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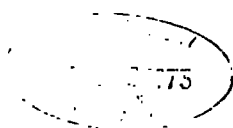
STUDIES ON PROTEIN BIOSYNTHESIS IN
CELL-FREE SYSTEMS FROM VICIA FABA (L)

by

J. N. Yarwood, B.Sc.(Dunelm)

A thesis submitted in accordance with
the requirements for the degree of
Doctor of Philosophy in the
University of Durham.

August 1973.



" The capacity to synthesise protein at 37 °C
in a dilute aqueous environment is virtually
half the definition of life."

Nature, 227, 659, (1970).

ABSTRACT

Selected aspects of the mechanism of protein synthesis on plant 80S ribosomes were investigated using amino acid incorporating systems isolated from the plumules of germinated seeds and the developing cotyledons of Vicia faba (L). Complete and transfer amino acid incorporating systems were characterised using ribosomes and enzyme fraction prepared in a variety of ways.

The soluble fraction obtained from the developing cotyledons was partially resolved by chromatography on Sepharose 4B into two complementary fractions required for peptide chain elongation. Chromatography on Sephadex-G200 failed to resolve the enzyme fraction into complementary fractions. Further purification of the partially resolved fractions was eliminated by the extreme lability of the fractions.

An attempt was made to demonstrate the synthesis of the storage globulin, legumin, by a microsomal system from 60 day developing cotyledons. In addition to the expected [³⁵S] methionyl tryptic peptides a number of additional fragments were obtained suggesting that peptides other than the constituent peptides of legumin were synthesised in the endogenous mRNA directed system. The possible nature of these products is discussed.

Chromatography on BD-cellulose or DEAE-cellulose was used to resolve the tRNA^{met} species present in the total tRNA from developing cotyledons. In both cases two major tRNA^{met}

species were resolved, one of which appeared to function as a chain initiator as demonstrated by data from AUG dependent binding, the AUG dependent reaction with puromycin, and N-terminal analyses of the products of Poly(AUG), Poly(UG) or endogenous mRNA directed incorporation. In addition BD-cellulose chromatography partially resolved a third tRNA^{met} species probably equivalent to the initiator tRNA of cell organelles. Although this system contains an active transformylase activity, the cytoplasmic initiator tRNA is not formylatable in contrast to that of animal and microbial systems.

ABBREVIATIONS

The abbreviations, symbols and conventions used in this thesis are in accordance with the recommendations of the Biochemical Journal 131 (1973) 1-20, with the following exceptions and additions:-

| | |
|--------|-------------------------------------|
| hr | hour(s) |
| S | sedimentation coefficient |
| sec | second(s) |
| TCA | trichloroacetic acid |
| GMPPCP | 5' guanylyl-methylene-diphosphonate |

Occasional abbreviations have been defined where they occur in the text.

In addition, the following conventions have been adopted:-

| | |
|--|--|
| tRNA ^{Met} | Unresolved mixture of deacylated methionine accepting tRNA species. |
| met-tRNA | Unresolved mixture of methionine accepting tRNA species amino-acylated with methionine |
| Similarly for tRNA ^{Phe} and phe-tRNA | |
| met-tRNA _i | Generalised eukaryotic initiator tRNA species. |

TF₁ has been used throughout to denote the eukaryotic amino-acyl tRNA binding enzyme, and is equivalent to Transferase I of Moldave (1968) the binding enzyme of McKeehan and Hardesty (1969) and to T₁ of Legocki and Marcus (1970).

TF₂ has been used throughout to denote the eukaryotic translocase enzyme (ribosomal peptidyl transferase), and is equivalent to Transferase II of Moldave (1968), transfer factor II of McKeehan and Hardesty (1969) and to T₂ of Legocki and Marcus (1970).

F₁, F₂ and F₃ have been used throughout to denote the

bacterial initiation factors and are equivalent to A, C and B respectively of Revel and Gros (1966). Since the eukaryotic initiation factors remain incompletely characterised the nomenclature adopted by individual workers has been retained throughout.

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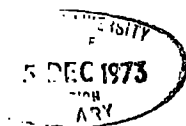
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INTRODUCTION

The biosynthesis of protein can be investigated at a variety of levels from the in vivo situation using whole organisms to the in vitro level using the purified components in strictly defined conditions. Each level of investigation recommends itself to the solution of a different type of problem and each has its associated advantages and disadvantages.

Studies at the whole plant level have been concerned mainly with the dynamics of protein turnover (Vickery et al., 1940; Hevesy et al., 1940; and Hall & Cocking, 1966), and the effect of environmental variables upon the yield of total protein (witness the reviews of McKee, 1958).

For studies on mechanistic and developmental aspects of protein synthesis, the whole organism has several disadvantages. The most insurmountable difficulty is that presented by the wide variety of tissue types present, each characterised by an assortment of specialised cells. Interpretation in terms of synthesis of specific proteins by specific cells under strictly defined conditions becomes impossible and all that can be followed is change in total protein content. Since the total organism is composed of such a wealth of cell types each synthesising its own particular types of protein, the problem of separation and characterisation of the newly synthesised proteins becomes immense. It may be pertinent, however, to stress that despite the vast number of types of protein synthesised by the different cell types, there is an underlying biochemical



unity in that all proteins are synthesised by the same fundamental mechanism (Crick, 1968) using information stored, transcribed and translated by a universal process.

The study of both developmental and mechanistic aspects of protein synthesis requires that substances be supplied to the system under rigidly specified conditions and the effect upon the process noted. At the in vivo level interpretation of the effects of exogenously supplied substances is complicated by problems of rate-limiting uptake, subsequent modification and problems of internal concentration gradients. It may appear at first sight that such difficulties could be overcome by the use of unicellular organisms. However, even in this case such problems of uptake and modification occur, as well as the complexity associated with a system in which different proteins are synthesised at specific intracellular sites. However, Peterson & Torrey (1968) using Fucus embryos found that exogenously supplied [^{14}C] leucine did not equilibrate with the main soluble leucine pool before incorporation and that uptake was proportional to the external concentration. These authors used studies of [^{14}C] leucine uptake in vivo to follow protein synthesis following fertilization of Fucus eggs. However, the results of such studies are confined to speculation upon the possible sites and role of the newly synthesised protein. A major disadvantage of using cultures of unicellular organisms is that resulting from the heterogeneity of the population with respect to position in the life cycle. However, using the technique of synchronous

culture (Tamiya, 1966; Pirson & Lorenzen, 1966), a homogeneous population can be obtained. Although such culturing techniques are readily available for many unicellular organisms, the use of such cultures in vivo has contributed little to our knowledge of the mechanism of protein synthesis but has found extensive application in the elucidation of the problems associated with the sequence of events involved in the timing of DNA synthesis in the life cycle (Cummins, 1969). In vivo studies have demonstrated that proteins are synthesised de novo in most, if not all, tissues (von der Decken, 1967), and that organs preferentially synthesising protein for export are characterised by high rates of protein synthesis (Campbell & Work, 1952). Rapidly growing cells and cells synthesising storage proteins exhibit high rates of protein synthesis, whilst organs synthesising proteins for intracellular utilisation show only low relative synthetic activities (von der Decken, 1967).

Knowledge of the reactions involved in protein synthesis in vivo has been considerably extended by the use of in vitro amino acid incorporating systems. It appears to be accepted (with certain reservations) that the in vitro situation will largely reflect the in vivo mechanism and the use of in vitro systems has made it possible to elucidate to a considerable extent the reactions involved in the synthesis of protein and the mechanism whereby protein synthesis is regulated.

In vitro investigations of protein synthesis can be conducted at the cellular and subcellular level. The perfusion technique used in animal systems, whereby an

isolated organ is continually perfused with a physiological fluid supplemented with a labelled amino acid, together with all the requirements for its normal metabolic function, has no simple equivalent in plant systems because of the nature of the plant conductive tissues. The perfused organs reflect the activity of the original organ without being affected by the metabolism of the whole animal and provides a means of isolating specific functional units. However, this technique has limited application in mechanistic studies because of its inherent complexity. The method has been used with animal systems to elucidate the site of synthesis within perfused organs of various types of protein. Using such a technique Miller et al. (1951) demonstrated the implication of the liver in the synthesis of plasma proteins and subsequently using a technique of continuous perfusion of the non-hepatic, caudal part of the rat, demonstrated that α -globulin was synthesised in the bone marrow and lymph nodes (Miller et al., 1954).

The tissue slice system has been widely used in animal systems to study the incorporation of amino acids into protein (Askonas & Humphrey, 1958; Perlmann et al., 1959; Hultin et al., 1960). As with perfused organs the activity of the tissue slices reflects the activity of the original tissue independently of the metabolism of the whole animal and has the advantage that the suspending medium can be more strictly defined. The technique involves the preparation of thin slices of tissue which are suspended in a strictly defined physiologically balanced medium supplemented with

(for the study of protein synthesis) a radioactively labelled amino acid. The uptake and incorporation into protein of the labelled amino acid is followed. However, interpretation of the results of such investigations are often complicated by such factors as problems of uptake and subsequent modification, leakage of enzymes, coenzymes, minerals, and osmotic uptake of water. It must be stressed that in such systems it is not always possible to extrapolate from the external bathing medium to the intracellular environment. Several investigators have used tissue slices from mammalian sources to investigate problems of sites of production of specific proteins, in most cases the protein studied could be detected by specific techniques which did not require extensive fractionation and purification procedures. Askonas & Humphrey (1958) using slice systems from various organs, investigated the sites of production of α -globulin and antibodies in immunised rats. The labelled antibodies synthesised were located by precipitation by their specific antigens. Perlmann et al. (1959) used agar diffusion methods (Ouchterlony, 1949) to characterise the labelled antigenic proteins after incorporation of radioactive amino acids by liver slices. Hanking & Roberts (1964) used a slice system to study the effect of intracellular levels of amino acids on rates of protein synthesis and turnover.

There are few recorded instances of slice systems from plant material. Bailey et al. (1970), using cotyledon slices for the incorporation of radioactive precursors into synthesised protein, in combination with electron-microscope

autoradiography, demonstrated that in Vicia faba, globulin is synthesised on the rough endoplasmic reticulum and is transported to the developing protein bodies via the endoplasmic reticulum and not across the cytoplasm. Thus, such a system lends itself to the study of developmental rather than mechanistic aspects of protein synthesis.

One of the inherent difficulties of the in vivo method, and of the perfusion and slice techniques, is the heterogeneous nature of the cell populations. When incorporation of amino acid is measured it is not certain which of the cell types are most active in protein synthesis. In order to minimise the problems of interpretation resulting from the use of a heterogeneous cell population, attempts have been made to separate the different cell types in rat liver and to use these homogeneous cell populations to determine the sites of synthesis of specific proteins (Perlmann et al., 1964; Lundkvist et al., 1964). One of the most readily available homogeneous cell populations is the mammalian reticulocyte. These immature blood cells actively synthesise protein and possess the great advantage that 80% of the soluble protein synthesised is haemaglobin which is readily isolated and purified. Dintzis (1961) used isolated reticulocytes to study the incorporation of [^{14}C] leucine into haemaglobin. However, since reticulocytes are incomplete cells, lacking nuclei, their application is limited to problems of protein synthesis at the translational level.

A further type of homogeneous cell population is that presented by excised cells grown in cell culture. Several

tissue culture systems using plant cells have been described (refer to Street, 1969; Steward et al., 1969). However, it must be stressed that results obtained from the use of such homogeneous cell populations cannot be applied without due consideration to the intact organism from which the original cells were obtained. A mass of undifferentiated callus may appear superficially similar to a developing cotyledon in its early stages. However, in many cotyledons at some specific point in the development, the synthesis of massive amounts of storage protein is initiated. Hence, comparison may be valid before this point but it is no longer possible to equate the situation in developing cotyledons with that in cell cultures of the corresponding material, once storage protein synthesis has been initiated. The problems of uptake, modification, isotopic dilution as well as those of characterisation of both the medium and the product synthesised confine the application of such cell cultures to developmental aspects of protein synthesis (Street, 1969; Steward et al., 1969).

In view of the problems associated with the use of in vivo and in vitro systems of the various types mentioned, it would seem that in order to study the regulation of and mechanism of assembly of amino acids into proteins, it is necessary to use highly purified preparations of the basic components, ribonucleoprotein particles/^(RNP)transfer ribonucleic acid (tRNA) and soluble enzymes supplemented by a variety of soluble factors in a strictly defined buffered medium. It is only by the disruption of the cell and subsequent

fractionation and purification of the components involved that it is possible to unequivocally equate observed effects with the presence, concentration, modification etc., of any factor involved in protein synthesis in the absence of other explanations involving uptake phenomena etc.

Whilst it must be agreed that our understanding of the mechanism of protein synthesis has been largely derived from the use of cell-free systems from micro-organisms, it is important to understand that subtle distinctions will exist and it is not possible to directly apply results obtained with the bacterial system to the cytoplasmic ribosomal system of eukaryotic organisms. A wide variety of sub-cellular in vitro systems from both plant (Boulter, 1970; Allende, 1969) and animal sources (von der Decken, 1967) have been described. Since protein synthesis in the eukaryotic cell occurs at sites other than the cytoplasmic ribosomes, such systems are classified as "organelle" or "cytoplasmic". The classification being based on the organelle (chloroplast, mitochondria, or nuclei) used or from which the components were derived. Ribosomal systems have been divided into two broad categories on the basis of their sedimentation coefficients. Those of the prokaryotic organisms (Stegeman et al., 1970), mitochondria (Kuntzel & Noll, 1967), and chloroplasts (Lyttleton, 1962; Boardman et al., 1965; Sager & Hamilton, 1967) are approximately 70S, whilst the eukaryotic cytoplasmic ribosomes are approximately 80S. However, there is no sharp distinction between the types and several exceptions have been described

(Schmitt, 1969; Morimoto et al., 1971; Rifkin et al., 1967). Ciferri & Parisi (1970) point out that organelle ribosomes with high sedimentation coefficients (80S) show greater functional similarities with the 70S type than with the 80S cytoplasmic type.

A wide variety of amino acid incorporation data using intact chloroplasts and mitochondria and amino acid incorporating systems extracted from these organelles are described (Boulter, 1970; von der Decken, 1967; Boulter et al., 1972). The preparation of such systems involves homogenisation of the cell, purification of the organelle, and subsequent isolation of the component involved in protein synthesis. In the preparation of both types of organelle the major difficulty is the rapidity with which lysis and subsequent lack of integrity occurs following homogenisation of the tissue, and precautions must be taken to ensure the fastest possible separation of the organelle from the remaining cytoplasmic matrix.

The study of amino acid incorporation by intact cell-free organelle preparations is complicated by the difficulty of isolating active preparations free of contaminating bacteria (App & Jagendorf, 1964; Lado & Schwendimann, 1966), and other organelles and experiments must be rigorously controlled to avoid misinterpretation of data.

Studies of amino acid incorporation by chloroplasts and mitochondria have highlighted the essential similarity between the mechanism of protein synthesis on prokaryotic and eukaryotic 70S type ribosomes (Ciferri & Parisi, 1970;

Bianchetti et al., 1971). The interchangeability of the components of the bacterial and eukaryotic 70S amino acid incorporating systems (Ciferri & Parisi, 1970; Morimoto et al., 1971) has been used as evidence of the endosymbiont hypothesis (Margulis, 1970). Studies on protein synthesis by organelles involving characterisation of the proteins synthesised may eventually resolve the problem of the autonomy of these organelles.

Several workers (refer to Boulter et al., 1972) have studied the effect of a variety of inhibitors upon amino acid incorporation by organelle preparations. However, caution must be exercised in interpreting such inhibitor studies, since protein synthesis may represent only one of a variety of interrelated processes carried out by the intact organelles, the inhibition of any one of which may result in a change in the amount or type of protein synthesised.

The bulk of the proteins synthesised in a eukaryotic cell (with the exception of the large sub-unit of Fraction 1 protein in green leaves), are synthesised not in the organelles but in the cytoplasm, upon free or membrane-bound 80S ribosomes, and in order to study the mechanism and regulation of protein synthesis the components must be purified, characterised and their interactions elucidated. Thus, for mechanistic studies, the end point of which must be a sequential understanding of the mechanism of protein synthesis on the 80S ribosomes, the cytoplasmic cell-free system remained, until very recently, unsurpassed. Such systems have been described from a variety of plant and animal tissues (von der

Decken, 1967; Boulter, 1970) and contain ribonucleoprotein particles, tRNA and various soluble enzymes. In all, some 120 different macromolecules are known to be involved in translation (Lengyel & Söll, 1969). In the presence of a suitable messenger RNA (mRNA), such systems incorporate radioactively labelled amino acids into polypeptide material. Such systems have been used to study developmental (Duffus, 1967; Mascarenhas & Bell, 1969; Payne, 1970) and mechanistic problems (Lengyel & Söll, 1969).

For a variety of problems cell-free systems possess severe limitations. Most cell-free amino acid systems described are active for only a limited period of time, incorporation being complete under standard conditions in 20-30 min. Exceptions have, however, been described. Using red beet microsomes, Ellis & MacDonald (1967) found that incorporation continued at a linear rate for 1 hr, whilst Payne (1970) using microsomes from developing cotyledons of Vicia faba describes a complete amino acid incorporating system, in which incorporation was linear over a period of 2 hr. The more usually reported instability of such systems probably results from accumulative degradative, structural and functional changes in the components taking place during the incubation period at temperatures of 25-37°. However, in some cases it may be that changes in the concentration of components of the incubation mixture may result in one or more components becoming limiting and the rate of reaction will therefore progressively decrease (Boulter, 1970). In an attempt to eliminate such concentration changes, Coleman

(1969) has devised a "dynamic" assay system. A polysome-tRNA-enzyme mixture is eluted down a Sephadex-G25 column equilibrated with a radioactive amino acid mixture, GTP and ATP using a Tris, Mg^{2+} , KCl buffer. The polysome-tRNA-enzyme mixture is excluded from the gel particles and is in continual contact with a fresh and relatively constant supply of amino acids, GTP and ATP. Whilst at the same time, low molecular weight degradation products enter the gel particles and therefore, move down the column at a slower rate than the polysome-tRNA-enzyme mixture. Coleman compared $[^{14}C]$ -amino acid incorporation into peptidyl form in a typical "static" cell-free system from Bacillus amyloliquefaciens to incorporation achieved using the same components in a Sephadex column "dynamic" system under aseptic conditions. Over a 40 min incubation period incorporation in the "dynamic" system was four-fold greater than that in the static system. Coleman suggests that the tail off in incorporation after 20 min was due to exhaustion of the polysome supply, since levels of low molecular weight precursors were not limiting.

A severe criticism of the usual static in vitro amino acid incorporating system is the low overall incorporation obtained as compared to that obtained in the intact organism. In most cases the rate barely approaches that found in vivo. Payne (1970) found that the rate of incorporation of $[^{14}C]$ phenylalanine into peptidyl material in response to endogenous mRNA by a V. faba cell-free system under standard conditions was 1% of that found in vivo.

Most tissues lend themselves to some form of homogenisation

technique but not all yield active amino acid incorporating systems. The lack of activity probably reflects the activity of hydrolytic enzymes and inhibitory substances liberated during the homogenisation technique, and may result from the rupture of vacuoles, lysosomes etc., in which the enzymes have been previously isolated from their substrates. There have been several attempts to reduce degradative activities to a minimum during the homogenisation technique, the most popular being the use of nuclease inhibitors. Watts & Mathias (1967) and Tester & Dure (1966) used bentonite to selectively absorb ribonuclease (RNase) during extraction of plant polysomes. Murthy & Rappoport (1965) report that the incorporation ability of rat brain microsomes and isolated RNP particles was enhanced when bentonite, an inhibitor of RNase activity was present during preparation. However, Lonsdale (1972) found that inclusion of bentonite in the extraction medium did not inhibit degradation of rRNA during extraction of polysomes from cotyledons of V. faba. Also, Tester & Dure (1966) demonstrated that bentonite was capable of absorbing significant quantities of ribosomes, thus effectively decreasing the yield.

Two other RNase inhibitors have been described, polyvinylsulphate (Moller & Boedtker, 1962) and diethylpyrocarbonate (Weeks & Marcus, 1969; Anderson & Key, 1970; Lonsdale, 1972). However, these inhibitors are severely limited in their application since they inhibit in vitro ribosomal amino acid incorporation (Weeks & Marcus, 1969). The RNase inhibitors described above are all non-physiological,

have a range of effects and are of doubtful general usefulness. Roth (1956) has described the use of a natural nuclease inhibitor from rat liver which has been used with some success in the preparation of polysomes from a wide range of material (Northup et al., 1966).

Since the use of RNase inhibitors is not always completely successful, perhaps the most attractive solution to the problem of the liberation of degradative enzymes following homogenisation, is a system whereby homogenisation is eliminated. Several such systems have been prepared from bacterial sources; Schaechter et al. (1965) describe a lysate system from Bacillus megaterium, whilst Scheinbuks et al. (1969) prepared a cell-free amino acid incorporating system from Azotobacter vinelandii. Using an osmotic shock technique to disrupt cells, they showed that 80-90% of the cellular ribosomes released were in the form of polyribosomes. Tissue from more complex organisms does not lend itself readily to such techniques because of the difficulty associated with the cellular structure, as well as that of obtaining homogeneous cell cultures. However, recent application of the technique of lysis to reticulocyte preparations by Hunt et al. (1972) yielded a completely unfractionated system in which the initial rate of globin synthesis was close to the steady state rate in intact reticulocytes, degradation being reduced to a minimum. No matter how gentle the extraction techniques may be, the intricate spatial relationship between the cell and a host of factors present in the intact organism is lost when the cells are removed from their environment in the

organism and it must be with caution that findings made in what may be an artefactual in vitro system, are extrapolated to the in vivo situation.

