

SOME ASPECTS OF THE RÔLE OF RAT LIVER RIBOSOMES IN
PROTEIN BIOSYNTHESIS

by

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DECLARATION

No part of this thesis has been presented for another degree at any University.

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ABBREVIATIONS AND SYMBOLS

For the abbreviations of the chemicals used see Appendix at the end of the thesis.

ATP = adenosine triphosphate

c = curi

cpm = counts per minute

EDTA = ethylenediamine tetraacetic acid

E_{260} or E_{280} = extinction coefficient measured at 260 or 280 m μ
 = absorbancy of a solution containing 1 g per ml in
 a cell 1 cm thick

g = gram

\underline{g} = acceleration due to gravity = 980 cm sec⁻²

GSH = glutathione

GTP = guanosine triphosphate

mRNA = messenger ribonucleic acid

PEP = phosphoenolpyruvate

PEP-K = potassium salt of PEP

PEP-(CHA)₃ = tri-Cyclohexylammonium salt of PEP

phe-tRNA = phenylalanine transfer ribonucleic acid

poly U = polyuridylic acid

RNA = ribonucleic acid

RNase = ribonuclease

rRNA = ribosomal ribonucleic acid

S = Svedberg, or unit of sedimentation velocity = 10⁻¹³ sec

TF and TF-AS = partially purified transfer enzyme fractions

TCA = trichloroacetic acid

tRNA = transfer ribonucleic acid

tris = tris (hydroxymethyl) aminomethane

YPH = yeast protein hydrolysate

CHAPTER 1

INTRODUCTION

The work described in this thesis was carried out in a laboratory where a study was being made on the effect of 4-dimethylamino-3'-methylazobenzene, a rat liver carcinogen, on the biosynthesis of proteins of the liver. This work was started by Dr. A.O. Hawtrey who, with his collaborators, found that in vitro protein synthesis with microsomes was considerably stimulated 40 hours after an intraperitoneal injection of the dye. This stimulation was shown to correlate with the binding of the dye to the cytoplasmic proteins (Hawtrey, Shirren & Dijkstra, 1963). A similar stimulation of synthesis by the carcinogen was then found to take place at the level of polysomes which suggested that there was control of protein synthesis even at the level of polysomes (Hawtrey & Nourse, 1966). It was because of this finding that it was decided to study the role of polysomes and the factors associated with them in the synthesis of protein. The results described here are part of this study, and include only those which were obtained solely by the author. The work done in collaboration with Hawtrey has been published and will be referred to in the text.

The work is described in three parts. The first part (Chapter 3) deals with the characterization of polysomes and the study of their breakdown by various treatments and chemicals. This work was begun in 1963, shortly after polysomes were discovered and when little was known about them. The primary objective was to use the polysomes to prepare single ribosomes free of transfer enzymes so that the part played by these enzymes in protein biosynthesis could be studied. An interesting aspect of this work was the close parallel between the actual breakdown of polysomes and that which could be predicted from the most recent concepts of protein biosynthesis.

The second part (Chapter 4) embraces the investigation of various methods of preparing single ribosomes from polysomes and the study of their properties. Three different methods were used, namely, one developed by the author and the other two adapted from methods described in the literature. When these ribosomes were tested for their activity in polypeptide synthesis they were found to differ and it was shown that the differences were not because of their content of associated transfer enzymes. The reason for these differences was therefore sought. The original idea of studying the role of the transfer enzymes was abandoned because it was clear that work on the transfer enzymes was

It seemed most probable that the differences in activity of the three preparations of ribosomes were a result of the removal of different structural parts of the ribosomes, either RNA or protein, during their preparation. Since it had been shown by many research workers that 5S ribonucleic acid (5S RNA) is a component of ribosomes, it was decided to investigate the content of this RNA in the different preparations. The third and major part (Chapter 5) of this thesis deals with the above aspect. In chapter 5 methods are described whereby 5S RNA was removed from the ribosomes and the influence of this removal on the activity of ribosomes in protein synthesis was studied. Results are also given on the effect produced by adding 5S RNA to these deficient ribosomes. Since it has been reported that a protein is required for the attachment of 5S RNA to the ribosome, the effect of these treatments on the ribosomal proteins was also studied.

The study of the steps involved in protein synthesis has attracted a large number of workers throughout the world and numerous papers on the subject are published every month. Consequently there are parts of the present study which were done independently by other research groups and published while this study was in progress or subsequent to its completion. An attempt has been made in each section to show what was actually known at the time work was started and to indicate how the results obtained correlate with findings published elsewhere.

CHAPTER 2

A REVIEW OF THE LITERATURE ON PROTEIN BIOSYNTHESIS

2.1 INTRODUCTION

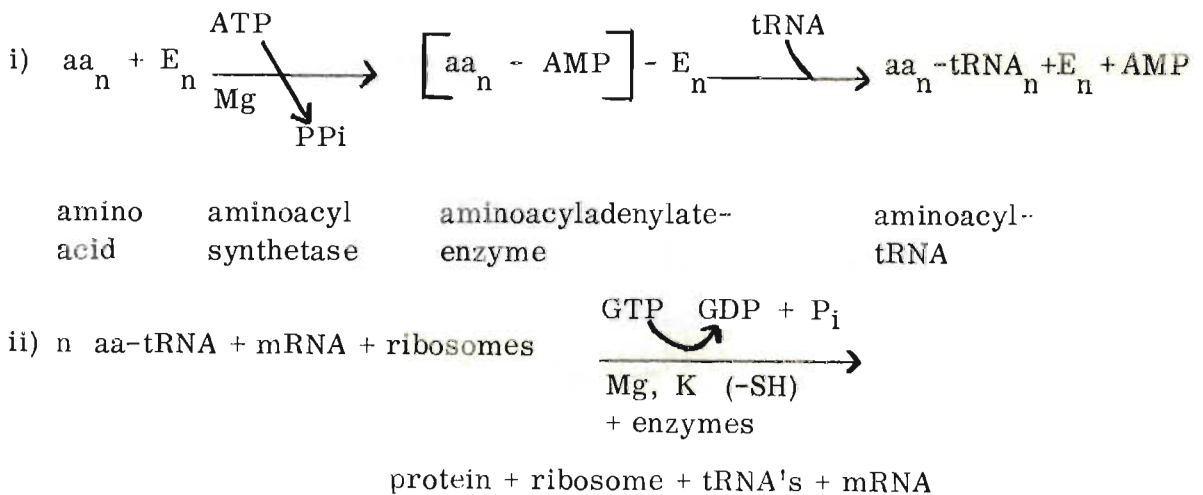
In the early 1950's it was shown that the cellular site of protein synthesis was the microsome, a lipoprotein membrane containing spherical nucleoprotein particles (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950). In about the mid-1950's it was shown that of the whole microsome structure only the spherical nucleoprotein particles, now known as ribosomes, were active participants in this synthesis (Allfrey, Daly & Mirsky, 1953; Littlefield, Keller, Gross & Zamecnik, 1955). An in vitro system for the measurement of protein synthesis was developed at about the same time by Zamecnik and his collaborators (Zamecnik & Keller, 1954; Littlefield, Keller, Gross & Zamecnik, 1955; Keller & Zamecnik, 1956; Littlefield & Keller, 1957). There was some elapse of time before it became evident that the active form of the ribosome was the polysome consisting of a number of ribosomes joined by a strand of RNA, called mRNA (see Chapter 3). Development in this field has been rapid and now an almost complete understanding of the different steps has been achieved.

Polysomes for the study of protein synthesis have been obtained from two distinctly different types of cells, namely those with a nucleus, such as yeast (haploid) and rat liver (diploid) and those without a nucleus of which Escherichia coli is an example. Most of the polysomes of the nucleated species are situated on the endoplasmic reticulum in the cytoplasm of the cell. Some occur in the free state or are found in the mitochondrion. In the anucleate species, the polysomes exist either in the free state or are associated with the membrane of the cell wall. The historical background and general features of protein synthesis are well covered in different reviews and will not be discussed in this chapter unless they have a definite bearing on later developments or are not comprehensively covered elsewhere (Watson, 1963 & 1964; Moldave, 1965; Elson, 1965; Singer & Leder, 1966; Schweet & Heintz, 1966; Novelli, 1967).

Those steps in the biosynthesis of proteins which proceed after activation of amino acids up to the completion of the polypeptide chain are outlined here. It should be mentioned that this is not the complete story as such

proteins, the attainment of the correct three dimensional configuration of the different proteins and the dispersion of the proteins within cells, are all part of this biological phenomenon. This thesis, however, deals only with the steps involved in actual peptide bond formation on the ribosome. Most of the advances were made during in vitro studies of the different systems and recently, especially with E.coli systems. Although much has been contributed by investigations using rat liver and reticulocyte systems, the stages in the process revealed in the studies on E.coli are used, though not exclusively, as a basis for the outline given here.

The steps in the biosynthesis of proteins can be summarized as follows:



In the first phase amino acids are activated by reactions with ATP followed by their transfer to transfer ribonucleic acid (tRNA). The tRNA for each amino acid is specific, there being one, two and sometimes more of these for each individual amino acid. Both steps of the reaction require magnesium ions and the aminoacyl synthetases, enzymes which are specific for each amino acid. As this reaction is well documented in most text books, it will not be dealt with further. A lot of work has still to be done on the mechanism of the recognition of the amino acids by their specific aminoacyl synthetases and of these complexes by the different tRNA's.

The second phase is the transfer of amino acids from tRNA to the growing peptide chain. This involves (1) messenger ribonucleic acid (mRNA), which carries the code to be translated, (2) ribosomes, which contain the catalytic sites at which all reactions involved in peptide synthesis take place, (3) monovalent and divalent cations, and (4) enzymes. During this reaction guanosine triphosphate (GTP) is split into guanosine diphosphate (GDP) and

phosphate with the liberation of energy. The processes in reaction (ii) above can be subdivided into the following steps:

- (a) initiation of protein synthesis,
- (b) chain elongation, and
- (c) chain termination and release.

These steps are discussed in more detail below.

2.2 INITIATION OF PROTEIN SYNTHESIS

Initiation of protein synthesis involves the attachment of mRNA to the ribosome, followed by the binding of a modified aminoacyl-tRNA, placement of this tRNA on its correct site and finally the binding of the next aminoacyl-tRNA, as coded by the mRNA, on an adjacent ribosomal site. The reactions representing this initiation and the factors participating in the reaction are shown in Fig. 2.1. The structure of the ribosome plays a very important part here. This is discussed in detail later (section 2.3.4.1). It is sufficient at this stage to mention that it consists of two subunits, a smaller (30S) unit and a larger (50S) unit which associate to give the active (70S) ribosome.

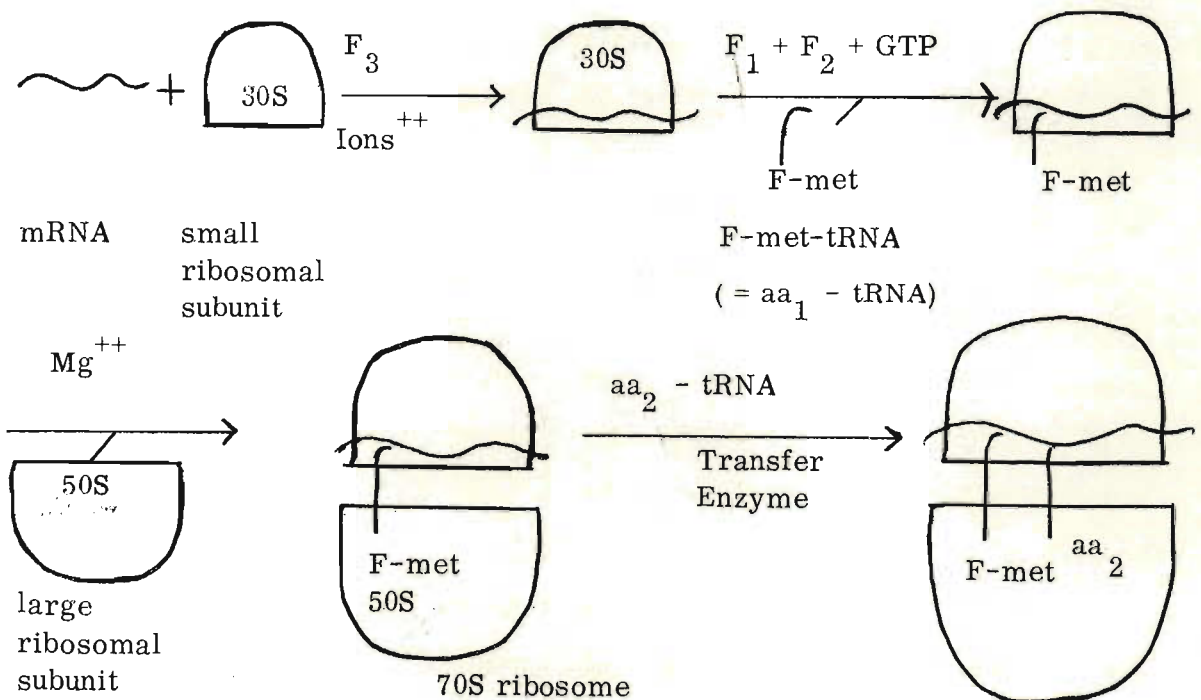


Fig. 2.1

Initiation of protein synthesis

F_1 , F_2 and F_3 = initiation factors.

F-met-tRNA = formylmethionyl-tRNA

2.2.1 The Attachment of mRNA to Ribosomes

The interaction between mRNA and ribosomes was studied using the following three systems: (i) in vitro with synthetic polyribonucleotides, (ii) in vitro with natural mRNA's, both homologous and heterologous to the system, and (iii) in vivo or in conditions closely simulating those of an in vivo situation.

Using polyuridylic acid (poly U) in the E. coli system, Okamoto and Takanami (1963) reported the association of the synthetic polyribonucleotide with both 70S ribosomes and with 30S ribosomal subunits. No binding to the 50S subunit was obtained. Takanami & Zubay (1964) showed that poly U readily binds at random to a number of ribosomes, yielding complexes with sedimentation constants larger than 70S ribosomes. Other synthetic polyribonucleotides also have been shown to bind to ribosomes (Takanami & Okamoto, 1963a & 1963b; Moore, 1966a).

Similar binding was subsequently shown between natural mRNA's and ribosomes. Joklik & Becker (1965) using HeLa cell homogenates showed an association between the RNA of vaccinia virus and ribosomes. A similar association between coliphage RNA and 70S ribosomes of E. coli was shown by Takanami, Yan & Jukes (1965). More extensive studies on this interaction by Godson & Sinsheimer (1967) revealed that the association was first between that of the phage RNA and the 30S subunit. Similar results were obtained by Eisenstadt & Brawerman (1967) using a different phage RNA. In these homologous systems of E. coli, no association between the phage RNA and 70S ribosomes or the 50S ribosomal subunits was found. In heterologous systems using ribosomes from E. coli and RNA's from unrelated systems as message, binding occurred between both 70S ribosomes and 30S ribosomal subunits (Van Duin, Pley, Bonnet-Smits & Bosch, 1968).

Thus the attachment of mRNA's to ribosomes was found possible, and in homologous systems the binding was specific. It was also found that magnesium ions were necessary for the binding of mRNA. Furthermore, Moore (1966a) and Gordon (1966) showed that, besides magnesium, other divalent cations like manganese, calcium and spermidine could be used. Moore (1966b), on proposing that the binding of mRNA to ribosomes depended on hydrogen bond formation between the amino groups of ribosomal RNA (rRNA) and the phosphates of mRNA (poly U in his experiments), suggested that the rôle of magnesium ions was that of neutralizing negative phosphate ions. Less magnesium was required in homologous systems because of the positive attraction between the mRNA and

been selectively modified by various chemical means. These results suggested a mode of attachment between mRNA's and ribosomes.

A more selective initial association between mRNA and ribosomes however, must exist for the synthesis of physiologically functional proteins. It has been shown that translation of mRNA occurs from the 5'- to the 3'-terminus (Salas, Smith, Stanley, Wahba & Ochoa, 1965). The observations, which have shown that the 5'-terminus of many natural and synthesized mRNA's is a nucleoside-5'-triphosphate, have suggested the general occurrence of a triphosphate at the commencement of the genetic message (Takanami, 1966; Roblin, 1968; Watanabe & August, 1968; Maitra & Hurwitz, 1965).

Finally, in E. coli the binding of homologous mRNA to ribosomes was also shown to be dependent on a protein factor located on the 30S subunit (Eisenstadt & Brawerman, 1966; Brawerman & Eisenstadt, 1966; Revel & Gros, 1966; Revel, Herzberg, Becarevic & Gros, 1968; Iwasaki, Sabol, Wahba & Ochoa, 1968). This enzymic binding of the mRNA to the 30S subunit appears to be linked to the transcription of RNA by RNA-polymerase (Shin & Moldave, 1966; Revel & Gros, 1967; Brown & Doty, 1968; Revel, Herzberg, Becarevic & Gros, 1968).

Similar binding of synthetic mRNA's (Williamson, Hausmann, Heintz & Schweet, 1967; Hawtrey & Nourse, 1968) and of natural mRNA's (Joklik & Becker, 1965; Shelly Beard & Armentrout, 1967) to the ribosomes of mammalian cells has also been shown. Beyond this not much is known because less research has been conducted in this sphere and the presence of a nucleus in these cells introduces further complications. As it is believed that all of the cytoplasmic mRNA's are transcribed from the DNA of the chromatin situated in the nucleus, the transcribed RNA must first be transported from this site to the cytoplasm before it is attached to the ribosomes of the cytoplasm where mRNA is translated. It now seems that particles called informers and not ribosomes or their subunits are involved in this transport, although it is not yet clear how these operate (Henshaw, Revel & Hiatt, 1965; McConkey & Hopkins, 1965). From these observations it is obvious that the whole sequence of events for the attachment of mRNA to ribosomes is different in mammalian cells.

2.2.2 The Binding of Initiator Aminoacyl-tRNA to Ribosomes

An understanding of chain initiation and the binding of initiator-tRNA by

of formylmethionyl-tRNA (F-met-tRNA) in *E. coli*. Using countercurrent distribution it was shown that two methionyl-tRNA species exist, one which can be enzymically formylated (methionyl-tRNA_F^{*}) and the other not (methionyl-tRNA_M). Noll (1966) suggested that the formylation of the amino group might facilitate peptide bond formation and then Adams & Capecchi (1966) and Webster, Engelhardt & Zindler (1966) showed that it functioned in peptide chain initiation. It was established that the initiation codons for the binding of methionyl-tRNA_F were AUG, UUG and GUG (Clark & Marcker, 1966b; Kolakofsky & Nakamoto, 1966) whilst only the codon AUG stimulated the binding of methionyl-tRNA_M (Clark & Marcker, 1966a). It also seemed that the presence of these initiation codons fixed the reading frame of the message and prevented out-of-phase translation ("out-of-phase" meaning where triplets are read as overlapping those coded for by DNA) because it was found that the codon AUG suppressed the reading of codons which partially overlapped its sequence. It also stimulated the reading of the adjacent 3'-codon (Sundararajan & Thach, 1966).

Once it was established that a modified aminoacyl-tRNA and specific codons were involved in the binding of aminoacyl-tRNA the sequence of reactions resulting in, and the factors required for the binding, were determined. Nomura & Lowry (1967) and Nomura, Lowry & Guthrie (1967) showed that formylmethionyl-tRNA directed by the AUG codon of polyribonucleotides bound first to the 30S ribosomal subunit in the absence of the 50S subunit. This binding was followed by the attachment of the 50S subunit to the complex in the presence of magnesium ions. The binding was also dependent on ribosomal factors, designated F1 and F2, as shown by Salas, Hille, Last, Wahba & Ochoa (1967), and was most pronounced at low magnesium concentrations. The binding of phage RNA's to ribosomes was similar to that described above (Nomura & Lowry, 1967; Clark, 1967). It may be noted that non-formylated aminoacyl-tRNA bound only to 70S ribosomes, while the process did not require factors F1 and F2, but higher concentrations of magnesium (Nomura & Lowry, 1967; Salas, Hille, Last, Wahba & Ochoa, 1967). GTP was also found to be necessary for the binding of formylmethionyl-tRNA (Anderson, Bretscher, Clark & Marcker, 1967; Anderson, Dahlberg, Bretscher, Revel & Clark, 1967). The dependence on GTP was also influenced by the concentration of magnesium, because at high concentrations (10-20 mM) there was no requirement (Bretscher & Marker, 1966; Zamir, Leder & Elson, 1966) while at low concentrations (5 mM) the

requirement was absolute (Anderson, Dahlberg, Bretscher, Revel & Clark, 1967; Ohta, Sarkar & Thach, 1967). GTP was not hydrolysed during the binding which occurred at high concentrations of magnesium.

Very little is known about chain initiation in mammalian systems. No formylated-aminocyl-tRNA has yet been shown to be present and with reticulocytes it seems that this form is not required (Rich, Eikenberry & Malkin, 1966). Baliga, Pronczuk & Munro (1968) concluded from their studies on the regulation of the aggregation of polysomes by the supply of amino acids that an enzyme factor is necessary for chain initiation. Also Shaeffer, Arlinghaus & Schweet (1968) have shown a requirement for GTP and enzyme factors during chain initiation with reticulocyte ribosomes. These studies were carried out using the poly U system at low concentrations of magnesium ions. In the homologous reticulocyte system, where the de novo synthesis of haemoglobin was studied, a factor for chain initiation was isolated by Miller & Schweet (1968). From this scanty evidence it seems that, while some aspects of chain initiation in mammalian systems differ from those in E. coli, many of the requirements are similar.

2.2.3 The Binding of Aminoacyl-tRNA to the Initiation Complex

Before peptide bond formation can take place, an additional aminoacyl-tRNA must become attached to the ribosome at a site adjacent to the one at which formylmethionyl-tRNA is attached. Two main ideas for this reaction have been suggested, with variations within each. Bretscher & Marker (1966) proposed that formylmethionyl-tRNA enters the condensing site directly leaving the decoding site open for the attachment of the incoming aminoacyl-tRNA (approximately as drawing (iv) of Fig. 2.2). Ohta, Sarkar & Thach (1967) proposed that formylmethionyl-tRNA initially binds to the second decoding site in the presence of GTP but without its hydrolysis (site 2 in Fig. 2.2 (ii)). This was followed by the shift of the formylmethionyl-tRNA to the condensing site with the concomitant hydrolysis of GTP (this is the transition in Fig. 2.2 from (ii) to (iv)). This model is supported by evidence which showed that, at low (5 mM) concentrations of magnesium, hydrolysis of GTP occurred when the complex (F-met-tRNA-mRNA-ribosome) reacted with puromycin to form formylmethionylpuromycin (Anderson, Dahlberg, Bretscher, Revel & Clark, 1967; Ohta, Sarkar & Thach, 1967; Leder & Nau, 1967). This reaction is a little different when formylmethionyl-tRNA or peptidyl-

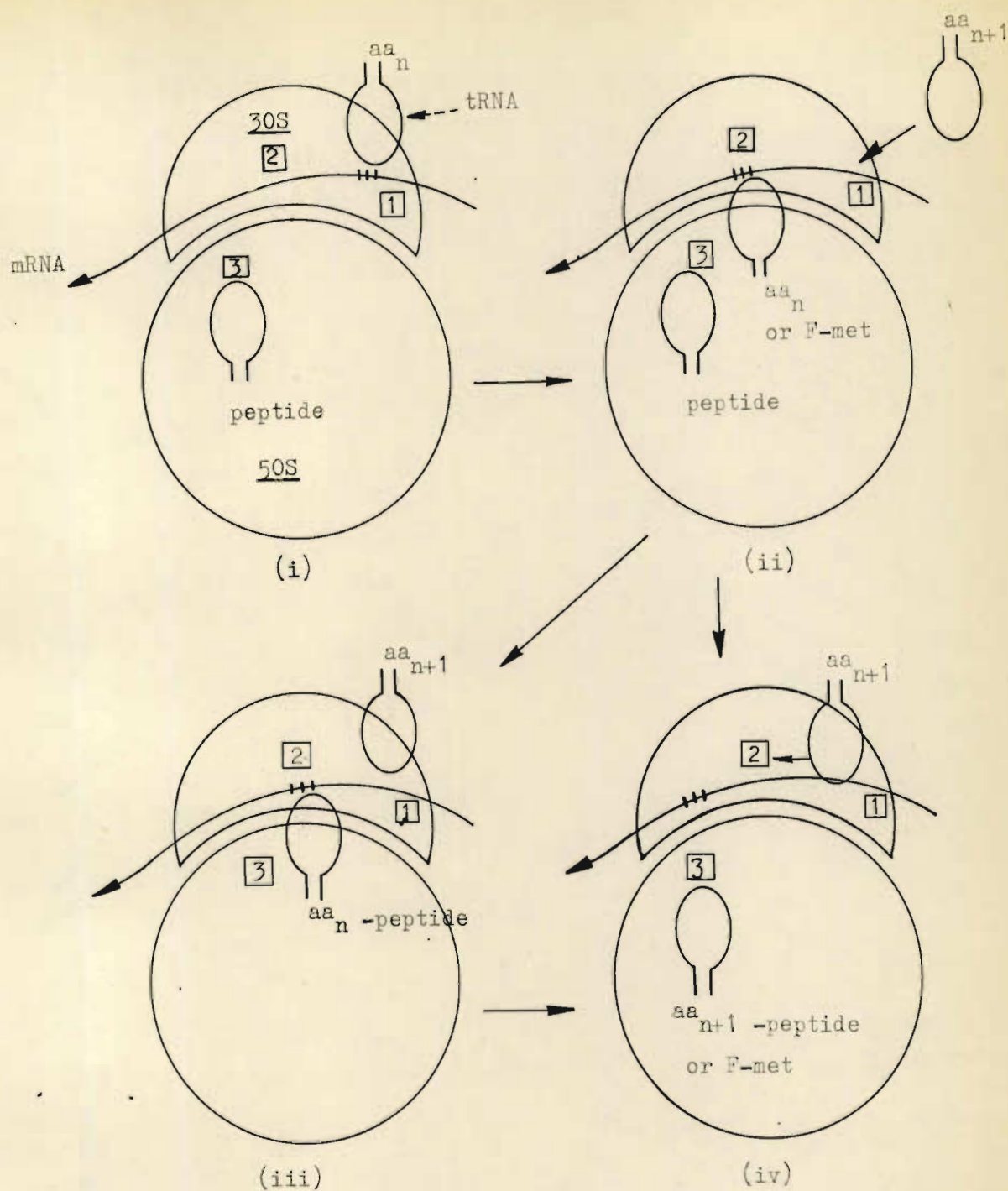


Figure 2.2 Binding sites of tRNA

- 1** = 1st decoding site
- 2** = 2nd decoding site
- 3** = condensing site

aa = amino acid

+++ = placed codon of mRNA

(See section 3.3.3 where the release of labelled peptide chains in the presence of puromycin is discussed).

Bretscher (1968) put forward another model which, he claimed, explains the reactions which occur when high concentrations of magnesium (10 mM or more) are used. In this he suggested that there were at least two sites which recognized aminoacyl-tRNA's on the 30S subunit. Non-formylated aminoacyl-tRNA first entered one site requiring the presence of GTP (site 1 of Fig. 2.2 (i).) and then was shifted to the second site (site 2 of Fig. 2.2 (ii)) with the concomitant hydrolysis of GTP. On the other hand formylmethionyl-tRNA entered the second site directly, requiring the presence but not the hydrolysis of GTP. The second site was the one on which peptide bond formation took place and this site was able to recognize a tRNA carrying a blocked amino group (either a peptide or the formylated amino acid). The formylmethionyl-tRNA was moved to the condensing site on the 50S subunit (site 3 of Fig. 2.2 (iv)) by the translocase enzyme. This model fits all the requirements for GTP and does not differ from the proposed sequence of reactions occurring during normal peptide bond formation (see section 2.3). This is well depicted by the model given by Bretscher (1968).

The above model postulated that it was the presence of the initiation codon(s) which brought about the onset of chain initiation, but more recent evidence by Guthrie & Nomura (1968) has shown that it is the specificity of the interaction between the small ribosomal subunit and formylmethionyl-tRNA which determines this initiation.

Given above are the most recent concepts of in-phase chain initiation (the reading of the triplets of the genetic message as coded for by DNA starting at the initiation codon). These are the requirements necessary for peptide bond formation to proceed at physiological ion concentrations. Peptide bond formation however, can take place in the absence of many of these initiation requirements, but then higher concentrations of magnesium are necessary, especially during initiation. For peptide bond formation to proceed at higher concentrations of magnesium, only GTP and the transfer enzymes are necessary (see section 2.3). There is no requirement for an initiation codon or initiation factors. This type of chain initiation would be able to proceed out-of-phase, and is of significance here because it is that which has been used for the studies to be presented in later chapters. Binding at high concentrations of Mg^{++} cannot be simulated in vivo but was used with in vitro studies because it eliminated the need for initiation factors while it was not expected to affect peptide bond formation.

2.3 CHAIN ELONGATION

Chain elongation is a series of reactions which are repeated during the extension of the polypeptide chain, one amino acid at a time. The reactions, which take place on the ribosome, can be divided into three distinct, though not necessarily independent steps. They are, (i) the binding of aminoacyl-tRNA to ribosomes, (ii) the formation of the peptide bond, and (iii) the translocation of the peptidyl-tRNA from the site on the 30S subunit to the condensing site on the 50S subunit. The reactions require soluble protein fractions, the transfer enzymes, GTP and sulphydryl groups. These steps are described below.

2.3.1 The Binding of Aminoacyl-tRNA to Ribosomes

This reaction is analogous to that described in section 2.2.3, except that in this instance the condensing site is occupied by a tRNA to which the growing peptide chain is attached. Much work has been done to determine the factors necessary for the binding of aminoacyl-tRNA to ribosomes. In the earlier work evidence was presented both for (Arlinghaus, Schaeffer & Schweet, 1964) and against (Kaji & Kaji, 1964; Spyrides, 1964; Heredia & Halvorson, 1966) the participation of an enzyme factor and GTP in addition to divalent (magnesium) and monovalent (potassium or ammonium) cations. Differences in the types of binding were observed. Non-enzymic binding, which was optimum at higher concentrations of magnesium, was inhibited by deacylated tRNA (Kaji & Kaji, 1964; Seeds & Conway, 1966; Kurland, 1966). On the other hand enzymic binding which was usually measured at physiological ion concentrations, was not inhibited by deacylated tRNA (Arlinghaus, Favelukes & Schweet, 1963). All the recent evidence is in favour of the requirement of both an enzyme factor and GTP for the binding at physiological ion concentrations (Ravel, Mosteller & Hardesty, 1966; Ravel, 1967; Mosteller, Culp & Hardesty, 1967; Ibuki & Moldave, 1968; Felicetti & Lipmann, 1968).

Much evidence has now accumulated as to the rôle of the transfer enzyme(s) and GTP in the binding of aminoacyl-tRNA to ribosomes. An association between one of the transfer enzymes and aminoacyl-tRNA has been shown to take place in the presence of GTP but without its hydrolysis (Ibuki, Gasior & Moldave, 1966; Hardesty, Lin & Culp, 1967; Gordon, 1967; Ravel, Thorey, Froehner & Shive, 1968). Ravel, Thorey, Froehner & Shive (1968)

and Gordon (1968) showed that the association is first between the transfer enzyme and GTP, which then associate with aminoacyl-tRNA. This complex then binds to the ribosome. For binding to take place it is also necessary that the correct codon of the mRNA is aligned adjacent to the initial binding site on the ribosome (1st decoding site = site 1 of Fig. 2.2).

Jost, Shoemaker & Noll (1968) were then able to show that only the 30S subunit of the ribosome was necessary for the initial binding. They were able to form a complex between the 30S subunit of ribosomes, mRNA and aminoacyl-tRNA in the presence of one of the transfer enzymes and GTP, the latter two being necessary for complex formation at low concentrations of magnesium. This is the structure shown by drawing (i) of Fig. 2.2 without the 50S subunit. The resulting complex was able to participate in peptide bond formation in the presence of the 50S subunit which was shown to stabilize it.

It was subsequently found that the GTP analogue, 5'-guanylmethylenediphosphate, was able to replace GTP in the enzyme-dependent binding of aminoacyl-tRNA to ribosomes (Ibuki & Moldave, 1968). Peptide bond formation was not possible, however, when the enzyme-dependent binding was carried out with the analogue instead of GTP (Hershey & Monro, 1966; Skogerson & Moldave, 1968). This evidence led Moldave to infer that binding of aminoacyl-tRNA was a two-step reaction. Both of the steps require GTP, the first without its hydrolysis and the second probably with its hydrolysis (see rôle of GTP under section 2.3.4). The positions are on sites 1 and 2 depicted by (i) and (ii) respectively in Fig. 2.2. A two-step reaction was also implied by the findings of Jost, Shoemaker & Noll (1968) which have already been discussed. These results suggest that the two binding sites for aminoacyl-tRNA's are either both on the 30S subunit or one on the 30S subunit with the other requiring both the subunits.

Complex formation between aminoacyl-tRNA and 70S ribosomes was also shown to take place at high concentrations of magnesium in the absence of a transfer enzyme and GTP. The reaction nevertheless required the placing of the correct codon of the mRNA (Nirenberg & Leder, 1964; Suzuka, Kaji & Kaji, 1966). Again binding occurred on the 30S subunit alone which was stimulated two-fold on addition of the 50S subunit. Therefore there are also two sites available for the non-enzymic binding of aminoacyl-tRNA to ribosomes which are

probably analogous to those for enzymic binding. Heintz, Salas & Schweet (1968) have shown that the aminoacyl-tRNA bound non-enzymically is able to participate in peptide bond formation without the addition of the binding enzyme. This suggested that polypeptide synthesis, measured at high concentrations of magnesium and without the requirement of transfer enzymes, was hardly different from that occurring under physiological conditions (see formation of peptide bond under section 2.3.2).

Ravel, Thorey, Froehner & Shive (1968) have shown that enzymic binding of aminoacyl-tRNA to ribosomes is stimulated by sulphydryl compounds. It is uncertain, however, whether the sulphydryl group is necessary for this or a later step, the translocase reaction, where its participation is also required (see section 2.3.3).

To sum up; the binding of aminoacyl-tRNA to ribosomes at physiological ion concentrations has been shown to require a transfer enzyme, GTP, as well as the placing of the correct codon on the ribosomes. Furthermore, this reaction appears to proceed in two distinct steps. Similar results have been obtained with both *E. coli* and mammalian systems.

2.3.2 The Formation of the Peptide Bond

With aminoacyl-tRNA now bound to the decoding site, and the peptidyl-tRNA already on the condensing site, the 70S ribosome-complex is primed for peptide bond formation (drawing (ii) of Fig. 2.2), which has been found to occur spontaneously without the addition of supernatant or other factors (Allen & Zamecnik, 1962; Traut & Monro, 1964; Leder & Bursztyn, 1966; Bretscher & Marcker, 1966; Hultin, 1966; Hawtrey, Nourse & King, 1966; Skogerson & Moldave, 1968). The above result indicated that the enzyme catalyzing peptide bond formation was associated with ribosomes. The non-requirement of GTP in peptide bond formation is not surprising as the energy available in the ester linkage of aminoacyl-tRNA is approximately -9 kcal/mole and that in peptidyl-tRNA -7 kcal/mole. Formation of the peptide bond requires about -3 kcal/mole (Jencks, Cordes & Carriuolo, 1960) and it is seen that this requirement is easily met by that present in the two acyl ester bonds. The various requirements of GTP will be discussed in section 2.3.4.

Once peptide bond formation has taken place, the peptide chain becomes attached to the tRNA located on the decoding site, and the tRNA on the condensing

site which contains the growing peptide is released (Noll, personal communication; Jost, Shoemaker & Noll, 1968; Skogerson & Moldave, 1968).

Skogerson & Moldave proved the existence of the complex, by which the peptide was initially bound to the decoding site (site 2 in drawing (iii) of Fig. 2.2), by showing that the amino acid of the aminoacyl-tRNA bound to ribosomes became incorporated into a peptide bond (polypeptide) in the absence of the second transfer enzyme. Jost, Shoemaker & Noll proved this by showing that peptidyl-tRNA was able to bind to the 30S subunit in the presence of an enzyme, but in the absence of the 50S subunit (possibly as site 2 of drawing (iii) of Fig. 2.2 without the 50S subunit). This complex is stabilized on addition of the 50S subunit (site 2 of drawing (iii) of Fig. 2.2) and after addition of the second transfer enzyme the peptidyl-tRNA was moved to the condensing site (site 3 of drawing (iv) of Fig. 2.2). Only after translocation (see section 2.3.3) could peptidyl-tRNA be located on the 50S subunit when the 70S ribosome was dissociated into its subunits. When the author visited North Western University in Evanston, U.S.A. (1967), Noll told of how he had succeeded in separating peptidyl-tRNA bound to the 30S subunit on a gradient (unexplained) from a system active in peptide synthesis, thus showing the existence of this complex during polypeptide synthesis. At this position the ribosome complex is primed for the next step.

