in the initiation of mitochondrial

protein synthesis. Work is still in progress to determine the initiator of protein synthesis on 80s ribosomes, although it appears that a methionyl-tRNA may be involved here also (Smith and Marcker, 1970).

Since the discovery of precursors for rRNA, a tRNA precursor has been looked for. In 1967, Lal and Burdon reported the discovery of an unstable rapidly labelled RNA in Krebs II ascites cells. This RNA elutes from Sephadex G-100 in a position intermediate between 5s and tRNA and it can be detected in the cytoplasm in as short a time as three minutes after the addition of the RNA precursor to the cell suspension. At this time it contributes the major fraction of the low molecular weight RNA. As the exposure time is increased, the proportion of radioactivity present as tRNA and 5s RNA increases greatly until, after about 2h, the rapidly labelled RNA can not be discerned. Such a pattern of labelling would be expected with a precursor of tRNA; this suggestion is strengthened when, after the initial short labelling period, RNA synthesis is completely inhibited using actinomycin D and the incubation is continued for 45min. Under these conditions the radioactivity, present in control cultures as the rapidly labelled RNA, disappears and a similar amount appears in the 4s

RNA position. Incorporation of labelled methionine, the source of methyl groups in RNA (Mandel & Borek, 1961band 63), indicates that the precursor RNA is less methylated than mature tRNA (Lal & Burdon, 1967).

In further reports, Burdon and his colleagues (Burdon, Martin & Lal, 1967; Burdon, 1967a; Burdon & Clason, 1969) give additional properties of the presumed tRNA precursor and studies on its intracellular location. No evidence could be found for its existence in the nucleus of Krebs II ascites cells; it appeared to be present free in the cell sap unassociated with mitochondria or microsomes. Since the tRNA precursor is undermethylated compared to tRNA, it is obviously important to determine the location of the tRNA methylases; Burdon et al (1967) showed that these enzymes exist mainly in the cytoplasm where they would be expected to occur if the tRNA precursor functioned as their substrate.

Lindahl, Adams & Fresco (1966) reported that tRNA molecules can exist in two forms, native and denatured. The latter cannot accept amino acids and elutes from Sephadex G-100 before the active form. Heating to 60°C in the presence of magnesium ions allows the conversion of the denatured tRNA to the native form. Burdon (1967a)

used this treatment to determine whether the difference in elution characteristics of the tRNA precursor and the mature tRNA could be explained solely by altered conformation. Although some change in the elution pattern does occur, the precursor RNA still elutes from Sephadex before the mature tRNA. This suggests that the precursor may be slightly longer than tRNA as well as having a more open configuration. To confirm this, isolated radioactive tRNA precursor, together with non-radioactive Krebs II ascites cell cytoplasmic RNA, was treated with formaldehyde; the treated RNAs were fractionated on Sephadex G-100. This formaldehyde treatment has been reported to eliminate the contribution made by double-stranded, hydrogen-bonded segments to the conformation of the tRNA molecules (Boedtker, 1967 and 1968). The remaining secondary structure of the molecules is therefore due to single-stranded base-stacking. Even so, the precursor RNA still elutes from Sephadex G-100 before the mature tRNA. From these results, Burdon and Clason (1969) suggest that the tRNA precursor is probably about 15 nucleotides longer than completed tRNA.

In 1969, Bernhardt and Darnell published evidence for the existence of a tRNA precursor in Hela cells. Using polyacrylamide gel electrophoresis, a rapidly-labelled RNA fraction appeared between the 5s and 4s RNA bands when the cells were pulse-labelled with RNA precursors. From kinetics of labelling studies, this RNA seems to be a precursor of tRNA and the authors referred to it as pre-tRNA. Its properties are the same as those of Burdon's precursor RNA. Since pre-tRNA is undermethylated compared to the finished tRNA,

Berndardt and Darnell deprived the Hela cells of methionine; this treatment reduces the rate of conversion to tRNA, however, as it also reduces the rate of synthesis of the pre-tRNA, no accumulation occurs. It appears that this effect is not due to a requirement for protein synthesis as valine starvation did not affect the processing of the tRNA precursor nor did cycloheximide treatment.

Kay and Cooper (1969) have been studying rapidly-labelled cytoplasmic RNA in human lymphocytes. In vitro these cells are relatively inert metabolically unless stimulated by certain agents. One of the most effective of these is the bean extract phytohaemagglutinin (PHA) which stimulates the cells to synthesise RNA, protein and DNA, and then to divide. Kay and Cooper report that one of the first RNA types to be synthesised has properties very similar to the tRNA precursor discussed above. It is found free in the cell sap and is undermethylated compared to tRNA. It appears to be much more susceptible to degradation by snake venom phosphodiesterase than is mature tRNA. These authors attempted to confirm its precursor relationship to tRNA by DNA-RNA hybridisation. However they could only conclude that under conditions where nuclear heterodisperse RNA showed hybridisation to the DNA, the precursor resembled tRNA in that no hybridisation could be demonstrated. This was due to technical difficulties which prevented the preparation of sufficient quantities of highly labelled precursor RNA and tRNA.

This apparent tRNA precursor also occurs in insect cells; in

1969, Egyházi, Daneholt, Edström, Lambert and Ringborg observed it in cells of the salivary glands of Chironomus tentans. They isolated this rapidly-labelled RNA from chromosomal and nuclear sap fractions as well as the cytoplasm of these cells. This difference in distribution found in the insect cells compared to the mammalian cells may be due to different isolation techniques or to differences in the biological material and further investigations will be required to resolve this problem.

It therefore appears that, like rRNA, eukaryotic tRNA is first transcribed from the DNA as a precursor molecule requiring certain modifications before it acquires the properties of the functional tRNA.

IV. RNA Methylation

Three types of RNA are known to contain methylated nucleosides; these are tRNA, rRNA and its precursors and the low molecular weight nuclear RNA of eukaryotes. The existence of these minor constituents was quite unsuspected as the functioning of RNA in the protein synthesising system appeared to be explained completely in terms of the 4 major nucleosides. In fact more than 10 years after their discovery, the function of the minor nucleosides is still a matter for discussion.

Another problem is their origin, for there seems to be no method for producing such a variety of methylated compounds from a sequence of DNA which contains only 5-methyl cytidine and perhaps two other minor nucleosides (Culp, Dore and Brown, 1970) in addition to the 4 major nucleosides. Mandel and Borek (1961 a and b, 1963) demonstrated that the methyl groups in Esch. coli RNA arose directly from L-methionine.

In 1962 Fleissner and Borek reported that cell-free extracts of Esch. coli could catalyse the transfer of methyl groups from S-adenosylmethionine to methyl-deficient Esch. coli tRNA and the existence of enzymes capable of methylating tRNA at the polynucleotide level was rapidly confirmed (Starr, 1963; Gold, Hurwitz and Anders, 1963). The latter group reported the identification of 6 enzymes distinguishable by the bases used as substrate and by the products formed (Hurwitz, Gold and Anders 1964 a&b).

Since each methylated nucleoside is present in such small amounts, there must be some reason why the enzymes do not modify all of the nucleosides for which they are specific. Either most nucleosides are protected from the action of the enzymes by their position in the tertiary structure of the tRNA molecules, or the methylases recognise the sequence surrounding the particular nucleoside to be methylated. Whether or not the first mechanism is important, there is evidence to support the second. Baguley and Staehelin (1968) have isolated a methylase from rat liver which is specific for the 2-N position of guanine and another for the 1 position of adenine. When they used these enzymes to methylate methyl-deficient Esch. coli tRNA in vitro, they found methyl groups contained in only 3 of the oligonucleotides released by digestion with pancreatic ribonuclease. 2-N-methyl guanine was present in one sequence while 1-methyl adenine occurred in the other two. This suggests that the sequences surrounding the nucleoside to be methylated are important for its identification. Another indication that some factor other than the tertiary structure

is involved in the methylation of RNA comes from the discovery that although the enzymes can not methylate normal RNA extracted from the same source, heterologous enzymes can add extra methyl groups. (Srinivasan and Borek, 1963; Gold et al 1963 a and b). This also shows that the complement of RNA methylases varies from species to species.

This species specificity is interesting as it shows that tRNA may be hypermethylated. The effect of the absence of methyl groups on the functioning of tRNA has already been considered; extra methyl groups may also affect the structure of tRNA. However this would be of limited interest if hypermethylation were only an in vitro phenomenon; in fact it has been reported also in in vivo situations. Infection of Esch. coli with T2 phage causes an increase in tRNA methylation and also differences in the pattern of bases methylated (Wainfan, Srinivasan and Borek, 1965). Not all virus infections cause an increase of tRNA methylation, for example T3 and T4 phage infections reduce the number of methyl groups in tRNA (Gold, Hausmann, Maitra and Hurwitz, 1964; Boezi, Armstrong and De Backer, 1967). In mammalian cells too there is a variation in the degree of methylation of tRNA. Bergquist and Matthews (1962) found the highest levels of methylated nucleosides in tRNA extracted from tumours. Borek's group has been studying the methylating enzymes present in extracts of various tissues; they report elevated levels, 2 to 10 times normal, in tumour extracts (Borek, 1963; Borek and Srinivasan,

1966; Ttsutsui, Srinivasan and Borek, 1966) and suggest that hypermethylation may play some role in carcinogenesis. This could be brought about by the invasion of the cell by a foreign methylating system. Virus particles are known to enter cells and direct them to synthesise virus-specific proteins. Some of these proteins may even be detected under conditions which do not permit the synthesis of new virus particles (Habel,1965; Benjamin, 1966; Fleissner, 1970). If one of these proteins were a tRNA methylase, it could be capable of hypermethylating the tRNA of the host. The altered tRNAs could then affect the normal control of protein synthesis with the production of a tumour cell.

Unfortunately the evidence for this interesting hypothesis may have to be re-examined in the light of the results of Kaye and Leboy (1968). They observed that both the degree of methylation and the pattern of nucleosides methylated *in vitro* depended critically on the concentration of ions present in the assay system. Using optimal conditions, they could not distinguish between the methylases of normal and malignant tissue. *In vivo* studies may give a more realistic comparison of the methylation of tRNA in various cell types.

V. Control of RNA Synthesis

Messenger RNA molecules appear to be very short-lived in bacterial cells. This means that continued RNA synthesis is required for the production of protein. Surprisingly it appears that the reverse is also true. In 1952, Sands and Roberts reported the results

of growth experiments carried out with mutants of Esch. coli which require a supply of tryptophan and histidine for growth. When the two amino acids are removed from the medium, protein synthesis ceases as expected but the rate of RNA formation is also greatly reduced. Since these amino acids are not required for the production of RNA, some control mechanism must operate when protein synthesis cannot proceed due to lack of amino acids. Stent and Brenner (1961) suggested that uncharged tRNA molecules, present during amino acid starvation, might inhibit RNA polymerase. When the amino acid is returned to the medium, the tRNA molecules are all loaded and the inhibition is removed allowing RNA synthesis to continue. Kurland and Maaløe (1962) put forward a similar model to explain the effect of chloramphenicol on protein and RNA synthesis. This drug prevents the production of protein but unlike with amino acid deprivation, RNA synthesis may even be stimulated (Osawa, 1965). This can be explained by Stent and Brenner's model; the tRNA molecules are kept charged by amino acids derived from turnover of cellular proteins, the chloramphenicol preventing their reutilisation in protein synthesis. However some evidence stands against the theory that RNA synthesis is controlled directly by an inhibition of RNA polymerase by uncharged tRNA molecules. Bremer, Yegian and Konrad (1966), while confirming that tRNA molecules do inhibit the polymerase in vitro, report that the difference between tRNA and aminoacyl-tRNA is insufficient to account for the rapid shut off of

RNA synthesis observed on the removal of only one amino acid. The drug trimethoprim is believed to inhibit protein synthesis by preventing the formylation of methionyl-tRNA (Eisenstadt and Lengyel, 1966); it also prevents RNA synthesis (Shih, Eisenstadt and Lengyel, 1966). This means that although protein synthesis itself is not necessary, as shown by the chloramphenicol results, some step after the formation of aminoacyl-tRNA is required for the maintenance of RNA synthesis.

Other models for the control of RNA formation have also been suggested, for example that RNA polymerase is inhibited by the presence of free ribosomes caused by the breakdown of polysomes in the absence of an essential amino acid (Morris and De Moss, 1966). However, like the loaded tRNA theory, they do not fully explain the many features of the control of RNA synthesis in bacteria and much more information is obviously required.

Recent work with Esch. coli has shown that RNA polymerase can be separated into two parts, the core enzyme and the sigma factor (σ) (Burgess, Travers, Dunn and Bautz, 1969). The purified core enzyme is capable of transcribing DNA templates e.g. from calf thymus or T4 bacteriophage (Burgess et al, 1969; Sugiura, Okamoto and Takanami, 1970) but the products formed are very heterogeneous. With added σ factor, the core enzyme transcribes T4 DNA forming a uniform product very similar to that isolated from infected Esch. coli (Sugiura et al, 1970). The σ factor therefore seems to ensure initiation of RNA

synthesis at specific sites (Sugiura et al, 1970; Travers & Burgess, 1969). Sugiura et al (1970) also report that σ factor is unstable to storing. It is therefore possible that normal RNA synthesis may be controlled by the availability of active σ factor.

Mammalian cells in tissue culture grow in a very rapid manner, similar to bacteria, and amino acid deprivation of Hela cells causes a reduction in the rate of RNA synthesis (Maden, Vaughan, Warner & Darnell, 1969) but the exact response is different from that in bacteria. Except with methionine, which is known to be directly involved in RNA synthesis, the RNA production is only reduced and not completely shut off on amino acid starvation. In fact even in the absence of methionine, 45s and 32s RNA is still produced although 28s and 18s rRNA are not formed. (Vaughan, Soeiro, Warner & Darnell, 1967). In further contrast to the bacterial system, cycloheximide inhibits not only protein but also RNA synthesis (Willems, Penman & Penman, 1969). It appears that complete protein synthesis may be required to allow RNA production to continue in Hela cells. However, in L929 cells, RNA production is not affected by amino acid starvation unless the serum is also omitted from the growth medium (Sköld & Zetterberg, 1969). This suggests that stringent amino acid regulation of RNA synthesis does not exist in these tissue culture cells.

In the intact animal, many different cell types are controlled in a co-ordinated manner. This might be expected to make the situation even more complex. Unlike in the in vitro tissue culture

situation, many examples are known of the stimulation of RNA production. This difference is probably due to the fact that in the whole animal, the metabolism of the cell is continuously regulated while in tissue culture only control systems which relate to the life of the individual cell are maintained.

The rate of ribosome production varies greatly *in vivo*. During early embryogenesis of *Xenopus laevis*, no rRNA is synthesised but at later stages of development rapid synthesis does occur (Brown and Gurdon, 1964). Normal liver cells produce large quantities of proteins and there is a considerable turnover of ribosomes (Loeb, Howell and Tomkins, 1965). Even so this production is small compared to the RNA synthesis which occurs during regeneration after partial hepatectomy (Chaudhuri and Lieberman, 1968).

Injection of 17β - oestradiol causes growth of the uterus in immature rats. The first chemical change so far observed is an increased rate of RNA synthesis (Gorski, 1964). In a more normal situation, great variations occur in the rate of RNA production during the oestrus cycle of the hamster (Warren and Barker, 1967). A final example of the variation of RNA formation comes from PHA stimulated lymphocytes. Non-dividing lymphocytes produce very little RNA. After stimulation with PHA, the first observable change is once again an increased rate of RNA synthesis. In this case the new RNA has been analysed by ultracentrifugation and it is found that 4s RNA appears first followed by rRNA (Rubin and Cooper, 1965). Obviously a complex

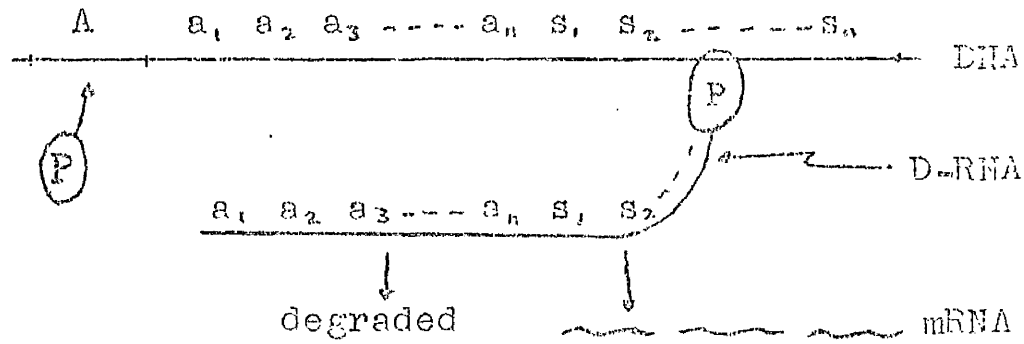
system of control involving many factors functions in eukaryotic cells *in vivo*.

The existence of RNA precursors is very interesting and particularly well documented for eukaryotic cells. In these the modification of a precursor molecule appears to be involved in the synthesis of all cytoplasmic RNA molecules, with the possible exception of 5s RNA formation for which a model has not yet been determined. It has been suggested that even mRNA is produced via much longer precursor molecules as mentioned in the section on high molecular weight nuclear RNA. DNA-RNA hybridisation studies indicate that much of this nuclear RNA consists of non-messenger sequences (Georgiev, 1969; Soeiro and Darnell, 1970). Georgiev (1969) suggests that these extra sequences could be involved in controlling gene transcription. Using a Jacob and Monod type model, he suggests that a molecule of RNA polymerase attaches to the DNA at a special acceptor region of the gene. It travels along the DNA synthesising RNA at first from further acceptor genes then from structural or functional genes. These structural genes contain the information for the synthesis of proteins i.e. when transcribed they produce mRNA. Transcription of these genes depends on the transcription of the acceptor genes as they lie between the functional genes and the polymerase acceptor site. It is proposed that the acceptor genes are capable of combining with regulatory molecules thus preventing the passage of the RNA polymerase and controlling the transcription of the

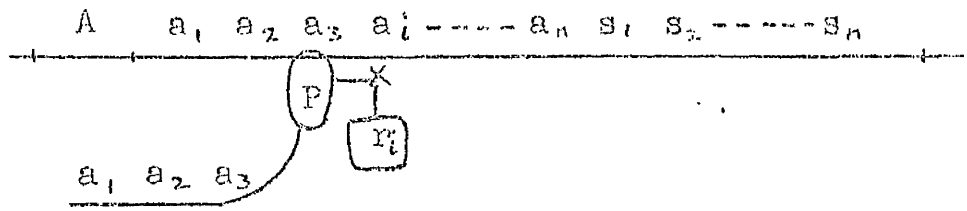
Fig. I.5.

Control of RNA synthesis.

a.) normal synthesis.



b.) inhibition.



A - RNA polymerase attachment site.

$a_1 - a_n$ - acceptor genes capable of binding with regulatory molecules.

$s_1 - s_n$ - structural genes give rise to mRNA for protein synthesis.

(P) - RNA polymerase.

(r_i) - regulator molecule which binds specifically to acceptor gene a_i and prevents transcription of DNA

D-RNA - high molecular weight DNA-like RNA.

structural genes. (See Fig I.5). Since the rapidly-labelled nuclear RNA is of such high molecular weight, many acceptor genes could be placed between the polymerase attachment site and the structural genes. This would allow for a complex control system presumably required by the highly ordered eukaryotic cells. Britten and Davidson (1969) gave a more detailed description of an essentially identical model for gene regulation in the higher cells.

Since the 45s ribosomal RNA precursor contains a large proportion of non-ribosomal sequences a similar mechanism may control the synthesis of rRNA. Although there also appears to be a precursor for tRNA in higher cells, insufficient is known about it to suggest how its synthesis may be controlled.

VI. Present Work

From the foregoing discussions it can be seen that many functions have been suggested for the small tRNA molecules. Some of these suggestions are backed by considerable evidence while others still have to be confirmed. In spite of this, compared to ribosomal RNA, very little is known of the synthesis of tRNA let alone how it may be controlled or exert an influence on the synthesis of other molecules. It was therefore decided to further characterise the tRNA precursor molecule and if possible to examine the steps involved in its conversion to mature tRNA. The system chosen for this study consisted of a single cell line grown in tissue culture. With this system it was hoped to rule out difficulties due to different cell types responding to the treatments in different ways. The external environment could

easily be altered and drugs such as actinomycin D could be added in a defined and reproducible manner.

This tissue culture system appeared to have certain advantages over work with whole animals when an examination of Borek's theory of carcinogenesis was attempted. Due to the invasive properties of malignant cells, they may travel through the blood stream to form tumours some distance from their site of origin (Willis, 1952; Engell, 1955). Therefore the comparison of the properties of tumour tissue with the surrounding normal organ may not give a true picture. Malignant tissue culture cells may be derived from a normal cell line by virus transformation. Normally when a virus particle enters a cell many progeny virus are produced but another response to infection by certain viruses has been observed; this is transformation. The virus is absorbed into the cell but without the appearance of new virus particles; instead the growth characteristics of the cell are altered and, unlike the parent cell line, the transformed cells will cause tumours on injection into animals. Thus using this tissue culture system definite control cells may be used as the origin of the cells is known.

M A T E R I A L S

A N D

M E T H O D S .

MATERIALS

A. Biological

The BHK21/C13, SR8/V1, SR8/V5, SR8/R1, and B5 cell lines were a gift from Dr. I. A. Macpherson, Imperial Cancer Research Fund, Lincoln's Inn Field, London. The PyY cells were ~~gifted~~ given by Dr. J. D. Pitts, Biochemistry Department, Glasgow University. Eagle's medium (Glasgow Modification) and filtered calf serum were purchased from the Institute of Virology, Glasgow University. Brain heart infusion broth and Sabouraud medium were obtained from Oxoid Ltd., London. Difco Laboratories, Detroit, Michigan supplied trypsin and the Agar and PPLO broth required for the PPLO agar plates. Penicillin/streptomycin was purchased from Flow Laboratories Inc., Irvine.

Composition of Media

Eagle's medium (Glasgow Modification):- This was prepared by the method of Busby, House and MacDonald (1964). It contained the antibiotics streptomycin (100µg/ml), penicillin (100 units/ml) and the antimycotic agent n-butyl, p-hydroxy benzoate. Buffered Salt Solution (BSS):- This consisted of 0.116 M-NaCl, 5.4 mM-KCl, 1 mM-$MgSO_4$, 1 mM-NaH_2PO_4, 1.8 mM-$CaCl_2$ and 0.002% (w/v) phenol red and was adjusted to pH 7.0 with 8.4% (w/v) $NaHCO_3$. Versene :- 0.6 mM-EDTA in phosphate buffered saline (PBS) to which 0.002% (w/v) had been added. PBS consisted of 0.17 M-NaCl, 3.4 mM-KCl, 10 mM-Na_2HPO_4 and 2 mM-KH_2PO_4 at pH 7.4. Trypsin/Citrate :- Trypsin was made up as a 0.25% (w/v) solution in citrate buffer which was constituted as follows :-

10.5mM-NaCl, 1mM-sodium citrate and 0.002% (W/v) phenol red. The pH was adjusted to 7.8 using NaOH.

Trypsin/versene:- 1 part trypsin/citrate to 4 parts versene.

B. Chemical

Liquid scintillation materials:-

Hyamine hydroxide, 1M in methanol, Naphthalene and Dioxan were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh. Koch-Light Laboratories Ltd. supplied 2,5 diphenyl oxazole. Cellulose acetate filters, 0.45 μpore size (HAWP 02500) were obtained from Millipore (U.K.) Ltd., Wembley, Middlesex.

Radiochemicals:-

Guanosine -8-T (4.9c/mm); adenosine -T(G) (11.2c/mm); uridine-T(G) (4.25c/mm); cytidine -5-T (26.8c/mm) and L-methionine (methyl -C14) (56.8 mc/mm) were purchased from The Radiochemical Centre, Amersham, England.

Materials for Chromatography:-

Whatman No.1 and 3MM paper and also DE 81 (DEAE-paper) were obtained from H. Reeve Angel & Co. Ltd., London. Sephadex G-100 was supplied by Pharmacia (G.B.) Ltd., London. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex and kieselguhr (Hyflo Super Cel) from Koch-Light Laboratories Ltd.

Materials for Autoradiography:-

Industrex X-ray film type D was purchased from Kodak Ltd.,London. Gevaert G5c developer was obtained from Agfa-Gevaert Ltd.,Brentford,

Middlesex and Amfix from May and Baker Ltd., Cumbernauld, Dumbartonshire.

Miscellaneous:-

Bentonite powder and sodium dodecyl sulphate were obtained from British Drug Houses Ltd., Poole, Dorset. Actinomycin D was purchased from Merck, Sharpe and Dohme Inc., Rahway, New Jersey. Toyocamycin was a gift from Dr. G. Acs, Institute for Muscle Disease, New York. Beckman Spinco, Palo Alto, California supplied cellulose nitrate tubes ($\frac{1}{2}$in x 2in). Snake venom phosphodiesterase was purchased from Worthington Biochemical Corporation, New Jersey. Esch. coli (strain B) soluble RNA was obtained from Calbiochem Ltd. while Sigma Chemical Company supplied the 5' monophosphates of the 4 major nucleosides and also s-adenosylmethionine.

METHODS

A. Biological

1. Growth and Passage of Cells

BHK21/C13 cells and several cell lines derived from them by viral transformation (see following section), were maintained as follows:-

The cells were grown as monolayers in 80 ounce Winchester bottles which rotated about their long axes in a 37°C warm room. (House and Wildy, 1965). The medium used was Eagle's (Glasgow modification) (Busby, House and MacDonald, 1964), to which 10% (v/v) filtered calf serum had been added (referred to as EC10). This medium contains 100 units/ml penicillin and 100 μg/ml streptomycin. 180ml of medium were used per Winchester, the buffering capacity being maintained by replacing the air with an atmosphere of 5% (v/v) CO_2 in air. For serial passage, the cells were removed from the glass with trypsin-citrate solution (described in Materials section). For experimental work, 2 x 10^7 cells/bottle were grown for two days before the addition of radioactive tracers. At this time the cells appeared to be growing logarithmically (see Fig II.1, curve a), and almost covered the glass surface with a uniform monolayer of cells.

Although the medium contained antibiotics, cell lines routinely grown were tested for possible contamination with microorganisms. Blood agar plates and brain heart infusion medium were used to test for bacterial infection. To test for fungi, sabouraud medium was used. Contamination with pleuropneumonia-like organisms (PPLO) was monitored for using PPLO agar plates and incubating for 14 days at

Fig. II.1

Growth of C13 cells under various conditions.

Several cultures of C13 cells were set up at 1.5×10^6 cells/4 ounce bottle in 20ml Eagle's medium containing 10%(^{v}/v) calf serum (EC_{10}). At the times indicated, the cells were removed from 2 bottles using trypsin/versene and counted using a Coulter counter. The average values were plotted against the time of incubation at 37°C. At 49h after the cultures were set up, the remaining bottles were divided into 3 groups and treated as follows:-

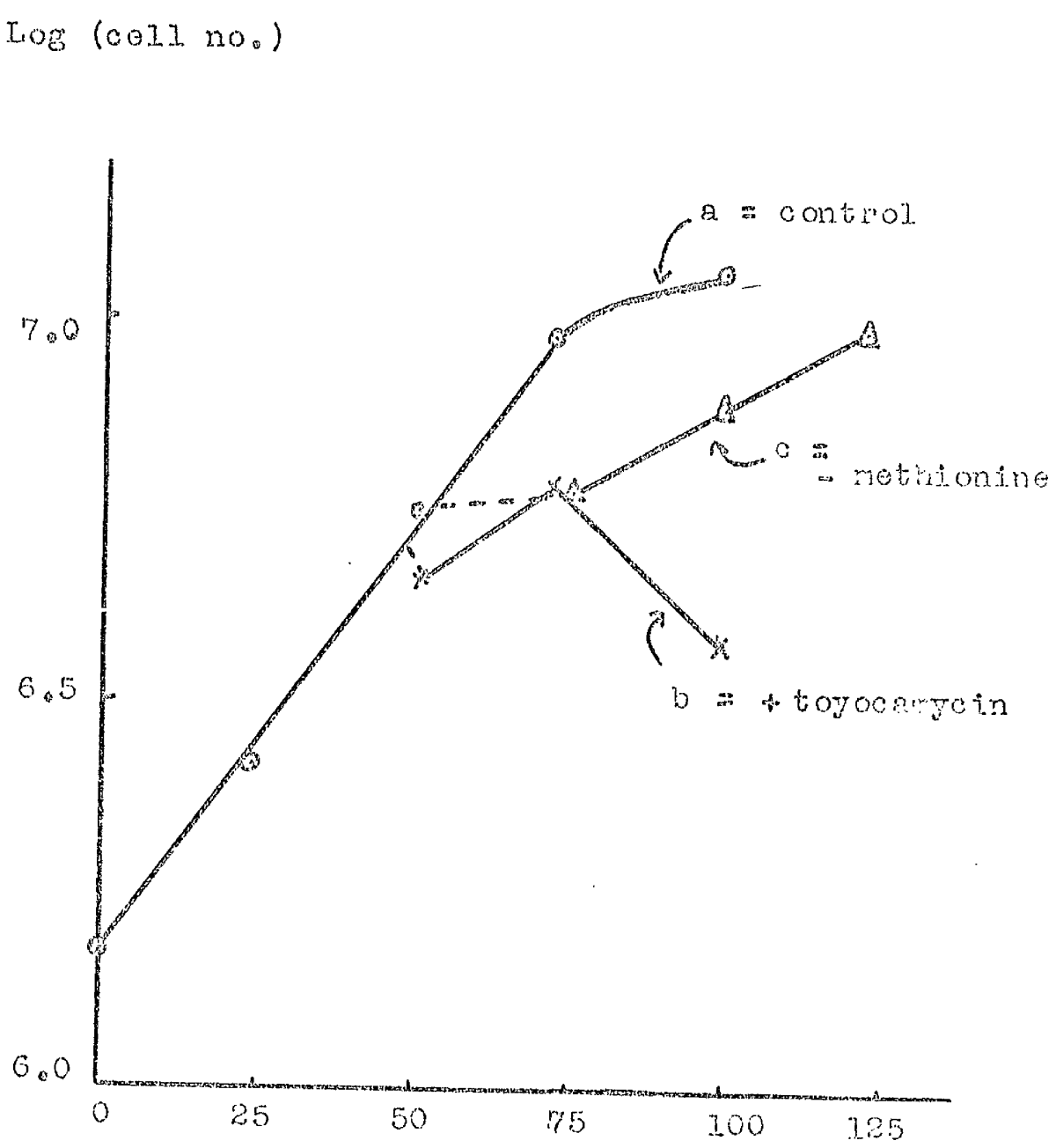
a) The medium was removed and replaced with 10ml of EC_{10} o——o

b) The medium was replaced with 10ml of Eagle's medium, plus 2.5%(^{v}/v) calf serum, to which toyocamycin had been added to a concentration of 1µg/ml. After 2.5h incubation, this medium was removed and the cell sheet washed with 10ml BSS. 10ml of EC_{10} was then added and the incubation continued x——x

c) After removal of the medium, the cell sheet was washed with 10ml of Eagle's medium lacking L-methionine, then 10ml of this deficient medium supplemented with 2.5%(^{v}/v) calf serum was added. The incubation was continued for 26h and then this medium was replaced by 10ml of EC_{10} Δ——Δ

The continuous line indicates growth in EC_{10} while the broken line shows the length of incubation in methionineless medium or with toyocamycin.

FIGURE II. 1.


Log (cell no.)
a = control
c = + methionine
b = + toyocaryein
7.0
6.5
6.0
0
25
50
75
100
125
Time (h) after sub-culturing.

37°C in an atmosphere of nitrogen and also by staining thin cell sheets with orcein stain and examining microscopically for colonies of the organisms - the method of Fogh and Fogh (1964).

All glassware used for tissue culture work was soaked in chloros, washed, rinsed in distilled water and sterilised by autoclaving.

2. Cell Lines Used

The cell line mainly used was BHK21/C13 (referred to as C13). This line was derived from baby hamster kidney cells by Macpherson and Stoker (1962). Colonies of these cells show a well-defined parallel orientation (Fig II.2).

When C13 cells are infected with polyoma or Rous sarcoma virus, some cells are transformed and grow in a disarrayed manner, quite unlike the parent cells (Fig II.2). A small proportion of the cells transformed with Rous sarcoma virus spontaneously return to the ordered growth pattern of the C13 cells. These cells are termed revertants.

When samples of each cell line are injected into hamsters, the transformed cells show a high degree of tumour production, while the original C13 cells and the revertants do not. This is shown in Table II.3.

Transformation of C13 cells with BRYAN strain of Rous sarcoma virus gave rise to the B5 cell line. SR8/V1 and SR8/V5 cell lines were derived from C13 by transformation with the SCHMIDT-RUPPIN strain

FIG II.2

NORMAL HAMSTER FIBROBLASTS

CLONE 7

TRANSFORMED HAMSTER FIBROBLASTS

CLONE 7T

TABLE II.3

Cell Line	Proportion of Animals Developing Tumours		
	Dose 10^6 cells	10^4 cells	10^2 cells
C13	3/16	0/8	-
Polyoma Transformed	5/6	8/8	7/8
Rous sarcoma Transformed	8/8	7/8	5/8
Revertant	2/8	0/8	0/8

Values taken from Macpherson I.A.
(1965) Science 148, 1731

of the same virus. SR8/R1 was a revertant cell line derived from SR8/V5 cells. All these cell lines were a gift from Dr I. Macpherson. PyY cells arose by transformation of C13 cells with polyoma virus and were gifted by Dr J.D.Pitts.

All the cell lines mentioned above were cloned shortly after their isolation and therefore should contain only one type of cell.

3. Preparation of Primary Cultures

Primary cultures of hamster embryo cells were prepared as follows:-

A pregnant hamster (nearly full term) was killed by ether anaesthetisation and the uterus removed aseptically. The embryos were then removed under sterile conditions and washed with a buffered salt solution (BSS) containing 100 units /ml penicillin and 100µg/ml streptomycin. They were then minced with a sterile razor and washed three times with BSS plus penicillin and streptomycin to remove red blood cells. The tissue pieces were washed once with trypsin-citrate to remove trypsin inhibitors and then incubated for 30 min at 37°C with fresh trypsin-citrate. To neutralise the trypsin and prevent lysis of the cells, an equal volume of calf serum was added and mixed with a wide-bore 10ml pipette. Separated cells were removed from undigested tissue debris by filtering through sterile muslin. The cell suspension was centrifuged at 800xg for 10 min and the

cell pellet resuspended in ECl0. This cell suspension was counted using a Coulter counter and diluted in ECl0 to a concentration of $5x10^6$ cells/ml. 180 ml of this suspension was dispensed into 80 ounce Winchester bottles and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air, for 2 days.

4. Drug Treatments

a) Actinomycin D

Actinomycin D was dissolved in distilled water to give a concentration of 5µg/ml. This stock solution was stored in the dark at -20°C until required and then added to the growth medium to give the desired concentration.

To inhibit ribosomal RNA synthesis only, actinomycin D was added to a final concentration of 0.04µg/ml and the cells incubated at 37°C for 10 min before the addition of labelled RNA precursors. This treatment has been reported to inhibit nucleolar RNA synthesis without affecting the production of other types of RNA (Perry, 1962).

When the complete inhibition of all RNA synthesis was desired, a much higher concentration of actinomycin D was required. The actinomycin D powder was dissolved in a small amount of medium and added to the growth medium to give a final concentration of 5µg/ml. This concentration is reported to inhibit all RNA synthesis within a few minutes of its addition(Scherrer, Latham and Darnell,1963).

b) Toyocamycin

Toyocamycin, (4-amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo (2,3-d)-pyrimidine), is an analogue of adenosine (see Fig II.4) and has been reported to inhibit ribosomal RNA synthesis by preventing the breakdown of the 45s precursor RNA (Tavitian, Uretsky and Acs 1968).

A stock solution of Toyocamycin, 40 μg/ml, was made up in Eagle's medium and stored at 4°C. The stock solution was added to the growth medium to give the required concentration. For maximum effect, the cells were incubated with toyocamycin for 30min at 37°C before the addition of radioactive RNA precursors.

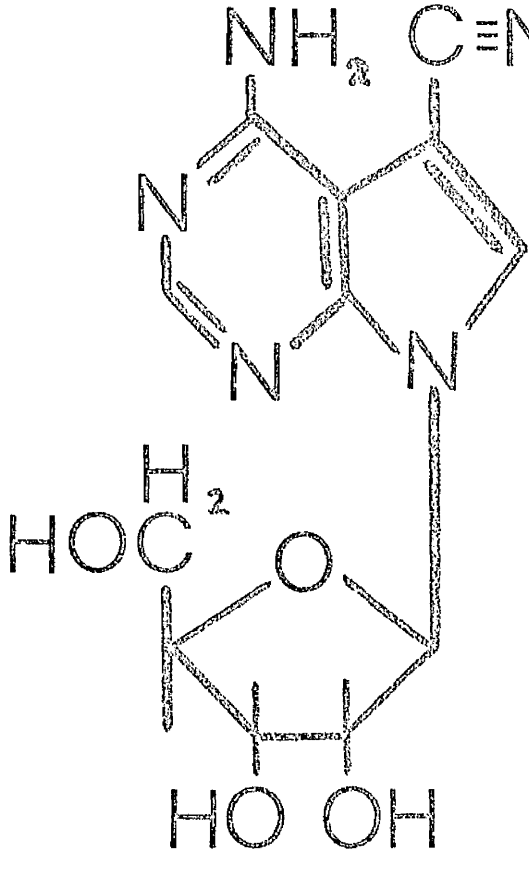
On microscopic examination of the cell sheets just prior to RNA extraction, the cells appeared perfectly normal. However, as seen in Fig II.1 (curve b), although the cells begin to divide on removal of the drug, after 24h the number of viable cells falls off sharply. It therefore appears that toyocamycin, at this concentration (1 μg/ml), causes irreversible damage to the cell.

Indeed Heine (1969) has reported electron microscopic studies of the effect of toyocamycin on nuclear structure. At low concentrations, up to 1 μg/ml, considerable disorganisation of the nucleolus may occur but with no apparent effect on the rest of the nucleus. However the author does not report whether or not these changes are reversed by removal of the drug.

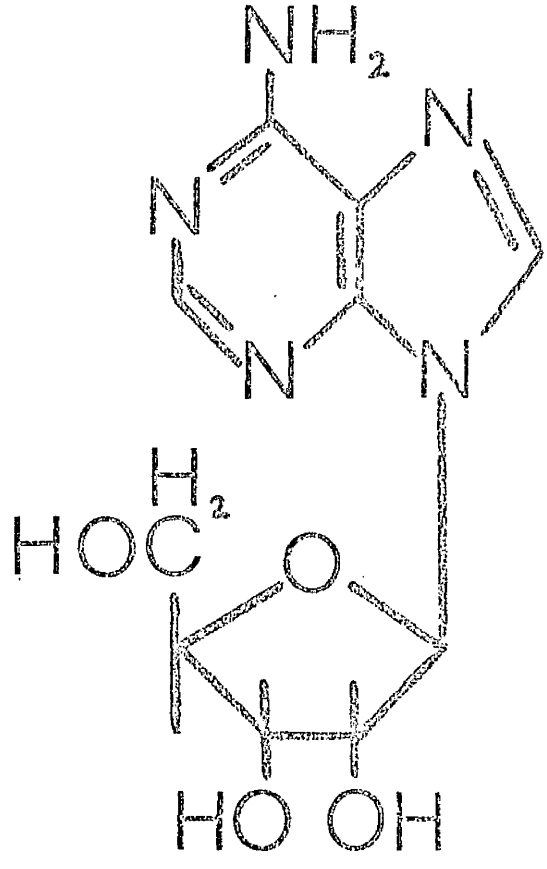
5. Methionine starvation

Cultures of C13 cells were depleted of the essential amino acid L-methionine by growing them for 24h in 100 ml of Eagle's medium minus

FIGURE II.4.



toyocamycin



adenosine

L-methionine but supplemented with 2.5% ($^{v}/v$) calf serum. Although methionine is essential in the medium for growth of mammalian cells, this treatment does not completely deplete the intracellular pool. This is due to the breakdown of proteins both intracellular (Eagle, Piez, Fleischman and Oyama, 1959) and from the serum contained in the medium (Piez, Oyama, Levintow and Eagle, 1960). The cells do not divide during this treatment although they appear quite normal when examined microscopically. When the deficient medium is replaced with complete Eagle's medium plus 10% ($^{v}/v$) calf serum, the cells start to divide again though a little slower than the untreated control cells (see Fig II.1, Curve c). This suggests that most of the cells are still viable after 24h methionine starvation.

6. Resting Cells

C13 cells were set to resting using the technique described by Fried and Pitts (1968). A confluent Winchester bottle of cells was washed once with 20 ml of versene and then treated with 25 ml of trypsin/versene at room temperature. The cells were shaken off the glass and gently dispersed into single cells. The cell suspension was immediately transferred into 80 ml of ice-cold Eagle's medium and centrifuged at 500×g for 10 min. The cell pellet was resuspended in Eagle's medium containing 0.5% ($^{v}/v$) calf serum and seeded into a Winchester at 1.5×10^{8} cells per bottle. The culture was gassed with 5% ($^{v}/v$) CO_2 in air and incubated at 37°C for 5 days.

B. Biochemical

7. Preparation of Bentonite

Bentonite was purified according to the method of Fraenkel-Conrat,

Singer and Tsugita (1961). Bentonite powder (BDH) was suspended in 20 volumes distilled water and centrifuged at 1,200 x g for 15 min. The precipitate was discarded and the supernatant fluid centrifuged at 9,000 x g for 20 min, The pellet was suspended in 0.1M-EDTA, pH 7.0, and allowed to stand at 20°C for 48h. The suspension was again centrifuged differentially and the 9,000 x g sediment suspended in 0.01M - ammonium acetate, pH 6.0. After centrifuging again at 9,000 x g the bentonite was suspended in the ammonium acetate buffer at a concentration of 1-1.5% (w/v) and sterilised by autoclaving at 15lb/in^2 for 20 min. The purified bentonite was then stored at 4°C.

8. Preparation of RNA

After growth of the cells and incubation with the appropriate radioactive precursors, the cell monolayer was washed three times with 50 ml portions of ice-cold non-radioactive Eagle's medium. RNA was then extracted by one of the following methods.

a) Cold Phenol Technique

This was essentially the method used by Burdon, Martin & Lal (1967). These authors showed that the species of RNA isolated by this method appear to be identical to those present in isolated cytoplasm. All solutions used were ice-cold and unless otherwise stated, all operations were carried out at 0-4°C. Glassware was thoroughly heated in a bunsen flame before use. This treatment was necessary to destroy any ribonuclease present on the glass as this enzyme is known to be very stable to moderate heating (Anfinsen and White, 1961).

The cells were removed from the glass by shaking vigorously with 5 ml of 0.05M - ammonium acetate, pH 5.1, made 0.25% (w/v) with respect

to bentonite, and an equal volume of 80% ($^{w}/v$) phenol, equilibrated with the same buffer. The Winchester bottle was washed with a further 10 ml portion of the ammonium acetate - bentonite - phenol mixture. The two portions were combined and shaken for 5 min at room temperature using a mechanical shaker. The resulting emulsion was separated by centrifugation at 10,000 x g. The aqueous phase was removed, re-extracted once with phenol and additional bentonite, and three times with ether to remove any traces of phenol. The RNA was precipitated with two volumes absolute ethanol and collected, after at least 2 h at -20°C, by centrifugation at 10,000 x g for 10 min. The RNA samples were dissolved in 0.05M - ammonium acetate, pH 5.1, and stored at -20°C until analysed.

8.b.) Hot Phenol Technique

Total cell RNA can be prepared by this technique (Scherrer and Darnell, 1962). The treatment with sodium dodecyl sulphate and heating to 60°C releases the nuclear RNA (Warner et al, 1966).