The intracellular site of protein synthesis is the ribosome. However, ribosomes may be segregated into those which are free in the cytoplasm (free ribosomes) or those which are attached to the membranes of the endoplasmic reticulum, membrane-bound ribosomes (mb-ribosomes) (e.g. Palade, 1958; Campbell, 1960; Moulié, 1964; Blobel & Potter, 1966; Campbell & Sargent, 1967; Campbell & Lawford, 1967). In the establishment of a cell-free amino acid incorporating system, a choice exists between types of ribosomes to use and this must be based upon the type of problems to which the system will be applied. Since cells which secrete large amounts of protein, e.g. liver, often have most of their ribosomes bound to membranes, it has been assumed that protein synthesised for "export" is synthesised on membrane-bound ribosomes. Table 1 shows the site of synthesis of a variety of proteins and indicates that in several cases mb-ribosomes synthesise proteins distinct from those synthesised on free polysomes. However, it must not be assumed that all proteins synthesised on mb-ribosomes are for export. Attardi et al. (1969) showed that 10-20% of the polysomes of HeLa cells, which have no apparent secretory function, are membrane-bound, whilst Andrews & Tata (1971) have shown that rat cerebral cortex and skeletal muscle have a minimum of 22% and 9% of their ribosomes bound to membranes, despite the fact that neither are secretory

TABLE 1.

Differential synthesis of specific proteins by free and membrane-bound polysomes

| System | Protein | Site of synthesis | | Reference |
|--------------|------------------------------|-------------------|---------------|---|
| | | free poly-somes | mb-poly-somes | |
| Rat liver | serum albumin | - | + | Campbell <i>et al.</i> (1960); Ganoza & Williams (1969); Redman (1968). |
| | membrane protein | - | + | Dallner <i>et al.</i> (1966); |
| | non-serum protein | + | - | Ganoza & Williams (1969); |
| | NADPH-cytochrome c reductase | + | + | Ragnotti <i>et al.</i> (1969); |
| Reticulocyte | globin | + | - | Bulova & Burka (1970); |
| | non-globin protein | - | + | " " |
| Pancreas | amylase | - | + | Redman <i>et al.</i> (1966). |

Results were obtained from in vitro studies.

tissues. It is likely that in these instances, as in that reported by Dallner et al. (1966) in rat liver, the proteins synthesised on such mb-ribosomes may be involved in membrane structure. In order to study aspects of the synthesis and transport of such exported proteins, a system in which the intimate relationship between membrane and ribosome is preserved, is essential.

It is unlikely that the relationship between the membrane and its attached ribosomes is simply a topographical one in which the ribosomes lie at random on the surface of the membrane. Redman & Sabatini (1966) have shown that, unlike free polysomes from both secretory and non-secretory tissue which release their nascent proteins into the surrounding fluid matrix, the nascent protein synthesised on mb-ribosomes from secretory tissue was released into the lumen of the microsomal vesicles, indicating some intimate and precise relationship between the membrane and the attached ribosomes. A considerable body of evidence now suggests that, when a protein is not synthesised in situ, an intracellular transport mechanism involving the endoplasmic reticulum (and in some instances the Golgi bodies) is involved in its translocation (for animal systems see Palay, 1958; Caro & Palade, 1964; Jamieson & Palade, 1965; Schramm, 1967; Beams & Kessel, 1968; and for plant systems, Bain & Mercer, 1966; Engleman, 1966; Opik, 1968; Bailey et al., 1970). Since vectorial release of nascent proteins synthesised on mb-ribosomes does occur, this presents a further problem to the use of such mb-ribosomes in cell-free systems, especially where

the end point is the characterisation of the specific protein synthesised. In this case the protein must be released from the lumen of the membrane or vesicles before it can be characterised.

To what extent free and mb-ribosomes do represent distinct classes of ribosomes is uncertain. The evidence of Payne (1968) and Payne & Boulter (1969) indicates that the dramatic increase in the number of mb-ribosomes following the initiation of storage globulin synthesis in V. faba, is due to a preferential synthesis of new mb-ribosomes, rather than the attachment of pre-existing ribosomes to membranes. Both the above workers and O'Neal Nicholson & Flamm (1965) have, however, demonstrated an increase in free ribosomes under conditions of decreased metabolic activity, as a result of a detachment of mb-ribosomes and not of de novo synthesis. In contrast, some meristematic cells which are rapidly dividing have mostly free ribosomes, which are later bound to the membrane as the cell matures (Whaley et al., 1960). Data consistent with the idea of a lack of exchange between free and mb-ribosomes comes from observations on the mechanism of subunit assembly on the membrane. Using mouse myeloma cells in tissue culture, Baglioni et al. (1971) showed that the 60S large ribosomal subunit binds directly to the membrane of the endoplasmic reticulum (ER), probably at a specific binding site (Sunshine et al., 1971). The initiation complex between the mRNA, 40S subunit and initiator tRNA and initiation factors takes place in the cytoplasm and is apparently capable of distinguishing between free and membrane-bound

60S subunits. The nature of the factor(s) responsible for this specific attachment remains uncertain but the possibility cannot be excluded that the specificity may reside in specific mRNA molecules. That such a specific recognition may occur between subunits, membrane and possibly mRNA, suggests the possible dangers associated with removal of membranes from a system in which protein synthesis is normally a membrane-associated process.

One of the major difficulties associated with the use of mb-ribosome or microsome preparations is the heterogeneous nature of the preparation. Not only are there present the attached ribosomes and their associated protein and nucleic acid components, but a wide variety of enzymatic activities have been shown to be associated with the ER (Dowben, 1969). It is possible that some of these enzymes or factors may be stimulatory to protein synthesis by mb-ribosomes and their removal would significantly reduce the activity of such preparations. However, others are most certainly degradative. For the study of the steps involved solely in the elongation of polypeptide chains in response to a synthetic mRNA molecule, it is unlikely that removal of ribosomes from the membrane will have adverse effects; there has been no reported evidence of a specific association between the factors involved in peptide chain elongation and the ER. However, in order to study the synthesis, under the direction of an endogenous message, of a specific protein, then a preparation in which ribosomes and polysomes are attached to the ER would appear the ideal choice, especially if

synthesis in vivo occurs on mb-polysomes.

The general procedure for the preparation of microsomes involves homogenisation of the tissue, removal of debris by low-speed centrifugation, removal of mitochondria and other organelles sedimenting at 30,000 x g for 20 min, and sedimentation from the post-mitochondrial supernatant of a microsomal fraction at 105,000 x g for 60 min (Campbell & Sargent, 1967). However, this microsomal pellet is heterogeneous in nature containing the following constituents:-

- (1) fragments of rough-surfaced ER;
- (2) fragments of the smooth-surfaced ER;
- (3) free ribosomes which are unattached to membranes;
- (4) free polysomes which are unattached to fragments of the rough-surfaced ER.

It is this heterogeneous microsomal preparation which is often used in the study of developmental aspects of protein synthesis (Payne, 1970; Redman et al., 1966), since the proportion of the various classes of ribosomes probably approximates closely to the in vivo distribution. It should be stressed that the original scheme of differential centrifugation described by Claude (1943) and extended by Hogeboom et al. (1948), is not applicable to all tissues. Siekevitz (1962) found that heart microsomes isolated by this method showed biochemical properties of both mitochondria and ER. Care must be taken to fully characterise, by both biochemical and structural analyses, such preparations before incorporation data can be meaningful. However, interpretation of amino acid incorporation data in terms of a specific type(s) of

particle(s) by such preparations is complicated by the heterogeneous nature of the pellet, since biochemical studies suggest that both the membrane-bound ribosomes and free ribosomes are engaged in the synthesis of specific proteins. In rat liver the bulk of the serum albumin has been shown to be synthesised on mb-polysomes, whilst ferritin was synthesised on free polysomes (Ganoza & Williams, 1969; Hicks et al., 1969; Redman, 1969).

Alternative methods of preparation are required for the preparation of homogeneous samples of mb-ribosomes and mb-polysomes or free ribosomes or polysomes. The free ribosomes and polysomes in the microsome fraction can be separated from the bound ribosomes and polysomes by sucrose density gradient centrifugation. Rosbash & Penman (1971) describe the separation on a 15-30% sucrose density gradient of mb-ribosomes and mb-polysomes from the free ribosomes and polysomes of HeLa cells and have demonstrated that mb-ribosomes may be classified as "loosely" or "tightly" bound, according to the ease of dissociation from the membrane by EDTA, puromycin or RNase. Such techniques are useful in implicating the synthesis of a specific protein with a specific class of ribosomes.

For the study of steps involved in polypeptide chain elongation, a wide variety of systems prepared from a range of tissues having widely different proportions of free or mb-ribosomes, have been used and in the majority of instances the ribosomes and polysomes have been removed from the membrane using one of a selection of detergents (Mans &

Novelli, 1964b; Moldave & Skogerson, 1967; Rich, 1967;

Eisenstadt & Brawerman, 1967a). Littlefield et al. (1955) employed the detergent, sodium deoxycholate (DOC) to isolate ribosomes etc. from rat liver microsomes. However, because of the association with membranes of significant amounts of nucleases (Lawford et al., 1966; Blobel & Potter, 1966), it has been found more practicable to liberate ribosomes etc. from the membranes prior to sedimentation at 105,000 x g. Campbell & Sargent (1967) suggest that when the microsomal fraction is treated with detergent in the presence of the cell sap, an endogenous ribonuclease inhibitor may prevent polysome degradation. It is essential that excess deoxycholate be removed from the preparation prior to use in amino acid incorporation systems since deoxycholate is inhibitory to cell-free systems. Preparations made by the use of deoxycholate and other detergents, for example, Lubrol W (Rendi & Hultin, 1960), iso-octane (Hawtry & Schirren, 1962), Triton X100 (Lonsdale, 1972), have been shown to contain free ribosomes, polysomes and subunits.

It is the use of such still heterogeneous preparations that has, to a considerable extent, elucidated our present knowledge of the mechanism of protein synthesis. However, the precise role which each type of particle plays in protein synthesis is still unclear. The polysome, a collection of monomeric ribosomes associated with a strand of messenger RNA, which several biochemical studies suggest is located between the subunits (Moore, 1966a; Takanami & Zubay, 1964; Takanami et al., 1965), is the active unit in protein synthesis

(Warner et al., 1962; Warner et al., 1963). Polysomes have been demonstrated in a variety of tissues and consist of ribosomes in the process of translating an endogenous mRNA. In order to study the synthesis of a specific protein, it is essential to preserve polysomes intact and for such studies gentle extraction and purification techniques must be employed. The best method of preserving intact the in vivo polysome profile is that used by Warner et al. (1962) with reticulocytes. Following gentle lysis in hypotonic buffer, it was demonstrated that a high proportion of polysomes contained 5-6 ribosomes. The use of polysome preparations to elucidate the steps involved in peptide chain elongation would appear to be infinitely preferable to the use of synthetic messengers, for example, polyuridylic acid (poly(U)), but it, unfortunately, is limited by the extreme lability of such preparations. However, it must be pointed out that results obtained in such studies would be superior to those obtained in an artefactual situation.

The role of free ribosomes is still confused; it appears to be now generally accepted that initiation of protein synthesis involves the formation of an initiation complex involving the small ribosome subunit, to which the larger subunit is subsequently joined to produce a functional unit active in translating the mRNA. After the mRNA has been translated it appears to be the general situation that the functional unit (ribosome) is released and then dissociated into its component subunits ready for the next round of initiation. This ribosome cycle will be discussed in more detail later

but it poses a problem as to the relative in vivo significance and synthetic function of the monomeric ribosome which has been so widely used in elucidating the steps involved in protein synthesis. The inference would appear to be that the monomeric ribosome population which is unattached to mRNA, represents merely an inactive pool of ribosomal subunits.

The typical "complete" cell-free ribosomal system capable of amino acid incorporation has been summarised by Boulter (1970), and consists of the following components: monovalent cation (usually K^+ or NH_4^+), Mg^{2+} , ATP and an ATP regenerating system, GTP, tRNA's, supernatant enzyme fraction, ribosomes (or microsomes), one or more radioactively labelled amino acids, and is buffered for autoxidation and for hydrogen ion content. In addition, some form of mRNA is essential. This may be supplied, (a) endogenously as an integral component of a polysome preparation; (b) by the addition of a synthetic polyribonucleotide, e.g. poly(U) or poly(A) (Lengyel et al., 1961; Nirenberg & Matthaei, 1961a); or (c) by the addition of an RNA fraction which possesses the properties and characteristics of mRNA. In the complete system described above, aminoacylation of the tRNA takes place during the assay. In the so-called 'transfer system' a $[^{14}C]$ or other radioactively labelled aminoacyl-tRNA is added to the other components listed and the labelled amino acid and ATP regenerating system omitted. This system possesses the advantage that fewer radioactive counts need to be added and investigation of steps involved in peptide chain elongation can be carried out independently of tRNA aminoacylation.

It is now accepted that the process of protein synthesis in living cells involves two stages, transcription and translation. The DNA sequence is transcribed into an RNA intermediate, which has a ribonucleotide sequence complementary with the deoxyribonucleotide sequence of one of the strands of DNA. In this transcriptional stage the DNA acts as an informational template whilst the mRNA acts as an information carrying intermediate or messenger, hence its designation mRNA. In the translational step, the mRNA attaches to the ribonucleoprotein particles, the ribosomes, which are the site of protein synthesis. The mRNA determines the order of polymerisation of amino acids into protein (Attardi, 1967; Matthaei et al., 1968; Ochoa, 1968). mRNA is translated in the 5' to 3' direction (Ochoa, 1968). Hence the synthesis of a protein is initiated at the amino-terminal amino acid (N-terminus) and proceeds in a stepwise fashion towards the carboxy-terminal amino acid (Attardi, 1967; Bishop et al., 1960; Dintzis, 1961; Matthaei et al., 1968; Ochoa, 1968). Translation begins at a specific group of three adjacent nucleotides (codon) in the mRNA and subsequent codons are translated in a sequential manner. It is the group of three bases, or codon, which specifies the identity of the amino acid which is to be attached to the growing peptide chain. The codons which specify the twenty 'protein' amino acids have been elucidated and constitute the Genetic Code (refer to the Cold Spring Harb. Symp. quant. Biol., 1966). The linear sequence of amino acids which constitute the primary structure of a polypeptide chain, possess all the information

required for the production of the three-dimensional structure of the native protein molecule (Anfinsen, 1967).

In discussing the mechanism of protein synthesis, it is convenient to divide the process into:-

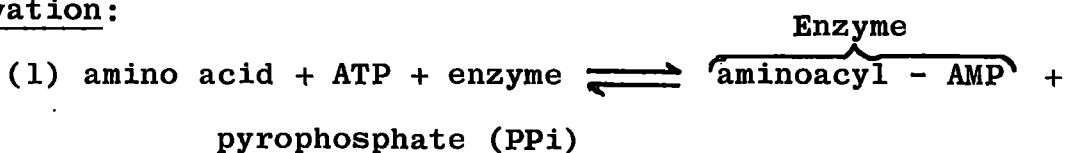
- (i) amino acid activation and formation of aminoacyl-tRNA;
- (ii) peptide chain initiation;
- (iii) peptide chain elongation;
- (iv) peptide chain termination.

It must be stressed that the process is, however, normally a continuous one and its division into arbitrary steps occurring independently of one another, in no way reflects the situation in vivo.

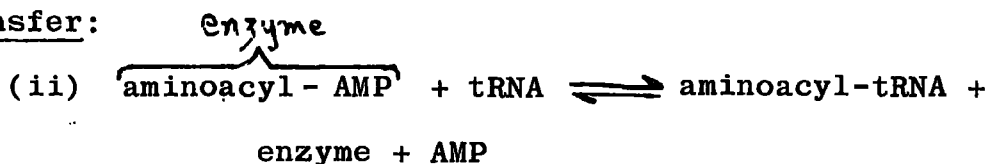
- (i) Amino acid activation and formation of aminoacyl-tRNA

The initial step of protein synthesis proper is the formation of aminoacyl-tRNA. The formation of the aminoacyl-tRNA is catalysed by the aminoacyl-tRNA synthetase or ligase (AA-tRNA synthetase) and has been, until recently, considered to be a two-step reaction involving activation of the amino acids by the formation of enzyme-bound aminoacyl-adenylates (Stulberg & Novelli, 1962; Allende et al., 1966; Cassio, 1968; Rouget & Chapeville, 1968), and transfer of the aminoacyl-adenylate to a specific tRNA molecule (transacylation) (Allende & Allende, 1964; Norris & Berg, 1964). The generally accepted overall reaction may be represented:-

Activation:



Transfer:



The overall reaction is usually studied by measuring the aminoacylation of tRNA by radioactively labelled amino acids. The initial activation reaction can be studied independently from the transfer reaction by the amino acid dependent exchange of $[^{32}\text{P}]$ -labelled pyrophosphate $[^{32}\text{P}]\text{PPi}$ with ATP, or by the ATP dependent synthesis of amino acid hydroxymates (Stulberg & Novelli, 1962).

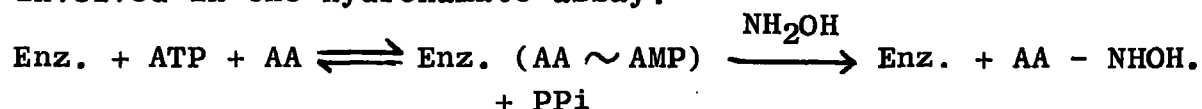
Rouget & Chapeville (1971) suggest this scheme may, in fact, be an oversimplification of the in vivo situation. These workers have shown that with leucyl-tRNA synthetase of E. coli the binding of ATP to the enzyme is the first step of leucine activation and the binding of the amino acid occurs after the formation of an enzyme-ATP complex. Using threonyl-tRNA synthetase, Allende et al. (1970), Berry & Grunberg-Manago (1970) with lysyl-tRNA synthetase, Parin et al. (1970) with tryptophanyl-tRNA synthetase, have obtained similar results.

Loftfield (1972) however, suggests that recent evidence throws doubt on the generally accepted two-step mechanism. He suggests that: (1) the physiological concentrations of enzyme and tRNA and the association constants of these macromolecules suggest that essentially all the enzyme is present in the cell as Enz. tRNA complex. In the cases of arginine, glutamine and glutamate, the tRNA-free enzyme is totally unable to form enzyme bound aminoacyl-adenylates Enz. (AA \sim AMP); (2) The reaction of Enz. (AA \sim AMP) with tRNA in vitro is so

slow and so incomplete that this reaction fails by a factor of 100 or 1000 to account for the in vivo esterification of tRNA or the in vivo rate of protein synthesis; (3) With many enzyme - tRNA systems, it is possible to obtain aminoacyl-tRNAs under circumstances where there is no evidence of an Enz. (AA ~ AMP) complex. For instance, spermine replaces Mg^{2+} as a catalyst for the esterification of many tRNAs but fails to catalyse ATP:PPi exchange. Similarly, dATP supports the synthesis of most tRNA esters, while participating only in the phenylalanine-catalysed dATP:PPi; (4) Several studies show that the addition of substrates to the enzyme must be random, depending only on the concentration and Kd's (dissociation constants) of the substrates (because of which the tRNA will generally add first); (5) General bases, such as imidazole, ammonia and hydroxylamine, all stimulate the esterification of tRNA while being potent inhibitors of ATP:PPi exchange [and by inference Enz. (AA ~ AMP) synthesis]. Balance studies show that base inhibition of ATP:PPi exchange and PPi inhibition of tRNA esterification or amino acid hydroxamate formation cannot result from competition for the activated amino acid, the enzyme bound aminoacyl-adenylate.