2.3.3 Translocation of Peptidyl-tRNA to the Condensing Site

This is the reaction whereby the peptidyl-tRNA on the decoding site on the 30S subunit is moved to the condensing site on the 50S subunit (from site 2 of drawing (iii) to site 3 of drawing (iv) of Fig. 2.2). It is analogous to the reaction whereby formyl-methionyl-tRNA is transferred to the condensing site during chain initiation. This reaction is probably coupled with peptide bond formation because peptidyl-tRNA is normally associated with the 50S subunit which is isolated after incubation of the complete system.

Both GTP and one of the transfer enzymes are needed for the translocation reaction (Leder & Nau, 1967; Ohta, Sarkar & Thach, 1967; Hille, Miller, Iwasaki & Wahba, 1967; Jost, Shoemaker & Noll, 1968; Skogerson & Moldave, 1968). Sulphydryl compounds have also been shown to stimulate the reaction (Skogerson & Moldave, 1967). It is probable that the transfer enzyme and sulphydryl compounds are connected with the movement of the mRNA/peptidyl-tRNA complex, the energy for this reaction being supplied by that which is

There is no indication as yet as to how the codons of the mRNA are placed during this series of reactions and the codon depicted in Fig. 2.2 by the three perpendicular lines on the mRNA indicates only that the message has moved on one codon. Probably the positions of the codons depicted by Bretscher (1968) are near to the truth.

2.3.4 The Rôle of the Different Factors Required in Chain Elongation

It has been shown that monovalent (K^+ or NH_4^+) and divalent (mainly Mg^{++}) cations, GTP, sulphhydryl groups and the transfer enzymes are needed in this reaction. The reaction takes place on the surface of the ribosome and therefore the ribosome is of the utmost importance.

2.3.4.1 The Structure and Rôle of the Ribosome. - The structure of the ribosome will be only briefly reviewed here because this has been well covered in the literature (Petermann, 1964; Watson, 1964; Chedd, 1968). The synthesis of the ribosome is described in a paper by Vaughan, Warner and Darnell (1967) and will therefore be excluded from this account.

Ribosomes from different species differ in size and are generally classified into two principal groups according to their sedimentation coefficients, namely those of the 70S class which are found in some bacteria, chloroplasts and mitochondria, and 80S class which are found in yeast, higher plants and mammalian cells (those from *E. coli* are 70S while those from rat liver are 76S). Loening (1967) more recently classified ribosomes by the size of their RNA. The different species cover a whole range of sizes, with those of *E. coli*, the smallest, at one end of the scale and those from mammalian cells at the other. Watson (1964) says, "Two important facts must always be considered when thinking about ribosomes. The first is that they are chemically very complex. The second is that they are always constructed from two dissociable subunits, one approximately twice the size of the other." These two subunits are 30S and 50S from the 70S class of ribosomes, and 40S and 60S from the 80S class of ribosomes. It is conventional to refer to the smaller and larger subunits as 30S and 50S, respectively, irrespective of their actual size, as has been done in the present work. Each subunit consists of protein and RNA. According to the most recent findings, it appears that the large subunit contains 40 or more proteins (R.R. Traut, personal communication) and two sizes of RNA, namely 23-28S (see Loening, 1967) and 5S RNA (see Chapter 5). The small subunit consists of about 20 different proteins (Traut.

Moore, Delius, Noller & Tissières, 1967) and 16-18S RNA (see Loening, 1967). Most studies on the number of proteins in ribosomes have been carried out using ribosomes from E. coli, but it is not expected that this number will differ much in the ribosomes from mammalian cells.

The ribosome is genetically unspecific because it can be used as the site of synthesis of any cellular protein, and the genetic information to order proteins is not present in its RNA component. Its functions in the biosynthetic process for the synthesis of proteins are numerous as is shown in other sections of this chapter. The principal rôles of the ribosome and its constituent molecules are as follows: (i) the ribosome provides the structure and contains the catalytic sites upon which all the reactions relating to peptide bond formation and possibly the folding of the different proteins, takes place; (ii) the constituent molecules act as catalysts to many of the reactions taking place and contain peptide synthetase which is responsible for peptide bond formation; (iii) the ribosome affords protection to the various active sites of the molecules participating in peptide bond formation.

2.3.4.2 The Rôle of Cations - Although it is known that the different cations function in the different reactions taking place during peptide bond formation, the exact manner in which they function is not known. It is possible that magnesium plays some rôle in the neutralization of the negative phosphate groups of the ribose phosphate backbone of RNA, as is suggested by Moore (1966b), in the binding of mRNA to ribosomes (see section 2.2). Skogerson & Moldave (1968) have shown that the requirement for monovalent cations differs during the different steps of peptide bond formation. Since the different monovalent cations, as well as the different divalent cations, are interchangeable and each sort can often be replaced by suitable organic ions (for example, spermidine can be used in the place of magnesium ions), it does seem that their main rôle seems to be of an ionic nature. How they function in this capacity is not known but their rôle is most probably connected with the maintenance of the configuration of enzymes or ribosomal sites, neutralization of negative charges, and the formation of complexes.

2.3.4.3 The Rôle of the Transfer Enzymes. - The isolation and rôle of the transfer enzymes has been the subject of more conflicting reports in the literature than perhaps any of the other facets of protein biosynthesis. Only some measure

of agreement about the number, properties and function of these enzymes is beginning to emerge. Their isolation from the different cells has been thoroughly reviewed in recent papers and will not be discussed here, although the best method for their isolation still leaves much to be desired. The most relevant references in this field, feature in a special issue of Archives of Biochemistry and Biophysics dedicated to the memory of Richard S. Schweet (Hardesty, 1968).

At least two protein fractions, which are present in the particle-free supernatant of the homogenates of the different cells, have been shown to be necessary for chain elongation in polypeptide synthesis (Allende, Monro & Lipmann, 1964, and Nishizuka & Lipmann, 1966, from E. coli; Richter & Klink, 1967, from yeast; Arlinghaus, Shaeffer & Schweet, 1964, from reticulocytes; Gasior & Moldave, 1965a & 1965b; Klink, Nour & Aepinus, 1963; Klink, Kramer, Nour & Petersen, 1967, from different livers). A third factor is present on the ribosomes. It has been shown that in E. coli more than two supernatant enzymes may be involved in this reaction (Lucas-Lenard & Lipmann, 1966). A possible interpretation of the last mentioned result is that the transfer system is a large complex, consisting of different reactive sites which may be separated from each other during the different isolation and fractionation procedures.

Much of the controversy as to the rôles of the two supernatant transfer enzymes has now been resolved, and according to the most recent evidence, the functions of these are in the binding of aminoacyl-tRNA and in the movement of peptidyl-tRNA. The binding enzyme has numerous functions for which it requires GTP and monovalent as well as divalent cations. The enzyme which participates in the transfer of peptidyl-tRNA from the decoding site (site 2 of drawing (iii) of Fig. 2.2) to the condensing site (site 3 of drawing (iv) of Fig. 2.2) has been called the translocase enzyme and for action it requires GTP and sulphhydryl groups. The manner in which these two transfer enzymes participate in the different reactions is covered in the previous sections and in the two following sections on the action of sulphhydryl groups and GTP.

2.3.4.4 The Rôle of Sulphydryl Groups. - The manner in which sulphhydryl groups function in polypeptide synthesis is unknown, although it has been shown that they are necessary. The longer the duration of incubation in an in vitro system, the greater was the requirement for these sulphhydryl groups

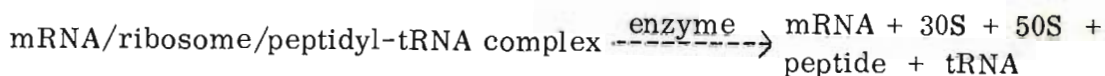
(Arlinghaus, Shaeffer & Schweet, 1964; Fessenden & Moldave, 1963; Conway, 1964; Nishizuka & Lipmann, 1966; Mosteller, Ravel & Hardesty, 1966). The function of sulphhydryl groups seems to be related to the rôle of the enzyme participating in the translocase reaction and their action is closely tied up with that of GTP. This was realized from the results of Sutter & Moldave (1966) who showed that polypeptide synthesis was markedly stimulated when their transferase II was incubated with a sulphhydryl compound, ribosomes, ammonium ions and GTP. Finally, Skogerson & Moldave (1968) showed that these compounds had a stimulatory action in the translocation of peptidyl-tRNA from the decoding to the condensing site. Stimulation of the binding of aminoacyl-tRNA to ribosomes was also caused by the presence of sulphhydryl compounds (Ravel, Thorey, Froehner & Shive, 1968). It is likely that this effect was connected with that associated with the translocase reaction because it was shown that different fractions of the transfer enzymes separate differentially according to the method of preparation (Klink, Kloppstech, Kramer & Dimigen, 1967; Richter & Klink, 1967; Lucas-Lenard & Lipmann, 1966).

2.3.4.5 The Rôle of GTP - GTP, it seems, participates in numerous steps during polypeptide synthesis. The first reaction in which GTP participates is the binding of aminoacyl-tRNA to the ribosome (Gordon, 1968; Skogerson & Moldave, 1967 & 1968; Ravel, Thorey, Froehner & Shive, 1968). This reaction is precipitated initially by an association between GTP and the binding enzyme, followed by the association of the complex with tRNA, after which it becomes bound to the ribosome. No hydrolysis of GTP occurs during this binding. The binding seems to be primarily to a site on the 30S subunit (as depicted by the binding of aminoacyl-tRNA to site 1 in drawing (i) of Fig. 2.2). This is followed by a reaction in which GTP is hydrolysed and the aminoacyl-tRNA becomes more tightly bound to the ribosome in the presence of the 50S subunit (site 2 as in drawing (ii) of Fig. 2.2). The next reaction which requires GTP is the translocase reaction, in which peptidyl-tRNA is transferred by the translocase enzyme from the decoding site to the condensing site with concomitant hydrolysis of GTP and release of inorganic phosphate (site 2 of drawing (iii) to site 3 of drawing (iv) of Fig. 2.2) (Skogerson & Moldave, 1968; Jost, Shoemaker & Noll, 1968). These findings suggest that two molecules of GTP are hydrolysed during peptide bond formation, but, according to Nishizuka & Lipmann (1966), only one molecule of GTP is hydrolysed in the overall

reaction of the formation of each peptide bond during which the molecule is broken down to GDP and inorganic phosphate. This discrepancy may be due to an in vitro artefact caused by the separation of the different steps. It is possible, however, that two molecules of GTP are in fact hydrolysed, the one being resynthesized on the ribosome from the excess of energy (-13 kcal/mole) released during the formation of the peptide bond, which is well in excess of that required for the formation of the phosphate bond between GDP and inorganic phosphate.

2.4 PEPTIDE CHAIN TERMINATION AND RELEASE

This process is not yet well understood, except that in E. coli an enzyme factor is necessary for release and the codons which give the signal for chain termination have been determined. The overall reaction is illustrated in the following equation:

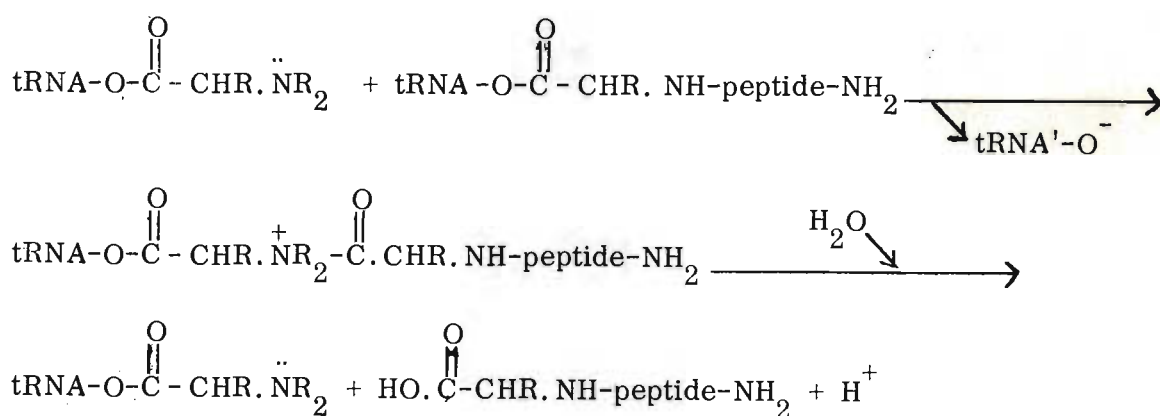


The information for chain termination in E. coli was obtained by use of mutant strains of bacteriophage RNA's. The RNA's from the "amber" and "ochre" mutants showed that there are two chain terminating codons, UAG and UAA respectively (Sarabhai, Stretton, Brenner & Bolle, 1964; Brenner, Stretton & Kaplan, 1965). There is evidence that no specific tRNA recognizes these chain terminating codons (Bretscher, 1968). Ganoza (1966) showed that a soluble enzyme fraction (R-factor) was necessary for chain release. when she found that a concurrent decrease in the release of polypeptides from ribosomes occurred with each step in the purification of the transfer factors, without a concomitant decrease in amino acid polymerization. The so-called R-factor was not of ribosomal origin. It was later isolated by Cappechi (1967) who used DEAE-Sephadex to separate this protein from the fractions containing transfer activity. With the aid of an amber mutant, which synthesizes a short hepta-peptide, he was able to show the participation of this protein in chain termination.

The means of scission of the ester bond between the polypeptide and the tRNA has not yet been determined. Cuzin, Kretchmer, Greenberg, Hurwitz & Chapeville (1967) showed that hydrolysis of the ester bond could be obtained using an enzyme (hydrolase). They achieved this using isolated N-substituted aminoacyl-tRNA's and peptidyl-tRNA. It is not known whether the hydrolase is

seems to exclude this. It is also not known whether the enzyme, hydrolase, functions at the level of ribosomes.

Termination and release of the peptide chain can be made to occur by the addition of puromycin to a system active in peptide synthesis. Release occurs because of the similarity of puromycin to aminoacyl-tRNA's, where a reaction between its amino group and the ester bond of the peptidyl-tRNA, bound to the condensing site, occurs (Smith, Traut, Blackburn & Monro, 1965; Wettstein & Noll, 1965; Hawtrey, Nourse & King, 1966). Release of the peptide chain, attached to puromycin occurs because puromycin has no site for attachment to the ribosomes. Hawtrey & Biedron (1966) have suggested the novel idea that a tertiary amino acid (possibly attached to a tRNA) is involved in chain release. This idea is presented in the equations below:



Peptide bond formation occurs between the tertiary amino acid and the ester linkage of peptidyl-tRNA to give a quaternary linkage. Under aqueous conditions this linkage would be unstable and would readily hydrolyse to the free peptide and tertiary aminoacyl-tRNA. Hawtrey & Biedron showed that this happens when the tertiary analogue of puromycin is used.

Since no tRNA has yet been shown to participate in chain release, it could be that some unknown compound containing a tertiary amino group is able to participate in a reaction similar to that proposed by Hawtrey & Biedron (1966). This compound may be Capecchi's enzyme, but, in the case of an enzyme, it would have to be assumed that the enzyme can recognise the releasing codons.

It has been shown that the 70S ribosome of *E. Coli* dissociates into its subunits after chain release. Mangiarotti & Schlessinger (1966) & (1967) found that rapidly lysed cells yield extracts containing almost exclusively polysomes and ribosomal subunits, with few or no 70S monosomes.

Mangiarotti & Apirion (1967) obtained a similar result on studying the release of nascent polypeptides in the presence of puromycin. These results suggested that ribosomal particles cycle through a free pool of 50S and 30S ribosomal subunits. The subunits reassociate to form 70S particles only during chain initiation. A similar suggestion for reticulocyte ribosomes was proposed by Bishop (1966). Conclusive evidence for this was obtained by Kempfer, Meselson & Raskas (1968). They differentially labelled ribosomes during the growth period and analysed the distribution of isotopic label between the ribosomes and subunits. The subunits formed during chain release differ from those formed by subjection of ribosomes to low concentrations of magnesium in that they are unable to reassociate into 70S monomers. Nomura, Lowry & Guthrie (1967) have shown an effect of initiation factors on the dissociation of 70S ribosomes. These results all suggest the participation of a protein in the dissociation and reformation of 70S ribosomes.

2.5 DISCUSSION

In concluding the review on the biosynthesis of proteins it is relevant to give a brief outline of the problems still to be solved in respect of the reactions which have been discussed. Even though the main sequence of events is known, much work has still to be carried out before the sequence in all the different species of cells is known. The main problems which remain unsolved are the following: (i) how chain initiation functions in nucleated cells. (It is not even known whether chain initiation in all anucleate cells is similar to that occurring in E. coli); (ii) how chain termination in the different cells occurs (There is some understanding of how chain termination occurs in E. coli. Although it is probable that the terminating codons of all cell types are the same, this step may differ widely in anucleate and nucleate cells, as appears to be the case with chain initiation); (iii) how the ribosomes carry out their various functions, and (iv) how the different reactions are controlled. Since the problems connected with chain initiation and chain termination have been briefly dealt with in the review they will not be considered further. The participation of the ribosome in protein synthesis will be considered in its relation to the control of the rate of protein synthesis.

With respect to the control of the rate of protein synthesis it is most likely that it occurs at the level of the reactions connected with mRNA and the ribosome. With mRNA, those reactions concerned with its transcription, transport

Control at the level of the reactions connected with the ribosome would be the most easy to effect because of the vast number of molecules that make up this structure; therefore much of the current research on protein synthesis is aimed at attempting to unravel the structure of the ribosome. In contrast to the earlier belief that the ribosome merely provided a surface on which the different reactions of protein synthesis took place, it is now believed that the translation of mRNA and the reactions associated with translation are to a large extent controlled by the ribosome. Much of the present research therefore, is aimed at elucidating the controlling influence of the different molecules comprising the ribosome. The work described in the following chapters deals with just this aspect of the research.

CHAPTER 3

STUDIES ON POLYSOMES OF RAT LIVER

3.1 INTRODUCTION

The work reported in this chapter was carried out during the period from 1963 to 1965. A review of what was known at that time on ribosomes and their participation in protein synthesis is given by Watson (1963).

It was shown as early as 1950 that the cellular site of protein synthesis was the microsomal component (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950). Relatively soon thereafter it was established that the ribosome, a component of the microsome, was the actual site on which this synthesis took place (Allfrey, Daly & Mirsky, 1953; Littlefield, Keller, Gross & Zamecnik, 1955). It was some time before it was shown that the active form of the ribosome was the polysome because of the confusion resulting from the assumption that genetic information was carried from the DNA template by ribosomal-RNA.

Polysomes were first discovered by Warner, Rich & Hall (1962), working with the reticulocyte system, and by Wettstein, Staehelin & Noll (1963) in the rat liver system. The involvement of polysomes was demonstrated by incubating reticulocytes in vitro with a mixture of ^{14}C -labelled-amino acids, then lysing the cells, and centrifuging the lysate through sucrose density gradients. The different fractions of the gradient were collected and then analysed by different methods including electron microscopy. Analysis revealed that labelled polypeptides were associated only with the clusters of ribosomes which became known as polysomes. In the rat liver preparations, polysomes were demonstrated by showing that, of the isolated fractions on sucrose gradients, only those ribosomes consisting of clusters were capable of polypeptide synthesis and that, if these clusters were degraded to single units they lost their capacity for synthesis. It was shown that the ribosomes in the clusters were joined together by a strand of RNA, now known to be mRNA, when it was observed that their treatment with RNases caused their rapid fragmentation into single ribosomes. Similarly during the incorporation of labelled amino acids into polypeptides, the aggregates diminished in size with an increase in single ribosomes. The integrity of the polysome also depends on the presence of monovalent and divalent cations. tRNA

to which they are linked by ester bonds and various protein fractions (Gilbert, 1963 a and b; Warner, Rich & Hall, 1962; Warner, Knopf & Rich, 1963; Goodman & Rich, 1963; Wettstein, Staehelin & Noll, 1963; Staehelin, Wettstein & Noll, 1963; Noll, Staehelin & Wettstein, 1963). A review on the organization of polysomes has been given by Noll (1965). A brief outline of the finer structure of ribosomes is given in Chapter 2 in section 2.3.4.1. . .

Methods used for the isolation of rat liver polysomes were described by Wettstein, Staehelin & Noll (1963). They showed that a much higher proportion of polysomes was present if during the isolation of ribosomes by the method of Korner (1961), the temperature was maintained at about 0°C.

Furthermore polysomes free of monomer particles could be prepared by centrifuging the mitochondrial supernatant treated with deoxycholate layered over dense sucrose layers, the most suitable being a 2M layer overlaid with a 0.5 M layer. This method remains the one most widely used for the preparation of polysomes from rat liver.

The initial object of this thesis was to study the function of the factors participating in protein synthesis, especially the transfer enzymes, for which polysomes or single ribosomes free of the transfer enzymes were required. It was considered advantageous to work with single ribosomes, and, since polysomes could serve as a useful source of active ribosomes, it was decided that the preparation of single ribosomes would be best from this source. The properties of polysomes were therefore studied, special attention being focussed on the means by which they could be broken down into single ribosomes. Two approaches were used, namely; (i) the hydrolysis of the mRNA strand connecting the different ribosomes, either by the RNases associated with ribosomes, or an exogenous source of this enzyme, and (ii) the release of single ribosomes from the end of the mRNA during protein synthesis. Both these methods were investigated.

Elucidation of the function of the factors participating in protein synthesis was attempted by studying the breakdown of polysomes in the presence of the factors participating in and affecting protein synthesis. The factors which were of interest here were ATP, GTP, a thiol group, different ions, the transfer enzymes, and puromycin which is the antibiotic that causes the premature release of peptides from the ribosome. These studies led to the development of a very good method for the preparation of single ribosomes, based on the use of a specific enzyme which

relevant studies discussed in relation to the type of breakdown caused by each of the factors investigated and the agreement between this breakdown and some of the most recent concepts of the steps involved in chain elongation during protein synthesis.

3.2 METHODS AND MATERIALS

For clarity and ease of reference a complete list of reagents and solutions used for the work described in different chapters is given in the Appendix.

3.2.1 Preparation of Homogenate and Fractions from Rat Liver

Female albino rats of the Wistar strain, weighing between 140-200 grams, were used throughout this study. The rats were obtained from the National Nutrition Research Institute of the C.S.I.R., Pretoria. The animals were housed in wire cages at 20°C and fed ad lib. on the Institute's stock diet (protein 20%, ash 7.4%; main component: maize meal 56%) and tap water.

3.2.1.1 Homogenates. - Homogenates were prepared by the method of Hawtrey, Schirren & Dijkstra (1963). Rats were killed by decapitation and drained of the bulk of their blood. The livers were then quickly removed, freed from adhering lipid and fibrous material, placed in ice-cold beakers and weighed. All further manipulations were carried out at 0°C. The livers were rinsed 4 or 5 times in Medium A (0.25 M-sucrose, 50 mM-tris-HCl, pH 7.6, 25 mM-KCl and 5 mM-MgCl₂), dried on Whatman No. 1 filter paper and transferred to a mortar. The tissue was cut up with scissors and pulped to a fine paste which was then homogenised in an all-glass Dounce homogenizer (supplied by Blaessig Glass Specialities, Rochester, N.Y., U.S.A.) with two volumes of Medium A using 8-10 strokes of the loose plunger (clearance about 0.0015 inch). This was immediately used in the further treatment.

Some of the rats used were starved 20-24 hours prior to decapitation depending on the purpose for which the homogenate was to be used. The purpose of starvation was to avoid the presence of glycogen. Livers from unstarved rats usually weighed from 5-6 grams, whilst the weight of the starved rats was decreased by about 1 gram.

3.2.1.2 Polysomes. - Polysomes were prepared from rat liver homogenates

Noll (1963) following the procedure described by Hawtrey & Nourse (1966). All manipulations were carried out at 0°C . Liver homogenate (90 ml) prepared as in section 3.2.1.1 from rats which had been starved for 24 hours, was centrifuged at $1,400g$ for 10 minutes to remove cell debris and nuclei. The resulting supernatant was centrifuged at $14,370g$ in the 30 rotor of a Spinco Model L ultracentrifuge for 20 minutes to remove mitochondria. To every 13 ml of the resulting supernatant was added 1.5 ml of 13% (w/v) sodium deoxycholate (freshly prepared in distilled water, final concentration between 1.3-1.4%). This was allowed to stand for 5-10 minutes before being carefully layered over a preformed double layer made up of 9 ml of 2 M-sucrose (lower) and 12 ml of 0.5 M-sucrose (upper). Both sucrose layers contained 50 mM-tris-HCl buffer, pH 7.6, 25 mM-KCl and 5 mM- MgCl_2 . This was centrifuged at $99,000g$ (30 rotor) for 240 minutes. After centrifugation, the solution above the pelleted polysomes was carefully removed by suction and the sides of the tubes carefully wiped with filter paper. The pellets were suspended in 1.5 ml of Medium A and allowed to stand for at least one hour before use (they were usually left overnight at 0°C to become suspended). The final suspension of ribosomes was centrifuged at $1,400g$ for 10 minutes to remove aggregated and denatured material. The suspension of polysomes was kept at 0°C and usually used within 5 days.

By determining the dry weight and extinction of a suspension of polysomes, it was found that 1 mg of polysomes in a volume of 1 ml gave an $E_{1\text{ cm}}^{260\text{ m}\mu}$ of 13.5 ($E_{260} = 13.5$).

3.2.1.3 Microsomes.- Starved rats were used for this preparation and all manipulations were carried out at 0°C . 60 ml of homogenate prepared as in section 3.2.1.1 was centrifuged at $1,400g$ for 10 minutes and the resulting supernatant at $14,370g$ for 20 minutes. The $14,370g$ supernatant was then centrifuged at $105,000g$ for 60 minutes (Spinco, 40 rotor) to pellet the microsome fraction. The supernatant (cell sap) was discarded or reserved for the preparation of pH 5 precipitate and supernatant if required, the sides of the tubes cleaned with tissue paper and the pellets suspended in 1 ml each of Medium A. Denatured material was removed by low speed centrifugation.

If a cleaner preparation of microsomes was required, they were washed by diluting the suspension 10-fold and then recentrifuging and suspending the pellets as before.

3.2.1.4 pH5 Precipitate. - For this preparation it was not necessary that the rats be starved but both starved and unstarved rats were used. All manipulations were carried out at 0°C . To the cell sap, obtained from the centrifugation of the 14,370g supernatant at 105,000g for 60 minutes to remove the microsomes, was added cold acetic acid (1-N) until the pH was 5.2. This was allowed to stand for 30 minutes to allow complete precipitation. The precipitate which contained tRNA and the aminoacyl-tRNA synthetases was recovered by centrifugation at 2,000g for 15 minutes. It was washed by suspension in 50 volumes of distilled water, using gentle homogenization, and again centrifuged as before. The final pellet was dissolved in Medium A (20 ml per 60 g rat liver used). When the precipitate had dissolved, it was centrifuged at 10,000g for 5 minutes to remove denatured material. The preparation was stored in the frozen state.

3.2.1.5 pH5 Supernatant. - This is the supernatant which remained after the removal of the pH5 precipitate. After neutralization with KOH, the supernatant was kept frozen and in this state, it remained active for as long as six months. It lost activity, however, on standing at 0°C , and each time it was frozen and thawed. In the experiments described below the supernatant was therefore frozen immediately after preparation, thawed just before use, and thereafter discarded.

3.2.2 Preparation of ^{14}C -aminoacyl-tRNA's

The following were incubated together at 37°C for 15 minutes in a total volume of 60 ml made up by the addition of distilled water.

40 ml pH5 precipitate in Medium A (from 120 gram rat liver).

12 ml solution containing 330 mg ATP in distilled water which had been adjusted to pH 7.6 with KOH, and to which was then added 2.0 ml of 0.3 M- MgCl_2 after which it was again adjusted to pH 7.6.

0.5 ml 0.3 M- MgCl_2 .

4 ml ^{14}C -phenylalanine (40 μc , specific activity 335 $\mu\text{c}/\mu\text{mole}$) or

5 ml ^{14}C -yeast protein hydrolysate (YPH) (50 μc , specific activity 850-1,500 $\mu\text{c}/\text{mg}$).

After incubation the mixture was cooled in ice and adjusted to pH 4.5 with 1 N-acetic acid. This was left to stand for 15 minutes at 0°C to allow

flocculation before being centrifuged at 2,000g for 15 minutes. The resulting precipitate was washed by suspending it in 30 ml iced water and centrifuged as before. The washed precipitate was dissolved in 25 ml of Medium A and 25 ml of cold distilled water was added, after which it was shaken with 50 ml of phenol for 60 minutes at room temperature to remove the protein. After shaking, the mixture was centrifuged at 2,000g for 25 minutes so as to break the emulsion and separate the layers. The clear aqueous phase was recovered and the RNA was precipitated by addition of 0.1 volume of 20% (w/v) potassium acetate and 2.5 volumes of cold ethyl alcohol. (An increase of 50% in yield was obtained if the phenol layer was reshaken with water.) This mixture was left to stand at -15°C overnight. The RNA precipitate was collected by low speed centrifugation and redissolved in 10 ml iced water. The RNA was then re-precipitated as before and the final precipitate dissolved in iced distilled water and dialysed against the same water for 24 hours (5 changes). The resultant solution of aminoacyl-tRNA was stored frozen as 1 ml aliquots.

For ^{14}C -phenylalanine-tRNA from E. coli see appendix.

3.2.3 Preparation of Linear Sucrose Gradients

Linear sucrose gradients were prepared as described by Stead, Nourse & Hawtrey (1964).

3.2.4 Ultracentrifugation

Preparative ultracentrifugation was carried out in either a Beckman Model L or Model L4 ultracentrifuge. The g values which are given are average.

Analytical ultracentrifugation was carried out in a Beckman Model E ultracentrifuge, using Schlieren optics and was performed by Mr. N. van der Walt of the department of Physical Chemistry at the N. C. R. L.

3.2.5 Preparation and Study of Electronmicrographs

The different preparations were examined using both the shadowing technique and a method for negative staining. The grids containing the ribosomes were prepared as follows: a drop of ribosomes at a concentration 4-10 μg ribonucleoprotein/ml, was placed on the grid which was carefully blotted and washed in a series of similar solutions of decreasing concentration, starting with that in which the preparation of ribosomes was suspended. The solutions

were diluted 1x, 2x, 4x, 8x, and 16x. The grids were finally washed in distilled water for the shadowing technique and in a solution of 1% phosphotungstic acid (contact time 15 seconds) followed by distilled water when negatively stained. The grids were dried in air. Palladium was used for shadowing. In another preparation the ribosomes were suspended in gelatin at a concentration of 50 μg per ml, the gelatin allowed to gel, and thin sections made of the gel which were placed on grids. The different grids were then examined under the electron microscope (Hitachi Model Hu 11B), studied and photographed at various magnifications.

Some of the preparations described here were suitable for the purpose of the experiment but others were not. However, no further attempts were made to improve the technique because time on the electron microscope was limited.

3.2.6 Methods for the Assay of Polypeptide Synthesis

This was done using either labelled free amino acids and a cell fraction containing tRNA's and aminoacyl-tRNA synthetases, or labelled aminoacyl-tRNA's and a source of the transfer factors. The methods used are given below.

3.2.6.1 Method 1: Using the cell sap or the pH5 precipitate and free ^{14}C -amino acids. - The reaction medium contained tris-HCl buffer, pH 7.6 (50 mM); KCl (25 mM); MgCl_2 (8.5 mM); sucrose (0.25 M); ATP (0.62 μmoles), GTP (0.25 μmoles), PEP-K (5 μmoles), GSH (0.3 μmoles), all adjusted to pH 7.6 with KOH; phosphoenolpyruvate kinase (30 μg); ^{14}C -labelled yeast protein hydrolysate of specific activity 850-1,500 $\mu\text{c}/\text{mg}$ (0.7 μc); or ^{14}C -phenylalanine of specific activity 325 $\mu\text{c}/\mu\text{mole}$ (0.30 μc) and poly U (50 μg); polysomes (0.2-0.3 mg of ribonucleoprotein); cell sap (2-3 mg) or pH5 precipitate (1-2 mg). The final reaction volume was 0.6 ml. Incubations were carried out in test tubes at 37°C for various times.

3.2.6.2 Method 2: Using ^{14}C -labelled aminoacyl-tRNA's and a source of transfer enzymes. - The reaction medium contained in a volume of 400 μl ; tris-HCl buffer, pH 7.6 (50 mM), KCl (25 mM), MgCl_2 (8.5 or 13 mM), sucrose (0.25 M) (all final concentrations); GTP (0.16 μmole), PEP-K (4 μmoles), GSH (2 μmoles) (all neutralized); phosphoenolpyruvate kinase (30 μg); ^{14}C -YPH-tRNA's from rat liver (6,000-12,000 cpm) or ^{14}C -phe-tRNA from rat liver (2,000-6,000 cpm) or from *E. coli* (15,000 cpm) added with poly U (50 μg) (40-60 μg of

aminoacyl-tRNA was used); ribonucleoprotein (0.2-0.3 mg); pH5 supernatant (2-3 mg) or partially purified transfer factor (TF, for preparation see under 4.2.4) (0.2-0.4 mg). Incubations were carried out in test tubes at 37°C for various times.

3.2.7 Isolation and Counting of ^{14}C -labelled Proteins

After incubation the reaction was stopped by immersing the tubes in ice and adding 8 ml of ice-cold 5% trichloroacetic acid (TCA). The tubes were left overnight at 2°C to allow proper precipitation and were then washed by one of the two methods below.

3.2.7.1 Centrifugation method. - The TCA-precipitates were centrifuged down and washed twice more with cold 5% TCA using the same procedure. **The precipitates in 5% TCA were then heated at 90°C for 15 minutes.** (This treatment causes the hydrolysis of aminoacyl-tRNA with solubilization of amino acids and short peptides.) The tubes were then cooled and centrifuged as before. Using the same procedure, the precipitates were washed twice more in cold 5% TCA and then treated in either of the following ways:

(a) using the centrifugation method, washed twice in ethanol (96%), once in ethanol/ether (50/50) and once in ether, after which they were passed onto planchettes, dried, weighed, and counted in a Phillips electronic counter (tube PW 41491, counting efficiency 8-10%).

(b) passed on to Millipore filters (HA 0.45), washed once on the filter with cold 5% TCA, the filter dried, and counted in the Packard Scintillation Spectrometer as described in the method below.

3.2.7.2 Millipore method. - The precipitates in 5% TCA were heated at 90°C for 15 minutes, after which the tubes were cooled in ice. When cold, the contents of the tubes were passed through Millipore filters (HA 0.45) and washed thrice on the filter with cold 5% TCA and thrice with 50 mM-tris-HCl buffer, pH 7.6, containing 1 mM-MgCl₂ (Medium X). The filters were then dried and counted in a Packard Scintillation Spectrometer using 0.03% dimethyl-POPOP and 0.5% PPO (Packard Scintillation grades) in 15 ml toluene. Counting efficiency was 80%.

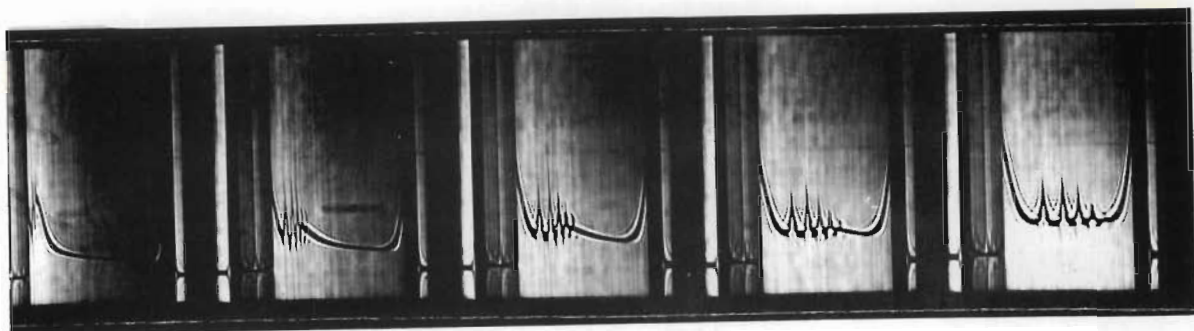
The Millipore method was the method of choice and was used for all the experiments described unless otherwise stated.