The cells were removed from the glass with trypsin/versene (see Materials section). The cell suspension was cooled in ice and centrifuged at 800 x g and the pellet was suspended in 6 ml 0.05 M - ammonium acetate, pH 5.1, containing 0.25% ($^{w}/v$) bentonite. Sodium dodecyl sulphate was added to 0.5% ($^{w}/v$) and mixed. An equal volume of 80% ($^{w}/v$) phenol equilibrated in acetate buffer, pH 5.1, was added and thoroughly mixed at room temperature. The mixture was then held at 60°C with constant shaking for 3 min. After mixing thoroughly for a further minute, the emulsion was cooled in ice and separated by centrifugation at 10,000 x g. The aqueous layer was re-extracted once at room temperature

with 5 ml 80% ($^{W}/v$) phenol in ammonium acetate buffer and 1 ml 1% ($^{W}/v$) bentonite. Traces of phenol were removed from the separated aqueous layer by extracting 3 times with ether. The RNA was precipitated with 2 volumes of absolute ethanol and collected by centrifugation at 10,000 x g for 10 min, after standing for at least 2 h at -20°C. The RNA was dissolved in 0.05 M - ammonium acetate, pH 5.1, and stored at -20°C. Unless otherwise stated, all operations were carried out at 0 - 4°C.

9. RNA Fractionation

a.) Gel Filtration

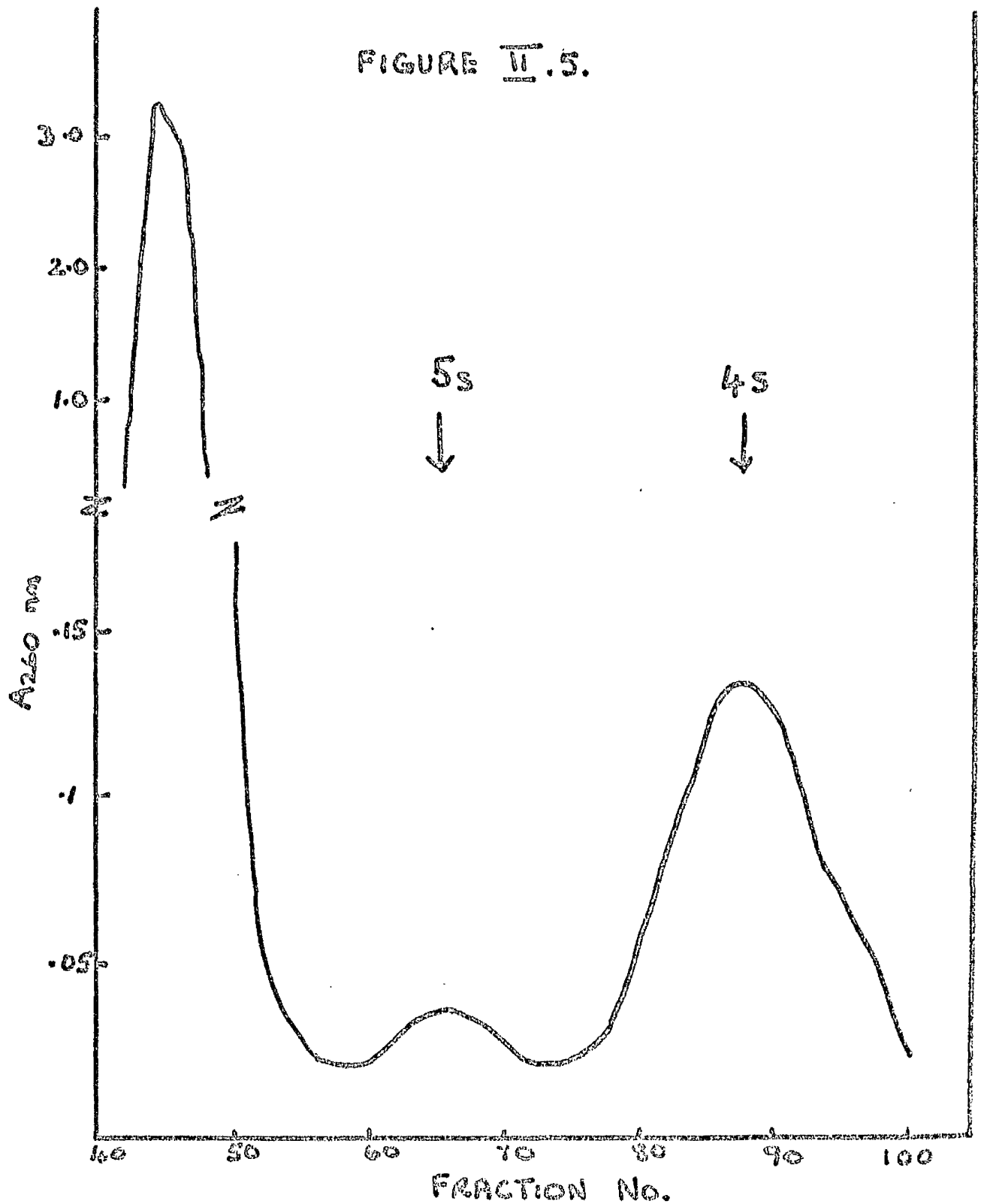
This method was used first by Virmaux, Mandel and Urban (1964). Gel filtration was carried out with Sephadex G-100 equilibrated in 0.05 M - ammonium acetate, pH 5.1. Columns 90 cm long and 2.5 cm in diameter were used. 1 - 1.5 mg of RNA dissolved in 1 or 2 ml of 0.05 M - ammonium acetate, pH 5.1, were applied to the column and eluted with the same acetate buffer. All operations were carried out in 4°C cold room. 3 ml fractions were collected in tubes containing 1 drop of 1% ($^{W}/v$) sodium dodecyl sulphate to prevent degradation of the RNA by ribonuclease (Burdon et al, 1967). The samples were examined for extinction at 260 nm and assayed for radioactivity (see Methods section 16). The separation obtained is shown in Fig II.5. High molecular weight RNA's e.g. 28s and 18s are excluded from the gel and appear as one large peak at about fraction 45. 5s RNA and tRNA are retarded by the gel and elute about fractions 65 and 85 respectively.

Fig. II.5

Fractionation of RNA by gel filtration.

RNA was applied to a column of Sephadex G-100 and eluted with 0.05M - ammonium acetate, pH5.1. 3ml fractions were collected and the u.v. absorbing material measured.

FIGURE II.5.



9.b.) Chromatography on columns of methylated albumin-coated kieselguhr(MAK)

i.) Preparation of methylated albumin.

Methylated albumin was prepared by the method of Sueoka and Cheng (1962). 5 g Bovine albumin powder (fraction V from bovine plasma - Armour Pharmaceuticals) was dissolved in 500 ml methanol and acidified with 4.2 ml 12 M-HCl. The mixture was allowed to stand in the dark for 3 days during which time the albumin reprecipitated. The methylated albumin was collected by centrifugation, washed in methanol, dried in ether and ground to a powder which was stored at -20°C.

ii.) Preparation of MAK stock for the middle layer of the column.

8 g of kieselguhr were boiled in 40 ml of 0.1 M - sodium chloride in 0.05 M - sodium phosphate, pH 6.8, to remove air. A 1% (w/v) solution of methylated albumin was made up in distilled water. 2 ml of this solution was added dropwise, with constant stirring, to the cooled, boiled kieselguhr. 30 ml of 0.1 M - sodium chloride in 0.05 M - sodium phosphate buffer, pH 6.8, were then stirred into the suspension to ensure complete precipitation of the methylated albumin. This suspension of MAK was washed by pouring it into a glass column 3 cm in diameter and eluting with 100 ml of 0.4 M - NaCl in phosphate buffer, pH 6.8, under a pressure of 3 lb/in^2. The washed MAK was suspended in 50 ml 0.4 M - NaCl in 0.05 M - sodium phosphate, pH 6.8, and stored at 0°C for several weeks.

iii.) Preparation of a 3 layer column.

The column was prepared by the method of Mandell and Hershey (1960) as follows:-

1). 4 g of kieselguhr were boiled in 20 ml 0.1 M - NaCl in 0.05 M - sodium

phosphate, pH 6.8.

2). 3 g kieselguhr were boiled in 20 ml 0.4 M - NaCl in phosphate buffer, pH 6.8.

3). 0.5 g kieselguhr was boiled in 5 ml 0.4 M - NaCl in phosphate buffer.

After the suspensions had cooled, 1 ml of a 1% ($^{w}/_{v}$) solution of methylated albumin in water was added dropwise to 1). 15 ml of 0.1 M - NaCl solution were then added and stirred in. The suspension of MAK was gently poured on top of a layer of acid washed sand in a 3 cm Quickfit column. The layer of sand prevented penetration of the kieselguhr into the glass sinter. The MAK was packed under atmospheric pressure and washed with 0.1 M - NaCl in sodium phosphate buffer, pH 6.8. 5 ml of MAK stock suspension were mixed with 2) and this was packed on top of layer 1). The column was completed by the addition of 0.5 g kieselguhr - layer 3), and washed with about 80 ml of 0.2 M - NaCl in 0.05 M - sodium phosphate, pH 6.8 (applied under a pressure of 3 lb/in^2).

iv.) Application of the sample.

The RNA was applied to the column in dilute solution (about 20μg/ml) in SSC (0.15 M - NaCl, 0.015 M - sodium citrate). All unattached u.v. absorbing material was washed off the column with 0.2 M - NaCl in sodium phosphate buffer, pH 6.8. RNA was then eluted with a non-linear gradient of NaCl in 0.05 M - sodium phosphate, pH 6.8. The gradient was formed by mixing 200 ml 0.9 M - NaCl from a reservoir with 200 ml 0.2 M - NaCl in a mixing vessel.

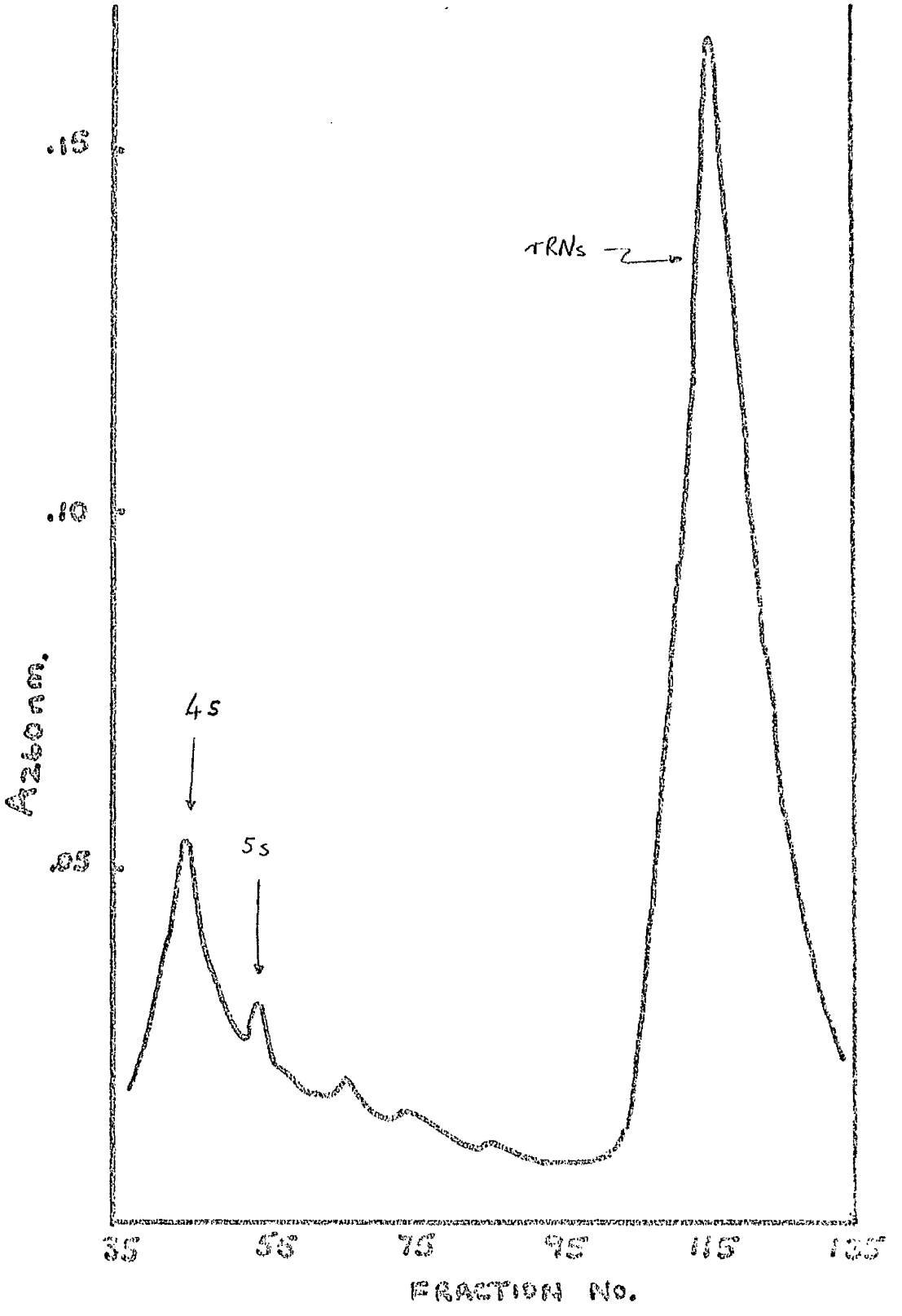
2 ml fractions were collected and analysed for extinction at 260 nm and for radioactivity (Methods, section 16). The separation obtained is

Fig. II.6

Fractionation of RNA by chromatography on MAK.

The sample of RNA was applied to the MAK column in SSC (0.15M-NaCl, 0.015M-sodium citrate) at a concentration of 20μg/ml. Unattached u.v. absorbing material was washed off with 0.2M-NaCl in 0.05M-sodium phosphate, pH6.8. The RNA was eluted from the column with a non linear gradient of sodium chloride in 0.05M-sodium phosphate, pH6.8. The gradient was formed by mixing 200ml 0.9M-NaCl from a reservoir with 200ml 0.2M-NaCl in a mixing vessel. The gradient was applied under pressure (3lb/in^2) and 2ml fractions were collected.

FIGURE II. b.



shown in Fig II.6.

tRNA is eluted at low salt concentration followed immediately by 5s RNA. 28s and 18s RNA are eluted together at a much higher salt concentration.

As not all the RNA was eluted with the NaCl gradient used, it was thought desirable to prepare a fresh MAK column for each RNA sample to be analysed. This also solved the problem of "channelling" caused by bubbles which appeared in the MAK if the column were stored for more than 1 or 2 days.

9.c.) Zonal Ultracentrifugation

This method, first reported by Britten and Roberts (1960), is useful for studying high molecular weight RNA e.g. 28s and 18s, but does not separate the small RNA species which appear as a single peak near the top of the gradient (see Fig II.7).

Solutions of 5% ($^{w}/_{v}$) sucrose were made up in 0.05 M - ammonium acetate, pH 5.1, containing 0.01% ($^{w}/_{v}$) sodium dodecyl sulphate. These solutions were then sterilised by autoclaving at 15 lb/in^2 for 15 min as this treatment appears to destroy any ribonuclease present in the solutions (Burdon 1967b). 4.6 ml 5% - 20% sucrose gradients were prepared in $\frac{1}{2}$" x 2" cellulose nitrate tubes and 0.3 ml of 0.05 M - ammonium acetate, pH 5.1, containing 0.5 - 1 mg RNA, was carefully layered on top of the gradient.

After centrifugation at 40,000 rev/min in the SW50 rotor of the Spinco Model L Ultracentrifuge for 3.25 h, the tube was punctured with a hypodermic needle and two drop fractions were collected into 2 ml portions

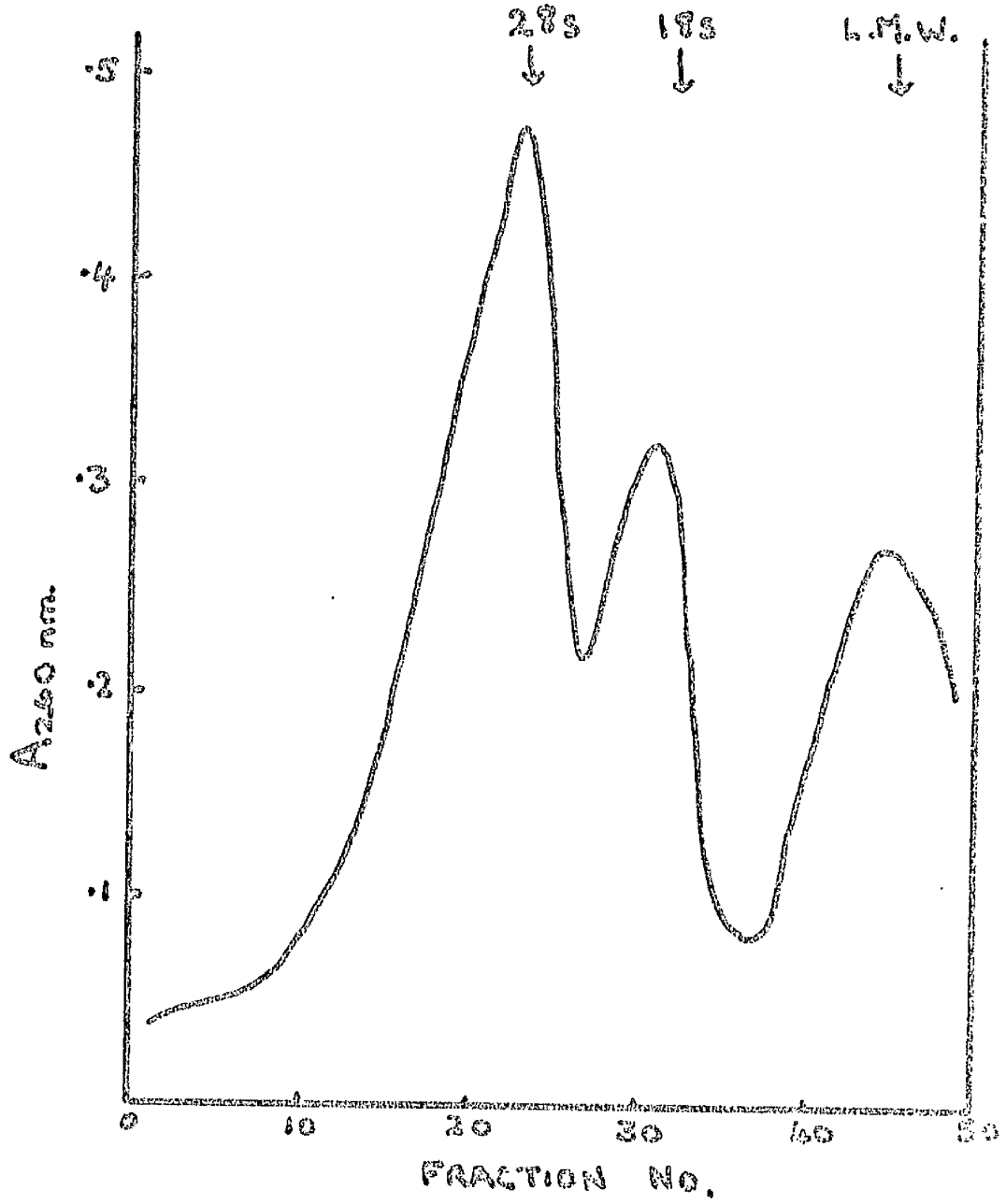
Fig. II.7

Analysis of RNA by zonal ultracentrifugation.

The RNA sample was applied to the top of a 5-20%(^{w}/v) sucrose gradient and centrifuged for 3.25h at 40,000 rev/min in the SW50 rotor of the Spinco Model L Ultracentrifuge. 2 drop fractions were collected from the bottom of the tube using a hypodermic needle.

Low molecular weight material (LMW) contains 28*s*A, 5*s*, 4*s* RNA and small oligonucleotides.

FIGURE II.7.



of 0.05 M - ammonium acetate, pH 5.1, containing 0.01% ($^{w}/_{v}$) sodium dodecyl sulphate. The samples were then analysed for extinction at 260 nm and acid - insoluble radioactivity (Methods section 16).

10. Partial Purification of pre-tRNA.

Cultures of C13 cells were set up in 10 - 20 Winchester bottles and grown for 2 days to obtain confluent monolayers. The medium was removed and the cell sheet washed once with 20 ml versene. The cells were then shaken off the glass with 15 ml of a solution of trypsin in versene. The action of trypsin was inhibited by the addition of 0.5 vol. calf serum to prevent lysis of the cells. The cells were then collected by centrifugation at 800 x g for 10 min and suspended in complete growth medium at a concentration of 5×10^{7} cells/ml. All the above operations were carried out using aseptic technique.

The cell suspension was placed in a sterile, stoppered bottle and shaken in a 37°C water bath for 30 min. The appropriate radioactive RNA precursor was added and the incubation continued for 10 min. At the end of the incubation, the cell suspension was rapidly cooled by the addition of 5 volumes of iced medium and the cells were spun out at 800 x g for 10 min. The RNA was then extracted from the cell pellet using the cold phenol technique (Methods section 8.a).

The RNA obtained was fractionated on Sephadex G-100 (Section 9.a). The fractions were measured for extinction at 260 nm; a small portion of each was removed and assayed for radioactivity. The fractions between 5s and 4s which contained the high specific activity pre-tRNA were then pooled (Fig II.8). This RNA along with non-radioactive cytoplasmic RNA

Fig. II.8.

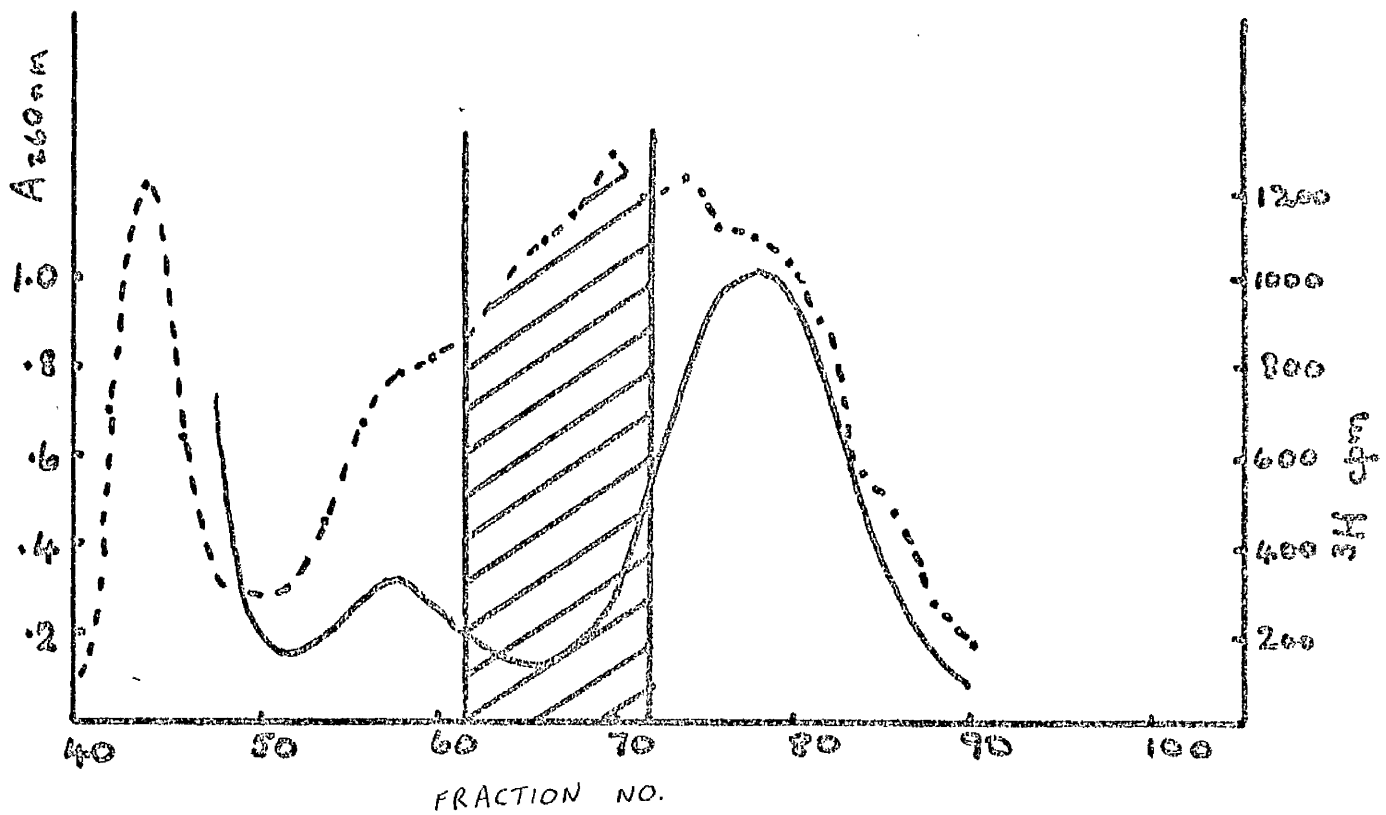
The partial purification of pre-tRNA:

The radioactive RNA, from C13 cells which had been incubated for 10min with radioactive nucleosides, was eluted from Sephadex G-100. 3ml fractions were collected and the extinction at 260nm was measured. A sample was removed from each fraction and assayed for total radioactivity. The fractions indicated by the shaded area were pooled, precipitated with non-radioactive cytoplasmic RNA and used when partially purified pre-tRNA was required

Extinction at 260nm (———)

Radioactivity (—o—o—)

FIGURE II. 8.


A260nm
1.0
.8
.6
.4
.2
40
50
60
70
80
90
100
FRACTION NO.
1200
1000
800
600
400
200
mμ

was precipitated by the addition of 0.1 volumes of 1 M - NaCl and 2 volumes absolute alcohol. The RNA was collected by centrifugation at 10,000 x g for 10 min. It was dissolved in 0.05 M - ammonium acetate, pH 5.1, and stored at -20°C.

11. Preparation of Crude Cell Extract.

The cells from 6 confluent Winchester bottles of C13 were removed as described in the preparation of pre-tRNA (section 10). Instead of being suspended in growth medium, however, the cells were washed once with Eagle's medium minus calf serum and packed at 800 x g for 10 min.

The packed cells were suspended in 5 volumes of distilled water and disrupted with 10 strokes of a Potter homogeniser. The suspension was then made 0.02 M with respect of tris/HCl buffer, pH 7.8 and dialysed against 800 ml of the same buffer for 3 h at 4°C. After dialysis, the cell homogenate was centrifuged at 800 x g for 10 min. This removed large particulate matter and produced a homogeneous suspension which was tested directly for its action on pre-tRNA. Estimation of the protein in the extract was carried out using the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin (fraction V, Armour Pharmaceuticals) as standard.

b.) Assay for conversion of pre-tRNA to 4s material

The activity of the cell extract was tested as follows:- 0.5 ml of the extract (about 5 mg protein) was incubated at 37°C with 0.5 ml pre-tRNA solution and 50μl 0.2 M - $MgCl_2$ for 1 or 2 h and the mixture frozen. The protein was removed from the reaction mixture by following the cold phenol extraction procedure (Methods section 8.a). Traces of ether remaining in the solution were removed in a stream of

nitrogen.

The conversion of pre-tRNA to 4s material was determined by gel filtration on Sephadex G-100. Release of radioactivity from the pre-tRNA in the shape of mononucleotides or short oligonucleotides, during the conversion, was determined using DEAE-paper chromatography or two-dimensional chromatography on Whatman 3 MM paper (Methods section 13 and 15 respectively).

12. Digestion of RNA with snake venom phosphodiesterase

Samples of RNA (0.5 - 1 mg) to be treated with snake venom phosphodiesterase (orthophosphoric diester phosphohydrolase, E.C. 3.1.4.1) were precipitated with 2 volumes of ethanol, washed in absolute ethanol, ether and dried in a stream of nitrogen.

A stock solution of snake venom phosphodiesterase was made up to 5 mg/ml in distilled water and stored at -20°C. When required the enzyme was diluted 1 + 4 with .02 M - tris/HCl pH 8.5, containing 0.01 M - $MgCl_2$.

The sample was dissolved in 200 μl of the diluted enzyme and incubated at 37°C; samples were removed at various times. The reaction was stopped by adding 0.5 volume 6 mM - EDTA, which is reported to inhibit the venom phosphodiesterase (Razzell, 1963), and freezing the sample.

The samples were later analysed by two-dimensional paper chromatography (Methods section 15). The 5' monophosphates of adenosine, guanosine, uridine and cytidine were cochromatographed to serve as markers. These were detected under u.v. light and the appropriate areas of the chromatograms were cut out and assayed for

radioactivity as described in Methods section 16.

13. DEAE-paper chromatography

This method, reported by Furlong (1965) is useful for separating short oligonucleotides according to length.

The sample to be analysed was applied as a streak 2 cm long at one end of the DEAE-paper strip. Ascending chromatography was carried out with 0.75 M - ammonium bicarbonate, pH 8.6, until the solvent front was 20 cm above the origin. The DEAE-paper was dried and cut up into 0.5 cm strips and assayed for radioactivity (Methods section 16).

Marker 5' mononucleotides were again cochromatographed to indicate the positions of the purine and pyrimidine mononucleotides. They were detected under u.v. light and were found to run just behind the solvent front.

14. Hydrolysis of RNA

Unlabelled Esch. coli B sRNA was added to the radioactive RNA to give a specific activity of about $1\text{-}2 \times 10^5$ c.p.m./mg RNA. The RNA was precipitated with 2 volumes absolute ethanol and, after 2h at $-20^{\circ}C$, it was collected by centrifugation at 10,000 x g for 10 min.

The RNA was hydrolysed to its constituent nucleoside 2' (or 3') monophosphates by incubation at $37^{\circ}C$ for 18h in 0.3 M - KOH. The pH was then adjusted to 7.0 by the addition of 1.2 N -perchloric acid. After 15 min at $0^{\circ}C$, the resulting precipitate of $KClO_4$ was removed by centrifugation. The supernatant solution was dried

down as droplets (about 100 µl) on polythene sheets in a stream of warm air. The nucleotides were then taken up in 100 µl of distilled water and used for subsequent analysis.

15. Two-dimensional Paper Chromatography of Nucleotides

This method, reported by Hayashi, Osawa & Miura (1966) was used for the analysis of RNA hydrolysates or the samples from the snake venom phosphodiesterase digestion.

50 µl of the RNA hydrolysate or snake venom digest with added 5' monophosphate markers, were spotted 10 cm in from one corner of a 46 cm x 46 cm sheet of Whatman No. 1 or 3 MM chromatography paper. Descending chromatography was carried out for 18h in the first solvent system:- isobutyric acid : 0.5 M -NH_4OH (50:30 v/v). The chromatogram was dried and developed in the second dimension for 20h. Once again descending chromatography was used, the solvent system being isopropanol : 6 N - HCL (65:35 v/v). The chromatogram was dried and the u.v. absorbing spots, from the unlabelled marker nucleotides, were marked. The major nucleotides were identified by comparison with published data. (Hayashi et al, 1966).

When chromatography had been carried out with hydrolysed RNA derived from cells incubated with L-[^{14}C-methyl]-methionine, the positions of the methylated bases could be located by autoradiography. This was carried out as follows:-

The dried chromatogram was stapled to an X-ray film and sealed in a light-tight, lead-lined folder. When 1 - 2 x 10^5 c.p.m. of ^{14}radioactivity were applied to the chromatogram, the X-ray film

was exposed for 3 weeks. The film was then developed for 10 min in Gevaert G-5c developer, fixed in Amfix, washed and dried.

The X-ray film could be aligned with the chromatogram by means of the staple marks. This allowed the positions of the methylated nucleotides to be compared with those of the major nucleotides.

16. Assay of Radioactivity

Aqueous samples, arising from analyses of RNA by gel filtration, chromatography on MAK etc., to be assayed for acid insoluble radioactivit were made 5% (w/v) with respect of ice-cold trichloracetic acid. The precipitated RNA was collected on Millipore filters (HAWP 025CO, 45 μpore size). The filters were dried at 160° for 5 - 10 min and then immersed in 10 ml of the toluene - based scintillation fluid. (see later in this section).

In the experiments where the cells had been incubated with L-[^{14}C-methyl] -methionine to label the methylated bases present in rRNA and tRNA, the samples were not immediately treated with trichloracetic acid. First they were incubated with 0.5 volumes of 1M - tris/HCl, pH 10.0 at 37°C for 1 h. This step was necessary since, during growth of the cells with ^{14}C-methionine, the methionine tRNA would become loaded with radioactive L-methionine. Unless this was removed, a falsely high value for the degree of methylation of tRNA would have been obtained. Incubation at 37°C in alkaline medium has been reported to release amino acids loaded onto tRNA (Burdon, 1967a). After the addition of sufficient 50% (w/v) trichloracetic acid to bring the pH to 1, the samples were treated as described above.

Occasionally the radioactivity in an aqueous sample was assayed without prior trichloracetic acid precipitation. This was achieved by adding a portion of the sample to 9.5 ml of dioxan-based scintillation fluid (see later in the section). The solution was made up to 10 ml with distilled water.

After DEAE or 3 MM paper chromatography, the following procedure was required to determine the amount of radioactivity present in various parts of the chromatograms. Either areas corresponding to the added marker nucleotides were cut out or the whole chromatogram was sectioned into small squares. These pieces were then placed in scintillation vials with 0.5 ml hyamine hydroxide in methanol. The vials were tightly capped and heated to 60°C for 10 min to remove the radioactivity from the paper. 9.5 ml of toluene-based scintillation fluid were then added and mixed.

The samples prepared by one of the above methods were then counted using a liquid scintillation spectrometer. Three machines were available viz.:- Nuclear Chicago 720, Packard Tri-Carb Series 400 and Philips Liquid Scintillation Analyser. The latter two were calibrated to count both ^{3}H and ^{14}C radioactivity contained in a single sample.

16.b.) Scintillation Fluids

1) Toluene based.

5 g of 2, 5 diphenyl oxazole were dissolved in 1 litre Analar Toluene and stored at room temperature.

2. Dioxan based

7 g 2, 5 diphenyl oxazole and 100 g of naphthalene were dissolved in 1 litre of dioxan. This scintillation fluid was stored under nitrogen at room temperature.

R E S U L T S .

III. RESULTS

A. Synthesis of transfer RNA in C13 cells

To date there have been several reports pointing to the existence of a precursor to tRNA in eukaryotes. However this study was started following the initial discovery by Lal and Burdon (1967) of a possible tRNA precursor in Krebs II ascites cells. Since these cells appear to metabolise actively for only a few hours after their removal from the mouse, it was decided to investigate the synthesis of tRNA further using a tissue culture cell line (BHK21/C13 or C13). The first step therefore in examining tRNA synthesis in the C13 cells must be to confirm the existence of the precursor and then to examine its properties.

1. Properties of the rapidly labelled low molecular weight RNA in C13 cells

a) Kinetics of labelling

Following the method described by Lal and Burdon (1967) for the isolation of the tRNA precursor (pre-tRNA) from Krebs II cells, exponentially growing C13 cells were exposed to [^{3}H]-guanosine for a short time and RNA was extracted using the cold phenol technique (Methods section 8a). Time intervals of from 5min to 2h were used and the RNA preparations were fractionated on Sephadex G-100 (Fig III.1a-f). With the 5min labelling time, the major peak of radioactivity, apart from that excluded from the gel, eluted between the 5s and 4s RNA shown in the pattern of optical density at 260nm (Fig III.1a). By

Fig. III.1

Kinetics of synthesis of low molecular weight cytoplasmic RNA.

Elution patterns of C13 RNA from Sephadex G-100 in 0.05M-ammonium acetate, pH5.1. The RNA was prepared by cold phenol extraction of cells incubated at 37°C with [8-^{3}H]-guanosine for various times.

a) 5min incubation with 2µc/ml [8-^{3}H]-guanosine

b) 10min " " 1.5µc/ml "

c) 20min " " 1µc/ml "

d) 20min, e) 60min and f) 120min incubation with 0.5µc/ml [8-^{3}H]-guanosine.

[e) and f) are shown on next page]

Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-)

FIGURE III. 1.

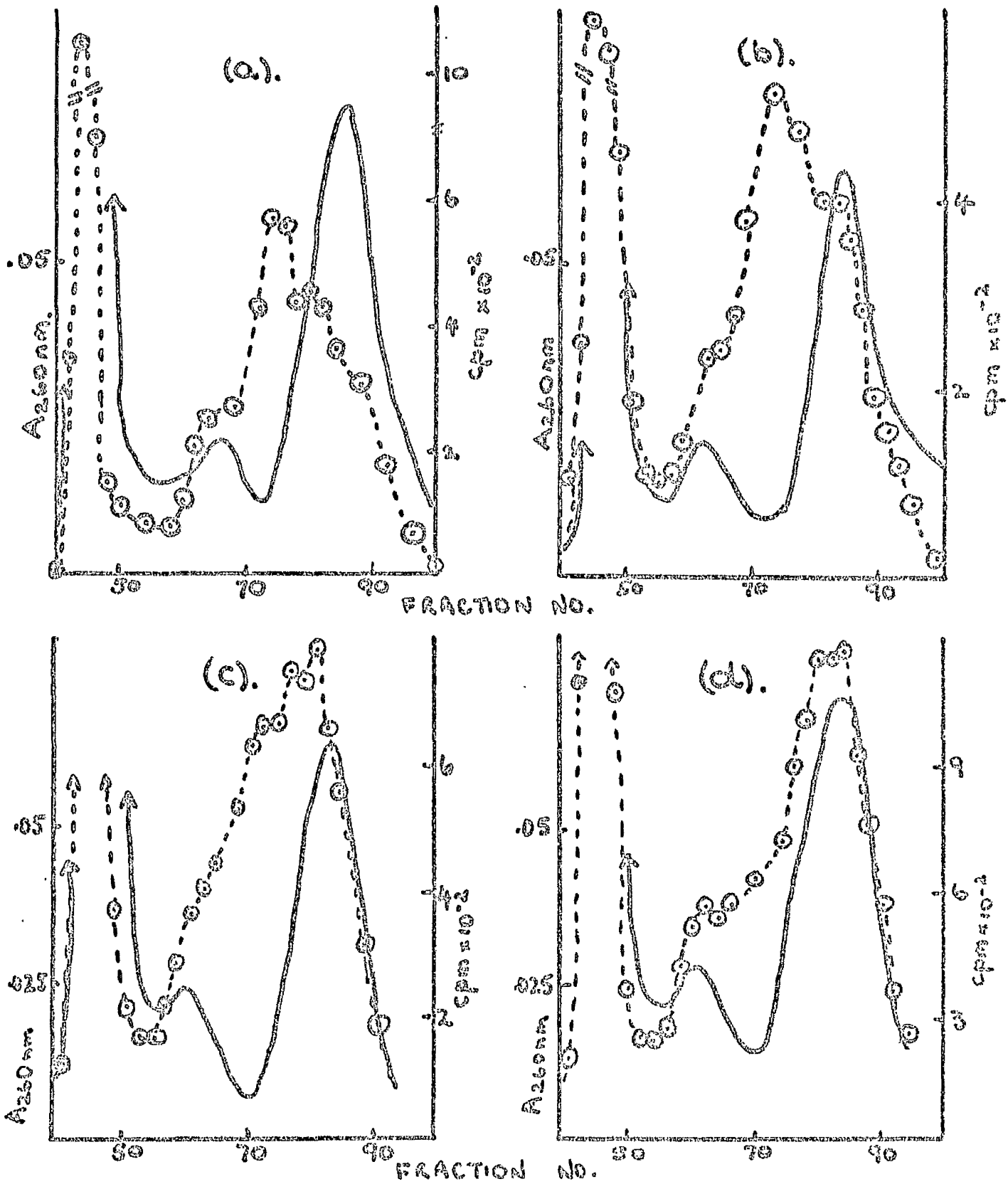
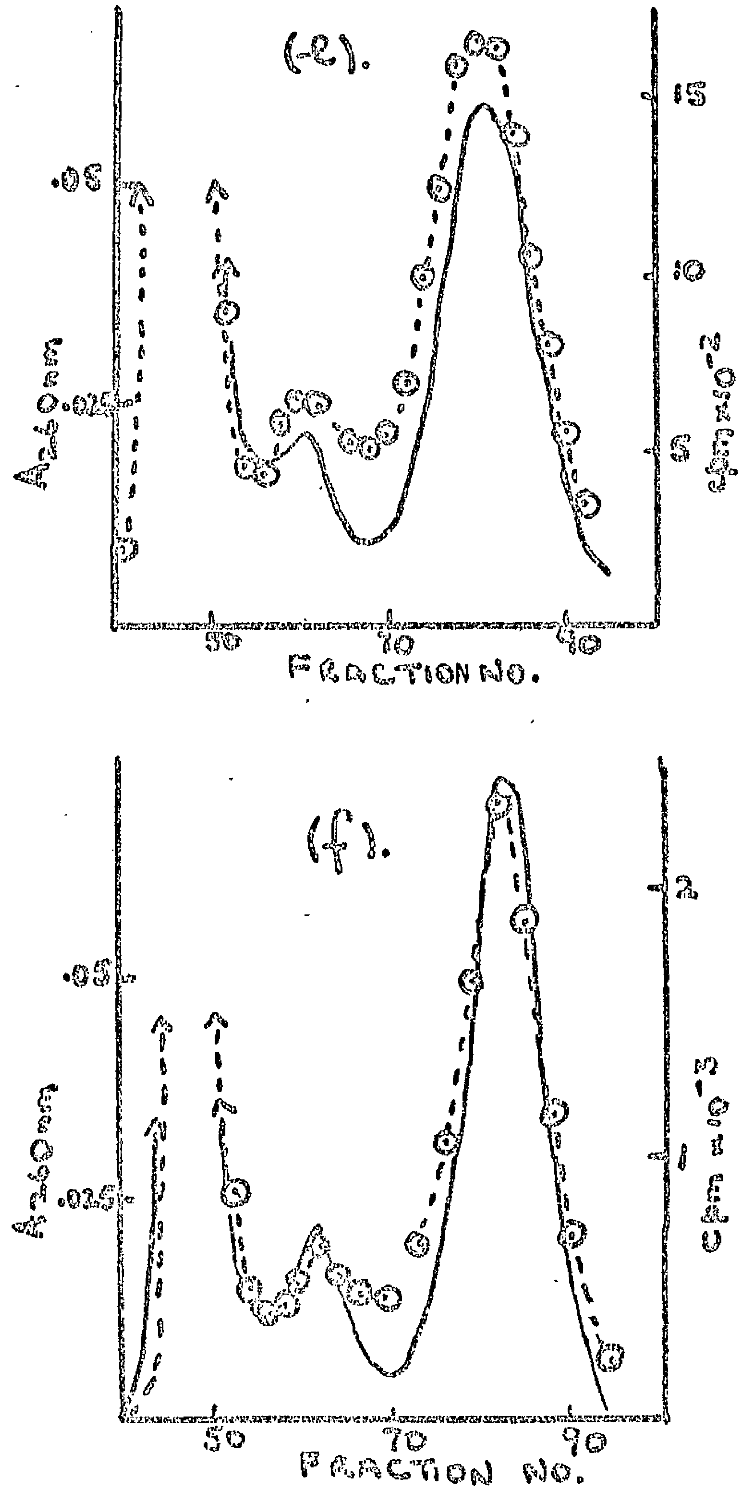


FIGURE III.1.


(e).
·05
·025
15
10
5
50
70
90
FRACTION NO.
(f).
·05
·025
2
1
50
70
90
FRACTION NO.

10min, a considerable proportion of the radioactivity eluted from the Sephadex gel with the 4s RNA (Fig III.1b). The fraction of the radioactivity eluting between the 4s and 5s RNA peaks gradually reduced with the longer labelling times while the amount of label in the 4s RNA increased until by 2h the radioactivity followed exactly the profile of optical density at 260nm. (Fig III.1f). A similar picture was obtained by Lal and Burdon (1967). It is the pattern of labelling which would be expected of a precursor molecule:- rapidly labelled but contributing a lessening proportion of the total radioactivity as the amount of product increases.

b.) Effect of actinomycin D on RNA synthesis in C13 cells

If this rapidly labelled RNA is indeed a precursor of tRNA, it must be converted to tRNA and it should be possible to observe this if RNA synthesis is completely inhibited just after the rapidly labelled RNA has been formed. There is another possible explanation for the occurrence of this low molecular weight RNA - that it is due to the breakdown of high molecular weight RNA e.g. rRNA precursors. This second possibility could be ruled out if the pre-tRNA were still produced when the synthesis of high molecular weight RNA had been prevented. The following experiments were designed to investigate both these possibilities.