Loftfield submits, therefore, that the generally accepted mechanism of tRNA esterification is of doubtful application in the case of many amino acids, and that although under non-physiological conditions the synthetases do catalyse the synthesis of Enz. (AA ~ AMP), that this complex is not ordinarily an intermediate in protein synthesis. He suggests that in the physiological situation, where the concentrations of spermine

may be greater than Mg^{2+} , and where almost all enzyme is associated with tRNA, a concerted reaction in which tRNA, amino acid, free enzyme and ATP react to form aminoacyl-tRNA, AMP, PPi and free enzyme with no discrete intermediates, may be involved. Such a reaction mechanism is represented diagrammatically in Fig. 1, and explains also the formation of the amino acid hydroxamate in the presence of hydroxylamine as involved in the hydroxamate assay:-

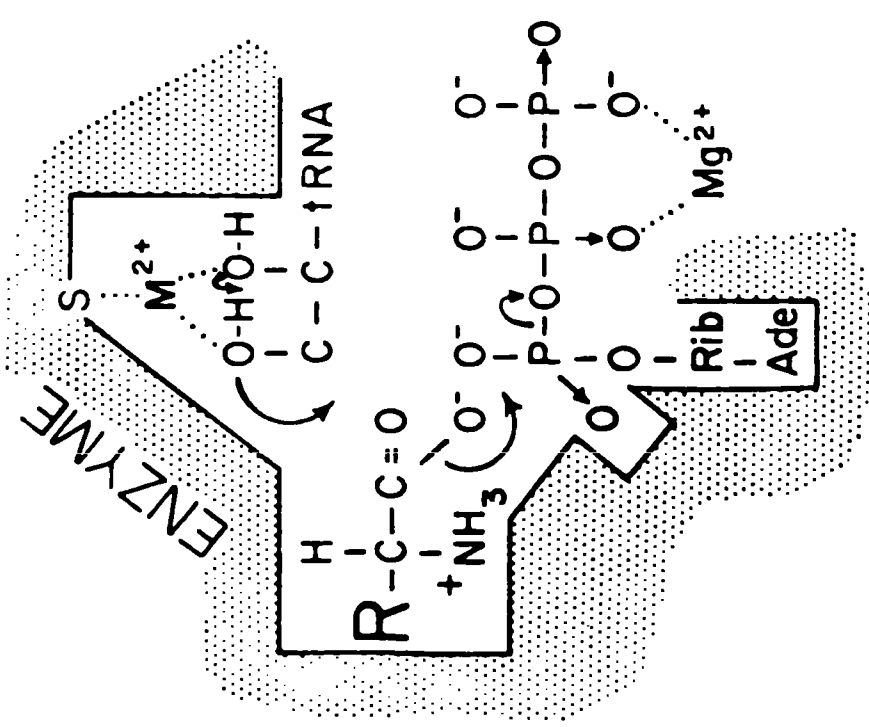
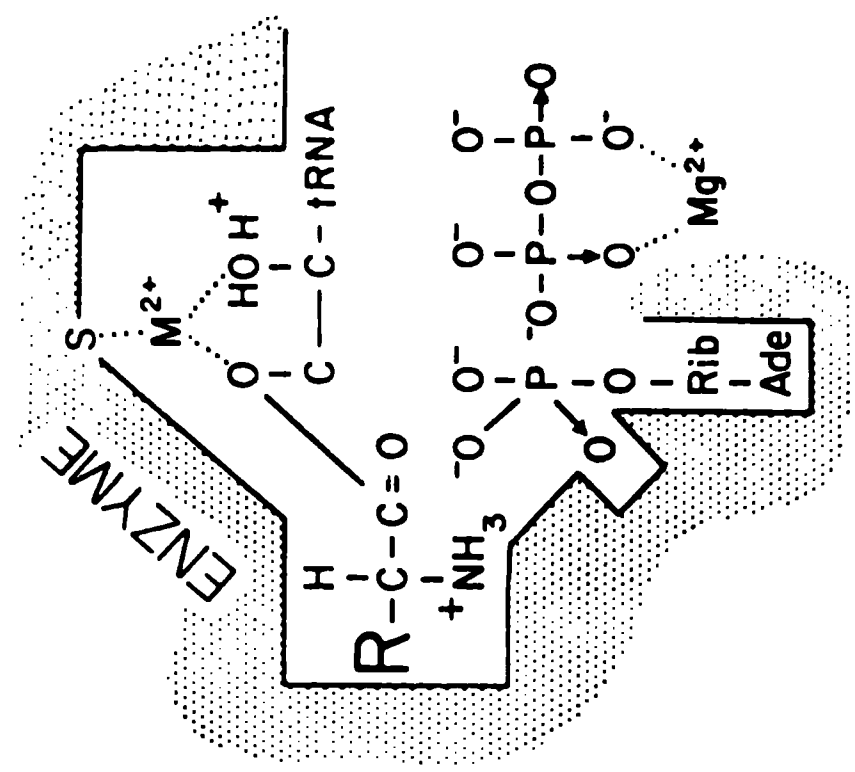


In every organism in which it has been tested, there exist at least 20 aminoacyl-tRNA synthetases which link the correct amino acid to the 3'-hydroxyl (McLaughlin & Ingram, 1964) of the terminal adenosine of a specific tRNA molecule. In order to maintain fidelity of protein synthesis the

AA-tRNA synthetases must maintain a high degree of specificity (Loftfield & Eigner, 1966; Novelli, 1967; Peterson, 1967). Specificity must be exhibited at the level of amino acid activation, as well as in the transfer step, although specificity at the latter is higher than at the former. Using material from E. coli, it was shown besides activating their cognate amino acids, isoleucyl-tRNA synthetase activates valine, and valine-tRNA synthetase activates threonine (Baldwin & Berg, 1966; Bergmann et al., 1961; Hirsch & Lipmann, 1968). These 'wrong' amino acids are not then transferred to the tRNA. Loftfield (1972) suggests that in such cases the 'mistakes' may result from allosteric effects, resulting from non-physiological conditions. The isoleucine enzyme of Bacillus stearthermophilus catalyses the attachment of valine to

Fig.1. A schematic representation showing how tRNA, amino acid and ATP react to form aminoacyl-tRNA, AMP and PPI. It is proposed that a metal ion (M^{2+}) bound tightly through a protein sulphur assists in orienting the substrate and delocalising developing charges. The 2'-OH of the tRNA serves as a general base accepting the proton from the 3'-OH of adenosine. The now very nucleophilic 3'-O⁻ of the adenosine attacks the amino acid carboxyl, displacing an oxygen to the α -P - of ATP, in turn forcing the release of the β - γ P's as $MgP_2O_7^{2-}$. The reaction is fully reversible so that exogenous $[^{32}P]$ PPI may become incorporated into ATP. In the event that NH_2OH occupies the M^{2+} site, the same series of reactions occur, with the concerted formation of amino acid hydroxamate, AMP and PPI, however, in this case, thermodynamic considerations make the reaction irreversible.

(Reproduced from Leftfield (1972) by kind permission of Academic Press).



tRNA^{Ile} at extreme temperatures (Arca et al., 1967) or in 3M urea or 10% ethanol (Arca et al., 1968).

Although regarded then as being generally highly specific, these enzymes do show a much reduced specificity against amino acids not normally present in the cell. Azetidine-2-carboxylic acid, a lower homologue of proline, is activated and transferred to tRNA^{Pro} by prolyl-tRNA synthetases from rat liver (Atherly & Bell, 1964), E. coli (Fowden & Richmond, 1963) and mungbean (Peterson & Fowden, 1965). However, in Convalaria and Polygonatum, where azetidine-2-carboxylic acid occurs naturally, the enzyme distinguishes between the two compounds and azetidine-2-carboxylic acid is not incorporated into an aminoacyl-tRNA compound. Such findings may be correlated with the in vivo toxicity of this compound to mungbean, where it is incorporated into proteins in place of proline, but not to Polygonatum. In other cases, amino acid homologues either bind to the enzyme or form aminoacyl-adenylates without being esterified to tRNA. Thus, 2-amino-4-methylhex-4-enoic acid, a homologue of phenylalanine occurring naturally in Aesculus californica, inhibits the formation of Phe-tRNA^{Phe} to the point of toxicity in microorganisms (Anderson & Fowden, 1970), while it is only activated but not transferred to tRNA by the Aesculus californica system. There is only one reported instance of a D-amino acid (D-tyrosine) being attached to a tRNA (Calender & Berg, 1966); this is thoroughly documented and must, therefore, be accepted.

Specificity between synthetase and tRNA from the same organism (i.e. homologous system) is very high; however,

misrecognition between enzyme and tRNA has been observed in heterologous systems. Since Doctor & Mudd (1963) studied the cross reaction between synthetases and tRNAs from E. coli, yeast and rat liver, and showed that both homologies and differences existed between species, several workers have investigated the species specificity of plant synthetases. Using purified valyl-, phenylalanyl-, and leucyl-tRNA synthetases prepared from yeast and E. coli, Largerkvist & Waldenström (1964) studied aminoacylation of tRNAs in the heterologous systems. The yeast enzymes all charged E. coli tRNA, although the leucyl-tRNA synthetase would only charge 60% of the E. coli tRNA compared with the homologous enzyme. In contrast, of the three E. coli enzymes, only the valyl-tRNA synthetase would aminoacylate yeast tRNA. The possibility should not be excluded that it was mitochondrial derived synthetases from yeast which aminoacylated the E. coli tRNA, and it was mitochondrial tRNA^{Val} that was charged by the E. coli enzyme. Barnett and co-workers (Barnett, 1965; Barnett & Epler, 1966; Barnett & Brown, 1967; Barnett et al., 1967) demonstrated that phenylalanyl-tRNA synthetase from the mitochondria of Neurospora and from E. coli were interchangeable, whilst the cytoplasmic Neurospora synthetase was specific for phenylalanine only with the homologous tRNA and aminoacylated both tRNA^{Val} and tRNA^{Ala} from E. coli with phenylalanine. It has been generally assumed that whenever free interchange of synthetases and whole tRNA preparations have been reported, fidelity of acceptor tRNA is maintained; the work of Barnett et al. cautions against such assumptions.

Species specificity in the synthetases of higher plants has been less thoroughly investigated. Ermokhima et al. (1965), using crude preparations of synthetases and of tRNA from pea, yeast and algae, showed that whilst there was little species specificity for tRNA^{Phe} and synthetases, the tRNA^{Met} and enzymes from pea and algae, though themselves completely interchangeable, could not effectively replace the yeast components; mixed systems resulted in only 40-50% of the charging obtained with the homologous system. Moustafa (1966) showed that the enzymes from pea seed and wheat-germ catalysed aminoacylation with homologous tRNA faster than with heterologous tRNA and that tRNA aminoacylated with lysine in the presence of lysyl-tRNA synthetase from one organism would not accept further lysine in the presence of the enzymes from the other, although both enzymes were chromatographically distinguishable on calcium phosphate gels.

One of the main problems still unresolved at the present time is specificity of aminoacyl-tRNA synthetases with respect to isoaccepting tRNA molecules (i.e. different tRNA species accepting the same amino acid). It is not known whether in an organism having a number of isoaccepting tRNA molecules, there is an equivalent number of specific synthetases. There are six different leucine-accepting tRNA species in soyabean, each of which may have a specific synthetase activity. Anderson & Cherry (1969) found that while soyabean cotyledons contained six tRNA^{Leu} species aminoacylatable with homologous synthetases, only four major leucyl-tRNA species are found when a homologous hypocotyl system was used, although there

were traces of the other two (leucyl-tRNA₅ and leucyl-tRNA₆). These two isoaccepting species were, however, more readily acylated by means of cotyledon synthetases. In contrast, the hypocotyl synthetase did not acylate cotyledon tRNA₅^{Leu} and tRNA₆^{Leu}. The differences could not be correlated with differences in the amounts of mitochondria or other organelles between the two tissues. The leucyl-tRNA synthetase activity of soyabean cotyledon has now been resolved by Kanabus & Cherry (1971), into 3 fractions. One of these fractions exclusively aminoacylates tRNA₅^{Leu} and tRNA₆^{Leu}, whilst the remaining two aminoacylate the other four tRNAs equally well. Since the former fraction is absent from the hypocotyl it may be concluded that not only does soyabean contain three leucyl-tRNA synthetases but also that they display a considerable degree of organ specificity.

A number of workers (Kelmers et al., 1971; Shugart & Novelli, 1971; Gillam & Tener, 1971) have described and resolved chromatographically separable isoaccepting tRNA species, however, Sueoka & Kano-Sueoka (1970) point out that the number of chromatographically separable tRNA species capable of accepting a particular amino acid may be misleading, since not all may be able to transfer the amino acid into protein. It is suggested that artefacts of preparation and chromatography may result in aggregation or modification of the tRNA molecules such that the acceptor capacity remains unimpaired, but the ability to donate the amino acid is lost.

It appears to have been generally accepted, despite a lack of concrete evidence, that the codons (i.e. the sequence of

three bases in the mRNA which specify a particular amino acid) used by higher plants are the same codons used by microorganisms. B asilio et al. (1966) demonstrated that the amino acid codons recognised by wheat-germ ribosomes directed by uridylic-rich polynucleotides coincided almost perfectly with those reported for E. coli.

The structure of tRNA and its interaction with the synthetases has been extensively reviewed (Miura, 1967; Holley, 1968; Mehler, 1970; Chambers, 1971; Jacobson, 1971). The tRNA molecules are all approximately the same size, containing between 75-80 nucleotides. Since they co-crystallise easily they must have a similar shape (Blake et al., 1970) and the distance from the anticodon to -C-C-A terminus must be almost identical for all species.

Despite numerous efforts to elucidate the recognition sites, i.e. those atoms or groups of atoms that inform the activating enzyme that a tRNA is specific for the correct amino acid, it now appears that no one atom, nucleotide or group of nucleotides is essential to recognition but that almost all are involved. It is now obvious that the minor (or rare) bases play no general determinative role in enzyme tRNA recognition (Loftfield, 1972). Cramer ^{et al.} (1968) describe numerous experiments which show that, except for the anticodon and the terminal adenosine, the vast majority of bases are sequestered, protected from chemical attack. Loftfield (1972) argues that, whatever structure should be established, tRNA is a molecule whose outer surface is a monotonously repeating series of ribose and phosphate with most of the bases directed

inwards and that, with the exception of the anticodon, there are no exposed bases that are not common to all or many tRNAs. Since the anticodon can be deleted or modified without modification of the amino acid specificity (Bayev et al., 1967; Yoshida et al., 1968), it is unlikely that it is involved in tRNA•enzyme recognition. It appears, therefore, that the specificity with respect to synthetase recognition is the result of interaction of all those parameters which determine the tertiary structure of the molecule. Changes in the physical properties of tRNA which may modify its tertiary structure can alter its interaction with the synthetase enzymes. Cramer et al. (1968) and Schlimme et al. (1969), report that temperature dependent aminoacylation of tRNA^{Ser} from yeast shows a marked drop at 39°, which is not due to denaturation of the enzyme which occurs only at 45° and above. The recently published x-ray crystallographic data of Kim et al. (1973) on the three-dimensional structure of tRNA^{Phe} from yeast will perhaps resolve the problems associated with the mode of action of this molecule. The polynucleotide chain has a secondary structure which is consistent with the cloverleaf conformation of Holley (1968); however, its folding is different from that proposed in any model (Cramer et al., 1968; Levitt, 1969; Connors et al., 1969). The molecule is made of two double-stranded helical regions oriented at right angles to each other, in the shape of an L. One end of the L has the -CCA acceptor; the anticodon loop is at the other end, and the dihydrouridine and T ψ C loop form the corner. The CCA stem projects out and appears free from contact with the rest of the molecule. It is postulated that this stem may be capable

of changing its orientation and such an effect may be important during the process of protein synthesis. However, elucidation of the tertiary structure of tRNA has not yet revealed the relationship between the molecules structure and function.

Evidence from differential inactivation of synthetases suggests that the activation and transfer functions of AA-tRNA synthetases may be in some way separable and may involve different enzymatic sites (Cassio, 1968; Papas & Mehler, 1968; Yaniv & Gros, 1969). In contrast, Yarus & Berg (1969), Helene et al. (1971) have suggested an interaction between the two catalytic sites of these enzymes.

The ribosome is the site at which amino acids are polymerised to form protein both in vivo and in vitro (Littlefield et al., 1955). As mentioned earlier, two different size classes of ribosomes exist: 70S which occur in prokaryotic organisms, mitochondria and chloroplasts; and 80S which occur in the cytoplasm of eukaryotes. Separation of ribosomes into these board groups is convenient though arbitrary. The ribosome consists of two unequal subunits; 30S and 50S in 70S ribosomes (Tissieres & Watson, 1958), and 40S and 60S in 80S ribosomes (Chao, 1957). The smaller subunit of the 70S type ribosome contains a single molecule of 16S RNA and about 20-30 structural polypeptide chain; whilst that of the 80S type contains a single 18S polyribonucleotide chain and about 60 structural polypeptide chains (Spirin, 1969). The large subunit of the 70S ribosome contains two molecules of ribosomal RNA (rRNA), 23S and 5S and 40-43 polypeptide chains of structural ribosomal protein. The 60S subunit also contains two molecules of rRNA, 28-30S and 5S and about 100 structural polypeptide chains

(Spirin, 1969).

Both 70S and 80S type ribosomes perform essentially the same function in protein synthesis, suggesting that the general features of structure have been preserved irrespective of differences in size and chemical composition. Only the complete ribosome, consisting of both large and small subunits, can bring about the process of translation. Neither of the ribosomal subunits individually can replace the complete ribosome in the synthesis of polypeptide chains (Gilbert, 1963a; Pestka & Nirenberg, 1966). However, Shakulov et al. (1962) pointed out that the formal integrity of a ribosome, as established by physical methods, does not always serve as a criterion for functional activity of a ribosome. Kúcan (1966) showed that even a single break in rRNA within a ribosome is sufficient to render it inactive in protein synthesis.

Although translation is a function of the complete ribosome, certain specific functions can, in fact, be assigned to the subunits. A region on the small ribosome subunit is known to interact with the template polynucleotide (mRNA) (Takanami & Okamoto, 1963a; Okamoto & Takanami, 1963; Dahlberg & Haselkorn, 1967). The small ribosome subunit has a specific aminoacyl-tRNA binding site (Matthaei et al., 1964; Suzuka et al., 1965; Kaji & Kaji, 1966) and, as described later, it possesses the specific "adherence sites", to which the initiation factors and initiator tRNA bind (Eisenstadt & Brawerman, 1967b; Nomura & Lowry, 1967; Hille et al., 1967). Since interaction of the small ribosomal subunit with GTP gives it competence to bind the initiator tRNA, it is likely that there is a specific region on the small subunit capable of recognising GTP

(Lipmann et al., 1967; Lucus-Lenard & Lipmann, 1967). One of the flattened surfaces of the small ribosome subunit must possess a special affinity for a definite surface of the large ribosomal subunit which allows them to associate into a complete ribosome. This contacting surface must bear groups which selectively bind the large subunit into the specific functional conformation. There is indirect evidence in favour of binding by interaction between both rRNAs (Marcot-Queiroz & Monier, 1965a, b; Moore, 1966b) and ribosomal proteins (Morgan et al., 1963; Zak et al., 1966; Tamaoki & Miyazawa, 1967). However, the possibility remains that the subunits are held together by the participation of peptidyl-tRNA, which is probably positioned in the translating ribosome on the border between the units (Schlessinger et al., 1967).

Spirin (1969) suggests that the complexity of the protein component of the large subunit has a direct relation to the formation of the important functional centres localised thereupon. The large subunit bears the site of "non specific" binding of tRNA which is a loose association of tRNA in the absence of mRNA (Cannon et al., 1963; Cannon, 1967). A definite small fraction of structural ribosomal protein has been implicated in this binding (Spirin, 1969; Raskas & Staehelin, 1967). If the functioning ribosome, synthesising polypeptide, is dissociated into the constituent subunits, then the peptidyl-tRNA remains firmly bound on the large subunit (Gilbert, 1963b), suggesting that the site of peptidyl-tRNA-binding site (P site) is on the large subunit. Cannon (1967) has shown that this site is identical to that involved in "non-specific" binding of aminoacyl-tRNA, and it

is this site which carries out the function of holding and appropriately orientating the growing peptide; attached to tRNA in a translating ribosome. It has been shown that the large subunit possesses the peptidyl-transferase enzyme involved in peptide bond formation, and that the peptidyl-transferase centre is an integral part of the subunit structure (Traut & Monro, 1964; Rychlik, 1966; Zamir et al., 1966; Monro & Marcker, 1967; Monro, 1967). It is most probable that this activity resides in a protein component of the large subunit. As will be amplified later, the 50S subunit participates in the formation of a GTP-ase centre, in combination with the transfer factor G, and the 30S subunit.

The function of the 5S RNA, which is bound to the 23S RNA by four proteins (Monier, 1972), remains uncertain but Comb & Sarkar, (1967) suggest it is involved in stabilising the association between the large and small subunits, whilst Forget & Reynier (1970) suggest a purely structural role. Erdmann et al. (1971) have shown that reconstituted ribosomes in which the 5S RNA has been omitted, are deficient in (a) polypeptide synthesis; (b) peptidyl transfer; (c) $[^3\text{H}]$ -UAA dependent binding of R_1 termination factor; (d) G-dependent $[^3\text{H}]$ -GTP binding; (e) codon directed tRNA binding as assayed in the presence of 30S subunits.

Numerous investigators have studied the structure and arrangement of rRNA and protein in the ribosomes in attempts to elucidate more precisely the role of the various components in the process of protein synthesis (Nanninga, 1963; Cotter et al., 1967; Malkin & Rich, 1967; Peterman & Pavlovec, 1969).

The structure of the ribosome is generally assumed to be a slightly prolate ellipsoid of revolution, the most characteristic feature of which is the presence of a groove perpendicular to the long axis which divides the complete ribosomes (as seen in the electron microscope) into the two equal parts corresponding to the subunits. The shape of ribosomes is universal and does not vary from species to species. Cox & Bonanou (1969) suggest that the helical regions of rRNA are arranged on the inside and outside of the ribosomal subunits, and are linked by the single stranded non-helical regions. The proteins are associated with the non-helical regions of the rRNA, and their location is specified by the nucleotide sequence of the rRNA (Osawa, 1968; Cox & Bonanou, 1969). Nomura et al. (1968) showed that alteration of only 6-8 bases of the 16S RNA by nitrous acid, in the absence of any degradation, resulted in rRNA that could no longer form a functional 30S subunit with 30S ribosomal protein. However, the persistence of 70S and 80S ribosomes as distinct structural entities after extensive hydrolysis of the RNA moiety indicates the importance of protein:protein interactions in maintaining the secondary structure of ribosomes (Santer, 1963; Cox, 1969), despite complete loss of synthetic activity. The inaccessibility of a segment of mRNA of about 25 nucleotides length (Takanami & Zubay, 1964; Takanami et al., 1965) and the protection of about 30-35 amino acids of the growing polypeptide chain from proteolytic activity (Malkin & Rich, 1967), suggests that the site at which peptide bond formation and polypeptide elongation occur is a protected region within the

ribosome. This has been assigned to the cleft between the two subunits in the functional ribosome (Spirin, 1969).