3.3 RESULTS

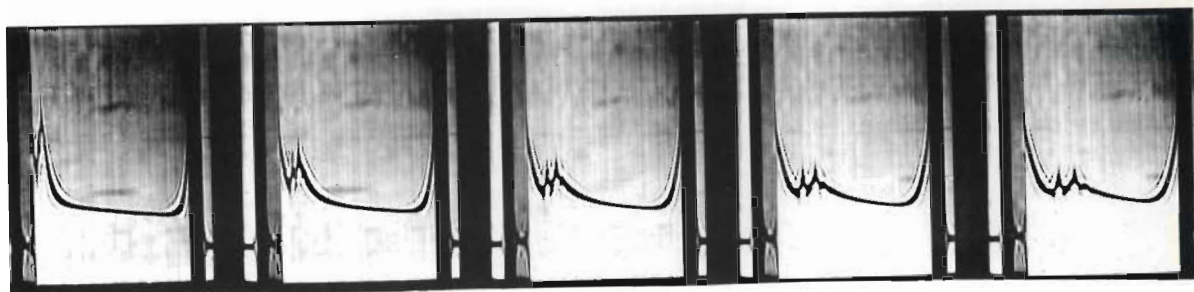
3.3.1 Characterization of Polysomes

3.3.1.1 Analyses of Polysomes on Sucrose Gradients. - The analysis of polysomes in Medium A on linear sucrose gradients containing various amounts of MgCl_2 is shown in Fig. 3.1. The recovery of polysomes placed on the three gradients was usually between 25-30% when the concentration of magnesium in the gradient was 10 mM (a), 35-40% when 5 mM (b) and 50-60% when 1 mM (c). Slightly higher recoveries were usually obtained if the polysomes were aged because of an increase in the lighter fractions due to breakdown (see section 3.3.2.1). The size distribution of polysomes varied over a wide range, from monomers to complexes containing up to 20 or more ribosomes per strand of mRNA (calculated from position on gradients and observed in electron micrographs, see d of Plate 3.2), with a maximum in complexes consisting of 5-7 ribosomes. The distribution patterns were similar when using either 5 mM- MgCl_2 or 10 mM- MgCl_2 (a), except that with the latter more material moved through the gradient. Possibly this was due to the aggregation of polysomes in the presence of the higher concentration of magnesium ions. Although the distribution pattern of polysomes with the gradient in c, containing 1 mM- MgCl_2 , did not appear to differ much from those obtained with the gradients containing higher concentrations of MgCl_2 , the increase in the two lighter peaks (monomer and dimer) did indicate that breakdown of polysomes had occurred. It would seem that the monomers (seen with the gradient used in c) resulted from the breakdown of the polysomes during their centrifugation in the low concentration of magnesium. The results of Wettstein, Staehelin & Noll (1963) which showed that no single ribosomes passed through the layer of 2 M-sucrose used in the isolation procedure support this supposition. It was found that when polysomes were suspended in a buffer which contained only 1 mM- MgCl_2 and then centrifuged over gradients like those used in a and b, patterns similar to those obtained with c resulted. This was a further indication that breakdown of polysomes occurred at this concentration (1 mM) of magnesium ions.

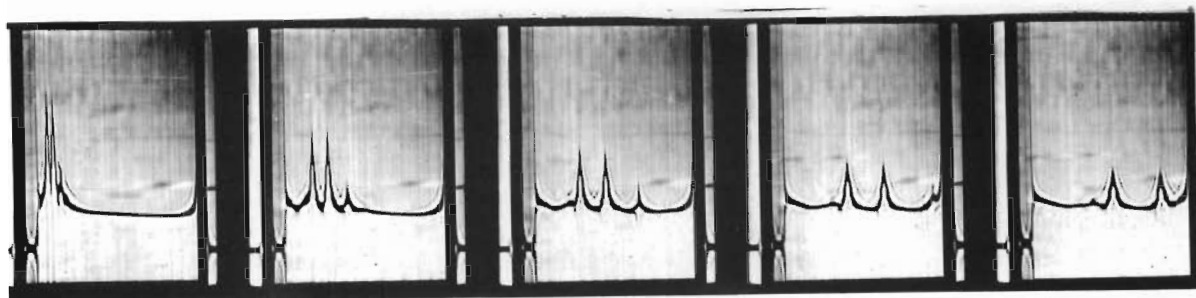
The small amount of monomeric ribosomes that was obtained in these preparations probably resulted from handling. With more stringent handling, less of this lighter material was obtained on the gradients (see effect of temperature in section 3.2.2).



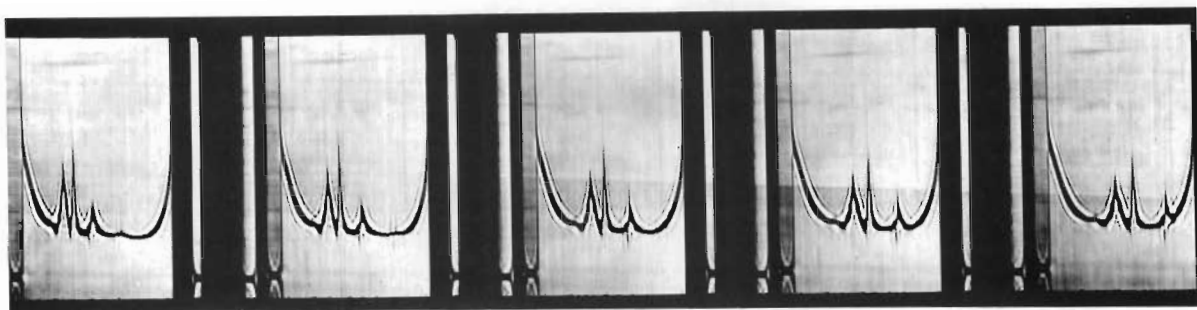
a



b



c



d

**PLATE 3.1 SEDIMENTATION VELOCITY PATTERNS OF RIBOSOMES
IN THE ANALYTICAL ULTRACENTRIFUGE**

Schlieren optics were used and the runs were done at 20°C.

- a Polysomes in Med A. Speed 31,410 r.p.m. Pictures taken at 4 minute intervals.
- b M-ribosomes in Solution M. Speed 31,410 r.p.m. Pictures at 4 minute intervals.
- c R-ribosomes in Solution R. Speed 31,410 r.p.m. Pictures at 8 minute intervals.
- d S-ribosomes in 0.25 M sucrose. Speed 39,460 r.p.m. Pictures at 4 minute intervals.

which contained 10 mM- and 5 mM-MgCl₂ respectively, were considered satisfactory and were used for the studies on the breakdown of polysomes.

There were no apparent differences in the profiles obtained when the concentration of tris-HCl in the gradient was varied between 10-50 mM or if KCl was excluded (results not shown).

3.3.1.2 Analyses of Polysomes in the Analytical Ultracentrifuge. - Plate 3.1a shows the differential pattern of polysomes in Medium A, centrifuged in the analytical ultracentrifuge using Schlieren optics. Sedimentation was from left to right in the photographs. The aim of the experiment was not to determine the S values but to obtain another picture of the different sized particles. It can be seen that a typical polysome profile was obtained except that in this instance there was a higher proportion of smaller particles than was observed with sucrose gradients. This was most likely due to the breakdown caused by the higher temperature (20°C) at which this run was made (see effect of temperature in section 3.3.2).

3.3.1.3 Polysomes Under the Electron Microscope. - Electron micrographs of positively and negatively stained polysomes are shown in Plate 3.2. In Plate 3.2a, an electron micrograph of low magnification (7,500x), it is seen that the polysomes were free of other cytoplasmic particles. Other pictures taken at random were similarly free of other cytoplasmic particles. If the preparation had been contaminated with mitochondria or lysosomes, particles 100-200 times as large as those shown would have been present. The size of the mitochondrion is about 2 μ, while that of single ribosomes is from 10-15 mμ.

The majority of the particles existed as polysomal clusters of two or more, and a typical picture of a polysome consisting of about 20 ribosomes is seen in Plate 3.2d, an electron micrograph at a magnification of 270,000. This shows the space pattern common to all specimens, in which ribosomes are regularly aligned in staggered positions of parallel double rows (Behnke, 1963; Waddington & Perry, 1963; Rifkind, Danon & Marks, 1964; Perl, 1964; Weiss & Grover, 1968). The polysomes in the pictures of Plate 3.2 are classified according to the requirements given by Weiss and Grover (1968), which were: (i) groups of four, said to be located at the corners of "tetrads". (In Plate 3.2 the tetrads are indicated by the perpendicular lines)

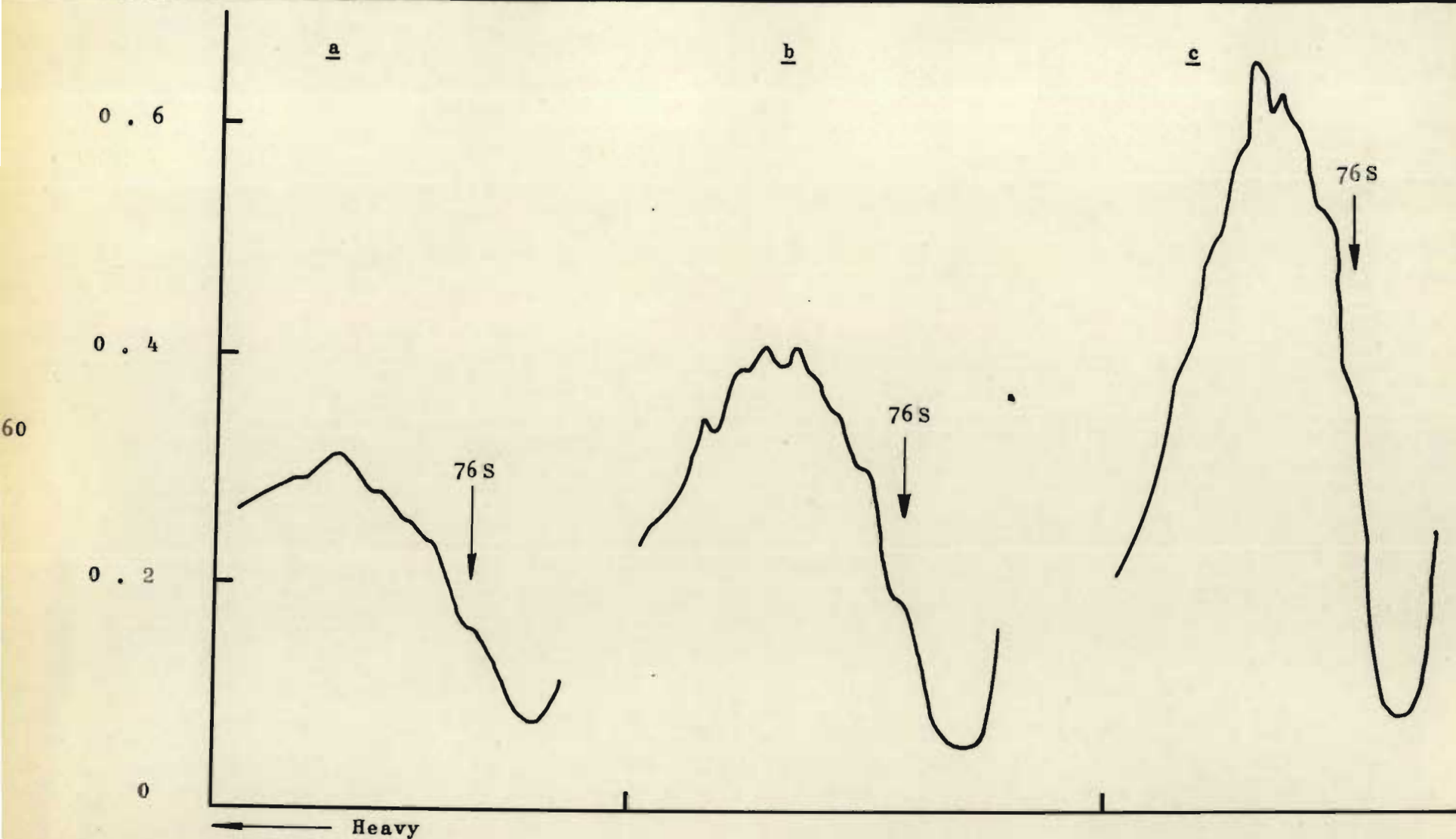


Figure 3.1

Analysis of polysomes on sucrose gradients

4 mg Polysomes were placed on different 15-30% (w/v) linear sucrose gradients containing 50 mM tris-HCl, pH 7.6, 25 mM 1 and the following amounts of $MgCl_2$; 10 mM in a, 5 mM in b and 1 mM in c. Centrifugation was for 150 minutes at 100,000g in a Beckman SW 25.1 rotor.

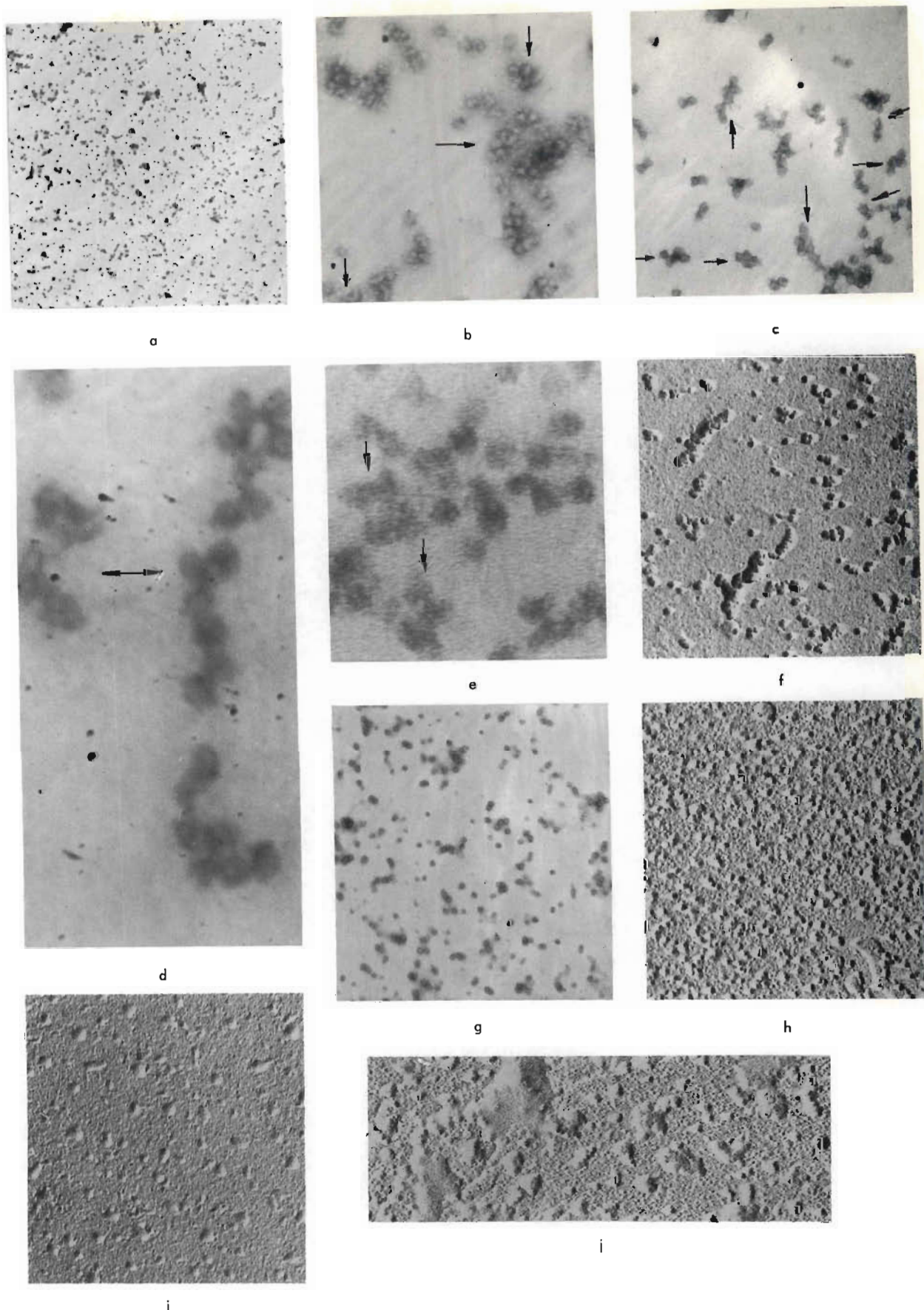


PLATE 3.2 ELECTRON MICROGRAPHS OF RIBOSOMES

POLYSOMES *NEGATIVE STAIN:* a = 7,500, b = 135,000, d = 270,000;
POSITIVE STAIN: c = 135,000;
NEGATIVE STAIN IMBEDDED IN GELATINE: e = 300,000;
SHADOWED: f = 30,000.

R-RIBOSOMES: *NEGATIVE STAIN:* g = 30,000; *SHADOWED:* h = 30,000.

S-RIBOSOMES: *SHADOWED:* i = 30,000.

M-RIBOSOMES: *SHADOWED:* i = 30,000.

down in b, c and especially e.); (ii) zigzag strands of various lengths. (These are shown by the horizontal arrows in b, c and d of Plate 3.2;) It was difficult to show the presence of mRNA strands in the electron micrographs where negative staining of polysomes occurred, but these strands were sometimes seen when positive staining was present which is demonstrated by the angled arrow pointing up in c of Plate 3.2. This description of the assumed space pattern of polysomes and the presence of a stained mRNA strand in the positively stained electron micrographs have been used as a means to differentiate between polysomes and clusters of ribosomes due to aggregation caused by magnesium. Nearly all the electron micrographs of preparations of polysomes, where the aggregates consisted of four or more single ribosomes, have confirmed this interpretation.

3.3.1.4 Activity of Polysomes in Different "in vitro" Systems . - Activity here is defined as the in vitro incorporation of labelled amino acids into polypeptides. This was measured as the amount of label which was stable in 5% hot TCA, a method which gives values slightly lower than those expected because short polypeptides would be lost during the method used for washing the ribosomes.

The time course of polypeptide synthesis by polysomes with different systems is shown in Fig. 3.2. Assay systems, in which the cell sap or the pH5 precipitate were used (enzyme fractions which contained tRNA, aminoacyl-tRNA synthetases and the transfer enzymes), were capable of synthesizing polypeptides from free amino acids (curves 1 and 2 respectively). The systems which contained only the transfer enzymes and which were devoid of tRNA required activated aminoacyl-tRNA. The polysomes were active in all the assay systems that were tried. The rate of the reaction was most rapid during the early stages (first 5 minutes) with a gradual tailing off until a plateau was reached. The more purified the system used, the sooner was the tailing off of the rate of the reaction to a plateau. The purity of the assay systems which were used increased in the following order: Curve 1 (cell sap), curve 2 (pH5 supernatant), and curves 4 and 5 (a partially purified transfer fraction, TF). As expected, the reaction stopped even sooner if the polysomes were first pre-incubated. If a lower concentration of the enzyme fraction was used the initial rate of the reaction was less, as was the total incorporation, but the time required to reach a plateau was only slightly increased.

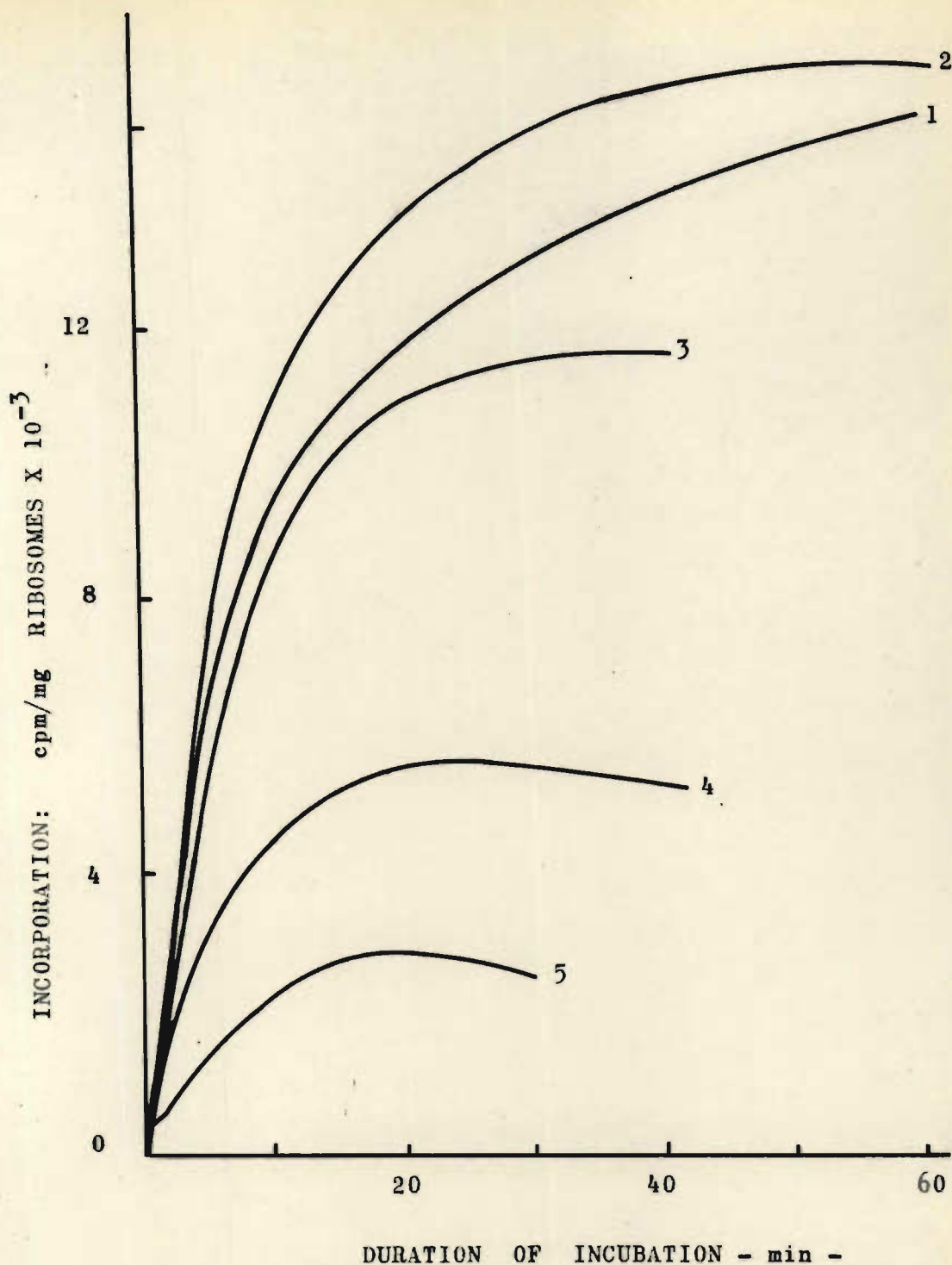


Figure 3.2

Kinetics of polypeptide synthesis

In curves 1 and 2 assays were done according to Method 1 as described under 3.2.6, using the cell sap and pH5 precipitate respectively. In 3, 4 and 5 assays were done according to Method 2. For 3 the pH5 supernatant was used while with curves 4 and 5 a purified fraction containing transfer enzymes (TF see Chapter 4) was used. With 5 the polysomes were pre-incubated for 2 minutes in the complete assay system for protein synthesis minus ^{14}C -label. ^{14}C -yeast protein hydrolysate was used in all experiments.

The reason for the tailing off of the rate of the reaction is not known (see Noll, 1965, page 85, for a discussion on this). Under optimum conditions of protein synthesis, as pertains in vivo, the rate of release of ribosomes from the message should equal the rate of attachment at the initiation site. This would keep the rate of the reaction constant and the polysomes intact. During the measurement of in vitro protein synthesis, however, this would not be expected and it was actually shown in section 3.3.3 that the release of ribosomes from the message was more rapid than reattachment when the breakdown of polysomes during protein biosynthesis was studied (see Figs. 3.5 and 3.6). However, this should not have caused complete cessation of the reaction. This cessation of the reaction was not caused by a lack of labelled ^{14}C -YPH amino acids, especially in curves 1 and 2, because the amount of label added to the systems was 10-fold and upward of that amount incorporated. Nevertheless, a lack of labelled aminoacyl-tRNA was probably a contributory cause for the decrease in the rate of the reaction in the systems where preformed aminoacyl-tRNA was used, because of their hydrolysis during the course of the incubation. The two most likely reasons for this decrease would be the hydrolysis of the mRNA by RNases and a loss in some factor(s) necessary for chain initiation. The result shown in Fig. 3.2 is in accordance with both these explanations as is discussed in the next paragraph.

It has been shown that RNase inhibitors are present in the cell sap of rat liver (Sugano, Watanabe & Ogata, 1967; Lawford, Sadowski & Schachter, 1967), and that microsomes contain RNases (Lawford, Langford & Schachter, 1966) which are activated by deoxycholate. It is therefore likely that these RNases are located on the ribosomes of rat liver as was shown with those of E. coli. If this is so, it would be expected that the less purified systems should continue synthesizing polypeptides for longer periods than the more purified systems. The results shown in Fig. 3.2 were in agreement with this suggestion. These results, however, also favour the suggestion proposed by Baliga, Pronczuk & Munro (1968) who suggested that the system ceased to incorporate amino acids because of the lack of or destruction of some chain initiation-factor required for the interaction of free ribosomes with mRNA. Again, with the more purified systems the reaction would cease earlier because of the lower concentration of initiation factor in the enzyme fraction used. Experiments with the purified ribosomes, described in Chapter 4 of this thesis,

favour the suggestion that the decrease in the rate of protein synthesis was due to the loss of the initiation factor. This was shown when these ribosomes were used to measure the rate of reaction at increased concentrations of magnesium ions which avoided the requirement of initiation factors in polypeptide synthesis. In this case the rate of the reaction continued in a linear fashion for nearly 60 minutes.

The above results show that the polysomes used in this study were active in the two main systems used to assay protein synthesis in vitro, namely (i) that with the cell sap or pH5 enzymes using ^{14}C -labelled amino acids, and (ii) that with transfer enzymes, supplied either as the unpurified pH5 supernatant or as the partially purified fraction, using ^{14}C -aminoacyl-tRNA. The kinetics of the systems used are similar to those that have been described for mammalian systems by others since the completion of this work.

3.3.2 Factors Effecting the Breakdown of Polysomes as Studied on Sucrose Gradients

The effects of a number of treatments on the breakdown of polysomes were investigated and the results of the experiments are presented in Figs. 3.3 to 3.8. In order to obtain information on the most effective centrifugation times for the analysis of the polysome preparations, the centrifugation times used in conjunction with different experiments varied from 120 to 150 to 200 minutes. It was found that centrifugation for longer times gave a better separation of the individual peaks, but with more material passing through the gradients.

The distribution patterns obtained proved adequate for the purpose of the experiments, irrespective of the centrifugation period employed. No attempt was made to repeat the series of experiments using a common centrifugation time.

3.3.2.1 Time and Temperature of Incubation in Medium A - The effect of incubating polysomes, suspended in Medium A, at 37°C for various times is shown in Fig. 3.3, while Fig. 3.4 shows the effect of incubation for 10 minutes at different temperatures. These treatments caused the breakdown of polysomes. A similar breakdown of polysomes, though less marked,

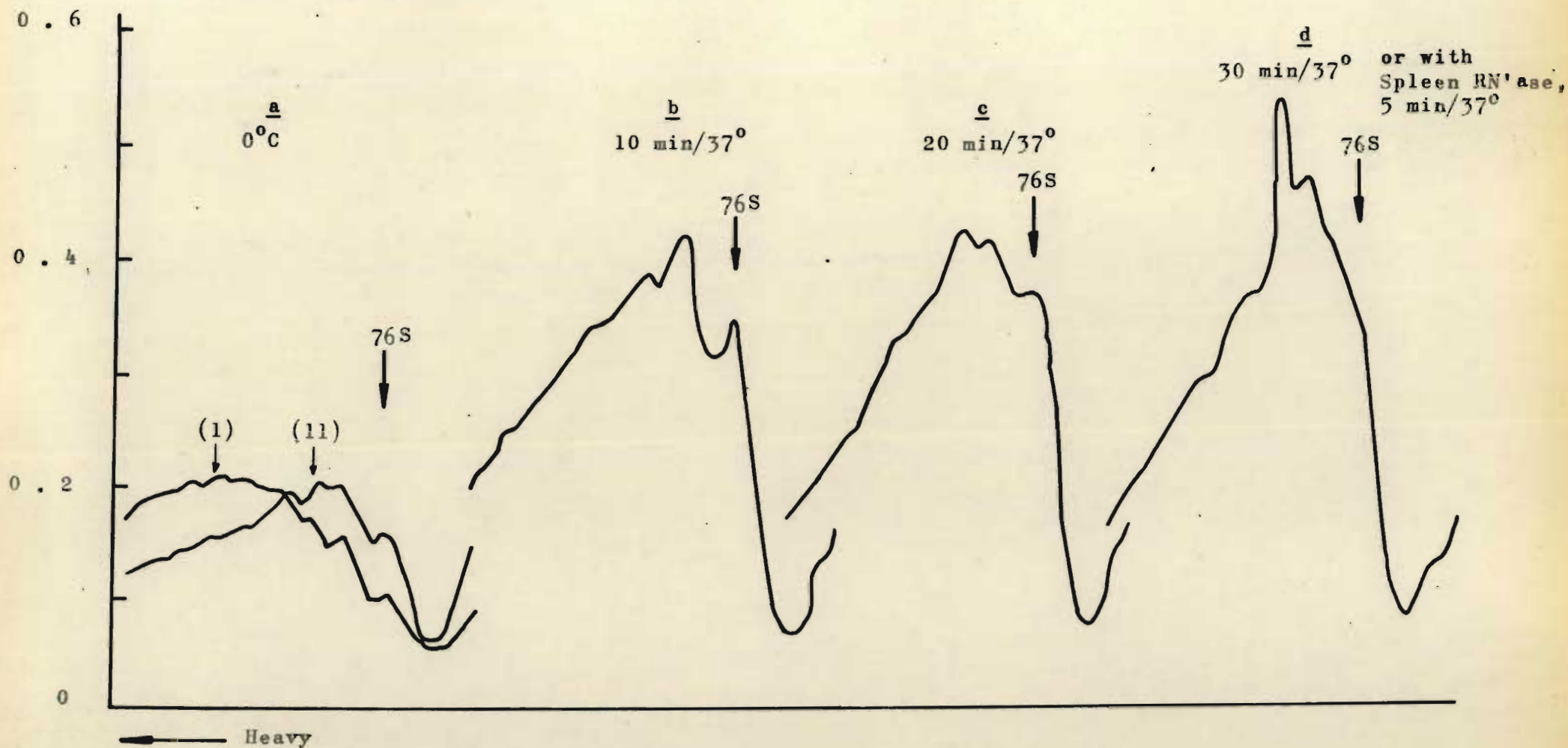


Figure 3.3
Effect of time of incubation on breakdown of polysomes in Medium A

1 mg of polysomes in Medium A was incubated for the times indicated. In a(i), b, c, and d the polysomes were used within 1 day of preparation. In a(ii) the polysomes were aged for 5 days at 0°C before use. After incubation the samples were layered in ice and layered over 15-30% (w/v) sucrose gradients containing 10 mM tris-HCl, pH 7.6 and 10 mM MgCl_2 . Centrifugation was at 63,000g for 120 minutes.

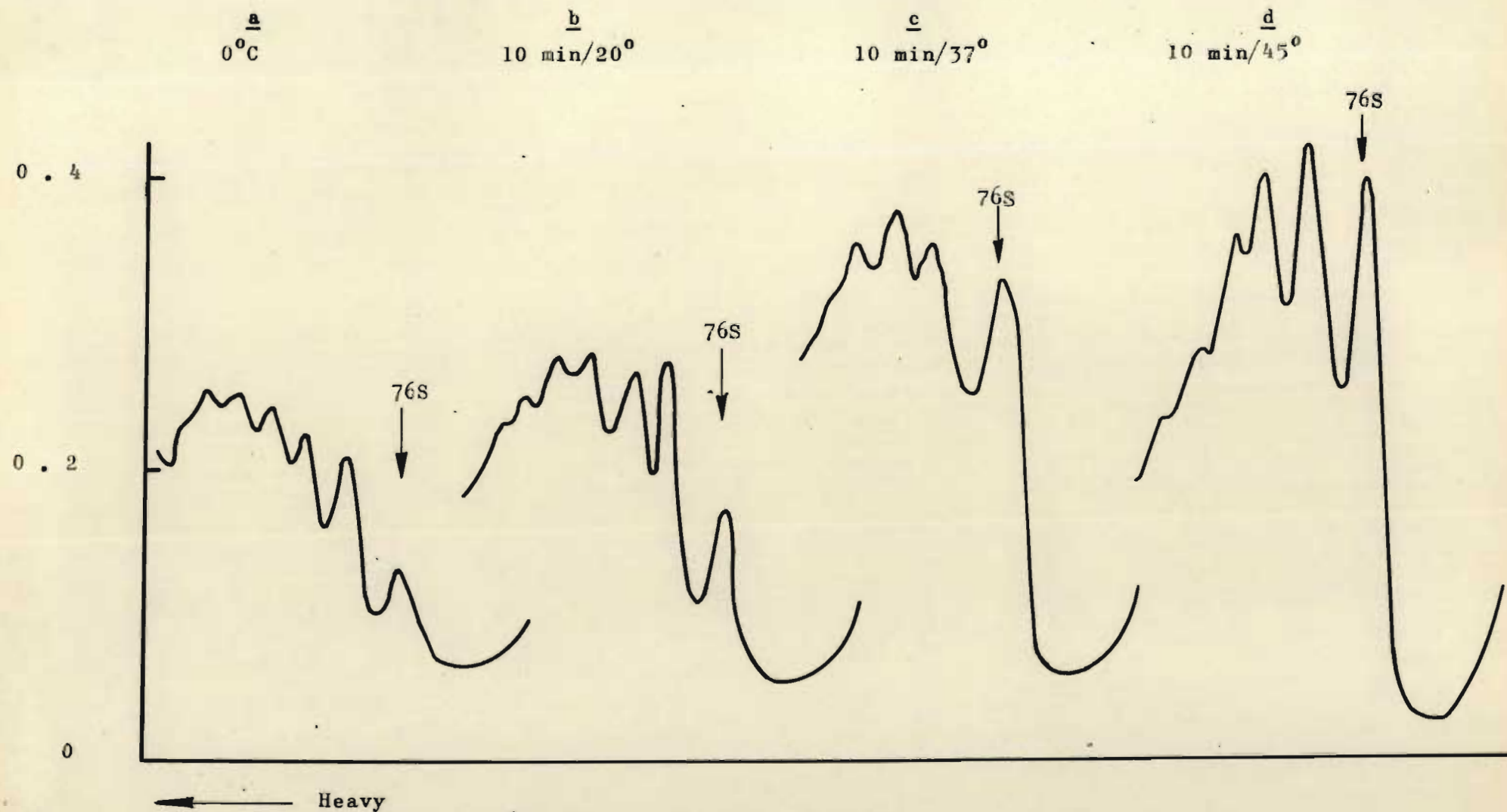


Figure 3.4
Effect of temperature of incubation on breakdown of polysomes in Medium A

1.2 mg of polysomes in Medium A was incubated for 10 minutes at the temperature indicated. Gradients similar to those in Figure 3.3 were used. Centrifugation was at 63,000g for 120 minutes.

during their ageing for 5 days at 0°C , as is shown in curve (ii) of Fig. 3.3a. The polysomes fragmented into smaller particles which depended on both the length and temperature of incubation, the size of the fragments decreasing with an increase in both the time of incubation at a fixed temperature and the temperature (up to 45°C) at which the incubation was carried out. The breakdown caused at temperatures higher than 45°C was not studied. If the incubation was allowed to continue for longer, practically all the material moved in the area of the smaller particles which indicated a breakdown of polysomes to single ribosomes or aggregates of these, aggregation being caused by the association of monomeric ribosomes in the presence of magnesium. The pattern of breakdown of polysomes observed here was similar to that obtained when spleen RNase was added (d of Fig. 3.3), except that the latter reaction was much faster. This suggested that the breakdown in Medium A was due to RNases associated with the ribosomes which cause the scission of the mRNA connecting the individual ribosomes.