Perry (1962) has reported that low concentrations of actinomycin D, e.g. 0.04μ g/ml, will completely inhibit the synthesis of rRNA without affecting the production of the other types of RNA.

To test this with C13 cells, two cultures were set up. One was preincubated at 37°C with 0.04µg/ml actinomycin D for 10 min, as described by Perry, then both cultures were incubated with [^{3}H]-guanosine for 2h before the total cell RNA was extracted by the hot phenol technique (Methods section 8b). Total RNA was isolated rather than cytoplasmic to see if any nuclear RNAs, e.g. 45s RNA, were synthesised which could be broken down to give low molecular weight molecules in the cytoplasm. Both RNA preparations were fractionated on sucrose gradients (Methods 9c). Fractions were collected and every second was assayed for extinction at 260nm and acid-insoluble radioactivity. The remaining fractions in the low molecular weight peak were pooled and applied to a column of Sephadex G-100 along with added unlabelled cytoplasmic RNA to act as marker of the 4s and 5s RNA peaks. This second fractionation was necessary to observe the range of RNAs synthesised because the low molecular weight peak obtained from the sucrose gradients contains several different types of RNA e.g. 5s and tRNA.

Fig III. 2a and b shows the species of RNA synthesised in the untreated control cells. The sucrose gradient analysis gave 3 peaks containing 28s, 18s and low molecular weight RNA, with both the extinction and radioactivity measurements. Fractionation of the low molecular weight material on Sephadex G-100 gave a separation into 3 types of radioactive RNA viz. 5s, 4s and material excluded from the gel (peak a). No attempt was made to identify this excluded material

Fig. III.2

Species of RNA synthesised in C13

A culture of C13 cells was incubated at 37°C for 2h with 0.2μc/ml [8-^{3}H]-guanosine and total cell RNA was extracted by the hot phenol technique (Methods 8b).

a) The RNA preparation was analysed by zonal ultracentrifugation (Methods section 9c). Every second fraction was assayed for extinction at 260nm and acid insoluble radioactivity.

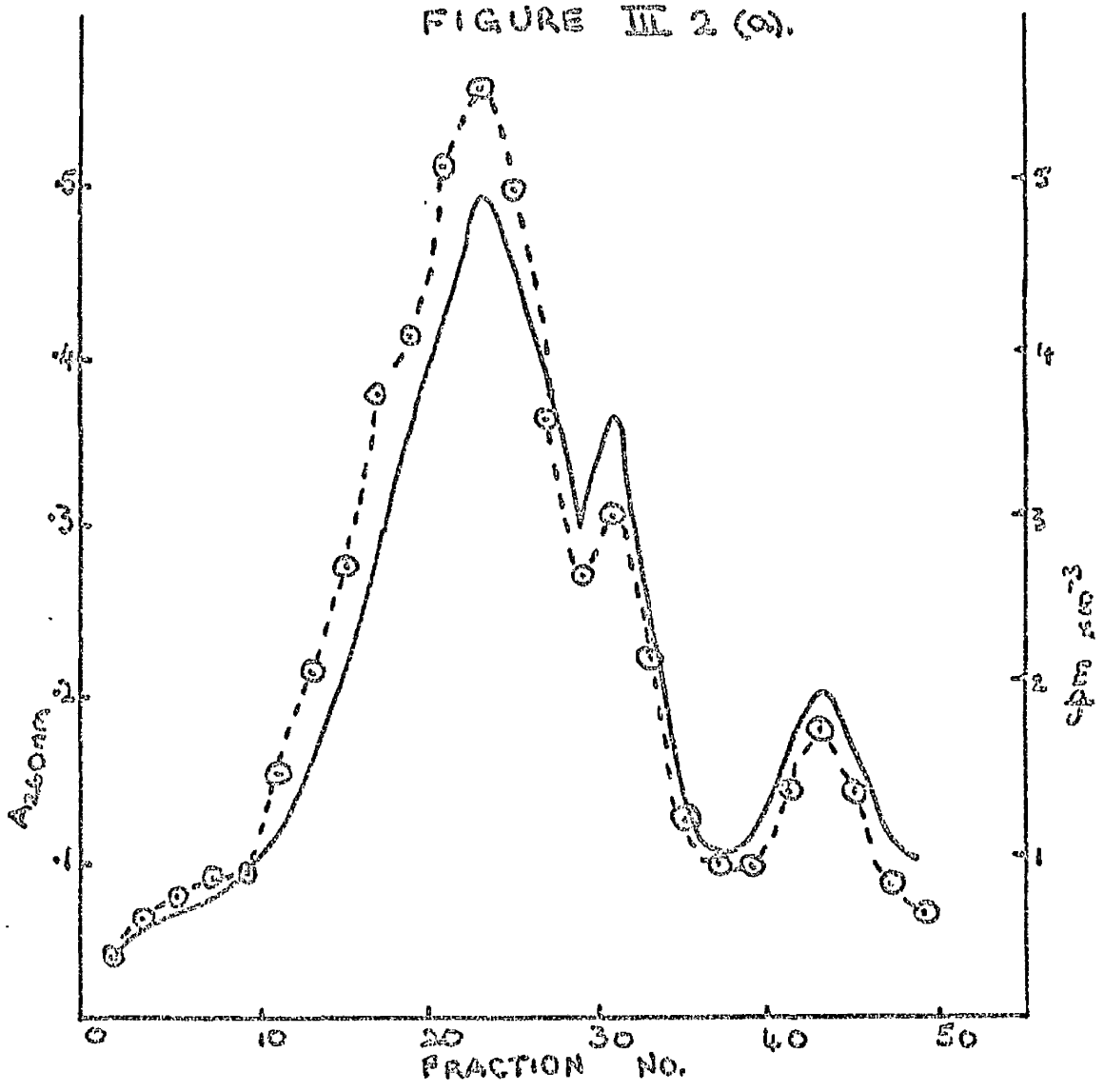
The remaining fractions in the low molecular weight region (tubes 39-51) were pooled and non-radioactive, cytoplasmic C13 RNA was added to act as marker of extinction at 260nm.

b) The pooled fractions from the sucrose gradient were analysed on Sephadex G-100 in 0.05M-ammonium acetate, pH5.1

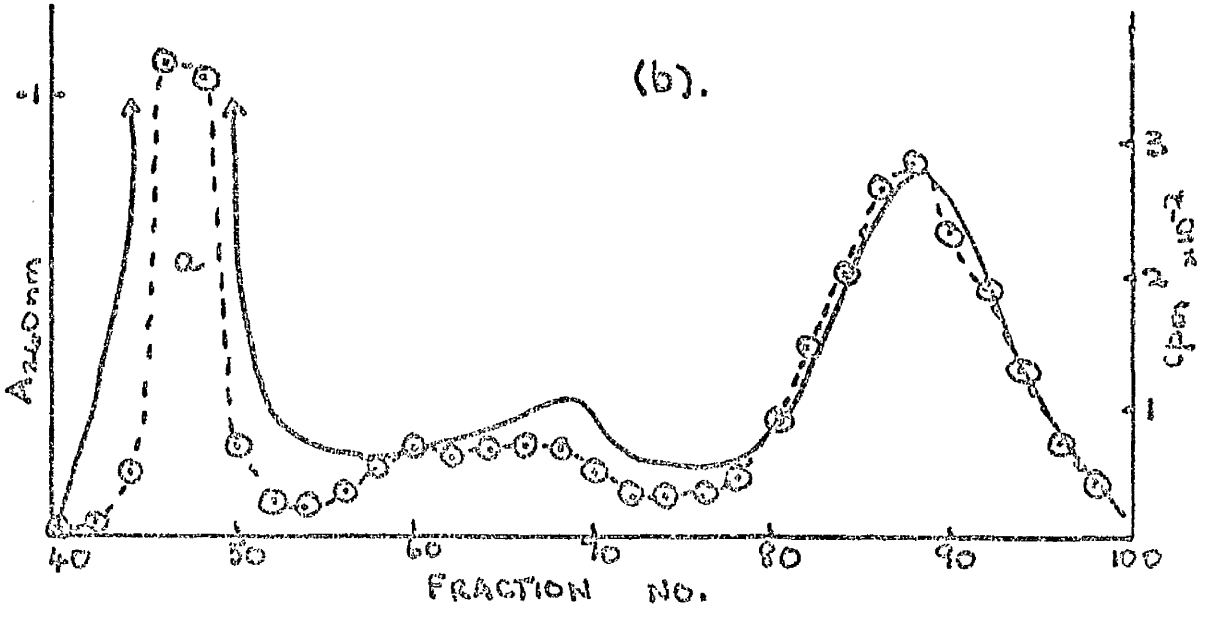
Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-)

FIGURE III 2 (a).



(b).



but it is possible that it is 28sA RNA which is known to dissociate from the 28s rRNA on heating to 60°C (Pene et al, 1968) - a treatment which is included in the hot phenol extraction procedure used, as just mentioned, to isolate the total RNA from C13 cells.

The results of the analyses of the RNA preparation obtained from the actinomycin D treated cells are presented in Fig III. 3a and b. From the sucrose gradient fractionation, it can be seen that 28s and 18s RNA were not synthesised nor were their high molecular weight precursors. Some heterodisperse material, probably high molecular weight nuclear RNA, was spread over the entire gradient but the only definite peak of radioactivity occurred in the low molecular weight region. When this radioactive RNA was analysed on Sephadex G-100 only 5s and 4s RNA were labelled; virtually no radioactivity was excluded from the gel. This is in agreement with the suggestion that this material probably is 28sA RNA which like the 28s ribosomal RNA arises from the 45s ribosomal RNA precursor.

These experiments show that while 28s, 18s, 28sA, 5s and 4s RNA are all synthesised in the control cells, only 5s and 4s RNA with some heterodisperse material are formed in the cells treated with 0.04 μg/ml actinomycin D. Therefore if the rapidly labelled RNA is formed in the presence of this concentration of actinomycin D, it cannot be due to the degradation of rRNA or its precursors. To test this a monolayer culture of C13 cells was preincubated for 10min with actinomycin D (0.04 μg/ml); [^{3}H]- guanosine was added and the

Fig. III.3

Species of RNA synthesised in presence of actinomycin D

Cl3 cells were incubated at 37°C with 0.04µg/ml actinomycin D for 10min. Following the addition of 0.2µc/ml [8-^{3}H]-guanosine, the incubation was continued for 2h before the extraction of total cell RNA using the hot phenol technique. The RNA preparation was analysed by zonal ultracentrifugation. Every second fraction in the low molecular weight region (tubes 36-48) was pooled and, together with non-radioactive cytoplasmic RNA marker, applied to a column of Sephadex G-100. 0.05M-ammonium acetate,pH5.1, was used to elute the RNA from the column.

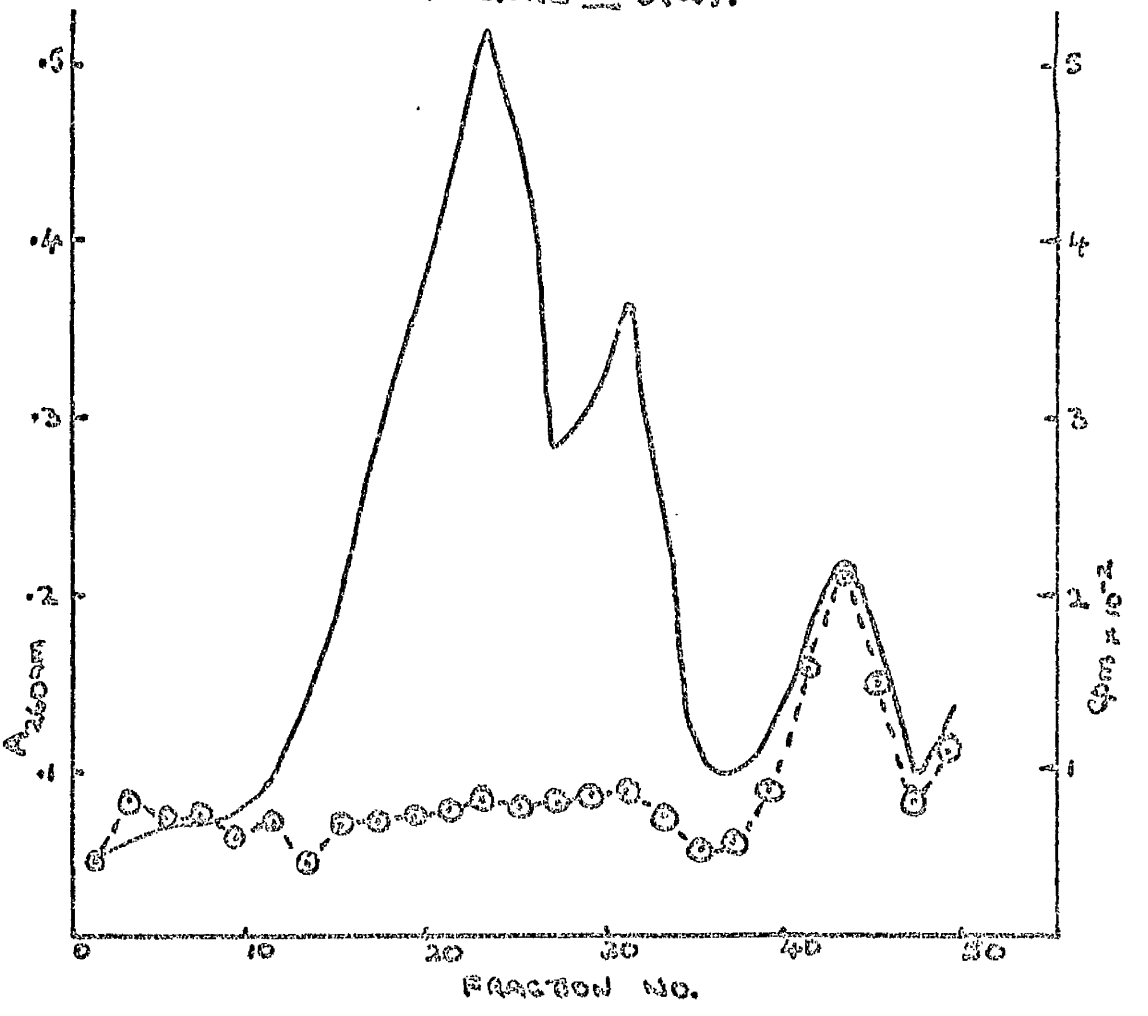
a) Sucrose density-gradient analysis of total cell RNA.

b) Fractionation, by gel filtration, of low molecular weight RNA.

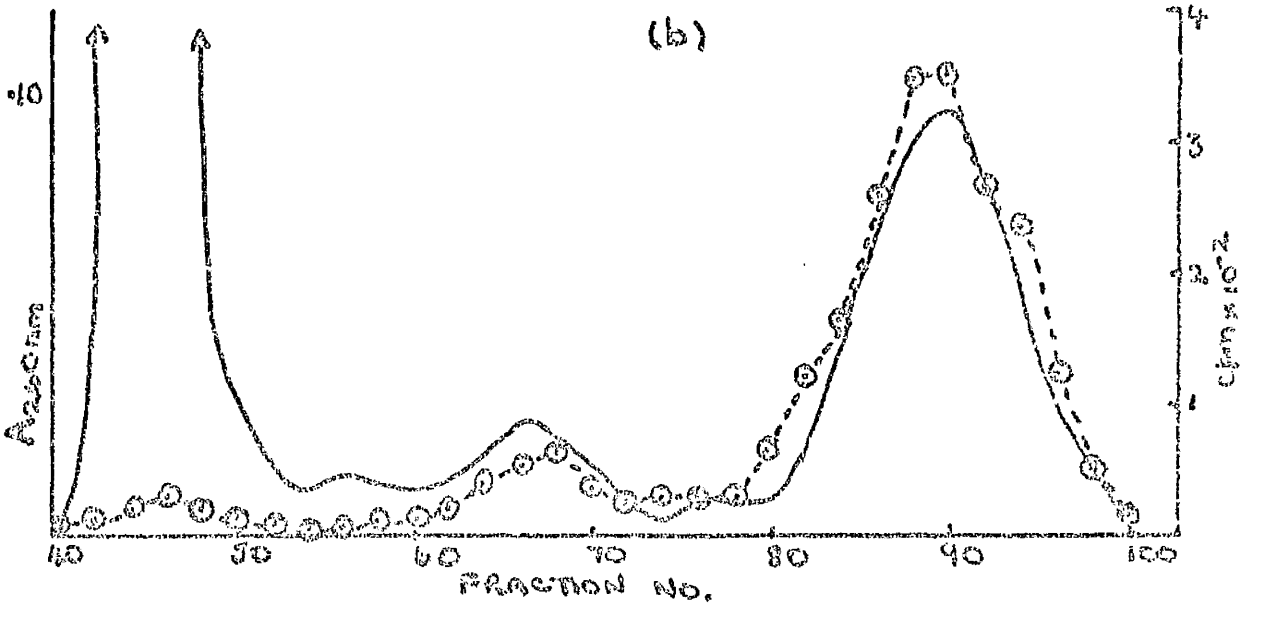
Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-)

FIGURE III 3(a).



(b)



incubation continued for 10 min. Cytoplasmic RNA was extracted and analysed as before by ultracentrifugation and gel filtration (Fig III. 4a and b). The fractionation on the sucrose gradient confirmed that rRNA was not synthesised, however some heterodisperse material sedimenting around the 18s region still appeared, and it is possible that this corresponds to cytoplasmic mRNA. When the low molecular weight peak was analysed on Sephadex G-100, the main peak of radioactivity appeared between 5s and 4s RNA - in the position of pre-tRNA. Shoulders on this peak indicated the presence of material running with the 5s and 4s RNA. Some heterodisperse material eluted from the column between the excluded peak and the 5s RNA. This experiment proves that pre-tRNA cannot be a breakdown product of rRNA or its precursors as it is formed under conditions which prevent the transcription of the rRNA genes.

It remains to decide whether or not pre-tRNA is converted to tRNA. A culture of C13 cells was treated with actinomycin D as above, but after the 10 min incubation with [^{3}H]- guanosine, the medium was removed and replaced with fresh medium containing 5 µg/ml actinomycin D, - sufficient to prevent all further RNA synthesis (Scherrer et al. 1963). The incubation was continued for 2h before cytoplasmic RNA was extracted using the cold phenol technique. The RNA preparation was analysed by zonal ultracentrifugation and gel filtration, (Fig III, 5a and b). On comparing this figure with Fig III. 4a and b, it can be seen that the heterodisperse RNA has disappeared but, more important, the

Fig. III.4

Effect of actinomycin D on the synthesis of rapidly-labelled cytoplasmic RNA.

A culture of Cl3 cells was treated with 0.04μg/ml actinomycin D for 10min before the addition of [8-^{3}H]-guanosine (2μc/ml). The incubation at 37°C was continued for 10min and then cytoplasmic RNA was extracted by the cold phenol method. After zonal ultracentrifugation, the low molecular weight RNA, (contained in tubes 38-50) was analysed by gel filtration on Sephadex G-100, non-radioactive cytoplasmic RNA having been added to mark the peaks of extinction at 260nm.

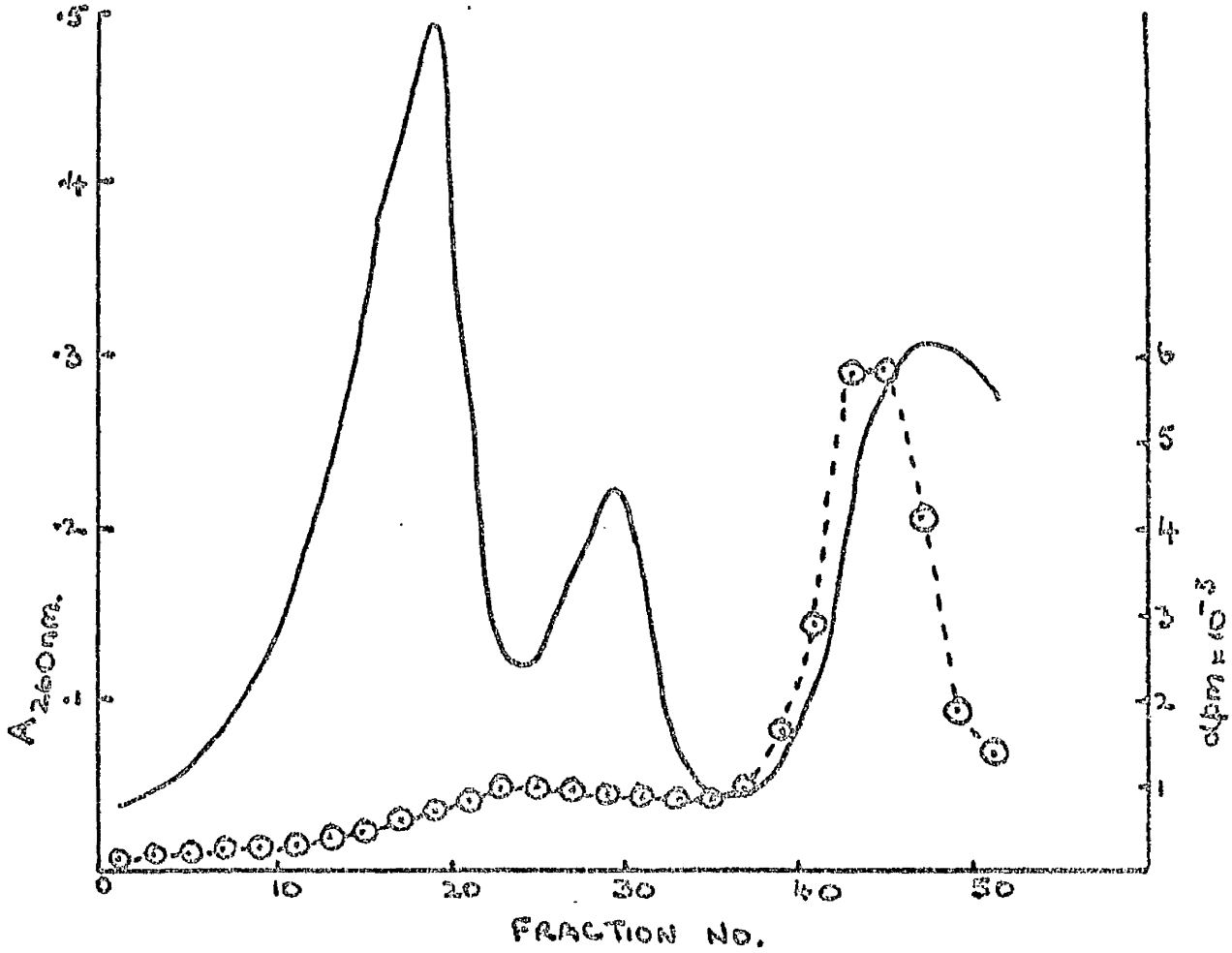
a) zonal ultracentrifugation of cytoplasmic RNA.

b) gel filtration of low molecular weight RNA.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III 4(a).



(b).

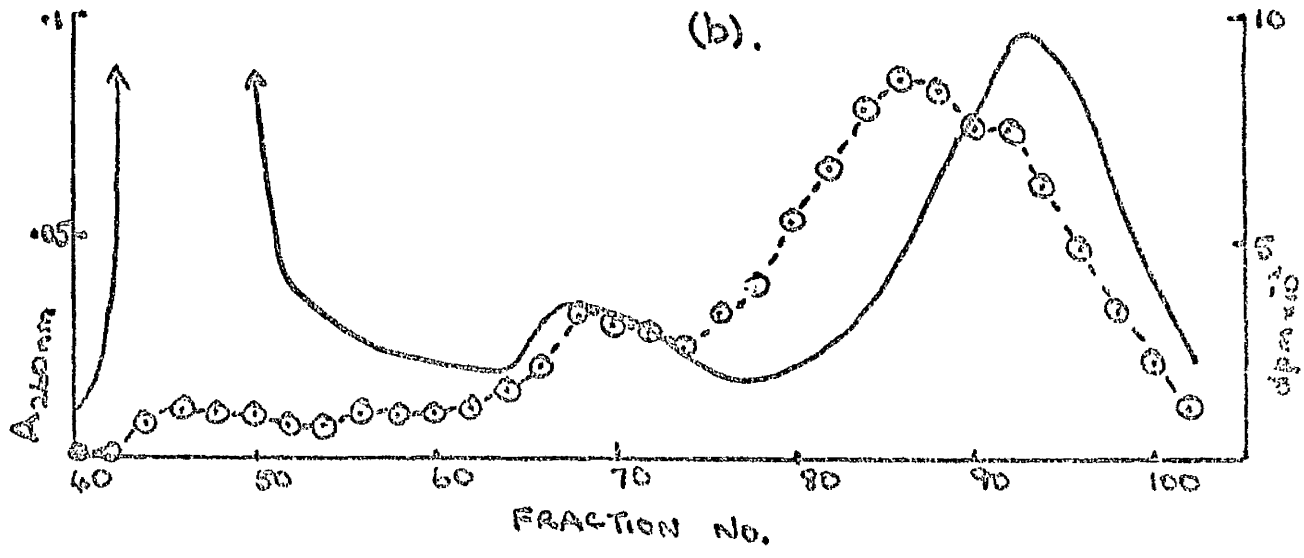


Fig.III.5

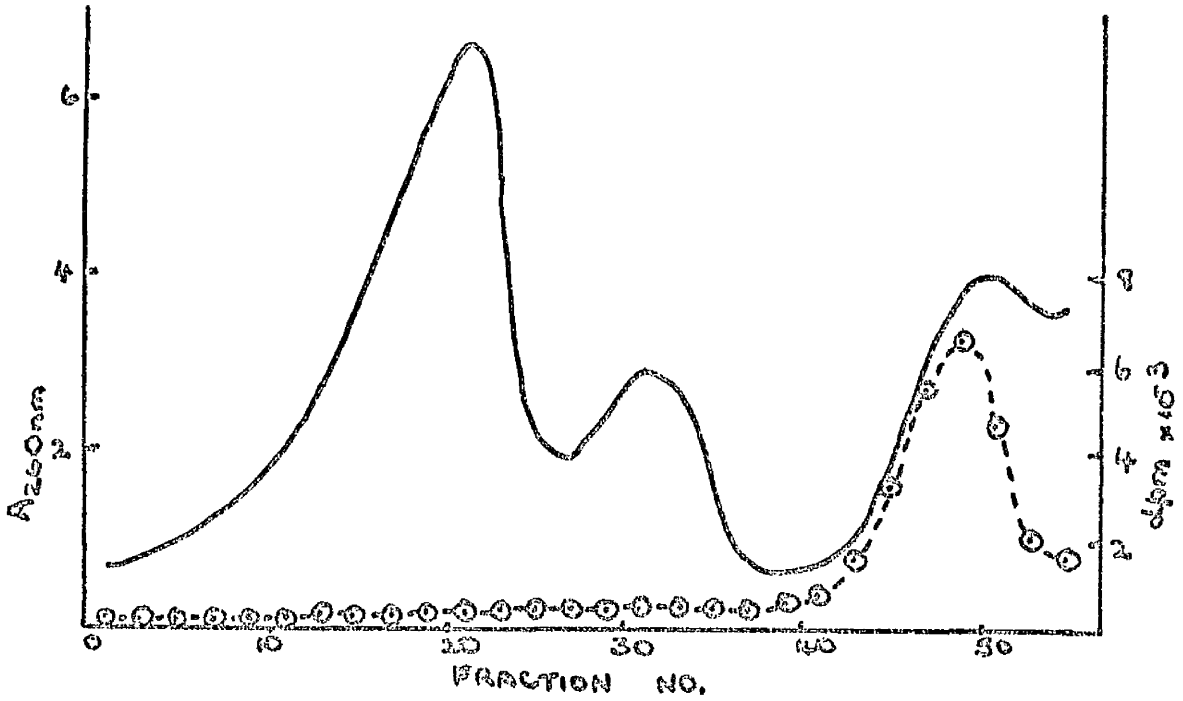
Stability of rapidly-labelled RNA to incubation with actinomycin D.

A culture of C13 cells was preincubated for 10min with 0.04μg/ml actinomycin D followed by a 10min incubation with 2μc/ml [8-^{3}H]-guanosine. The radioactive medium was then removed and replaced with fresh medium containing 5μg/ml actinomycin D. After 2h incubation, cytoplasmic RNA was extracted and analysed first by zonal ultracentrifugation (shown in part a.) and then the low molecular weight RNA was fractionated by gel filtration on Sephadex G-100 (part b).

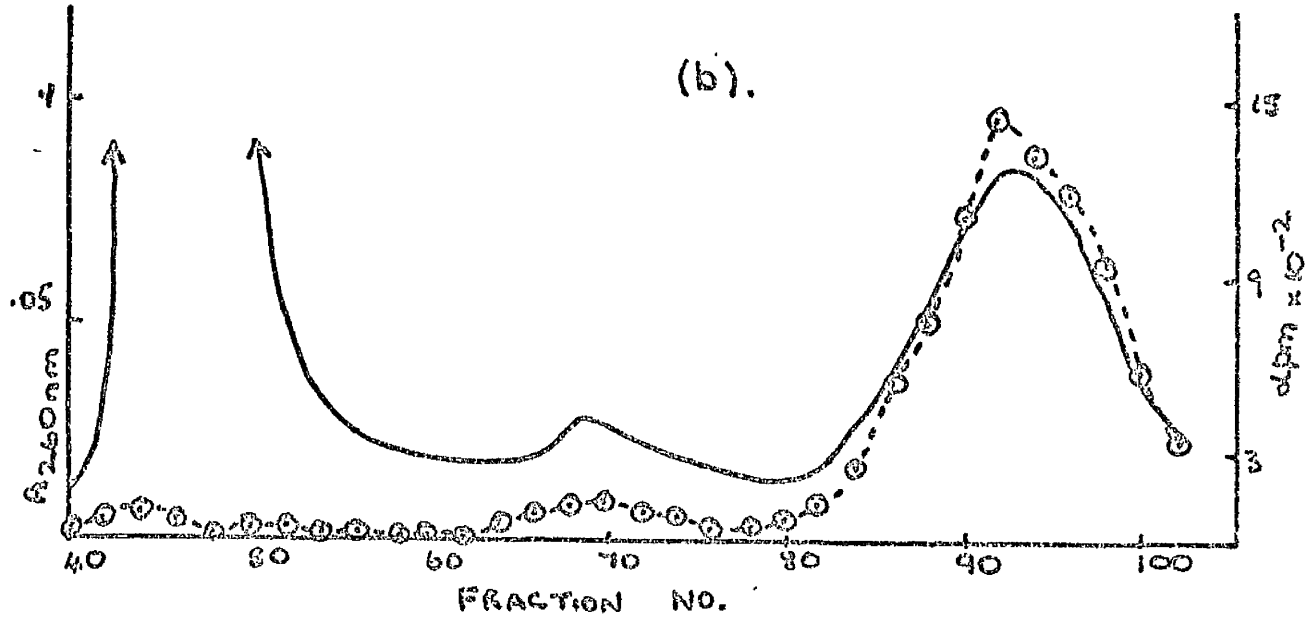
Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-)

FIGURE III 5 (a).



(b).



pre-tRNA which is so obvious in Fig III. 4b, has completely disappeared leaving only 5s RNA and a large amount of 4s RNA. In order to confirm definitely that the pre-tRNA is converted to tRNA, it is necessary to quantitate the results obtained in the last two experiments. This is difficult partly because separate monolayer cultures are not directly comparable but mainly because of the still relatively poor separation of 5s, 4s and pre-tRNA. In an attempt to estimate the amount of pre-tRNA, it was assumed that the radioactivity eluting with the optical density peaks was not pre-tRNA but was newly synthesised 5s and 4s RNA. Symmetrical peaks were drawn to follow the optical density profile and the remaining radioactivity in fractions 73-90 (Fig III.4b) was assumed to be pre-tRNA. This assumption may underestimate the amount of pre-tRNA, nevertheless it contributed 21% of the radioactive material of the size of tRNA and larger. A further 10% appeared as the heterodisperse material eluting before 5s RNA. The actinomycin D "chase" experiment (Fig III.5a and b) shows that both the heterodisperse material and the pre-tRNA are unstable. If they were completely degraded to much smaller molecules, 31% of the total radioactive material formed in 10 min should be lost during the "chase". In fact, after allowing for the different amounts of material obtained from the two sucrose gradients, the recovery of radioactivity from the Sephadex G-100 analysis of the actinomycin D "chased" preparation was 85% of that obtained with the control "unchased" RNA preparation, i.e. only 15% of the radioactive

material was lost. Therefore at least part of the pre-tRNA must be converted to 4s RNA; as much as 75% of the pre-tRNA may appear as 4s RNA after the 2h chase with actinomycin D, if all the heterodisperse RNA is completely degraded. Bearing in mind that a minimum value for the amount of pre-tRNA has been used, it appears that a considerable proportion of this rapidly labelled RNA is converted to 4s RNA in the absence of RNA synthesis.

c.) Chromatography on columns of methylated albumin kieselguhr(MAK).

Since, using Sephadex G-100, a clear separation of pre-tRNA from 5s and 4s RNA was not achieved it was decided to try a different fractionation technique. Chromatography on MAK columns was chosen in the hope of obtaining better resolution of these 3 low molecular weight components of the RNA samples. While only the size and configuration of the molecules determine the elution pattern with Sephadex, the base composition also influences the separation obtained with MAK; thus molecules with high G+C content elute at lower salt concentrations than similarly sized molecules with a high content of A+U. However the molecular size is still important, large molecules are bound more tightly to the protein of the column and therefore require a higher concentration of salt to elute them.

To test this method of fractionation, cytoplasmic RNA was prepared from C13 cells labelled with [^{3}H]- guanosine for 2h. When this RNA was eluted from the MAK column (see Methods 9b), the acid insoluble radioactivity followed the optical density profile almost exactly; the 3 peaks, corresponding to tRNA, 5s and rRNAs, were uniformly labelled

Fig. III.6

Fractionation of cytoplasmic RNA by chromatography on methylated albumin kieselguhr (MAK).

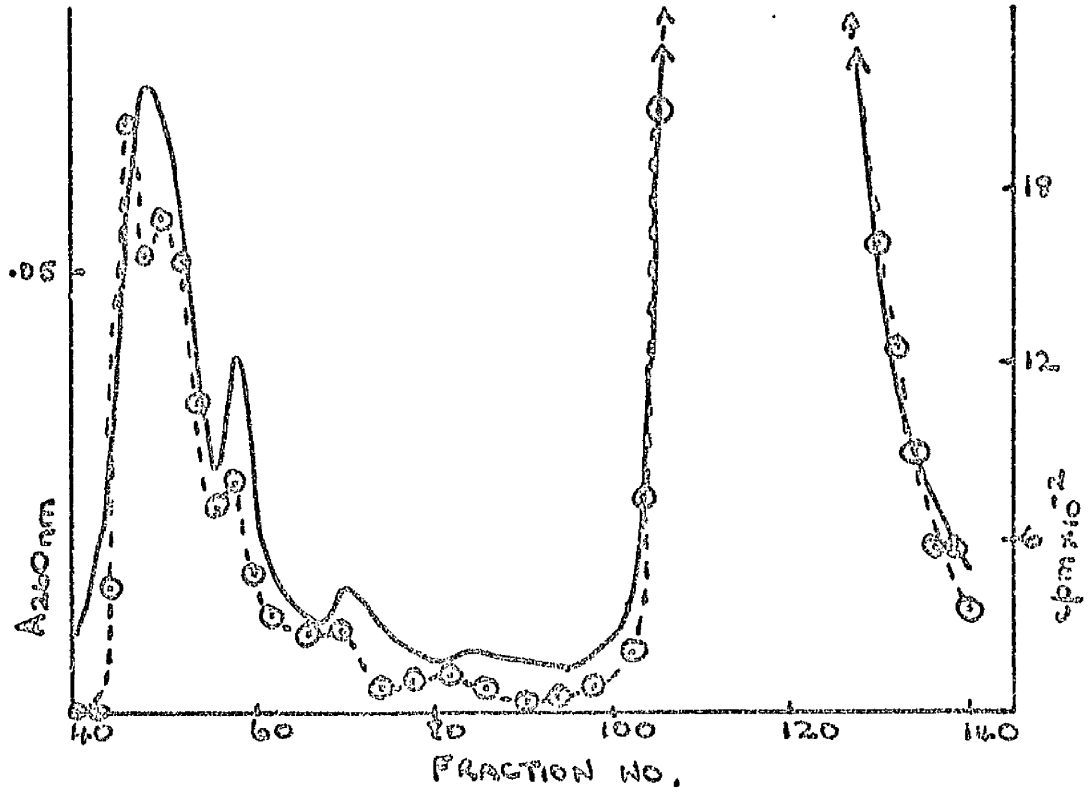
Cytoplasmic RNA was extracted from Cl3 cells using the cold phenol technique, and fractionated on a MAK column using a gradient of sodium chloride in 0.05M-sodium phosphate buffer, pH6.8 (see methods section 9b).

a) 2h; b)10min incubation with 2µc/ml [8-^{3}H]-guanosine.

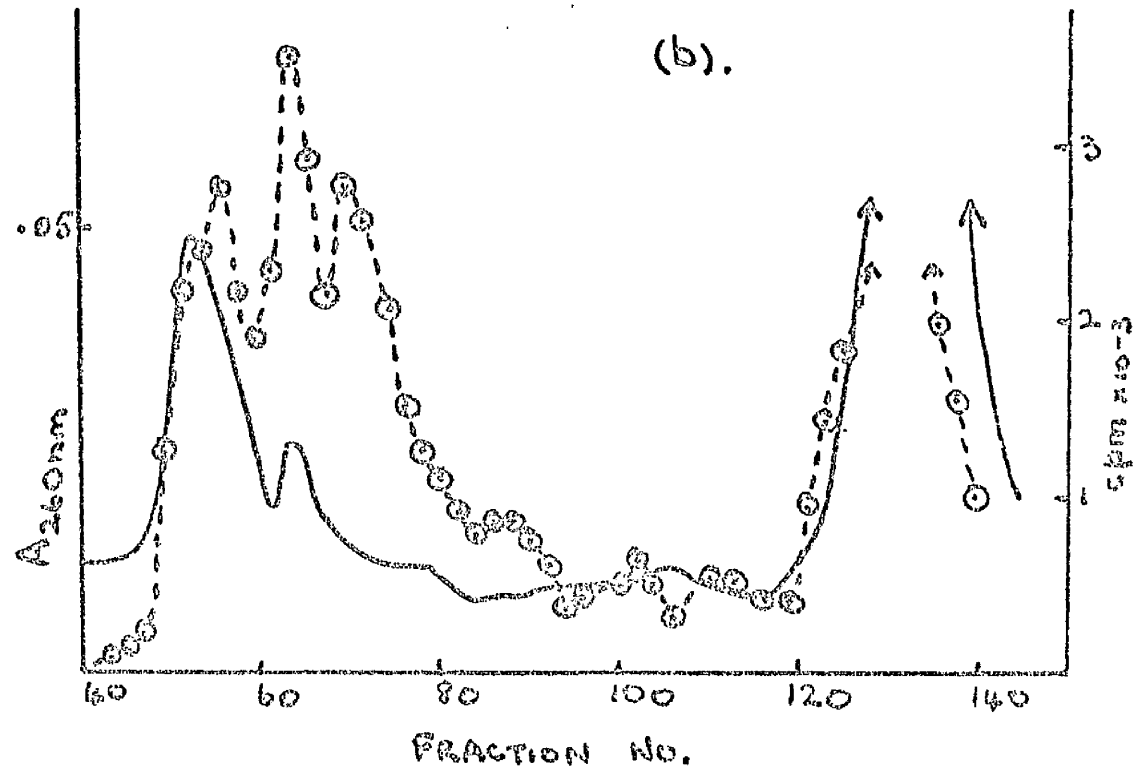
Extinction at 260nm (——)

Acid insoluble radioactivity (--o-o-)

FIGURE III 6(a).



(b).



(Fig III. 6a). On reducing the incubation with the tritiated RNA precursor to 10min, the pattern shown in Fig III.6b was obtained. A small amount of radioactive material eluted with the peak of rRNA and also some with the tRNA but most of tritium was incorporated into a high specific activity material eluting from the MAK after the tRNA. To decide if this were indeed pre-tRNA, the separation of 10min labelled RNA was repeated and fractions were pooled as indicated in Fig III. 7a; they were applied to Sephadex G-100 columns with added cytoplasmic RNA to act as marker of the 5s and 4s RNA peaks. The results are shown in Fig III. 7b-g where it can be seen that fractions 1 and 2 contain only 4s RNA. Fractions 3 and 4 consisted of labelled RNA which eluted with 5s and 4s RNA, some pre-tRNA was also present especially in fraction 4. The pre-tRNA supplied the largest proportion of the radioactivity in fraction 5. In fact this fraction had a distribution of radioactivity very similar to that of the RNA preparation prior to its elution from the MAK column (see Fig III. 1b), except that little material was excluded from the gel; these larger RNA species were present in fraction 6. These results show that pre-tRNA elutes from MAK after the 5s RNA but contaminated with it as well as with 4s RNA. Therefore fractionation on MAK gives no better separation than is obtained with Sephadex G-100. Since the latter technique did not involve the use of salt gradients nor the preparation of fresh columns for each experiment, it allowed better comparison between experiments and it was decided to continue using gel filtration to study pre-tRNA synthesis.

Fig. III.7

Comparison of fractionation on MAK and Sephadex G-100 of rapidly labelled cytoplasmic RNA.

After a 10min incubation with [8-^{3}H]-guanosine, cytoplasmic RNA was extracted from C13 cells using the cold phenol technique and fractionated on a MAK column using a gradient of sodium chloride in 0.05M-sodium phosphate, pH6.8.

a) A 0.2ml sample was removed from each fraction and assayed for total radioactivity as described in Methods section 16. The fractions indicated were pooled and reprecipitated with ethanol in the presence of non-radioactive, cytoplasmic RNA which served as marker of the peaks of extinction at 260nm. The elution profiles of these fractions from Sephadex G-100 in 0.05M-ammonium acetate, pH5.1, are shown in the remaining graphs of this figure as follows:-
b) fraction 1 including tubes 44-51 from the MAK column; c) fraction 2, tubes 52-59; d) fraction 3, tubes 60-66; e) fraction 4 including tubes 67-72; f) fraction 5 namely tubes 73-80.

Extinction at 260nm (——)

Total radioactivity present in $^{1}/_{10}$ of the fraction (-Δ-Δ-)

Acid insoluble radioactivity present in each fraction (-o-o-).

FIGURE III 7.