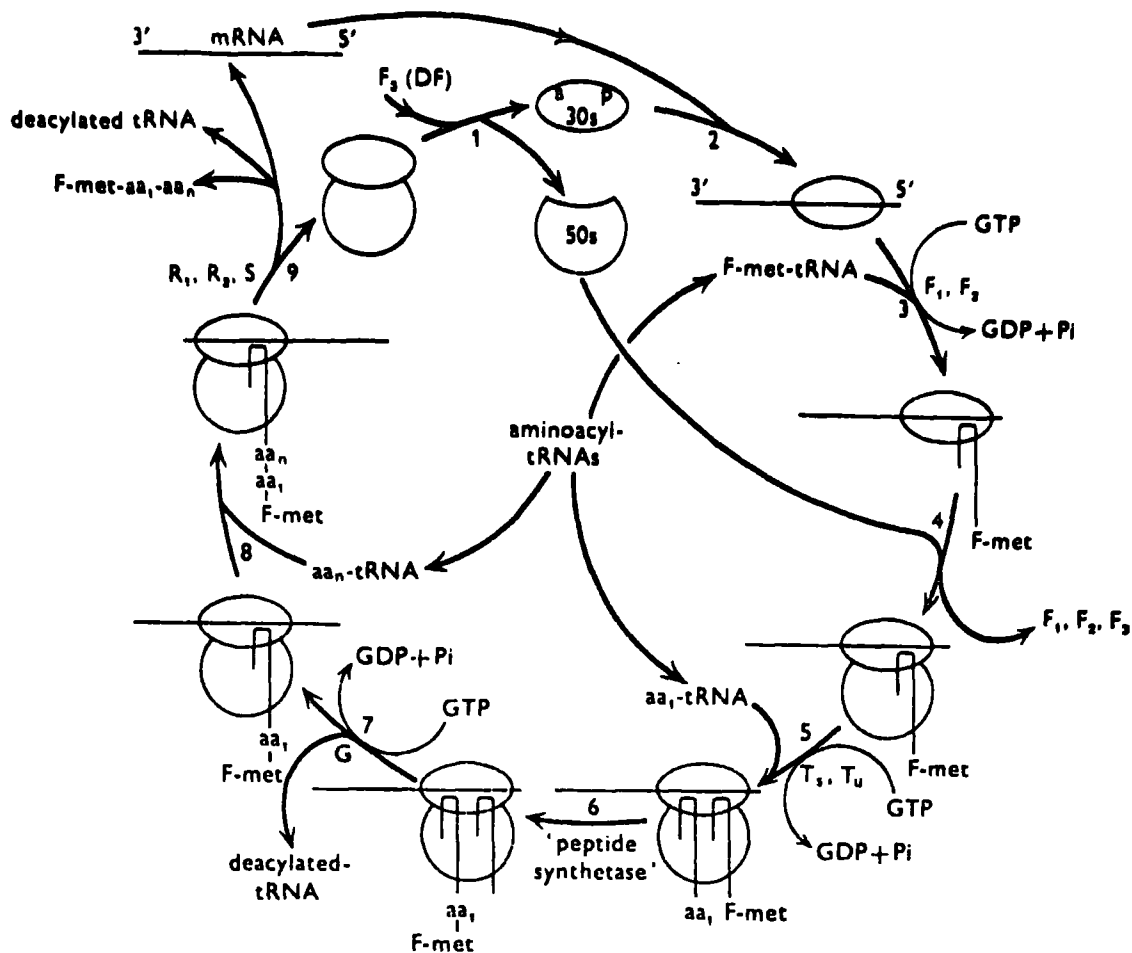
The ribosome has been described as the "work bench" for protein synthesis, but it should perhaps be stressed that it carries out its function in protein synthesis in association with the other components of the protein synthesising system. The so-called "transfer factors" involved in peptide chain elongation and the three initiation factors may be present in a permanent, albeit, labile association with the ribosome, whilst the polynucleotide template and the aminoacyl-tRNAs are only temporarily associated with it during the process of functioning. During the course of translation, the ribosome does not accommodate the entire mRNA, but draws it, in a step-wise fashion, from one end to the other, at each given moment being bound to only a discrete section of the template (Watson, 1963; Warner et al., 1963b; Gierer, 1963; Noll et al., 1963; Spirin, 1969). Simultaneously with the drawing of the mRNA through the ribosome, the polypeptide chain is synthesised. The ribosome translates the information encoded in the form of the linear sequence of bases of the mRNA chain, continuously being displaced relative to this chain (Spirin, 1969).

(ii) Peptide chain initiation

The mechanism of polypeptide chain initiation on 70S microbial ribosomes has been clearly established for several years and is summarised in Fig. 2.

The original implication of methionine in bacterial polypeptide chain initiation came from the work of Waller (1963),

Fig.2. The mechanism of protein synthesis on the 70S microbial ribosome.



Mechanism of protein synthesis on the 70S microbial ribosome.

Initiation

1, 2: Dissociation of monomer into native subunits requires initiation factor F_3 (dissociation factor DF), which also promotes binding of mRNA to 30S subunit. 'a' and 'p' indicate relative positions of aminoacyl- and peptidyl-sites respectively.

3: Formation of initiation complex requires factors F_1 and F_2 , GTP and f-met-tRNA. The latter is shown entering directly into the 'p' site, alternatively it may enter the 'a' site and then move to the 'p' site.

4: Functional ribosome formed by addition of 50S subunit; initiation factors released.

Chain elongation

5: Transfer factors T_a and T_u and GTP required for binding of aminoacyl-tRNA in 'a' site.

6: Peptide bond formation requires peptide synthetase (peptidyl-transferase) thought to be a function of the ribosome.

7: GTP and transfer factor G are required for displacement of discharged tRNA and translocation of peptidyl-tRNA to the 'p' site. Although often referred to as the 'translocase', factor G appears to promote the release (uncoupled from translocation) of the deacylated tRNA molecule from the 'a' site, while translocation *per se* seems to be a function of the 50S subunit (Roufa, Skogerson & Leder, 1970; Tanaka, Lin & Okuyama, 1971; however, cf. Lucas-Lenard & Haenni, 1969).

8: Chain elongation proceeds by repetition of steps 5, 6 and 7.

Chain termination

9: Requires release factors R_1 , R_2 and S.

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who found that the N-terminal amino acids of most E. coli proteins appeared to be restricted to methionine, alanine and serine, accounting for 45%, 30% and 15% respectively, of the total N-terminal residues, despite the fact that methionine constitutes only 3% (Sueoka, 1961) of the total amino acid composition of E. coli proteins. Over 60% of the methionine residues attached to E. coli tRNA have their α -amino group formylated and no other N-formylated aminoacyl-tRNA has been described (Lengyel & Söll, 1969). There are two methionine accepting tRNAs in E. coli designated tRNA_F and tRNA_M, both are aminoacylated by the same synthetase enzyme (Heinrikson & Hartley, 1967). Methionyl-tRNA_F (Met-tRNA_F) can be formylated by a supernatant enzyme, methionyl-tRNA_F transformylase (Dickerman et al., 1967) using N¹⁰-formyl tetrahydrofolate as the formyl donor (Adams & Capecchi, 1966; Dickerman et al., 1967; Marcker, 1965). The formylation of the α -amino group of methionine occurs after esterification by tRNA^{met} (Marcker & Sanger, 1964). Methionyl-tRNA_M (Met-tRNA_M) cannot be formylated (Clark & Marcker, 1965; 1966b). The tRNA_F has unusual base pairing near the 3' and 5' ends (Dube et al., 1968), which distinguishes it from all other tRNAs whose sequences have been determined.

Met-tRNA_M has been shown to bind to E. coli ribosomes in the presence of the trinucleoside diphosphate, AUG; the Mg²⁺ optimum for this binding, like that for other tRNA binding, is 25 mM. Met-tRNA_F and formyl methionyl-tRNA_F (f-met-tRNA_F) are bound to ribosomes by the triplets AUG, GUG, and, less well, by UUG. This binding is optimal at 10 mM Mg²⁺. Both the

low magnesium ion concentration and the degeneracy (i.e. recognition of both the normal methionyl codon, AUG, and recognition, when placed N-terminally, of GUG, the codon normally specifying valine within a peptide chain) are unique features of tRNA_F (Clark & Marcker, 1966a; Kellogg et al., 1966; Sundararajan & Thach, 1966; Ghosh et al., 1967). Both f-met-tRNA_F and met-tRNA_F can donate methionine into trichloroacetic acid (TCA) insoluble polypeptides at 10 mM Mg²⁺ in response to the synthetic random messengers, poly (UG) or poly (UAG), in an E. coli in vitro system. The formyl methionine and methionine occupy exclusively N-terminal positions. However, met-tRNA_M can also donate methionine into polypeptides in response to random poly (UAG) and this methionine is found only in internal positions (Clark & Marcker, 1965; 1966a).

The RNA of bacteriophage f₂ and R₁₇, acting as a messenger, directs the synthesis of at least three virus-specific proteins in an E. coli in vitro system (Capecchi & Gussin, 1965; Nathans et al., 1962). Each of these proteins has N-formyl methionine as the N-terminal amino acid (Adams & Capecchi, 1966; Webster et al., 1966; Vineula et al., 1967; Lodish, 1968), indicating the implication of f-met-tRNA_F in chain initiation using natural messengers. The dependence of f₂ RNA-directed in vitro protein synthesis by extracts of Trimethoprim (an inhibitor of dihydrofolate reductase, Burchall & Hitchings, 1965) treated cells on f-met-tRNA was shown by Eisenstadt & Lengyl (1966), who showed that f₂ RNA-directed protein synthesis was dependent upon added f-met-tRNA_F or N¹⁰-formyl tetrahydrofolate. This dependence was found only

at low Mg^{2+} concentration (4-8 mM); at higher Mg^{2+} the incorporation was only partially or not at all dependent on f-met-tRNA_F (Kolakofsky & Nakamoto, 1966). Capecchi (1966), using a cell-free extract of E. coli in which protein synthesis was directed by an endogenous mRNA, demonstrated that one f-met residue was incorporated per 150 amino acid residues and that formyl methionine was the only formylated amino acid. Thus, there appears to be substantial evidence for the implication of f-met-tRNA_F in chain initiation in bacterial systems under physiological conditions (i.e. low magnesium ion concentration).

In a consideration of the steps involved in chain initiation it is convenient to accept the basic, but somewhat controversial, premise that two ribosome binding sites exist. The acceptor or aminoacyl site (A site) and the donor of peptidyl site (P site). Binding of aminoacyl-tRNA to the A site is inhibited by the antibiotic tetracycline (Gottesman, 1967), whilst puromycin, an analogue of the terminal aminoacyladenine part of aminoacyl-tRNA (Gilbert, 1963b) can react with peptidyl-tRNA present in the P site (Morris & Scheet, 1961), giving rise to peptidyl-puromycin and releasing free tRNA (Monro et al., 1967). Hence, it is possible to distinguish between different tRNA binding sites on the ribosomes by judicious use of such antibiotics.

Initiation of natural mRNA in E. coli has been shown to require the presence of at least three protein factors, designated F₁, F₂ and F₃, or A, C and B respectively (Brawerman & Eisenstadt, 1966; Eisenstadt & Brawerman, 1966; Revel &

Gros, 1966; Stanley et al., 1966; Salas et al., 1967). Unfortunately, the different factors isolated by the different groups do not appear to bear any simple relationship to one another. Stanley et al. (1966) recover their initiation factors from the supernatant fraction of ribosomes washed in high salt (0.5-1.0 M ammonium chloride), and these factors are not normally present in the supernatant enzyme fraction. Eisenstadt & Brawerman (1966) obtain their factors from the supernatant fractions, whilst factors B and C of Revel & Gros (1966) are associated with a DNA-rich fraction of a crude extract. Initiation factors are, however, most frequently obtained from the supernatant after washing ribosomes with 1.0 M-ammonium chloride. The crude wash fraction has been resolved in three components, F_1 , F_2 and F_3 , which are essential for translation of mRNA with initiator codons at low Mg^{2+} concentration.

The initial step in polypeptide chain initiation is the binding of mRNA to the 'native' (see later) 30S subunit. The binding of mRNA to the 30S subunit is dependent upon the presence of the initiation factors, F_1 and F_3 (Sabol et al., 1970), and is independent of F_2 . Greenshpan & Revel (1969) implicate F_2 in mRNA binding; however, Sabol et al. (1970) suggest that such an activity of F_2 was the result of contaminating impurities or was due to non-specific interactions.

In the presence of initiation factors F_1 and F_2 , and GTP, f-met-tRNA_F is bound to the mRNA-30S subunit to form complex I (Allende & Weissbach, 1967; Anderson et al., 1967; Eisenstadt & Brawerman, 1967b; Hershey & Thach, 1967;

Lucas—Lenard & Lipmann, 1967; Nomura & Lowrey, 1967).

The initiation complex I can be retained on Millipore filter (Lengyel & Söll, 1969). 5'-Guanylyl-methylene-diphosphate (GMPPCP), a GTP analogue having a methylene bridge between the β and γ phosphorus atoms and hence not enzymatically cleaved into GDP + Pi (Herschey & Monro, 1966), can substitute for the latter in the formation of complex I (Anderson et al., 1967; Mukundan et al., 1968), indicating that GTP cleavage does not occur during the formation of complex I. The complex I formed in the presence of GMPPCP is stable to filtration through a Millipore filter, but largely dissociates in the course of centrifugation through a sucrose gradient (Kondo et al., 1968).

The precise ribosomal site at which f-met-tRNA_F is initially bound is still uncertain; unlike other aminoacyl-tRNAs, it may enter the P site directly or it may enter initially at the A site and be subsequently translocated to the P site. Bretscher (1969) suggests that since f-met-tRNA_F and met-tRNA_F bound to ribosomes with the triplet AUG at 10 mM magnesium, yielded formyl-methionyl-puromycin and methionyl-puromycin respectively with puromycin (Bretscher, 1966; Bretscher & Marcker, 1966), the f-met-tRNA_F can enter directly at the peptidyl site. Bretscher suggests that the dual coding of AUG and GUG for f-met-tRNA_F may be a feature of the P site, whose codon-anticodon recognition may be less specific than in the A site and may be a device whereby GUG (a codon for valine) does not get confused with formyl-methionine and why AUG does not code for f-met-tRNA_F in the interior of polypeptide chains. However, the above work does not exclude the

possibility that f-met-tRNA_F enters initially at the A site. Ohta et al. (1967) have demonstrated that f-met-tRNA_F in initiation complex I formed in the presence of GMPPCP, does not react with puromycin, suggesting that the initiator tRNA is not bound to the P site under these conditions. Furthermore, tetracycline was found to block the binding of f-met-tRNA_F to ribosomes in the presence of GTP, mRNA and initiation factors (Sarkar & Thach, 1968), indicating that f-met-tRNA is bound, at least initially, to the A site. Kuechler (1971) investigating the role of GTP hydrolysis in the positioning of f-met-tRNA on the 30S subunit, suggests f-met-tRNA_F is directly bound to the P site in a state insensitive to puromycin, or that it enters the A site and is translocated by GTP hydrolysis which is not accompanied by a relative movement of ribosome and mRNA.

The precise role and identification of the initiation factors responsible for the formation of complex I, is still unresolved. Factor F₃ has been implicated (Iwasaki et al., 1968; Revel et al., 1970) in messenger selection since E. coli ribosomes recognise specifically natural mRNA from other polynucleotide templates only in its presence. It has been postulated that F₃ represents a kind of specific recognition factor permitting the ribosomes to start translating natural messengers at defined sequences. Although F₃ has been shown to bind to purified mRNA (Iwasaki et al., 1968), it cannot be assessed to what extent such binding, outside of ribosomes, is specific and related with its initiation factor actively proper. It is likely, however, that F₃ recognises some

specific initiator sequence of natural mRNAs, most probably a sequence much larger than the AUG initiator codon. Groner & Revel (1971) have established the role of a complex between initiation factors F_2 and F_3 in the selective recognition and binding of f-met-tRNA_F.

Evidence that the 30S subunit was involved in initiation complex I was obtained in experiments in which "heavy" 70S ribosomes, labelled with $[^{15}\text{N}]$ and deuterium were mixed with "light" 50S subunits, poly AUG, f-met-tRNA_F and initiation factors. f-met-tRNA_F was found to be bound to hybrid 70S ribosomes, consisting of a heavy 30S subunit and a light 50S subunit. It is argued that these hybrid ribosomes could only have arisen if the heavy 70S ribosomes dissociated, liberating heavy 30S subunits, which after the formation of the initiation complex, would have complexed with light 50S subunits (Guthrie & Nomura, 1968). More recently, Slobin (1972) has provided more concrete evidence for the implication of the 30S subunit in the formation of the initiation complex I. This author demonstrated that using ribosomes in which the 30S and 50S subunits had been cross-linked covalently by the bifunctional imidoester, dimethylsuberimidate, the initiation complex could not be formed with f_2 RNA and f-met-tRNA_F. However, cross-linked ribosomes retained 80% of the activity of unmodified ribosomes for polyphenylalanine formation in a poly U-dependent system requiring initiation factors and N-acetylphenylalanyl-tRNA. As well as indicating the necessity of the 30S subunit in the formation of the "proper" initiation complex I, these results indicate the artefactual situation

which obtains in the 70S poly(U) system. It is suggested that the two tRNAs bind to different sites. It is most probable that the N-actylphenylalanyl-tRNA binds to the P site, whilst the f-met-tRNA_F may enter at a form of P site, the true entry site.

Subsequent to the formation of initiation complex I, the 50S subunit is attached to complex I to form complex II. This junction does not require the hydrolysis of GTP (Kolakofsky et al., 1968a). The f-met-tRNA_F in complex II is thought to be bound at the A site of the ribosome (Ghosh & Khorana, 1967; Nomura et al., 1967; Kolakofsky et al., 1968a; Kondo et al., 1968; Sarkar & Thach, 1968). If the analogue GMPPCP is used instead of GTP in the formation of complex I, the 50S subunit is still joined to the complex to form the 70S complex, but the f-met-tRNA_F is unreactive towards puromycin or other aminoacyl-tRNAs (Kolakofsky et al., 1968a; Ohta & Thach, 1968). GTP hydrolysis is, therefore, required to convert the bound f-met-tRNA_F from an unreactive to a reactive state; this conversion occurs on the 70S ribosome (Kolakofsky et al., 1969). When the 50S subunit joins to the complex I initiation factor F₁ is released (Hershey et al., 1969). However, factor F₂ appears to remain attached to the new complex II at least until GTP hydrolysis has taken place. F₂ has been demonstrated (Kolakofsky et al., 1968b) to catalyse the ribosome-dependent hydrolysis of GTP and Kolakofsky and his co-workers suggest that the GTPase activity of factor F₂ may be involved with the translocation of f-met-tRNA_F from its unreactive state in the A site into the P site, ready for peptide bond formation.

Since initiation factors are not found on either 70S ribosomes or free 50S subunits (Parenti-Rosina et al., 1969), it is concluded that F_2 is released from the 70S initiation complex after the hydrolysis of GTP and the translocation of f-met-tRNA_F into the peptidyl site. However, the exact mode and site of action of both F_2 and the GTPase activity remains unclear. Once f-met-tRNA_F has been translocated to the P site, the A site remains free to accept another aminoacyl-tRNA, and chain initiation is complete.

Unformylated met-tRNA_F cannot initiate protein synthesis in E. coli at low Mg^{2+} concentration (Kolakofsky & Nakamoto, 1966) and in vivo protein synthesis stops if formylation of met-tRNA_F is inhibited (Shih et al., 1966).

Another implication of the importance of N-blocked aminoacyl-tRNA in chain initiation comes from the work of Lucas-Lenard & Lipmann (1967). Using Pseudomonas fluorescens, these workers reported that N-acetylphenylalanine could act as a chain initiator in the poly(U) system at low magnesium concentration (4 mM). This finding has been confirmed for both 70S and 80S ribosomes. Igarashi (1970) reports that any N-blocked peptidyl-like tRNA^{Phe} will act as an initiator for the poly(U) dependent polyphenylalanine system, stressing further the artefactual nature of such a system.

Although the basic mechanism of polypeptide initiation on 70S ribosomes has been understood for several years, it is only recently that information regarding the mechanism in eukaryotic cells has been forthcoming. Several workers have demonstrated that f-met-tRNA_F is the initiator tRNA in

mitochondria and chloroplasts (Schwartz et al., 1967; Smith & Marcker, 1968; Burkard et al., 1969; Galper & Darnell, 1969; Marcker & Smith, 1969; Bianchetti et al., 1971), and it has been proposed (Smith & Marcker, 1968; Marcker & Smith, 1969), that f-met-tRNA_F is the universal initiator of protein synthesis on 70S ribosomes. However, there is no unequivocal evidence of the presence of f-met-tRNA_F in the cytoplasm of eukaryotes.

A number of workers have, however, demonstrated two major methionine-accepting tRNA species in the cytoplasm of mammals (Caskey et al., 1967; RajBandary & Ghosh, 1969; Smith & Marcker, 1970) and in yeast (Takeishi et al., 1968). Since the cytoplasm of these species lacks an active transformylase, neither of these species are formylated under normal conditions, either in vivo or in vitro, using the homologous enzyme preparation.

One of these tRNA^{Met} species, designated tRNA_{F*}^{Met}, can be formylated in the presence of added E. coli transformylase and the coding properties of the product are indistinguishable from those of f-met-tRNA_F (Caskey et al., 1968; Marcker & Smith, 1969). Despite a difference their primary structures (Marcker & Smith, 1969) the tRNA_{F*}^{Met} can substitute for the E. coli initiator in the E. coli cell-free system (Takeishi et al., 1968). This suggests that the unique structural feature of f-met-tRNA_F, necessary for its function as the specific initiator has been conserved in yeast and mammalian met-tRNA_{F*}. The presence of such a met-tRNA_{F*} species in the cytoplasm of these eukaryotes suggests that initiation on mammalian and

yeast 80S ribosomes involves a formylatable (in the presence of E. coli transformylase), but unformylated tRNA^{Met} species. Initiation of haemoglobin synthesis has been conclusively demonstrated to involve an unformylated tRNA^{Met} species as the initiator tRNA (Bhaduri et al., 1970; Gupta et al., 1970; Houseman et al., 1970; Jackson & Hunter, 1970; Wilson & Dintzis, 1970). This is in direct contradiction of earlier work implicating a valyl-tRNA species in initiation of haemoglobin (Rahamimoff & Arnstein, 1969).

The situation with respect to the initiator tRNA in plant systems is less well documented. A number of workers (Leis & Keller, 1970; Marcus et al., 1970b; Tarrago et al., 1970; Ghosh et al., 1971) report the occurrence, in the monocotyledon wheat, of two major and one minor tRNA^{Met} species. Evidence is presented in this thesis for a similar situation in Vicia faba, a dicotyledon. Despite the presence in the supernatant of an active transformylase (Leis & Keller, 1970) capable of formylating E. coli met tRNA_F, neither of the major tRNA^{Met} species from wheat-germ are formylatable either by homologous or by E. coli transformylase. One of the tRNA^{Met} species has however, been identified as the specific initiator tRNA on the basis of AUG dependent-binding (Tarrago et al., 1970), AUG dependent reaction with puromycin (Leis & Keller, 1970), and N-terminal analyses of the product of tobacco mosaic viral RNA-directed (TMV RNA-directed) incorporation (Marcus et al., 1970b).

The precise reaction sequence whereby the specific initiator methionine tRNA (met-tRNA_i) interacts with the 40S subunit and

subsequently the 60S subunit to form the initiation complex has not yet been clarified. However, there are indications that although essentially similar factors may be involved, the sequence of events may be distinct from that observed in 70S systems. Using crude lysates of rabbit reticulocytes which synthesize protein at a rate comparable with that in the intact cell, Legon et al. (1973) present evidence that the initial step in initiation in this system is the association of the initiator tRNA (met-tRNA_i) with the 40S subunit, in the absence of mRNA, to form a methionyl-tRNA_i-40S subunit complex. These authors believe that bound GTP is an integral component of the complex. An essentially similar result has been obtained by Schreier & Staehelin (1973a) using a fractionated mammalian system, the efficiency of which approached that of unfractionated crude cell-free extracts (Schreier & Staehelin, 1973a). These authors have shown that globin mRNA does not promote the binding of methionyl-tRNA_i to 40S subunits, but that the initiator tRNA associates with the 40S subunit in the absence of mRNA under the direction of the appropriate initiation factors. It should perhaps be stressed that although both groups of workers were investigating initiation of globin synthesis, they arrived at the conclusion using very different experimental techniques. These results are, however, in striking contrast to those of Weeks et al. (1972). Using 40S and 60S subunits derived from messenger-free wheat-germ ribosomes and TMV-RNA, they present evidence that initiation involves the formation of a 40S subunit-mRNA-met-tRNA complex (Complex I), which subsequently combines with a 60S ribosomal

subunit to form on 80S monoribosome (complex II) functional in protein synthesis. Burgess & Mach (1971) also interpret investigations of met-tRNA_i binding to washed eukaryotic ribosomes in terms of the model proposed for the bacterial system. Legon et al. (1973) question the significance of such results obtained with many fractionated eukaryotic systems on the basis of their very low efficiency.