Breakdown of polysomes in this way would be expected to yield single ribosomes containing fragments of mRNA that were undegraded because of the protection afforded by the width of the ribosome to which it was bound. Such fragments have been shown to be protected by polysomes which were treated with RNases (Brentani, Brentani & Raw, 1966; Brentani, Brentani, Raw, Cunha & Wrotschincky, 1968). Although the presence of this fragment of mRNA attached to ribosomes had not been shown when these experiments were carried out, its possible presence was discussed in this laboratory while this work was being done. Therefore, single ribosomes prepared in this way were expected to be unsuitable for studies with an exogenous source of mRNA because the attached fragment would inhibit the binding of the added mRNA. The presence of this fragment on ribosomes in the above experiments was not determined, but it was found that polysomes which were incubated in Medium A for 45 minutes, or in the presence of spleen RNase for 10 minutes, were not as active in the synthesis of polyphenylalanine, dependent on the presence of poly U, as a preparation which was incubated in an active protein synthesizing system (see Section 3.3.2.2). These results are shown in Table 3.1.

Table 3.1 Activity of Single Ribosomes

Single ribosomes were prepared by the incubation of polysomes in the system shown in the Table below. After incubation, the mixtures were diluted 20-fold with cold Medium A and the ribosomes re-isolated by centrifugation. The ribosome pellets were dissolved and assayed for their activity in the synthesis of polyphenylalanine as described under Method 1 of section 3.2.6.1, using ^{14}C -phenylalanine and poly U. The centrifugation method of section 3.2.7.1 and the Phillips counter were used for the isolation and counting of the labelled polypeptides.

Method of preparation of single ribosomes	Incorporation of ^{14}C -phenylalanine. (cts/mg nucleoprotein)
a - Polysomes incubated in Medium A	86
b - Polysome incubated with spleen RNase	70
c - Polysomes incubated in the system for protein synthesis (Method 1 of Section 3.2.6.1)	152

3.3.2.2 Incubation in Complete System for Amino Acid Incorporation. - The effect of the incubation of polysomes in the complete amino acid incorporation system is shown in Figs. 3.5 and 3.6. This incubation resulted in their rapid breakdown to monomers. The large increase in the dimer and trimer peaks, which also accumulated as the result of this type of incubation, was attributed to the aggregation of monomers caused by the presence of magnesium. The increase in the number of monomers was best illustrated in Fig. 3.6c in which the gradient containing 1 mM- MgCl_2 was used.

It is noteworthy that the profile of polysomes on gradients showing breakdown during protein synthesis contained only monomers (and small aggregates of these) together with the normal pattern of polysomes. There was no increase in the numbers of polysomes of intermediate size (as for example tetramers and larger) as seen in the previous section. This is because the ribosomes were run off one at a time as they reached the end of the message during protein synthesis, in contrast to the arbitrary scission of the connecting mRNA in the previous method leaving variable numbers of ribosomes joined together.

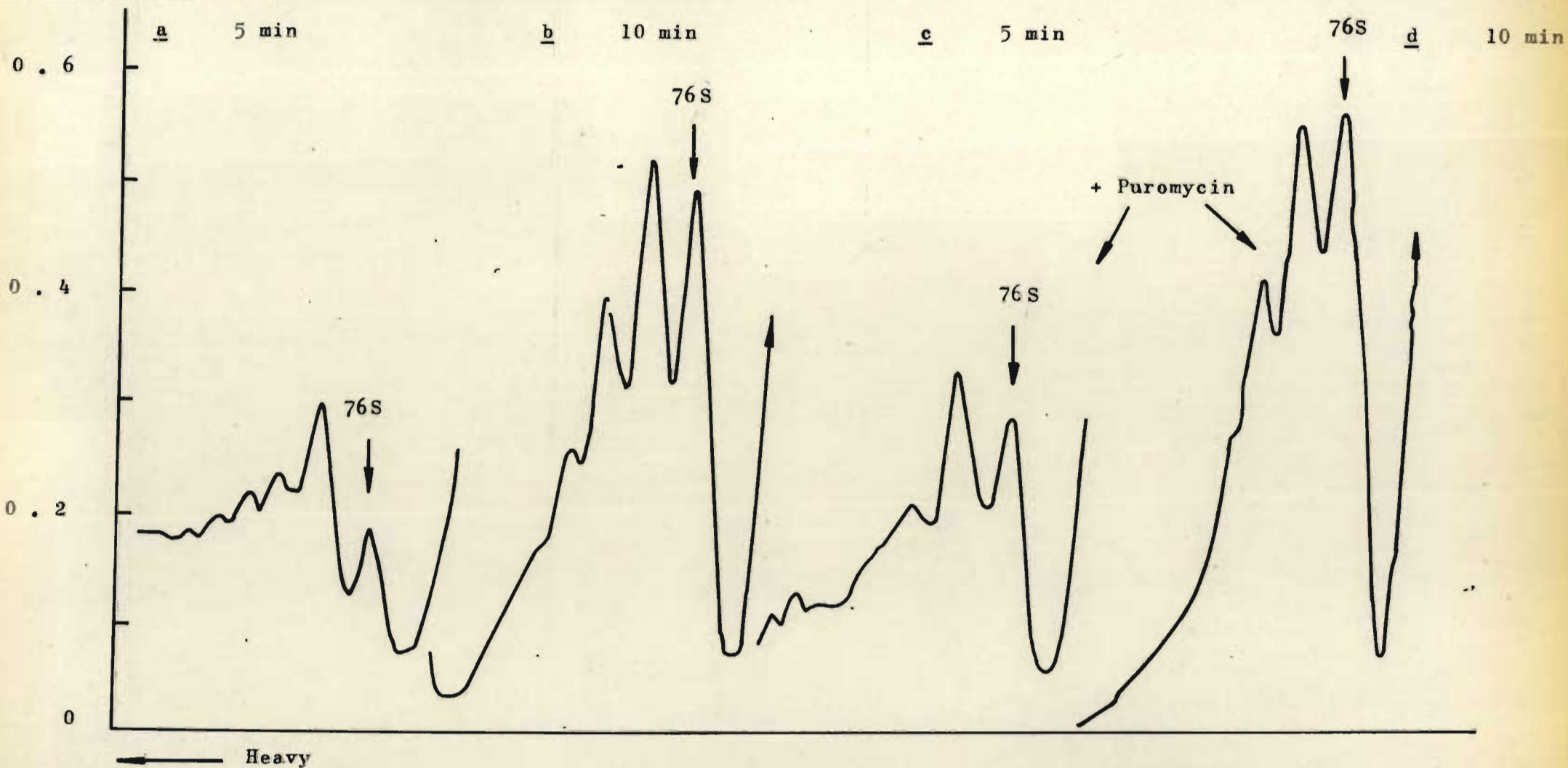
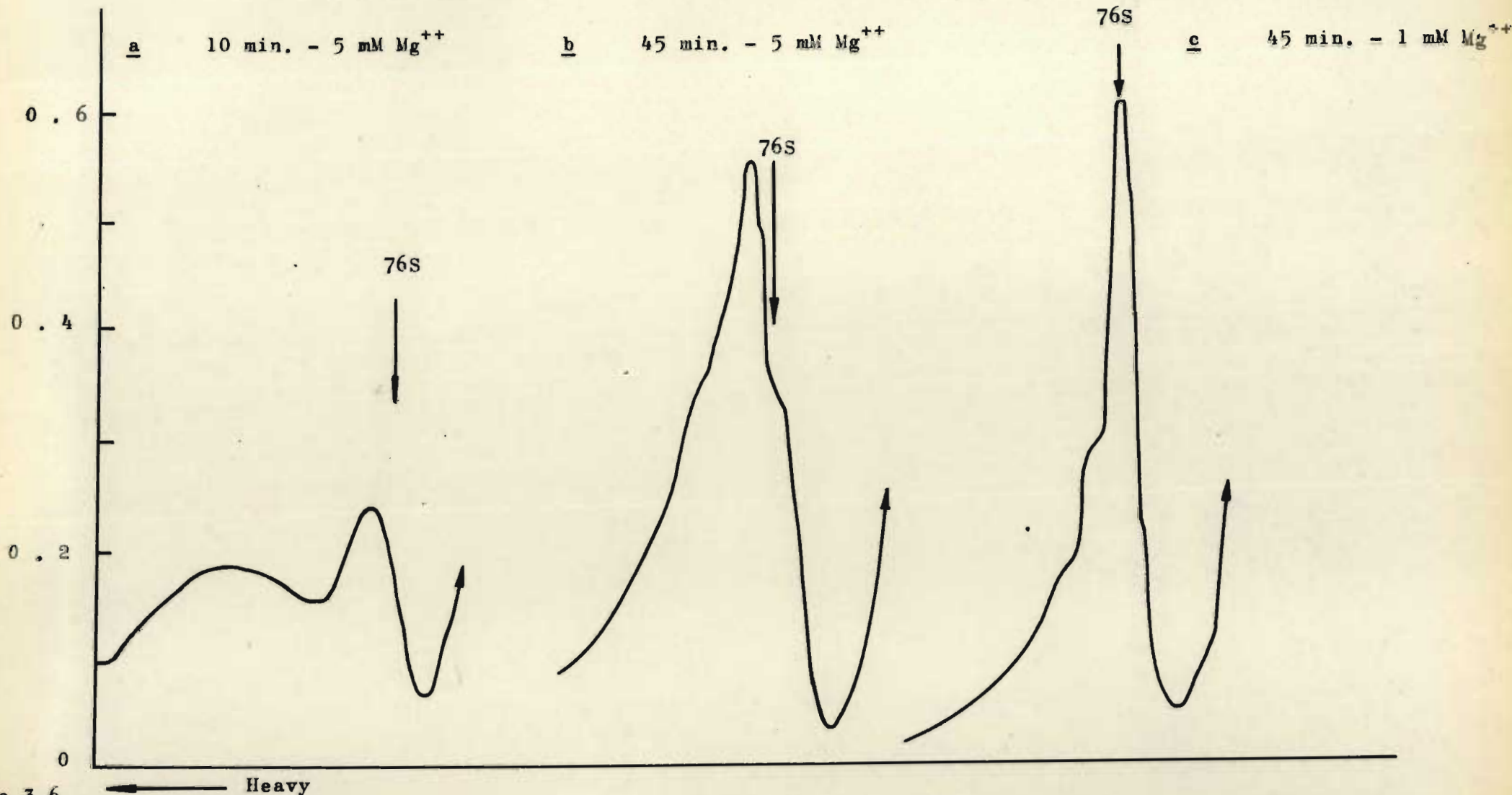


Figure 3.5
 Breakdown of polysomes during protein synthesis and the effect of puromycin
 Polysomes (0.6 mg) were incubated at 37°C for the times indicated in the complete assay system for protein synthesis using the pH5 precipitate as described for Method 1 under 3.2.6. With c and d puromycin (0.5 mg) was included. After incubation the tubes containing the samples were cooled in ice and then layered over gradients similar to those used in Figure 3.3. Centrifugation was for 200 minutes at 63,000g.



e 3.6
down of polysomes during protein synthesis
 omes (0.5 mg) were incubated at 37°C for the times indicated using the pH5 precipitate as described in Method 1 under
 . After incubation the samples were cooled in ice and then layered over 15-30% (w/v) sucrose gradients containing
 M tris-HCl, pH 7.6, 25 mM KCl and different amounts of $MgCl_2$ as shown. Centrifugation was for 150 minutes at 63,000g.

The results of the experiments discussed in section 3.3.1.4 clearly showed that the release of ribosomes in these experiments was associated with protein synthesis because of the incorporation of ^{14}C -amino acids into polypeptides stable in hot TCA with the same system. For this reason the effect of adding an inhibitor of protein synthesis, namely puromycin, was examined. The results in Fig. 3.5 (c) and (d) show that inclusion of puromycin in the medium did not inhibit the release of ribosomes, but in fact accelerated it slightly. The first of these findings was not unexpected because, although it was known that puromycin prevented the synthesis of long peptide chains, it does not prevent the formation of peptide bonds. The amino group of puromycin intervenes by reacting with the ester bond of peptidyl-tRNA attached to the condensing site on the ribosome instead of the amino group of the aminoacyl-tRNA attached to the decoding site (Nathans & Lipmann, 1961; Hultin, 1961; Allen & Zamecnik, 1962; Morris, Favelukes, Arlinghaus & Schweet, 1962; Morris, Arlinghaus, Favelukes & Schweet, 1963). This reaction does not however stop the readout of the genetic message, but actually seems to increase its rate, as was confirmed by the increase in the rate of breakdown of polysomes (see c and d of Fig. 3.5). In experiments described later in this chapter it was shown that the incubation of polysomes with puromycin alone did not increase the rate of breakdown over those incubated in its absence (see section 3.3.2.3). The experiments of Noll (1965), which were published soon after the completion of this work, confirmed these results. The effect of puromycin was examined further and is discussed below.

3.3.2.3 Incubation with the Factors which Participate in Protein Synthesis but without Peptide Synthesis. -

The reasons for doing this work have been discussed in section 3.1. . . The breakdown in the presence of the factors which participate in protein synthesis was studied in the absence of added amino acids and tRNA, which are required for continued polypeptide bond formation, but in the presence of those factors necessary for chain elongation and of puromycin which causes chain release. The factors which were of interest here are ATP, GTP, a sulphydryl compound (GSH in these experiments) and a source of the transfer enzymes (the pH5 supernatant was used here). These results are shown in Figs. 3.7 and 3.8, and include those in which polysomes were incubated with

described in this section the breakdown which occurred is compared to that where polysomes were incubated with Medium A only.

The results in Fig. 3.7 show the incubation with each factor alone. Incubation of polysomes with the pH5 supernatant had a somewhat protective effect (b). Their incubation with ATP and GSH had no effect (a), while that with GTP and puromycin caused only a slight breakdown (c); that with GTP being slightly more than that with puromycin. However, the effect of the incubation of polysomes with any of these factors alone was not very pronounced so the effect of these in combination was determined and the results are shown in Fig. 3.8.

The addition of puromycin together with any of GSH (b) and particularly GTP (c) or both (d), with or without the pH5 supernatant, caused a far greater release of monomers than puromycin alone (a). The breakdown effected when GTP was included with puromycin, with or without GSH and the pH5 supernatant, was much more rapid and complete than that which occurred in the presence of the complete protein synthesizing system. Not much breakdown, above that which took place in the presence of the factors alone, occurred in any of the combinations which did not contain puromycin.

As will be shown below (section 3.4), these findings can be fully explained in terms of current knowledge of protein synthesis, although, at the time they were obtained, it was possible to form only an incomplete hypothesis as to the reasons for the results.

3.3.3 Release of Pre-labelled Peptide Chains from Polysomes

When the results reported in the previous section were obtained, it was surmised that they were related to the release of peptide chains during incubation. To study this further, polysomes were labelled with ^{14}C -yeast protein hydrolysate as described in Fig. 3.9. During the subsequent isolation of the polysomes, radioactivity associated with free amino acids and short peptides should have been removed so that any label attached to the polysomes would have been in the form of peptides of about 10 or more amino acids.

The breakdown of the labelled polysomes was then studied. The results in Fig. 3.9 show that more than 95% of the counts remained with the polysomes when they were incubated alone, there being very little of the label released (see a). Similar results were obtained when the labelled polysomes

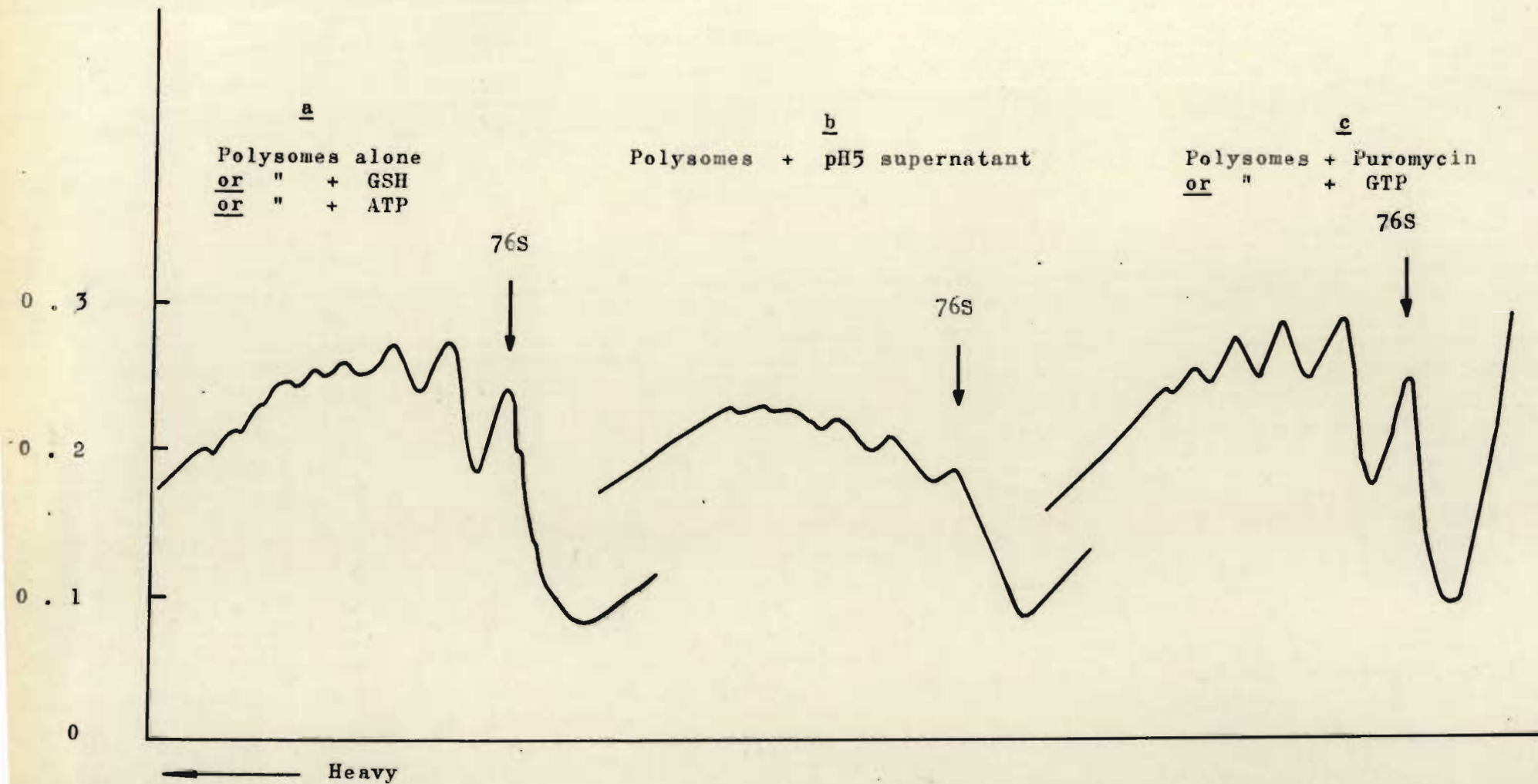


Figure 3.7
 The effect of incubation of polysomes separately, with puromycin and the factors which participate in protein synthesis. Polysomes in Medium A were incubated for 10 minutes at 37°C with each of the different factors as indicated. After incubation the samples were cooled in ice and layered over 10-30% sucrose gradients containing 10 mM tris-HCl, pH 7.6, 5 mM KCl and 5 mM MgCl₂. Centrifugation was for 150 minutes at 63,000g.

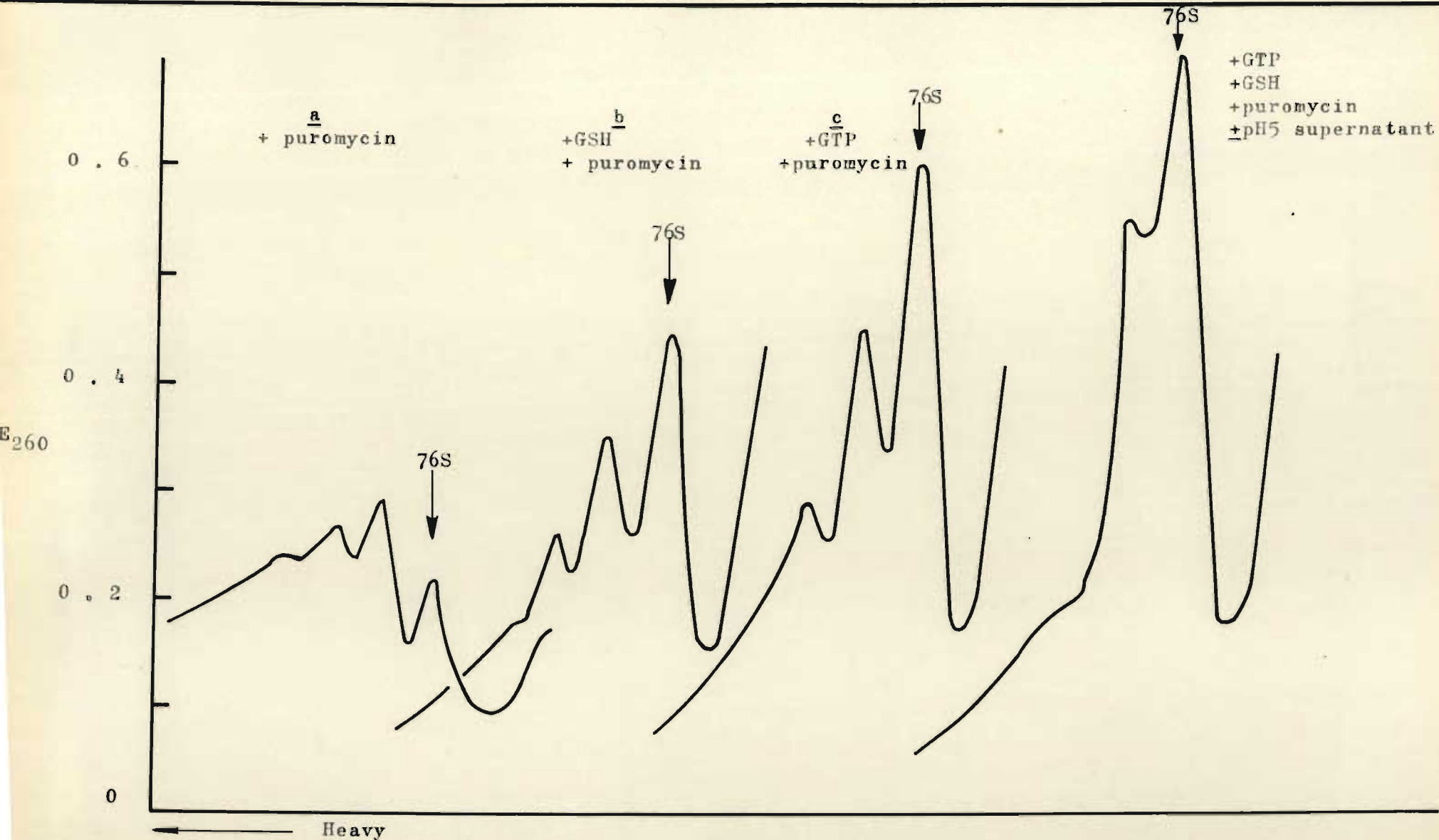


Figure 3.8

incubation of polysomes with combinations of different factors

Polysomes were incubated with the factors as indicated using the experimental conditions described under Figure 3.7 except that centrifugation was for 200 minutes.

GTP was included, especially in a system containing the pH5 supernatant and GSH, some release (about 20%) of the label was obtained. When pre-labelled polysomes were incubated with puromycin alone, about 30-35% of the label remained with the polysomes indicating a release of between 65-70% of labelled peptide chains (see b). This is in agreement with the findings of Traut & Monro (1964) who used ribosomes from E. coli. Considerably more of the label was removed when the factors necessary for the translocase reaction (enzymes, GTP and GSH) were included with puromycin, in which case only about 10-15% of the label remained with the polysomes (c). These results on the release of prelabelled peptide chains can be related to those on the observed breakdown of polysomes which was shown to take place using the same systems. Heintz, Salas & Schweet (1968) have recently described similar results with reticulocyte ribosomes.

The interpretation of these results in the light of the most recent ideas on protein biosynthesis is given in section 3.4.

3.3.4 Choice of Method of Breaking Down Polysomes to Single Ribosomes

It was shown that polysomes could be broken down by three distinctly different methods, namely those resulting from

- (i) the hydrolysis of the mRNA connecting different ribosomes, as seen when polysomes were incubated either in Medium A or with RNase,
- (ii) the release of single ribosomes from the end of the mRNA strand during protein synthesis, and
- (iii) the collapse of the structure of the polysomes when incubated in the presence of both the factors causing chain release (due to puromycin) and those allowing for translocation to take place (GTP, GSH and pH5 supernatant).

The type of single ribosomes obtained as a result of their release from the end of the message during protein synthesis (ii) was considered to be more suitable for the purpose of this work than those obtained as the result of hydrolysis of mRNA (i) because of the presence of the suspected fragment of mRNA with the latter. The type of single ribosome obtained as a result of both protein synthesis (ii) and translocation with chain release (iii) seemed to be similar. Although ribosomal release by method (iii) did appear to be better, it was not considered as a practical method when this work

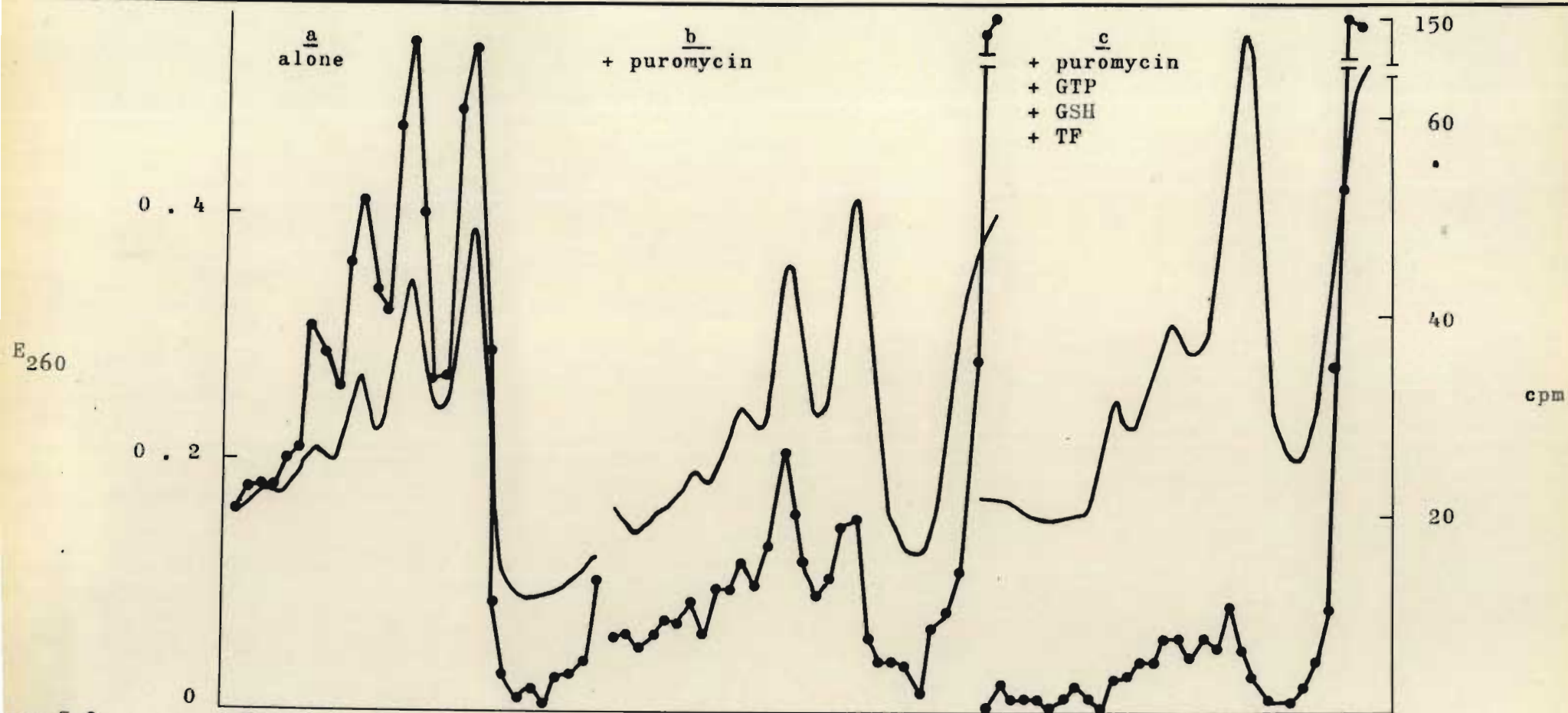


Figure 3.9
Effect of the incubation of ^{14}C -labelled polysomes with various fractions
 Polysomes were pre-labelled by incubating them with ^{14}C -yeast protein hydrolysate for 5 minutes at 37°C using Method 1 described under 3.2.6. After incubation the reaction was stopped by addition of 20 volumes of ice-cold Medium A, labelled polysomes isolated by high speed centrifugation and dissolved in Medium A. These labelled polysomes (1000 cpm each) were then incubated with the fractions shown at 37°C for 10 minutes, after which they were placed on gradients similar to those used in Figure 3.7 and centrifuged at $63,000g$ for 200 minutes. The fractions were then collected and assayed for E_{260} and precipitated with cold 5% TCA. The precipitates were collected, dissolved in ammonia and passed on to planchets where they were dried and counted for ^{14}C -label using a Phillips Electronic counter as under 3.7. B=cpm which passed through the gradient to the bottom of the tube. TF = partially purified transfer fraction (Chapter 4)

was done because puromycin was difficult to obtain at the time, and because the reaction with puromycin was still not completely understood. In the light of present day knowledge it is realized that the production of single ribosomes by the incubation of polysomes with puromycin and the factors necessary for translocation would be most suitable for preparing ribosomes with which to study the transfer factors. Also, in the light of the results presented above, the most convenient method would be to incubate polysomes only with puromycin, GTP and GSH, in the absence of an added source of the transfer enzymes, since breakdown was unaffected by their absence or presence (see d of Fig. 3.8). It is not known to what extent the single ribosomes thus obtained need be washed further to remove adhering transfer enzymes, but it is expected that this washing need be less vigorous if the pH5 supernatant is not included during the preparation of single ribosomes. At the stage that it was possible to come to this conclusion, suitable methods for the preparation of single ribosomes free of the transfer enzymes had already been worked out, as is discussed in Chapter 4, and no further time was spent on this.

3.4 INTERPRETATION OF RESULTS

As shown in Chapter 2, aminoacyl-tRNA's play an important role in the attachment of mRNA to ribosomes (once chain initiation has commenced) and in keeping the subunits of ribosomes together. They must therefore be present on ribosomes in order to keep the structure of the polysome intact. The binding of aminoacyl-tRNA's to the first decoding site in the presence of mRNA is influenced by GTP and one of the transfer enzymes. Binding at this site has little influence in holding the two subunits together because it can take place in the absence of the large subunit. It influences the structure of the polysome only in that it aids in the attachment of mRNA which links the different ribosomes together. Binding of aminoacyl-tRNA's to the second decoding site, besides requiring mRNA, is influenced by the presence of GTP, one of the transfer enzymes, the large ribosomal subunit and possibly a sulphhydryl group. Since binding of aminoacyl-tRNA to this site requires the linking up of the larger subunit, it is expected to influence the integrity of the polysomes in the holding of the subunits together, besides its rôle in the linking of the different ribosomes as a result of its influence on the binding of mRNA. The binding of peptidyl-tRNA, briefly, to the second decoding site just

because of its recognition of the codon of mRNA and its attachment to both subunits of ribosomes. The influence here is even stronger because of the influence of the growing peptide chain in holding the subunits together. The binding of the peptidyl-tRNA to the condensing site influences the integrity of the polysome only because of the effect of the attached growing peptide in the binding of the subunits. No effect of the attachment of mRNA is noticed because its binding is solely to the large subunit and independent of mRNA.

It is thus evident that removal of aminoacyl-tRNA from the first decoding site and from the condensing site undermines the stability of the polysome only slightly, whereas its removal from the second decoding site leads to almost complete breakdown. The observation that treatment with puromycin alone causes very little breakdown of polysomes is thus explained, since only the peptidyl-tRNA attached to the condensing site is removed. Clearing of the vital second decoding site by translocation can take place only if the condensing site is first emptied. In the absence of peptide bond formation this cannot occur, except as the result of the action of puromycin. Hence, the addition of factors inducing translocation can have no effect on the stability of the polysomes in the absence of puromycin, except to the very limited extent allowed by the few condensing sites which happened to be open at the time the polysome was isolated, as was indeed found here (Fig. 3.7c). Only where chain release by puromycin and the translocase reaction occur simultaneously can it be expected that breakdown of polysomes will result. The translocase reaction can take place in systems containing the pH5 supernatant, GTP & GSH (see Chapter 2). In the experiment, where polysomes were incubated with all of these factors and puromycin, extensive breakdown occurred (see d of Fig. 3.8). The results on the release of polypeptide chains from polysomes, in Fig. 3.9, confirmed this, since, in the experiment where the factors necessary for translocation were added with puromycin there was both an increase in the amount of label released (showing translocation) and breakdown of polysomes. Furthermore, this breakdown was more extensive than that occurring as a result of protein synthesis (compare the result in Fig. 3.8d with that of Fig. 3.6a).

Since the polysomes used here have been shown to contain a small complement of the transfer enzymes (see Chapter 4), their incubation with only puromycin, GSH and GTP should also result in their extensive breakdown as only one translocase reaction would be needed for this. This was found to

breakdown as that which occurred in its presence (also d of Fig. 3.8). If GSH and GTP were omitted from the system it would be expected that this breakdown would be much less. Indeed this was found to be so (c and d respectively of Fig. 3.8). Even in the absence of GSH or GTP it is seen that fairly extensive breakdown occurred. This breakdown in the absence of GSH would be expected when using unwashed polysomes, as the requirement for sulphydryl groups during protein synthesis by polysomes was only measured after some time had elapsed (see references by Moldave and collaborators). This probably indicates that the reducing rôle of sulphydryl compounds is related to some priming function associated with the translocase enzyme (Moldave's transferase II) which has been shown to be stabilized by GSH (Sutter, Gasior & Moldave, 1965; Sutter & Moldave, 1966). This rôle of GSH is discussed further below in an attempt to explain the breakdown which occurred in the absence of GTP.

It is generally believed that both GTP and sulphydryl groups are concerned with the movement of peptidyl-tRNA's from the decoding to the condensing site on ribosomes. It is the view of the writer that GSH functions by reducing some compound connected with the polysome and translocase enzyme, which subsequently reacts with a high energy compound, possibly associated with a transfer enzyme which was formed during the hydrolysis of GTP. This would then be the reaction which drags the peptidyl-tRNA on the second decoding site to the condensing site, at the same time moving the mRNA one codon forward. (This is the reaction depicted by (iii) to (iv) of Fig. 2.2 in Chapter 2). The exact nature of these compounds and the reactions taking place are not known, but for the purpose of explaining the breakdown of polysomes which occurred in the experiments described above (b and c of Fig. 3.8), it is further suggested that the two compounds which form in the presence of GSH and GTP, the reduced and high energy compounds respectively, do so independently of one another. To explain this in terms of polysome structure, there would thus be individual ribosomes in the isolated polysome which contained either the reduced or high energy compounds (or both) which were formed on the ribosomes just prior to their isolation. On this basis, the breakdown obtained when polysomes were incubated with puromycin and GSH (b of Fig. 3.8) would be the result of the presence of the high energy compound at the time of isolation of the polysomes. The breakdown obtained with puromycin and GTP (c of this figure) was because of the presence of the reduced

compound. The type of breakdown in both these instances was the same as that which took place in the presence of all the factors, as in d of Fig. 3.8. The results obtained in these experiments agree with this explanation, and, if correct, would pin-point the actions of sulphydryl groups in protein synthesis. The greater amount of breakdown obtained when puromycin was incubated with GTP (c of Fig. 3.8) than when incubated with GSH (b of the same figure) may be due to the greater stability of the reduced compound which was formed by the action of sulphydryl groups, rather than that of the activated compound formed by the action of GTP.