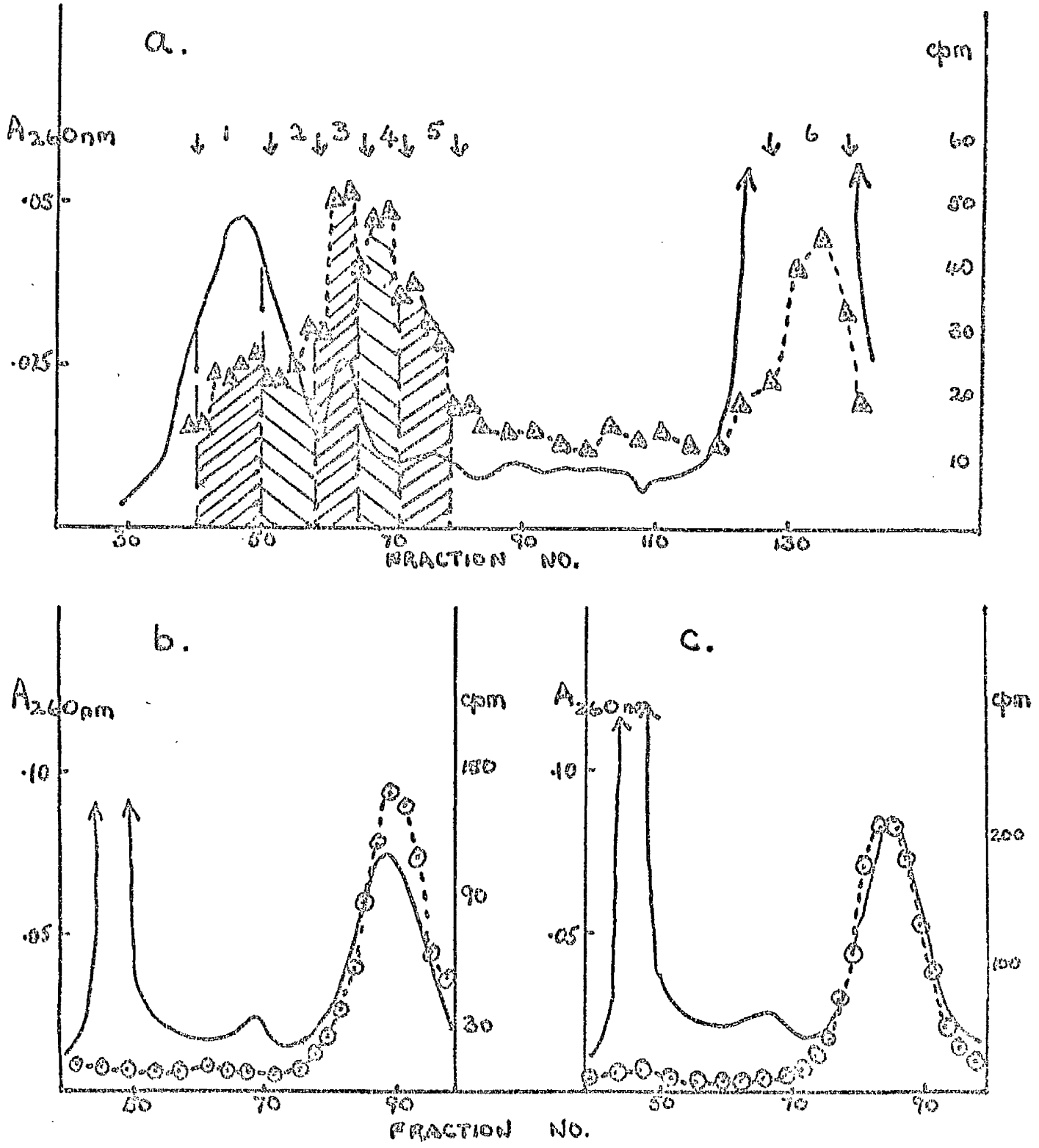
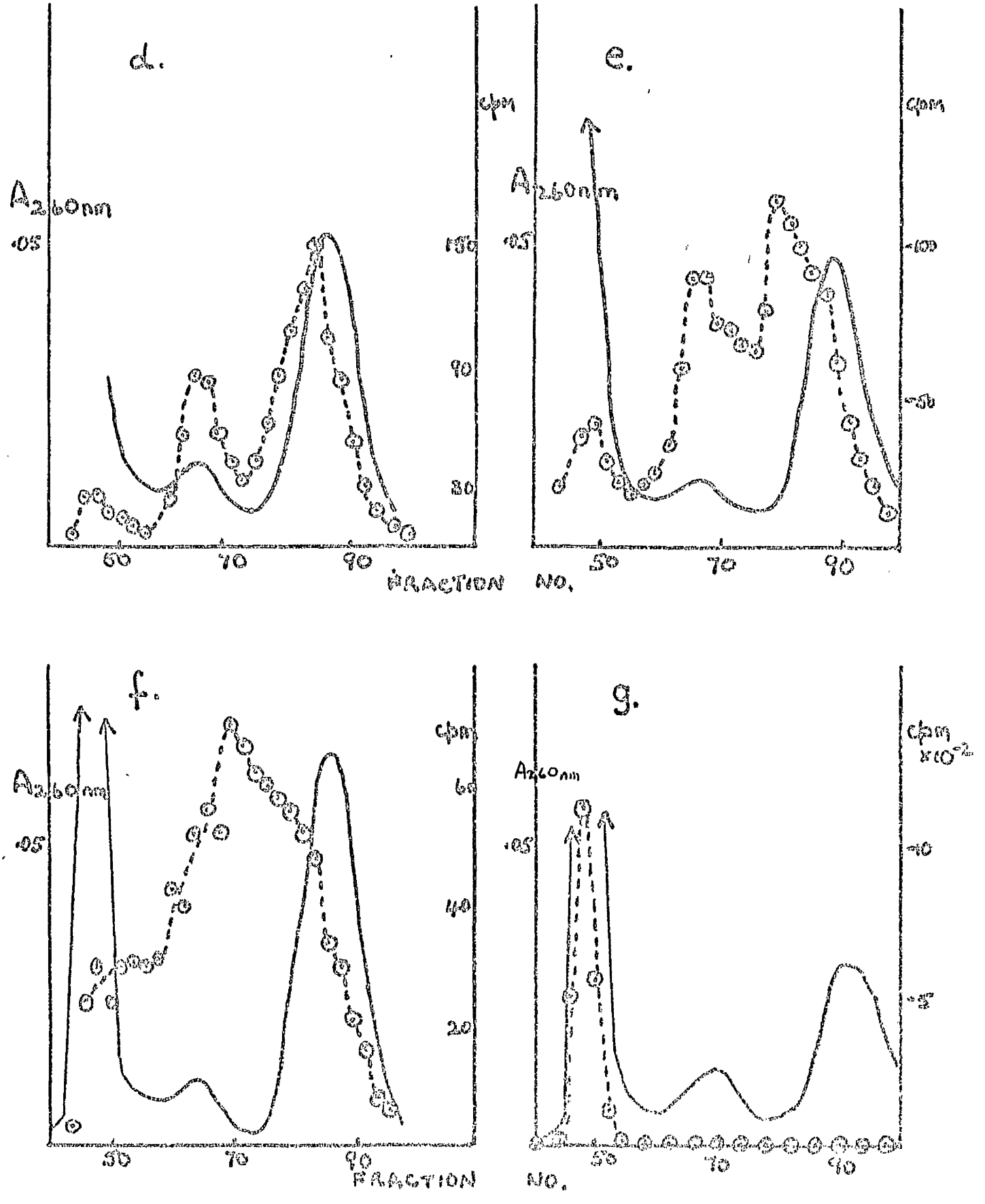

a.
A260nm
cpm
1
2
3
4
5
6
.05
.025
60
50
40
30
20
10
50
60
70
90
110
130
FRACTION NO.
b.
A260nm
cpm
.10
.05
150
90
30
50
70
90
c.
A260nm
cpm
.10
.05
200
100
50
70
90
FRACTION NO.

FIGURE III 7


d.
e.
f.
g.
A260nm
cpm
.05
150
90
80
100
50
40
20
60
70
90
FRACTION NO.
cpm x10^-2
10
5

d. Methylation of pre-tRNA & 4s RNA

As mentioned in the introduction, tRNA is known to contain methylated nucleotides which are formed, after the completion of the polynucleotide chain, by the action of specific tRNA methylases. The substrates for these enzymes must be unmethylated precursors of tRNA. An attempt was made to compare the degree of methylation of C13 tRNA and pre-tRNA to decide if the latter could be the substrate for the methylases.

Cultures of C13 cells were grown in the presence of L-[^{14}C-methyl]-methionine, to indicate the incorporation of methyl groups, and [^{3}H]-guanosine, to measure RNA synthesis. 20mM-sodium formate was added to prevent the radioactive methyl groups from entering the one carbon pool (Winocour, Kaye and Stollar, 1965) otherwise they would become incorporated into the purine ring system (Kit, Beck, Graham & Gross, 1958). It is necessary to use this dual isotope technique because RNA is methylated soon after its synthesis and only newly formed RNA will accept the ^{14}C labelled methyl groups (Burdon 1966). The [^{3}H]- guanosine therefore indicates the relative amounts of tRNA and pre-tRNA synthesised after the addition of the radioactive methionine. After 20min incubation with the isotopically labelled precursors, cytoplasmic RNA was extracted and Fig III.8 shows the fractionation of this RNA on Sephadex G-100. The longer incubation than usual in the preparation of pre-tRNA was chosen in order that a larger number of methyl groups could be incorporated. Although at this time the major peak of ^{3}H radioactivity in the low molecular weight RNA was in the 4s

Fig. III.8.

Methylation of pre-tRNA and 4sRNA in C13 cells.

Cells were incubated at 37°C for 20min with L-[^{14}C-methyl]-methionine (0.5μc/ml) and [8-^{3}H]-guanosine (0.2μc/ml) in medium made 20mM with respect to sodium formate. The RNA was extracted by the cold phenol technique and analysed by elution from Sephadex G-100 with 0.05M-ammonium acetate, pH5.1. Each fraction was mixed with 0.5 volumes 1M-tris/HCl buffer, pH10.0, and incubated at 37°C for 1h before it was assayed for acid insoluble radioactivity. This procedure was necessary to remove any radioactive methionine which had been bound as methionyl-tRNA during the incubation period.

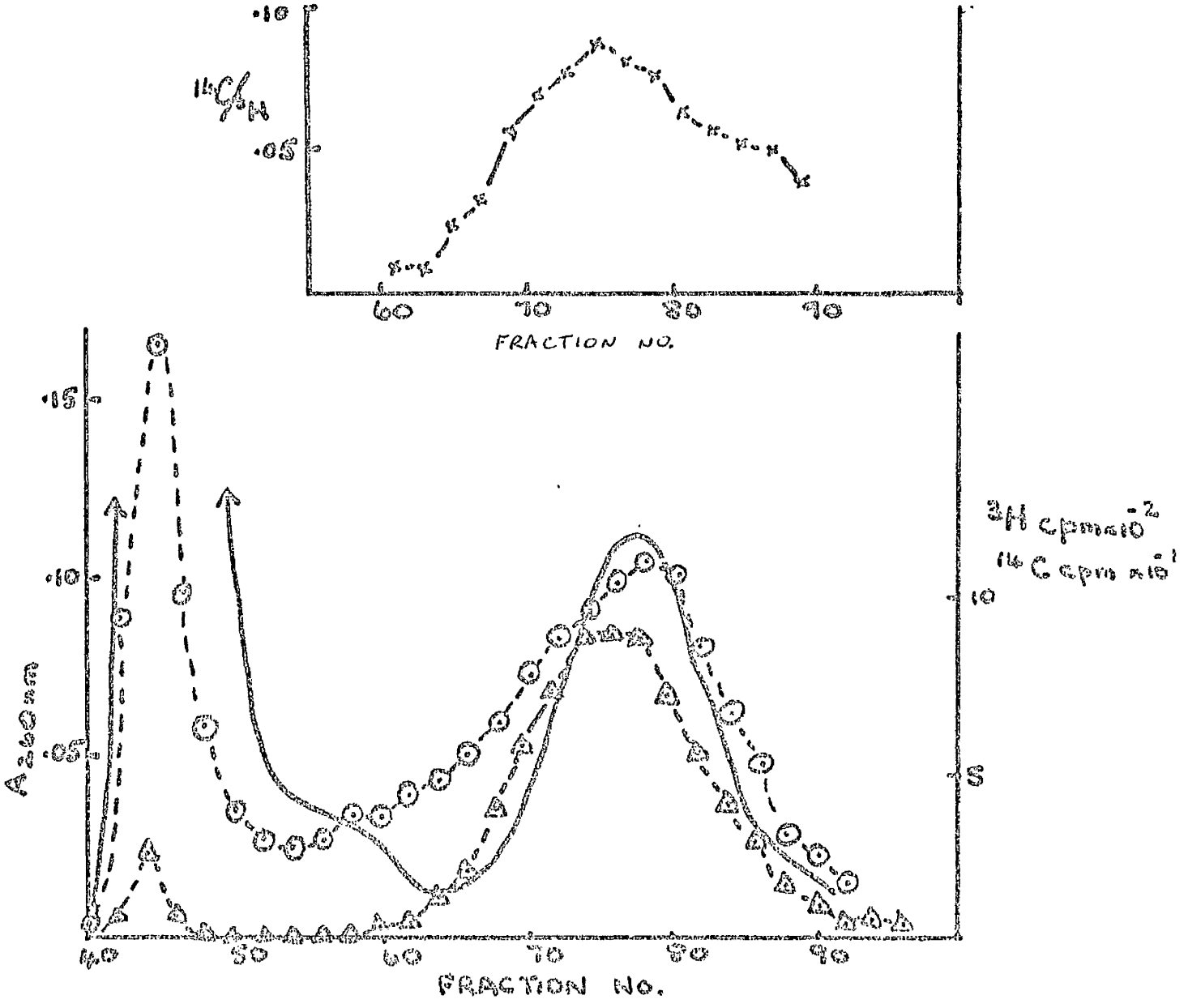
Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-)

Acid insoluble ^{14}C radioactivity (-Δ--Δ-)

Ratio ^{14}C c.p.m./^{3}Hc.p.m. (x—x—x)

FIGURE III 8.


.10
.05
60
70
80
90
FRACTION NO.
.15
.10
.05
40
50
60
70
80
90
FRACTION NO.
10
5

position, there was still evidence of pre-tRNA running between 4s and 5s RNA. The ^{14}C radioactivity on the other hand showed a small peak in the excluded material and a much larger peak following the extinction profile of 4s RNA. Much fewer ^{14}C counts relative to ^{3}H were found in the pre-tRNA region than in the 4s RNA peak. This suggests that C13 pre-tRNA is less methylated than is mature tRNA. A similar result had been obtained by Lal and Burdon (1967) using Krebs II ascites cells.

2. Accumulation of pre-tRNA

To examine the chemical properties of pre-tRNA further, it would be a great advantage if a method could be found to accumulate it, since at present the level of radioactivity is somewhat low. Three treatments were tried, two of them having been found to accumulate rRNA precursors in mammalian cells.

a) Methionine starvation

The first method used was methionine starvation. This treatment had been reported to block rRNA precursor maturation at the 32s level in Hela cells (Vaughan et al 1967); 28s and 18s RNA did not appear in the cytoplasm. Since pre-tRNA appeared to require further methylation before its final conversion to mature tRNA, it seemed reasonable to expect that lack of methionine - the source of methyl groups for RNA methylation - might prevent the conversions and hence cause a build up of the precursor molecule.

C13 cells were starved of methionine as described in Methods section 5. However preliminary experiments proved that although this

treatment reduced the rate of total RNA synthesis to no more than $\frac{1}{3}$ of the control level, sucrose gradient analyses proved that 18s and 28s RNA were still appearing normally in the cytoplasm. Despite this it was decided to test the effect of methionine starvation on tRNA synthesis. Cultures of methionine starved $_{\Lambda}$C13 cells were incubated with $[^{3}H]$ - guanosine for various times before extraction of the cytoplasmic RNA which was fractionated on Sephadex G-100 (Fig III.9a-c). With a 10 min labelling period (Fig III.9a) radioactivity is incorporated into 5s, 4s and pre-tRNA giving a pattern very similar to that obtained with the untreated control cells (Fig III.1b) except that the material excluded from the gel was greatly reduced. When the incubation with $[^{3}H]$ - guanosine was increased to 1h the amount of pre-tRNA decreased relative to tRNA, however it was still quite significant. Even when the labelling time was lengthened to 2h the acid-insoluble radioactivity did not follow the extinction profile exactly, and a considerable amount of RNA still eluted in the pre-tRNA position. Unfortunately, although this treatment undoubtedly lengthened the time during which it was possible to identify pre-tRNA as distinct from 4s RNA, it did not completely block the conversion. Therefore a purification of pre-tRNA from mature tRNA was not achieved.

b) Toyocamycin treatment

Tavitian, Uretsky and Acs (1968) reported that toyocamycin, an analogue of adenosine (Fig II.4), while allowing the production of 45s RNA, completely blocked its maturation to rRNAs. They reported that tRNA synthesis was unaffected, however they only analysed their RNA

Fig. III.9

Effect of methionine deficiency on the synthesis of low molecular weight cytoplasmic RNA.

Cultures of C13 cells were maintained in methionine deficient medium for 24h (see Methods section 5) before being exposed to [8-^{3}H]-guanosine for various periods. Cytoplasmic RNA was then extracted and analysed by gel filtration on Sephadex G-100

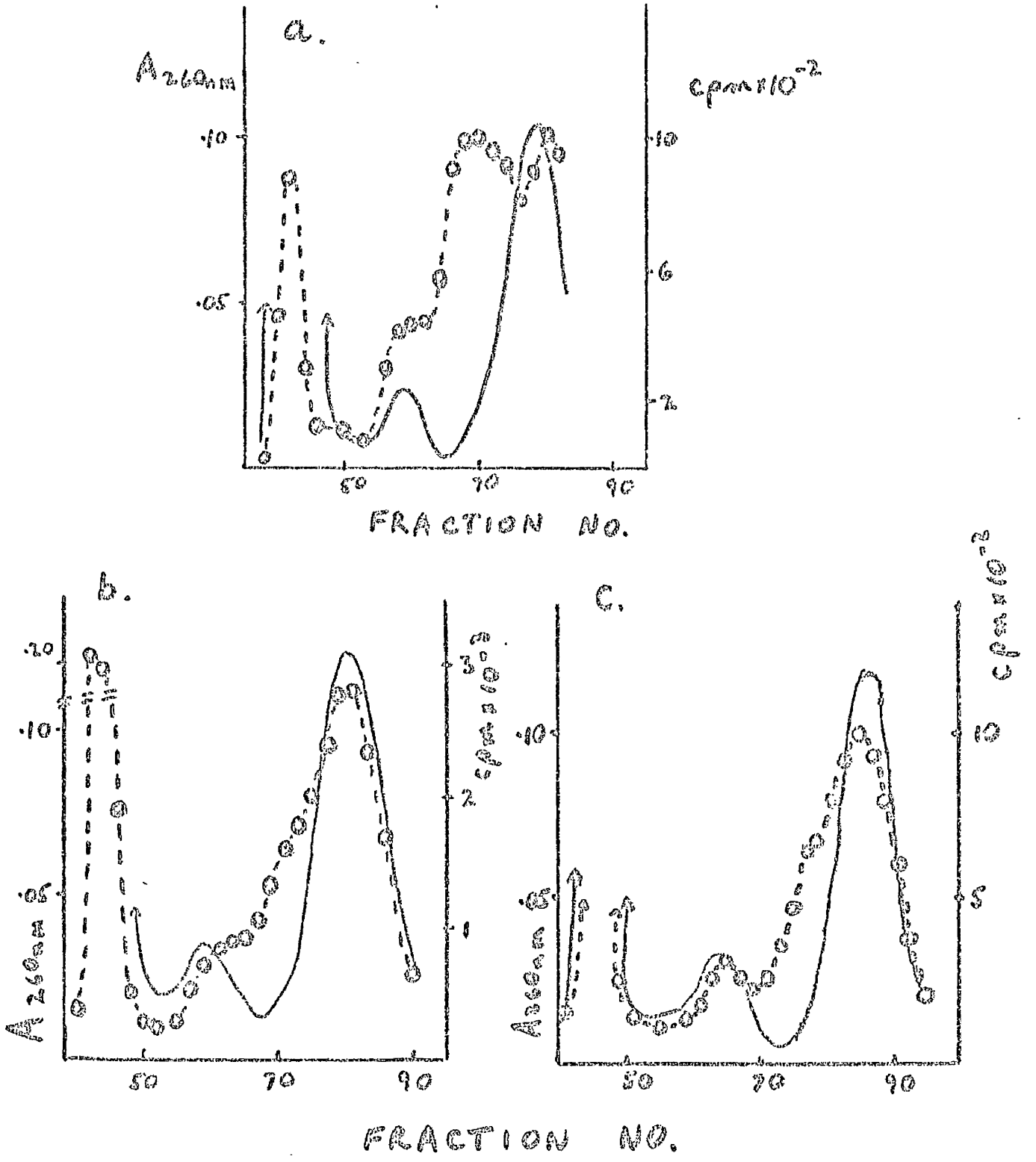
a) 10min incubation with 1.5µc/ml [8-^{3}H]-guanosine

b) 60min and c) 120min incubation with 0.5µc/ml [8-^{3}H]-guanosine.

Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-).

FIGURE III. 9


a.
A_{260nm}
cpm x 10⁻²
.10
.05
10
6
2
50
70
90
FRACTION NO.
b.
c.
A_{260nm}
cpm x 10⁻³
.20
.10
.05
3
2
1
50
70
90
FRACTION NO.

preparations using sucrose gradients. This method of fractionation, although useful for studying rRNAs, does not separate tRNA from other low molecular weight RNAs. Therefore when the action of the drug on C13 cells was tested, the samples were analysed for the presence of pre-tRNA using gel filtration.

Cell cultures were preincubated for 30min with various concentrations of toyocamycin (0.1-0.8 μg/ml) before the addition of [^{3}H] - guanosine (Methods section 4b). After 2h incubation with the labelled precursor, total RNA was prepared by the hot phenol method.

When the cells were incubated with 0.1 μg/ml toyocamycin (Fig III. 10a) the radioactivity appearing in the 28s and 18s regions of the gradient was reduced compared to the pattern obtained with the untreated control cells (Fig III. 2a). The incorporation of [^{3}H] - guanosine into the low molecular weight RNA was relatively unaffected. An additional small peak of radioactivity appeared near the bottom of the gradient (peak A), this was not present in the control RNA preparation and could be due to the expected accumulation of 4.5s RNA caused by the presence of the drug. The fractionation of part of this preparation of total cell RNA on Sephadex G-100 showed that as well as the high molecular weight RNA, 5s and 4s RNA had also been synthesised (Fig III. 10b).

After treatment with 0.4 μg/ml toyocamycin, it was found that no 28s or 18s RNA had been formed but a considerable amount of acid-insoluble radioactivity was present at the top and bottom of the sucrose

Fig. III.10

RNA species synthesised in the presence of 0.1µg/ml toyocamycin.

Cl3 cells were incubated for 30min with 0.1µg/ml toyocamycin. 0.2µc/ml [8-^{3}H]-guanosine was added and the incubation continued for 2h. Total cell RNA was extracted using the hot phenol technique; the RNA preparation was divided into two and treated as follows:-

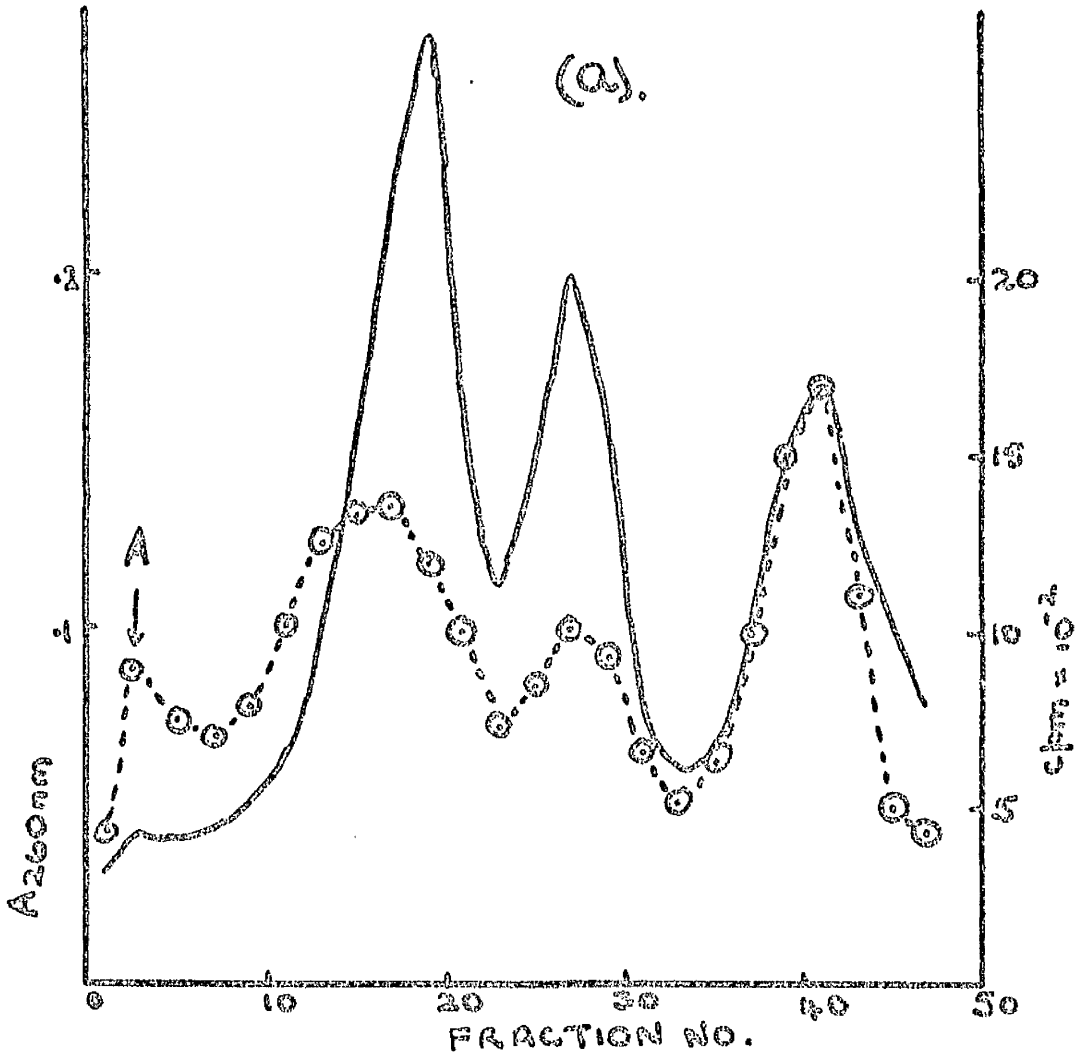
a) Fractionated by zonal ultracentrifugation.

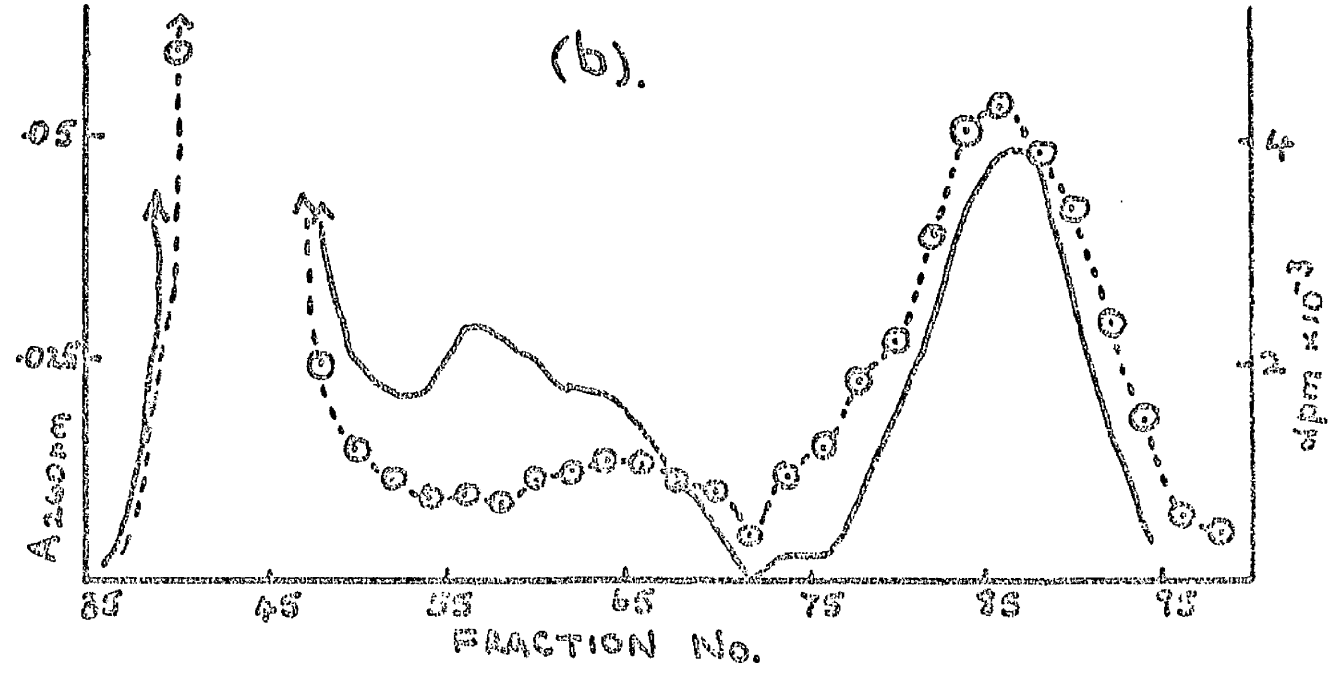
b) Analysed by gel filtration using Sephadex G-100.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.10.





gradient; The elution pattern from Sephadex G-100, showed that 4s RNA and a small amount of 5s RNA were synthesised as well as the very high molecular weight RNA observed on the sucrose gradient. When the toyocamycin concentration was raised to 0.8μg/ml, more than half of the radioactivity was present as high molecular weight RNA, while the remainder occurred at the top of the gradient (Fig III. 11a). Every second sample in this peak of low molecular weight RNA was pooled and, with added cytoplasmic marker RNA, it was fractionated on Sephadex G-100 (Fig III 11b); this showed that the low molecular weight peak contained a small amount of 5s RNA, 4s RNA and some material eluting in front of the 4s RNA - in the pre-tRNA position. At the higher toyocamycin concentration of 2μg/ml the amount of pre-tRNA still dectectable after 2h was slightly greater (Fig III. 12).

It seems therefore that when the toyocamycin concentration was increased to a sufficiently high level, not only was rRNA synthesis affected but so also was the formation of mature tRNA. However, as in the case of methionine starvation, toyocamycin treatment did not prevent conversion of pre-tRNA, it only slowed down the processing. Thus once again an accumulation of pre-tRNA was not achieved. Nevertheless it was decided to use this technique of delaying the processing of pre-tRNA in an attempt to measure the degree of methylation of this RNA species. Since, with toyocamycin treatment, pre-tRNA could be identified for up to 2h

Fig. III.11

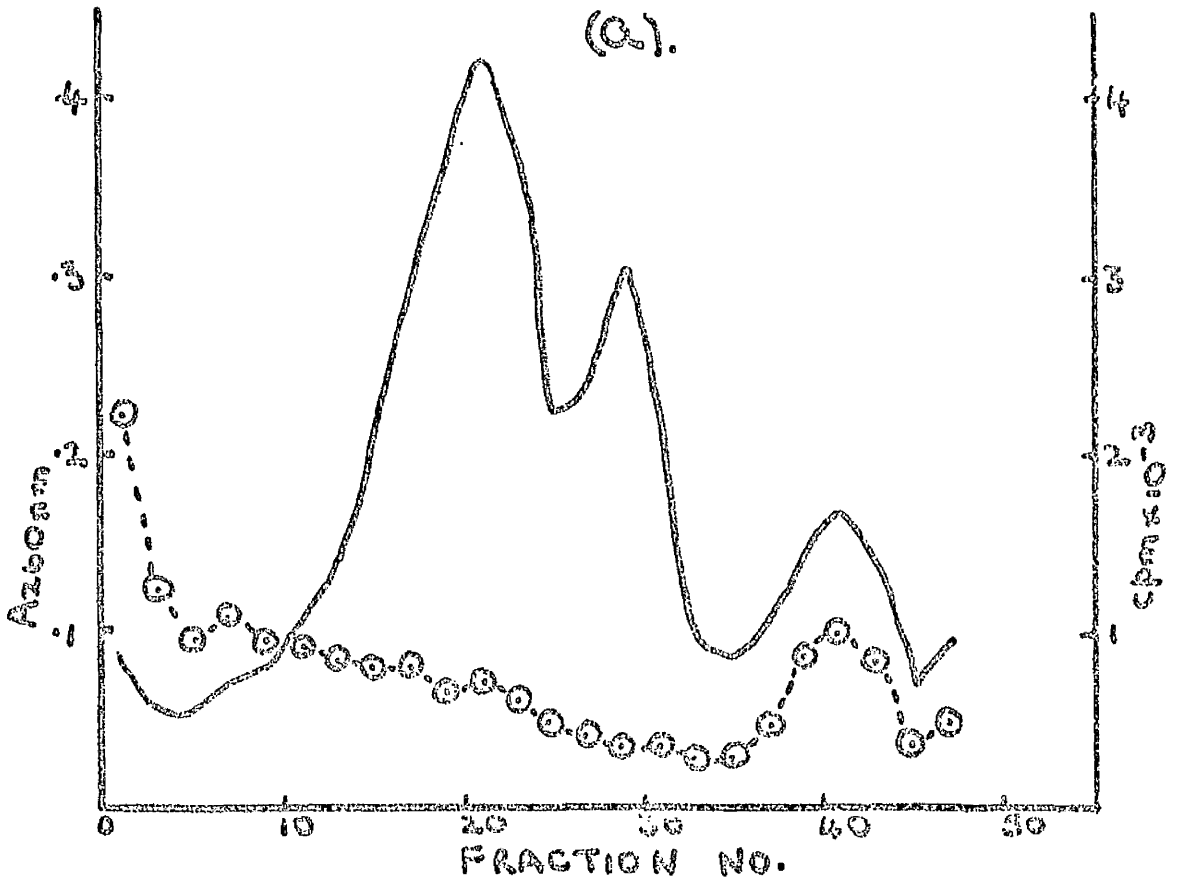
RNA species formed in the presence of 0.8µg/ml toyocamycin.

A culture of C13 cells was preincubated for 30min with 0.8µg/ml toyocamycin before 0.2µc/ml [8-^{3}H]-guanosine was added and the incubation continued for 2h. The hot phenol technique was used to extract total cell RNA which was analysed by zonal ultracentrifugation. Every second fraction was assayed for extinction at 260nm and acid insoluble radioactivity (part a). The remaining fractions were pooled and analysed by gel filtration along with added cytoplasmic RNA marker (part b).

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.11.



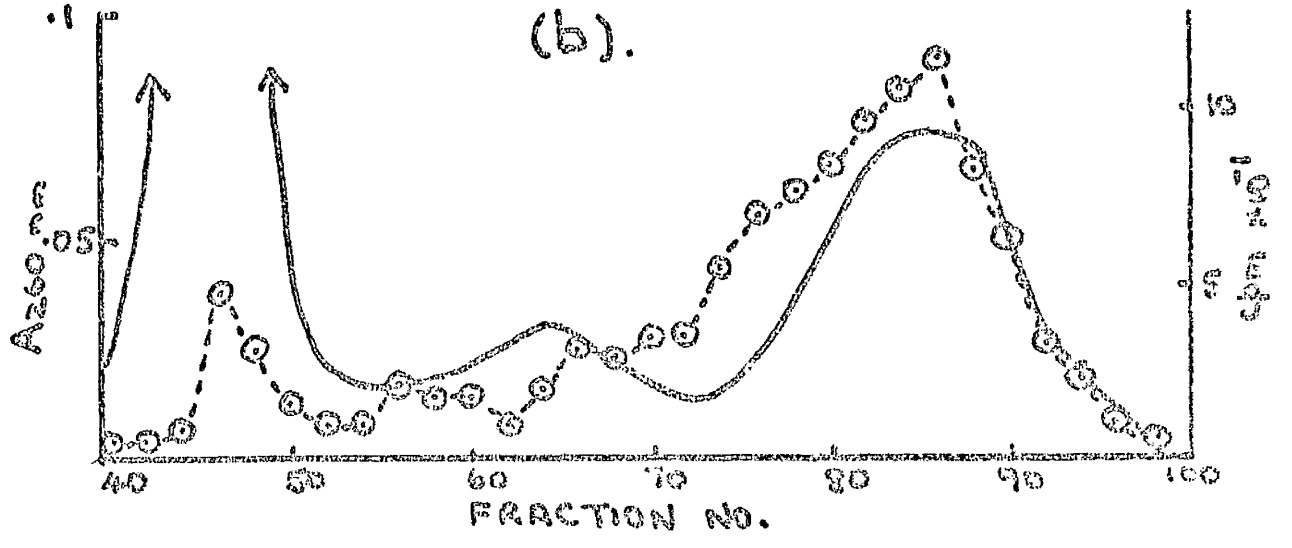


Fig. III.12

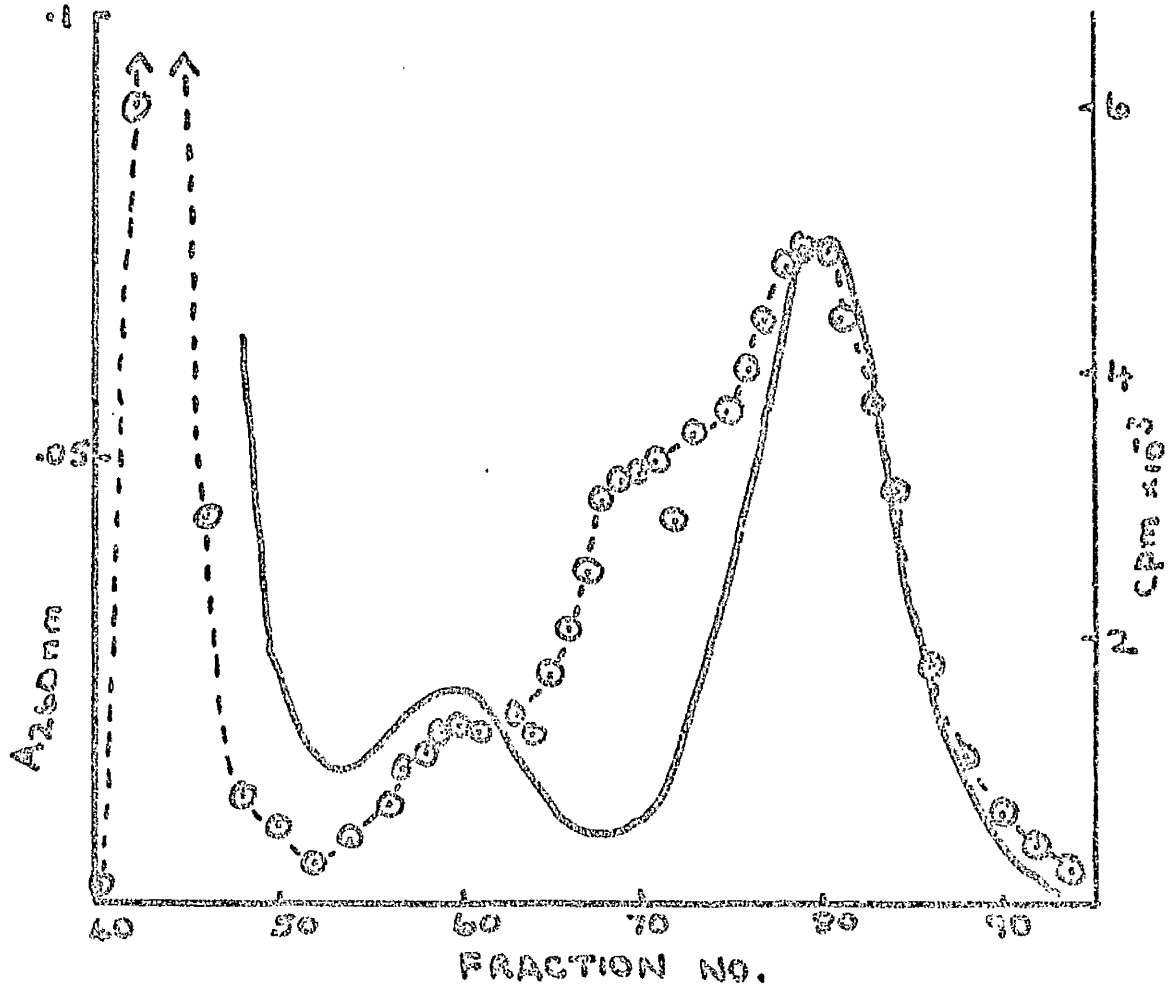
Species of RNA synthesised in the presence of 2µg/ml toyocamycin.

Cl3 cells were preincubated with 2µg/ml toyocamycin for 30min before the addition of 0.5µc/ml [8-^{3}H]-guanosine. Total cell RNA was prepared and analysed by gel filtration on Sephadex G-100

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.12.



after the addition of [^{3}H]- guanosine, it was hoped that more [^{14}C]-methionine would be incorporated into any methyl groups present in pre-tRNA. It had been thought that the short labelling times required to observe pre-tRNA in untreated cells might not allow equilibration of the L-[^{14}C-methyl]-methionine with the intracellular pool of unlabelled methionine; this would explain the very low incorporation of radioactivity which was obtained with the normally growing cells. A culture of C13 cells was treated with .8μg/ml toyocamycin and incubated with [^{3}H]- guanosine, [^{14}C-methyl]-methionine and 20mM-sodium formate for 2h. Total RNA was extracted using the hot phenol technique and fractionated on Sephadex G-100. The fractions were "stripped" of amino acids by incubating in .5M-tris/HCl,pH10 (see Methods 16) and acid-insoluble radioactivity was measured. Fig III.13 shows that the ^{14}C radioactivity coincided with 4s RNA peak of optical density; the radioactivity due to ^{3}H however did not follow the optical density pattern exactly - as expected "extra" counts were present in the pre-tRNA region. This result therefore adds weight to that obtained with the 20min incubation carried out with normally growing cells, namely that pre-tRNA is less methylated than mature tRNA.

c) Resting Cells

When C13 cells are maintained in low serum (0.5% ^{v}/v) medium for 5 days they do not grow and divide; the activities of several enzymes are reduced to very low levels by the 5th day (Fried and Pitts, 1968). Not all enzymes are reduced at the same rate and it is

Fig. III.13

Methylation of RNA formed in the presence of toyocamycin.

The culture of C13 cells was incubated for 30min with 0.8µg/ml toyocamycin. 0.2µc/ml [8-^{3}H]-guanosine and 0.6µc/ml L-[^{14}C-methyl]-methionine were added to the growth medium which was made 20mM with respect to sodium formate. The incubation was continued for 2h before the extraction of total cell RNA. The low molecular weight RNA was analysed by gel filtration on Sephadex G-100. To remove any radioactive methionine bound as methionyl-tRNA, 0.5 volume 1M-tris/HCl, pH10.0, was added to each fraction and incubated at 37°C for 1h before the acid-insoluble radioactivity was assayed. (Methods section 16).

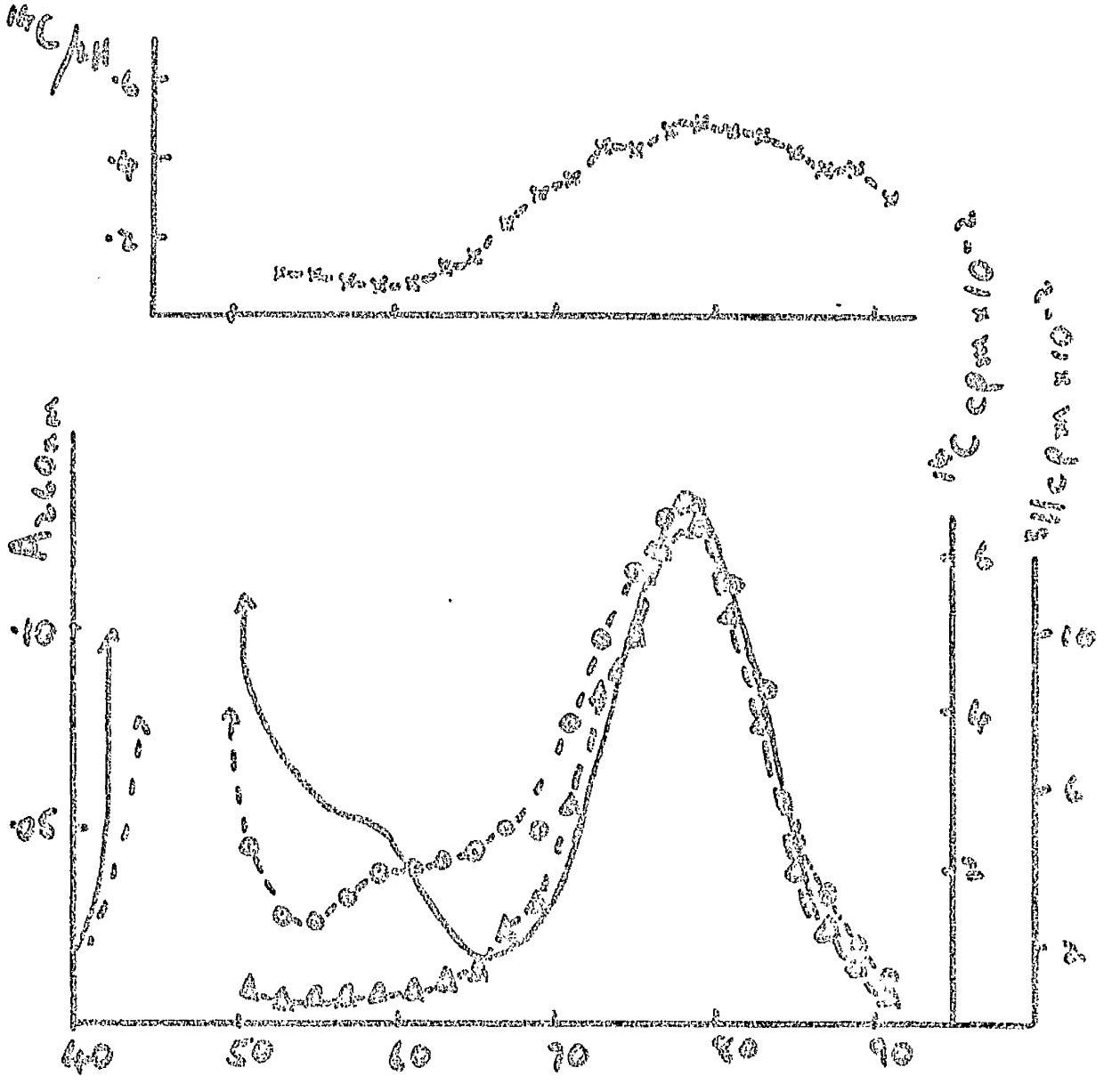
Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-)

Ratio ^{14}C c.p.m./^{3}Hc.p.m. (x-x-x)

Acid insoluble ^{14}C radioactivity (-Δ--Δ-).