There is evidence for the participation of specific initiation factors in eukaryotic systems. In plants, Marcus (1970a, b) reports the formation of an initiation complex between radioactive TMV-RNA and wheat-germ ribosomes, which requires the presence of ATP and two protein factors isolated by chromatography on DEAE-Cellulose from the supernatant fluid after high-speed centrifugation. The requirement for ATP was initially thought to be absolute; however, Liddell (1972) reports that GTP can replace ATP. When tRNA was added to the initiation complex, peptide synthesis occurred and the radioactivity was found to be associated with polysomes.

Confirmation of the existence of two such initiation factors comes from studies using the inhibitor aurintricarboxylic acid (ATA), which has been shown to specifically inhibit the attachment of f₂ RNA to E. coli ribosomes (Grollman & Stewart, 1968; Webster & Zinder, 1969; Stewart et al., 1971). Marcus et al. (1970a) have shown that ATA inhibits the formation of the initiation complex in wheat-germ. This data suggest that in wheat-germ at least, the initial step in the formation of the initiation complex is independent of met-tRNA_i or indeed any other aminoacyl-tRNA. This is in strict contradiction

to the work of Legon et al. (1973), who suggest that the initial step in the formation of the initiation complex is independent of mRNA and involves GTP and met-tRNA_i; and, initiation factors only.

Three protein factors, M₁, M₂ and M₃, isolated from a 0.5M-potassium chloride rabbit reticulocyte ribosomal wash fraction, have been shown to be required for initiation in cell-free haemoglobin synthesis at 3 mM magnesium (Shafritz et al., 1970; Pritchard et al., 1970; Kerwar et al., 1970). The factors, M₁ and M₂, bind met-tRNA_i but not met-tRNA_M to reticulocyte ribosomes in the presence of AUG.

There is evidence that one of the initiation factors may be messenger specific. Heywood (1969) demonstrated the inability of myosin mRNA from chick muscle to function with washed reticulocyte ribosomes, unless muscle, not reticulocyte, initiation factors were present. Washed muscle ribosomes with initiation factors from reticulocytes also cannot translate myosin mRNA. This specificity may be due to inability of myosin mRNA to bind to reticulocyte ribosomes in the presence of reticulocyte initiation factors. Such data is highly suggestive of the existence of cell-specific messenger recognition factors present on the ribosomes, which may be involved in regulation of mRNA binding. Naora & Kodaira (1970) also present evidence suggesting that mRNA from one tissue cannot be translated by ribosomes and factors from another.

If a methionyl-tRNA species is the universal initiator tRNA, it would not be unreasonable to expect all polypeptide

chains to carry an N-terminal methionyl residue. That this is not so, attests to the presence in many cells of specific enzymes capable of removing the N-terminal (and perhaps other terminally situated) amino acids. However, there should be evidence that at sometime shortly after their initiation, newly synthesised polypeptide chains (perhaps while still attached as peptidyl-tRNA) will bear an N-terminal methionine residue. The majority of E. coli proteins have methionine, alanine, serine or threonine, as the N-terminal amino acid. On the other hand, most, if not all, E. coli proteins are initiated by N-formylmethionine, suggesting that E. coli and, presumably, other bacteria have an enzyme(s) capable of removing the formyl residue, and in some cases the terminal methionine residue. Enzymes (deformylases) cleaving f-met peptides into formate and methionyl-peptides have been recognised in E. coli, B. stearothermophilus and B. subtilis, and are found to have those characteristics of specificity required of an enzyme whose function in vivo is to remove formyl residues from nascent peptides (Adams, 1968; Fry & Lamborg, 1968; Takeda & Webster, 1968). Since f-met-tRNA_F is not a substrate for the enzyme, it is concluded that deformylation occurs after the incorporation of the formylmethionyl residue into a peptide. The existence in E. coli and B. subtilis of specific enzymes (aminopeptidases) capable of removing methionine residue from the N-terminal hexapeptide of f₂ coat protein (but only after deformylation), suggests that removal of the N-terminal N-formylmethionyl residue occurs in two distinct steps (Takeda & Webster, 1968). Evidence (to be discussed

later) exists that a similar situation exists in eukaryotic cells, where N-terminal methionyl residues are cleaved from the nascent protein.

Translation in vitro requires the presence of an initiating codon at the 5' end of the RNA sequence to be translated. However, considerable evidence exists that the initiating triplet does not occupy the 5' terminus of mRNA molecules but is preceded by a sequence of untranslated codons. In the case of polycistronic messages, one or more initiating regions probably occur intramolecularly. Sequence determinations on bacteriophage MS₂-RNA (De Wachter et al., 1971) indicate that the initiating codon, AUG, is located in nucleotide positions 130-132. Adams & Cory (1970) have deduced that the initiating codon in bacteriophage-R₁₇-RNA is preceded by an untranslated sequence of at least 91 nucleotides, whilst Billeter et al. (1969) report an untranslated sequence of 61 nucleotides in the RNA of bacteriophage-Q β .

The presence of such untranslated N-terminal sequences in eukaryotic mRNAs is less well documented. Gaskill & Kabat (1971) have shown that the haemoglobin mRNA contains at least 200 more nucleotides than are required to code for the globin chains. Smith (1972) has described the isolation and properties of a fragment of RNA from Encephalomyocarditis (EMC) virus with the properties of the initiation site, and found a region rich in adenosine occurring on the 5' side of the initiation site. The fragment had an unusual primary and secondary structure which, it is suggested, functions as a messenger recognition site for initiation factors or ribosomes.

A number of workers (Kates, 1970; Lim & Canellakis, 1970; Edmonds et al., 1971; Darnell et al., 1971) report the presence within mammalian viral, polysomal and nuclear RNA species of regions rich in adenylic acid residues, the so-called poly(A) regions. These regions have been demonstrated on the 3' end of a number of mRNAs. The immunity from mutation and conservation throughout evolution of such untranslated sequences of nucleotides, implies a strict requirement for that region. They may be involved in the process of regulation of gene expression; however, their function, as yet, remains obscure. Kwan & Brawerman (1972) present evidence for the association of a particle with these poly(A) regions. The particle is identified as composed mainly of protein. The situation of such a particle on the 3' end of the mRNA indicates that it is not involved in the initiation process; its function is still unclear.

Since polycistronic mRNAs contain internal initiating codons, initiation may either be sequential, i.e. begin only at the 5' terminus and proceed towards the 3' terminus or ribosomes may attach at any of the internal initiation sites. Lodish (1968) has shown that the addition of bacteriophage-f₂-RNA to an E. coli in vitro system results in the simultaneous synthesis of two different polypeptides, and Rekosh et al. (1970) have shown that an E. coli system with polio-virus-RNA initiates at least eight distinct sites. In the case of the histidine operon of Salmonella typhimurium (coding for 10 enzymes involved in histidine biosynthesis), sequential translation has been shown to occur under conditions where histidine was limiting

(derepression) (Berberich et al., 1967) and the intracellular formyltetrahydrofolate pool was low. However, when the intracellular formyltetrahydrofolate pool was large, simultaneous synthesis of all the enzymes of the histidine operon resulted.

The bacterial initiation factors have been implicated as, at least, part of the translational control mechanism involved in the alteration in mRNA translation brought about by T₄ phage infection (Hsu & Weiss, 1969; Dube & Rudland, 1970; Schedl et al., 1970; Steitz et al., 1970). Hattman (1970) reports the superinfection, with T₄ phage, of E. coli cells previously infected with an RNA phage such as f₂ or M₁₂, results in inhibition of synthesis of the RNA phage coat protein. A similar situation was observed in vitro. When ribosomes from T₄-infected cells were allowed to translate E. coli mRNA, MS₂ RNA or f₂ RNA they translated E. coli mRNA only 50% as efficiently, and RNA phage RNA less than 20% as efficiently as normal (Hsu & Weiss, 1969). Chloramphenicol treated ribosomes behaved like ribosomes from uninfected cells indicating that such a change in ribosome-mRNA specificity was dependent upon protein synthesis. A heat-labile factor extracted from infected ribosomes conferred the restriction on T₄-infected ribosomes and when added to normal ribosomes, the factor conferred upon them a specificity for T₄ RNA translation. Schedl et al. (1970) suggest that a change occurs in the initiation factor F₂, whilst Dube & Rudland (1970) cite a change in F₃ as responsible for the specificity. The capacity of uninfected E. coli ribosomes to bind to the three different R17 initiation signals can be altered by the

addition of an initiation factor preparation from T₄ phage-infected ribosomes (Steitz et al., 1970). Ribosomes with the initiation factors from uninfected cells bound to all three cistrons of the R17 RNA, whereas those with initiation factors from infected cells bound only to the initiation point of the maturation protein cistron. Such results suggest that following T₄ infection, a new initiation factor(s) is made, specified by the infecting phage, or a modification of pre-existing host factors results, such that ribosomes now recognise the starting signals of only T₄ mRNA. There is little data on such changes in mRNA specificity following viral infection of eukaryotes, but such changes may be important in the loss of cellular control following the infection by carcinogenic viruses.

(iii) Peptide Chain Elongation

After the 'priming' of the ribosome by the formation of the initiation complex (f-met-tRNA-mRNA-30S subunit) and its coupling with a 50S subunit to form a 70S monosome, the process of peptide chain elongation can occur by the stepwise addition of one amino acid at a time in a repetitive cycle.

The elongation cycle involves three distinct events:-
(1) codon-directed binding of aminoacyl-tRNA to a ribosomal site (A) adjacent to that occupied by the initiator or peptidyl-tRNA (P); (2) peptidyl transfer between the newly bound aminoacyl-tRNA and the initiator or peptidyl-tRNA; and (3) translocation of both the mRNA and the newly synthesised peptidyl-tRNA from the acceptor (A) to the donor (peptidyl, P) site on the ribosome, thus, freeing the A site for further rounds of elongation. Concomitant with the translocation

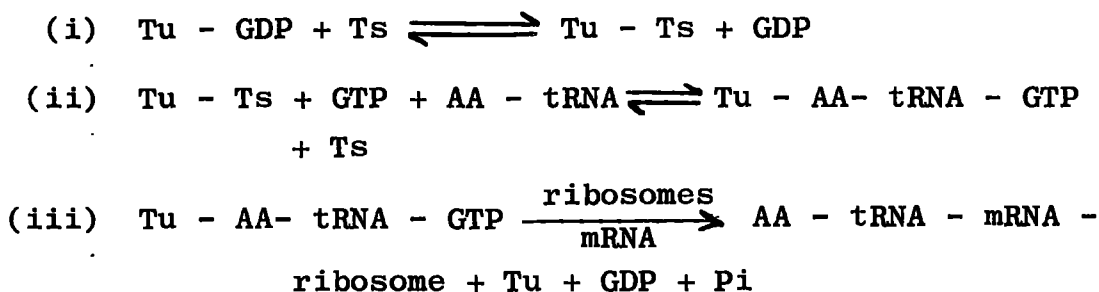
step the tRNA which donated the peptidyl moiety is released. The events involved in peptide chain elongation on the 70S ribosome will be described since they have been more completely elucidated than those taking place on 80S ribosomes.

The basic mechanism on 70S and 80S ribosomes is identical; however, slight differences do exist and will be discussed. Elongation of peptide chains requires K^+ , Mg^{2+} , GTP and charged tRNA and a soluble enzyme fraction. Three protein factors involved in peptide chain elongation have been resolved from the soluble fraction of the cell. They have been isolated from a range of microorganisms by the variety of workers, each assigning their specific nomenclature to what appeared to be analogous proteins. Lucas-Lenard & Lipmann (1966) isolated from E. coli and P. fluorescens, three factors, Ts, Tu and G. These appear to correspond to the factors S₁, S₃ and S₂ isolated by Skoultchi et al. (1968) from B. stearothermophilus and the factors FI_s, FI_u and FII of Ravel & Shorey (1969). In the absence of a uniformity of nomenclature the Ts notation will be used throughout the description of the events involved in peptide chain elongation. Tu and Ts together constitute the 'transfer' factor T, whilst factor G functions as a translocase.

The adapter hypothesis (Crick, 1957) requires only the intervention of tRNA between the mRNA codon and the amino acids that are polymerised into the nascent protein. However, the mechanism whereby the aminoacyl-tRNA is attached to the ribosomes is more complex than was initially proposed, and involves the participation of the factors Ts, Tu and GTP. There is considerable evidence that aminoacyl-tRNA is brought

to the vacant A site in the form of a complex involving factor Tu and GTP. In the soluble fraction of the cell, the Tu and Ts factors are associated and together constitute the T factor (Nishizuka & Lipmann, 1966b). Tu, in the presence of Mg^{2+} , forms a complex with GDP or GTP, which is retained on Millipore filters. However, in the presence of aminoacyl-tRNA and Ts, the binding specificity of the Tu is changed (Cooper & Gordan, 1969) and Tu forms a ternary complex with GTP and aminoacyl-tRNA, and no longer binds GDP or Ts (Ravel et al., 1967; 1968; Gordan, 1968). This complex formation is specific for aminoacyl-tRNA which cannot be replaced by uncharged tRNA (Gordan, 1967; Ravel et al., 1967), N-acetylated aminoacyl-tRNA (Ravel et al., 1967), f-met-tRNA (Ono et al., 1968), or deaminated aminoacyl-tRNA (Jerez et al., 1969). f-met-tRNA_F does not normally form a complex with Tu. The GTP analogue, GMPPCP, can form the ternary complex, indicating that GTP hydrolysis is not involved in the formation of the complex. GTP and aminoacyl-tRNA are present in a ratio of 1:1 (Haenni et al., 1968; Ono et al., 1969a). Addition of the T-GTP-aminoacyl-tRNA complex to initiated ribosomes results in rapid binding to the ribosomes of the complex and the formation of dipeptidyl-tRNA between the initiator tRNA and the aminoacyl-tRNA (Lucas-Lenard & Haenni, 1968). The rate of reaction is considerably greater when the preformed ternary complex is added to the initiated ribosomes, than when all the constituents were added at zero time suggested that the complex is an intermediate in the binding of aminoacyl-tRNA to the ribosomes and that Tu is effectively a carrier of aminoacyl-tRNAs to

ribosomes (Lucas-Lenard & Haenni, 1968; Skoultchi et al., 1969).



Although GMPPCP can replace GTP in the formation of the T-GTP-aminoacyl-tRNA complex and in the binding of aminoacyl-tRNA, binding of such a complex does not result in the formation of a dipeptide between the newly bound aminoacyl-tRNA and the adjacent initiator tRNA. Although GTP remains unhydrolysed during the formation of the ternary complex (Skoultchi et al., 1968), it is hydrolysed to GDP and inorganic phosphate in the transfer of aminoacyl-tRNA to the ribosomes, and there is a stoichiometric breakdown of GTP compared to the amount of aminoacyl-tRNA bound to the ribosome (Shorey et al., 1969; Lucas-Lenard et al., 1969; Ono et al., 1969b). GTP hydrolysis was insensitive to inhibition by chlortetracycline (Hierowski, 1965), an inhibitor of aminoacyl-tRNA binding, and to sparsomycin (Goldberg & Mitsugi, 1967), an inhibitor of peptidyl transferase, and to fusidic acid, an inhibitor of factor G mediated translocation, indicating that the GTP hydrolysis was not required for either binding of aminoacyl-tRNA or for peptide bond formation or for translocation of the bound aminoacyl-tRNA. When the aminoacyl-tRNA-GMPPCP-Tu complex is bound to ribosomes, the Tu and GMPPCP remain attached to the ribosomes (Lucas-Lenard et al., 1969; Skoultchi et al., 1970), whereas when the GTP containing complex is bound the Tu and GDP are

removed from the ribosome as a Tu-GDP complex (Ravel et al., 1969; Skoultchi et al., 1969; Hachmann et al., 1971). Ts displaces Tu from the Tu-GDP complex as indicated in equation (i), regenerating the Tu-Ts complex (Miller & Weissbach, 1970).

Although the role of Tu in transporting the aminoacyl-tRNA to the ribosome is clearly understood, there seems to be little information on the mechanism whereby the aminoacyl-tRNA is bound to the appropriate codon. Kurland (1972) summarises the evidence indicating that the codon-anticodon interaction is insufficiently strong to account for the stability and fidelity of aminoacyl-tRNA binding. The author reviews the evidence for the implication of both ribosomal RNAs and proteins in the binding and concludes that interactions involving ribosomal proteins are most likely involved in the stabilisation of the codon-anticodon interaction.

Kurland (1971) has proposed a model in which the codon and a group of proteins provide a kinetic barrier through which the most likely tRNA to pass is that with an anticodon complementary to the codon; the tRNA which passes this barrier is then non-specifically bound to the ribosome.

The situation concerned aminoacyl-tRNA binding in eukaryotes is essentially similar to that described for prokaryotes. A transfer or elongation factor, equivalent to the Tu + Ts complex, has been purified from a variety of sources, and in the absence of a uniform nomenclature will be referred to as TF_I . TF_I activity has been demonstrated in reticulocytes (McKeehan & Hardesty, 1969), rat liver (Gasior & Moldave, 1965), calf liver (Kloppstech et al., 1969) and from the plant sources,

wheat embryo (Jerez et al., 1969), yeast (Ayuso & Heredia, 1968; Richter & Klink, 1967).

A complex analogous to the bacterial Tu-GTP-aminoacyl-tRNA complex has been demonstrated (Ibuki et al., 1966) in rat liver, reticulocyte, wheat and yeast systems. The eukaryotic TF_1 factor can be replaced by the bacterial T factor in the binding of aminoacyl-tRNA to eukaryotic ribosomes and in polymerisation when supplemented with the eukaryotic translocase factor TF_2 (Krisko et al., 1969). However, eukaryotic TF_1 will not substitute for bacterial T factor on bacterial ribosomes. The eukaryotic TF_1 factor, like the T factor, has an aminoacyl-tRNA and ribosomal dependent GTPase.

After the attachment of the aminoacyl-tRNA to the ribosome, the α -amino group of the aminoacyl-tRNA is immediately involved in peptide bond formation with the carboxyl-terminal residue of the neighbouring initiator or peptidyl-tRNA present in the donor (P) site. The reaction is catalysed by the enzyme, peptidyl-transferase, which is a constituent protein of the 50S subunit (Monro et al., 1967; Maden et al., 1968). The reaction is completely independent of GTP or supernatant factors (Monro et al., 1967). Peptide bond formation has been shown to be independent of the 30S subunit by the use of methanol (33% v/v) treated 50S subunits. The alcohol causes an uncoupling of polymerisation from sequence control and other reactions in protein synthesis, since under these conditions peptidyl transfer between f-met-tRNA_f and aminoacyl-tRNA, and between f-met-aminoacyl-tRNA and another aminoacyl-tRNA, can occur giving rise to a series of random di-, tri-,

and tetrapeptidyl tRNAs (Monro, 1969).

When f-met-tRNA was digested with ribonuclease T1, a series of fragments were isolated containing various portions of 3' terminal-CAACCA-(f-met) fragment. Using such fragments in the so-called "fragment reaction" it was demonstrated that the minimum fragment able to attach to the peptidyl site and participate in peptidyl transfer, was the -CCA-(f-met). It was concluded that the interaction at the donor site of the peptidyl-transferase involved the 3'-terminal CCA moiety of the aminoacyl-tRNA species (Monro et al., 1968). The peptidyl-transferase activity has been assigned to the core of ribosomal proteins but is only active in the presence of the rest of the 50S subunit (Staehelin et al., 1969).

The peptidyl-transferase activity of rabbit reticulocytes was originally thought to be a function of the soluble transfer factor TF_2 (Arlinghaus et al., 1964). It has now been clearly demonstrated in both mammalian (Skorgerson & Moldave, 1968a; Vazquez et al., 1969) and plant/^{systems}(Neth et al., 1970) to be an integral part of the 50S subunit and is capable of carrying out the fragment reaction.

Following binding of the aminoacyl-tRNA and the formation of the first peptide bond, the peptidyl-tRNA is attached to the acceptor site and elongation stops since no further aminoacyl-tRNA can bind until the acceptor site is vacated and a new mRNA codon is exposed. Incubation of these ribosomes with factor G and GTP results in the binding of further aminoacyl-tRNA or puromycin, suggesting that G factor and GTP are involved in translocating the peptidyl-tRNA from the

acceptor to the donor site (subsequently releasing the free tRNA held on the donor site) (Haenni & Lucas-Lenard, 1968). The G mediated translocation is inhibited by fusidic acid (Haenni & Lucas-Lenard, 1968) and GMPPCP cannot substitute for GTP in the formation of peptidyl puromycin, hence GTP hydrolysis must be required for translocation. The role of G factor as a translocase was elegantly demonstrated (Erbe & Leder, 1968; Erbe et al., 1969), using the synthetic messenger AUG-UUUUUU as a template. Insertion of f-met in response to the AUG codon was shown to require purified initiation factors, and GTP. The formation of f-met-Phe by translation of the second codon and peptide bond formation required, in addition, T factor and GTP. Unless factor G was present the tripeptide f-met-Phe-Phe was not formed. Translation of the third codon required T factor, GTP and G factor. Thus, it was concluded that the G factor was essential for making the third codon available by the translocation of the peptidyl-tRNA and mRNA. A similar conclusion has been reached by Roufa et al. (1970), using Q β virus. Modolell et al. (1971) demonstrated that the G factor interacts with the 50S ribosomal subunit at a site which is distinct from the peptidyl-transferase, and that it enters and leaves the site during each round of protein synthesis.