The results of the release of prelabelled peptides during the breakdown of polysomes, obtained in section 5.3.3, support this explanation. If all the peptidyl-tRNA occupied the condensing site, treatment with puromycin alone should remove all the labelled peptides. That it did not do so, as shown in Fig. 3.9b, was probably because the peptidyl-tRNA's which had just participated in peptide bond formation were located on the decoding site, the site where they do not react with puromycin. Thus, at any time, part of the labelled peptide would not be expected to be available for reaction with puromycin. In the presence of the factors necessary for the translocase reaction, namely GTP, GSH and the translocase enzyme, this would subsequently move across to the condensing site and be removed by puromycin as seen in Fig. 3.9 c. These results have been interpreted in accordance with the very recent findings of Jost, Shoemaker & Noll (1968) and Skogerson & Moldave (1968), who, by different methods, showed a similar release of labelled peptides from polysomes and that peptidyl-tRNA bound to some site on the ribosomes was not released by puromycin.

CHAPTER 4

PREPARATION OF WASHED RIBOSOMES FREE OF THE TRANSFER ENZYMES

4.1 INTRODUCTION

To study the rôle of the factors present in the cell sap in the biosynthesis of proteins, it was necessary that ribosomes be prepared which were free of transfer enzymes. It was important to distinguish between those proteins which were of ribosomal origin and those that originated in the supernatant. In the light of the knowledge on protein synthesis in 1963, when this work was started, it seemed that the ideal ribosomes for the study of de novo synthesis of polypeptides were those which contained all their constituent parts and to which could be added mRNA, aminoacyl-tRNA's, the ions and co-factor requirements and the transfer enzymes. At the time nothing was known about the enzymes required for chain initiation, but by using high concentrations of magnesium (8-10 mM or more) the need for initiation enzymes, unknowingly, had been eliminated. It has been shown that at these high concentrations of magnesium there is no need for the initiation enzymes (see Chapter 2).

By mid-1963 a number of ways had been worked out for freeing ribosomes of contaminating transfer enzymes. The general practice of the groups working in this field was to make use of solutions containing monovalent or divalent cations. Lipmann and co-workers, working with E. coli, washed the ribosomes with solutions containing either magnesium or ammonium ions (Nathans & Lipmann, 1961; Allende, Monro, Nathans & Lipmann, 1962; Spyrides & Lipmann, 1962; Nakamoto, Conway, Allende, Spyrides & Lipmann, 1963) while Schweet and co-workers washed the ribosomes from reticulocytes with solutions of KCl (Arlinghaus & Schweet, 1962). Various buffers, deoxycholate and other substances were also used to wash ribosomes (Fessenden & Moldave, 1962 & 1963, using rat liver). A more comprehensive review on the removal of extraneous protein from ribosomes is given by Petermann (1964, p43).

A method of preparing ribosomes free of mRNA was developed by the author based on the results of the work reported in Chapter 3. In addition the released ribosomes were washed with solutions of KCl because this salt is known to remove bound proteins and was used by Arlinghaus & Schweet (1962) to wash the transfer enzymes from reticulocyte ribosomes. It was also decided

to buffer the solutions of KCl at a slightly alkaline pH in the hope that this would aid in releasing the non-ribosomal proteins, including the transfer enzymes. This was done because ribosomal proteins were known to be basic (Crampton & Petermann, 1959) and it was believed by the author that an alkaline wash would preferentially release the non basic, therefore non-ribosomal, proteins. The idea of using an alkaline buffer to wash ribosomes was also based on the findings of Kuff & Zeigel (1960) who showed that at more alkaline pH haemoglobin did not readily attach to ribosomes, whereas, around, pH 7 or less attachment readily occurred with the precipitation of the complex. Only the method finally decided on is described under "methods" (Section 4.2.1.1) while the various modifications tried during the course of this work are discussed in the results. While this work was in progress other methods were described for the preparation of washed ribosomes which were said to be ideal for studying the transfer enzymes. Two of these methods were then also studied and are described here, namely an adaption of the method used by Arlinghaus, Shaeffer & Schweet (1964) with reticulocytes, and that of Gasior & Moldave (1965a) from rat liver. The characteristics of these various ribosomes were compared, particularly with respect to (i) their physical properties, such as their size when studied on sucrose gradients, in the analytical ultracentrifuge and under the electron microscope, and (ii) their activity and requirements for the transfer enzymes and the optimal conditions needed to measure this activity. These ribosomes were free of the transfer enzymes and therefore satisfactory for the work for which they were intended. However, the work showed in addition that the treatments also removed some other factor(s) needed for protein synthesis since the different ribosomes differed in their activity, and this difference could not be alleviated by the addition of transfer enzymes, a finding which had not been reported previously.

4.2 METHODS

A list of the reagents and solutions used are given in the appendix.

4.2.1. Preparation of Washed Ribosomes

Three methods for the preparation of washed ribosomes are described here. Polysomes, prepared as described in Chapter 3, were used as starting material for all three of these.

Unless otherwise stated all procedures were carried out in the cold (about 0-4°C).

4.2.1.1 R-ribosomes. - This method was developed by the author.

Polysomes were incubated in the optimal protein synthesizing system for 45 minutes (see Section 3.2.5.1) Method 1; the pH5 precipitate and free non-radioactive amino acids were used). After incubation, the reaction mixture was cooled in ice and three volumes of an ice-cold solution, containing 50 mM-tris-HCl, pH 8.3, 50 mM-KCl and 1 mM-MgCl₂ (solution-R1) added. This mixture was then left for 30 minutes after which it was centrifuged at 105,000g for 90 minutes (Spinco 40 rotor) to pellet the ribosomes. Each ribosome pellet was then suspended in 1 ml distilled water, and when the ribosome pellets were completely in suspension, an equal volume of double strength solution-R1 was added so that the final concentration was that of solution-R1. After standing for 30 minutes, 2 ml amounts were layered over 10 ml of a 1 M-sucrose solution, containing the same buffer and ions as solution-R1, in a 40 rotor centrifuge tube. These were centrifuged at 105,000g for 3 hours. The ribosomes were again first dispersed in distilled water (0.5 ml per pellet) and an equal volume of double strength solution-R added so that the final concentration was that of solution-R (50 mM-tris-HCl, pH 7.6, 25 mM-KCl, 1 mM-MgCl₂). This suspension of ribosomes was left overnight before the undispersed material was removed by low speed centrifugation. This ribosome suspension was stored at 0°C and used within one week.

4.2.1.2 S-ribosomes. - These were prepared essentially by the method of Arlinghaus, Shaeffer & Schweet, (1964), who used "KCl-shock" treatment to remove the transfer factors from the ribosomes of reticulocytes. The method was as follows:

To 3 ml polysomes (rat liver) in Medium A was added 0.56 ml of 0.6 M-KCl (final concentration 0.15 M) and 1 ml of a solution containing 70 mg GSH which had been adjusted to pH 7.6 with KOH. This mixture was incubated at 37°C for 25 minutes, after which was added the rest of the system which allows for optimum protein synthesis (as with R-ribosomes, see section 4.2.1.1). This was then incubated for an additional 40 minutes at 37°C. After incubation the mixture was cooled in ice and diluted 8-fold with a solution containing 0.25 M-sucrose, 2 mM-MgCl₂, 17.5 mM-KHCO₃ and 100 mM-KCl. This mixture

pellet was rinsed with a solution of 0.25 M-sucrose and then suspended in 2 ml of the same sucrose solution. The suspension was made 1% (final concentration) with respect to deoxycholate and incubated for 3 minutes at 37°C. After incubation the mixture was cooled in ice and diluted with 10 volumes of a solution containing 0.25 M-sucrose, 17.5 mM-KHCO₃ and 2 mM-MgCl₂, and centrifuged at 105,000g for 3 hours (40 rotor). The resulting pellets of ribosomes were rinsed with a solution of 0.25 M-sucrose and finally suspended in a total volume of 1.5 ml of the same sucrose solution. This was left overnight and the undispersed material removed by low speed centrifugation. The suspension of ribosomes was stored at 0°C and used within one week.

4.2.1.3 M-ribosomes. - These were prepared essentially according to the method of Gasior & Moldave (1965).

To 10 ml polysomes (80-90 mg) in Medium A was added 12.5 ml Medium X and 2.5 ml 3% sodium deoxycholate (final concentration 0.3%). The mixture was stirred gently for 15 minutes after which it was centrifuged at 105,000g for 60 minutes (40 rotor). The pellets were suspended in 2 ml 1 mM-MgCl₂ by gentle homogenisation and then made 50 mM with respect to MgCl₂ in a total volume of 12 ml. This suspension was again stirred and centrifuged as before. The ribosome pellet was then washed three times with a solution of 10 mM-MgCl₂ by the same procedure. The final pellet was rinsed and suspended in 5 ml of solution-M (0.35 M-sucrose, 50 mM-tris-HCl, pH 7.6, and 4 mM-MgCl₂) and dialysed against the same solution for 12 hours. After dialysis the suspension of ribosomes was centrifuged at 1,500g for 10 minutes to remove undispersed material. This suspension of ribosomes was stored at 0°C and used within one week.

All three of these preparations, R-, S- and M-ribosomes, can be stored at -20°C as the pellet obtained after the final centrifugation for as long as 3 months and perhaps longer.

4.2.2 The Preparation of Partially Purified Transfer Enzyme Fractions

Here the total transfer enzyme fraction in the pH5 supernatant was either partially purified and concentrated as a whole, or separated into fractions containing the different transfer enzymes.

4.2.2.1 The Fractionation and Concentration of the Total Transfer Fraction . -

pH 6.8, by stirring the mixture (3 mg gel per 3 mg protein in solution) for 15 minutes. (This gel was prepared by the method of Keilin & Hartree, 1938.) The gel was then centrifuged at 10,000g for 10 minutes, the supernatant discarded and the precipitate successively extracted with 0.03 M-potassium phosphate buffer, pH 6.8, and 0.2 M-potassium phosphate buffer, pH 6.8, containing 15% (w/v) ammonium sulphate. These extractions were carried out by adding the buffer to the gel and then homogenising this with a Dounce homogeniser, after which the mixture was centrifuged at 10,000g for 10 minutes to separate the extract from the gel. The second extract, that extracted with 0.2 M-potassium phosphate containing 15% ammonium sulphate, was then treated in one of two ways. It was either

- (a) dialysed against Medium X for 12 hours, after which it was centrifuged at low speed to remove the undissolved material, the dialysed solution being used as a source of partially purified transfer factor (TF in text); or
- (b) precipitated with ammonium sulphate at 70% saturation and the precipitate dissolved in a minimal volume of Medium X. This was then dialysed against 500 volumes of Medium X for 24 hours with three changes of buffer. After removing the denatured material this solution was used as a source of partially purified transfer factor (TF-AS in text).

These two partially purified transfer fractions were stored in the frozen state and could be kept as such for as long as six months. They rapidly lost activity on continual freezing and thawing and were therefore used only once after freezing.

4.2.2.2 Separation on Sephadex Columns of Fractions Containing the

Transfer Enzymes. - Sephadex G-200, prepared in 20 mM-potassium phosphate buffer, pH 7.2, containing 50 mM-KCl, was packed under the pressure exerted by 200-300 ml of the same buffer at a height of 600-900 cm during continuous flow of the buffer in a glass column 10 x 2.5 cm. The partially purified transfer fraction, TF-AS (6 mg), was placed on the column and eluted with 20 mM-potassium phosphate buffer, pH 7.2, containing 0.2 M-KCl. 2 ml fractions were collected, their E_{260} and E_{280} measured and then assayed for their transfer activity. Two main protein peaks were obtained as shown in Fig. 4.1. The fractions under these peaks were pooled separately and precipitated with ammonium sulphate at 70% saturation. The resulting precipitates were dissolved in Medium X and dialysed against the same solution.

4.2.3 Method of Assay for the Binding of ^{14}C -phe-tRNA by Ribosomes (Binding Reaction)

The following were incubated in glass tubes in a total volume of 300 μl : assay buffer (50 mM-tris-HCl, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl and 20 mM-MgCl₂; all final concentrations); 0.2 mg ribosomes; 0.06-0.08 mg ^{14}C -phe-tRNA (3,000-7,000 cpm rat liver preparation or 15,000 cpm *E. coli* preparation); 50 μg poly U (Miles Laboratories). The mixture was incubated for 15 minutes at 37°C and the reaction stopped by the addition of 5 ml ice-cold assay buffer. The contents of each tube were then passed onto millipore filters (HA 0.45, Millipore Filter Corp., Bedford, Mass.) and washed on the filter according to the method of Nirenberg & Leder (1964), with three aliquots of 20 ml of a solution containing 10 mM-tris-HCl, pH 7.6, 25 mM-KCl & 20 mM-MgCl₂. The filters were then dried and counted in a Packard Scintillation Counter using PPO & POPOP dissolved in toluene as described under section 3.2.6.2.

4.3 RESULTS AND DISCUSSION

Since this work was started with the intention of preparing ribosomes free of the transfer enzymes, the results of this chapter are presented, with that purpose in mind, in the following order:

- (i) recovery of ribonucleoprotein (It was necessary to know this in order to ensure that enough material with which to work was obtained to facilitate planning of experiments),
- (ii) activity (It was necessary to determine whether these ribosomes were active in polypeptide synthesis and to determine whether they contained any transfer enzymes),
- (iii) content of mRNA and stimulation of polypeptide synthesis on addition of poly U,
- (iv) stability of preparations,
- (v) ability to bind aminoacyl-tRNA (This was necessary because peptide synthesis depended on the binding of aminoacyl-tRNA by ribosomes and also because this binding may have been dependent on a transfer enzyme), and finally,
- (vi) the physical characteristics of the different ribosomes were determined to characterize their conformation and to determine

Table 4.1 The recovery of ribonucleoprotein with different preparations of ribosomes

Preparation	% recovery
S-ribosomes	33-40
M-ribosomes	35-50
R-ribosomes prepared as described in methods	55-60
" without prior suspension in water	20-40
" washed at pH 7.6 instead of 8.3	60
" washing included deoxycholate treatment	45-55

these were in agreement with the conclusions drawn from measurement of their activity.

It must be pointed out that the sequence in which the experiments were done did not necessarily follow the sequence presented here and much back tracking was done to check certain points once new parameters were established.

4.3.1 Yield of Ribosomes

The recovery of the total nucleoprotein of ribosomes was determined from their absorption at 260 m μ (E_{260}) and expressed as a percentage of the absorption of the original sample of polysomes from which they were prepared. The results are shown in Table 4.1.

It was generally found that the recovery of nucleoprotein with R-ribosomes was higher than that with either M- or S-ribosomes, which were of the same order. If R-ribosomes were prepared without the prior dissolution of the pellets from each centrifugation in distilled water, the amount of nucleoprotein recovered was noticeably reduced, although this did not affect the activity of the preparation. If R-ribosomes were washed in a buffer of pH 7.6 instead of pH 8.3 the recovery of nucleoprotein was not much affected, despite the increase in activity which was obtained (see section 4.3.2). However these ribosomes had a higher residual activity (activity in the absence of added transfer enzymes, see section 4.3.2). If the method used for the washing of R-ribosomes

included treatment with deoxycholate, as was done with M-ribosomes, then the recovery of nucleoprotein and activity was decreased. An increase in the yield of S-ribosomes could be obtained if the pellet resulting from the first washing procedure was dissolved in a sucrose solution containing the buffer at pH 8.3 as was used with R-ribosomes. However, in this instance the activity of the ribosomes was greatly reduced. The possible reasons for these differences will be discussed in Chapter 5. From the above results and those presented in section 4.3.2 it was concluded that the three most suitable methods for the preparation of ribosomes for the study of the transfer enzymes were those described under the methods of this chapter.

4.3.2 Activity of Ribosomes

The activities of R-, S- and M-ribosomes are shown in Table 4.2. In these experiments the ability of the ribosomes to incorporate labelled amino acids into polypeptides was measured only with the system starting with aminoacyl-tRNA's because only the reactions subsequent to acylation of tRNA were of interest here.

The results in Table 4.2 show that in comparison to polysomes, R-, S- and M-ribosomes had little residual activity, and that they were all active in the presence of an added source of the transfer enzymes. The three preparations were active in the absence of added mRNA, but the activity of all three was much stimulated on addition of poly U. These results suggest that the ribosomes were not free of mRNA, especially M-ribosomes of which a large proportion have the characteristics of polysomes when analysed by physical means (see section 4.3.6). The preparations of R- and S-ribosomes did not contain many particles which moved as polysomes when analysed on sucrose gradients or in the analytical ultra-centrifuge (see section 4.3.6). The activity found with R- and especially S-ribosomes in the absence of poly U may be due to the presence of mRNA in the pH5 supernatant which was used in the assay, since, when a partially purified transfer fraction (TF) was used as the source of transfer enzymes, the activity in the absence of poly U was less and the stimulation by the polynucleotide was more (see Fig. 4.2). Whether the residual activity of these ribosomes in the absence of poly U was due to the presence of mRNA in the supernatant was not determined.

Table 4.2 Polypeptide synthesis by different ribosomes

This synthesis was measured by incubating ribosomes in the complete amino acid incorporating system, described in Method 2 in section 3.2.6. The pH5 supernatant was used as the source of transfer factors. The concentration of magnesium ions in the assay system used for polysomes, R- and M-ribosomes was 8.5 mM and for S-ribosomes, 13 mM. Incubation was for 15 min. at 37°C.

Preparation	Activity (cpm/0.2 mg ribosome)			
	alone		+ pH5 supernatant	
	¹⁴ C-YPH-tRNA	¹⁴ C-phe-tRNA + Poly U	¹⁴ C-YPH-tRNA	¹⁴ C-phe-tRNA + Poly U
Polysomes	500	216	2,150	2,387
R-ribosomes	35	80	626	2,610
M-ribosomes	40	60	1,748	2,540
S-ribosomes	18	40	480	3,600

Although the results in Table 4.2 showed that the ribosomes had little activity in the absence of an added source of transfer enzymes, this did not prove that they were devoid of all transfer enzymes, since at least two of these enzymes are necessary for peptide bond formation in rat liver (see Chapter 2) and the absence of just one would render the ribosome inactive. Rigorous proof thus required the separation of the two transfer factors and the study of the activity of the ribosomes in the presence of each singly and the two in combination. Separation of two transfer enzymes was obtained by molecular exclusion of TF-AS, the partially purified transfer fraction which had been treated with ammonium sulphate, on Sephadex G-200 (Fig. 4.1). The measurement of peptide synthesis in the presence or absence of these factors is shown in Table 4.3. These results show that the ribosomes were relatively inactive with either of the two enzymes alone and required the addition of both fractions to synthesize polyphenylalanine. This proved that all three ribosomal preparations were free of the two transfer enzymes necessary for peptide bond formation and showed that the differences in activity were not due to different amounts of transfer enzymes associated with them.

Table 4.3 Activity of ribosomes in the presence of different transfer enzymes

Polyphenylalanine synthesis was measured for 15 minutes as described in Method 2 of section 3.2.6. ^{14}C -phe-tRNA, prepared from rat liver as described in section 3.2.2, was used and the ^{14}C -labelled peptides counted in the Phillips electronic counter as described for the centrifugation method of section 3.2.7. The partially purified transfer fraction, TF-AS, was prepared as described in section 4.2.2. The fractions, A and B, were prepared from TF-AS passed through Sephadex G-200 columns as described under Fig. 4.1. Approximately 20 μg each of fractions A and B were used. (These amounts were not sufficient to saturate the systems but because of the low concentration of protein in these fractions no more could be added to the assay systems because of the dilution factor.).

Added transfer fraction	Polyphenylalanine synthesis (cpm/0.2 mg ribosome)		
	R-ribosomes	S-ribosomes	M-ribosomes
None	6	5	7
TF-AS	255	357	190
A alone	30	42	32
B alone	12	9	10
A + B together	115	129	114

4.3.3. Optimum Requirements for the Synthesis of Polyphenylalanine Starting from ^{14}C -phe-tRNA in the Presence of Poly U

For most of this work R-ribosomes and the partially purified transfer fraction, TF, were used. The following factors were tested for their effect on this synthesis: concentration of GTP and GSH, concentration and type of phosphoenol pyruvate, concentration of poly U and concentration of magnesium ions. The concentrations used by Staehelin, Wettstein & Noll (1963) with polysomes were used as a guide for these studies. These were 8 mM-MgCl₂, 0.4 μ moles GTP, 5 μ moles GSH and 5-10 μ moles PEP with 10 μg phosphoenol pyruvate kinase in a total volume of 1.0 ml. In the experiments described below 0.2 mg ribosomes in a total volume of 0.5 ml was used and the concentration of these

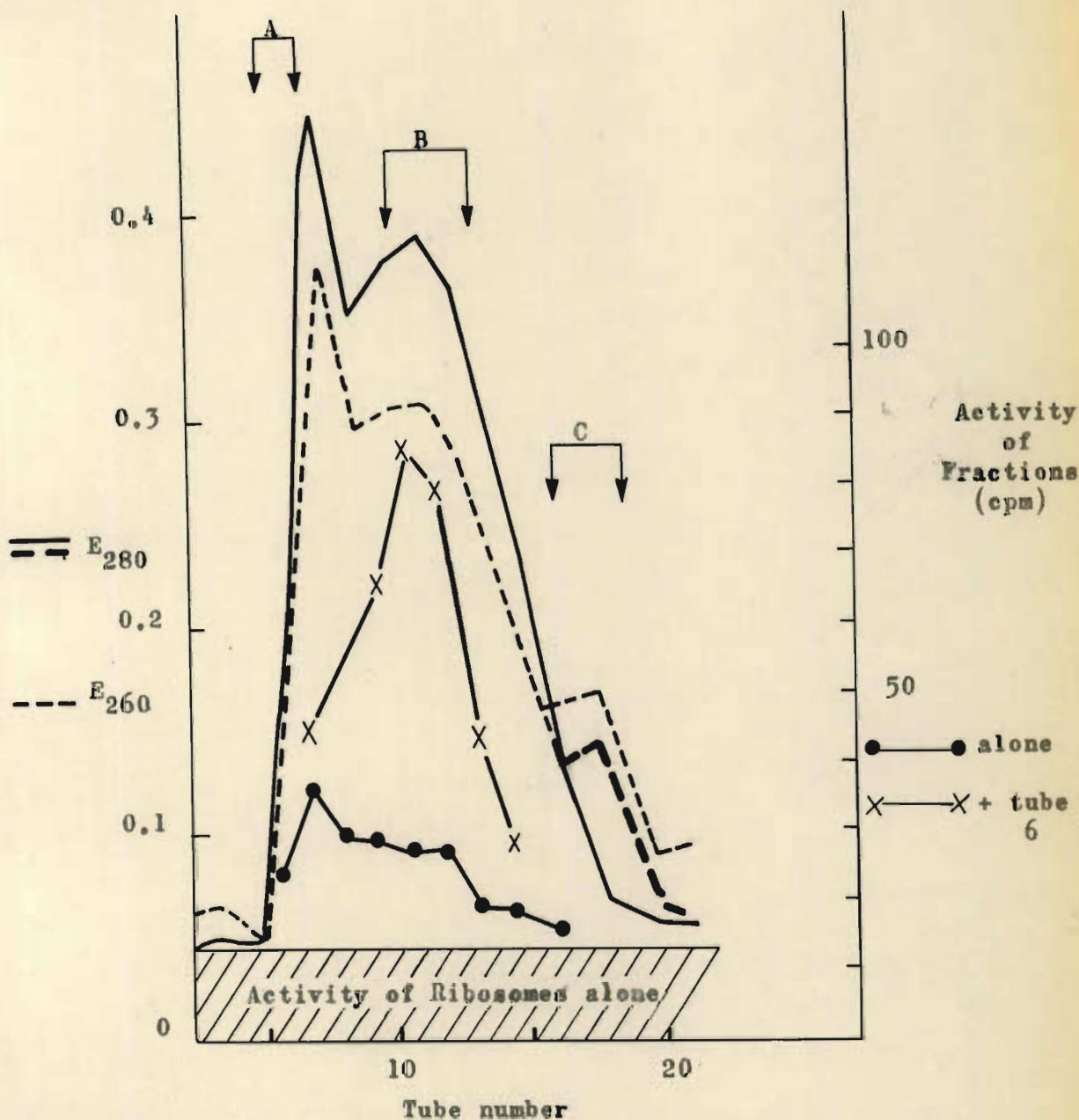


Figure 4.1

Fractionation of TF-AS by gel filtration

Partially purified transfer factor (TF-AS) was passed through Sephadex G-200 as described under Methods. Transfer activity was measured by Method 2 of section 3.2.6 using ^{14}C -phe-tRNA of rat liver and R-ribosomes. Incubation was for 60 minutes. 0.1 ml Aliquots of the different fractions in a total assay volume of 0.8 ml were used. When the fractions were assayed alone 0.1 ml of the elution buffer was included. The final concentration of KCl used with these assays was 50 mM.

4.3.3.1 Concentration of GTP and GSH. - There was no difference in polyphenylalanine synthesis if the concentration of GTP was varied between 0.1 to 0.4 μ moles per total volume of 0.5 ml. Higher concentrations of GTP than this were not tested. With concentrations of GTP of less than 0.1 μ moles/0.5 ml there was a decrease in synthesis. A concentration of 0.2 μ moles in a total volume of 0.5 ml was therefore used.

No difference in polyphenylalanine synthesis was observed when the concentration of GSH was varied between 2-5 μ moles per total volume of 0.5 ml. When GSH was omitted from the assay system polyphenylalanine synthesis was very much decreased. A concentration of 2.5 μ moles of GSH per total volume of 0.5 ml was therefore used.

4.3.3.2 Concentration and Type of PEP. - Two types of PEP were tried, namely the trihexylammonium salt, which was neutral when dissolved in water, and the monovalent potassium salt which was strongly acidic when dissolved in water.

When using the potassium salt a concentration of 5 μ moles per total volume of 0.5 ml was found not to be limiting under any conditions for incubation times of up to 1 hour. Sometimes a concentration of half this amount would become limiting after an incubation time of 1 hour.

The first experiments, using the trihexylammonium salt, showed that a concentration of 2 μ moles per total volume of 0.5 ml was slightly superior to that of 5 μ moles. However, this was found to be so only for incubations of 15-20 minutes or less, as this synthesis over longer periods of incubation was higher with the higher concentration of the salt used.

In the experiments where the ratio of poly U/ribosomes was low (about 1-2) neither type of PEP seemed superior to the other. On the other hand, as the concentration of poly U was raised to optimum, a higher rate of polyphenylalanine synthesis was obtained with the potassium salt. A concentration of 5 μ moles of the potassium salt per total volume of 0.5 ml was therefore used.

4.3.3.3. Optimum Concentration of Poly U. - The optimum requirements of poly U in the various systems used for assaying polyphenylalanine synthesis is shown in Fig. 4.2. The assay systems tested were those using the pH5 precipitate with free ¹⁴C-phenylalanine and also those using the pH5 super-

nant or the partially purified transfer fraction, TF, with ^{14}C -phe-tRNA. Optimal incorporation was obtained when the concentration of poly U was between 25-50 μg for every 0.2 mg ribosomes used in the assay. By taking the molecular weight of the ribosomes as 6×10^{-6} and that of the poly U as about $1-2 \times 10^{-4}$ this gave a ratio of poly U/ribosomes as 50 to 100. The optimal concentration of poly U which was required for this reaction did however vary since more was needed for longer incubations, but, as the total label incorporated did not differ much in this instance, a concentration of 50 μg per 0.2 mg ribosomes in the assay was decided on.

4.3.3.4 Optimum Concentration of Magnesium Ions. - The system by which the synthesis of polyphenylalanine was measured can be considered as the de novo synthesis of polypeptides in a heterologous system (see Chapter 2). As the reports in the literature have shown, the concentration of magnesium ions required for the assay of polypeptides in the various systems depended on the mRNA and ribosomes used. It was therefore necessary to determine the optimum concentration of magnesium ions required in the assay for the synthesis of polyphenylalanine by the three preparations of ribosomes used here, namely R-, S- and M-ribosomes. These results are shown in Fig. 4.3.

When a sample of ^{14}C -phe-tRNA made from rat liver was used, the optimum for R- and M-ribosomes was about 7-10 mM while that for S-ribosomes was from 15-20 mM. However, when ^{14}C -phe-tRNA from E.coli (obtained from Schwarz Biochemicals) was used the optimum with S-ribosomes was not so high, being between 7-12 mM. For these studies therefore the concentration of MgCl_2 which was used in the assay systems with R- and M-ribosomes was 8.5 mM while that for S-ribosomes was generally 13 mM.

4.3.4 Stability of Ribosomes

All three of the suspensions, R-, M- and S-ribosomes, could be stored at 0°C for 10-12 days with very little decrease in activity. After that they started to precipitate, probably because of denaturation, and lost activity. The effect of freezing suspensions of ribosomes differed between the three preparations. Only M-ribosomes were stable to any extent. R-ribosomes were the least stable in this case. However, if the ribosomes were stored as a pellet at -15 to -20°C they could be kept for much longer periods.

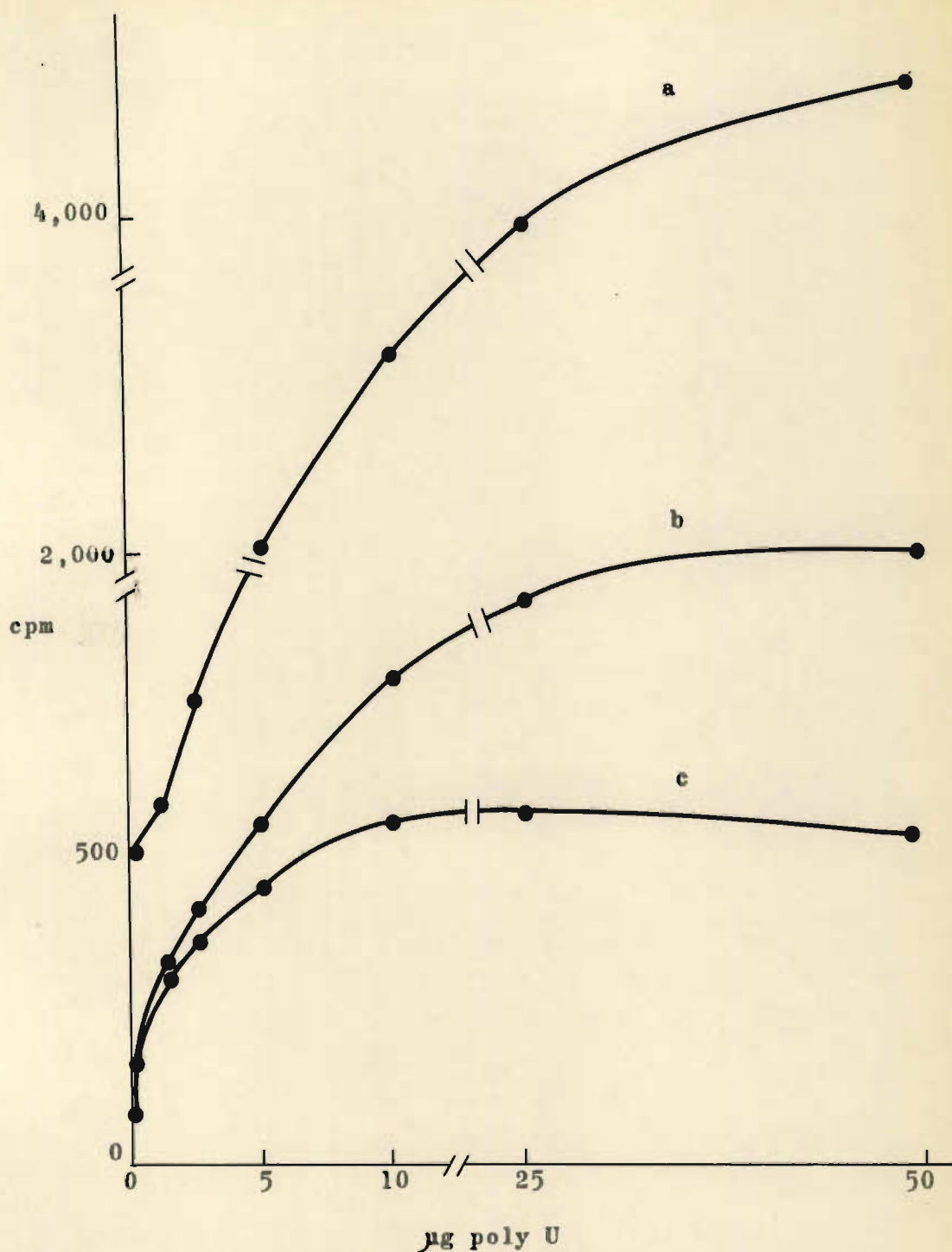


Figure 4.2 Optimum concentration of poly U required for polyphenylalanine synthesis

Assays were carried out as described in section 3.2.6 using R-ribosomes, 0.2 mg. The assay systems tested were those using the pH5 precipitate with ^{14}C -phenylalanine (a) (Method 1) and the pH5 supernatant (b) or the partially purified transfer fraction, TF (c), with ^{14}C -phenyl-tRNA from rat liver (Method 2). Incubation was for 15 minutes.

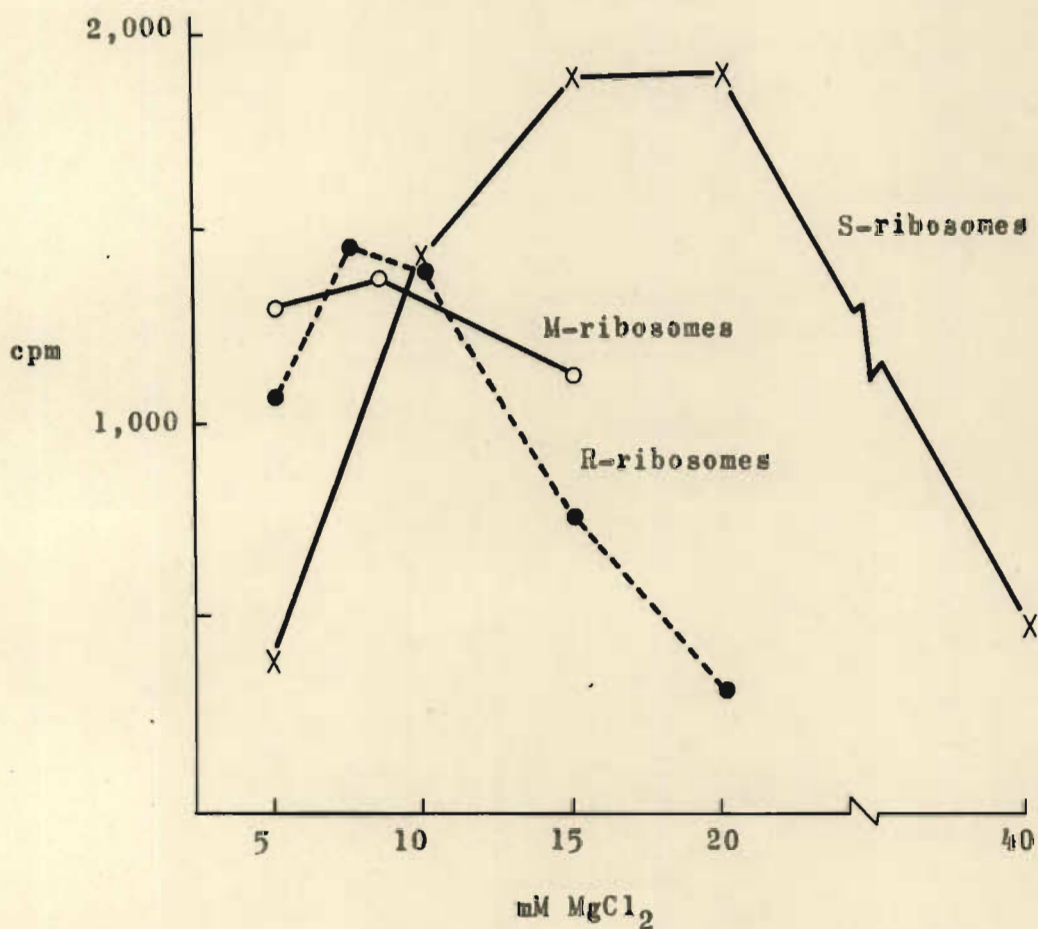


Figure 4.3 Optimum requirement of magnesium for polyphenylalanine synthesis

Assays were carried out as in Method 2 of section 3.2.6, using the pH5 supernatant and ^{14}C -phe-tRNA of rat liver. Incubation was for 15 minutes.