FIGURE III.13.



possible that they do not all increase in activity immediately after the addition of normal growth medium containing 10% ($^{v}/v$) serum. In particular it was hoped that this treatment might reduce the rate of pre-tRNA conversion to tRNA, relative to the rate of synthesis of the pre-tRNA molecules and thus an accumulation of them would occur.

Cultures of C13 cells were set up as described in Methods section 6. Following 5 days incubation at 37°C in the low serum medium, [^{3}H]- guanosine was added and after 2h, cytoplasmic RNA was extracted by the cold phenol technique. This RNA preparation was analysed on Sephadex G-100 (Fig III. 14) and found to contain 5s and 4s RNA and material excluded from the gel - the same species of RNA as can be obtained from actively growing cells after a 2h labelling period (Fig III. 1e). A similar result was observed if the low serum medium was replaced with normal growth medium, containing 10% ($^{v}/v$) serum, at the same time as the [^{3}H]- guanosine was added (Fig III. 15). In other words no pre-tRNA could be detected either in resting cells or immediately after their stimulation when a 2h incubation period was used. These experiments indicate that a large change in the relative rates of synthesis and processing of pre-tRNA does not occur in resting cells, but a more detailed study of this system might prove interesting, particularly so because of the results of Kay and Cooper (1969). These authors used human lymphocytes with which they studied the stimulation of resting cells to an actively dividing state; they did indeed find pre-tRNA in these cells after stimulation with PHA.

Fig. III.14

RNA synthesised in resting C13 cells

C13 cells were set up in Eagle's medium containing 0.5%(^{v}/v) calf serum (Methods section 6) and maintained at 37°C for 5 days. 0.2µc/ml[8-^{3}H]-guanosine was added and the incubation continued for 2h. Cytoplasmic RNA was prepared and analysed by gel filtration.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

Fig. III.15

RNA synthesised in resting C13 after the addition of normal growth medium.

C13 cells were set to resting as described in Methods section 6. After 5 days, the medium was replaced with normal growth medium containing 10% (^{v}/v) calf serum. 0.2µc/ml [8-^{3}H]-guanosine was added and the incubation continued for 2h; cytoplasmic RNA was extracted and analysed on Sephadex G-100.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.14.

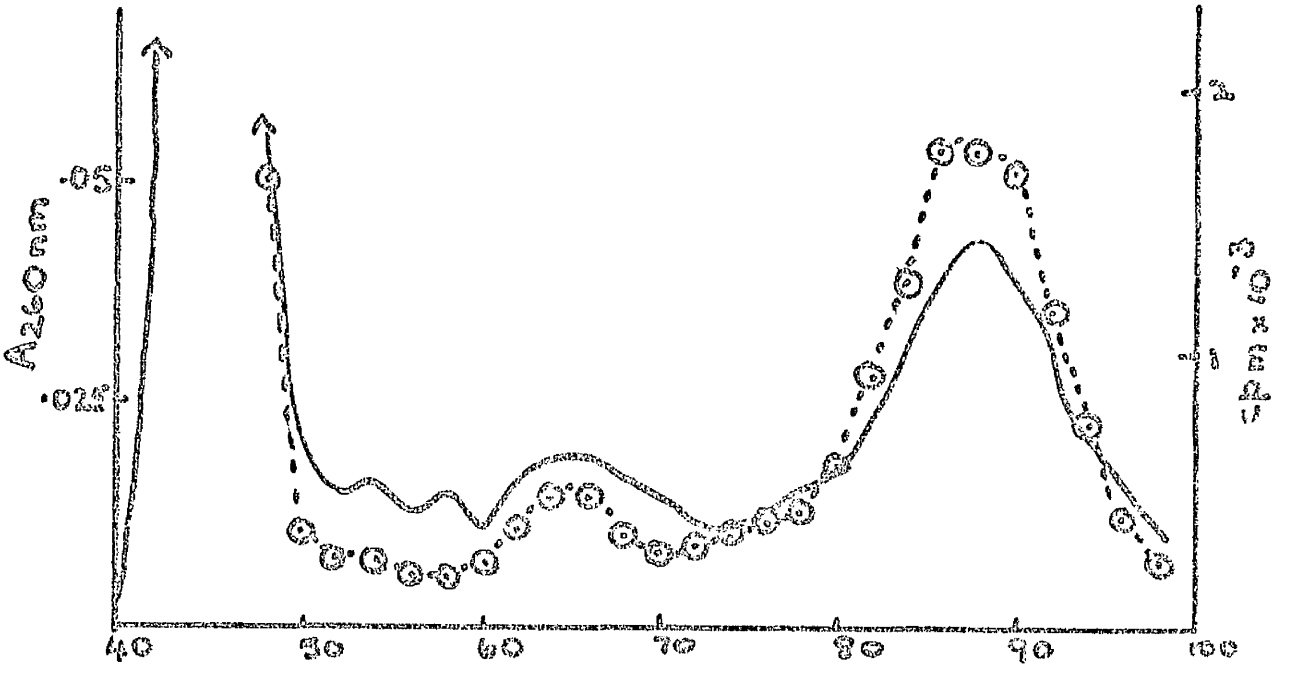
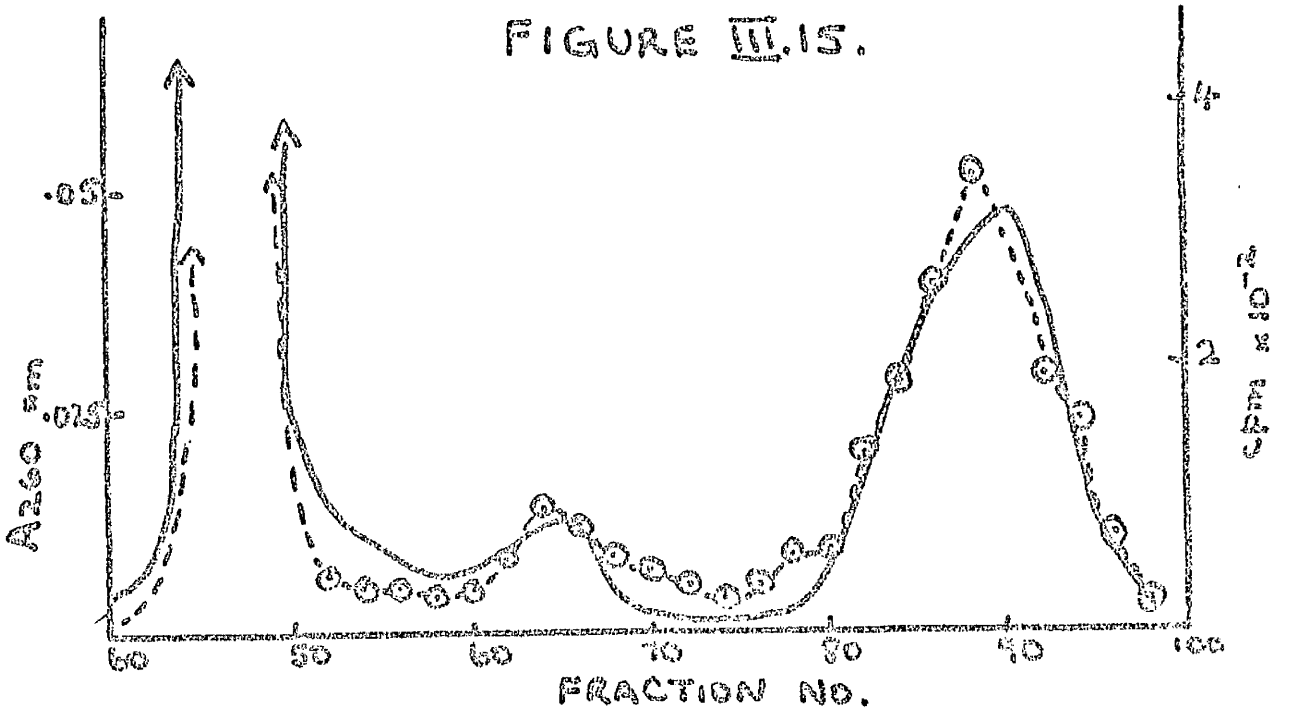


FIGURE III.15.



3. In vitro studies of pre-tRNA

Since no simple method of accumulating pre-tRNA could be found, attempts to make a direct comparison of the nucleotide sequences of pre-tRNA and mature tRNA had to be abandoned. Instead the in vitro conversion of pre-tRNA to 4s material was examined. For these investigations, pre-tRNA was partially purified from 5s and 4s material as described in Methods section 10.

a) Enzymic conversion of pre-tRNA to 4s material

A crude cell extract was prepared from actively growing C13 cells as described in Methods section 11, except that the dialysis step was omitted. Samples of the pre-tRNA preparation were incubated with the cell extract made 10mM with respect to $MgCl_2$ (Methods section 11). After the incubation, protein was removed by phenol extraction, unlabelled cytoplasmic RNA was added to act as marker of the 5s and 4s peaks and the samples were analysed on Sephadex G-100. To act as a control for comparison, pre-tRNA was incubated at 37°C with tris buffer and $MgCl_2$ but without the addition of the cell extract. Fig III. 16a shows that as expected the radioactivity eluted between 5s and 4s RNA - the position of pre-tRNA. After 1h incubation with the extract, the peak of radioactivity coincided with the 4s peak of the marker RNA, but a large proportion of the counts still appeared in front of the 4s RNA (Fig III. 16b). The radioactivity followed the optical density pattern quite closely in the 4s region after a 2h incubation with the cell extract (Fig III. 16c). These results suggest that the extract had converted the pre-tRNA to material which eluted from Sephadex G-100

Fig. III.16

Pre-tRNA incubated with the cell extract for various times.

a) Partially purified pre-tRNA (Methods section 10) was mixed with 1 volume of 0.02M-tris/HCl, pH7.8 and made 10mM with respect to $MgCl_2$. After 2h incubation at 37°C, the RNA was extracted using the cold phenol technique, cytoplasmic RNA was added as marker of the 5s and 4s RNA peaks and the RNA preparation was analysed by gel filtration.

b) The pre-tRNA preparation was incubated with an equal volume of an extract of C13 cells (Methods section 11) in 0.02M-tris/HCl, pH7.8, $MgCl_2$ was added to a final concentration of 10mM. After 1h incubation at 37°C, the RNA was extracted and analysed by gel filtration, non-radioactive cytoplasmic RNA being added to mark the 5s and 4s RNA peaks.

c) as b) except that the incubation was continued for 2h.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III. 16a.

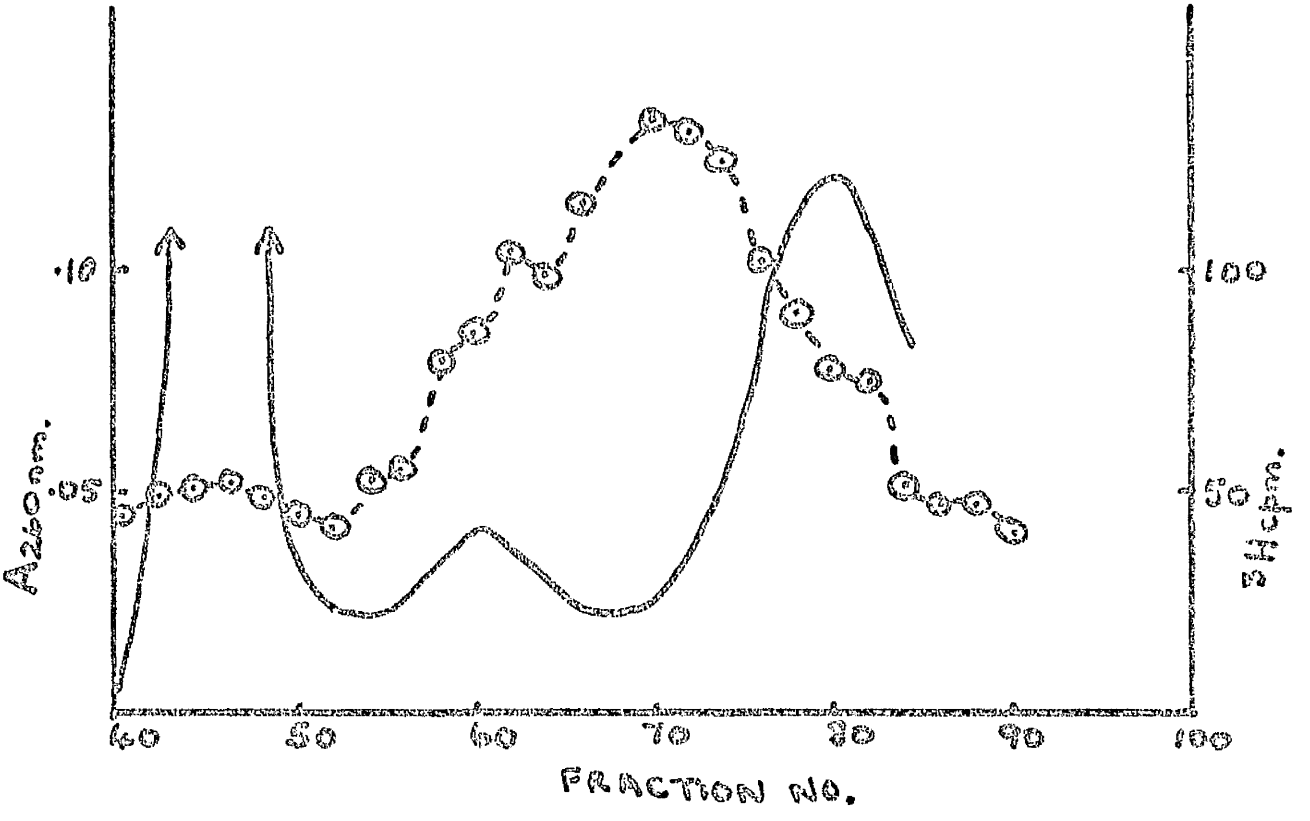
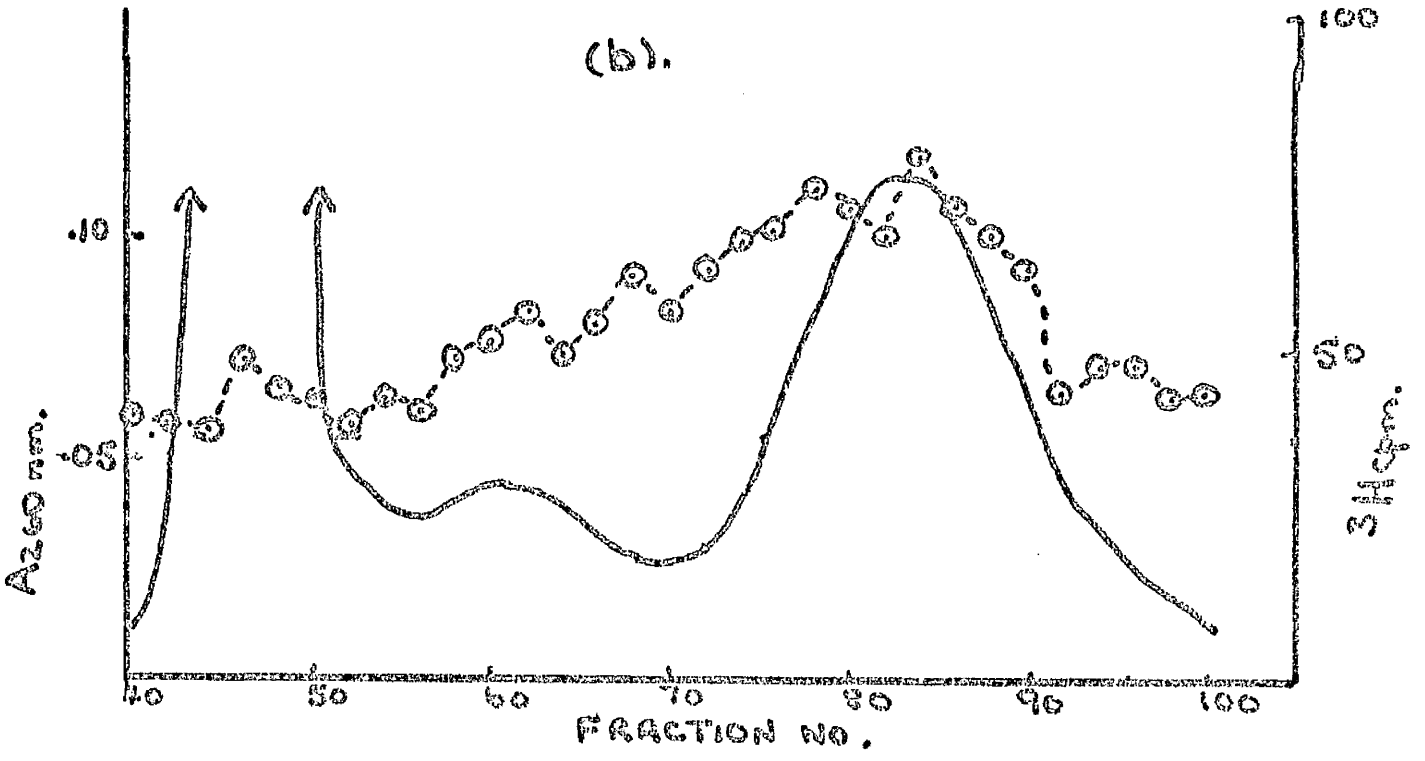
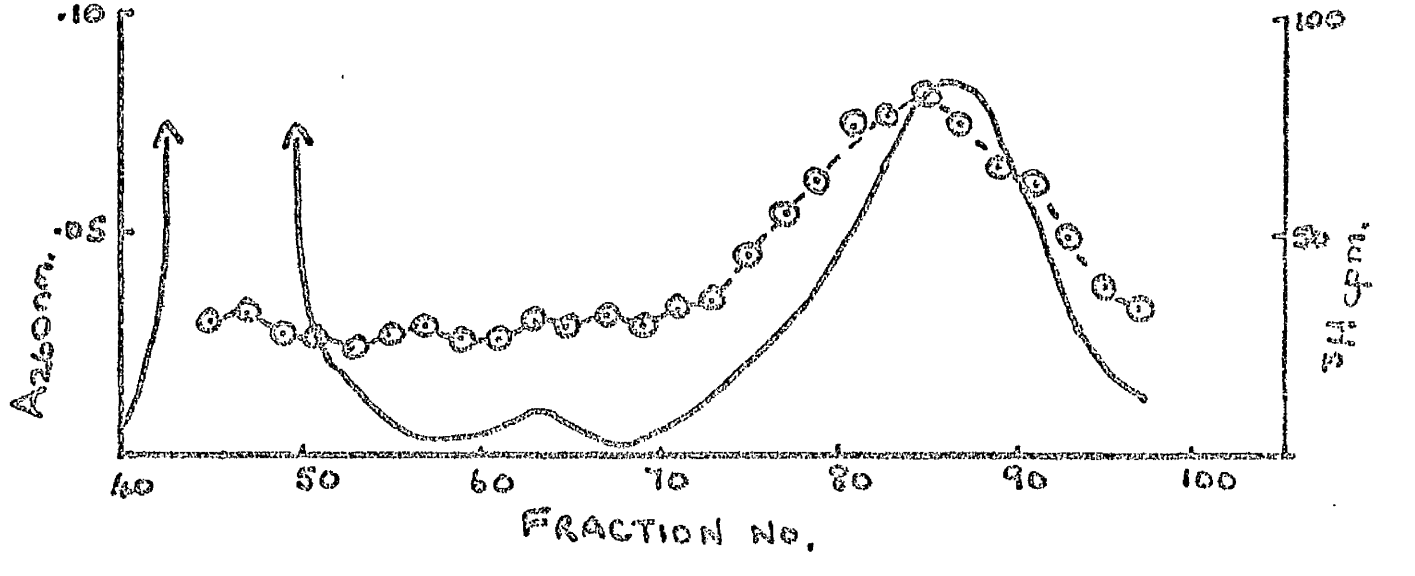


FIGURE III .1b.

(b).



(c).



with tRNA. It is of course possible that this change in elution pattern was due to an alteration in the conformation of pre-tRNA brought about by incubation with certain ions present in the extract and not to a specific enzymic activity. To examine this possibility, a sample of the cell extract was heated in a boiling water bath for 5min and cooled in ice before its addition to the pre-tRNA incubation mixture. This treatment should destroy most enzymes with the possible exception of RNase which possesses an unusual stability to heat - the enzyme isolated from Taka-Diastase has been reported to retain its activity after 10min at 100°C (Uchida and Egami, 1966). When the heat denatured extract of C13 cells was examined for its effect on pre-tRNA, it was found that the radioactivity eluted from Sephadex G-100 in front of the 4s RNA marker, even after a 2h incubation period(Fig III. 17). Therefore the altered elution pattern of pre-tRNA was due to the action of certain enzymes present in the extract. However since the system was obviously very crude, it was necessary to try to decide if the altered elution of the radioactive material were due to the action of a specific enzyme on pre-tRNA or to random degradation of the RNA. The effect of incubating tRNA with the extract was therefore tested. A sample of radioactive tRNA was prepared in a similar manner to that described in Methods section 10 for the preparation of pre-tRNA except that the cells used had been incubated with [8-^{3}H] guanosine for at least 2h. This tRNA

Fig. III.17

Effect of heat on the activity of C13 cell extract.

The extract of C13 cells was heated in a boiling water bath for 5min, then cooled in ice before it was added to the pre-tRNA; $MgCl_2$ was added to a concentration of 10mM. After 2h incubation at 37°C, the RNA was extracted and analysed by gel filtration on Sephadex G-100. Cytoplasmic RNA was added as marker.

Extinction (——)

Acid insoluble radioactivity (-o-o-).

Fig. III.18

tRNA incubated with the extract of C13.

tRNA was prepared from cells grown for 2h with [8-^{3}H]-guanosine. A sample of this tRNA preparation was incubated for 2h at 37°C with an equal volume of the cell extract from C13 cells and $MgCl_2$ was added to a concentration of 10mM. After the extraction of the radioactive RNA from the incubation mixture, non-radioactive cytoplasmic RNA was added to mark the 4s RNA and the RNA preparation was analysed using Sephadex G-100.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.17.

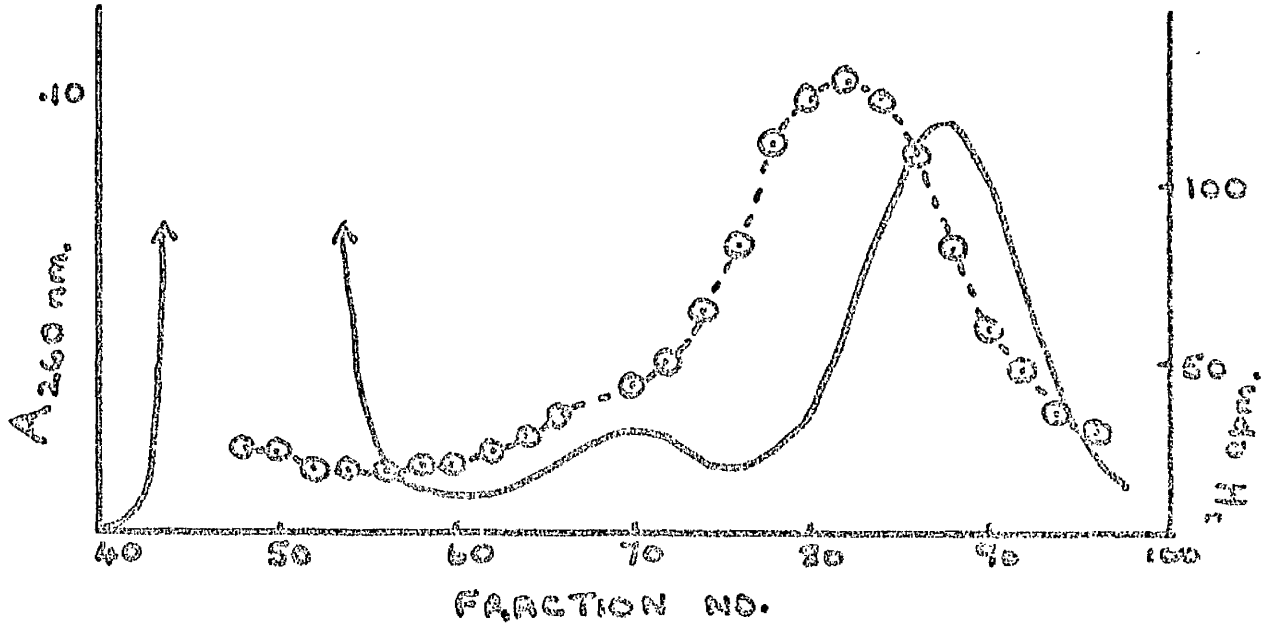
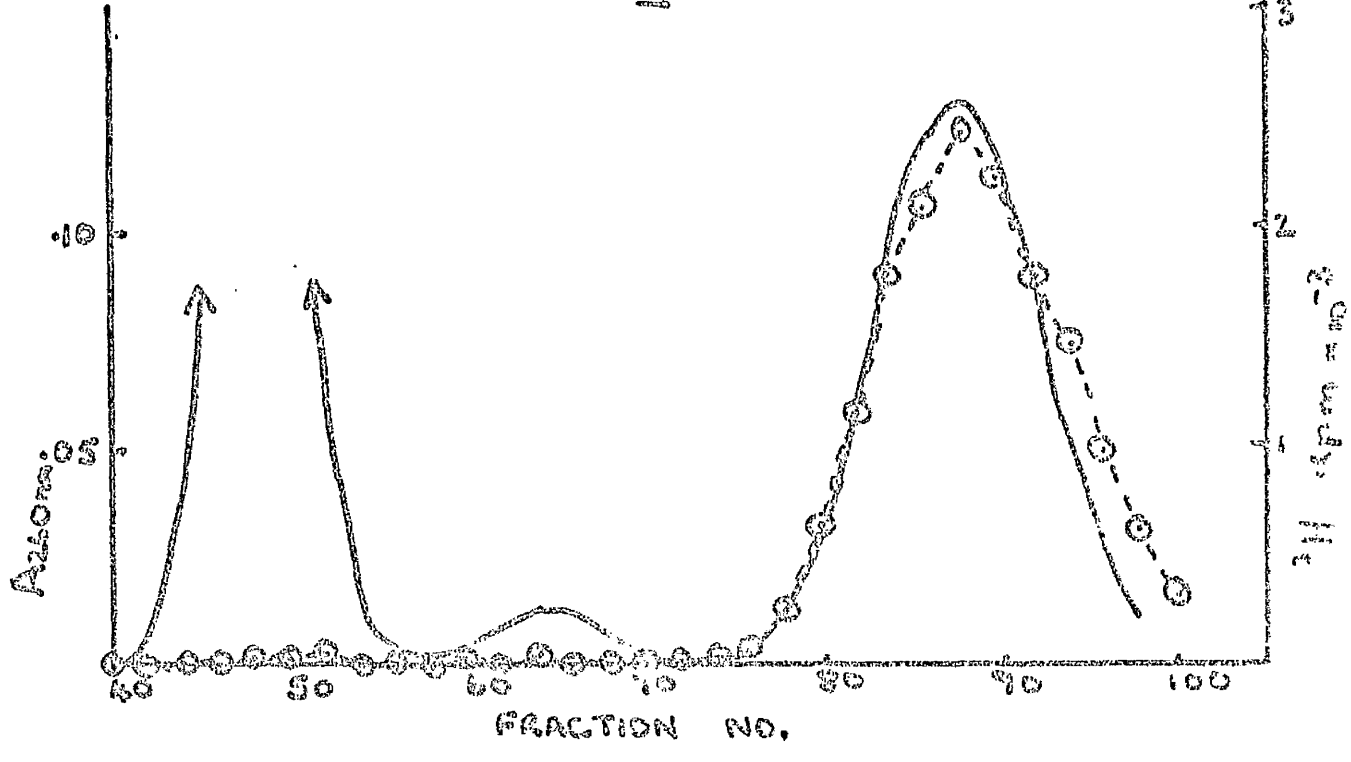


FIGURE III.18.



preparation was then incubated with the cell extract for 2h at 37°C; the radioactive RNA was extracted using the cold phenol method and non-radioactive cytoplasmic RNA was added to act as marker of the 4s RNA. Fig III.18 shows that the radioactivity eluted from the Sephadex G-100 column exactly with marker 4s RNA which had not been treated with the cell extract. Therefore, under the same conditions which allowed the conversion of pre-tRNA to 4s material, incubation with the cell extract caused no alteration in the elution pattern of tRNA. This indicates that a specific effect on pre-tRNA was being observed.

Another question to be answered was whether or not specific cofactors were required to allow the conversion to proceed. Obviously any ions present in the extract must be removed before a determination can be made of those which are essential for the activity of the enzyme responsible for the conversion of pre-tRNA to 4s material. The cell extract was therefore dialysed against tris/HCl buffer, pH 7.8, as described in Methods section 11. Since the dialysis could remove inhibitory ions as well as those required for the optimum activity of the enzyme, a 1h incubation period was chosen so that either an increase or reduction in the rate of conversion could be determined. The fractionation on Sephadex G-100 of the radioactive RNA after incubation with the dialysed extract is presented in Fig III. 19 and comparison with Fig III.16b indicates that dialysing the extract caused virtually no difference in the rate of conversion of

Fig. III.19

Activity of the Cl3 extract after dialysis.

The extract of Cl3 cells was dialysed against 0.02M-tris/HCl, pH7.8, before its addition to the pre-tRNA incubation mixture containing $MgCl_2$ (10mM). After 1h incubation at 37°C, RNA was extracted and analysed with Sephadex G-100. The pattern of extinction at 260nm is due to added cytoplasmic RNA.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

Fig. III.20

Effect of adding s-adenosylmethionine to the cell extract.

0.5ml of the dialysed extract of Cl3 cells was added to an equal volume of pre-tRNA containing $MgCl_2$ (10mM). 12.5µmoles of non-radioactive s-adenosylmethionine was added, a 1h incubation at 37°C was carried out and the extracted RNA was analysed by gel filtration. Marker cytoplasmic RNA was added.

Extinction at 260nm (——)

Acid insoluble radioactivity (-o-o-).

FIGURE III.19.

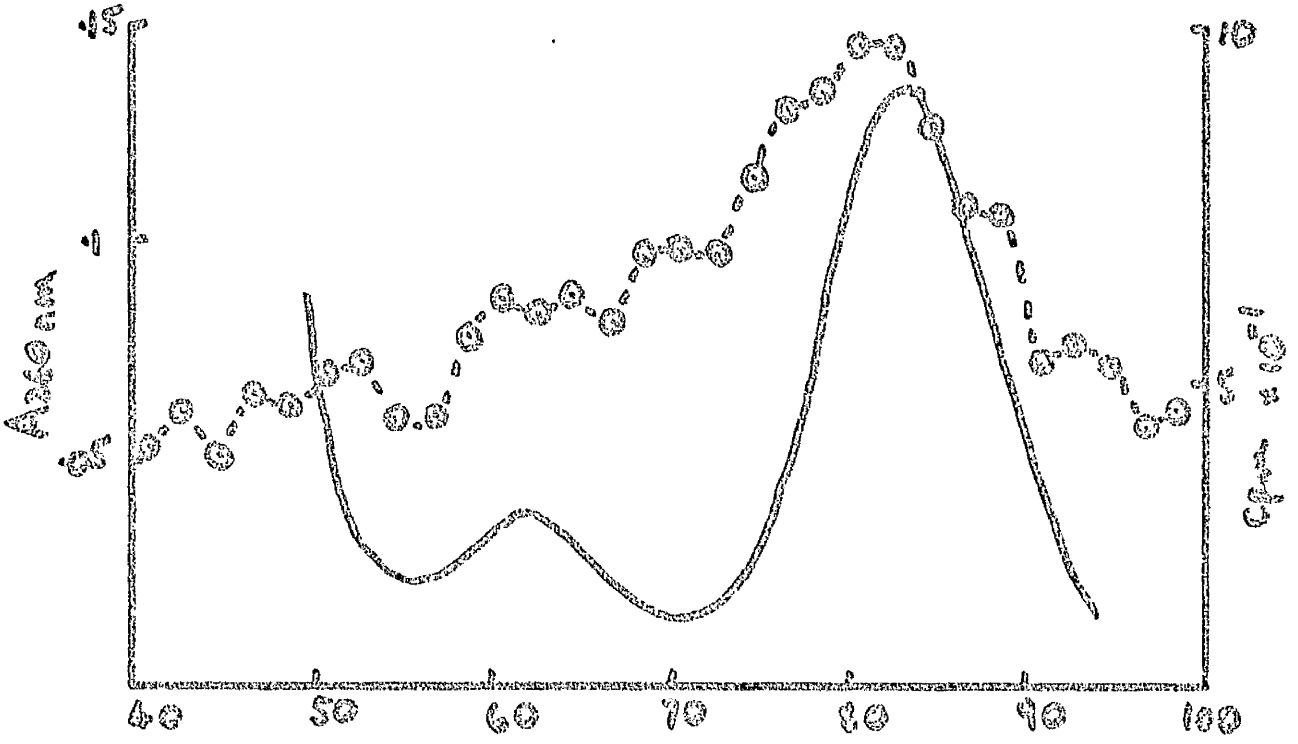
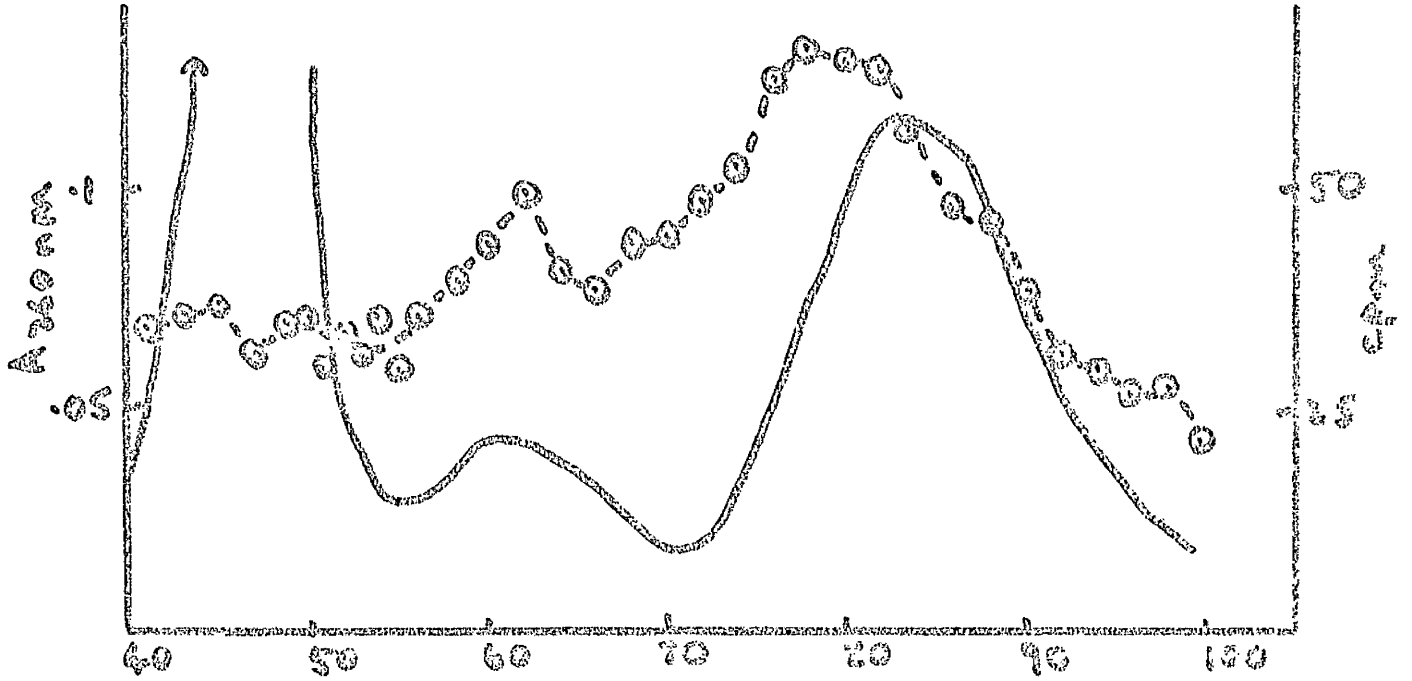


FIGURE III.20.



pre-tRNA to 4s material. While there appear to be no ions essential for the conversion of pre-tRNA to 4s material it is possible that the addition of certain ions could increase the activity of the enzyme, however, due to the complex nature of the crude cell extract, it was decided that these investigations would be more meaningful if delayed until a purified preparation of the enzyme had been prepared.

b) Modifications occurring during the conversion of pre-tRNA to 4s material

i) Methylation

Since pre-tRNA has been shown to be less methylated than mature tRNA, it must acquire methyl groups during its conversion to tRNA. In the absence of methods for accumulating pre-tRNA, it is difficult to prepare sufficient material to measure directly the incorporation of labelled methyl groups during the incubation with the cell extract. An indirect approach was therefore taken to decide whether or not methylation occurred during the in vitro incubation. It is known that s-adenosylmethionine is the precursor of the methyl groups present in RNA (Fleissner and Borek, 1963); therefore it would be expected to increase the rate of conversion of pre-tRNA to tRNA if methylation were involved in the reaction. An incubation mixture containing pre-tRNA and dialysed cell extract was prepared and s-adenosylmethionine was added to the same concentration as was required for assaying tRNA methylase (Burdon, Martin and Lal, 1967). A 1h incubation at 37°C was again used so that any increase in the rate of conversion could be observed. Comparison of Fig III. 20 with Fig III. 16b. shows that no increase in the rate of conversion of

pre-tRNA to 4s material did occur following the addition of s-adenosylmethionine and indicates that methylation is not necessary to allow the conversion to take place. This result is in agreement with the initial investigation of pre-tRNA carried out by Lal and Burdon (1967). These authors showed that inhibition of tRNA methylation by the addition of ethionine did not prevent the conversion of pre-tRNA to 4s RNA *in vivo*.

ii) Pseudouridine formation

tRNA is known to contain pseudouridine (Ψ), in fact as much as 20-25% of the uridine in tRNA may be present as pseudouridine. Unlike the methylated nucleosides, the method of formation of pseudouridine is not yet decided; two hypothesis have been put forward, namely the rearrangement theory in which pseudouridine is formed from uridine already incorporated into the polynucleotide chain, and the incorporation theory which suggests that pseudouridine is incorporated directly during the formation of the polynucleotide. So far no enzyme has been isolated which is capable of modifying uridine already present in the polynucleotide chain, although Weiss and Legault - Demare (1965) have reported studies with *Esch. coli* spheroplasts which suggest that RNA may be an intermediate in the conversion of uridine to pseudouridine. On the other hand attempts to incorporate ΨTP directly into RNA have proved contradictory (for a review see Goldwasser and Heinrikson, 1966). Obviously a knowledge of the pseudouridine content of pre-tRNA should allow a decision to be

made between these hypotheses. A sample of pre-tRNA was therefore prepared using [G-^{3}H] - uridine as the labelled RNA precursor; it was purified as before (Methods section 10) and hydrolysed to its constituent nucleotides (Methods section 14). The hydrolysate was analysed by two-dimensional paper chromatography using the solvents described in Methods section 15. UMP-2' (or 3') and ΨMP-2' (or 3') could be identified under u.v. light due to the Esch. coli B sRNA which had been added to act as an internal marker; the appropriate areas were cut out and assayed for radioactivity (Methods section 16). It was found that C13 pre-tRNA contained a negligible amount of pseudouridylate, less than 1% of the total uridylate. Although the pseudouridine content of C13 tRNA has not been reported, this value for pre-tRNA is much lower than the 20% normally found in tRNA.

Siddiqui et al (1970) have shown that cyanoethylation of the only pseudouridine present in Esch. coli formylmethioine tRNA alters the tertiary structure of the molecule; the complete lack of this minor nucleoside may also reduce the stability of the normal tRNA configuration. It is therefore conceivable that the formation of pseudouridine during the incubation of pre-tRNA with the cell extract could cause the altered elution pattern which was observed. A sample of pre-tRNA labelled with [G-^{3}H] - uridine was incubated with the dialysed cell extract for 2h, the acid precipitable radioactivity was prepared and alkali hydrolysed; the hydrolysate was analysed for UMP and ΨMP as before. No increase in the pseudouridine content could be detected. Lack of pseudouridine therefore does not cause the different

positions of elution from Sephadex G-100 of pre-tRNA and tRNA.

iii) Alteration in length

From his studies with Krebs II ascites cell pre-tRNA, Burdon (1967a) has concluded that a change in conformation alone is not sufficient to account for the earlier elution from Sephadex G-100 of pre-tRNA compared to tRNA - a reduction in molecular weight appeared also to be required. The results of Bernhardt and Darnell (1969) were in agreement with this conclusion. If pre-tRNA does have a longer polynucleotide chain than tRNA, the extra nucleotides should be removed during the conversion of pre-tRNA to 4s material. No acid-insoluble material could be detected eluting from Sephadex G-100 after the peak of 4s RNA. Likewise no material smaller than 4s RNA could be detected when portions of each fraction were assayed for total radioactivity (see Methods 16). These negative results may be explained by the large dilution with buffer which is expected when small molecules are eluted from a column of the size used for these experiments. A different technique was obviously required if any low molecular weight material were to be detected following the incubation of pre-tRNA with the cell extract. Chromatography on DEAE-paper has been shown to separate oligonucleotides according to size, large molecules remaining at the origin while mononucleotides travel up the paper with R_F values of from 0.6 for purine to 0.9 for pyrimidine mononucleotides (Furlong, 1965).

As it is obviously possible that the extra nucleotides could

Fig. III.21

Effect of incubating ^{3}H-C labelled pre-tRNA with the crude extract of C13 cells.