Translocation of the peptidyl-tRNA from the acceptor to the donor site involves the removal, from the donor site, of the tRNA which has donated its peptidyl moiety. Lucas-Lenard & Haenni (1969) have demonstrated that G factor and GTP are involved in removal of the free tRNA and that discharge tRNA cannot be released from the ribosome unless

translocation takes place simultaneously. The rate of release of discharged tRNA was the same as the rate of translocation of the peptidyl-tRNA. Tanaka et al. (1971) studying the differential inhibition of the translocation and G factor catalysed release of deacylated tRNA by the antibiotics, fusidic acid and bottromycin A₂, suggest that G factor primarily participate in the release of deacylated tRNA and that translocation per se is catalysed by the 50S ribosomal subunit.

The mechanism whereby G factor interacts with the ribosome, mRNA and peptidyl-tRNA, to cause translocation, is uncertain. A ribosome-GTP-G factor complex has been observed by Millipore binding and gel filtration (Brot et al., 1969); however, its functions are uncertain.

One of the major problems still unresolved is the stoichiometry, role and site of GTP hydrolysis during peptide chain elongation. It was initially assumed that the energy liberated by factor G mediated GTPase was used to boost the carboxyl activation of amino acids, but the energy required for linking a peptide bond appears to be amply supplied by the energy-rich ester link between the amino acid or peptidyl-carboxyl group and the 3'-terminal adenosine in the tRNA (Nishizuka & Lipmann, 1966a). Assuming one molecule of GTP is hydrolysed during aminoacyl-tRNA binding and another is required for translocation, then considering only peptide chain elongation a stoichiometry of two molecules of GTP hydrolysed per peptide bond formed, would be expected. However, a ratio of 1:1 has been reported (Nishizuka & Lipmann, 1966b). This could be explained if the GTP hydrolysed in

aminoacyl-tRNA binding is coupled to translocation; this has, however, been disputed (Ono et al., 1969b).

The mammalian translocase factor, TF_2 , is in most respects similar to the G factor in that it complements TF_1 in polymerisation, it possesses a ribosome-dependent GTPase (Kaziro et al., 1969), is inhibited by fusidic acid, and is required, in conjunction with GTP, in the translocation reaction. In eukaryotic systems the donor site is reported to be filled only with peptidyl-tRNA as a result of translocation and peptidyl or analogues of peptidyl-tRNA cannot directly enter the donor site (Siler & Moldave, 1969). Baliga & Munro (1971) have shown that a TF_2 -GTP complex binds specifically at the acceptor site.

TF_2 is specific for mammalian ribosomes and cannot substitute for or be substituted by, bacterial G factor (Krisko et al., 1969). Unlike bacterial G factor, all mammalian TF_2 factors examined have been shown to be inhibited by diphtheria toxin in the presence of NAD (Lucas-Lenard & Lipmann, 1971).

Evidence obtained by the use of diphtheria toxin inhibition in the presence of factor TF_2 , suggests that less than 10% of the cells calculated content of TF_2 molecules are found in the high-speed supernatant. It is suggested that TF_2 remains attached to the ribosome and its release is dependent upon the movement of ribosomes along mRNA and perhaps upon dissociation of the ribosomes following chain termination (Gill et al., 1969).

The nature of the GTP-dependent mechanism whereby translocation of the peptidyl-tRNA from the acceptor to donor site

occurs, remains unclear. Woese (1970) has proposed a model based on the Fuller-Hodgson (1967) model of the anticodon loop of tRNA involving a GTP-mediated change in ribosome conformation allowing transition between two conformational states of the tRNA. There is, as yet, no evidence for such a model.

In addition to the transfer factors mentioned above Kuwano & Schlessinger (1970) have implicated cyclic-AMP (cAMP) in chain elongation in E. coli. cAMP associates with the G-factor and GTP and the above workers suggest that its role may be one of inhibition of activation of a ribosomal nuclease (ribonuclease-V) by factor G, GTPase, hence, suggesting the role of cAMP in regulating the balance between mRNA translocation and degradation by ribosomes.

(iv) Peptide chain termination

Following several rounds of peptide chain elongation the synthesis of the nascent protein is complete. It is, however, not released immediately upon completion, release of the nascent protein has been shown to be a dynamic process involving specific termination codons and a variety of protein factors.

The initial evidence for the implication of specific termination codons comes from the ochre and amber (nonsense) mutations of E. coli in which premature chain termination occurred (Brenner & Beckwith, 1965; Stretton & Brenner, 1965), as a result of gene mutations. Using suppressor mutations, i.e. strains of mutant E. coli capable of translating the nonsense mutations as an amino acid rather than as a terminator codon, the amber mutation was identified as UAG (Stretton &

Brenner, 1965) and the ochre mutation as UAA (Brenner & Beckwith, 1965). Brenner et al. (1967) later identified UGA as a terminator codon.

All attempts to find a specific terminator tRNA(s) which recognised the terminator codons were unsuccessful (Capecchi, 1967; Bretscher, 1968; Fox & Ganoza, 1968). Using an amber mutation in the phage R17 RNA which caused premature termination of the coat protein giving^a hexapeptide, Capecchi (1967) demonstrated the requirement for release of this fragment for a fraction (R) distinct from the elongation factors. The R factor was resolved into two components, R1 which translated UAA and UAG, and R2 which was required to translate UAA and UGA (Scolnick et al., 1968). An additional factor, S, was later identified by Milman et al. (1969). This factor alone had no release activity but stimulated the rate of release dependent upon a particular R factor and the correct codon.

Since R factors form a complex with ribosomes and the appropriate trinucleotide codon (Scolnick & Caskey, 1969), it is likely that an R factor-terminator-codon-70S ribosome intermediate is involved in terminator codon recognition.

There is some evidence for the participation of peptidyl-transferase in termination. Antibiotics that inhibit the peptidyl-transferase also inhibit the release reaction (Capecchi & Klein, 1969). Lucas-Lenard & Lipmann (1971) suggest that the termination step can be divided into a terminator codon-dependent R-factor binding reaction and a hydrolytic reaction in which the R factor alters the substrate specificity of the peptidyl-transferase so that it, in fact,

acts as a hydrolase, causing the transfer of the peptidyl moiety of peptidyl-tRNA to water rather than to aminoacyl-tRNA.

Evidence now suggests that following termination the completed polypeptide is released into the supernatant as the free peptide rather than attached to a tRNA molecule as peptidyl-tRNA. The free tRNA is thus left on the ribosome in the donor site. The means by which this is removed remains controversial. Lucas-Lenard & Haenni (1969) suggest that the G factor and GTP cannot release this tRNA, since there is no peptidyl-tRNA adjacent to it on the acceptor site to synchronise translocation with removal, and there is no evidence for a GTP or G requirement for termination. However, data of Tanaka et al. (1971) suggest that G factor is responsible for removing free tRNA from the peptidyl site. A factor (TR) has been isolated that releases tRNA from ribosomes when no translocation is involved (Kaji et al., 1969). However, the site at which such a factor is active remains unclear, whether it acts before dissociation of the 70S monosome or after, and its possible relationship to the dissociation factor (described later) (Davis, 1971), are yet to be resolved.

Whilst the terminator codons must be towards the 3' end of the sequence of the message to be translated, it is not a prerequisite that they, in fact, lie at the 3' terminus of the mRNA chain. Sequence studies of portions of the bacteriophage-R17-RNA reveal an intercistronic region of 10 codons between the 2, 3' terminal termination codons for the coat protein cistron and the 5' terminus of the synthetase cistron (Nichols, 1970).

Beaudet & Caskey (1971) using release factor preparations from reticulocytes, Chinese hamster liver or guinea-pig liver, in combination with rabbit reticulocytes, have demonstrated that the same three terminator codons probably act in the same way in eukaryotes. Essential differences have been noted, however, between the situation in prokaryotic and eukaryotic systems. All attempts to resolve the single mammalian release factor into different components have failed; the possibility exists however, that it is made up of subunits. Khairallah & Pitot (1967) have demonstrated a requirement for energy in the release of polysome-bound proteins of rat liver, whilst Goldstein et al. (1970) have shown that GTP stimulates the R-dependent release of f-met from an f-met-tRNA-ribosome complex.

Despite substantial evidence, mammalian peptide termination appears to proceed by a mechanism essentially similar to that in bacteria. There is no data available about the mechanism of protein chain termination in plant systems.

The participation of the 30S ribosomal subunit in the formation of the initiation complex and the subsequent translation of the mRNA by the 70S monomer, raises the problem as to the source of the 30S subunits involved in initiation. In order to maintain a pool of 30S subunits capable of initiation, it is necessary to postulate the dissociation of 70S monosomes after the completion of a round of protein synthesis, i.e. a sub-unit cycle in which dissociation of the 70S monosome yields a pool of subunits which are available for the initiation of a new round of protein synthesis and

which associate in the presence of mRNA forming the translating monosome which after termination is again dissociated. The subunit cycle in E. coli is well documented, but the precise role of the factors involved is still controversial.

Early studies by Tissieres et al. (1959) revealed the reversible dissociation of the 70S ribosome into a 30S and 50S subunit under conditions of low magnesium concentration. These "derived" subunits differed from the "native" subunits found in lysates of E. coli prepared in the presence of adequate magnesium in that they associated to form 70S ribosomes at increased magnesium concentrations. Both Tissieres et al. (1960) and Watson (1964) postulated some form of ribosomal cycle involving dissociation and re-association of subunits.

The first unequivocal demonstration of a physiological dissociation of the ribosome was provided by Kaempfer et al. (1968). An E. coli culture was grown initially in a medium containing heavy isotopes ($[^3\text{H}] [^{15}\text{N}]$) and then transferred to a medium containing light isotopes. Analysis of the ribosomes indicated extensive subunit exchange had occurred. Closer kinetic evidence with protein-synthesising extracts suggested that the ribosome undergoes subunit exchange in each round of translation (Kaempfer, 1968; Kaempfer & Meselson, 1969).

The unequivocal demonstration of a subunit cycle did not however, reveal the function of such a cycle. The observation that the initiation of translation of bacteriophage-f2-RNA takes place on the 30S subunit, and not on the 70S ribosome as had been supposed, offered an explanation for subunit exchange (Nomura & Lowry, 1967; Nomura et al., 1967). Nomura et al.

proposed a model of initiation in which the 30S subunit selectively recognises the initiation codon on mRNA and binds to it; f-met-tRNA then attaches to the 30S subunit to form the initiation complex. Evidence for the dissociation of 70S monosomes prior to initiation was obtained in experiments in which "heavy" 70S ribosomes were mixed with "light" 50S subunits, poly (AUG), f-met-tRNA and initiation factors. The presence of f-met-tRNA bound to hybrid 70S monosomes could only be explained by the dissociation of the "heavy" 70S monosomes and the association of a heavy 30S subunit with a "light" 50S subunit (Guthrie & Nomura, 1968).

That a subunit cycle linked to rounds of protein synthesis does exist is now generally accepted. What, however, remains controversial is the mechanism of and sequence of dissociation of the subunits.

One of the paramount difficulties has been the preparation of lysates which reveal the in vivo distribution of polysomes, monosomes and subunits.

The early work using drastic methods of disrupting bacterial cells has been criticised in that they yielded exceedingly high proportions of monosomes resulting from polysome degradation. Mangiarotti & Schlessinger (1966) described a gentle method of lysis which yielded only polysomes and subunits. It was assumed that other "gentle" methods which revealed significant proportions of 70S monosomes, resulted in degradation of polysomes and that the 70S monosome was completely artefactual. Because the cell appeared to lack 70S ribosomes it was concluded that a ribosome released at the end of a round of translation

immediately and spontaneously dissociated into a pair of subunits.

In order to test the assumption that ribosomes after their runoff from polysomes dissociate completely, Kohler et al. (1968) investigated the relative distribution of monosomes, subunits and polysomes under various conditions where runoff was increased. In all cases the decrease in polysome peak was paralleled by an increase in the 70S peak rather than in the subunits. Using other methods of runoff, MacDonald & Yeater (1968) and Algranati et al. (1969) independently obtained similar results. These runoff 70S monosomes lacked nascent polypeptides and mRNA and are more dissociatable than 70S monosomes derived from polysomal fragmentation. Phillips & Franklin (1969) suggest that runoff yields only free subunits and that the K^+ used in the preparation of lysates, unlike the physiological ion, Na^+ allowed the formation of 70S initiation complexes. Despite a considerable difference in the proportion of 70S monosomes obtained by different investigators, it now appears that the steady state 70S peak in growing cells is a mixture of free and complexed ribosomes (i.e. held together by the presence of peptidyl-tRNA). A further complication has been introduced by the demonstration by Infante & Krauss (1971) that high hydrostatic pressure in the centrifuge tube promotes ribosome dissociation.

Despite an increase in the 70S monosome peak following runoff, the subunit peak remained approximately constant suggesting that runoff ribosomes are not in spontaneous equilibrium with native subunits, but that the intervention of some factor is required for their dissociation. The factor has been called dissociation factor (DF), and it is argued (Davis,

1971) that since ammonium chloride treated ribosomes can initiate effectively when supplied with the known initiation factors, and because this process requires dissociation, that one of the initiation factors must function in dissociation. Subramanian et al. (1968) demonstrated that runoff but not complexed ribosomes were dissociated by a crude initiation factor preparation. Subramanian et al. (1969) identified DF as initiation factor F₃ and calculate that the concentration in the cell is in the correct order for one molecule per small subunit and its low affinity for the ribosome is consistent with a cyclic function in which it is bound at one stage and displaced at another.

The recognition of a dissociation factor however, does not resolve the conflict as to whether the runoff 70S monosomes were the initial product of runoff or were secondary products and that the initial product of termination was subunits. Kaempfer (1970) allowed "heavy" and "light" polysomes to run off together, the resulting 70S monosomes were of hybrid density, indicating that subunit exchange had occurred and that runoff yields subunits and that stable 70S monosomes are formed only when initiation is prevented. Although the specific point in the cycle at which dissociation occurs remains controversial, it seems that free monosomes are in rapid equilibrium, under physiological ionic conditions, with a very low concentration of free subunits. Such a conclusion has been reached independently by Infante & Baierlein (1971), who studied sedimentation patterns obtained under high hydrostatic pressures.

In contrast to the identification of DF as initiation

it is suggested by Miall et al. (1970) factor F_3 by Subramanian and Davis (1971), that DF is identical to initiation factor F_1 . These workers suggest that DF tends to aggregate in the absence of urea and under such conditions it would be retained on DEAE-cellulose along with F_3 , hence the DF activity in the F_3 fraction.

Kaempfer (1971) has presented data, based on density gradient profiles, to support the hypothesis that initiation factor F_3 does not function as a ribosomal dissociation factor as suggested by Subramanian & Davis (1971), but rather functions as an anti-association factor which prevents the reassociation of 30S and 50S subunits to form inactive 70S monosomes. Under conditions favouring synthesis, run-off from polysomes results in subunits directly. These subunits are prevented from re-associating by F_3 . When conditions do not favour protein synthesis, the supply of subunits exceeds that of F_3 , and they recombine and are sidetracked into an inactive pool of single monosomes which only slowly dissociate into subunits.

Subramanian & Davis (1971) however, using glutaraldehyde fixed ribosomes, have demonstrated a rapid exchange of free monosomes and subunits, an obvious contradiction to the slow dissociation proposed by Kaempfer. They postulate that F_3 can act reversibly, so it cannot only dissociate ribosomes but can catalyse an exchange of subunits by mediating a subunit association to form monosomes.

The aim of the present work was to investigate some aspects of the mechanism of protein synthesis in an in vitro system prepared from Vicia faba (L). Previous work in the Botany Departments of the Universities of Liverpool and Durham

has resulted in the development of a crude, homologous, complete amino acid incorporating system prepared from either the developing cotyledon or plumules of germinated beans. This system utilises a crude microsomal preparation containing free and membrane-bound ribosomes and polysomes, and the unfractionated 105,000 x g supernatant as a source of the enzymes involved in protein synthesis. In addition the crude tRNA used is of low and variable acceptor capacity. Work was initially directed towards the partial purification and characterisation of these components. Since developing beans are available for only a limited period throughout the year, germinated beans have been used as a source of material and the interchangeability of components from various sources investigated. An attempt was made to resolve the enzymes involved in peptide chain elongation; however, in view of the limited success of other works in this field, an attempt was made simultaneously to investigate the mechanism of initiation on the 80S ribosome of Vicia faba (L).

MATERIALS AND METHODS

1. Biological Materials

A. Developing seeds

Seeds of Vicia faba (L) cv. Triple White, were obtained from Tyneside Seed Co. Ltd., Sunderland. Plants were grown in the Botanical Gardens, University of Durham. Seeds planted prior to the end of March were sown in pots in heated greenhouses, and the seedlings planted outdoors at the end of March. Sowings after this date were made directly in the open. Daily observations were made and the flowers labelled on the first day of opening. Pods were collected immediately prior to use and the material standardised by discarding material differing significantly in size from the norm. The material was further standardised by selecting seeds of approximately the same size from each pod. All such selections were subjective. It was assumed that pollination occurred on the day that the flower opened and hence the age of the material given in the text refers to the number of days that the pod had developed after flower opening. Previous work (Wheeler, 1965; Briarty, 1967) has shown that this method results in material which is on average the same age.

B. Germinating seeds

Dry seeds of V. faba (L) cv. Triple White, were surface sterilised by immersing in calcium hypochlorite solution containing 3.5% available chlorine, for two min. The hypochlorite solution was decanted and the seeds further washed with cold running tap water for 8 hours. The seeds

were then soaked overnight in fresh, still tap water at room temperature. The total washing/soaking time was 24 hr.

Prior to sowing the seeds were again surface sterilised and washed with sterile tap water until they no longer smelled of chlorine. The seeds were sown in seed trays lined with absorbent paper towelling moistened with sterile tap water, in such a way that there was no contact between neighbouring seeds. The trays were covered with glass and germination allowed to proceed in the dark at 30° for 72 hr, after which time the plumules had emerged from beneath the testa and were about 1 cm in length.

The plumule was cut off just behind the plumular hook and the region including plumular hook and leaves collected in a beaker on ice. Plumules were either used immediately or quick frozen in liquid nitrogen and stored at -70°. There was no significant deterioration in amino acid incorporating activity of components prepared from such material after three months storage.

C. Rats

Male, albino rats, type Wistar, were obtained through the Department of Zoology, University of Durham.

D. Rabbit reticulocyte fractions

Regular and deoxycholate treated ribosomes, 40/70 enzyme fraction and deacylated tRNA prepared from rabbit reticulocytes were a gift of Dr. Boyd Hardesty, Clayton Foundation Biochemical Institute and Department of Chemistry, the University of Texas, Austin, Texas, U.S.A. (Mosteller et al., 1967; Lin et al.,

1966; Ravel et al., 1966).

E. A frozen paste of Escherichia coli strain M.R.E. 600 was a gift of Professor K. Marcker, University of Aarhus, Denmark.

2. Chemicals and Reagents

With the exceptions listed below, all chemicals were obtained from British Drug Houses (B.D.H.) Ltd., Poole, Dorset, and were of analytical grade:-

Adenosine-5'-triphosphate, disodium salt (ATP),

Guanosine-5'-triphosphate, sodium salt (GTP),

Creatine phosphate,

Phosphocreatine kinase (PCK),

Triton X100,

2-Mercaptoethanol (BME),

Polyuridylic acid, ammonium or potassium salt (poly(U)),

Trizma base (Tris), Analytical grade,

Bovine serum albumin Fraction V (BSA),

Ribonuclease A from Bovine Pancreas Type 1A,

T₁ ribonuclease,

Calcium phosphate gel,

Puromycin

Ribonuclease T₁ from Aspergillus oryzae (Grade 111)

were obtained from The Sigma Chemical Co. Ltd., London.

L-amino acid, "A" grade,

Dithiothreitol (DTT),

Soluble ribonucleic acid from Escherichia coli (E. coli), were obtained from The California Corporation for Biochemical Research, Los Angeles, U.S.A.

2,5-Diphenyloxazole (PPO),
1,4-bis(2-(5-phenyloxazolyl))-benzene (POPOP),
Ninhydrin,
Glutathione, reduced (GSH),
were obtained from Koch-Light Laboratories Ltd., Colnbrook,
Bucks.

Radiochemicals were obtained from The Radiochemical Centre,
Aylesbury, Bucks. and were:-

| | |
|--|-------------------------------|
| L-phenyl(alanine)-1- [^{14}C] | Sp. act. 50 mc/mM |
| Lysine | Sp. act. 310 mc/mM |
| L-methionine- [^{35}S] | Sp. act. - as quoted in text. |

Soluble ribonucleic acid from Brewer's yeast was obtained
from Boehringer Mannheim Biochemicals, London.

Rhodium on alumina was obtained from Johnson Matthey
Chemicals Ltd., London.

R.B.S. 25 from Chemical Concentrates Ltd., London.

Repelcote from Hopkin and Williams.

Polyadenylic acid (poly(A)), mol. wt. 100,000,

Poly-DL-lysine,

Poly(AUG) (1:2:5:1),

Poly(UG) (1:1),

ApUpG

were obtained from Miles Labs. Inc., Elkhart, Indiana, U.S.A.

Trypsin from Worthington Biochemical Comp., Freehold, New
Jersey, U.S.A.

Other materials

Visking dialysis tubing was obtained from The Scientific
Instrument Centre Ltd., Leeke St., London, W.C.1.

Millipore filter HAWP 00010 from Millipore Corporation, Bedford, Mass., U.S.A.

Sephadex G25, G200,

DEAE-Sephadex A50,

Sepharose 4B,

were obtained from Pharmacia, Uppsala, Sweden.

Chromatography and filter paper from Whatman.

Benzoylated DEAE-Cellulose (BD-cellulose), 50-100 mesh, was obtained from Schwarz Bioresearch Ltd., Orangeburg, New York, U.S.A.

Amicon PM30 ultrafiltration membranes were obtained from Amicon (N.V.), Oosterhout (N.B.), Holland.

3. Preparation of reagents and solutions.

Solutions used in the isolation of sub-cellular particles.

Extractant A.

0.05M-Tris/HCl buffer, pH 7.6 at 0°; 0.5M-sucrose, 5mM-magnesium chloride; 25mM-potassium chloride and 5mM-2-mercaptoethanol. The mercaptoethanol was added immediately before use.