4.3.5 The Binding of ^{14}C -phe-tRNA by Different Ribosomes

Soon after these studies were started it was shown by Arlinghaus, Shaeffer & Schweet (1964) that one of the transfer enzymes of the reticulocyte system was involved in the binding of aminoacyl-tRNA to ribosomes. It was therefore necessary to measure this binding during the study of the transfer enzymes and so the conditions for this were worked out here.

The method tried here was similar to that used by Nirenberg & Leder (1964) and also Kaji & Kaji (1964) with the ribosomes of E. coli. This binding was measured in the absence of enzymes, at high concentrations of magnesium ions and in the presence of poly U. The ability of R-, M- and S-ribosomes to bind ^{14}C -phe-tRNA under these conditions, and also their optimum requirement for magnesium ions in this reaction is shown in Fig. 4.4.

The optimum concentration of magnesium ions required for this binding by the three ribosomes was the same, namely, 15-20 mM. As in the synthesis of polyphenylalanine, it was found that S-ribosomes were the most active while R- and M-ribosomes were about equally active. No enzyme fraction was able to stimulate this binding when measured at the optimum concentration of magnesium ions.

Unknown at the time that this work was done, was the fact that the dependence of the binding of aminoacyl-tRNA on an enzyme factor was masked by the use of high concentrations of magnesium in this assay. However, the conditions worked out here were nevertheless suitable for the purpose of the experiments described in Chapter 5, and were therefore used throughout that work.

4.3.6 Physical Characteristics of Ribosomes

For the purpose of obtaining more knowledge about the preparations of ribosomes used here, and especially since it was found that they differed in activity in both the synthesis of polyphenylalanine and binding of phe-tRNA, some of their physical characteristics were measured. To do this they were analysed on sucrose gradients and in the analytical ultracentrifuge, and also studied under the electron microscope.

4.3.6.1 Analysis of Ribosomes on Sucrose Gradients. - The profiles of R-, M- and S-ribosomes, when centrifuged over sucrose gradients, are

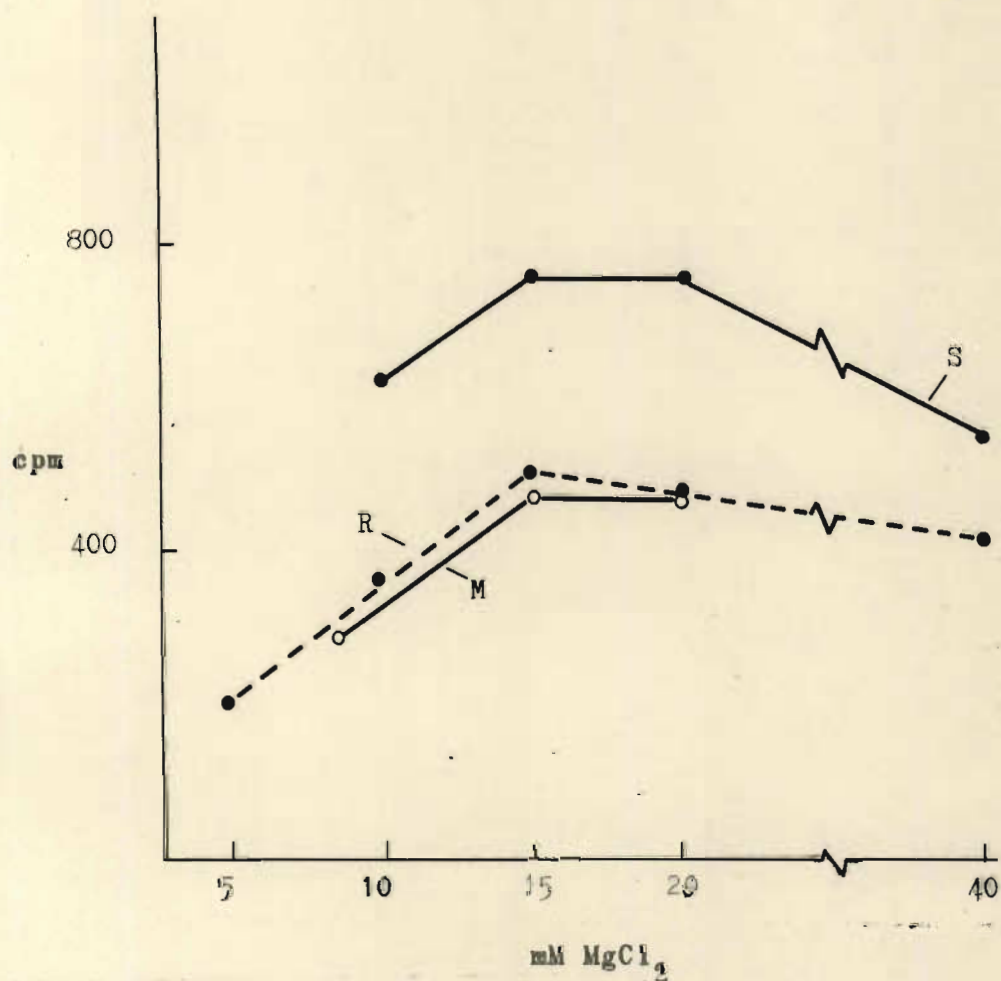


Figure 4.4 Optimum requirement of magnesium for the binding of ¹⁴C-phe-tRNA by different ribosomes

Assays were carried out as in section 4.2.3 with the final concentration of magnesium ions being that shown in the figure. ¹⁴C-phe-tRNA from rat liver was used.

shown in Fig. 4.5(i). It is seen that the size distribution of these ribosomes differ. The majority of S-ribosomes moved in a single peak as particles which were smaller than the 76S monomer. R-ribosomes moved mainly as three peaks, one smaller than, one equal to, and one larger than the 76S monomer. M-ribosomes moved as a broad peak covering the monomer, dimer and much of the polysome area of the gradient.

This result showed that S-ribosomes and a large proportion of R-ribosomes consisted of particles which were smaller than the monomer. Experiments were then done to determine whether these smaller particles were the subunits of ribosomes although no division of the slower moving peak into two peaks was noticeable, as was expected. R- and S-ribosomes were therefore centrifuged over 5-25% linear sucrose gradients, similar to those used by Pestka & Nirenberg (1966), for the identification of the subunits of E. coli. These results are shown in Fig.4.5(ii). Neither R-ribosomes (curve a) nor S-ribosomes (curve d) were found to move as the subunits of ribosomes. They both moved as particles which were equal to or slightly larger than the 50S subunit, which was in agreement with the result shown in Fig. 4.5(i). When these ribosomes were dialysed against N-buffer for 10 hours, still no separation into their respective subunits was obtained (curves b and e respectively) although less material moving in the 70S area of the gradients was obtained. This was the buffer used by Pestka & Nirenberg (1966) to obtain the subunits from the ribosomes of E. coli. Both R- and S-ribosomes were, however, shown to consist of the two subunits, and in equal proportions, when they were treated with EDTA. This showed that these preparations were no different in this respect to polysomes treated in the same way (Tashiro & Morimoto, 1966; Hawtrey & Nourse, 1968, and others).

A similar inability to separate the subunits of ribosomes of rat liver by dialysis against low concentrations of magnesium ions has been shown by Tashiro & Morimoto (1966). These workers explained that this was because of the polymerization of the individual subunits, especially of the 30S subunits, in the presence of even low concentrations of magnesium.

4.3.6.2 Analysis of Ribosomes in the Analytical Ultracentrifuge. - The results with sucrose gradients were partly confirmed when these ribosomes were analysed in the analytical ultracentrifuge, as shown in Plate 3.1, c-d, although a different picture was obtained for S-ribosomes.

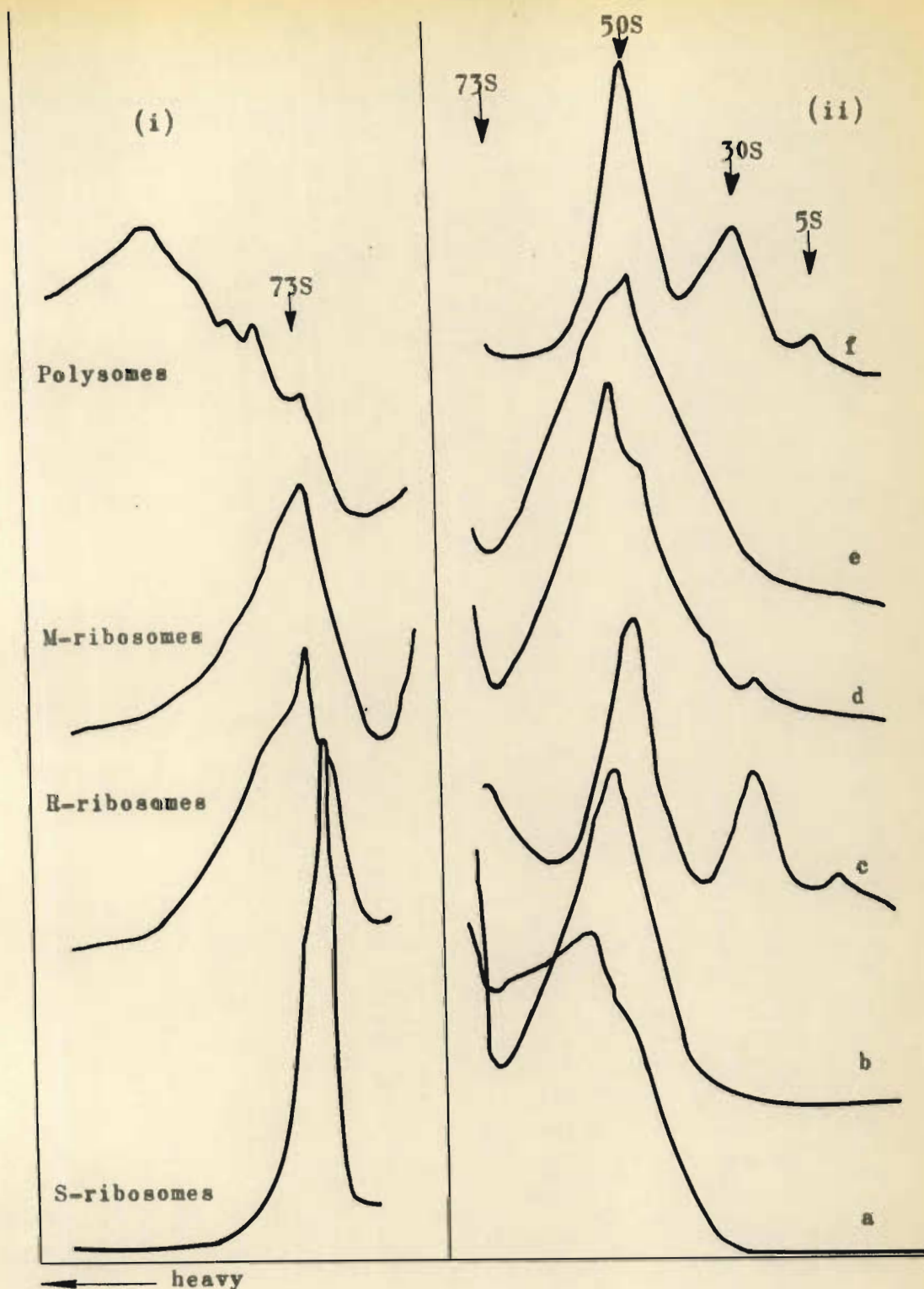


Figure 4.5

Analysis of ribosomes on sucrose gradients

In (i) 10-30% (w/v) linear sucrose gradients were used. The different gradients contained for polysomes, Medium A; for M-ribosomes, solution M; for R-ribosomes and S-ribosomes, solution R. Centrifugation was at 63,000g for 150 minutes.

In (ii) 5-25% (w/v) linear sucrose gradients containing N-buffer were used. R-ribosomes and S-ribosomes respectively in gradients a and d were used as described in methods; in b and e after dialysis against 100 volumes of N-buffer for 10 hours with 3 changes of buffer; and in d and f after making 5 mM with respect to EDTA. Centrifugation was at 60,000g for 10 hours.

M-ribosomes consisted of particles of 73S and larger, except for a fairly large proportion which moved as a badly defined band at 22S which may represent mRNA which had separated from the ribosomes. The pictures obtained with both R- and S-ribosomes was the same, since, R-ribosomes moved as four bands, 100S, 70S, 48S and 34S, as did S-ribosomes, 100S, 72S, 52S and 40S. Although these S values were not worked out to infinite dilution these four bands would seem to represent the dimer of the 76S monomer, the 76S monomer and the 60S and 40S subunits of ribosomes. Very little of the smaller 40S subunit was present, showing that dimerization of this particle had occurred and that it was moving with particles in the heavier region. This was borne out by the Schlieren patterns obtained, especially of 60S particles, which were not as sharp as was expected for single pure particles.

4.3.6.3 Studies of Ribosomes Under the Electron Microscope. - Electron micrographs of R-, M- and S-ribosomes are shown in Plate 3.2, g to k, of Chapter 3. The purpose of this work was to determine whether the preparations consisted of single particles or of polysomal-like clusters.

The electron micrograph in which R-ribosomes were negatively stained (g), shows that the majority of these particles existed as the monomers of ribosomes, smaller particles and clusters of two, three and even four ribosomes. Usually the space pattern exhibited by polysomes was absent with these preparations and it is possible that the clusters which were present were the result of aggregation of monomers caused by magnesium ions. When R-ribosomes were studied using the shadow technique (h) no polysomal like clusters were present, as was found with polysomes (f). However, the method used to prepare electronmicrographs by this shadow technique was found to give a high proportion of monomer ribosomes in the preparation with polysomes (f). It was therefore possible that this dissociation of the polysomes occurred to such an extent with the preparation of R-ribosomes that all the clusters of polysomes which were present were dissociated.

No electronmicrographs of S-ribosomes could be prepared by the method of negative staining used here. However, a good picture of these ribosomes was prepared by the shadowing technique (i). In this picture only 3 different sized single particles were clearly visible, which probably represent the 76S monomer or the dimer of the 40S subunit, the 60S subunit and the 40S subunit.

No good electronmicrographs of M-ribosomes were obtained by any of the methods used, although, those obtained by means of the shadow technique, seemed to show the presence of clusters of ribosomes and monomers (j).

In conclusion it is seen that the picture obtained with electron microscopy was similar to that obtained by analysis on density gradients and in the analytical ultracentrifuge. This is, that the samples of R- and S-ribosomes contain no polysomes and that M-ribosomes do contain a small proportion of these polysomes. Both R- and S-ribosomes are made up of monomers and subunits, with S-ribosomes containing a larger proportion of the subunits although different pictures were obtained by the different methods of analysis.

4.4 DISCUSSION

As mentioned previously it was planned to isolate and prepare ribosomes with which to study the transfer enzymes. The experiments reported above showed that three different preparations were suitable for this purpose in that they were inactive in the absence of either of the separated transfer enzymes alone but were active when both were added, either as a mixture of the separated factors or an unfractionated source of enzymes. However, it was decided not to proceed with the study of the transfer enzymes, since, by the time the work had reached this stage a number of publications had appeared which showed that other workers in at least two laboratories were well ahead with work on the problem in liver (Moldave and co-workers, Gasior & Moldave, 1965 a & b; Ibuki, Gasior & Moldave, 1966; Gasior, Ibuki & Moldave, 1966; Sutter & Moldave, 1966; and Klink and co-workers, Klink, Nour & Aepinus, 1963; Klink, Kloppstech & Netter, 1966; Klink, Kramer, Nour & Petersen, 1967; Klink, Kloppstech, Kramer & Dimigen, 1967). Instead, attention was focussed on the differences between the activities of the S-, M- and R-ribosomes, which could not be explained on the basis of any of the then known factors involved in protein synthesis. This work is described in the next chapter.

CHAPTER 5

THE RELATIONSHIP BETWEEN THE FACTORS, ESPECIALLY 5S RNA, REMOVED DURING WASHING OF RIBOSOMES AND PEPTIDE BOND FORMATION

5.1 INTRODUCTION

As shown in Chapter 4, ribosomes prepared by different procedures revealed differences in respect of their ability to bind *phe*-tRNA and synthesize polyphenylalanine. It was shown further that these differences could not be explained in terms of the presence of different amounts of transfer enzymes in the various preparations. The present chapter presents the results of a further investigation aimed at finding an explanation for these observed differences. These studies revealed a relationship between the content of 5S RNA of the preparations and their activity; this work representing, at the time it was initiated, the first attempt to relate 5S RNA to protein synthesis. It has since become known that Monier and co-workers in Marseilles and also Comb and co-workers at Harvard Medical School are also working in this direction. A further finding was that ribosomes deficient in 5S RNA could not be reactivated with a purified form of this RNA, but only with that which contained protein. While this work was in progress Aubert, Monier, Reynier & Scott (1968) showed that ribosomal protein was necessary for the reassociation of 5S RNA with ribosomes.

As this chapter deals mainly with 5S RNA and the proteins stripped from the ribosomes during the washing procedures, it is relevant to review the literature dealing with these two topics before proceeding to a presentation of the experiments conducted by the author.

The 5S species of ribosomal RNA was first reported by Rosset & Monier (1963) to be present in *E. coli*. Its existence was soon confirmed and shown to be present in other types of cells (Comb & Katz, 1964; Galibert, Larsen, Lelong & Boiron, 1965; Marcot-Queiroz, Julien, Rosset & Monier, 1965; Bachvaroff & Tongur, 1966; Virmaux, Mandel & Urban, 1964; Comb, Sarkar, De Vallet & Pinzino, 1965; Schleich & Goldstein, 1966; Reich, Forget, Weissman & Rose, 1966). Rosset, Monier & Julien (1964) showed that this RNA was of ribosomal origin and associated with the 50S subunit. They also showed that it contained no methylated bases and only minor amounts of pseudo-uridylic acid,

this being determined as 0.5 residues per molecule by Comb & Zehavi-Willner (1967). Comb & Katz (1964) at first suggested that this RNA may be a precursor of tRNA but this was shown to be false when most of the properties of 5S RNA were found to be different from tRNA. Recently Brownlee & Sanger (1967) have applied a two dimensional technique of electrophoresis to fractionate the nucleotides and determine their sequence in 5S RNA from E. coli. In a later paper they arranged this sequence in a specific manner forming a three leafed clover (Brownlee, Sanger & Barrel, 1967). Forget & Weissmann (1967) independently confirmed this and also determined the sequence of 5S RNA in KB cells. Forget & Weissmann (1968) and also Comb & Zehavi-Willner (1967) showed that 5S RNA can assume two forms which are interconvertible and can be separated on methylated albumin columns. Forget & Weissmann (1968) report that Hindly, of the Medical Research Council Laboratories of Molecular Biology, Cambridge, England, obtained similar results after separating the two forms by means of acrylamide gel electrophoresis. The significance of this work is not yet known but it indicates that 5S RNA is a fairly homogeneous type of RNA and that in E. coli and KB cells only two slightly different species exist and that these only differ in one base residue. According to Dr. Marshall Nirenberg (personal communication), the synthesis of two or more almost identical species of RNA does not indicate that the two RNA's have different functions but that there may be different sites on the gene(s) for the synthesis of one species of RNA. The multiplicity of genetic sites for the synthesis of any molecule is an indication of the essential role which the molecule has for the survival of an organism. This would suggest that 5S RNA is essential for the functioning of ribosomes.

The function of 5S RNA on ribosomes is not known. Comb & Sarkar (1967) conducted numerous studies whereby 5S RNA in solution was shown to exchange with 5S RNA on ribosomes. Aubert, Monier, Reynier & Scott (1968) showed that this association between 5S RNA and ribosomes, or 50S subunits, was specific and required the presence of proteins on the ribosomes which were of ribosomal origin. They did this by treating ribosomes with 2 M-LiCl which removed 5S RNA and some ribosomal proteins from them. They then showed that the 5S RNA obtained in this way did not reassociate with the ribosomes which had been washed with LiCl unless it was added with the protein which had been removed at the same time. They did not test these different particles

for activity in the synthesis of polypeptides and therefore it was not shown whether 5S RNA was necessary for the activity of these particles. The only work in which there has been some suggestion as to the function of 5S RNA on ribosomes was that by Comb & Sarkar (1967) who showed that 5S RNA possibly played some role in the association of the subunits. In this work they dissociated the ribosomal subunits and removed 5S RNA from the ribosomes with EDTA, and then, in agreement with the findings of Tashiro & Morimoto (1966), found that these subunits failed to re-associate. This suggested that 5S RNA played a role in the association of subunits but, as they themselves pointed out, this interpretation should be viewed with caution because of the damage caused to the 30S subunit on treatment with EDTA.

In view of the fact that a ribosomal protein is necessary for the re-association of 5S RNA with ribosomes it is pertinent to give a review of those ribosomal proteins which are related to the activity of ribosomes. Most of these studies on the ribosomal proteins have been carried out with bacterial ribosomes and an ever increasing number of proteins are constantly being shown to be present. The function of ribosomal proteins has been studied from two different angles. One has been to analyse the number and properties of the different proteins on the ribosome and the other has been to remove proteins (split-proteins) from ribosomes using different chemical compounds and then return these singly to see if they are connected with the functioning of the ribosome. Only the work carried out using the latter method will be reviewed here. Numerous chemicals have been used to split proteins from ribosomes. Those most frequently used have been LiCl and CsCl. The processes by which these chemicals remove proteins have been shown to be reversible (Meselson, Nomura, Brenner, Davern & Schlessinger, 1964; Gesteland & Staehelin, 1967; Traub, Nomura & Tu, 1966; Staehelin & Meselson, 1966; Hosokawa, Fujimura & Nomura, 1966; Spirin & Belitsina, 1966). Lerman, Spirin, Gavilova & Golov (1966) removed fractions from ribosomes in discreet steps, although in more recent work the different groups of proteins removed depended on the concentration and salt used. It was then shown that distinct proteins responsible for the binding of tRNA, the binding of mRNA and for amino acid polymerization could be removed with CsCl (Traub, Nomura & Tu, 1966) while those responsible for the binding of 5S RNA to ribosomes could be removed with LiCl (Aubert, Monier, Reynier & Scott, 1968). In more recent work Traub and Nomura (1968a) have been able to separate the

split-proteins into four groups, acidic and basic proteins from both the 30S and 50S subunits. They demonstrated that basic proteins from 30S subunits and acidic proteins from 50S subunits were those responsible for the activity of split fractions. This work has been followed up with numerous papers showing how these different fractions are connected with the activity of ribosomes and their reassociation (Traub, Soll & Nomura, 1968; Nomura & Traub, 1968; Traub & Nomura, 1968b). In none of the work in which the split-proteins have been studied by Traub and co-workers has any consideration been taken of the possible removal of 5S RNA, and it is difficult to see how this species of RNA fits in with this work. This is being considered in the work to be carried out in the author's laboratory but will not be included in this thesis.

5.2 METHODS

5.2.1 Preparation of Ribosomes Washed with Magnesium Chloride

M-, R- and S-ribosomes were prepared as described in Chapter 4, section 4.2.1.

R- and S-ribosomes were washed with solutions of MgCl_2 as follows: A suspension of ribosomes was diluted with three volumes of MgCl_2 so that the final concentration of MgCl_2 was either 10 mM or 100 mM. This suspension was left for 30 minutes before centrifuging at 100,000g for 30 minutes. If necessary, the ribosome pellet was washed in the desired concentration of MgCl_2 following the same procedure; the number and type of wash used is indicated in the tables of the text. After the last wash with MgCl_2 the pellets were rinsed and suspended in an excess of the solution in which they were originally suspended, centrifuged at 100,000g for 60 minutes and the final pellet rinsed and dissolved in the same solution as before.

To wash M-ribosomes with different solutions of MgCl_2 the ribosomes were prepared as described in section 4.2.1. except that the solution of MgCl_2 , with which the ribosomes were washed after treatment with deoxycholate, was changed as desired. Usually the solution of 10 mM- MgCl_2 was replaced with one of 100 mM.

5.2.2 Preparation of Ribosomes Washed with Alkaline Buffers

Details of the procedures used are given in conjunction with the relevant tables (5.4 and 5.6) which summarize the results.

5.2.3 Preparation of Transfer Enzyme Fractions

The pH5 supernatant was prepared as previously described (section 3.2.1). The partially purified fractions, TF and TF-AS, as described in section 4.2.2. and the preparation of fraction C from the Sephadex columns is outlined in the caption of Figure 4.1.

5.2.4 Preparation of 5S RNA

Two methods were used.

5.2.4.1 Sodium Lauryl Sulphate/Phenol Method. - A suspension of rat liver microsomes in Medium A (section 3.2.1) was dialysed against distilled water in the cold for 16 hours. It was then made 1% with respect to sodium lauryl sulphate (w/v) and incubated at 30°C for 5 minutes. The total RNA was extracted from this by using phenol, as was described for the preparation of tRNA (Section 3.2.2) and precipitated from the resulting water phase with ethyl alcohol. The RNA, freed of phenol by dialysis, was dissolved in 50 mM-NaCl at a concentration of 1.5 mg/ml solution. RNA of higher molecular weight was removed by precipitation at 1 M-NaCl at 0°C for 16 hours. The RNA remaining in the supernatant, after centrifugation at low speed, was precipitated with 3 volumes of ethyl alcohol at -20°C and dialysed against distilled water for 12 hours. Undissolved matter was removed from the dialysed preparation by low speed centrifugation. The RNA in solution was shown by gel electrophoresis to consist of 50% 5S RNA with lesser amounts of tRNA and other RNA. 5S RNA was purified from the mixture of RNA in solution as follows: aliquots containing 50 µg of RNA were placed over 5 x 1 cm 7% polyacrylamide gels made in buffer A of Method of assay of 5S RNA (see 5.2.7.2). One aliquot of the sample of RNA was lightly stained with acridin orange. A current of 5 m amp was applied to each gel. When a clear separation of the different RNA bands was evident in the sample which had been stained (after about 40-50 minutes), the gels were removed and the sections of the unstained gels which coincided with the area of the 5S RNA band of the stained gel were cut out and extracted twice with 5 volumes of a solution of 0.1 M-KCl, using a Dounce homogeniser to break up the sections of gel. The extracts were treated with ethyl alcohol to precipitate the RNA which was then dissolved in distilled water and shown to consist mostly of 5S RNA with a small trace of tRNA when analysed by the method described in 5.2.7.2 (see e of Figure 5.3).

5.2.4.2 EDTA Method. - A fraction containing 5S RNA was released from R-ribosomes by a modification of the method of Parish, Kirby & Klucis (1966), as follows: 10 mg of ribosomes were mixed with 20 mg sodium bentonite, 200 mg Na_2EDTA and 100 mg ethylurea in a total volume of 2.5 ml solution. This was then immediately layered over 10 ml 1.0 M-sucrose in solution R in a 65 rotor tube and centrifuged at 230,000g for 30 minutes. After centrifugation the top 3 ml in the tube was carefully sucked off and dialysed against 2 changes of 100 volumes of solution R for 120 minutes. The resulting solution which was used in the experiments described below was shown to consist predominately of 5S RNA with traces of larger species of RNA when analysed on acrylamide gels (see gel f of Figure 5.3).

The purpose of using the 10 ml sucrose layer here was to separate the released 5S RNA from the ribosomes which contained the RNases (it was hoped that these enzymes were not themselves released) and which moved into the sucrose layer. The bentonite should also pass into this layer. This method worked equally well in the absence of bentonite and ethyl urea.

5.2.5 Methods for the Assay of Activity of Ribosomes

The synthesis of polyphenylalanine (Synthesis reaction) was assayed for by Method 2 of section 3.2.6.2 using ^{14}C -phe-tRNA and different transfer fractions. Assays for the binding of phe-tRNA (Binding reaction) was carried out as described in section 4.2.3. In the majority of the experiments ^{14}C -phe-tRNA of rat liver was used (see section 3.2.2) which was found to contain no 5S (see Figure 5.3j). In contrast ^{14}C -phe-tRNA of *E. coli* did contain 5S RNA (see Figure 5.3i), but it is not expected that the presence of 5S RNA in this sample of tRNA should have interfered with the results because it is unlikely that 5S RNA from different species is interchangeable, as has been found with other rRNA (Traub & Nomura, 1968b).

5.2.6 Measurement of Binding of ^3H -poly U by Ribosomes

To 7 mg ribosomes and $0.125\ \mu\text{C}$ ^3H -poly U in a glass tube in ice was added an incubation solution to give the following final concentrations in a total volume of 0.7 ml, 0.25 M-sucrose, 10 mM-tris-HCl, pH 7.6, 60 mM-KCl and 2 mM- MgCl_2 . The reaction mixture was incubated for 15 minutes at 24°C . The reaction was stopped in ice and after 10 minutes the contents layered above linear 5-20% (w/v) sucrose gradients containing 10 mM-tris-HCl, pH 7.6,

86 mM-KCl and 17.5 mM-MgCl₂. They were centrifuged at 25,000 rpm in a Beckman SW 25.1 rotor for 1 hour, 0.5 ml fractions were drop-collected, the even numbers of tubes used for E₂₆₀ measurements and the odd tubes passed straight on to Millipore filters (HA 0.45), and washed twice with 10 mM-tris-HCl buffer, pH 7.6, containing 15 mM-MgCl₂, the millipores dried and counted as described for the Millipore method under section 3.2.7 using a counting efficiency of 50%.

5.2.7 Determination of 5S RNA in Ribosomes

5.2.7.1 Extraction of RNA from Ribosomes for Analysis on Acrylamide

Gels. - RNA was extracted from ribosomes by the method of Sporn & Dingman (1963) as follows: 1 volume of ribosomes was incubated at 25°C for 5 minutes with 1 volume of a solution of 0.2% sodium lauryl sulphate in 2 mM-potassium phosphate buffer, pH 7.0. The mixture was then shaken with 2 volumes of water-saturated redistilled phenol at room temperature for 1 hour. It was then centrifuged at 2,000g to break the emulsion, the water phase kept, and the phenol phase again shaken with 1 volume of distilled water. The resulting water phase was added to the original water phase and the combined water phases reshaken twice with equal volumes of phenol. To the final water phase was added 2.5 volumes of ethanol to precipitate the RNA. This was left at -15°C overnight and the precipitate which formed was collected by low speed centrifugation. The precipitate was dissolved in 0.1 M-potassium acetate buffer, pH 6.0, and then dialysed for 48 hours against four changes each of 500 volumes of the same buffer. The exact volume of the final solution was measured, its content of RNA determined by its E₂₆₀, the RNA again precipitated with 2.5 volumes of ethanol and the final precipitate dissolved in tris (3.3 mM)-DEBA (diethylbarbituric acid) (30 mM) buffer, pH 7.0, containing 0.35 M-sucrose at a final concentration of RNA of 1 mg/ml.

5.2.7.2 Method of Assay of 5S RNA. - 5S RNA was assayed by acrylamide disc gel electrophoresis according to the method of Richards, Coll & Gratzer (1965) as adapted for rat liver preparations by King (1967). A 5 cm cylindrical column of a 10% gel, overlaid with 3 mm of a 5% spacer gel, was prepared in a clean dry 6 cm glass tube, diameter 5 mm, using the following solutions:-

- A. Tris-HCl buffer, pH 8.5: 5.88 g tris + 10 ml 1N -HCl in a total volume of 25 ml,
- B. Temed (N, N, N', N'-tetramethylethylenediamine), 0.28 % (w/v),
- C. 40 % gel: 9.5 g acrylamide + 0.5 g N, N'-methylenebisacrylamide in a total volume of 25 ml.
- D. 140 mg ammonium persulphate in a total volume of 100 ml.
- E. Tris-HCl buffer, pH 7.6: 1.22 g tris + 10 ml 1 N-HCl in a total volume of 25 ml.
- F. Reservoir solution, tris (3.3 mM)-DEBA (30 mM) buffer, pH 7.0: 808 mg tris + 11.052 g DEBA in a total volume of 2 litres.

The 10% gel was prepared by mixing 1 part A + 1 part B + 2 parts C + 4 parts D.

This was placed under vacuum for 30 seconds to remove air bubbles.

The 5% spacer gel was prepared by mixing 1 part distilled water + 1 part E + 1 part B + 1 part C + 4 parts D. Air bubbles were removed as with the 10% gel.

The gels were prepared in glass tubes, placed vertically with bottoms stoppered, by filling up to the 5 cm mark with the 10% gel, while preventing the formation of air bubbles. The upper meniscus of the gel was straightened by overlaying it with about 0.05 ml of distilled water after which it was left in the light until set (10-20 minutes). The overlay of water was then removed, the gel blotted dry and a 3 mm layer of the 5% spacer gel formed above the 10% gel in the same way. The glass tubes containing the gels were then placed in the electrophoretic apparatus (Canalco) which contained the Reservoir solution from which all air bubbles were removed. The sample of RNA (50 or 100 μ l) was then placed over the gel, the current switched on (5 m amp per tube), and electrophoresis allowed to continue for 20 minutes with the positive electrode in the lower reservoir. After this the gels were extracted from the tubes using glycerol and a thin metal rod. The gels were stained for 12 hours in a solution containing 2% acridine orange, 1% lanthanum acetate and 15% acetic acid. The gels were destained in the same Canalco apparatus in 7% acetic acid under an electric current (10 m amp per tube). The gels were scanned in a Model F Canalco high-resolution electrophoresis microdensitometer and the concentration of the different 5S RNA bands compared by cutting out the densitometer tracings of these bands and weighing them.

The 5S RNA band was identified by the distance it travelled during electrophoresis. This was originally established for the apparatus and conditions

used in this laboratory by King (1967). Experience showed that the buffer in which the RNA was suspended had little effect on this distance, which was constant, provided the current and time applied were constant.

5.2.8 Examination of Proteins in Ribosomes

After preliminary studies to find the best method for extracting the proteins from the ribosomes and the optimum conditions for disc gel electrophoresis (see section 5.2.8.3) the following procedures were adopted.

5.2.8.1 Extraction of Proteins from Ribosomes. - This was based on the method of Spitnik-Elson (1965). To 9 ml ribonucleoprotein particles in Medium A (any of polysomes, ribosomes and ribosomal subunits at a concentration of between 3-6 mg per ml solution) was added 9 ml of a solution containing 6 M-LiCl and 8 M-urea. This was well mixed and left at 4°C for 24 hours. The precipitate which formed was centrifuged down at 6,000g for 10 minutes and the resulting supernatant retained. The precipitate was washed twice with a solution of 3 M-LiCl and 4 M-urea (10 ml per wash) and the washings added to the original supernatant. To this total supernatant fraction was added an equal volume of cold 20% TCA which was then left for 16 hours at 4°C. The precipitate which formed was collected by centrifugation and washed twice with ether. Hereafter all solutions were made up with deionised water. The final precipitate was dissolved in a solution of 0.1 M-HCl and 8 M-urea. (3 ml was used per 9 ml of the original suspension of ribonucleoprotein of which the concentration was 6 mg per ml. For other concentrations the volume was adjusted accordingly.) This solution of protein was then dialysed against 100 volumes of a solution of 0.1 M-HCl and 8 M-urea for 16 hours. After dialysis any undissolved material was removed by low speed centrifugation, and the clear solution was used for the examination of the proteins present.

5.2.8.2 Disc Gel Electrophoresis of Proteins. - Using the identical conditions and apparatus described in section 5.2.7.2, 15% gels 4 cm long were made and run using the following solutions which were all prepared using deionised water.

G. 5.6 g KOH + 4 ml Temed + acetic acid to pH 4.3 and H₂O to 100 ml.,

H. 60 g acrylamide + 0.4 g N, N'-methylenebisacrylamide + H₂O to 100 ml.,

I. 4 mg ammonium persulphate in 6 ml 10 M-urea (made freshly just

J. Reservoir solution: 12.48 g β -alanine + 3.34 g glycine + water and acetic acid to pH 4.4 and 1,000 ml.

The gels were prepared by mixing 1 part G with 2 parts H and 5 parts I using the procedure as described in section 5.2.7.2.

For analysis, 20 μ l of ribosomal proteins were placed on the gels and electrophoresis continued for 2 hours applying a current of 4 m amp per tube with the negative electrode in the lower reservoir. The gels were stained in a solution of 0.02% amido black in 7.5% acetic acid overnight (4 hours was sufficient). The gels were destained in an apparatus designed by the author and his colleague, Mr. Albrecht, and shown in Figure 5.1. The method of destaining was found to be superior to any other tried in this laboratory. The length of the apparatus and its different compartments is immaterial and can be made according to need. The division of the apparatus into compartments was done so that the full use of the applied current could be made when only 2 to 3 gels were being destained. A current of 100 m amp was capable of destaining 3 gels 4 cm long in a 15 cm compartment in 30 minutes without causing any damage to the gels.