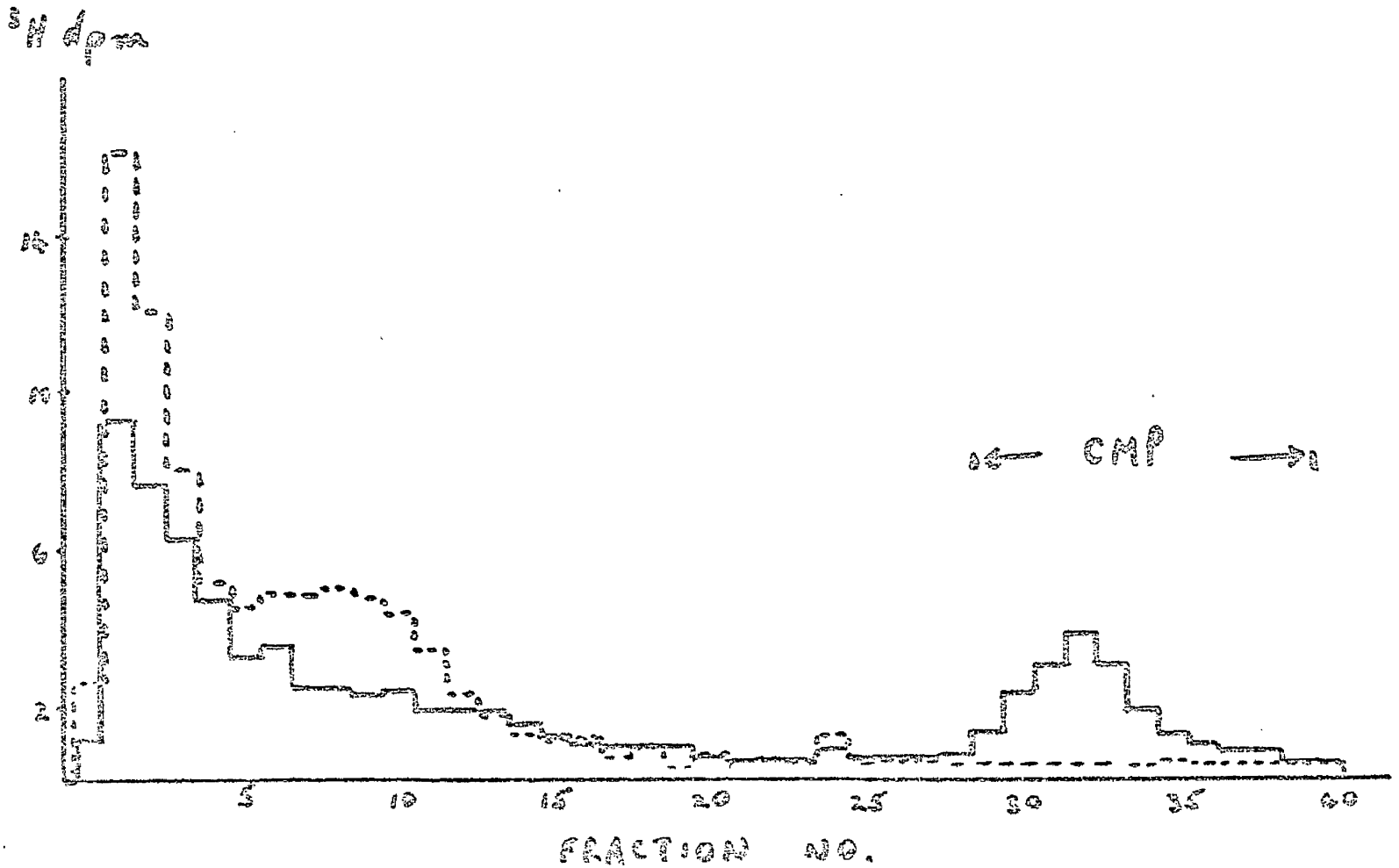
Partially purified pre-tRNA, extracted from C13 cells labelled for 10min with [^{3}H]-cytidine, was mixed with one volume of C13 cell extract in 0.02M-tris/HCl buffer, pH7.8, plus 10mM-$MgCl_2$ and incubated for 2h at 37°C. After the incubation, the protein was removed by one phenol extraction and the aqueous layer was analysed by DEAE-paper chromatography using 0.75M-ammonium bicarbonate, pH8.6, as solvent; non-radioactive CMP was cochromatographed to act as a marker and detected under u.v. irradiation. The dried chromatogram was cut up into 0.5cm strips and assayed for radioactivity (Methods 16)

^{3}H radioactivity (——).

To act as a control, a second sample of pre-tRNA was incubated with a heat denatured portion of the cell extract and analysed by DEAE-paper chromatography as before.

^{3}H radioactivity (- - - -).

FIGURE III. 21



consist of any combination of the 4 major nucleotides, it is necessary to prepare pre-tRNA labelled with each nucleotide and carry out separate analyses using the DEAE-paper chromatography technique described in Methods section 13. The appropriately labelled pre-tRNA samples were therefore incubated with the cell extract for 2h and the protein was removed by phenol extraction before the sample was applied to the DEAE-paper. When pre-tRNA, prepared from cells incubated with [^{3}H]- cytidine, was analysed, the result shown in Fig III. 21 was obtained. A small peak of radioactivity appeared in the position of the CMP marker, this amounted to 24.3% of the total radioactivity the remainder of which appeared at or near the origin. When the pre-tRNA was incubated with a portion of cell extract which had been heat inactivated, all the radioactivity remained near to the origin. Therefore during the incubation with the cell extract, which is known to convert pre-tRNA to 4s RNA, 24.3% of the cytidylic acid present in pre-tRNA is released and moves on DEAE-paper with CMP. The experiment was repeated using pre-tRNA labelled with [^{3}H]- guanosine and [^{14}C]- uridine (Fig III. 22 a and b). On this occasion 50% of the ^{14}C radioactivity appeared in the UMP position and once again the appearance of this peak of radioactivity depended on the activity of the enzymes present in the cell extract since with heat denatured extracts, all the ^{14}C radioactivity remained at the origin. A slightly different pattern was obtained with the ^{3}H radioactivity derived from [^{3}H]- guanosine. No peak of radioactivity was observed in the position

Fig. III. 22 a & b.

Effect of incubating ^{14}C-U and ^{3}H-G labelled pre-tRNA with the extract of Cl3 cells.

a) Partially purified pre-tRNA, extracted from Cl3 cells labelled for 10min with [^{3}H]-guanosine and [^{14}C]-uridine, was mixed with one volume of Cl3 cell extract made 10mM with respect to $MgCl_2$ and incubated for 2h at 37°C. After the incubation, the protein was removed by one phenol extraction and the aqueous layer was analysed by DEAE-paper chromatography using 0.75M-ammonium bicarbonate, pH8.6, as solvent; non-radioactive UMP and GMP were added as markers and detected under u.v. light. The dried chromatogram was cut up into 0.5cm strips and assayed for radioactivity (Methods section 16).

^{3}H radioactivity (——)

^{14}C radioactivity (- - - -).

b) (On next page) A second sample of the [^{3}H]-guanosine and [^{14}C]-uridine labelled pre-tRNA was incubated with a heat denatured portion of the cell extract and analysed by DEAE-paper chromatography as before.

^{3}H radioactivity (——)

^{14}C radioactivity (- - - -).

FIGURE III.22.(a).

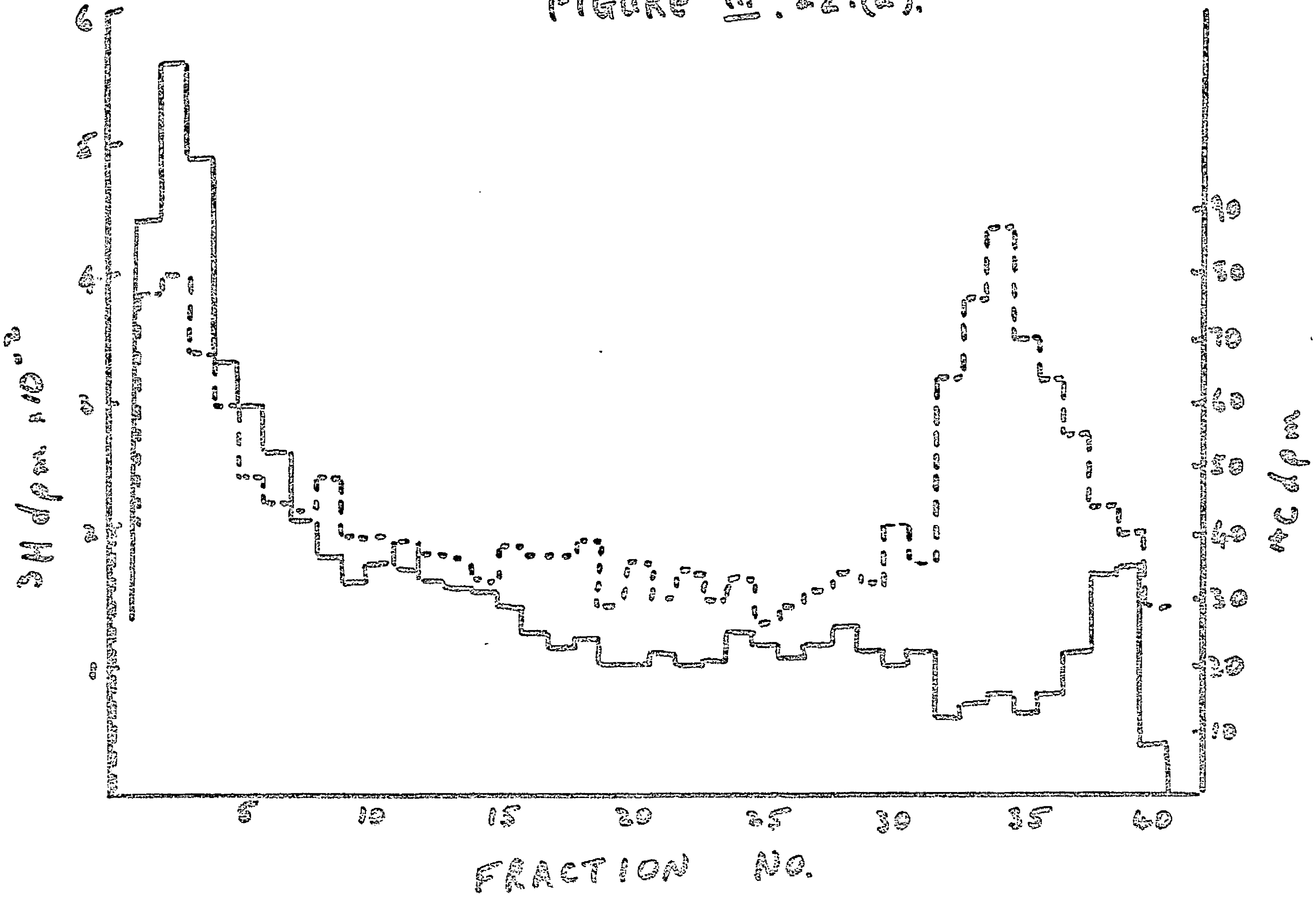
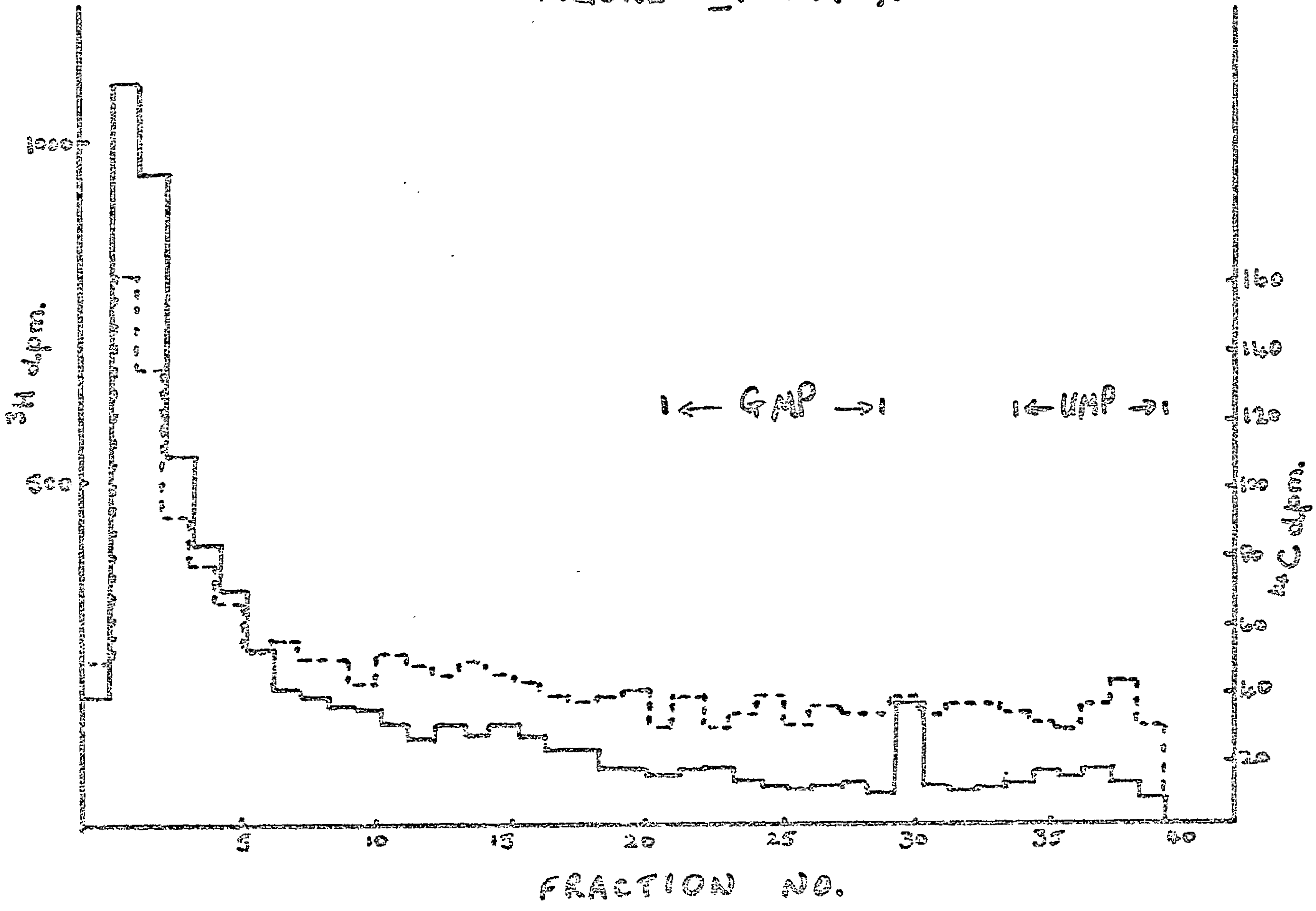


FIGURE III. 22.(b).



of the GMP marker, nevertheless 28% of the ^{3}H radioactivity had moved up the DEAE-paper as far as or further than GMP. When the incubation was carried out with the boiled cell extract, 13.8% of the ^{3}H radioactivity still appeared in this position. This release of mononucleotide material by the inactivated extract is surprising, particularly as it does not happen with either C or U. It is possible that during the preparation of the pre-tRNA some guanosine or its derivatives has remained absorbed to the RNA but it is released during the incubation at 37°C. But whatever the reason for this non-enzymatic release of radioactivity, this means that only 14.2% of the ^{3}H radioactivity is removed from the pre-tRNA during its conversion to 4s material. Pre-tRNA labelled with [^{3}H]- adenosine did not show this anomalous behaviour, 6% of the radioactivity being released as mononucleotides by a completely heat labile action of the extract of C13 cells (Fig III. 23). Therefore during the incubation of pre-tRNA with the cell extract, it is converted to material which elutes from Sephadex G-100 with tRNA and this conversion is accompanied by the release of 50% of the uridine, 24.3% of the cytidine, 14.2% of the guanosine and 6% of the adenosine which had been incorporated into the pre-tRNA. Assuming that pre-tRNA is converted to tRNA, it is possible to calculate the number of nucleotides which have been removed during the incubation. The composition of C13 tRNA is given in Table III. 24 Uridylic acid represents 18.3% of the nucleotides. Therefore assuming an average chain length of 80 nucleotides, tRNA contains 14.6 uridylate

Fig. III.23.

Effect of incubating ^{3}H-A labelled pre-tRNA with the extract of C13 cells.

Partially purified pre-tRNA prepared from C13 cells labelled for 10min with [^{3}H]-adenosine, was mixed with one volume of C13 cell extract made 10mM with respect to $MgCl_2$ and incubated at 37°C for 2h. After the incubation, the protein was removed by one phenol extraction and the aqueous layer was analysed by DEAE-paper chromatography using 0.75M-ammonium bicarbonate, pH8.6, as solvent; non-radioactive AMP was added as marker and detected under u.v. light. The dried chromatogram was cut up in strips and assayed for radioactivity (Methods section 16).

^{3}H radioactivity (——)

A second sample of [^{3}H]-adenosine labelled pre-tRNA was incubated with a heat denatured portion of the cell extract and analysed by DEAE-paper as before.

^{3}H radioactivity (----).

FIGURE III.23

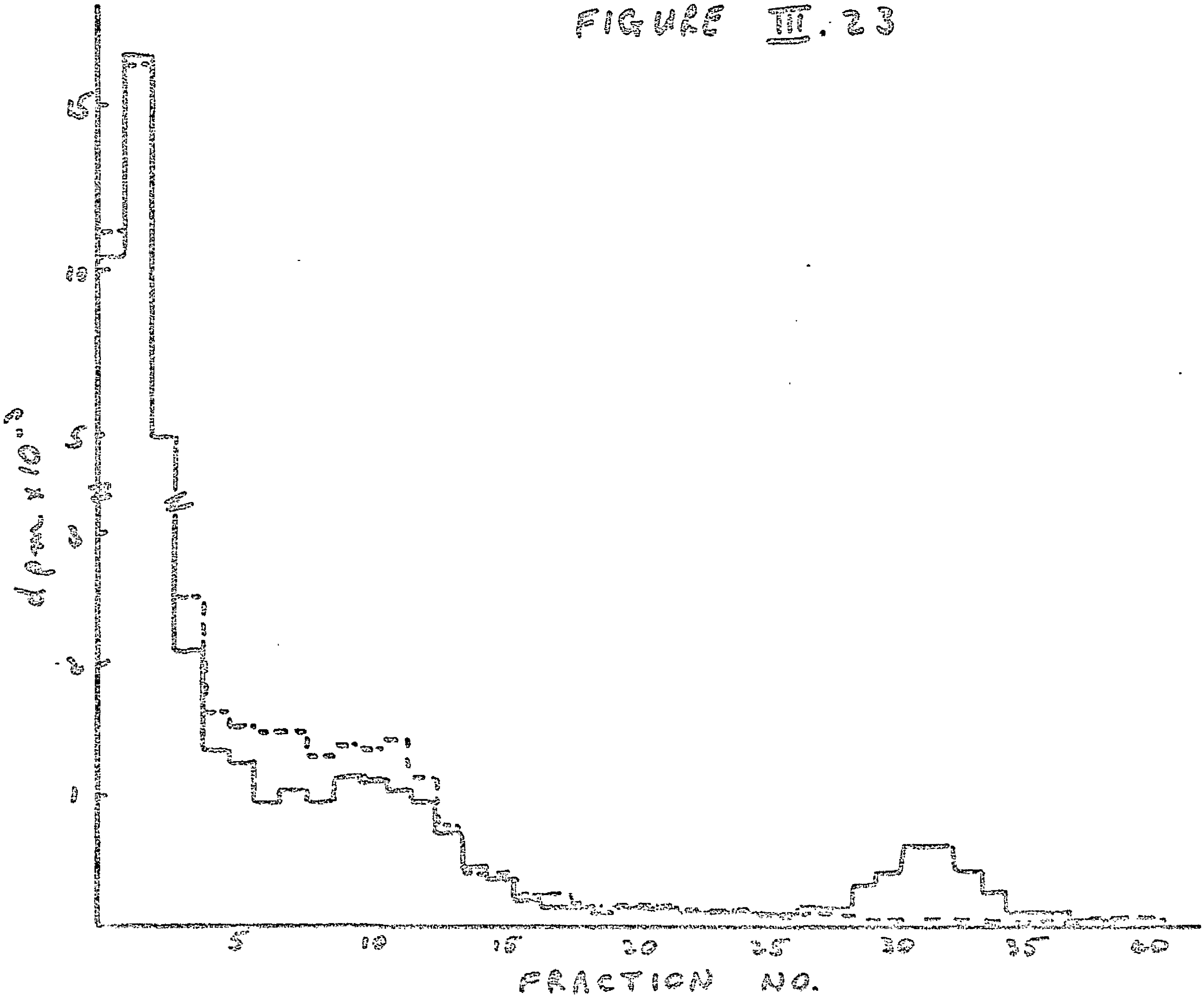


Table III.24

Nucleotide Composition of C13 tRNA and pre-tRNA.

		Nucleotides.			
		U	C	G	A
	% composition	18.3	28.3	31.6	21.8
tRNA	number per average chain length of 80 nucleotide	14.6	22.7	25.5	17.4
pre-tRNA	number of nucleotides calculated on the assumption that tRNA contains 80 nucleotides	29	30	29	18

residues. During the incubation with the extract, tRNA is assumed to be formed and 50% of the uridylate residues present in pre-tRNA are removed. This means that the 14.6 nucleotides present in tRNA represent the 50% of the uridylate which remains in the oligonucleotide fraction after the period of incubation. Similarly since 24.3% of the cytidylic acid in pre-tRNA is removed during the incubation, this means that 75.7% of the cytidylate in pre-tRNA is equivalent to 22.7 nucleotides or that 7 cytidylic acid residues are released. Using the same method of calculation 1 adenylate and 4 guanylate residues are found to be released.

Therefore pre-tRNA must contain 14U, 7C, 1A and 4G more than tRNA (Table III.24).

iv) Position of the extra nucleotides

Since pre-tRNA is not large enough to contain more than one tRNA, these extra nucleotides must be present at one or other or even at both ends of the precursor molecule. Some information of the positioning of these extra nucleotides can be gained if the pre-tRNA is partially digested by exonucleases. These enzymes conduct a stepwise degradation of nucleic acid and are specific for one or other end of the nucleotide chain. Snake venom phosphodiesterase removes 5' mononucleotides from the 3' end of RNA molecules (Razzell and Khorana, 1959; Razzell, 1963). Although this enzyme is more active when acting on short oligonucleotides, it will still degrade larger molecules; it is particularly useful since, unlike spleen

phosphodiesterase which attacks from the 5' end of RNA molecules, snake venom phosphodiesterase shows no specificity for the nucleotides attacked. (Razzell and Khorana, 1959; Brownlee and Sanger, 1967). In other words each nucleotide is released at the same rate and this simplifies the interpretation of the results since it means that if one nucleotide is released more rapidly than the others, it must be present at a higher concertration at the 3' end of the molecule.

To determine the position of the extra nucleotides, pre-tRNA was prepared from Cl3 cells which had been incubated for 10 min with [^{3}H]- cytidine, [^{3}H]- uridine and [^{3}H]- guanosine. Portions of this pre-tRNA were treated with snake venom phosphodiesterase as described in Methods section 12 and samples were removed at the times indicated. The reaction was stopped by mixing with EDTA (Razzell, 1963) and cooling in ice; the nucleotides released were analysed by two-dimensional paper chromatography (Methods 15) using the 5' monophosphates of the major nucleosides as markers. The fraction of the total counts present as each nucleotide was calculated. Knowing the distribution among the nucleotides of the radioactivity incorporated into the pre-tRNA (determined from an alkaline hydrolysate of the pre-tRNA) the fraction of each nucleotide released by the snake venom phosphodiesterase at each time can be calculated (Fig III.25). UMP and GMP were removed from the pre-tRNA molecule throughout the incubation with the enzyme but no CMP was released until after 30 min incubation. Since snake venom phosphodiesterase shows no preference for the different nucleotides, this

Fig. III.25.

Degradation of pre-tRNA by snake venom phosphodiesterase.

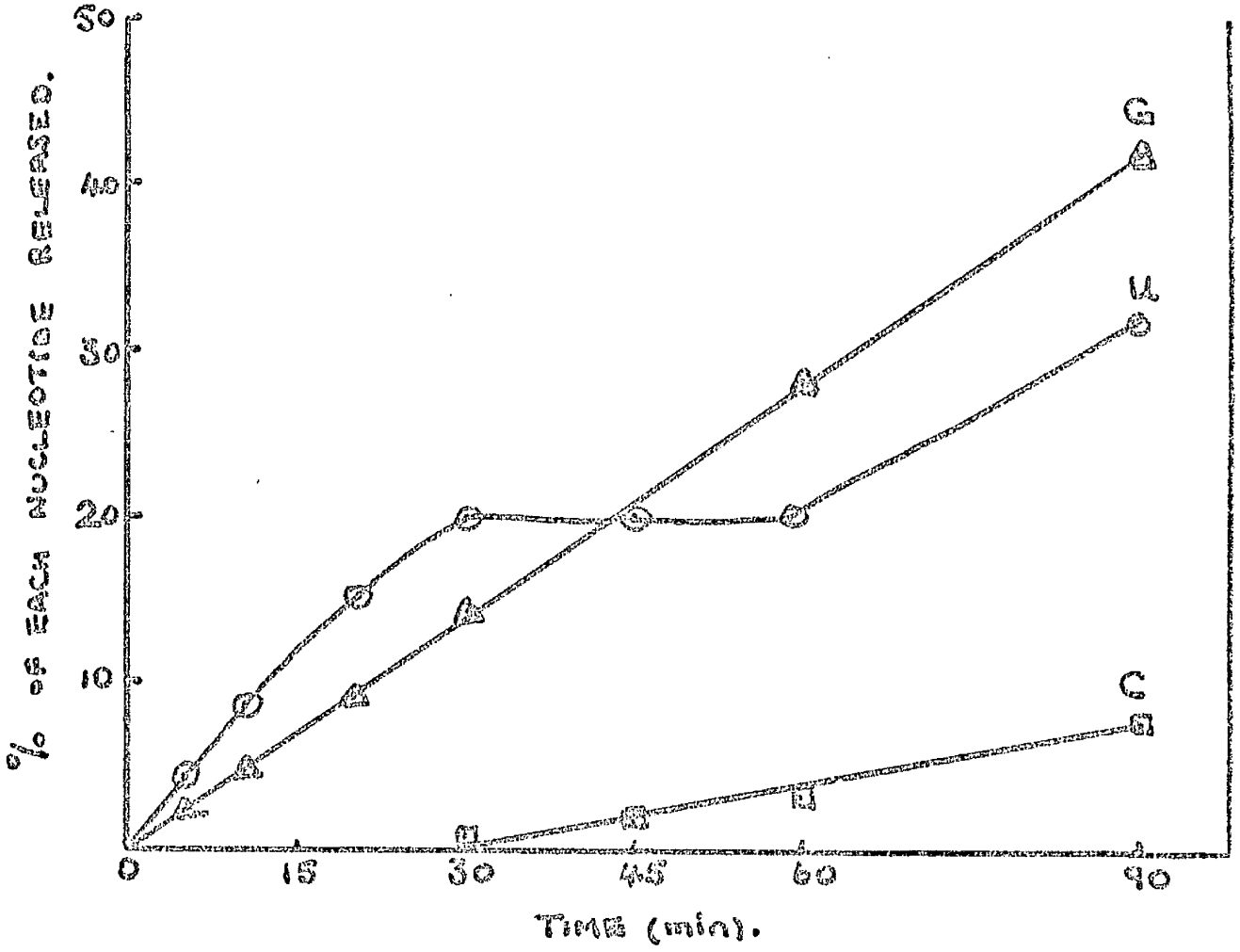
Partially purified pre-tRNA, prepared from C13 cells labelled with [^{3}H]-guanosine, [^{3}H]-cytidine and [^{3}H]-uridine, was digested with snake venom phosphodiesterase as described in Methods section 12. Samples were removed at various times after the addition of the enzyme and analysed by two-dimensional chromatography on Whatman 3MM paper (Methods section 15). The fraction of the total radioactivity present as each nucleotide was calculated. Knowing the distribution among the nucleotides of the radioactivity incorporated into pre-tRNA (obtained from an alkaline hydrolysate of a sample of the pre-tRNA preparation), the fraction of each nucleotide released by the snake venom phosphodiesterase at each sampling time could be calculated.

Per cent of guanylate residues of pre-tRNA released by phosphodiesterase action (△——△)

Per cent of cytidylate residues of pre-tRNA released by phosphodiesterase action (□——□)

Per cent of uridylate residues of pre-tRNA released by phosphodiesterase action (o——o).

FIGURE III.25.



must mean that very few Cs are close to the 3' end of the pre-tRNA molecule. An examination of the known tRNA sequences (Philipps, 1969) shows that the 3' terminal sequence in tRNA molecules contains a high proportion of C residues even after the removal of the pCpCpA sequence. Therefore pre-tRNA possesses a 3' terminal sequence unlike that from tRNA. Also, since snake venom phosphodiesterase is known to be inhibited by the presence of a phosphate on the 3' terminal nucleotide (Razzell and Khorana, 1959), pre-tRNA must possess a free 3' hydroxyl group.

While it is not yet possible to decide the exact sequence of these extra nucleotides, these preliminary results allow an estimate to be made of whether or not all the extra nucleotides are present at the 3' end of the pre-tRNA molecule. To do this the actual number of nucleotides released, rather than the percent value, is required and this can be calculated from the composition of pre-tRNA reported in the previous section (Table III. 24). The result is shown in Fig III. 26. The maximum number of guanylic acid residues which can be removed during the conversion of pre-tRNA to 4s material was found to be four. Thus during the digestion of pre-tRNA with snake venom phosphodiesterase, the first four guanylates released may be derived from the extra sequence but any further guanylates must have arisen from the tRNA sequence assumed to exist within the pre-tRNA molecule. Of course not all of these four extra guanylic acid residues need occur at the 3' end of the precursor molecule but with the data at present available it is only possible to estimate the maximum number of extra nucleotides at the 3'

Fig. III.26.

The number of each nucleotide released from pre-tRNA by the action of snake venom phosphodiesterase.

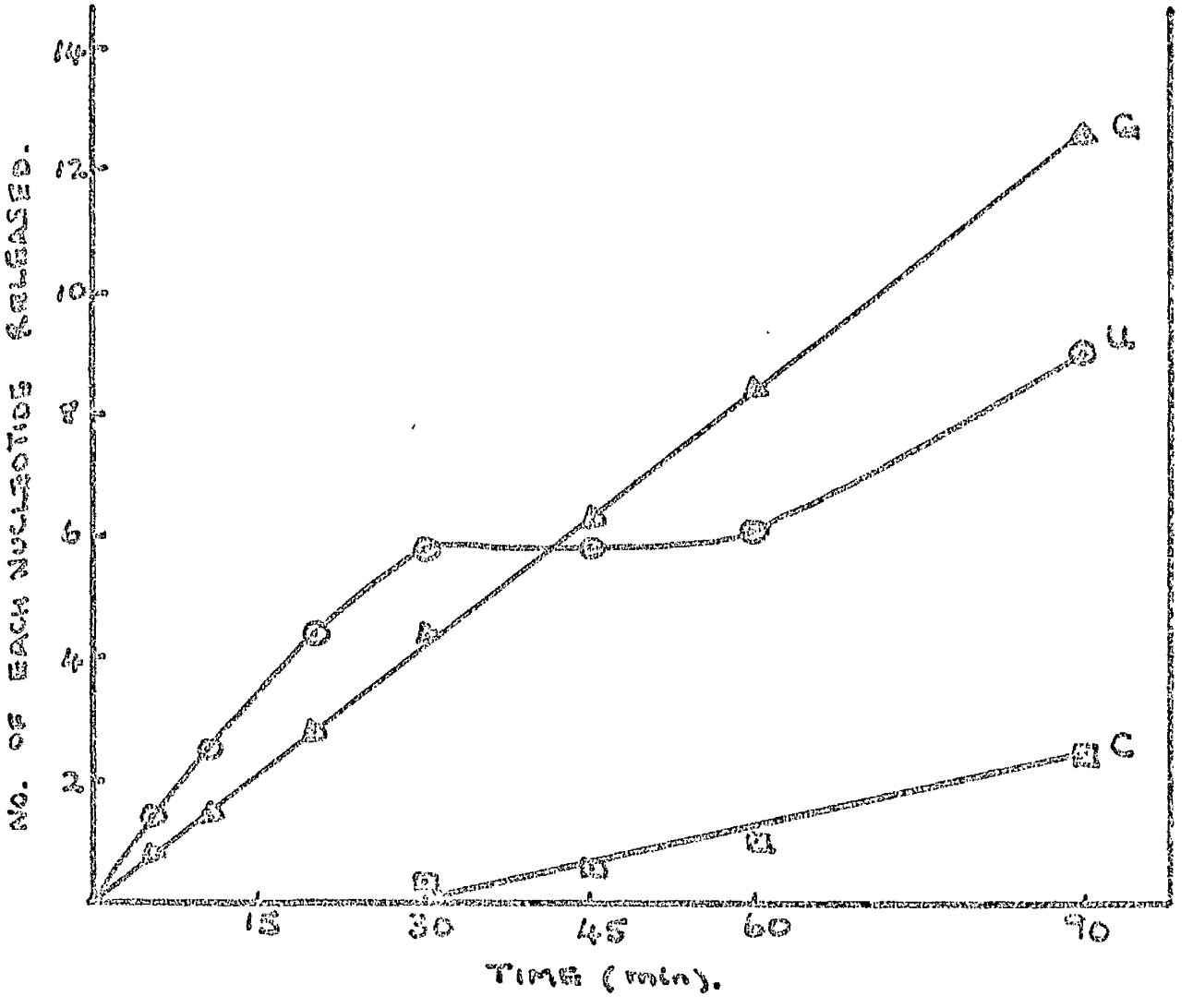
The results of Fig.III.25 were expressed as the number of each nucleotide released by the action of the phosphodiesterase. The values for the base composition of pre-tRNA shown in Fig. III.24 were used in this calculation.

Number of guanylic acid residues released (△—△)

Number of cytidylic acid residues released (□—□)

Number of uridylic acid residues released (o—o).

FIGURE III. 2b.



end of pre-tRNA. After 25min incubation with the phosphodiesterase, four guanylic acid residues have been released from pre-tRNA by the action of the phosphodiesterase (Fig III. 26) and during this time five uridylates but no cytidylates have been released. Thus probably not all the extra nucleotides occur at the 3' end of the pre-tRNA molecule; the maximum number at the 3' end may be (U_5G_4) while at least (U_9C_7) occur at the 5' end of the molecule. However as these figures rely on the calculation of the base composition of pre-tRNA, they must be considered only as estimates until they are confirmed by more direct evidence such as the determination of the base ratios of pre-tRNA or the results of degrading the molecule from the 5' end.

B. Methylation of tRNA and malignancy

Since 1963 Borek and his coworkers have carried out a study of the levels of tRNA methylating enzymes present in various mammalian tissues. They have come to the conclusion that tumours contain higher levels of methylase activity than do the adjacent normal tissues, in fact increases of up to ten times the normal level have been observed (Tsutsui et al, 1966). Borek suggested that this increase might be due to the invasion of the cell by a foreign, e.g. viral, tRNA methylase which could then cause hypermethylation of the tRNA present in the cell. By analogy with methyl-deficient tRNA, the addition of extra methyl groups might be expected to cause changes in the amino acid binding and coding properties of the tRNA molecules; this in turn could affect the delicately controlled protein synthesising system, resulting in the abnormal growth of the tumour cells.

Two points mar this hypothesis. Firstly although the tumours were compared with the adjacent tissues, this may not give an adequate control as malignant cells are known to be capable of migrating through the circulation (Willis, 1952; Engell, 1955) and thus produce tumours at some distance from their point of origin. Secondly the work of Kaye and Leboy (1968) has shown that the tRNA methylase activity of tissue extracts depends critically on the ionic composition of the assay medium; indeed under optimum conditions no difference could be found between extracts of normal and tumour cells.

In order to further investigate the problem of the relative

degrees of methylation of tRNA from normal and malignant cells it was decided to use a tissue culture system in which the relationships between the various cell lines was known exactly. Also comparison of the number of methyl groups actually incorporated into tRNA was thought to be of more importance than the levels of the enzyme extracted from the cells.

1. Levels of tRNA methylation in various cell lines

One disadvantage of using a tissue culture system is that only small amounts of material are available. It is therefore not possible to study the methylated nucleotides of tRNA by chemical methods e.g. by isolation and estimation by u.v. spectroscopy, however due to the easily controlled environment these cells are ideally suited to studies carried out with radioactive isotopes. Methionine is known to be the precursor of the methyl groups in RNA (Mandel and Borek, 1961b and 1963) therefore the addition to the growth medium of methionine labelled in the methyl group should allow an estimation to be made of the number of methyl groups present in tRNA. However two problems must be overcome before an accurate result can be obtained. Firstly, methionine is known to be a precursor not only of the methyl groups of RNA but, via the one carbon pool, it also forms the purines adenine and guanine (Kit et al, 1958) thus addition of methionine alone would give too high a value for the degree of methylation of RNA. This problem can be overcome by the addition of sodium formate to a concentration of 20mM as this prevents the incorporation of methyl groups from methionine into

the one carbon pool (Winocour, Kaye and Stollar, 1965). Secondly, since RNA is known to be methylated soon after it is synthesised, the majority of the tRNA molecules present in the cell are fully methylated and cannot accept the radioactive methyl groups from the added methionine. This makes it impossible to compare the degree of methylation of RNA from two different cell cultures by calculating the amount of radioactivity incorporated from L-[^{14}C-methyl]- methionine per mg of RNA since not only would too low a value be obtained but errors might arise due to differences in the rates of RNA synthesis. Obviously some means must be found to distinguish between the newly synthesised RNA molecules which are capable of accepting methyl groups and the mature fully methylated tRNAs. A dual isotope technique provides such a method - the addition of a labelled ribonucleoside e.g. [^{3}H]- uridine, allows the rate of RNA synthesis to be measured while the incorporation of methyl groups may be followed using L-[^{14}C-methyl]- methionine.

To test this system a culture of Cl3 cells was set up at a concentration of 10^7 cells per 80 oz Winchester bottle and grown for two days by which time the glass surface was approximately half covered with cells. The medium was then replaced by 50ml of Eagle's medium containing 0.5μc/ml L-[^{14}C-methyl] - methionine, 0.5μc/ml [G-^{3}H]- uridine and 20mM-sodium formate; the incubation at 37° was continued for 22h (approximately 1½ generations) and cytoplasmic RNA was prepared by the cold phenol method (Methods 8a). This RNA preparation was then

analysed both by zonal ultracentrifugation and by gel filtration so that an examination could be made of almost the complete range of cytoplasmic RNAs. Fig III. 27 shows the separation of 28s, 18s and low molecular weight RNA obtained by the sucrose density-gradient analysis. Tritium radioactivity, derived from the labelled uridine, was found in all three peaks indicating, as expected, that these species of RNA were synthesised during the incubation period. ^{14}C - radioactivity, arising from the incorporation of methyl groups, was also present in all three peaks. However, unlike the ^{3}H radioactivity, it did not follow exactly the pattern of extinction at 260nm indicating that these three fractions of RNA were not equally methylated. The ratio of the ^{14}C radioactivity to ^{3}H radioactivity was taken as the relative degree of methylation of the RNA present in each fraction (Fig III. 27a). From this it can be seen that 18s RNA contained about 1.33 times as many methyl groups per uridine as did 28s RNA; also the low molecular weight peak contained the most highly methylated material. Since the low molecular weight region is known to contain other species of RNA as well as tRNA, it was necessary to analyse the RNA preparation by gel filtration. Using this technique the 28s and 18s RNAs elute together but 5s and 4s RNA which cosediment in the sucrose density-gradient are clearly separated. When the RNA prepared from the cells incubated with [^{3}H]- uridine and L-[^{14}C-methyl]-methionine was analysed on Sephadex G-100 (Fig III. 28), the ^{3}H radioactivity once again followed exactly the extinction at 260nm but the ^{14}C radioactivity followed a very different pattern being

Fig. III.27.

Sucrose density-gradient analysis of cytoplasmic RNA from C13 cells labelled with [^{3}H]-uridine and L-[^{14}C-methyl]-methionine.

C13 cells were incubated for 22h with [^{3}H]-uridine (0.5μc/ml), L-[^{14}C-methyl]-methionine (0.5μc/ml) and 20mM-sodium formate. Cytoplasmic RNA was extracted by the cold phenol technique and analysed by zonal ultracentrifugation. The extinction at 260nm was measured but before each fraction was assayed for acid insoluble ^{3}H and ^{14}C radioactivity, 0.5vol. 1M-tris-HCl, pH10.0, was added to each fraction which was then incubated at 37°C for 1h. This treatment was necessary to remove any radioactive methionine which had been loaded on to the methionine tRNA present in the RNA preparation.

Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-)

Acid insoluble ^{14}C radioactivity (-Δ-Δ-)

^{14}C c.p.m./^{3}H c.p.m. (-x-x-).

FIGURE II.27.

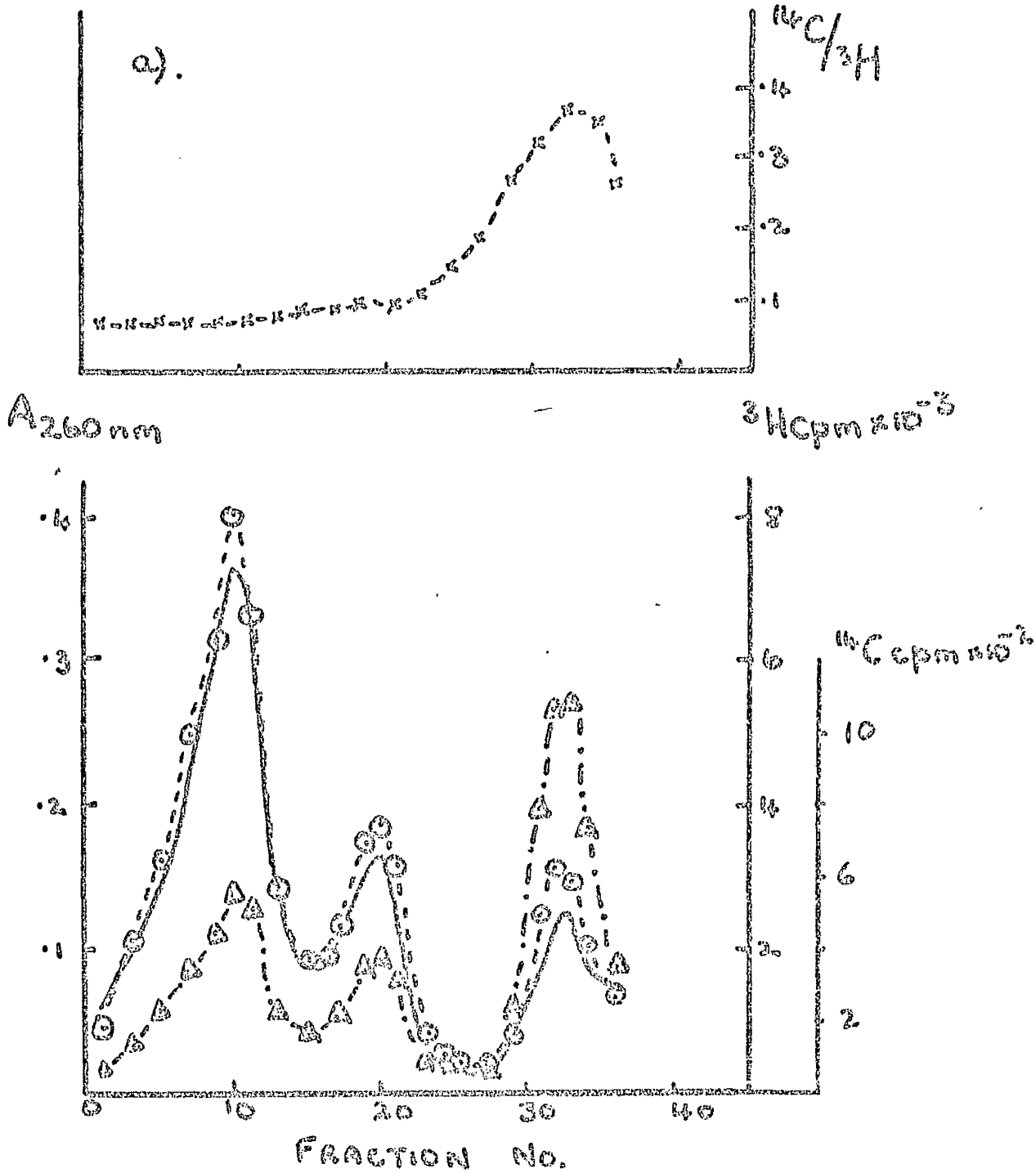

a).
$^{14}C/^{3}H$
1.4
1.3
1.2
1.1
$A_{260\,nm}$
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-3}
.4
.3
.2
.1
18
16
14
12
10
8
6
4
2
0
10
20
30
40
FRACTION No.