Resuspending Medium A.

0.01M-Tris/HCl buffer, pH 7.6 at 0°; 1mM-magnesium chloride, 25mM-potassium chloride, 5mM-reduced glutathione (GSH).

Resuspending Medium B.

This was identical to Resuspending medium A but mercaptoethanol was replaced by 5mM-dithiothreitol (DTT). This solution replaced Resuspending medium A in later experiments.

Ammonium chloride wash solution for ribosomes.

0.05M-Tris/HCl buffer, pH 7.6 at 0°; 10mM-magnesium chloride; 0.5M-ammonium chloride.

Solutions used in the preparation of *E. coli* DE52 supernatant.

Extractant.

10mM-Tris/HCl, pH 7.8 at 0°; 10mM-magnesium chloride; 60mM-potassium chloride, 5mM-2-mercaptoethanol. The mercaptoethanol was added just before use.

Scintillation fluid.

2,5-Diphenyloxazole 4.5 g
1,4-bis(2-(5-phenyloxazolyl))-benzene 0.1 g
Toluene to 1l

[¹²C]-protein amino acid mixture - phenylalanine

| | | | |
|-----------------|----------|------------|----------|
| Alanine | 0.00891g | Leucine | 0.01312g |
| Arginine | 0.02102g | Lysine | 0.01827g |
| Aspartic acid | 0.01331g | Methionine | 0.01492g |
| Asparagine | 0.01501g | Proline | 0.01151g |
| Cysteine | 0.01756g | Serine | 0.01051g |
| Glutamic acid | 0.01472g | Threonine | 0.01191g |
| Glutamine | 0.01462g | Tryptophan | 0.02042g |
| Glycine | 0.00751g | Tyrosine | 0.01812g |
| Histidine | 0.02096g | Valine | 0.01172g |
| Isoleucine | 0.01312g | | |
| Water to 100 ml | | | |

[¹²C]-protein amino acid mixture - lysine

The lysine in the above amino acid mixture was omitted and replaced by 0.01652 g of phenylalanine.

[¹²C]-protein amino acid mixture - methionine

The methionine in the former amino acid mixture was omitted and replaced by 0.01652 g of phenylalanine.

Ionophoresis buffer.

Pyridium acetate, pH 3.5 (Pyr:Acetic 0.3:3.33%)

| | |
|---------------------|---------|
| Pyridine | 10 ml |
| Glacial Acetic acid | 100 ml |
| Water | 2890 ml |

4. Extraction of sub-cellular particles

Precautions were taken throughout the preparation to minimise the risk of microbial contamination. Where possible, all glassware and solutions were sterilised before use. Glassware was autoclaved before use. Volumetric glassware was rinsed with "Analar" diethylether, and dried. Pipettes were plugged with cotton wool and autoclaved. All solutions were prepared using sterile distilled water.

A. Extraction of microsomes from the cotyledons of developing seeds of *V. faba*

All stages in the preparation were carried out at 0-4°. Beans were harvested as previously described. The pods were surface sterilised with alcohol, the seeds harvested and their testas removed using sterile scalpel and forceps. Cotyledons were rapidly ground in a 1:1 ratio with Extractant A, in an ice-cold mortar using cold acid washed sand. The resultant brei was centrifuged at 1,000 x g av. for 5 min in the 4 x 500 ml swingout rotor of an M.S.E. Mistral 4L centrifuge, and the supernatant decanted off. The pellet was reground with a further volume of Extractant A and again centrifuged. The

two supernatant fractions were combined and mitochondria and bacteria pelleted at 38,000 x g av. for 15 min in the 8 x 50 ml angle rotor of an M.S.E. 18 centrifuge. The supernatant (post-mitochondrial supernatant) was decanted through sterile gauze, previously dampened with cold Extractant A. Microsomes were pelleted at 105,000 x g av. for 90 min. in the 10 x 10 ml titanium angle rotor of an M.S.E. Superspeed 65 centrifuge. The supernatant (high-speed or 105,000 x g supernatant) was decanted through sterile gauze dampened with Extractant A, quick frozen in liquid nitrogen and stored until required at either -70° or in liquid nitrogen. The centrifuge tubes containing the pellets were inverted over absorbent paper and allowed to drain for 1 min, any remaining liquid was removed using tissue. The surface of the pellets and sides of the centrifuge tubes were washed twice with Resuspending medium A and finally the pellets were homogenised in Resuspending medium A using a manually operated Teflon homogeniser. The concentration was adjusted to 20 mg microsomes/ml as determined from the $E_{1\text{ cm}}^{260}$ measured in a Unicam SP800 spectrophotometer (see Section 5 below). The microsomes were stored in small batches at -70° or in liquid nitrogen.

B. Extraction of microsomes from the plumules of germinating seeds of *V. faba*

Plumules were removed from 72 hr germinated seedlings and batches of 100 were ground to a fine powder under liquid nitrogen in a mortar and pestle. Plumules which had been stored in liquid nitrogen were treated similarly, without allowing them to thaw. The frozen powder was transferred

to a rimless thick-walled Pyrex boiling tube and homogenised in 10 ml of Extractant A with 6 strokes of a Teflon homogeniser at a speed of 400-600 r.p.m. with a tube clearance of 0.25-0.33 mm. The combined homogenates were centrifuged at 1,000 x g av. for 5 min. The supernatant was decanted through sterile gauze previously moistened with the extractant. The pellet was rehomogenised, the homogenate centrifuged, and the supernatant filtered. Mitochondria and bacteria were removed from the combined supernatants as described for developing seeds, and microsomes pelleted at 105,000 x g av., as previously described. The centrifuge tube and pellets were washed in the same manner as for developing seeds, and microsomes were resuspended in Resuspending medium A at a concentration of 20 mg microsomes/ml.

C. Clarification of microsomal preparations

Before use, microsomal preparations from both developing and germinating seeds were clarified by centrifugation at 30,000 x g av. for 5 min. The concentration was determined as described in Methods, Section 5.

D. Extraction of ribosomes from the cotyledons of developing seeds and plumules of germinating seeds using Triton X100

The procedure was a modification of that described for the extraction of microsomes from cotyledons of developing seeds and from the plumules of germinating seeds. For the extraction of ribosomes from germinating seeds the filtered post-mitochondrial supernatant was adjusted to 1% (v/v) Triton X100 using a solution of 10% (w/w) Triton X100 in 0.05M-Tris, pH 7.6 at

4°, and the solution allowed to stand in ice for 15 min. In the case of developing seeds the post-mitochondrial supernatant was adjusted to 4% (v/v) Triton X100. Ribosomes were pelleted at 105,000 x g av. for 120 min in the 10 x 10 ml angle rotor of an M.S.E. Superspeed 65 centrifuge as previously described. The supernatant was decanted off and discarded, the pellets and sides of the tube were washed as before with Resuspending medium A. The pellets were pooled, resuspended in Resuspending medium A and ribosomes repelleted at 105,000 x g av. as above. The ribosomes were finally resuspended in Resuspending medium A at a concentration of 20 mg ribosomes/ml as determined by the $E_{1\text{ cm}}^{260}$, and stored in small batches at -70° or in liquid nitrogen.

E. Extraction of ribosomes from the cotyledons of developing seeds and from the plumules of germinating seeds using sodium deoxycholate

The procedure was based on that described for the isolation of microsomes from the cotyledons of developing seeds and from the plumules of germinating seeds. To the filtered post-mitochondrial supernatant was added, dropwise, sodium deoxycholate solution (5%, w/v) to a final concentration of 0.5% (v/v). The solution was allowed to stand in ice for 15 min; ribosomes were then pelleted at 105,000 x g av. for 120 min, as described above. The pellets were resuspended in Resuspending medium A and freed of remaining detergent by repelleting as described in Methods, Section 4D. The pellet was finally resuspended in Resuspending medium A at a concentration of 20 mg ribosomes/ml, and stored in liquid nitrogen or at -70° in small batches.

5. Determination of the concentration of microsomal and ribosomal suspensions

The concentration of microsomal and ribosomal suspensions was determined from the $E_{1\text{ cm}}^{260}$ of suitable dilutions, assuming an optical density of 11.3 for a 1 mg/ml suspension of microsomes or ribosomes (Ts'o & Vinograd, 1961).

6. Preparation of ammonium chloride washed ribosomes

Ribosomes were prepared as described in Methods, section 4D. After resuspension and washing in Resuspending medium A, the ribosomes were pelleted at 105,000 x g av. for 120 min, as previously described. The pellet was resuspended in ammonium chloride wash solution (approximately 60 mg ribosomes/20 ml wash fluid) and allowed to stand in ice for 15 min. The resultant white flocculent precipitate was removed by centrifugation at 30,000 x g av. for 5 min and the pellet discarded. Ribosomes were pelleted from the supernatant by centrifugation at 105,000 x g av. for 120 min. The ribosomal pellets were again resuspended in ammonium chloride wash solution, left in ice and the precipitate removed by centrifugation as above. Ribosomes were finally pelleted as above. The pellet was resuspended at a concentration of approximately 30 mg ribosomes/ml Resuspending medium A, using a manually-operated Teflon homogeniser. The ribosomal suspension was transferred to a pre-soaked dialysis sac and dialysed for 4 hr at 0° against 4 x 1 l 0.05M-Tris/HCl, pH 8.0 at 0°; 5mM-2-mercaptoethanol. The concentration of ribosomes was determined as described in section 5. Ribosomes were stored in small batches in liquid nitrogen.

7. Isolation of ribosomes from stored microsomal preparation

Attempts were made, using detergents, to isolate ribosomes from stored (4 months) preparations of microsomes from developing seeds.

A. Using sodium deoxycholate

The method used was based on that of Ravel et al. (1966). Previous work (Payne, 1968) showed that 0.5% (v/v) sodium deoxycholate was sufficient for complete solubilisation of microsomal membranes, and this concentration was used in preference to the 1% used by Ravel et al. (1966), where removal of transfer factors from the ribosomes was also involved.

All operations were carried out at 0°. Stored microsomes were allowed to thaw to 0° in ice, and the microsomal suspension slowly brought to 0.5% (v/v) sodium deoxycholate using 5% (w/v) sodium deoxycholate in Resuspending medium A. After standing in ice for 15 min the suspension was centrifuged at 30,000 x g av. for 5 min. The clarified supernatant was decanted off and ribosomes pelleted at 105,000 x g av. for 120 min. The pellet was washed as described in Methods, section 4D, resuspended by homogenisation in Resuspending medium A and ribosomes pelleted from the suspension at 105,000 x g av. for 120 min. The tube and pellet were washed as before and finally resuspended in Resuspending medium A at a concentration of 20 mg ribosomes/ml. The ribosomes were stored in small batches at -70° or in liquid nitrogen.

B. Using Triton X100

The procedure was carried out at 0°. Thawed microsomes

were diluted with Resuspending medium A to a concentration of 10 mg microsomes/ml. The suspension was constantly agitated while Triton X100 was added drop-wise to a concentration of 4% (v/v) and the solution was stirred in ice for 30 min. The suspension was clarified by centrifugation at 30,000 x g av. for 5 min, the supernatant decanted, and ribosomes pelleted at 105,000 x g av. for 120 min. The tubes and pellets were washed as previously described and ribosomes finally sedimented from the resuspended pellet. The ribosomes were stored at a concentration of 20 mg ribosomes/ml as previously.

8. Preparation of enzyme fractions from the 105,000 x g. supernatant fraction derived from microsomal preparations

A. Dialysis of supernatant

This method was applicable to either freshly prepared or stored 105,000 x g supernatant obtained from microsomal preparations from either developing or germinating seeds. All steps were carried out at 0-4°. Frozen supernatant was thawed at 0-4° in ice. Material was transferred to a previously soaked and washed dialysis sac and dialysed for 18 hr against a 1000-fold excess of buffer (5 mM-Tris/HCl, pH 7.6 at 4°, 1mM-EDTA and 1mM-2-mercaptoethanol). Any precipitate was removed by centrifugation for 15 min at 1,000 x g av. Protein was estimated by the Lowry Folin method (see Methods, section 10). The preparation, which is referred to as dialysed supernatant, was stored batchwise either in liquid nitrogen or at -70° until required.

B. Concentration of the 105,000 x g supernatant by freezing

The 105,000 x g supernatant fraction from microsomal

preparations was originally stored frozen at -20° in conical flasks. The initial freezing was carried out with the flasks in an inclined position to minimise the risk of breakage on expansion of the contents. Once the contents were frozen, the flasks were returned to the vertical position for ease of storage so that the frozen supernatant now formed a layer against one side of the flask and was inclined to the bottom of the flask at a steep angle. After one to two weeks storage the appearance of the flask contents changed as a viscous yellow component, originally trapped in the ice, drained down to the bottom of the flask leaving almost clear ice at the top of the flask. The viscous material which could be easily separated from the ice, appeared to be a concentrated extract of many of the components of the original supernatant in strong sucrose solution. Since preliminary experiments showed that this relatively concentrated protein/^{solution} contained all the soluble components (normally derived from the supernatant) required for in vitro incorporation of amino acids in the cell-free system, this differential freezing technique was adapted and employed as an initial concentrating step for those components. Large volumes of supernatant were frozen in measuring cylinders inclined a few degrees above the horizontal, and the cylinders returned to the vertical to allow the viscous unfrozen material to drain down. This was then collected and either used as the starting material for the various preparative procedures described below, or else stored at -70° until required.

C. Chromatography of the 105,000 x g supernatant on Sephadex G25

An attempt was made to further purify the 105,000 x g dialysed supernatant by chromatography on Sephadex G25. The "dialysed supernatant" was applied to a 4.0 x 60 cm column of Sephadex G25, previously equilibrated with 0.05M Tris/HCl, pH 7.6 at 0°, 1mM 2-mercaptoethanol. Protein was eluted at a flow rate of 240 ml/hr using the same buffer. An Isco u.v. monitor was used to follow the $E_{0.5}^{280}$ of the effluent and the broad peak of 280 nm absorbing material eluting immediately after the void volume was collected, the elution being continued until the O.D. fell to the base line value (Fig. 3). The concentration of protein in the eluant was determined using the Lowry Folin method (see Methods, section 10) and if necessary the solution was concentrated by ultrafiltration (see below). The protein solution was stored in small batches in liquid nitrogen or at -70°. The preparation was referred to as G25 supernatant.

D. Removal of nucleic acids from the 105,000 x g supernatant via chromatography on DEAE-cellulose

The method described was used for the removal of nucleic acids from either dialysed or G25 supernatant obtained from germinating or developing beans. The supernatant was absorbed on to a 2.4 x 15 cm column of DEAE-cellulose (Whatman DE52), chloride form, previously equilibrated with 0.05M Tris/HCl pH 7.6. Protein was eluted from the column at a flow rate of 15 ml/hr using 0.05M-Tris/HCl, pH 7.6; 0.15M-sodium chloride. The absorption at 280 nm was monitored using an Isco u.v. monitor and the broad peak of 280 nm absorbing material which eluted after the column volume, was collected (Fig. 4). Protein was estimated by the Lowry Folin method

Fig.3. Chromatography of dialysed 105,000xg supernatant on Sephadex G25. 50 ml of supernatant were applied to the column and eluted as described in the methods (Section 8c.)

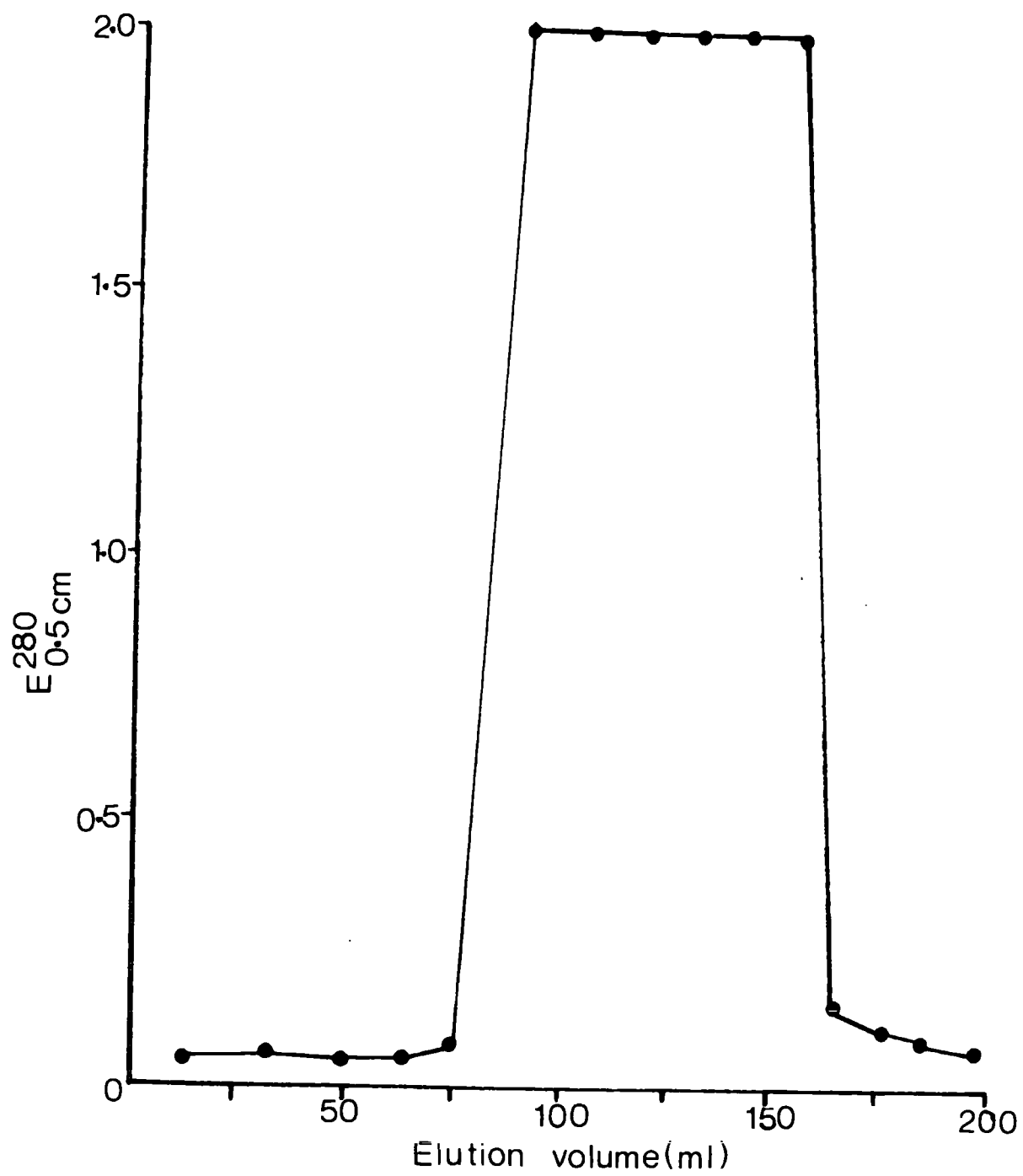
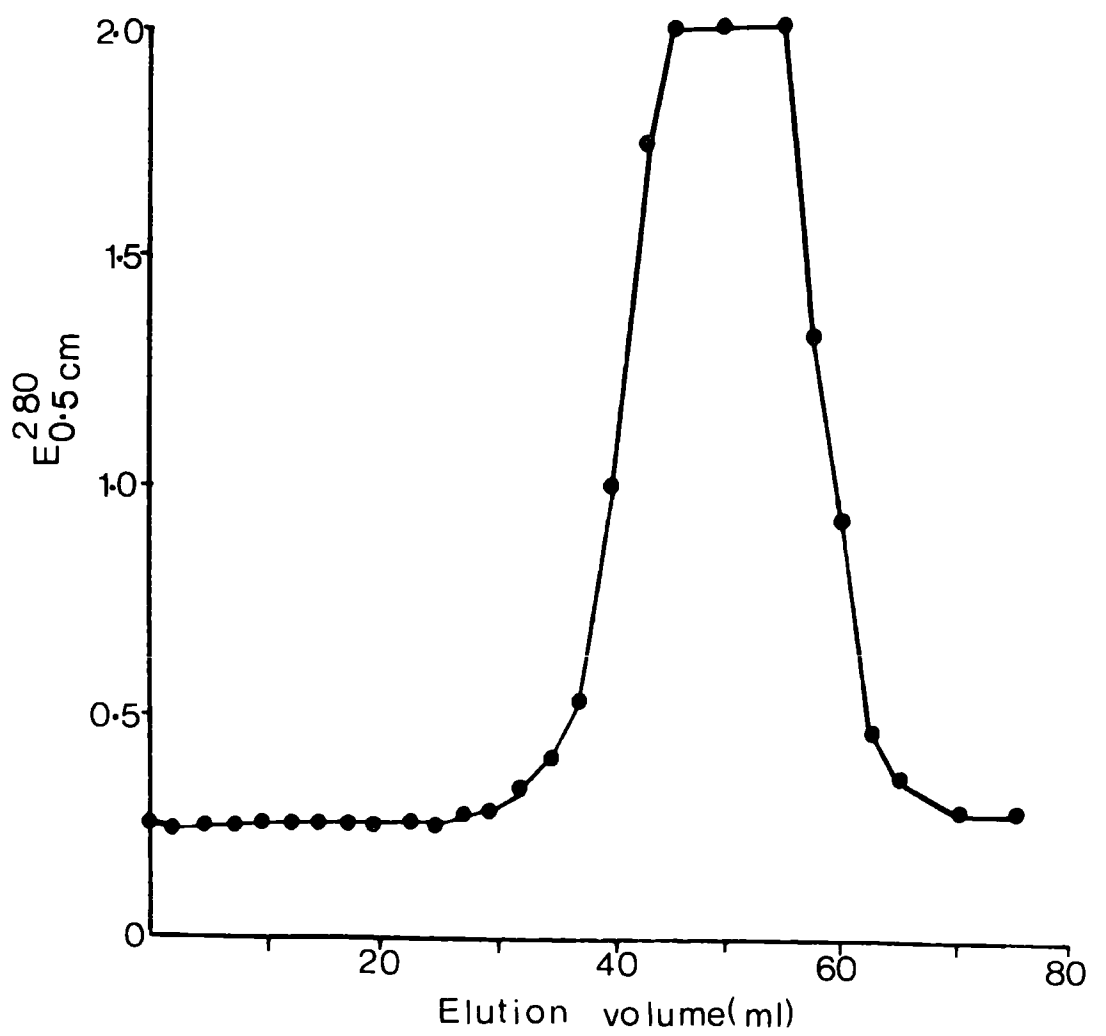
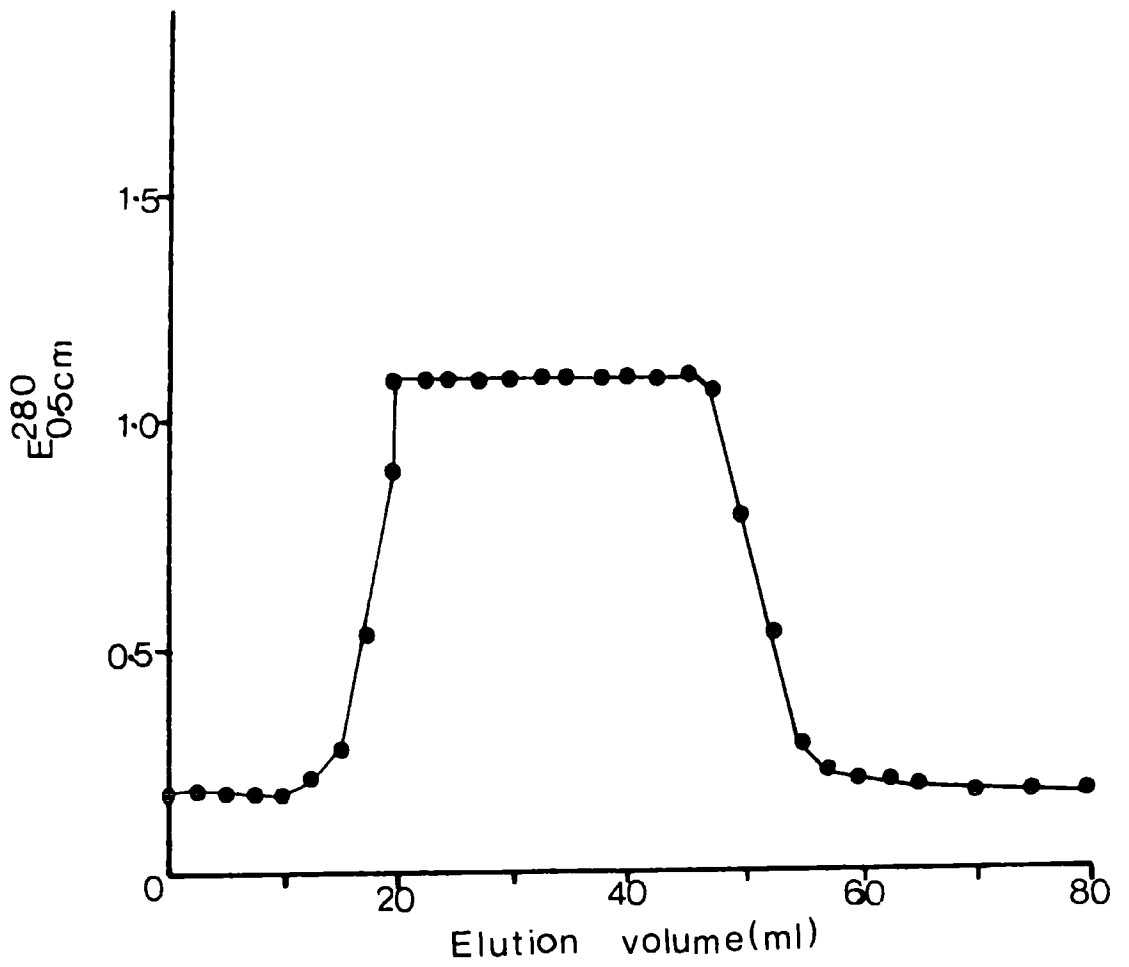