Gels prepared as described above were generally not scanned but photographed and compared visually. This was found sufficient for the purpose of these experiments which was to determine whether any of the proteins had been completely removed, or greatly reduced in concentration, from the ribosomes.

5.2.8.3 Discussion of the Methods Used to Prepare and Separate Ribosomal Proteins.— Since the methods by which the ribosomal proteins can be extracted and analysed on acrylamide gels are of primary importance here, a brief review of these is given below. A brief discussion of the different methods tried is also included here because although it is realized that they fall under results their inclusion in that section would detract from the main results.

When this work was started the methods available for the extraction of ribosomal proteins were the following: extraction with 66% acetic acid (Waller & Harris, 1961), dialysis against 6 M-urea (Spahr, 1962), treatment of ribosomes with 2 M-LiCl (Chao, 1961), dialysis against 1 M-NaCl (Spitnik-Elson, 1962) and treatment with 3 M-LiCl containing 4 M-urea (Spitnik-Elson, 1965). The extractants used reacted with the ribosomes from different cells in different ways, and some of the heterogeneity of the isolated proteins could be attributed to the effect of the chemicals used. The method in which 3 M-LiCl plus

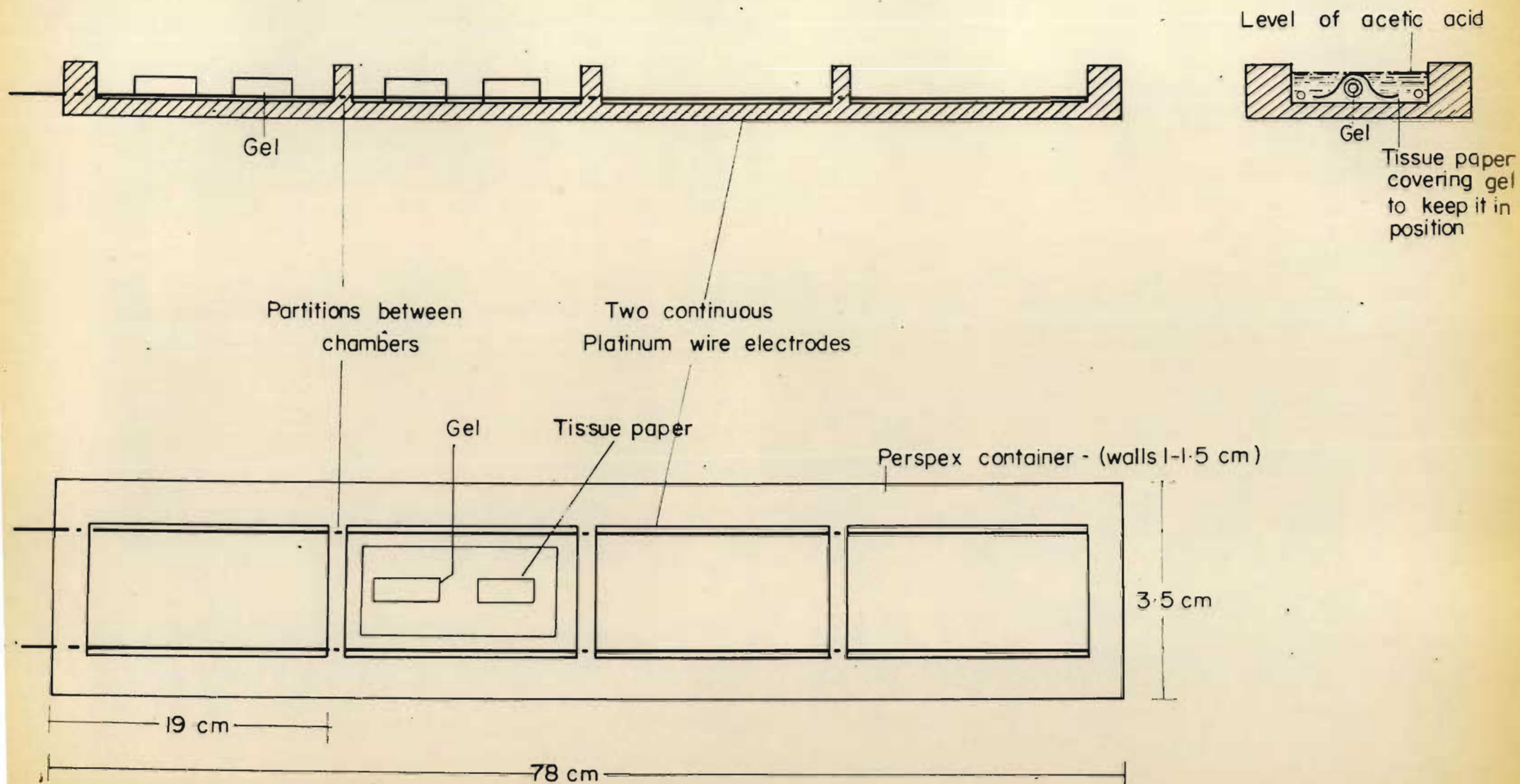


FIGURE 5.1
*Apparatus for destaining
 gel.*

4 M-urea was used gave the most consistent results and has been most widely used in recent work, and was therefore adopted in the present study. It causes virtually complete precipitation of ribosomal RNA and complete solubilization of the proteins. A second solution tried here for the extraction of ribosomal proteins, was 0.1 M-HCl plus 8 M-urea. This was used by Mackay, Hilgartner and Dounce (1968) for the extraction of proteins from nuclei. According to them this was superior to either 0.1 M-HCl or 8 M-urea alone, and since urea had also been used to extract the proteins from ribosomes, it was thought likely that 8 M-urea containing 0.1 M-HCl would be superior for this than urea alone.

In the method of Spitnik-Elson (1965) the final protein precipitate obtained was dissolved in 0.01 M-HCl. However such a solution was not suitable for direct analysis on acrylamide gels because of its low density. Therefore various modifications of this step were tried as described below:

- Modification 1. The proteins were dissolved in 0.01 M-HCl and dialysed against 0.1 M-HCl containing 8 M-urea.
- Modification 2. The proteins were dissolved in 0.1 M-HCl containing 8 M-urea instead of 0.01 M-HCl. This solution was then either not treated further or it was dialysed against 4 changes of 100 volumes of 0.1 M-HCl plus 8 M-urea for 24 hours. The dialysed solution was centrifuged at 5,000g to remove undissolved material.
- Modification 3. The proteins in 0.01 M-HCl were dialysed against 4 changes of 100 volumes of 0.125 M- H_2SO_4 for 24 hours and then precipitated with 10 volumes of acetone. (It was not possible to precipitate the ribosomal proteins dissolved in 0.01 M-HCl). This precipitate was then dried in air and weighed out amounts then dissolved in 0.1 M-HCl containing 8 M-urea. The purpose of drying the protein was to determine the exact amounts of ribosomal proteins added to the gels.

The analysis of the above solutions on different acrylamide gels is discussed later in this section.

Basic proteins, as the ribosomal proteins predominantly are, are usually analysed on gels containing ...

the cathode. Nearly all the work has been done using the method of Reisfeld, Lewis & Williams (1966) or a modification of this in which 6-8 M-urea was included in the gel and slightly different reservoir solutions were used. In the present work 3 variations of this procedure were tried as outlined below (Methods a-c). In addition the proteins were also run on a basic gel (Method d). All gels were 4.8 mm diameter, length 6-12 cm.

Method a: 7.5% gel, pH 4.3, overlaid with a 5 mm 3% spacer, pH 6.2. Reservoir solution contained 3.12% μ -alanine, 8 M-urea and was adjusted to pH 4.5 with glacial acetic acid. (0.4% bisacrylamide) (Traut 1966).

Method b: 15% gel, pH 4.3, overlaid with a 3 mm 3% spacer, pH 6.3. Reservoir solution contained 0.56% glycine and was adjusted to pH 4.3 with glacial acetic acid. (0.4% bisacrylamide). (Comings, 1967).

Method c: 15% gel, pH 4.3, no spacer. Reservoir solution contained 1.25% μ -alanine and 0.334 % glycine adjusted to pH 4.4 with glacial acetic acid. (0.4 % bisacrylamide).

Method d: 15 % gel, pH 8.6, no spacer, reservoir solution as for Method C. (2.0% bisacrylamide). This gel was similar to the small pore gel used for the analysis of RNA as described in section 5.2.7.2, except that it was double the concentration.

The values in the square brackets represent the concentration of N, N'-methylenebis-acrylamide used in the crosslinking of the gels. The concentrations of urea, temed, and other chemicals and preparation of the gels were as described in section 5.2.8.2.

To eliminate the possible loss of proteins during the preparation of washed ribosomes, these methods were tested out on polysomes. The results obtained by the different procedures are shown in Figure 5.2. Extraction with 3 M-LiCl plus 4 M-urea was superior to 0.1 M-HCl plus 8 M-urea (compare gels f and g) since the latter method extracted variable amounts and numbers of proteins. Thus in gel g a number of proteins, especially those which move faster than band 14, were missing. This was not always the case when this extractant was used, however, as can be seen in gel a where a full complement of bands was present. These inconsistencies were probably due to the buffering

effect of the ribosomal pellet, which, in turn, depended on how well the pellet was drained of the buffer used during the isolation, before the start of the extraction. This defect could possibly be overcome by dialysing the ribosome suspension against 0.1 M-HCl plus 8 M-urea.

There was nothing to choose between the 3 methods of dissolving the extracted proteins prior to placing them on the gel for electrophoresis. Therefore Modification 2, which was most convenient was used. The solution was usually dialysed against 0.1 M-HCl plus 8 M-urea and centrifuged before use to ensure that the pH was constant and not affected by any residual TCA in the pellet.

The gels prepared in acidic buffer (see c and d) were better than those in alkaline buffers (see h). There was no advantage in using long gels, like those used by Traut (1966), especially if the gels were to be photographed, because of the decrease in the definition and sharpness of the different bands in these cases. With all the gels tried however, even when using solutions from which undissolved material had been removed, there was a fairly high proportion of protein which did not move into the gels.

A spacer gel did not seem to offer any advantage so it was not used in routine work. In those instances where a spacer was tried it was apparent that not all the protein, which failed to move into those gels run without a spacer, was denatured, since a high proportion of this moved into the spacer (see gels a and b). The fraction therefore that did not move into the small pore gels needs investigation. Since polysomes were used in these preliminary tests, it may be that some of the protein was not of true ribosomal origin, e.g. the protein could have been nascent protein still attached to the ribosomes; this would not be of interest in the present work.

Of the three reservoir solutions tried, that with glycine alone (Method b) resulted in a better spread of the proteins in the gels (see gel a). The main disadvantage with this buffer, however, was that the sharpness of the bands was poor, especially those moving further down the gels. Another disadvantage was that this method was dependent on the pH of the added solution of proteins, and in the instances when this was above pH 5, many of the proteins moved with the buffer front. This latter disadvantage could be overcome by dialysing the solution of proteins against 0.1 M-HCl plus 8 M-urea. The reservoir solution which was found to be best, was the one containing both glycine and β -alanine

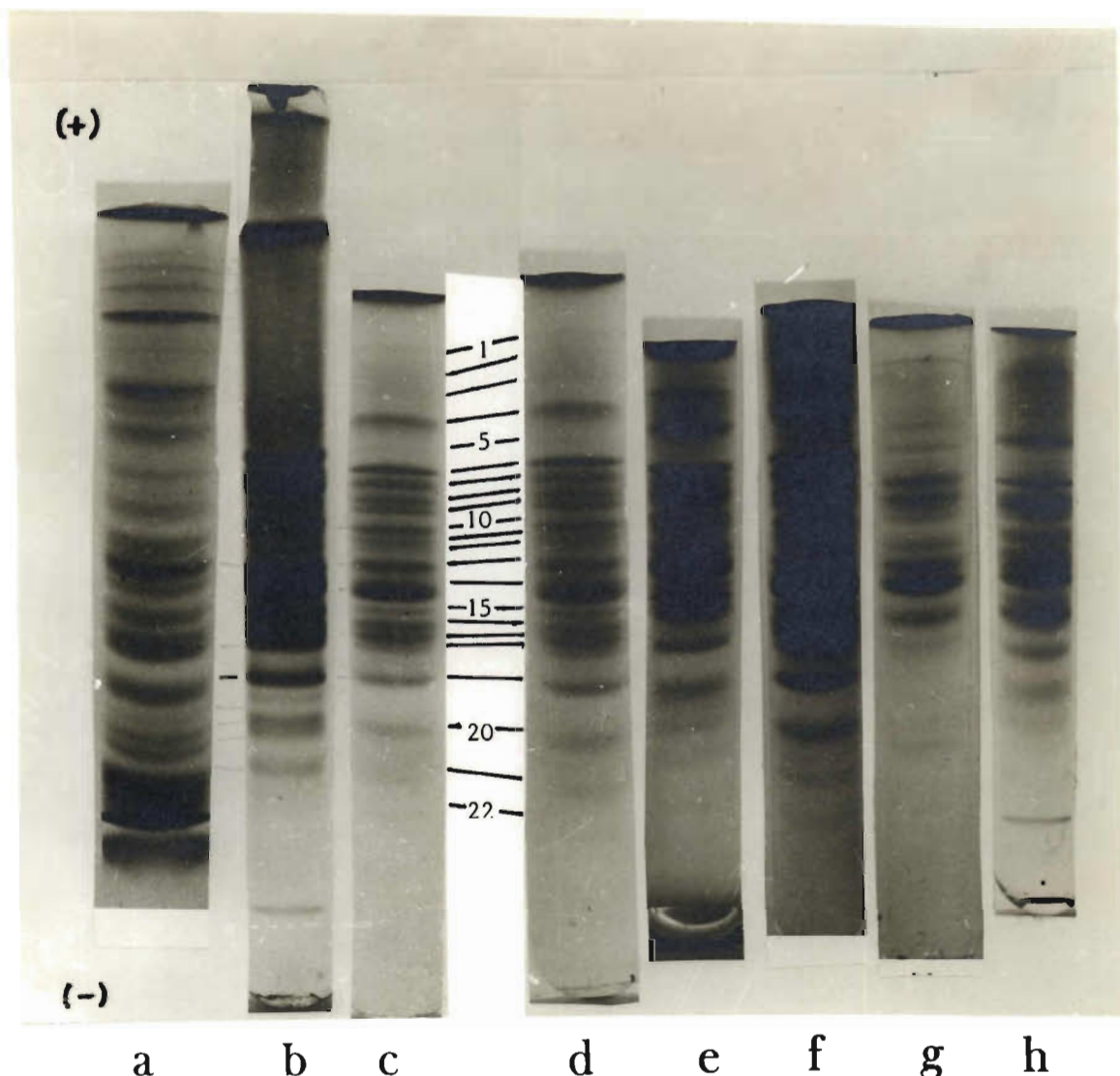


FIGURE 5.2 EXTRACTION OF POLYSOMAL PROTEINS BY DIFFERENT PROCEDURES AND ANALYSIS ON DIFFERENT ACRYLAMIDE GELS

The Modification and Methods referred to here are those described under 5.2.8.3. The different letters represent:

- a = Proteins extracted with 0.1M HCl + 8M urea analysed on a gel (6 cm) prepared by Method b.
- b = Proteins extracted with 3M LiCl + 4M urea using Modification 3 with 0.35M sucrose, and analysed on a gel (8 cm) prepared by Method a.
- c = Proteins extracted as b using Modification 2, analysed on a gel (5 cm) of Method c.
- d = Proteins extracted as b using Modification 1, analysed as c.
- e = Proteins extracted as b using Modification 1, analysed on gel (4 cm) of Method c but with reservoir solution of Method a.
- f = Proteins extracted as b using Modification 3 and analysed as e (gel 5 cm).
- g = Proteins extracted as a and analysed as e (gel 5 cm).
- h = Proteins extracted as b using Modification 2 and analysed on gel (4 cm) of Method d.

All gels, except b and h, were run using a current 4 m amp for 2 hours. With b this was 6 m amp for 45 minutes and h 4 m amp for 2.5 hours. Enlargement of photographs = 2.

(Method c) as judged by the good definition and sharpness of the different bands that was obtained (see gels c and d).

These observations led to the decision to routinely extract the proteins with 3 M-LiCl and 8 M-urea using Modification 2, and to analyse these proteins on gels without spacers in acidic buffer using the mixed reservoir solution of Method c above. The details of the methods used are described in section 5.2.8 above.

5.3 RESULTS AND DISCUSSION

5.3.1 A Fraction Separated by Molecular Exclusion with E_{260} Higher than E_{280} Found to Stimulate the Activity of Ribosomes

As has been mentioned, the purpose of this work was to determine the reasons for the differences in activity between R-, M- and S-ribosomes. A lead was taken from some odd results obtained during the fractionation of the partially purified transfer fractions, TF and TF-AS, on Sephadex columns. During this work it was sometimes found that a peak, with E_{260} higher than E_{280} , was eluted just after the main protein peaks. This peak is illustrated by the large dashed line under area C of Figure 4.1 of Chapter 4. With the initial idea that this fraction may have had something to do with the separation of a transfer factor it was tested for its effect on the activity of different ribosomes and the results of the experiment are shown in Table 5.1. When TF was a source of transfer enzymes, this fraction was able to stimulate the activity of R-ribosomes (17%) and especially of M-ribosomes which had been twice washed with 0.1 M-MgCl₂ (80%), while it had little or no effect on S-ribosomes (3%). The fraction was inactive by itself.

Shortly after this finding more care was taken to standardise the conditions used for fractionation of the partially purified transfer enzymes. When this was done the peak with high E_{260} (peak C of Figure 4.1) disappeared altogether and it was concluded that it was not a transfer enzyme. Once it was known that the differences in activity of the various ribosomes were not related to their content of transfer enzymes (see chapter 4) it was decided to look for the factor in the fraction from peak C of the original chromatogram which was responsible for the stimulation in activity of some ribosomes and not others. At first it was not possible to obtain this fraction except by chance. The reason for its spasmodic appearance was not then known. It was however then found

Table 5.1 Stimulatory Effect of a Fraction from Sephadex columns with a high E_{260} on the Activity of Different Ribosomes

Polyphenylalanine synthesis was assayed using TF as the source of transfer enzymes and ^{14}C -phe-tRNA (6,310 cpm added) prepared from rat liver. The fraction with high E_{260} was dialysed against 500 volumes of Medium X for 6 hours before 0.2 ml aliquots were used in the assay. Aliquots of 0.2 ml of Medium X were added to the controls. Incubation was for 15 minutes.

	Polyphenylalanine synthesis (cpm/0.2 mg ribosome)		% increase
	TF alone	TF + fraction with high E_{260}	
S-ribosomes	2,115	2,180	3
R-ribosomes	1,465	1,709	17
M-ribosomes washed twice with 0.1 M-MgCl ₂	515	925	80

that the fraction could be obtained by using a more alkaline buffer to prepare the liver homogenate from which the cell fractions were obtained. Because of the higher E_{260} , which suggested an increased amount of nucleic acid in the fraction, it was thought that fraction C might contain more RNA, and that this RNA might be responsible for the stimulation in the activity of ribosomes. For this reason an attempt was made to extract the RNA in this fraction by a method similar to that used for the extraction of tRNA as described in section 3.2.2, with a view to characterizing it on acrylamide gels.

No RNA however, could be extracted from fraction C, or for that matter from either of the partially purified transfer fractions, TF or TF-AS. When an excess of the p15 supernatant, prepared from liver homogenised in a buffer similar to Medium A except that the pH was adjusted to 9.6, was passed over a similar Sephadex column to that used in Figure 4.1 it was possible to extract a small amount of RNA from the area coinciding with area C of this figure. When this RNA was analysed on acrylamide gels several bands of RNA were obtained (a of Figure 5.3) and one of these coincided with the

band of ribosomal-RNA (b of Figure 5.3). Although the identity of the fraction with 5S RNA was not definitely established, it seemed possible that 5S RNA might be present in the particle-free supernatant of rat liver which was homogenised in an alkaline buffer.

5.3.2 Amount of 5S RNA Associated with Ribosomes

In view of the above results it was decided to investigate whether there was any relationship between the content of 5S RNA in ribosomes and their activity. The amount of 5S RNA in R-, M- and S-ribosomes was measured and compared with the activity of these ribosomes in both the synthesis of polyphenylalanine and the binding of phe-tRNA. These results are shown in Table 5.2. It was found that the three ribosomes differed considerably in their content of 5S RNA; R- and M-ribosomes normally contained less 5S RNA than did S-ribosomes, the relative amounts being nearly the same as the ratio of their activities. This relationship was therefore investigated further using methods which were fortuitously found to reduce the activity of ribosomes. This was done by the washing of ribosomes with solutions of magnesium chloride or with alkaline buffers.

5.3.2.1 The Effect of Washing Ribosomes with Solutions of Magnesium

Chloride. - It was found that the use of 0.1 M instead of 0.01 M-MgCl₂ during the washing procedure in the preparation of M-ribosomes, resulted in a reduction of their activity in both the synthesis and binding reactions (Table 5.3). It also resulted in a decrease in the amount of 5S RNA associated with the ribosomes. The effect of washing R- and S-ribosomes with solutions of MgCl₂ was then tested. These results are also shown in Table 5.3. Again it was found that this treatment resulted in a decrease in the activity of ribosomes in both reactions and in their content of 5S RNA. The effect however differed somewhat with these two preparations. With R-ribosomes the correlation between the loss of activity and the loss of 5S RNA was fairly good while that with S-ribosomes was bad. With the latter, the decrease in activity in both reactions was far in excess of the amount of 5S RNA removed. The reasons for this are not known and are discussed later.

By contrast, it was found that when using the same preparations of R-ribosomes as in Table 5.3, this washing with high concentrations of MgCl₂ had no effect on the capacity of the ribosomes to bind ³H-poly U (Figure 5.4). Thus, the decrease in the binding of phe-tRNA and polyphenylalanine synthesis did not

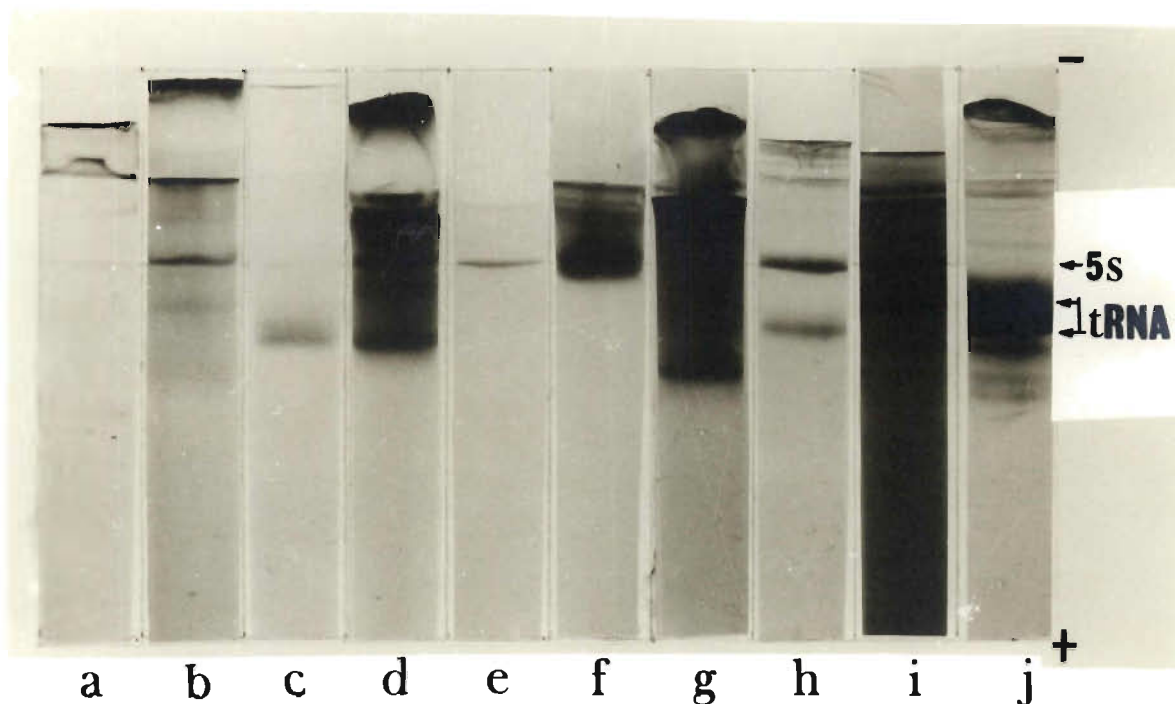


FIGURE 5.3 THE 5S RNA IN DIFFERENT FRACTIONS

Samples containing RNA were assayed for 5S RNA by acrylamide (disc) gel electrophoresis as under 5.2.7.2.

- a = RNA from pH 5 supernatant, extracted with phenol as under 3.2.2.
- b = RNA from S-ribosomes, extracted as under 5.2.7.1.
- c = Extract from S-ribosomes treated with tris-acetate buffer at pH 10.5 as under Table 5.7.
- d = RNA from R-ribosomes, extracted as under 5.2.7.1 but suspended in the tris-acetate buffer, pH 10.5 used in Table 5.7.
- e = Purified 5S RNA prepared by the sodium lauryl sulphate/phenol method under 5.2.4.1.
- f = Fraction released from 2 mg S-ribosomes with EDTA as under 5.2.4.2.
- g = Fraction released from 2 mg S-ribosomes with EDTA in the absence of bentonite and left at room temperature for 1 hour before continuing as under 5.2.4.2.
- h = RNA from R-ribosomes, extracted as under 5.2.7.1.
- i = ^{14}C -phe-tRNA from *E. coli* purchased from NEN.
- j = ^{14}C -phe-tRNA prepared from rat liver as under 3.2.2.

Gels h and j were run at 6 m amp for 25 minutes. In e, f and h the spacer gels are missing. These photos have been placed so that the 5S RNA bands coincide. Enlargements of photographs = 2.

TABLE 5.2 - Binding of ^{14}C -phe-tRNA and synthesis of ^{14}C -polyphenylalanine by different preparations of ribosomes

Ribosomes were prepared as described in section 4.2.1. The length of incubation for the synthesis reactions was 30 minutes using ^{14}C -phe-tRNA from rat liver. The concentrations of Mg^{++} used in the synthesis reactions with the three ribosome types were 13 mM for S-ribosomes, and 8.5 mM for both R- and M-ribosomes. The size of the 5S RNA peak was determined by cutting out the densitometer tracing of the peak under question and expressing the weight in mg.

Ribosome type	Size of 5S RNA peak (Weight of tracing-mg)	Activity		
		Binding of ^{14}C -phe-tRNA (cpm/0.4 mg ribosome)	Incorporation of ^{14}C -phenylalanine (cpm/0.2 mg ribosomes)	
			Source of transfer factor	
			TF	pH 5 supernatant
S-	10.3	745	3,320	3,600
R-	6.2	310	1,905	2,610
M-	6.1	278	1,868	2,540

5.3.2.2 The Effect of Washing Ribosomes with Alkaline Buffers. - It was at this stage of the work that it was observed that the pH of Medium A became more alkaline on standing, and often increased from pH 7.6 to pH 8.6 or more on standing for more than 20 days. Therefore, experiments were carried out in which the partially purified transfer fraction, TF, was made from homogenates prepared in more alkaline buffers and passed over Sephadex columns. In these cases the band with high E_{260} was obtained (C of Figure 4.1). This explained why this peak was not always obtained. The reason why the RNA in fraction C was not degraded by RNases was probably because of the protection afforded by the RNase inhibitor known to be present in the cell sap.

(Compare the action of RNase in the fractions washed from ribosomes by alkaline treatment, see section 5.3.4.) For this reason, and because a more alkaline buffer was used during the washing of R-ribosomes, which contained less 5S RNA than S-ribosomes, it was thought likely that treatment of ribosomes with alkaline buffers might remove 5S RNA from them. R- and S-ribosomes were therefore treated with buffers of different pH values and a comparison made of their activity and content of 5S RNA. These results are shown in Table 5.4. Dialysis of both R- and S-ribosomes against a buffer at pH 10.8, a fairly prolonged treatment, drastically reduced both the amount of 5S RNA associated with them and their activity. The results of experiments in which the ribosomes were treated in the same way except that a neutral buffer was used are included for comparison to show that the effect measured was caused by the alkaline solution and not just by the length of dialysis. (The role of RNases here is discussed in the next section). When S-ribosomes were exposed for shorter periods to pH values ranging from 8.0 to 10.0 when using weaker buffers, a graded reduction in the amount of 5S RNA retained on the ribosomes was obtained which nearly paralleled the decrease in their activity in the binding reaction. A lesser effect, above pH 8.6, was found to occur with the synthesis reaction.

All the above results, showing a correlation between the amount of 5S RNA on ribosomes and their activity gave the impression that 5S RNA may in some way be associated with this activity. If this were true, the experiments in which a decrease in both the activity and the content of 5S RNA of ribosomes was shown, but in which the correlation was bad, would also suggest that other factors connected with the activity of ribosomes were removed by the methods used to wash them. On the other hand, these experiments might be an indication that the noted correlation was only circumstantial and unrelated. Further experiments were carried out to test this and are described in the following section

5.3.3 Reversibility of the effect of loss of activity caused by the different washing procedures

It was the purpose here to determine whether the decrease in the activity of ribosomes caused by their washing with solutions of MgCl_2 or alkaline buffers could be reversed. The results in Tables 5.5 and 5.6 show that the decrease in the activity of ribosomes caused by washing them with solutions of MgCl_2 or alkaline buffers could be partially reversed by removing these solutions by

TABLE 5.3 - Effect of washing with $MgCl_2$ on Composition and Activity of Ribosomes

Ribosomes were treated with $MgCl_2$ as indicated. The final conc. of Mg^{++} during assay of the synthesis reaction was 8.5 mM in all cases: incubation was for 20 minutes (S- and R-ribosomes) or 30 minutes (M-ribosomes) in the presence of ^{14}C -phe-tRNA and pH 5 supernatant, both prepared from rat liver. Results for the different ribosomes cannot be compared because they were done on different occasions. The size of the 5S RNA peak was determined as in Table 5.2.

Ribosome type	No. of washes with $MgCl_2$		Size of 5S RNA peak (Weight of Tracing-mg)	Binding of ^{14}C -phe-tRNA (cpm/0.4 mg ribosome)	Incorporation of ^{14}C -phe (cpm/0.2 mg ribosome)
	0.01M	0.1M			
M-	3	0	6.1	278	2110
	1	2	2.6	49	600
R-	0	0	8.8	506	1555
	1	0	6.3	444	1280
	2	0	3.3	278	764
	0	2	1.9	203	225
S-	0	0	8.4	680	1429
	2	0	6.2	160	340
	0	1	6.8	168	366
	0	2	2.6	132	198

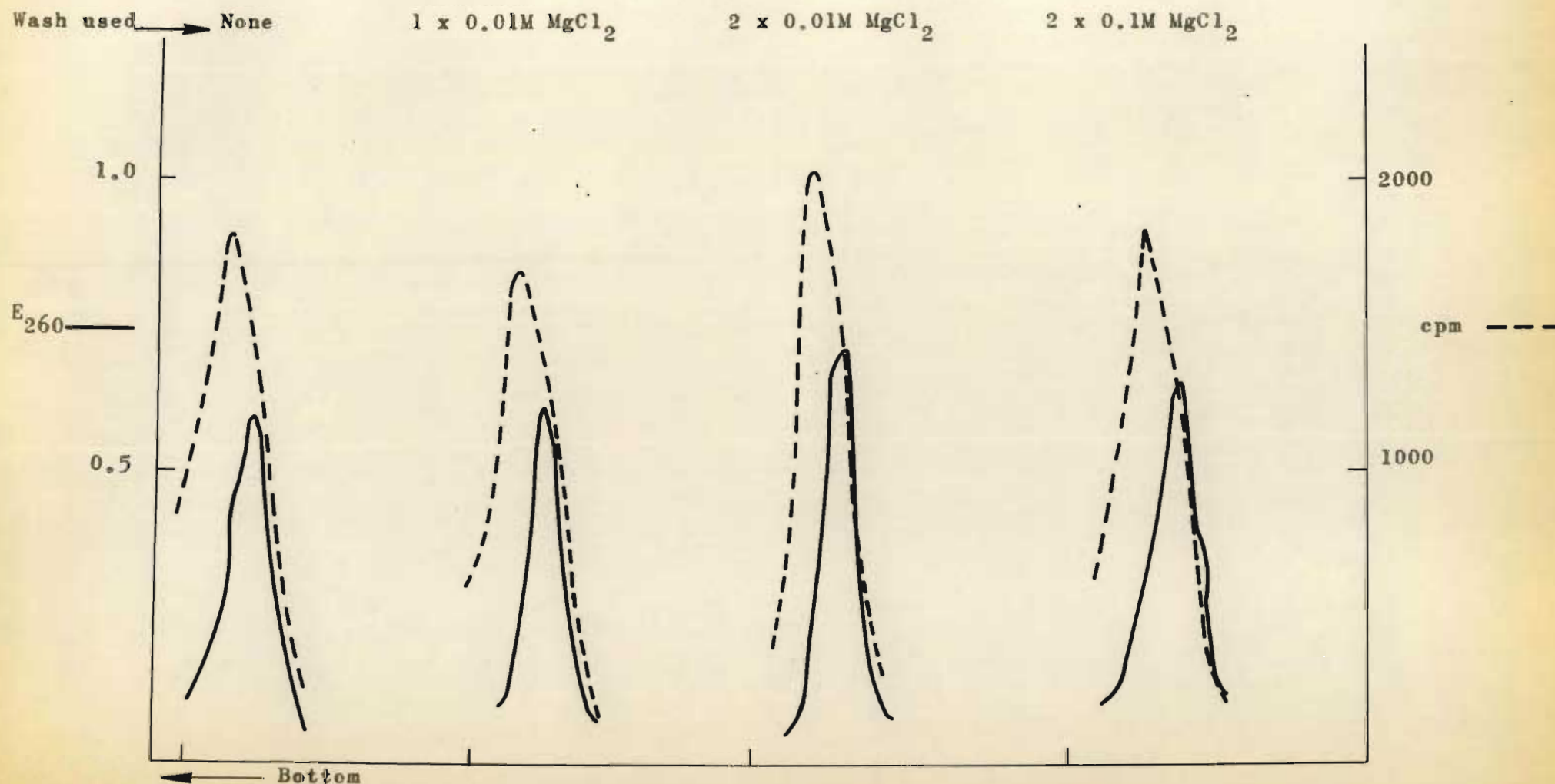


Figure 5.4 Binding of ^3H -poly U by R-ribosomes differentially washed in MgCl_2

This was done as described in section 5.2.6. The R-ribosomes used were the same as those of Table 5.3.

TABLE 5.4 - Effect of Treatment with Alkaline Buffers on
Composition and Activity of Ribosomes

In (a) ribosomes were dialysed for 3 hours against 200 vols. 10 mM-tris-acetate buffer, adjusted to the pH stated, which contained 100 mM-potassium acetate and 1 M-magnesium acetate. Ribosomes were then centrifuged down and resuspended in the buffers in which they were originally suspended.

In (b) ribosomes were prepared as usual, except that the solution containing 0.25 M-sucrose, 0.0175 M-KHCO₃ and 2 mM-MgCl₂ used for washing after treatment with deoxycholate was adjusted to the pH stated.

¹⁴C-phe-tRNA from rat liver was used in (a) and from E. coli in (b). Incubation in the synthesis reaction was for 15 minutes in the presence of pH 5 supernatant and a final concentration of Mg⁺⁺ of 10 mM. The amounts of 5S RNA were determined as in table 5.2.