Fig. III.28.

Analysis on Sephadex G-100 of C13 cytoplasmic RNA labelled with [^{3}H]-uridine and L-[^{14}C-methyl]-methionine.

C13 cells were incubated for 22h with [^{3}H]-uridine (0.5μc/ml), L-[^{14}C-methyl]-methionine (0.5μc/ml) and 20mM-sodium formate. Cytoplasmic RNA extracted by the cold phenol technique and analysed by gel filtration. The extinction at 260nm was measured and 0.5 volume 1M-tris/HCl, pH10.0, was mixed with each fraction. After 1h incubation at 37°C, the acid insoluble radioactivity was assayed as described in Methods section 16.

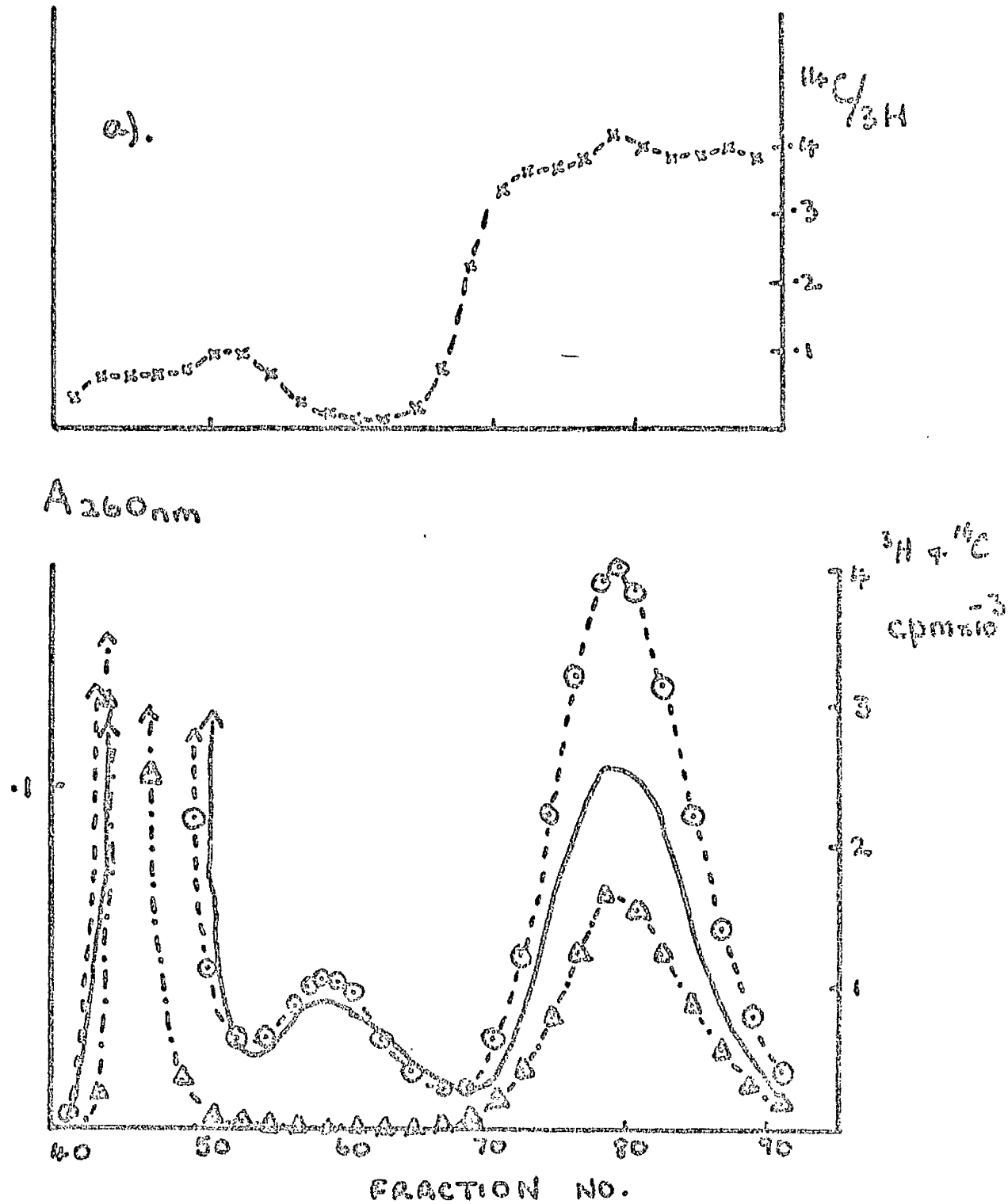
Extinction at 260nm (——)

Acid insoluble ^{3}H radioactivity (-o-o-)

Acid insoluble ^{14}C radioactivity (-Δ-Δ-)

^{14}C c.p.m./^{3}H c.p.m. (-x-x-).

FIGURE III.28.



present only in rRNA and 4s RNA. No peak of ^{14}C radioactivity could be detected in the 5s RNA indicating that this species of RNA contained no methylated nucleosides as had been proved by the work of Comb and Katz (1964), Brownlee et al (1967) and Forget and Weissman (1967). Since no ^{14}C radioactivity was found in the 5s RNA region, this confirms the belief that the addition of sodium formate prevents the methyl group of methionine from entering the purine ring system via the one carbon pool. The 4s RNA was found to be five times as methylated as the rRNAs. These analyses of C13 RNA species show that this dual isotope technique gives values for the relative degree of methylation for each RNA species which are very similar to those quoted in the literature (Darnell, 1968; Weinberg et al, 1967). This type of experiment was therefore used to compare the degree of methylation of tRNA in various different cells and since gel filtration gave a good separation of 5s and 4s RNA, this method was used to fractionate the RNA preparations.

Initially RNA was prepared from C13 and two cell lines derived from them. SR8/V1 cells were obtained by the transformation of the C13 cells with the SCHMIDT -RUPPIN strain of Rous sarcoma virus. These cells could be distinguished from the parent cell line by their altered growth characteristics (Fig II.2) and high tumour producing capacity (Table II.3). The SR8/R1 line arose by the spontaneous reversion of the transformed SR8/V1 to the characteristics of the C13 cells. The RNA was fractionated on Sephadex G-100 and the ratio of ^{14}C radioactivity

(representing methyl groups) to ^{3}H radioactivity (indicating RNA synthesis) was calculated for each fraction (Fig III. 29). C13 and SR8/R1 RNAs gave similar graphs while the SR8/V1 RNA had slightly lower values particularly in the 4s RNA region, but the difference was very small, about 10% less than the value for C13 RNA. Since such a slight difference was observed and also because, contrary to what was expected, the RNA from the more malignant cell had a lower value for the $^{14}C/^{3}H$ ratio than the other two cell lines, it was decided to test other cell lines (described in Methods sections 2 and 3). The values for the relative degree of methylation of rRNA and tRNA from the various cells are given in Table III. 30. From this table it can be seen that C13 tRNA contained more methyl groups per nucleotide than any other type of tRNA with the exception of RNA from the revertant cell, SR8/R1. This is the opposite relationship from what was expected but the variation was very slight and the degree of methylation of rRNA showed the same differences between species. These differences could simply be explained if the cell lines differed in the rate at which the labelled precursors were taken into the cell and equilibrated with the intracellular pools, which might indeed vary in size with the different cell lines. In particular the rate at which uridine is formed by the cells could affect the uptake of $[^{3}H]$- uridine from the medium. However Borek's theory suggests a specific effect on tRNA methylation and to decide if such an effect did indeed exist, the methylation of tRNA relative to rRNA was calculated by dividing the degree of methylation

Fig.III.29.

Relative degree of methylation of RNA from C13, SR8/V1 and SR8/R1 cells.

Cultures of C13, SR8/V1 and SR8/R1 cells were incubated for 22h with [^{3}H]-uridine (0.5μc/ml), L-[^{14}C-methyl]-methionine (0.5μc/ml) and 20mM-sodium formate. Cytoplasmic RNA was extracted by the cold phenol technique and analysed by gel filtration. The extinction at 260nm was measured and 0.5 volume 1M-tris/HCl, pH10.0, was mixed with each fraction. After 1h incubation at 37°C, the acid insoluble radioactivity was assayed as described in methods section 16. The ratio of ^{14}C radioactivity to ^{3}H radioactivity was calculated for each fraction and the graphs of C13, SR8/V1 and SR8/R1 RNA were superimposed.

Extinction at 260nm (——)

^{14}C c.p.m./^{3}H c.p.m. for C13 RNA (—o—o—)

" " SR8/V1 RNA (—△—△—)

" " SR8/R1 RNA (—×—×—).

FIGURE III. 29.

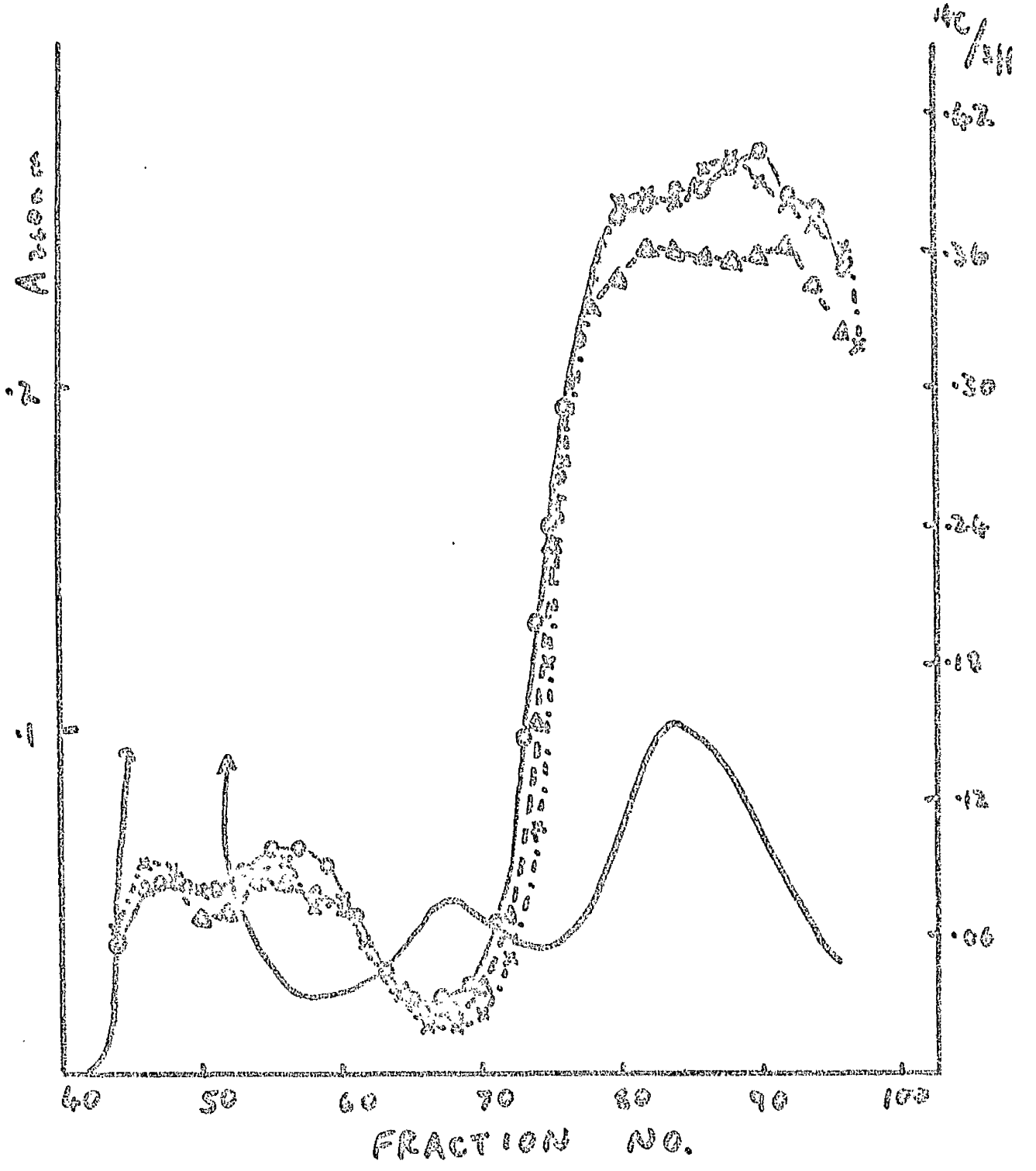


Table III.30

The relative degree of methylation of rRNA & tRNA from various hamster cells.

Cell Line	Relative Degree of Methylation (i.e. ^{14}C c.p.m./^{3}H c.p.m.)		
	rRNA	4sRNA	4sRNA/rRNA
C13	.080	.398	4.97
SR8/V1	.075	.358	4.77
SR8/R1	.087	.391	4.94
SR8/V5	.064	.306	4.78
B5	.060	.314	5.28
Baby Hamster Primary cells	.039	.207-.212	5.31-5.44

($^{14}C/^{3}H$) of tRNA by that of rRNA. This relative degree of methylation should rule out any effects due to differences among the cells of the rate of incorporation of the isotopes; the same figure should be obtained for each cell type unless an alteration in the pattern of methylation has occurred. The final column in Table III. 30 shows the values obtained for the methylation of tRNA relative to rRNA. Once again little variation was apparent; the mixed population of hamster embryo cells had in fact the highest value with almost the same for the cells transformed with the BRYAN strain of Rous sarcoma virus. When the C13 cells were transformed with the SCHMIDT-RUPPIN strain of the same virus a lower value was obtained, slightly lower even than that for the C13 cells. This indicates that the degree of tRNA methylation relative to rRNA methylation may be altered either up or down depending on the strain of the virus used to transform the cells. However the differences observed are very slight and the main conclusion to be drawn from these experiments is that no evidence could be found for the large increases in methylation of tRNA suggested by the work of Borek and his collaborators.

2. Analysis of the methylated nucleotides present in various cell lines

Although no large alteration in the number of methyl groups present in tRNA could be discovered after the cells were transformed by the Rous sarcoma virus, considerable changes in the pattern of methylation could remain undetected by the technique used in the previous

experiments. Therefore to test for any qualitative differences, RNA preparations were hydrolysed and the resultant nucleotides analysed by two-dimensional chromatography. When the cells had been incubated with L-[^{14}C-methyl]- methionine, the methylated nucleotides could be detected by autoradiography.

Three cell lines were used for these studies, namely C13, the SR8/V1 cell line derived from C13 by transformation with the SCHMIDT-RUPPIN strain of Rous sarcoma virus and a polyoma transformed C13 cell line - PyY. Cultures of each cell line were set up at a concentration of 10^7 cells per 80 ounce Winchester bottle and grown for 2 days at 37°C by which time the glass surface was approximately half covered with cells. L-[^{14}C-methyl]- methionine and sodium formate were then added and the incubation continued for 22h (1½ generations). Cytoplasmic RNA was extracted and analysed by gel filtration through Sephadex G-100. The fractions containing rRNA or tRNA were pooled together with a suitable amount of Esch. coli sRNA, precipitated with alcohol, and hydrolysed with alkali as described in Methods section 14. This treatment hydrolyses all phosphodiester bonds except those in which one of the nucleotides contains 2'-O-methylribose in place of ribose (Smith and Dunn, 1969; Morisawa and Chargaff, 1963); the RNA is thus reduced to 2' (or 3') mononucleotides, some of which may be methylated, and di- or trinucleotides containing methyl groups on the ribose and sometimes also on the base moieties. When this mixture of nucleotides had been fractionated by

two-dimensional paper chromatography, the major nucleotides arising from the carrier Esch. coli sRNA could be observed under u.v. light but the nucleotides labelled with the [^{14}C]-methyl groups could only be located by autoradiography (Methods 15). tRNA preparations from C13, SR8/V1 and PyY cells were fractionated by two-dimensional chromatography; autoradiographs were prepared and diagrams of these are presented in Fig III. 31. It is apparent that C13 and SR8/V1 tRNA differ in that the latter contains one extra radioactive spot; no other difference was obvious although it is possible that further alterations may have been obscured by insufficient separation of the methylated nucleotides. The identity of this extra methylated compound has not been determined but, from its position on the chromatogram, it would appear to be a methylated adenylic acid. Comparison of C13 and PyY tRNA shows further differences; the extra component present in SR8/V1 tRNA occurred also in the tRNA of PyY cells while a methylated nucleotide which was present in large amounts in both the other cell lines was almost completely absent from the autoradiograph of PyY tRNA. This nucleotide had a similar R_F to AMP in the isobutyric/NH_3 solvent but moved slightly faster in isopropanol /HCl and was probably the mononucleotide of a methylated adenosine.

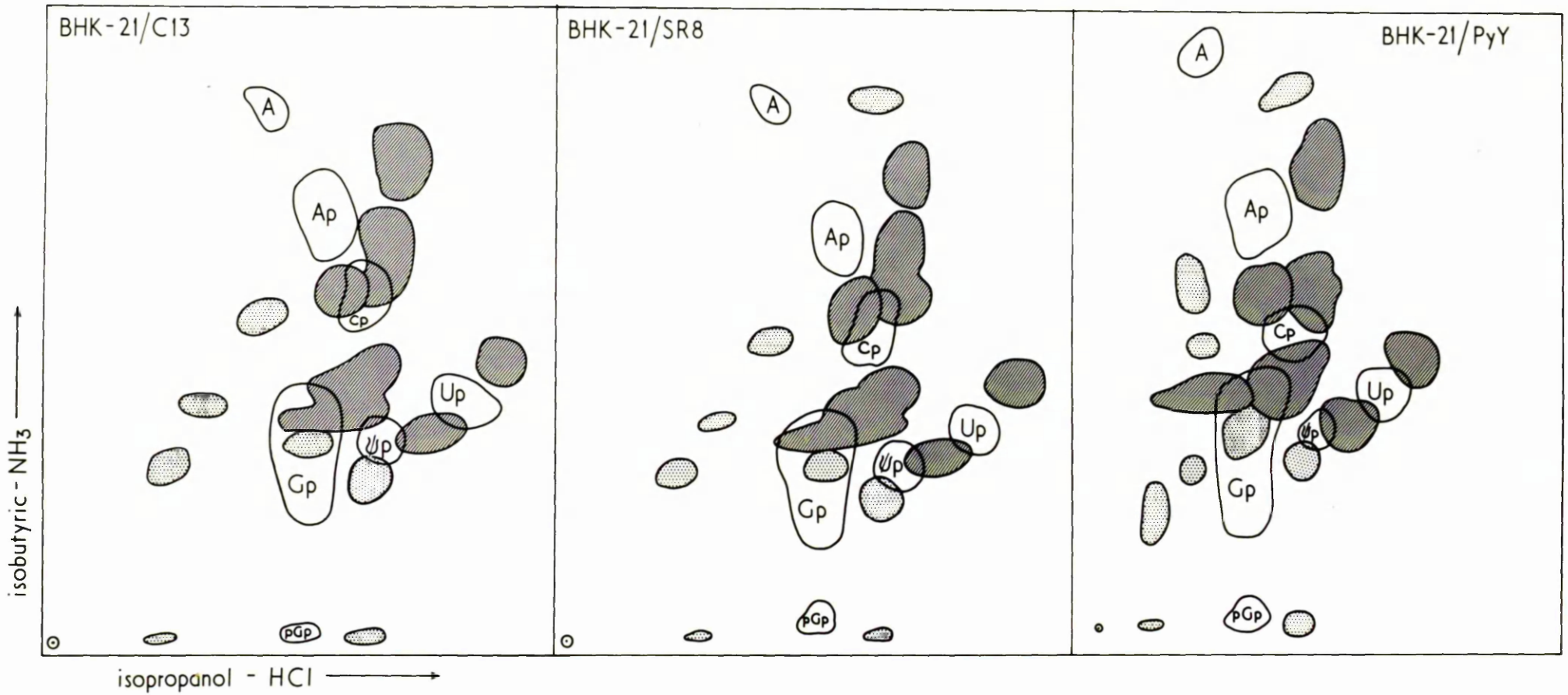
The radioactive spots with R_F values in the isopropanol/HCl solvent less than those of AMP or GMP are di-or trinucleotides (Hayashi et al, 1966) of which a great variety exist but only a few of these can be detected in tRNA. Transformation with polyoma virus

Fig. III.31

Methylated nucleotides present in tRNA from C13, SR8/V1 and PyY cells.

Samples of tRNA were prepared from C13, SR8/V1 and Py Y cells which had been incubated for 22h with L-[^{14}C-methyl]-methionine and 20mM-sodium formate. Esch. coli B soluble RNA was added to each radioactive tRNA preparation to adjust the specific activity to about 10^5c.p.m./mg RNA and the RNA was then hydrolysed as described in Methods section 14. The tRNA hydrolysates were analysed by two-dimensional chromatography and autoradiographs were prepared (methods section 15). Due to the added Esch. coli RNA, the four major nucleotides and Ψ could be detected under u.v. light and these were marked. Diagrams were prepared of the u.v. spots superimposed on the autoradiographs. The radioactive spots were divided into two groups, those of high intensity were represented by hatching and those of low intensity by stippling.

FIG III.31


BHK-21/C13
BHK-21/SR8
BHK-21/PyY
A
Ap
Cp
Up
Ψp
Gp
pGp
isobutyric - NH3
isopropanol - HCl

causes some alteration in the number of the radioactive spots representing these di- and trinucleotides. One new spot is definitely present with the possible addition of one or two others. Further slight differences may occur in the di- and trinucleotide fraction of the tRNA hydrolysates of Cl3 and PyY cells but due to the very low concentration of these radioactive compounds this cannot be determined exactly.

In contrast to the autoradiographs obtained with tRNA, those produced with the rRNA preparations from Cl3, 3R8/V1 and PyY cells were virtually identical (Fig III.32). The distribution of the radioactive material was quite different from that obtained with tRNA, as most of the radioactivity appeared in the position of di- and trinucleotides. This difference was of course anticipated as it is known that as much as 80% of the methyl groups in mammalian rRNA occur as 2'-O-methylribose (Brown and Attardi, 1965).

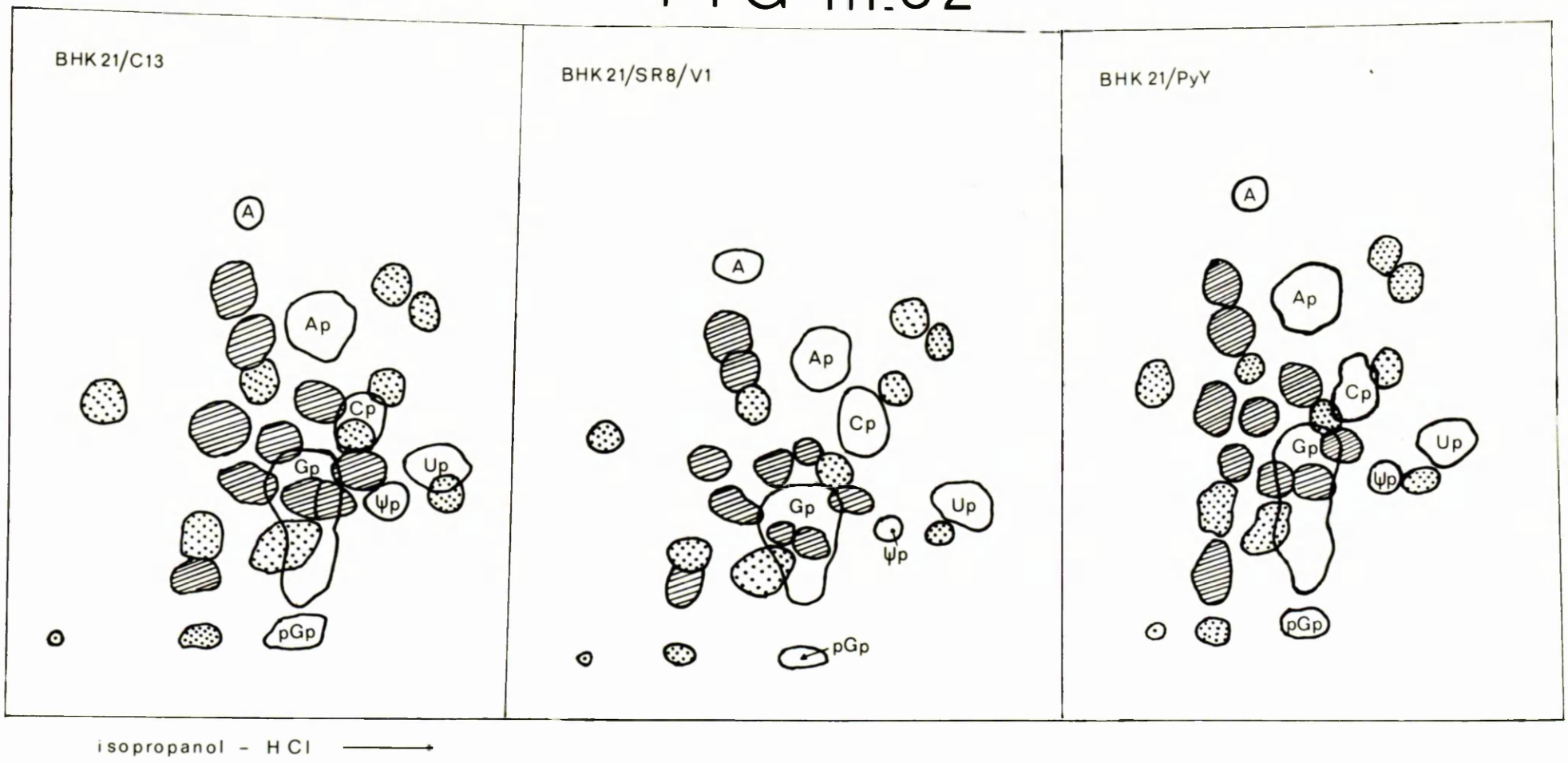
It seems therefore that transformation of Cl3 cells to highly malignant cells does alter the pattern of tRNA methylation but the number of differences may vary with the transforming virus. The methylation of rRNA is apparently unaffected by the transformation.

Fig. III.32.

Methylated nucleotides present in rRNA from C13, SR8/V1 and Py Y cells.

Samples of rRNA were prepared from C13, SR8/V1 and Py Y cells which had been incubated for 22h with L-[^{14}C-methyl]-methionine and 20mM-sodium formate. Esch. coli B soluble RNA was added to each radioactive rRNA preparation to adjust the specific activity to about 10^5c.p.m./mg RNA and the RNA was hydrolysed as described in Methods section 14. The rRNA hydrolysates were analysed by two-dimensional chromatography and autoradiographs were prepared (Methods section 15). The four major nucleotides and ψ were detected under u.v. light and marked. Diagrams were prepared of the u.v. spots superimposed on the autoradiographs. The radioactive spots were divided into two groups, those of high intensity being represented by hatching and those of low intensity by stippling.

FIG III.32


BHK 21/C13
A
Ap
Cp
Gp
Up
ψp
pGp
BHK 21/SR8/V1
A
Ap
Cp
Gp
Up
ψp
pGp
BHK 21/PyY
A
Ap
Cp
Gp
Up
ψp
pGp
isobutyric - NH3
isopropanol - HCl

D I S C U S S I O N .

DISCUSSION

1. Pre-tRNA as a tRNA precursor

With the exception of certain viral RNAs, all species of RNA are transcribed from a DNA template. However the mature functional molecules found in the cell are not necessarily the primary products of RNA polymerase action. This fact was brought to light by the discovery of precursors which are modified to form rRNAs both in prokaryotic and eukaryotic cells and it raises the question "are the mature tRNA molecules formed directly by transcription of the DNA?". The existence of enzymes capable of methylating tRNA in specific positions (Baguley and Staehelin, 1968) suggests that they are not. An unmethylated molecule would therefore be expected to exist as a precursor to tRNA. In 1963 Rosset and Monier reported the discovery of such a molecule - 5s RNA. Unlike tRNA, no definite function could be found, nor has yet been found, for this molecule. It contained neither methylated nucleosides nor pseudouridine and was slightly longer than tRNA - apparently an ideal tRNA precursor. However Galibert, Lelong, Larsen and Boiron (1967) studied the kinetics of synthesis of 5s RNA and tRNA in KB cells and came to the conclusion that a precursor - product relationship could not exist between these two RNA species, a result confirmed the same year by the determination of a unique primary sequence for 5s RNA (Brownlee *et al*, 1967; Forget and Weissman, 1967) thus ruling out the possibility of it giving rise to the many different tRNA molecules.

In their studies Galibert et al (1967) did observe a rapidly labelled species of RNA which eluted from MAK at a slightly higher salt concentration than tRNA. They put forward the idea that this might be a tRNA precursor but did not investigate this possibility further. About the same time, Lal and Burdon (1967) reported studies of a rapidly-labelled low molecular weight RNA with properties which indicated that it could be a tRNA precursor. This species of RNA (pre-tRNA) was isolated from Krebs II ascites cells using gel filtration through Sephadex G-100. During the work reported in this thesis, the existence of pre-tRNA in C13 cells was confirmed. Also the fractionation of the rapidly-labelled cytoplasmic RNA using gel filtration was compared with that obtained by chromatography on MAK and it was deduced that the rapidly-labelled RNA noted in KB cells by Galibert and his coworkers was indeed pre-tRNA. This species of RNA has been isolated from human lymphocytes using gel filtration (Kay and Cooper, 1969) and can also be detected as a high specific activity material migrating more slowly than tRNA on polyacrylamide gel electrophoresis of RNA prepared from Krebs II ascites cells (Burdon and Clason, 1969), Hela cells (Bernhardt and Darnell, 1969) and cells from the salivary glands of Chironomus tentans (Egyházi et al, 1969). Thus this rapidly-labelled low molecular weight RNA has been identified in at least 6 different eukaryotic cell types, from 4 different species and therefore appears to have a relatively widespread occurrence.

The kinetics of synthesis experiments carried out by each group are in agreement that pre-tRNA could be a precursor to tRNA.

Similarly when actinomycin D has been used to prevent further RNA synthesis, the general conclusion has been that pre-tRNA is converted to tRNA. However when the results of these actinomycin D chase experiments are examined, another explanation is possible, namely that pre-tRNA could arise from the breakdown of a high molecular weight RNA and, during the incubation with actinomycin D, further degradation could occur while tRNA is formed independently from an as yet unidentified precursor. Several high molecular weight RNAs are known which could break down to give pre-tRNA, these are rRNAs, ribosomal precursor RNAs and the heterodisperse DNA-like nuclear RNA, (Yoshikawa et al, 1964; Soeiro et al, 1966). Obviously if conditions could be found which inhibit the formation of these high molecular weight RNAs but still allow the formation of pre-tRNA, the original interpretation of the "actinomycin D chase" studies can be accepted - namely that pre-tRNA is converted to tRNA.

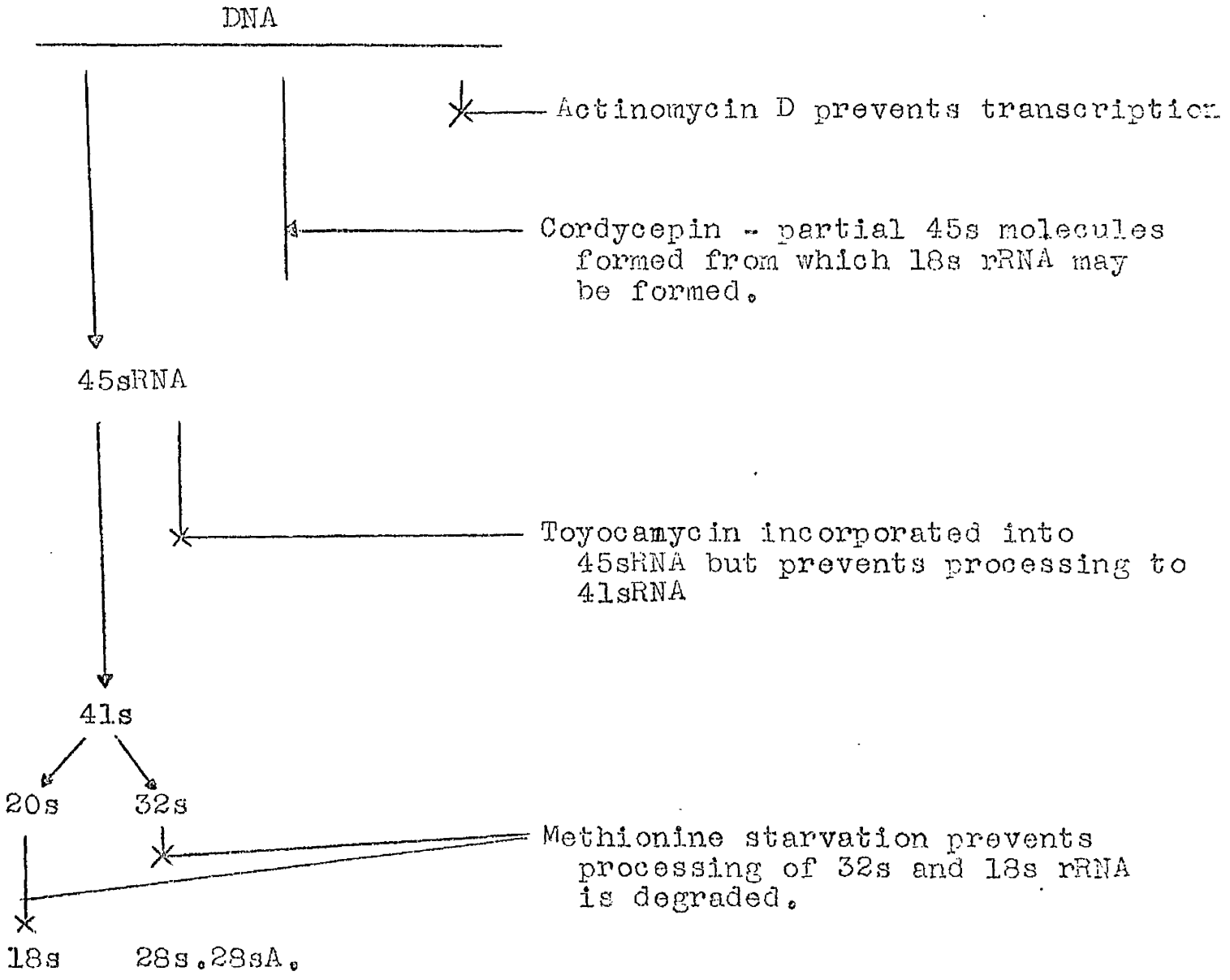
The first possibility that 28s and 18s RNA give rise to pre-tRNA is unlikely as very little mature rRNA should be formed during the short exposure to label required to observe pre-tRNA (Greenberg and Penman, 1966; Burdon, Martin and Lal, 1967). However the precursors to rRNA are rapidly synthesised and therefore could be broken down to give rise to pre-tRNA; indeed it is known that almost half of the 45s RNA is degraded during the processing in the nucleolus to form 28s and 18s RNA (Jeanteur et al, 1968; Jeanteur and Attardi, 1969). Several treatments have been reported to completely inhibit the appearance of

28s and 18s RNA without affecting the synthesis of the other species of RNA (see Fig IV. 1). Three of these treatments have been used during this work but the most conclusive results, as regards the origin of pre-tRNA, arise from the experiments using actinomycin D. This drug binds to DNA preventing its transcription by RNA polymerase (Hurwitz, Furth, Malamy and Alexander, 1962) and at very low concentrations the genes for rRNA are preferentially affected (Perry, 1962). Thus it is possible to maintain the synthesis of tRNA while preventing the formation of 45s RNA; since no precursor to rRNA is formed there can be no problem of the breakdown of a small proportion of the precursors, an objection which might be brought against the studies with toyocamycin. Due to the experiments reported here, it has been possible to prove that, under conditions which prevent 45s RNA synthesis, pre-tRNA is formed. It therefore cannot arise from the rRNAs or their precursors either as an artefact formed during the RNA extraction procedure or as the natural intermediate in the destruction of non-ribosomal sequences in the 45s RNA.

It should be noted that these experiments with actinomycin D also provide information about the synthesis of 5s RNA. Since this low molecular weight RNA occurs in ribosomes in a one to one ratio with 28s and 18s RNA, it might be expected to be formed from the same precursor. However when the synthesis of 45s RNA is completely inhibited by the addition of low concentrations of actinomycin D, 5s RNA is still formed. Using L cells, Perry and Kelley (1968) have also shown that 5s RNA is synthesised quite independently of 28s and

Fig IV.1

Inhibitors of eukaryotic rRNA synthesis.



18s RNA. These results support the conclusion drawn by Brown and Weber (1968) from their studies of mutants of Xenopus laevis; these workers showed that the genes of 5s RNA were not intermingled with those for the larger rRNAs in the nucleolar DNA.

To return to the origin of pre-tRNA, the heterodisperse DNA-like nuclear RNA is the third source of material which could be degraded to give rise to pre-tRNA. Unfortunately it is much more difficult to rule out this possibility because, unlike with rRNA, no selective inhibitor has been found which is capable of preventing the synthesis of this nuclear RNA while allowing the normal production of all other types of RNA. Kay and Cooper (1969) attempted to by-pass this problem by using the technique of DNA-RNA hybridisation. However due to technical difficulties in preparing sufficient pre-tRNA and tRNA from human lymphocytes, these authors found it impossible to draw definite conclusions from direct hybridisation studies let alone attempt the more definitive competitive hybridisation experiments such as were used by Jeanteur and Attardi (1969) to confirm that 45s RNA contained the sequences for 28s and 18s RNA.

In this work a different approach was used as it was considered more profitable to study further the conversion of pre-tRNA to tRNA. In vitro work is required for this as, due to the high concentration of tRNA molecules constantly present in growing cells, it cannot be proved in vivo that 4s RNA newly formed from pre-tRNA can accept amino acids -

the only conclusive proof that pre-tRNA is indeed a precursor to tRNA. However recent work has suggested that before a newly formed tRNA molecule can accept amino acids, several features of mature tRNA must have been formed. The pCpCpA terminal sequence is required for activity (Preiss, Dieckmann and Berg, 1961); methylation should have occurred since at least some of the tRNAs are inactive if lacking in methyl groups (Shugart et al, 1968) and the tRNA molecule must be present in an active configuration (Lindahl et al, 1966) which may depend on the presence of pseudouridine in the molecule (Siddiqui et al, 1970). To date there has been no report of the formation in vitro from pre-tRNA of a molecule which accepts amino acids. Nevertheless the initial studies reported here have shown that it is possible to convert pre-tRNA to a material which elutes from Sephadex G-100 with mature tRNA. Most important, this conversion depends on the activity of an enzyme, or enzymes, present in extracts of actively growing cells and under the conditions which allow this conversion, no effect on tRNA itself can be detected.

Pre-tRNA appears in the cytoplasm within a very short time of the addition of radioactive RNA precursors and in mammalian cells it has not been detected in the nucleus. There are several possible explanations of why this should be so. Firstly pre-tRNA may not be produced by transcription of the nuclear DNA; it could be formed in the mitochondria which are known to synthesise distinct species of RNA (Dubin, 1967; Barnett and Brown, 1967) including species of tRNA

which are less methylated than cytoplasmic tRNA (Dubin & Montenecourt, 1970). Although the technique used to extract mitochondrial tRNA differs from the cold phenol technique used here by the addition of sodium dodecyl sulphate, it is still possible that mitochondrial RNA is present in the cytoplasmic preparations used for studying pre-tRNA. However South and Mahler (1968) and Knight (1969) have reported that ethidium bromide selectively inhibits mitochondrial RNA synthesis while the production of nuclear RNA remains unaffected; pre-tRNA has been shown to be synthesised in C13 cells in the presence of ethidium bromide (Clason, 1970) and therefore it is unlikely to be of mitochondrial origin.

Alternatively pre-tRNA may be present in the nucleus, indeed it may exist exclusively within the nucleus, but during the extraction procedure, the nuclear pre-tRNA "escapes" into the cytoplasm; such a mechanism was suggested by Burdon & his colleagues (Burdon et al, 1967, Burdon & Clason, 1969) and is supported by the results of Egyházi et al (1969) who detected pre-tRNA in both nuclei and cytoplasm isolated by a non-aqueous microdissection technique from cells of Chironomus tentans.
A third explanation for the absence of pre-tRNA in the nucleus of mammalian cells is that it is synthesised from another precursor RNA of higher molecular weight which may occur only in the nucleus. Several pre-tRNA molecules might be formed as

a high molecular weight RNA in the nucleus and during its transport to the cytoplasm, this molecule could be split to give the small pre-tRNAs which would then be modified further in the cytoplasm to form mature tRNAs. The fact that the base composition of pre-tRNA - 56% G+C, is different from that of the DNA-like RNA, which has a G+C content of 44% (Soeiro et al, 1966) does not rule out this hypothesis as only a small fraction of these heterogeneous nuclear RNA molecules need function as precursors to pre-tRNA. Although there is no evidence as yet for such a large precursor, its existence would help to explain why it has not so far proved possible to inhibit completely the synthesis of the heterodisperse nuclear RNA without also preventing the formation of tRNA.

In concluding this section of the discussion, it may be said that although the final proof that pre-tRNA gives rise to an amino acid accepting molecule is missing, there have been no reports of experiments which rule out this possibility. On the contrary, considerable evidence has accumulated which supports the assumption that pre-tRNA is indeed a precursor to tRNA.

b) Length of pre-tRNA

Only two of the groups studying pre-tRNA have attempted to determine the relative lengths of pre-tRNA and tRNA. Bernhardt and Darnell (1969) treated the rapidly labelled RNA isolated from Hela cells with formaldehyde or 8M-urea. Both treatments are known to reduce the three dimensional structure of RNA molecules and in this way the authors hoped to remove any differences in

configuration which might confuse the comparison of the molecular weights of the two species of RNA. In fact after neither treatment was any difference detected in the relative mobilities of pre-tRNA and tRNA on polyacrylamide gel electrophoresis. This suggests that the molecular weights were indeed different. Burdon and Clason (1969) isolated pre-tRNA from Krebs II cells and tried to alter its conformation to that of tRNA by using the technique of Lindahl et al (1966) but they came to the conclusion that more than a change of configuration caused pre-tRNA to elute from Sephadex G-100 earlier than tRNA. They confirmed this result by treating the partially purified pre-tRNA plus added unlabelled cytoplasmic RNA with formaldehyde and observing that the relative positions of elution from Sephadex G-100 of pre-tRNA and tRNA were virtually unchanged indicating a difference in length of the two species of RNA. The in vitro work undertaken during this investigation of pre-tRNA in C13 cells has shown that although no alteration could be found in tRNA after incubation with the cell extract, pre-tRNA was converted to 4s material; moreover this conversion involved the release of nucleotides as would be expected when a precursor molecule was converted to a product of lower molecular weight.

During the incubation with the cell extract, about 26 nucleosides appear to be released compared to the 15 proposed by comparing the elution volumes from Sephadex of formaldehyde

treated pre-tRNA, 5s and tRNA (Burdon & Clason, 1969). These different values can be explained by the different techniques which were used for these determinations. With the C13 pre-tRNA, the fraction of each nucleotide released was calculated by comparing the amount of radioactivity recovered as mononucleotides with that remaining in the 4s RNA. Thus since any slight degradation, which might occur during the incubation, would increase the proportion of the radioactivity appearing as mononucleotides, the value obtained for the length of pre-tRNA will obviously be a maximum one. On the other hand, the value obtained by Burdon and Clason must be a minimum one since any degradation of the pre-tRNA would cause it to elute from the Sephadex closer to the tRNA than it should. Thus any errors which might occur in these calculations will be additive and the actual length of the extra sequence may lie between these values. The experiments involving the digestion of pre-tRNA with snake venom phosphodiesterase indicate that this extra material could occur as two short sequences one at each end of the molecule. It remains to be decided whether these sequences are removed by the action of endonucleases or exonucleases. Analysis by DEAE-paper chromatography showed the released radioactivity in the position of the marker mononucleotides, however this cannot be taken as proof of exonuclease action since, due to the complex nature of the incubation mixture, secondary reactions would be expected

to occur. It seems unlikely that an exonuclease would possess the necessary specificity to remove a certain number of nucleotides to leave the completed tRNA molecule, unless of course the secondary structure of the pre-tRNA plays a vital role in the reaction. Alternatively the extra sequences could be removed by the action of a specific endonuclease, in a manner analogous to the formation of chymotrypsin from its inactive precursor. The short oligonucleotides resulting from such endonucleolytic action could then be degraded rapidly by exonucleases to give the mononucleotides observed in the DEAE-paper chromatography.

It may be possible to fractionate the cell extract and remove the exonucleases as it has been reported (Razzell, 1961 a&b; Ereciñska, Gierakowska & Shugar, 1969) that these enzymes are mainly present in the microsomal and membrane fractions of the cells and these can easily be removed by high speed centrifugation. Therefore, assuming that an endonuclease is involved in adjusting the length of pre-tRNA and that it is not also attached to the microsomes, it should be possible to treat the pre-tRNA with a greatly purified enzyme preparation. Since it has already been shown that the enzyme required for the conversion of pre-tRNA remains active for at least several hours after the disruption of the cells, a further purification could be achieved by fractionating the preparation by gel filtration; also, as the enzyme is active after dialysis, the problem of losing activity

by the removal of essential ions during such a purification should not be encountered. With such a purified enzyme preparation it may be possible to detect the release of the extra sequences from the pre-tRNA as short oligonucleotides.

c) Methylation of pre-tRNA

Most of the reports of studies made with pre-tRNA have included a determination of the degree of methylation of this RNA species. Pre-tRNA from Krebs II ascites cells was found to be less methylated than tRNA (Lal & Burdon, 1967). In the present work it has been shown that the tRNA precursor in C13 cells is undermethylated compared to tRNA both in normally growing cultures and in cells in which the rate of RNA synthesis is severely reduced due to the action of toyocamycin (it should be noted that Tavitian, Uretsky and Acs (1968) reported that the methylation of RNA is unaffected by the presence of this drug). Kay and Cooper (1969) reported that human lymphocyte pre-tRNA was not significantly methylated nor was this species of RNA methylated in Chironomus tentans (Egyházi et al, 1969). In contrast, Bernhardt and Darnell (1969) claim not only that Hela cell pre-tRNA is methylated but also that it is methylated preferentially compared to tRNA. Since this is the only report not in agreement that pre-tRNA contains less methyl groups per nucleotide than tRNA, the evidence given in support of this conclusion should be examined more carefully.

Bernhardt and Darnell (1969) used the technique of polyacrylamide gel electrophoresis to study pre-tRNA in Hela cells. To overcome the difficulty in aligning radioactivity with u.v. absorbing material in the gel, these workers added marker RNA from cells which had been labelled for several hours with [^{3}H]-uridine since it is known that by this time the majority of radioactivity incorporated into low molecular weight RNA occurred in 5s and 4s RNA. In comparing the methylation of pre-tRNA and tRNA, Bernhardt and Darnell incubated Hela cells for 10 min with L-[methyl-C^{14}]-methionine, the RNA was extracted and analysed by gel electrophoresis; RNA from cells grown for 4 hours with [^{3}H]-uridine was added to locate the 4s RNA.

The ^{14}C radioactivity, representing the incorporated methyl groups, followed the ^{3}H radioactivity almost exactly in the 4s region but between the 4s and 5s RNA peaks, i.e. in the pre-tRNA position, the ratio of ^{14}C radioactivity to ^{3}H radioactivity was higher than in the tRNA region. The authors therefore concluded that the number of methyl groups incorporated per nucleotide was greater for pre-tRNA than for tRNA. However, in the same section of the paper, they show that only newly formed RNA was capable of accepting methyl groups since, when RNA synthesis was completely inhibited for 1 hour using a high dose of actinomycin D, no radioactive methyl groups were incorporated into the RNA. This must mean that at least three-quarters of the tRNA formed during

the 4h incubation with [^{3}H]-uridine was fully methylated and was not capable of accepting methyl groups. It is therefore not valid to use the [^{3}H]-uridine incorporation during a 4h incubation period as a measure of the amounts of pre-tRNA and tRNA which were capable of acting as substrates for the tRNA methylases. In fact in an earlier experiment Bernhardt and Darnell (1969) had shown that five times as much pre-tRNA as tRNA was formed during the time that the Hela cells were incubated with the radioactive methionine. When this figure is used to calculate the methyl groups incorporated per nucleotide, pre-tRNA in Hela cells appears to be considerably less methylated than tRNA. This illustrates a disadvantage of polyacrylamide gel electrophoresis compared to a method such as gel filtration. With the latter technique it is possible to mark the position of the RNA species exactly by measuring the u.v. absorbing material present in each fraction. This allows the dual isotope technique to be used to follow both the incorporation of methyl groups and the synthesis of the polynucleotide chains.

It therefore seems that pre-tRNA contains less methyl groups per nucleotide than does tRNA. However the in vitro experiments have shown that during the processing to form tRNA, about 30% of the nucleotides in pre-tRNA are removed. This could mean that, although pre-tRNA contains fewer methyl groups per nucleotide, it does in fact possess all the methylated nucleotides

present in mature tRNA i.e. the methylation could occur very soon after the synthesis of the precursor molecule and the subsequent modifications would then involve the removal of non-methylated nucleotides; such a situation is known to occur in the formation of the rRNAs (Greenberg & Penman 1966; Weinberg et al, 1967). Without a purified preparation of pre-tRNA an estimate must be made, from the Sephadex G-100 fractionation of cytoplasmic RNA, of how much of the ^{14}C radioactivity was present in tRNA and how much, if any, in pre-tRNA. If, as before, it is assumed that the newly synthesised tRNA eluted as a symmetrical peak with the mature tRNA which was marked in the profile of extinction at 260nm, then pre-tRNA contained a negligible amount of ^{14}C labelled methyl groups both in the 20 min incubation (Fig III.8) and the 2h incubation in the presence of toyocamycin (Fig III.14). It is therefore not necessary to allow for the loss of non-methylated nucleotides since no methyl groups exist in the molecule. If on the other hand it is assumed that all the radioactivity in the fractions between 5s and 4s RNA arise from pre-tRNA then an allowance must be made for the sequences which are not conserved. It should be noted that this method of calculating the degree of methylation of pre-tRNA will give a maximum value as contamination with methylated tRNA cannot be ruled out. With the 20 min incubation period very little ^{14}C radioactivity occurred in the pre-tRNA region but with the longer incubation

period made possible by the toyocamycin treatment a much higher incorporation of ^{14}C radioactivity occurred; this is probably due to better equilibration of the labelled methionine with the intracellular pool. In this experiment where the incorporation of methyl groups into pre-tRNA was determined in toyocamycin treated cells, the synthesis of RNA was followed by the addition of [^{3}H] guanosine to the medium. Bearing in mind that the series of in vitro experiments showed that 14% of the guanylic acid present in pre-tRNA was lost during the conversion to tRNA, it follows that only 86% of the ^{3}H radioactivity can be considered in the calculation of the number of methyl groups per nucleotide in pre-tRNA. When this calculation is carried out for fractions in the middle of the pre-tRNA region (i.e. the ones which are least likely to be contaminated by either 5s or tRNA) the average value for the ratio of ^{14}C radioactivity to the corrected ^{3}H radioactivity was less than half that obtained with tRNA. It therefore appears that even when the loss of nucleotides is taken into account, pre-tRNA contains fewer methyl groups per nucleotide than does tRNA.

Since pre-tRNA is less methylated than mature tRNA, it must acquire methyl groups during the maturation process. However this methylation step need not occur before the pre-tRNA is converted to material of the same size as tRNA.

This was first shown by Lal & Burdon (1967) who proved that

inhibition of tRNA methylation by incubation with ethionine did not prevent the maturation of pre-tRNA. Similarly the methionine starvation experiments reported here using C13 cells and the work of Bernhardt and Darnell (1969) with Hela cells both indicate the formation of 4s RNA in the absence of the source of methyl groups. In addition the in vitro conversion of C13 pre-tRNA to 4s material was not stimulated by the addition to the incubation medium of s-adenosylmethionine, suggesting that methylation is not an essential step in this conversion. However Bernhardt and Darnell (1969), during in vivo studies of the conversion of pre-tRNA, noted an increased rate of conversion when methionine was returned to starved cultures of Hela cells. This may reflect different requirements for methylation in C13 and Hela cells - such a difference has already been discovered in the processing of the rRNA precursors during methionine starvation and this will be discussed later.

d) Pseudouridine content of pre-tRNA

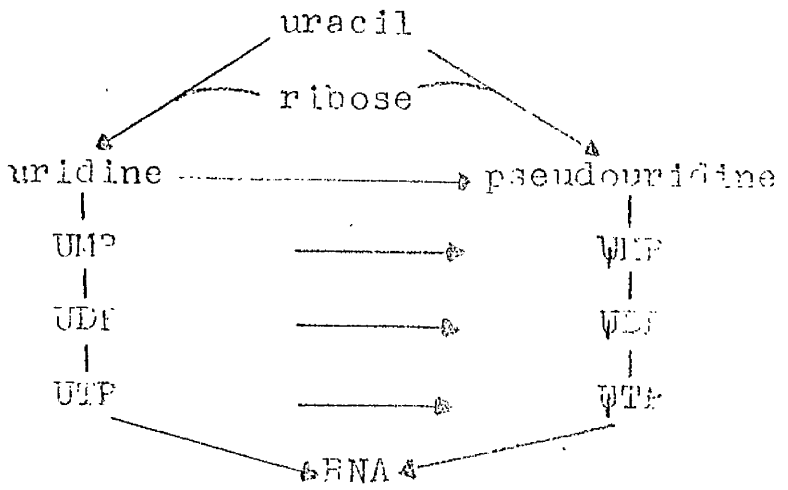
Besides the methylated nucleosides, tRNA contains other minor components, in fact the main single constituent apart from the four major nucleosides, is pseudouridine (Dunn, Smith & Spahr, 1960). Two alternative explanations are possible for the occurrence of pseudouridine in tRNA. In the form of ΨTP it may be accepted by the RNA polymerase and incorporated directly into tRNA during transcription of the DNA template. Although

so far no DNA nucleoside has been detected which could code for pseudouridine, it is quite possible that one might exist as the amount required would be too low to be detected by the analytical methods currently available. Alternatively, by analogy with the formation of the methylated nucleosides, it is possible that pseudouridine may be formed by the enzymic rearrangement of certain uridine residues present in the completed polynucleotide. However as yet no enzyme has been isolated which is capable of converting uridine to pseudouridine at the polynucleotide level, although Weiss and Legault-Demare (1965) have reported that RNA may act as an intermediate in the conversion of uridine to pseudouridine in Esch. coli spheroplasts. Obviously it is of great importance to determine the pseudouridine content of pre-tRNA as this could allow a decision to be made between the two mechanisms of pseudouridine formation in mammalian cells. Thus if pre-tRNA is found to lack pseudouridine, then it is likely that this nucleoside is formed by rearrangement at the polynucleotide level. On the other hand if pre-tRNA did contain the same number of pseudouridine residues as tRNA, no conclusion could be drawn about the origin of this minor nucleoside since it could be formed very soon after the transcription of the tRNA genes and before the appearance of pre-tRNA in the cytoplasm - such a situation occurs with 45s RNA which is methylated immediately after its synthesis.

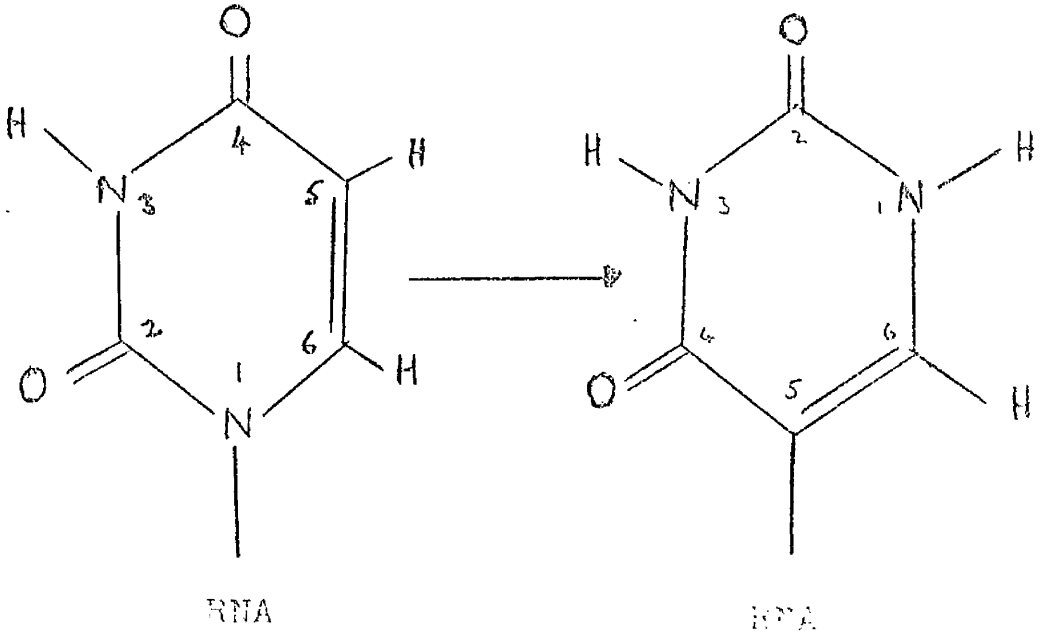
Fig IV.2

Origin of pseudouridine in RNA.

a) Incorporation hypothesis.



b) Rearrangement hypothesis.



If pseudouridine is formed by the direct incorporation method, the amount of it present in pre-tRNA can be calculated; by comparison with rat liver and yeast tRNA, pseudouridine should represent 20-25% of the total uridine. But pre-tRNA has been shown to be longer than tRNA, in particular this work has proved that as much as 50% of the uridylic acid residues present in pre-tRNA are removed during its conversion to 4s material. This must be taken into account when the relative amounts of uridine and pseudouridine in pre-tRNA are calculated. Assuming that all the pseudouridine present in pre-tRNA is conserved during the formation of mature tRNA, the ratio of pseudouridine to uridine will be doubled. Therefore, if pre-tRNA does contain all the pseudouridines present in tRNA, 10% of the uridines will be represented by pseudouridine and a higher value would be obtained if the extra sequences which are removed did indeed contain pseudouridine. The estimations which have been made using partially purified pre-tRNA show that less than 1% of the uracil is present in the form of pseudouridine. There are several possible explanations for this low level of radioactivity in pseudouridine. Firstly, if a rearrangement of the uridylic acid residue is involved in the conversion of uridine to pseudouridine, any tritium present in position 5 of the uridine would be lost as a result of linking the ribose moiety to the C6 of the pyrimidine ring. However as [6-^{3}H]-uridine was used in the preparation of pre-tRNA, this

loss of tritium from position 5 should not affect the formation of radioactive pseudouridine. Leaving aside the unlikely possibility that C13 tRNA contains no pseudouridine, there are two further explanations for the low pseudouridine content of pre-tRNA. It is possible that ΨTP is formed by a method which does not allow equilibration with the added radioactive uridine, the incorporation of unlabelled ΨTP could not be detected and it would appear that pseudouridine was not present in the pre-tRNA, but the work of Friedlander and Bounassisi (1970) makes such compartmentation of uridine and pseudouridine unlikely. Using mouse adrenal tumour cells in tissue culture, these workers noted considerable incorporation of radioactivity into pseudouridine of RNA during short periods of incubation with [^{3}H]-uridine. The only other alternative is that pseudouridine appears in tRNA by the rearrangement of certain uridylic acid residues in the RNA molecule. This work with pre-tRNA therefore supports the rearrangement hypothesis which is already in favour due to the studies carried out with bacterial RNAs (Dubin & Günalp, 1967; Ginsberg & Davis, 1968; Chirikdjian & Davis, 1970).

Pre-tRNA should therefore act as a substrate for pseudouridine-forming enzymes and the existence of such a substrate may allow this enzyme to be isolated. During the *in vitro* work carried out as part of this study, pre-tRNA has been converted to material which elutes from Sephadex G-100 with tRNA. It was hoped that

this conversion also involved the formation of pseudouridine but the pseudouridine content of the 4s RNA produced was found to be no higher than that of pre-tRNA. Thus, under the conditions used it has not so far proved possible to form pseudouridine from uridine present in pre-tRNA. The enzyme responsible for such a conversion may be very unstable and so would not remain active during the dialysis or incubation at 37°C - a lower temperature of incubation might allow its presence to be detected. Also since a dialysed cell extract was used in the determination of any formation of pseudouridine, it is very likely that some essential co-factor has been removed thus preventing the action of the enzyme.

Siddiqui *et al* (1970) have reported that modification of the pseudouridine of the GTΨCG sequence of *Esch.coli* formylmethionine tRNA alters the configuration to such an extent that the molecule is no longer active in accepting amino acids. If the absence of pseudouridine causes a similar disturbance of the conformation, it follows that the 4s RNA, formed from pre-tRNA by incubation with the cell extract, is not functional tRNA. As it is not possible to prove conclusively that pre-tRNA, or any other molecule, is the immediate precursor to tRNA unless it can be converted to an amino acid accepting molecule *in vitro*, it is of great importance that further studies should be carried out to find the conditions which will allow the formation of pseudouridine *in vitro*.

e) Accumulation of pre-tRNA

Several methods have been reported to inhibit rRNA production (see Fig IV.1) and they have proved extremely useful in elucidating the mechanism by which 16s RNA and 28s RNA are formed. Under slightly different conditions these treatments may help to answer problems in the formation of other species of cytoplasmic RNA and it is therefore worth considering what is known about the action of these inhibitors.

Actinomycin D acts by binding to the DNA and thus prevents the action of RNA polymerase. Surprisingly, low concentrations selectively inhibit transcription of the rRNA genes (Perry, 1962) while much higher concentrations prevent all RNA synthesis (Scharrer et al, 1963). The rRNA genes have a high G+C content and the recent work of Wells & Larsen (1970) indicates that G+C content and the sequence of the nucleotides is important in determining the degree to which actinomycin D will bind to DNA. Also evidence is accumulating that suggests there are two DNA - dependent RNA polymerases in eukaryotic cells, one in the nucleolus, the other in the nucleoplasm (Pogo, Littau & Alfrey & Mirsky 1967; Younger & Gelboin, 1970; Roeder & Rutter, 1970); these polymerases may show different sensitivities to the presence of the drug. A combination of both these effects may explain the selective action of low concentrations of actinomycin D. Pre-tRNA is synthesised and converted to 4s RNA normally during incubation of the cell cultures with low concentrations of actinomycin D and the

significance of this has already been discussed.

The other treatments shown in Fig IV.1 do not result in the complete inhibition of rRNA production but act by preventing the processing of the ribosomal precursor RNA at one stage or another and generally cause the accumulation of one or more of the intermediates leading to 18s and 28s RNA.

Two analogues of adenosine namely toyocamycin and cordycepin can be incorporated into RNA, (Tavitian et al, 1968; Siev, Weinberg & Penman, 1969). The 45s RNA molecules containing these modified nucleosides cannot be converted to 18s and 28s RNA. The effects are different however, for incorporation of cordycepin (3' deoxyadenosine) prevents the completion of the 45s RNA but some of the partially formed precursors may yield 18s RNA (Siev et al, 1969). From this it may be concluded that 18s RNA occurs near the 5' terminal of the 45s precursor molecule and 28s RNA near the 3' end. On the other hand when toyocamycin is incorporated in place of adenosine, 45s RNA synthesis continues to completion but the molecules formed cannot undergo the necessary processing to form either 18s or 28s RNA (Tavitian et al, 1968). In a later report the same authors show that the synthesis of 5s RNA and tRNA are not affected by the same concentration of toyocamycin which completely prevented the formation of rRNA in L929 cells (Tavitian, Uretsky & Acs, 1969). This is in agreement with the results reported in this thesis, although a higher concentration

of toyocamycin was required to completely inhibit rRNA synthesis in C13 cells. Since a similar selective effect had been observed with low concentrations of actinomycin D but a general inhibition of synthesis was obtained by increasing the concentration it was decided to test the effect of increasing the toyocamycin concentration. Some pre-tRNA did indeed appear after a 2h incubation whereas in untreated cultures it was virtually undetectable after 1h. However although the conversion of pre-tRNA to tRNA was delayed, it was not prevented as occurred with the conversion of 45s RNA to 28s and 18s RNA. Since toyocamycin has been shown to be incorporated into both 45s RNA and low molecular weight RNA (Tavitian et al, 1968), the problem arises as to why its action is selective in accumulating only the precursors to rRNA. One reason may be that the incorporation of toyocamycin in 45s RNA so alters its structure that further modification of the molecule is impossible; the conformation of pre-tRNA may not be altered to an inhibitory degree until a very high proportion of the adenosine residues has been substituted for by toyocamycin. Complete inhibition of the maturation of pre-tRNA may not be possible since high concentrations of this drug have been shown to be very toxic (Tavitian et al, 1968 and Fig II.1).

When Hela cells are grown in medium lacking the essential amino acid methionine, the formation of 28s and 18s RNA cannot be detected (Vaughan et al, 1967). Although growth in methionine

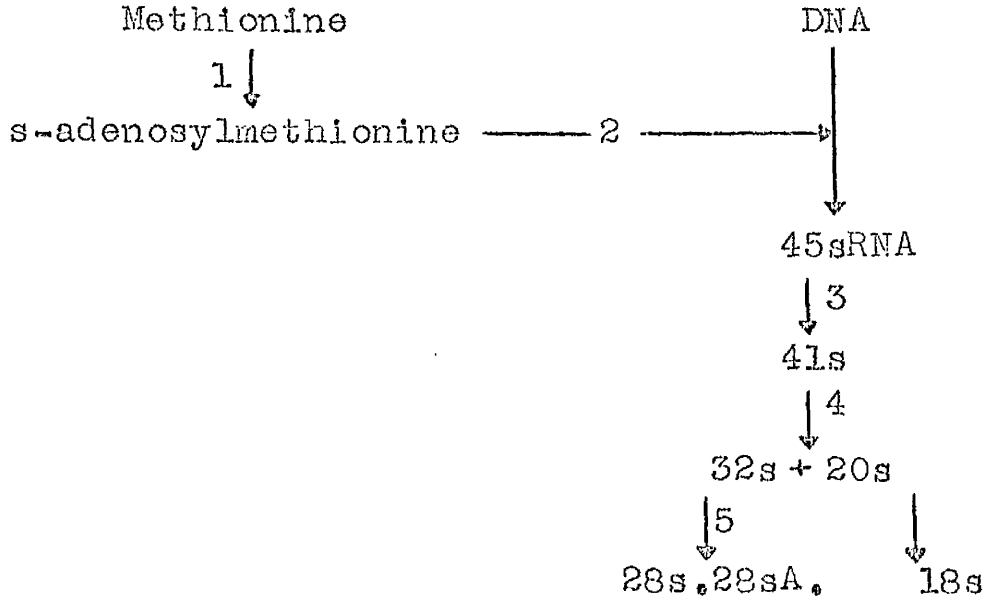
deficient medium does not cause complete depletion of the intracellular pool in mammalian cells (Eagle et al, 1959), the level of methionine is sufficiently reduced to prevent the methylation of 45s RNA. This severely undermethylated molecule can be split to form 32s RNA in an apparently normal manner but this precursor cannot undergo further processing to give 28s RNA. It is interesting that, unlike in the case of cordycepin treatment, the 18s RNA which must be formed during the processing of 45s RNA to 32s RNA cannot be detected. This may be due to an increased susceptibility to nuclease attack caused by the almost complete lack of 2' - O - methylribose (Vaughan et al, 1967). Bernhardt and Darnell (1969) have shown that methionine starvation of Hela cells also causes a reduction in the rates of synthesis of pre-tRNA and of its conversion to tRNA. A similar observation concerning the rates of pre-tRNA formation and processing was made with Cl3 cells grown for 24h in methionine deficient medium. However although similar results were observed with the synthesis of low molecular weight RNA in Cl3 and Hela cells, completely different effects occurred with rRNA. While the total RNA production was reduced to less than 1/3 of the normal, 28s and 18s RNA were still formed normally in Cl3 cells - a result confirmed by Salim(1970) and Clason(1970). Several reasons are possible for this discrepancy between the two cell lines. Firstly the intracellular pool of methionine may not have been so severely reduced in Cl3 cells if these cells have

have a higher rate of protein turnover than the Hela cells. Alternatively the methylase responsible for methylating 45s RNA may require a lower concentration of s-adenosylmethionine for optimal activity. In fact several enzymes are involved in the conversion of methionine to methyl groups on 28sRNA (Fig. IV.3) and a slight alteration in the activity of any one of these could allow the production of 28s RNA in methionine starved C13 cells. Since it has been shown that Hela cell 45s & 32s RNA are severely undermethylated, it is important that a determination be made of the level of methylation of the 28s RNA formed in the methionine deficient C13 cells; only a slight variation involving the addition of one or two extra methyl groups could account for the continued processing of the 32s RNA in these cells.

A further treatment causing inhibition of rRNA formation is incubation at an elevated temperature. Above 40°C mammalian cells cease to divide although they continue to metabolise for several days, (Warocquier & Scherrer, 1969). If Hela cells are maintained at 42°C for several hours, 28s and 18s RNA are no longer formed although 45s RNA is still synthesised (Warocquier & Scherrer, 1969). From the studies by these authors, the effect seems similar to that of methionine starvation but further work is necessary to pinpoint definitely the effect of this treatment. Obviously one of the enzymes required in the processing of 45s RNA is temperature sensitive, tRNA synthesis may also be affected but considerable

Fig. IV.3

Steps involved in formation of 28s rRNA.



1 Formation of s-adenosylmethionine by action of methionine adenosyltransferase.

2 Methylation of 45sRNA - several methylases involved.

3 Removal of non-ribosomal material - nucleases required.

4 Cleavage to form 32s and 20sRNA - endonuclease required.

5 Removal of non-ribosomal material to give 28s, 28sARNA - nucleases required.

investigation would be required to determine the conditions under which the enzymes responsible for processing pre-tRNA are no longer functional while those required for producing pre-tRNA are unaffected. Similar critical conditions may exist in the resting cell system as suggested by the work of Kay & Cooper (1969) in which they observed an increased synthesis of pre-tRNA in PHA - stimulated lymphocytes.

Finally virus infection is known to alter the production of host cell RNA. In particular infection of Hela cells with poliovirus causes an increase in the levels of several of the intermediates in rRNA synthesis. (Weinberg & Penman, 1970). Most important, it has recently been discovered that infection of C13 cells with pseudorabies virus greatly reduces the rate of conversion of pre-tRNA to tRNA (Shepherd, 1969). Although this work is still in progress, it has been suggested that the virus may specify the production of an inhibitor of one of the enzymes which is involved in the processing of pre-tRNA. Use of the *in vitro* system described in this work should enable a decision to be made on this point which is of great importance to the elucidation of the manner in which virus invasion usurps the synthetic apparatus of the host cell.

Function of pre-tRNA

In bacterial cells, precursors have been discovered for 23s and 16s rRNA (Hecht & Woese, 1968; Adesnik & Levinthal, 1970) and for 5s RNA (Forget & Jordan, 1970); these are slightly longer than

the mature molecules. The studies of the kinetics of synthesis of the stable RNAs of Esch. coli carried out by Pace, Peterson & Pace (1970) confirm the existence of these precursors and also indicate the probability of a tRNA precursor, although the molecule itself has not yet been identified. In eukaryotic cells, the two large rRNAs are transcribed together as a single precursor which is split and further modified to form the mature RNAs. The non-conserved portion of the precursor is larger than in the prokaryotic system, amounting to one fifth of the precursor in the lower animals and plants and almost one half in the higher animals. As mentioned in the introduction, it has also been suggested that the cytoplasmic mRNA may be formed by the modification of precursor molecules which could contribute to at least part of the heterodisperse DNA-like nuclear RNA (Britten & Davidson, 1969; Georgiev, 1969). As regards the formation of the low molecular weight cytoplasmic RNAs, no precursor has so far been identified for 5s RNA but one for tRNA, namely pre-tRNA, has been studied. The transcription of DNA as large molecules which are then modified therefore appears to be a general occurrence but does it represent the uncontrolled transcription of adjacent segments of DNA or is such a method of RNA synthesis of vital importance to the cell? The theories of Britten and Davidson (1969) and of Georgiev (1969) assume the latter possibility when they suggest that the non-conserved portions of the ribosomal precursors and the hetero-

disperse nuclear RNA represent the transcription of regulator portions of the operon. The larger proportion of non-conserved material present in the rRNA precursors of higher animals as compared to the bacterial RNAs would therefore be due to an increased number of regulator genes necessary for the complex regulation required by these cells within the whole animal.

During the synthesis of tRNA, 25% of the precursor is degraded; this is about the same proportion of the transcribed material which is removed during the formation of the large rRNAs or is degraded during the turnover of the heterodisperse nuclear RNA. However due to the small size of tRNA, this represents much less material and any possibility of a very complex system for regulating transcription is ruled out since the regulator genes must have a minimum size and therefore relatively few could exist in the segment of DNA from which pre-tRNA is transcribed. Also from the theories of regulator gene control, all the extra sequence would be expected to exist at the 5' end of the molecule but the work reported in this thesis with snake venom phosphodiesterase has indicated that at least some of the extra nucleotides could occur at the 3' end of the pre-tRNA molecule. While it is unlikely that the non-conserved portion of pre-tRNA arises as a direct product of a transcription control mechanism, the pre-tRNA molecule itself is ideally suited to supply a controlling influence on the synthesis of proteins. Active tRNA molecules are necessary

to allow protein synthesis to proceed, therefore systems which control the concentration of these molecules will affect the rate of protein synthesis. In this context it can be seen that the existence of pre-tRNA, which requires several modifications before it is converted to tRNA, allows a considerable degree of control to be exerted over the production of proteins. Since pre-tRNA has a longer nucleotide chain than its product, the first obvious point of control is the removal of the extra material. This could result in a general control of the amount of tRNA if all the terminal sequences were removed by a single nuclease; alternatively if there were several different sequences, each removed by a specific enzyme, a control could be exerted over the composition of the tRNA population. The recent discovery that the rate of conversion of pre-tRNA to 4s RNA is greatly reduced in C13 cells after infection with pseudorabies virus (Shepherd,1969) may be an indication of such a control system in action. Further investigation of this effect of viral action, using the in vitro system described in this thesis, is of great importance as it should supply information about the control of tRNA production in normal cells and also about the manner in which the invading virus takes over the synthetic machinery of the host cell.

Another indication that pre-tRNA may be involved in important control processes in the cell comes from the work of Kay and Cooper (1969), who showed that during the stimulation of resting lymphocytes

one of the earliest observable effects is an increased synthesis of pre-tRNA. Since lymphocyte stimulation is part of the immune response in mammals it is of obvious importance to acquire information about these early responses to stimulation.

Methylated nucleosides and pseudouridine have been shown to be lacking in pre-tRNA and must therefore be formed by post-transcriptional modifications which appear to occur after the length adjustment has taken place. Since there is evidence to suggest that the absence or alteration of these modified nucleosides prevents the normal functioning of at least some species of tRNA (Shugart et al, 1968; Peterkofsky, 1964; Revel & Littauer, 1966; Fleissner, 1967; Rake & Toner, 1966; Siddiqui et al, 1970), alterations in the activity of the enzymes responsible for producing these modifications provides further points for controlling the concentration of active tRNA molecules present in the cell. Once again the composition of the tRNA population may be altered when required, for example at least 6 tRNA methylases have been identified (Hurwitz et al, 1963 a&b) but the tRNA molecules contain on average 2-3 methyl groups (Hayashi et al, 1966, Dubin & Günalp, 1967). It therefore follows that each methylase cannot act on every tRNA species and an alteration in the activity of one or two methylases could alter selectively the composition of the active tRNAs present in the cell.

Since the existence of pre-tRNA affords so many possibilities

for controlling tRNA production and therefore protein synthesis, it is obviously of importance to study the conditions required to allow its conversion to functional tRNA.

2. Transfer RNA methylation and malignancy

Borek and his colleagues have been investigating the idea that alteration of the degree of methylation of tRNA molecules could affect the control of the protein synthesising system to such an extent that the cell might embark upon the aberrant growth of malignancy. In support of their theory, they have reported studies of the levels of tRNA methylases from various sources and show that greatly increased enzyme activities occur in extracts of tumour tissue as compared to the adjacent normal tissue (Tsutsui et al, 1966). Hancock (1965 & 67) also found more than twice the normal methylase activity in hepatoma extracts but only if s-adenosylmethionine were included in the assay medium; when methionine and ATP were added instead, the rate of tRNA methylation by the hepatoma extract was very low. The reason for this difference proved to be that although normal liver contained a large amount of s-adenosyltransferase, the enzyme required to form s-adenosylmethionine, the hepatoma showed very little transferase activity. It is possible that in the cell, the combination of these alterations in enzyme activity allow the normal methylation of tRNA to persist. From Hancock's work it follows that although the inclusion of s-adenosylmethionine in

the methylase assay will give an accurate result for the activity of the tRNA methylase, the *in vivo* situation is more complex involving the formation of this substrate. *In vivo* work is therefore required to confirm that elevated levels of the tRNA methylases do in fact mean hypermethylation of the RNA. Kaye and Leboy (1968) have also come to the conclusion that it is only from *in vivo* work that a final answer can be obtained to the problem of tRNA methylation and malignancy. This follows from their studies of the effect of the ionic environment on the activity of the tRNA methylases; they could detect no significant difference between extracts of normal tissue and tumours when the conditions used allowed optimum activity of the enzymes. Dialysed extracts were used by Kaye & Leboy (1968) while Borek's group used undialysed preparations and did not use the optimal concentrations of ions for the assay. However the conditions which provide optimal activity *in vitro* may not necessarily be equivalent to the optimal *in vivo* requirements. Also within the living cell many enzymes may be maintained at sub-optimal conditions so that stimulation as well as inhibition of their activities may be possible thus allowing a fine control to be exerted over the system. This difference between the *in vivo* and *in vitro* systems is particularly obvious from the results of Kaye and Leboy (1968) as they found the optimum temperature for tRNA methylation *in vitro* to be 5-6°C higher than the body temperature of the intact animal.

Viewed in the light of these results, Borek's work may indicate not the increased numbers of methylase enzymes but the stimulation of a normal amount of enzyme by altered ionic conditions within the tumour cell.

The system is obviously very complex but the important question at the moment is not whether or not tumour cells contain more enzyme than normal, but rather is the pattern of tRNA methylation altered. This problem can only be answered by direct comparisons of the RNA synthesised *in vivo*. In this work an attempt has been made to do this. Several cell lines of known origin were compared first to detect any overall quantitative alteration in the methylation of the RNA species after viral transformation and then a qualitative comparison was made of the various methylated nucleotides present in rRNA and tRNA from 3 cell lines. Contrary to the suggestions of Borek's group, no large increases were found in the degree of methylation of RNA in transformed cells as compared to the control Cl3 cells. However the control cells cannot be considered to be entirely normal since, although they are not highly malignant as are transformed cells, they do show a low rate of tumour production when injected into animals. For this reason the relative degree of methylation of RNA from hamster embryo primary cells was determined and lower values were indeed obtained both for tRNA and rRNA. This alteration in both RNA species could be explained by the rates of uptake of the two

labelled RNA precursors differing between the tissue culture cell lines and the cells freshly derived from the animal. Tissue culture cells are known to grow more rapidly than cells which have not been adapted to growth on glass and this could affect the rate at which the endogenous uridine was produced. A larger dilution of the radioactive uridine could therefore be expected in the tissue culture cells but, since methionine is not synthesised by mammalian cells, no similar effect should be observed with this RNA precursor. Such a selective reduction in the specific activity of uridine would cause an apparent increase in the degree of methylation of all RNA species in the more rapidly growing cells. Since similar variations were found in both tRNA and rRNA, it can be concluded that no specific increase in the degree of tRNA methylation occurred when cells were transformed to a malignant state by the viruses used in this study.

While Borek's theory was based on the high levels of tRNA methylase activity observed in tumour extracts in vitro, there is no reason to suppose that tumour cells are vastly different from normal cells. Small variations in the functioning of the control systems are possibly more feasible since large changes could lead to disorganisation and the death of the cells. Small differences in the methylation of tRNA might not be observed when the overall degree of methylation is determined either by the technique used in this work or by determining the activity of

methylases present in cell extracts, therefore the methylated nucleotides themselves must be analysed. In the system studied here the tRNAs from two transformed cell lines (namely SR8/V1 and PyY) were compared with the tRNA from the parent C13 line. The transforming viruses were unrelated, the Rous sarcoma virus containing RNA while polyoma is a DNA virus; their effects on the methylation of tRNA were slightly different. The tRNA of the cells transformed with the RNA virus (SR8/V1) contained at least one extra methylated nucleotide and therefore presumably one extra tRNA methylase. This extra enzyme could be present in large amounts in the cell but due to the substrate specificity shown by RNA methylases (Baguley & Staehelin, 1968), only limited sites on the RNA would be available for its action and no large overall increase in the level of tRNA methylation would be expected. The cells transformed by the DNA virus(PyY) showed further differences in the pattern of tRNA methylation. In addition to the extra methylated nucleotide found in the tRNA of the other transformed cell, differences were also apparent in the pattern of di- and trinucleotides formed by alkaline hydrolysis from PyY tRNA and C13 tRNA. Since this treatment yields only mononucleotides unless 2'-O- methylribose is present, the appearance of new spots in the di- and trinucleotide region of the PyY tRNA chromatogram must indicate that a new enzyme capable of adding methyl groups to the ribose of the molecule is present in these

transformed cells. As well as these extra methylations, the PyY tRNA lacked one of the methylated nucleotides which occurred in both the control and SR6/V1 tRNAs. This can be explained in two ways; either the material which had been observed there was accounted for by the appearance of the new spots - in which case they must contain two methyl groups / nucleotide; or else an inhibitor for one of the normal methylases must also be produced in these transformed cells. Therefore if these changes in the methylation of tRNA are caused by the appearance of new tRNA methylases, the cells transformed by the RNA virus possess one new enzyme while after transformation with the DNA virus, the cells contain at least two new methylases.

These results can of course be explained in another way; since the tRNA molecules contain different methyl groups, a variation in the relative proportion of the tRNAs present in the cells could cause alterations in the methylated nucleotides observed when the total tRNA of the cell is analysed. Such a variation in the tRNA population could account for both the addition and deletion of methylated nucleotides from the tRNA hydrolysate. Several groups have shown that virus infection can cause alterations in the tRNA population in bacteria (Smith, Abelson, Clark, Goodman and Brenner, 1966; Hsu, Foft & Weiss, 1967; Daniel, Sarid & Littauer, 1968; Weiss, Hsu, Foft & Scherberg, 1968) and Subak-Sharpe's group have also reported the appearance of new

tRNAs after viral infection of mammalian cells (Subak-Sharpe & Hay, 1965; Subak-Sharpe, Shepherd & Hay, 1966). While these alterations could be caused by the stimulation of inactive host genes, at least some of the new RNA hybridises with the viral DNA (Subak-Sharpe & Hay, 1966; Weiss et al, 1968; Daniel et al, 1968). Since the processes involved in viral transformation have not been fully elucidated, nor indeed have those of productive virus infection, it cannot be assumed that similar alterations of the tRNA population do occur in transformed cells but such alterations may be possible as it is known that transformed cells contain at least part of the genome of the transforming virus (Habel, 1965; Fleissner, 1970).

If an alteration in the tRNA population did indeed occur after transformation, the adaptor modification hypothesis proposed by Sueoka and Kano-Sueoka (1964) predicts that a change in the proteins synthesised by the cell could ensue. This theory depends on the fact that several aminoacyl tRNAs may be separated into different fractions; also due to the degeneracy of the genetic code, more than one codon specifies each amino acid. Although due to the 'wobble' hypothesis proposed by Crick (1966), each tRNA may not be specific for a different codon, it is possible that not all the tRNAs for a certain amino acid carry identical anticodons. For example there may be two codons specific for a certain amino acid and two tRNAs which contain the appropriate

anticodons, then mRNAs containing either codon will be translated but if the synthesis of one of the tRNAs is repressed, then only the mRNAs containing the codon equivalent to the other tRNA will be translated. Thus changes in the amount of just one tRNA molecule could alter the rate of synthesis of several proteins. Similarly if the altered methylation patterns are produced by the addition of supernumary methyl groups to an otherwise normal population of tRNA molecules, protein synthesis may be disturbed by miscoding caused by the altered methylation of the tRNAs. Thus whether the variations in the methylation of nucleotides present in tRNA arise from the synthesis of new tRNAs (Subak-Sharpe & Hay, 1965) or new tRNA methylases, these small differences could allow a large change in the composition of the cellular proteins which might account for the different growth characteristics of the transformed cells.

SUMMARY.

The Maturation of Low Molecular Weight RNA in Mammalian Cells.

by E. Janet Smillie.

Summary of the thesis presented for the degree of Doctor of Philosophy at the University of Glasgow, July, 1970.

In contrast to ribosomal RNA, little is known about the formation of transfer RNA although a great deal of information has been gathered about the structure and functioning of these small molecules. The properties of a possible tRNA precursor in cultured hamster cells have now been examined and some of the modifications required to form mature tRNA from it have been determined. An investigation has also been made regarding the occurrence of one of these tRNA modifications after the hamster cells have been transformed by tumour viruses.

It was found that when logarithmically growing hamster cells (BHK21/C13) were incubated with radioactive ribonucleosides for periods equivalent to 0.03 of their generation time, the majority of the label incorporated into low molecular weight RNA eluted from Sephadex G-100 at a position between 5s (ribosomal RNA) and 4s RNA (transfer RNA). This material, referred to as pre-tRNA and to be found predominantly in the cytoplasm, was synthesised under conditions which allowed tRNA formation but

completely inhibited the production of the precursors of rRNA. Also, when RNA synthesis was prevented by the addition of high concentrations of actinomycin D, previously formed pre-tRNA was converted to RNA eluting from Sephadex in the region of 4s RNA. Together with the kinetics of labelling studies, these observations confirm the possibility that pre-tRNA is a precursor to tRNA. It is known that, besides the 4 major nucleotides, tRNA contains methylated nucleotides and pseudouridylic acid but the content of these minor nucleotides in pre-tRNA was found to be very low. Treatment of the cells with the drug toyocamycin caused a reduction in the rate of conversion of pre-tRNA to 4s material and a similar effect was observed when the hamster cells were starved of the essential amino acid methionine. The formation of 5s RNA was not greatly affected by these treatments. It was also synthesised when the production of 45s RNA was completely inhibited by low concentrations of actinomycin D. Thus 5s RNA must be formed independently of the two large ribosomal RNAs.

The conversion of pre-tRNA to 4s material can also be brought about <u>in vitro</u>. A heat-labile activity was found to be present in cell-free extracts of actively growing hamster cells. No cofactors were essential for the conversions catalysed by the extract. Methylation of nucleotide residues was apparently not required in this process since the addition of s-adenosyl-methionine was not stimulatory. About 30% of the pre-tRNA

molecule was removed during the conversion to 4s material but with the extract used, no pseudouridine was formed; this of course may require some other cofactors. In summary, at least 3 different types of molecular modification must occur before pre-tRNA can be converted to functional tRNA, namely, reduction in molecular weight, formation of pseudouridine and methylation of certain nucleotide residues.

A comparison was also made of the degree of methylation of rRNA and tRNA in normal and Rous sarcoma virus transformed BHK21/C13 cells. Only slight variations could be found between the cell lines when a calculation was made of the number of methyl groups incorporated per uridine into rRNA and tRNA. Analyses were then made of the methylated nucleotides present in these species of RNA. Three cell lines were chosen for this study, namely BHK21/C13 and cell lines derived from them by transformation with Rous sarcoma virus (an RNA virus) and polyoma virus (containing DNA). While no difference could be detected in the methylated nucleotides present in rRNA, the pattern of tRNA methylation was slightly altered. One additional methylated nucleotide was detected in the tRNA of the cells transformed by the RNA virus while after transformation with the DNA virus three alterations in the methylation of tRNA were detected. Therefore specific alterations in the pattern of tRNA methylation were observed after viral transformation but the actual changes observed depended on the transforming virus.

R E F E R E N C E S .

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