System	Amounts of 5S RNA in ribosomes (weight of tracings-mg)	Activity (cpm/0.2 mg ribosome)	
		Binding reaction	Synthesis reaction
(a) S-ribosomes untreated	8.4	389	1116
dialysed at pH 7.4	8.0	234	1172
dialysed at pH 10.8	0.3	40	134
R-ribosomes untreated	6.2	274	892
dialysed at pH 7.4	5.8	215	441
dialysed at pH 10.8	0.2	66	60
(b) S-ribosomes normal, pH 8.0	6.6	1788	4855
washed at pH 8.6	6.2	1423	5094
washed at pH 9.2	4.9	1152	4336
washed at pH 10.0	4.3	651	4128

TABLE 5.5 - Reversibility of effect of high concentration of magnesium on the activity of ribosomes in the synthesis of polyphenylalanine

R-ribosomes in solution R were made 0.25M with respect to MgCl_2 and left to stand for 15 min. In (ii) ribosomes were then pelleted, suspended in an excess of solution R (1 mM- MgCl_2) for 14 hours, repelleted and re-suspended in solution R. In (iii) ribosomes were dialysed for 14 hours against 500 volumes solution R before pelleting and resuspending in solution R. Activity of ribosomes was measured after 15 minutes using ^{14}C -phe-tRNA from rat liver, pH 5 supernatant and 8.5 mM- Mg^{++} .

Treatment of ribosomes	Polyphenylalanine synthesis (cpm/0.2 mg ribosomes)
(i) Untreated ribosomes	956
(ii) Ribosomes pelleted at high Mg^{++} conc.	126
(iii) Ribosomes dialysed against low Mg^{++} conc. before pelleting	246

TABLE 5.6 - Reversibility of effect of alkaline pH on the activity of ribosomes in the synthesis of polyphenylalanine

R-ribosomes in solution R were dialysed for 3 hours against 500 volumes of the same buffer adjusted to pH 10.8 with KOH.

In (ii) ribosomes were then pelleted and resuspended in solution R. In (iii) ribosomes were first redialysed against 500 volumes of solution R before being pelleted and resuspended in solution R. Peptide synthesis was measured after 20 minutes incubation with ^{14}C -phe-tRNA from rat liver, pH 5 supernatant and 8.5 mM- Mg^{++} .

Treatment of ribosomes	^{14}C Incorporation of phe into peptide (cpm/0.2 mg ribosome)
(i) Untreated ribosomes	1020
(ii) Ribosomes pelleted at pH 10.8	46
(iii) Ribosomes redialysed against pH 7.6 buffer before pelleting	250

by dialysis prior to pelleting of the ribosomes. The effect was most striking in the case of the alkaline treatment, but in both cases the activity was restored to only about 25% of that of the original ribosomes. Probably these effects were not completely reversible because of damage to the ribosomes, as may happen if the ribonucleases, known to be associated with ribosomes, were to hydrolyse the ribosomal RNA during the unfolding of the ribosomal structure, which probably occurred.

5.3.4 Effect of Addition of 5S RNA and Other Fractions to depleted Ribosomes

When it was found that there was a correlation between the activity of some ribosomes and their content of 5S RNA, and that the loss of activity caused by some treatments could be reversed, the effect of addition of purified 5S RNA and of other fractions to ribosomes, known to be depleted of 5S RNA, was tested.

In the first experiments the effect of adding back the fractions obtained from ribosomes on treatment with MgCl_2 and alkaline buffers was measured. Rather conflicting results were obtained in this case, as in most instances it was found that the addition of these washings to depleted ribosomes either had no effect or actually caused a decrease in the activity of the ribosomes (see experiment c of Table 5.8). Only in a few instances did the added fractions have a stimulatory effect, and this was only when they were isolated rapidly and used without delay. Even under these conditions active fractions were obtained only on some occasions, and particularly seldom when the alkaline treatment was used. The results of an experiment where stimulation was found is given in Table 5.7. Here the fractions were prepared from S-ribosomes by treatment with 0.5 M- MgCl_2 and 0.4 M-tris-HCl, pH 10.5 (final concentrations) respectively. When these active fractions were analysed on acrylamide gels, faint bands were obtained which coincided with that of the 5S RNA band of ribosomal RNA (see c of Figure 5.3). Unluckily the gel in which the fraction obtained from treatment of S-ribosomes with 0.5 M- MgCl_2 was measured had dried up before it could be photographed, but it was similar to that of c in this figure. The fractions which had no stimulatory effect also showed no bands on electrophoresis. It is possible that the 5S RNA released from the ribosomes was destroyed by RNases which became active during the unfolding of the ribosome, especially that at high pH values. In the case where the fraction containing 5S RNA was prepared from the cell sap (see section 5.3.1)

it was protected by the presence of RNase inhibitors known to be present. These results are by no means conclusive but present further circumstantial evidence for a connection between the presence of 5S RNA on the ribosome and its activity in protein synthesis.

To try and get more proof of this, purified 5S RNA was added alone or with one of these fractions. The 5S RNA used here was considered to be pure because it moved as a single band when analysed by gel electrophoresis (e of Figure 5.3). Addition of 5S RNA to ribosomes depleted of this RNA did not give clear-cut stimulation in either of the binding or synthesis reactions. Instead, results typified by those in Table 5.8 were obtained. In these experiments binding was measured at both 0°C and 37°C . This was done because Nomura, Lowry & Guthrie (1967) claimed that much of the binding measured at 37°C in 20 mM-MgCl₂ was non-specific. This was shown in the experiments carried out here since it was calculated that as many as 4-6 of rat liver, or 8-12 of *E. coli*, ¹⁴C-phe-tRNA's were bound per ribosome at 20 mM-MgCl₂. It was thought that measurement of binding at 0°C would enable a correction to be applied. An alternative approach would have been to measure binding at 5 mM-MgCl₂. This was tried, but in all cases the binding was found to be lower when a source of transfer enzymes was added than without it; hence this approach was abandoned. As shown in Table 5.8 (a) and (c), addition of purified 5S RNA decreased the binding measured at 0°C while the binding at 37°C was unchanged, so that the net binding was more than 50% higher with 5S RNA. On the assumption that at 0°C 5S RNA and phe-tRNA competed for tRNA-binding sites and therefore binding of ¹⁴C-phe-tRNA was decreased in the presence of added 5S RNA, there could be two explanations for these results:

- (i) the increase measured at 37°C was due to the binding of 5S RNA to its available binding sites, this temperature being necessary for binding. As a result more sites were available for the binding of ¹⁴C-phe-tRNA than were present in the absence of bound 5S RNA,
- (ii) the increase at 37°C was due to the replacement of the 5S RNA on the tRNA binding sites by ¹⁴C-phe-tRNA because the latter had a higher affinity for this site at 37°C .

Table 5.7 - Effect of fractions washed from ribosomes able to partially stimulate the activity of ribosomes

Two fractions were prepared by making S-ribosomes respectively 0.5 M with respect to MgCl_2 or 0.4 M with respect to tris-HCl, pH 10.5, and immediately spinning these at 100,000g for 30 minutes and dialysing the resultant supernatants against 500 volumes of Medium X for 60 minutes to lower the concentrations of MgCl_2 or tris-HCl and also the pH of the latter. These fractions showed faint bands which coincided with those of 5S RNA when analysed by disc gel electrophoresis (see c of Figure 5.3). In the assays R-ribosomes washed twice with 10 mM- MgCl_2 were used and synthesis was measured after 15 minutes incubation at 37°C using ^{14}C -phe-tRNA from rat liver, pH5 supernatant and 8.5 mM- Mg^{++} .

Additions to assay system	Polyphenylalanine synthesis (cpm/0.2 mg ribosomes)
No additions	842
+ Mg-wash	1,035
+ pH 10-wash	931

To choose between these two explanations another experiment measuring the effect of 5S RNA was carried out and the results are shown in (b) of Table 5.8. Here ribosomes were pre-incubated at 37°C with 5S RNA and then re-isolated. When these ribosomes were assayed for activity a 25% increase in the total activity of the binding reaction at 37°C was obtained and nearly all this increase could be accounted for by the increase which took place at 0°C (an increase of 62%). This result was in agreement with the proposal given under (i) above, and shows that the incubation at 37°C was necessary for the binding of 5S RNA to its site on the ribosome and not for the binding of phe-tRNA, which was dependent on this attachment of 5S RNA. This pre-incubation of 5S RNA with ribosomes had little or no effect on the synthesis reaction. The inclusion of the pH5 supernatant, GTP or GSH in any of these reactions had no stimulatory effect. This indicated that other factors, responsible for the synthesis of peptides by ribosomes, were removed from ribosomes during their washing.

TABLE 5.8 - Effect of Purified 5S RNA on the Activity of
R-ribosomes

5S RNA was prepared by the sodium lauryl sulphate/phenol method as described in section 5.2.4. The amount added was equivalent to that expected to be associated with 1-2 equivalents of ribosomes. Mg-washings were prepared by making S-ribosomes 0.5 M with respect to MgCl_2 , allowing this to stand at 0°C for 20 minutes before centrifuging at 100,000g for 60 minutes, and dialysing the resultant supernatant against 500 volumes of Medium X for 90 minutes to lower its concentration of magnesium. This fraction was shown to contain no 5S RNA by gel electrophoresis. In (a) and (c) the 5S RNA was added at the beginning of the assay. In (b) and (d) ribosomes were first pre-incubated with 5S RNA in solution R for 15 minutes at 37°C , after which they were cooled in ice, the mixture diluted with 24 volumes of ice-cold solution R, pelleted and resuspended in solution R. Assays were carried out in the presence of the pH5 supernatant and Poly U. In (a) and (b), using one batch of ribosomes, ^{14}C -phe-tRNA from rat liver was used, while in (c) and (d), using different batches of ribosomes, that from *E. coli* was used. The final concentration of MgCl_2 in the synthesis reaction was 8mM. The synthesis is reaction at 0°C was negligible.

	Activity (cpm/0.2 mg ribosomes)			
	Binding reaction			Synthesis re- action 15'/37 $^\circ$
	0 $^\circ$	37 $^\circ$	Difference	
(a) <u>Not preincubated</u>				
R-ribosomes	90	170	80	1,002
R-ribosomes + 5S RNA	44	176	132	820
(b) <u>Preincubated</u>				
R-ribosomes	84	232	148	846
R-ribosomes + 5S RNA	136	290	154	822
(c) <u>Not preincubated</u>				
R-ribosomes alone	1,228	2,361	1,133	5,583
R-ribosomes + 5S RNA	743	2,302	1,559	5,324
R-ribosomes + Mg- washings	1,286	2,381	1,095	3,556
R-ribosomes + Mg- washings + 5S RNA	730	2,065	1,335	5,778
(d) <u>Preincubated</u>				
R-ribosomes + 5S RNA	1,602	3,122	1,520	4,933
R-ribosomes + 5S RNA				

In a discussion with Reynier during a visit to Marseille, France, in 1967, the author learned that a protein was removed with 5S RNA when the ribosomes of *E. coli* were treated with 2 M-LiCl (see also Aubert, Monier, Reynier & Scott, 1968). The presence of this protein was necessary for the reattachment of 5S RNA, removed by this method, to ribosomes. To determine whether this was the reason for the inability of the author to reactivate ribosomes with 5S RNA, the effect of adding 5S RNA together with the fraction removed from ribosomes by washing with MgCl_2 was tried. These results are shown in (c) of Table 5.8. The MgCl_2 -washings used in this experiment did not cause any stimulation of protein synthesis on its own, but instead inhibited both binding and synthesis by about one third (compare with result in Table 5.7). MgCl_2 -washings prepared in this manner always caused an inhibition. Addition of 5S RNA together with these washings increased the activity, but only to the level of that found without any additions. These MgCl_2 -washings have been found to contain small fragments of RNA. If these fragments include those from 5S RNA, as would be expected, then they and the 5S RNA which was added would most likely compete for the binding sites of 5S RNA on ribosomes. Since the binding of fragments of 5S RNA would most probably render those ribosomes inactive in peptide synthesis, it would only leave a fraction of the original ribosomes free to participate in polypeptide synthesis. If this was true, then the results obtained showed that 5S RNA can stimulate these reactions in the presence of a ribosomal fraction. The inhibition which was obtained, was probably caused by the addition of ribosomal protein to ribosomes already containing that complement of protein (see Nomura & Traub, 1968). However, until it can be shown that this was in fact what happened these results must be considered as inconclusive.

Since Forget & Weissmann (1968) and Sarkar & Comb (1969) had suggested that 5S RNA may exist as two or three complementary forms, there was a possibility that the active form might differ from that used above, and therefore other methods of preparing 5S RNA were necessary. As it had been shown that 5S RNA could be isolated from ribosomes using EDTA (Parish, Kirby & Klucis, 1966; Comb & Sarkar, 1967) this RNA was prepared by using a modification of the procedure used by Parish, Kirby & Klucis (1966), and its effect on the reaction under study tested. When the RNA in the fraction, obtained from S-ribosomes treated with EDTA, was analysed on acrylamide gels it was found to move as a broad band or two closely associated bands of 5S RNA

with traces of slower moving species of RNA (see Figure 5.3f). The fraction was capable of stimulating both binding of phe-tRNA and polyphenylalanine synthesis by R-ribosomes and R-ribosomes washed thrice with MgCl_2 by 23-37% (Table 5.9a).

As the fraction prepared using EDTA contained protein (see next section, 5.3.5, Figure 5.5b) as well as 5S RNA, the question arises as to which of these components is responsible for the observed stimulation. The presence of protein in a similar preparation has been shown by Keller, Cohen & Hollinshead-Beeley (1967). The best indication that it was the RNA in this fraction which was responsible for the stimulation came from the results obtained when it was prepared in the absence of bentonite and the mixture allowed to stand at room temperature for 1 hour before dialysing free of EDTA. The RNA in this preparation was degraded and moved as a smudge over the whole of the gel from the origin to the buffer front (Figure 5.3g). This fraction had no stimulatory action (Table 5.9b). In fact it inhibited peptide synthesis by 26%. In this respect it resembled the MgCl_2 -washings in which 5S RNA degradation had occurred (Table 5.8c). The possibility that the proteins in the extract were also degraded could not be excluded. The results presented in the next section however, show that a ribosomal protein connected with the loss of activity in R-ribosomes and S-ribosomes was not degraded in fractions free of 5S RNA when prepared from ribosomes treated both with EDTA or an alkaline buffer.

5.3.5 Ribosomal Proteins Removed during the Washing of Ribosomes

In view of the possible significance of the stripping off of protein during the washing of ribosomes (see previous section) this aspect was investigated further. The results of a preliminary analysis of the proteins remaining on the variously treated ribosomes is shown in Figure 5.5. There were slight differences in the intensities of the protein bands from ribosomes washed with the same solution on different occasions. This was presumably due to differences in the time the ribosomes were left in contact with the wash solution, which was not rigorously standardized since its importance was not realized initially. For this reason the results are only semi-quantitative, and only a visual comparison of the gels has been carried out here.

It is seen that treatment of the ribosome with alkaline buffer at pH 10.3 removed a large number of proteins almost completely (gels c.f and i). The

TABLE .9 - Effect of fractions washed from Ribosomes with EDTA on the activity of depleted Ribosomes

S- and R-ribosomes were prepared as described in section 4.2.1 and Mg-washed R-ribosomes were obtained from the latter by washing thrice with 10 mM-MgCl₂ in (a) and once with 100 mM- and twice with 10 mM-MgCl₂ in (b). In (a) the EDTA fraction was prepared from S-ribosomes as under .2.4.2, and was shown to contain 5S RNA (see Figure 5.3f), while in (b) sodium bentonite was omitted and the S-ribosome/EDTA mixture left at room temperature for 1 hour before continuing as under 5.2.4.2, and was shown to contain no 5S RNA. No 5S RNA remained on the ribosomes after this treatment with EDTA. Peptide synthesis was assayed by Method 1 of section 3.2.6 in the presence of the pH5 supernatant, ¹⁴C-phe-tRNA from rat liver and 8.5 mM-MgCl₂. Incubation was for 15 minutes. The EDTA fraction was inactive in the absence of ribosomes.

Assay fractions	Activity (cpm/0.2 mg ribosome)	
	Binding	Synthesis
(a) <u>Included bentonite, spun immediately</u>		
R-ribosomes	301	2,531
R-ribosomes + EDTA fraction	407	3,480
% increase	(33)	(37)
Mg-washed R-ribosomes	184	1,833
Mg-washed R-ribosomes + EDTA fraction	225	2,403
% increase	(23)	(31)
(b) <u>No bentonite, spun after 1 hour</u>		
Mg-washed R-ribosomes	128	302
Mg-washed R-ribosomes + EDTA fraction	131	224
% increase	(2)	(-26)

supernatant from the alkali treatment showed faint bands of the proteins which had been removed; these could be seen by direct examination of the gels but are not readily visible in the photographs (gels c_1 and f_1). The small amount of protein detectable in the supernatant may have been due to failure to precipitate them completely with TCA or to redissolve them in 0.1 M-HCl plus 8 M-urea afterwards. Alternatively, it may indicate a rapid degradation of the proteins in the supernatant similar to that found with 5S RNA. In view of the extensive removal of proteins by this treatment, which must have had a considerable effect on the structure of the ribosome, it is not possible to pin-point any specific cause for the loss of activity in polypeptide synthesis.

By contrast, washing with $MgCl_2$ brought about only small changes in the proteins of the ribosome. The only differences between untreated S- and R-ribosomes (gel d and g respectively) was that in the latter, which were less active in polypeptide synthesis (see Table 5.2), band 13 was often reduced and band 7 occasionally. The only proteins on both S- and R-ribosomes which were reduced by washing with solutions of $MgCl_2$ were those of bands 4 and 13 (see gels e and h). Thus, if the removal of a specific fraction is indeed related to the loss of activity of the ribosomes, the protein of band 13 appears to be most clearly implicated. This conclusion is supported by the results obtained with EDTA treatment. The concentration used here removed several proteins from polysomes and not just one as found by Keller, Cohen & Hollinshead-Beeley (1967) (gel b). Amongst those removed was band 13 but not band 4. Gel b_1 shows the proteins in the EDTA-extract. This particular extract was prepared by dialysing polysomes against 10 mM-EDTA for 20 hours in the absence of bentonite. It would be expected that, under these conditions, the fraction would not have any stimulatory effect. Nevertheless it showed a fairly strong band 13. Therefore it would seem that it was not degradation of the protein in the EDTA-fraction which was responsible for its loss in activity but that this was due to the destruction of the 5S RNA. Further evidence for this was presented when it was found that band 13 was present in the gels of the extracts from ribosomes treated with tris-acetate buffer, pH 10.3 (see c_1 and particularly f_1). Yet it was found that this extract prepared from S-ribosomes had no stimulatory effect on the activity of ribosomes. Also this extract contained no 5S RNA. It may be that the protein in band 13 is the one responsible for the loss of activity.

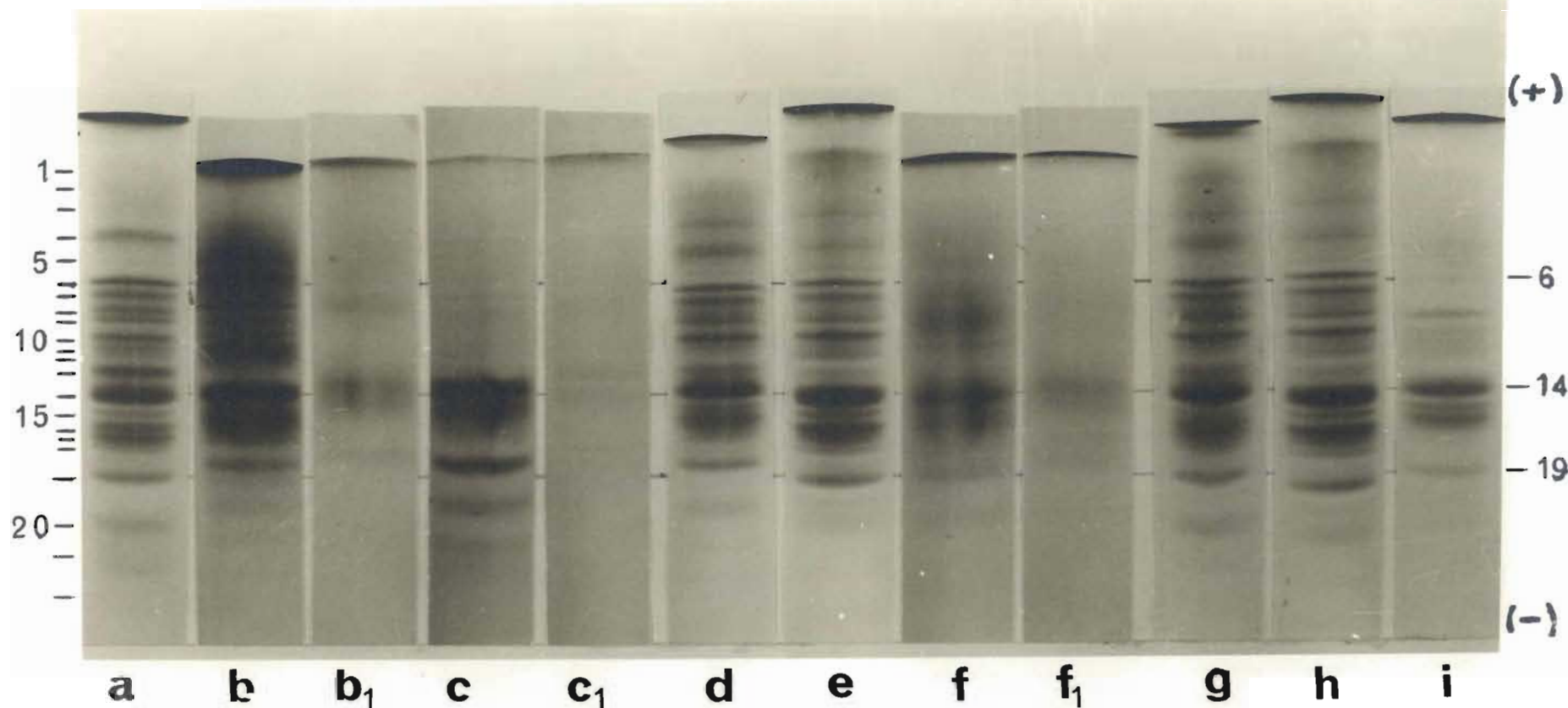


FIGURE 5.5 ANALYSIS OF RIBOSOMAL PROTEINS ON ACRYLAMIDE GELS

Extraction of proteins from ribosomes and their analysis on acrylamide gels was done as described under 5.2.8. The different letters represent: a = polysomes; b = polysomes dialysed against 100 volumes of 10 mM EDTA in 50 mM tris-HCl buffer, pH 7.6, for 20 hours; b₁ = the supernatant from b; c = polysomes dialysed against 100 volumes of a solution containing 50 mM tris-acetate, pH 10.3 + 50 mM potassium acetate, for 3 hours; c₁ = supernatant from c; d = S-ribosomes; e = S-ribosomes washed once with 10 mM and twice with 100 mM MgCl₂ as described under 5.2.1; f = S-ribosomes treated as c; f₁ = supernatant from f; g = R-ribosomes; h = R-ribosomes washed twice with 10 mM MgCl₂ as under 5.2.1; i = R-ribosomes treated as c except that dialysis was only for 1.5 hours. After treatment with EDTA or alkaline buffer the ribosomes were re-isolated by centrifugation at 100,000g for 3 hours and the resultant supernatants precipitated with trichloroacetic acid and the proteins extracted as under 5.2.8.1. Enlargements of photographs = 2.

of 5S RNA to the ribosome, since this is the only protein for which it could be shown that its loss from the ribosome paralleled the loss of 5S RNA and the decrease in activity. Much more work on this however, is needed before any final conclusions can be made.

5.3.6 Interpretations and Discussion of Results of the Removal of 5S RNA from Ribosomes

In much of the work reported in the literature to date it has tacitly been assumed that intact ribosomes prepared by various methods have the same complement of structural components and the same ability to carry out the various steps of protein synthesis. The present work has shown that this is not the case. Variable amounts of 5S RNA and ribosomal proteins are removed during the preparation of ribosomes by mild methods and this has an influence on their activity in protein synthesis.

It was not previously thought that 5S RNA was a labile component of the ribosome, although it was known that it could be removed by treatment with EDTA. Since the experimental work described here was completed, Sarkar & Comb (1969) have reported that ribosomal 5S RNA can exchange with external 5S RNA in vitro, but only at very low concentrations of magnesium (0.1 mM). No exchange was found in 10 mM-magnesium, whereas the present work has shown that repeated washings of the ribosomes with this concentration of $MgCl_2$ can decrease its content of 5S RNA. This decrease was even more pronounced when 100 mM- $MgCl_2$ was used. Sarkar and Comb assayed for exchange of 5S RNA by measuring the amount which came off labelled ribosomes. This was passed through millipore filters and the filters washed with the same concentration of $MgCl_2$ as used in the exchange reaction, namely 0.1 mM or 10 mM. In the author's opinion, the comparisons made when using these two concentrations of $MgCl_2$ are not valid, since it was his experience that when ribosomes and labelled tRNA were mixed with 1 mM or 15 mM- $MgCl_2$, at least 10 times more of the label remained on the filter when the higher concentration of $MgCl_2$ was used. It would seem therefore that much of the labelled 5S RNA was trapped on the filter when exchange at 10 mM- $MgCl_2$ was measured by Sarkar & Comb. The adsorption of any labelled 5S RNA released by 10 mM- $MgCl_2$ in the absence of externally added 5S RNA would be proportionally greater and would be virtually 100%. In the work presented here 5S RNA was also released from ribosomes treated

with tris buffers at various alkaline pH values. Generally the amount of release depended on the alkalinity of the buffer and the duration of contact with the buffer, more being released at the more alkaline pH values and the longer contact times.

In a number of the experiments presented here, but not all, the loss in activity of the ribosomes paralleled the removal of 5S RNA. Addition of isolated 5S RNA failed to restore the activity of such ribosomes, especially in the synthesis reaction, although it did restore that of the binding reaction to some extent. This however, does not mean that 5S RNA is not necessary for the activity of the ribosomes, since,

- (i) the form of 5S RNA added may have been unsuitable. When Sarkar & Comb (1969) found that ribosomes in which exchange of 5S RNA had taken place were inactive they suggested that this may have been due to an inactive form of this RNA being placed on the ribosome, since they had shown that at least three different configurations of this RNA existed. This seems somewhat unlikely, since Nomura & Traub (1968) showed that 16S rRNA isolated with phenol, and solubilized ribosomal proteins from 30S subunits, when mixed, were able spontaneously to adopt the right configuration to produce active 30S subunits. This is in line with current work, which has demonstrated that the primary structure of proteins carries all the information required for the production of the active tertiary structure,
- (ii) a protein was found to be necessary for the reattachment of the 5S RNA to the ribosome, possibly by making it assume the right configuration (Aubert, Monier, Reynier & Scott, 1968). This may be the protein which Sarkar & Comb (1969) showed was necessary for 5S RNA exchange, or possibly the protein which was removed with 5S RNA by treatment with EDTA,
- (iii) a protein may be required in addition to 5S RNA and independently of it. This will be considered further below.

A strong pointer that 5S RNA does play a role, in addition to protein, is the fact that activity could be restored to depleted ribosomes only when the added extracts, obtained from ribosomes that were treated with MgCl_2 , alkaline buffers and EDTA, contained 5S RNA. In particular, the fact that

bentonite, which is a RNase inhibitor, stabilizes the EDTA-extract argues that the 5S RNA in this was necessary for activity. It is also worthwhile noting that Traub, Nomura and co-workers in their experiments on the reconstruction of ribosomal subunits (see below), used a mutant of E. coli which was RNase negative, and so avoided the problems encountered here with the destruction of 5S RNA once it was removed from the ribosome.

There is no doubt that the ribosomal proteins play an important role in protein synthesis. This was convincingly demonstrated by Traub, Nomura and co-workers in a recent series of papers. They fractionated the 30S ribosomal unit into its constituent proteins and 16S rRNA and showed that an active unit could be reassembled from these isolated molecules only if certain proteins together with the RNA were present, namely the core proteins and the basic split proteins (Traub & Nomura, 1968b, see also Traub & Nomura, 1968a; Traub, Söll & Nomura, 1968). The two acidic split proteins were not essential but did stimulate this activity. To date the 50S subunit has only been reconstructed from the core (rRNA plus core proteins) plus the acidic and basic split proteins. With these units the acidic split proteins were essential for activity and the basic ones only stimulatory when added to the core plus acidic proteins. It is not possible to make a precise comparison of the proteins removed from rat liver in the work done here and those of the split proteins of the above workers, since ribosomal proteins have been shown to differ from different genera of bacteria and can therefore be expected to differ considerably in nucleated and non-nucleated species. When the patterns of ribosomal protein in Figure 5.5 is compared with those shown by Traub & Nomura (1968a), it is seen that there are many similarities. In particular, band 13 of Figure 5.5 seems to correspond to one of the basic proteins of the 50S subunit which is only partially split off with CsCl. In their work Traub and co-workers make no mention of the fate of 5S RNA in their reconstruction of particles. The moot point here is, would the addition of basic split proteins, particularly the one corresponding to band 13, be essential if they were added with 5S RNA and acidic proteins added to cores from 50S particles lacking 5S RNA? From the evidence presented here this could be the case. It is possible that 5S RNA was removed during the preparation of cores from 50S particles, and this was proportional to the amount of protein, corresponding to band 13, which was removed. The loss of 5S RNA is probably one of the main reasons why, during the reconstruction of 50S particles from

their cores and split proteins, only about 50% recovery of activity was obtained. In the reconstruction of the 30S particles as much as 100% recovery has been obtained (see Traub & Nomura, 1968b).

The answer to the question of the role of 5S RNA in proteins synthesis will undoubtedly be obtained when the 50S subunit is reconstituted from its constituent RNA's and proteins, in the same way as the 30S particles. As far as the author is aware this has not yet been done. The results presented here however, are interpreted as showing that with the washing methods used a protein was removed with 5S RNA. Both of these were responsible for the decrease in the activity of the ribosomes. It is possible that 5S RNA is concerned with keeping the ribosome in its correct configuration so that the ribosome can participate in protein synthesis. When added alone to depleted ribosomes 5S RNA can attach itself to the ribosomes, and thereby increase the sites available for the binding of tRNA. The further addition of a ribosomal protein(s) is necessary for the ribosome to assume its correct configuration in order that it may function in peptide bond formation.

SUMMARY

The work reported in this thesis was concerned with the relation between those factors, particularly 5S RNA, removed from ribosomes during their washing with different solutions; and the activity of these ribosomes was studied. Rat liver was used for this investigation and outlined below is a summary of how this work was set out and the results thereof.

1. Polysomes, the active protein synthesizing units, were prepared by a standard method from rat liver and used as starting material for all these studies. Polysomes were used to ensure uniformity and because the individual ribosomes in this organelle would most likely contain all the factors necessary for protein synthesis.
2. The best means of breaking down polysomes to single ribosomes was then studied. Three methods were investigated and the nature of the ribosomes produced by each was judged from the profile obtained by centrifugation on sucrose gradients. In the first method polysomes were incubated in tris-HCl buffer, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl and 5 mM-MgCl₂. This breakdown was considered to be similar to that occurring in the presence of ribonucleases, and yielded single ribosomes with fragments of mRNA still attached. In the second method polysomes were incubated in an active protein synthesizing system to yield predominantly single ribosomes free of mRNA while in the third, incubation was carried out in a system which allowed translocation and chain release to occur. The minimum requirements for this were GTP, GSH and puromycin. The addition of a source of transfer enzymes was not necessary because there were sufficient of these on the polysome for one translocase reaction, which, under the conditions used, was all that was needed for the complete disruption of the polysome. This method also yielded single ribosomes free of mRNA.
3. The breakdown of polysomes in the presence of the factors necessary for peptide bond formation was shown to occur in exact accordance with the most modern concepts of protein synthesis. Complete breakdown of polysomes into single ribosomes under conditions of peptide

synthesis occurs when all the macromolecules (mRNA and acylated-tRNA's) which keep their structure intact are removed. This breakdown is effected by translocation of all the aminoacyl-tRNA's, from the decoding sites to the condensing site, as peptides where they can be released. Translocation is effected in the presence of the transfer enzymes, GTP and GSH, and release is effected by puromycin.

4. Ribosomes free of the transfer enzymes were prepared by three different procedures. For two of these, single ribosomes produced by Method 2 above were used. In the first, which was developed by the author, the ribosomes were washed with a solution containing 50 mM KCl, 1 mM MgCl_2 and 50 mM tris-HCl, pH 8.3 after which they were passed through a layer of 1 M sucrose in the same solution. In the second, which was based on a method published while this work was in progress, single ribosomes, prepared from polysomes treated with GSH and KCl, were subjected to KCl-shock, a method whereby they were subjected to successive washes with solutions containing high concentrations of KCl. In the third, another method adapted from one published while this work was in progress, polysomes themselves were treated with deoxycholate and washed with solutions of MgCl_2 . The washed ribosomes prepared by each method were characterized in respect of both their physical and chemical properties. The most striking difference observed was in respect of their capacity to synthesize polypeptides which could not be related to their content of transfer enzymes or other extrinsic factors.
5. The reasons for the differences in activity between the three preparations were then sought. The main findings of this phase of the investigation were as follows:
 - (a) There was a correlation between the content of 5S RNA of the ribosomes and their activity both in the synthesis of polyphenylalanine and in the binding of phe-tRNA.
 - (b) 5S RNA attached to ribosomes could be partially or completely removed by washing with solutions of MgCl_2 (10 mM or 100 mM)

or alkaline buffers with a concomitant loss of activity of the ribosome. The effect of both treatments could be partially reversed.

- (c) A brief investigation of the ribosomal proteins removed by these treatments was made using the technique of disc gel electrophoresis. The regular disappearance of one of the bands obtained by this technique could be correlated with both the decreased activity and decreased content of 5S RNA of treated ribosomes.
- (d) 5S RNA alone was not capable of restoring the ability of ribosomes to synthesize polyphenylalanine, although it was to some extent able to restore their ability to bind phe-tRNA which reaction was measured in the presence of 20 mM-MgCl₂.
- (e) Fractions containing 5S RNA, prepared from ribosomes, were capable of restoring the ability of treated ribosomes both to synthesize polyphenylalanine and to bind phe-tRNA. These fractions also contained protein. It is not certain whether the stimulation obtained could in any way be attributed to the presence of 5S RNA or just to the content of protein in these fractions. When 5S RNA was absent in these fractions no stimulation occurred and this was taken as circumstantial proof that 5S RNA was at least one factor responsible for the stimulation that was obtained.

These results are interpreted as showing that with the washing methods used, a protein fraction was removed with 5S RNA. Both these were necessary for the activity of the ribosome. The 5S RNA functions at or before the binding of tRNA to ribosomes. It is suggested that 5S RNA in the presence of ribosomal protein acts in keeping the structure of the ribosome intact for activity. Although, when added alone to depleted ribosomes 5S RNA can to some extent attach itself to the ribosome, and thereby, increase the sites available for the binding of tRNA, the further addition of a ribosomal protein(s) is necessary for the ribosome to assume the configuration required for peptide bond formation.

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APPENDIX

Chemicals and enzymes

ATP (disodium salt), phosphoenolpyruvate (potassium, PEP-K, and tri-Cyclohexylammonium, PEP-(CHA)₃, salts), GSH and pyruvate kinase (crystalline suspension) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. GTP was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. ATP, GTP, GSH and PEP-K were neutralized with KOH before use. ¹⁴C-phenylalanine (225-360 mc/m mole) and ¹⁴C-Yeast Protein Hydrolysate (¹⁴C-YPH) (850 and 1,500 μ c/mg) was purchased from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A., ³H-polyuridylic acid (³H-poly U and unlabelled poly U) from Miles Laboratories, and ¹⁴C-phe-tRNA of *E. coli* (1 μ c/5.7 mg) from New England Nuclear Corp (NEN), 575 Albany Street, Boston, Mass. 02118, U.S.A. Scintillation Grades of 2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene (Dimethyl POPOP) were obtained from Packard Instrument Company, INC., 2200 Warrenville RD, Downers Grove, Illinois, U.S.A. All other chemicals used were of analytical grades.

Solutions of frequent use

Medium A: 0.25 M-sucrose, 50 mM-tris-HCl, pH 7.6, 25 mM-KCl and 5 mM-MgCl₂.

Medium X: 50 mM-tris-HCl, pH 7.6 and 1 mM-MgCl₂.

Solution R: 50 mM-tris-HCl, pH 7.6, 25 mM-KCl and 1 mM-MgCl₂.

Solution R-1: 50 mM-tris-HCl, pH 8.3, 50 mM-KCl and 1 mM-MgCl₂.

N-buffer: 10 mM-tris-acetate, pH 7.4, 50 mM-potassium acetate and 0.1 mM-magnesium acetate.

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