Fig.4. Chromatography of V.faba 105,000xg supernatant on DEAE-cellulose. 20 ml of either dialysed or G25 supernatant were applied to the column and eluted as described (Methods, section 8D).

Fig.5. Chromatography of E.coli S100 on DEAE-cellulose. 20 ml of S100 were applied to the column and eluted as described (Methods, section 9).



(see Methods, section 10) and the supernatant, free of nucleic acids, was stored in batches as previously. This preparation will be referred to as DE52 supernatant.

E. Concentration of enzyme fractions by ultrafiltration

The concentration of protein in the above supernatants was often too low to enable an optimum volume to be added to incubations, hence it was concentrated by ultrafiltration.

The procedure was carried out at 4°. The apparatus used was an Amicon Diaflo ultrafiltration cell, fitted with a washed PM 30 membrane. Ultrafiltration was effected at a pressure of 75 p.s.i. nitrogen. The volume was reduced by a calculated amount, the cell depressurised and the supernatant removed and, if necessary, clarified at 1,000 x g av. for 15 min, and the filtrate was discarded. The concentration of protein was estimated by the Lowry Folin method (see Methods, section 10), and the supernatant stored in small batches as previously.

F. Concentration of DE52 supernatant by precipitation with ammonium sulphate

DE52 treated 105,000 x g supernatant was brought to 70% saturation (at 0°) with respect to ammonium sulphate, by the slow addition, at constant pH, of solid ammonium sulphate. The solution was allowed to stand in ice for 30 min. The precipitated protein was pelleted at 38,000 x g av. for 15 min and the supernatant discarded. The pelleted protein was dissolved in buffer (5mM-Tris/HCl, pH 7.6 at 0°; 1mM-EDTA; 1mM 2-mercaptoethanol) and dialysed against three changes of a 1000-fold excess of the same buffer. The supernatant was

stored in small batches in liquid nitrogen. Protein was estimated as previously.

9. Preparation of Escherichia coli DE52 supernatant

A frozen paste of E. coli was thawed to room temperature and mixed with an equal weight of extractant until a homogeneous mixture was obtained. The bacteria were lysed by a process of freezing and thawing; the slurry was quick frozen in liquid nitrogen and thawed to 37°, the process was repeated four times. A further 4 volumes of extractant were added and the resultant slurry mixed well to a homogeneous suspension. The suspension was centrifuged at 105,000 x g av. for 120 min and the pellet discarded. The supernatant (S100) was decanted through previously wetted gauze and nucleic acids removed by chromatography on DEAE-cellulose (Whatman DE52). The S100 was absorbed onto a 1 x 19 cm column of DEAE-cellulose DE52, chloride form, which had been equilibrated overnight with extractant. Protein was eluted at a flow rate of 20 ml/hr using a buffer containing 0.05M-Tris/HCl, pH 7.5 at 0°; 0.15M-sodium chloride; 10mM-magnesium chloride; 60mM-potassium chloride and 5mM-2-mercaptoethanol. A broad peak of 280 nm absorbing material (Fig. 5), which eluted after the void volume, was collected. The $E_{0.5\text{cm}}^{280}$ of the effluent was monitored using an Isco u.v. monitor and elution continued until the optical density fell to a base line value. The effluent was dialysed overnight against a 1,000-fold excess of extractant. Protein was estimated using the Lowry Folin method. The solution, which was referred to as E. coli DE52 supernatant, was stored in small batches in liquid nitrogen.

10. Estimation of protein

Protein was determined by the method of Lowry et al. (1951). A calibration curve was prepared using standard solutions of bovine serum albumin (Fig. 6), and $E_{1\text{ cm}}^{750}$ was measured using a Hilger Watts Uvispek spectrophotometer.

11. Extraction of transfer ribonucleic acid from developing seeds of *Vicia faba*

Developing seeds of approximately 50-60 days old were harvested and the pods discarded. 500 g seeds were homogenised with 500 ml of a 1:1 mixture of 90% (w/w) aqueous phenol (redistilled): buffer (0.1M-Tris/HCl, pH 7.6 at 0°; 3mM-magnesium chloride; 24mM-potassium chloride and 5mM-2-mercaptoethanol) in a Waring blender at 0° for 5 x 30 sec at half-speed, followed by 3 x 30 sec at full-speed. The resultant brei was stirred at room temperature for 1 hr, cooled in ice for a further 1 hr, and centrifuged at 5,000 x g av. for 15 min to remove cell debris. The supernatant was decanted and the phases separated by centrifugation at 38,000 x g av. for 30 min. The upper aqueous phase was removed, care was taken not to disturb the precipitated protein at the interphase. 1.0M-potassium acetate, pH 6.0, was added to the separated aqueous phase to give a final concentration of 0.1M. Nucleic acids were precipitated by the addition of 2 volumes of 95% ethanol, the solution was stirred vigorously and precipitation allowed to proceed at -20° for 2 hr. The precipitate was recovered by centrifugation at 5,000 x g av. for 30 min, and the supernatant discarded. The pellet was drained of excess ethanol and dissolved in 200 ml. 0.1M-potassium acetate, pH 6.0; any

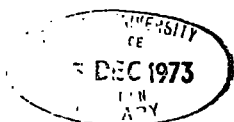
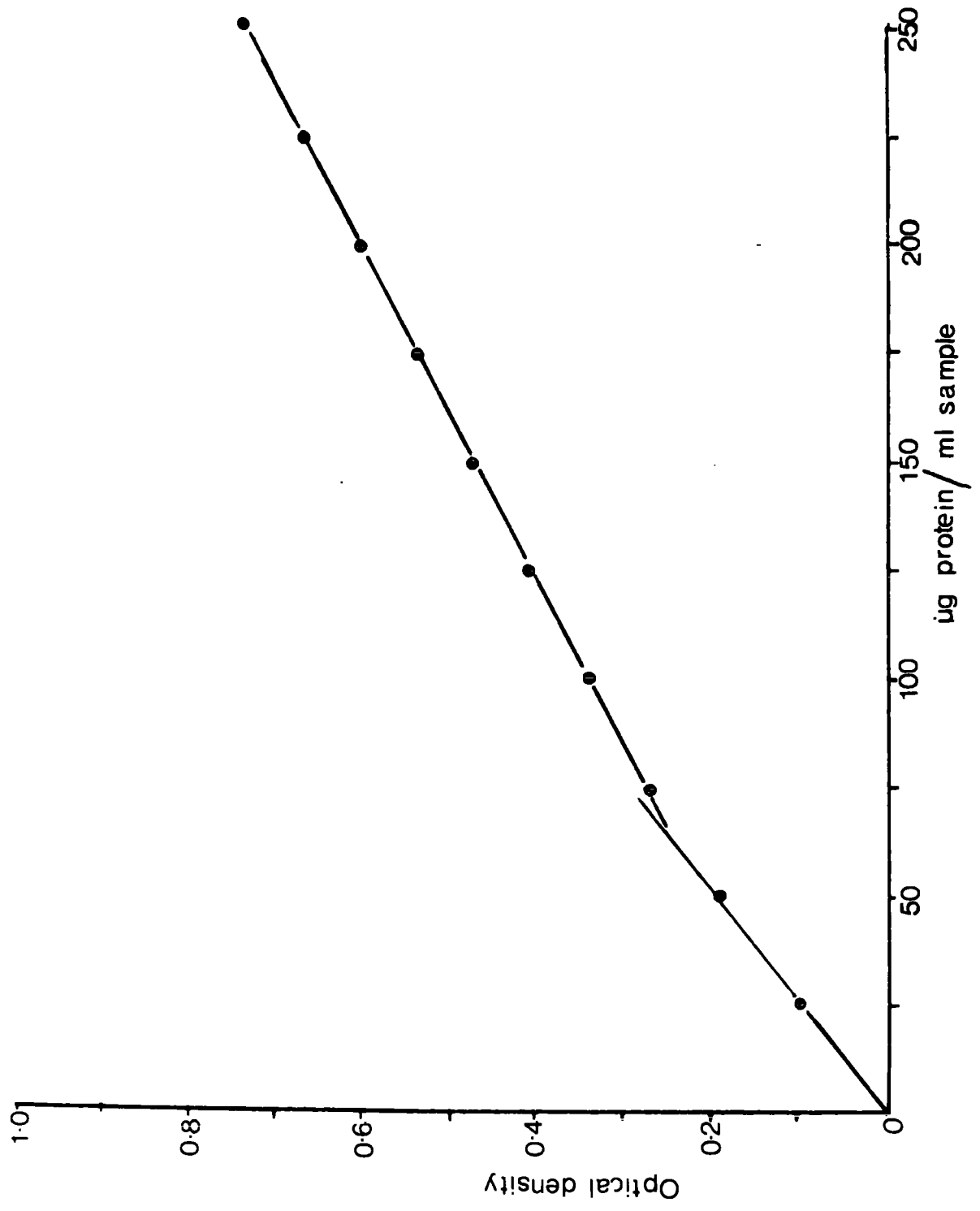


Fig.6. Calibration graph for the determination of protein by the method of Lowry et al.(1951).



insoluble material was removed by centrifugation at 5,000 x g av. for 30 min. Two volumes of ethanol were added to the supernatant and nucleic acids were precipitated at -20° for 2 hr. The pellet was recovered by centrifugation at 5,000 x g av. for 30 min and drained of excess ethanol. The pellet was dissolved in 1.8M-Tris/HCl, pH 8.9; 5mM-2-mercaptoethanol, and the tRNA deacylated by incubation at 37° for 45 min. Any insoluble material was removed by centrifugation at 5,000 x g av. for 30 min, and nucleic acids were precipitated by the addition of 2 volumes of 95% ethanol and precipitation completed at -20° overnight. The precipitated nucleic acids were recovered by centrifugation at 5,000 x g av. for 30 min and dissolved in a 50mM-Tris/HCl, pH 7.5 at 0° ; 5mM-2-mercaptoethanol. tRNA was separated from other nucleic acid species by chromatography on DEAE-cellulose, DE52. The nucleic acid solution was absorbed on to a 15 x 2.4 cm column of DE52, chloride form, equilibrated with 0.05M-Tris/HCl, pH 7.5 at 0° , 5mM-2-mercaptoethanol. The solution was washed into the column using the same buffer and the column was washed with the buffer until the $E_{0.5\text{cm}}^{260}$ fell to a background value. The $E_{0.5\text{cm}}^{260}$ of the column effluent was monitored using an Isco u.v. monitor. tRNA was eluted from the column with 1.0M-sodium chloride, 5mM-2-mercaptoethanol. The broad peak of 260 nm absorbing material was collected and tRNA precipitated by the addition of 2 volumes of 95% ethanol. The pellet was finally recovered by centrifugation at 5,000 x g av. for 30 min, drained of excess ethanol, and finally dissolved in 0.05M-Tris/HCl, pH 7.5 at 0° ; 5mM-2-mercaptoethanol, at a

concentration of 10 mg tRNA/ml. The tRNA was stored in liquid nitrogen until required.

12. Spectrophotometric estimation of tRNA concentration

tRNA samples were routinely dissolved in 0.05M-Tris/HCl, pH 7.5 at 0°, prior to charging. After bulk charging tRNA samples were dissolved in and dialysed against 0.01M-potassium acetate, pH 6.0 at 0°. The concentration of both types of tRNA samples was determined from the $E_{1\text{ cm}}^{260}$, using an SP800 spectrophotometer (Unicam Instruments Ltd.). An extinction coefficient of 24.0 (Yarwood, 1968) for a 1 mg/ml solution was used initially. However, in later experiments an extinction coefficient of 20.0 was adopted (Boulter, personal communication).

13. Standardisation of magnesium chloride solutions

All solutions of magnesium chloride used in the incubation of subcellular particles and in the aminoacylation of tRNA, were standardised by the method of Mohr, as described by Cumming & Kay (1939).

14. Characterisation of ribosome and microsome preparations

A. Sucrose density gradient centrifugation

The ribosome and microsome preparations were analysed on sucrose density gradients. Gradients were prepared using the apparatus described by Henderson (1969). The mixing chamber had an internal height of 6.0 cm and an internal diameter of 2.70 cm. Convex gradients were produced in 23 ml polycarbonate centrifuge tubes at 4°, and centrifuged in the 3 x 23 ml rotor in an MSE Superspeed 65 at 4°. Gradients were fractionated at

0.5 ml/min with 50% (w/v) sucrose, 0.5% (w/v) sodium benzoate using an Isco density gradient fractionator, Model 180. The $E_{0.5\text{ cm}}^{260}$ of the gradient was monitored using an Isco optical unit, and recorded on a Servoscribe potentiometric recorder, Model Re511. 20, at a chart speed of 12 cm/hr.

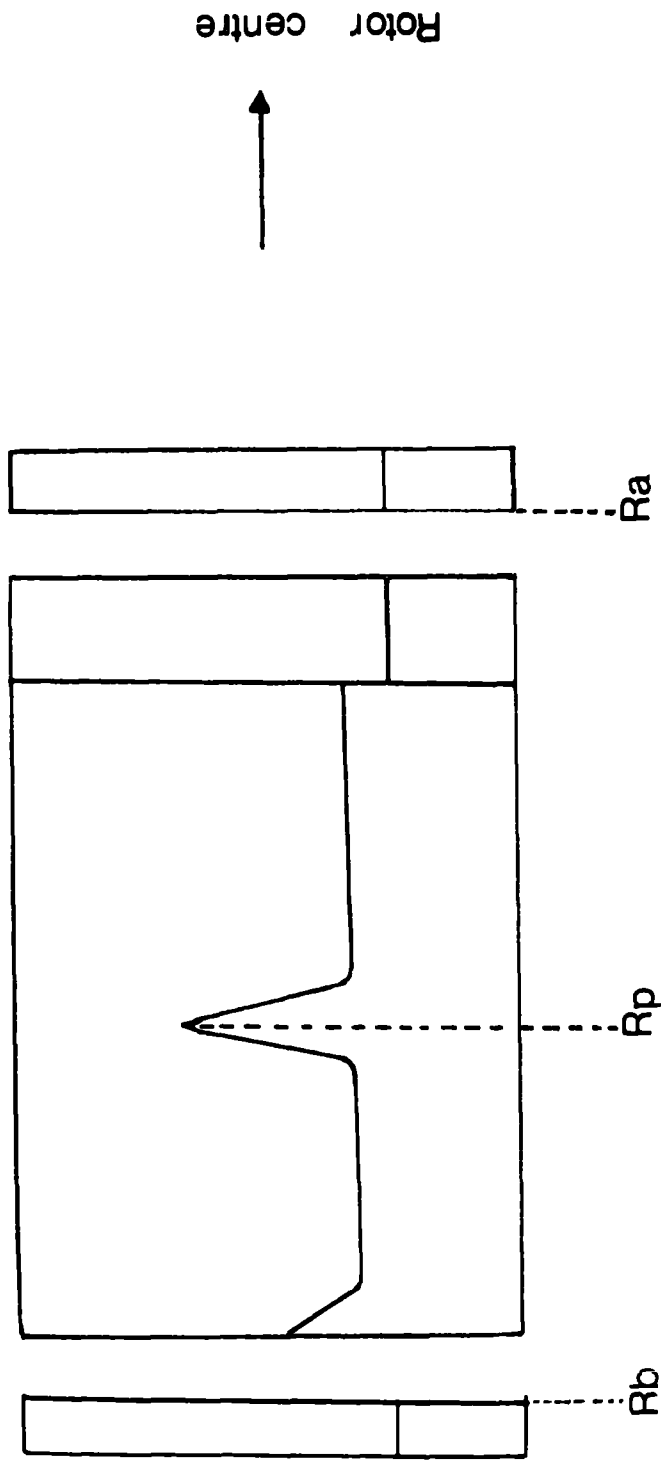
All sucrose used in the preparation of gradients was of Analar grade and had been sterilised in an autoclave at 0.7 kg/cm² for 15 min.

Sucrose volumes, slope of gradients and centrifugation parameters are given in the legends to the figures.

B. Examination of ribosome and microsome preparations in the analytical ultracentrifuge

The sedimentation coefficients of the major components of bean microsome and ribosome preparations were determined using a Beckman Model E ultracentrifuge using the sedimentation velocity technique. The dimensions of cell wedge, bar angle, and centrifugation data, exposure times, are given in the legend to the figures. A Shardlow micrometer was used to measure the distance from the maximum (peak) ordinate to the reference hole. The distances on the plate were converted to true distances in the cell by dividing them by the magnification factor of the camera lens (2.2) and added to 5.70, to give the true distance from the axis of rotation to the peak (r) (Fig. 7). The temperature rise throughout the run was assumed to be small. The sedimentation coefficient(s) is defined as the velocity of the sedimenting molecule per unit field:-

Fig.7. Dimensions of the Beckman Model E rotor used in the determination of sedimentation coefficients. 'a' and 'b' are respectively the inner and outer reference edges.



R_a to rotor centre 5.7cm

R_b 7.3cm

r is distance of centre of rotation to peak

$$r = 5.7 + \left(\frac{R_p - R_a}{m_x} \right)$$

m_x is magnification factor from cell to plate

$$S = \frac{1}{w^2} \times \frac{dx}{dt}$$

$$\frac{1}{w^2} \cdot \frac{d \ln x}{dt} = S$$

$$\frac{2.303}{w^2} \frac{d \log x}{dt} = S$$

$$\frac{2.303}{w^2} \frac{1}{60} \frac{d \log x}{dt} = S$$

Where x = distance of the boundary in cm from the axis of rotation.

t = time in seconds.

w = angular velocity

w = 2 r.p.sec.

Plot log x us t (min)

S^0 , the sedimentation coefficient at zero concentration was determined by performing S value determinations upon a series of dilutions and plotting S versus concentration and extrapolating to Co.

C. Estimation of RNA content or ribosomes and microsomes

The method used was the Schmidt Thannhauser procedure as described by Fleck & Munro (1962). Preparations containing approximately 5 mg of ribosomes or microsomes were diluted to a total volume of 0.5 ml with distilled water in a Piccolo centrifuge tube. An equal volume of ice cold 2.1N-perchloric acid was added and the tubes stood in ice for 15 min to complete precipitation. A further 1 drop of perchloric acid was added to ensure complete precipitation and the precipitate pelleted at full-speed for 5 min in a Piccolo centrifuge. The pellet was washed by resuspension with 0.5 ml 0.7N-perchloric acid, the precipitate was repelleted and the washing procedure repeated twice. To the washed pellet was added 1.3 ml 1N-potassium hydroxide and 3 ml of distilled water to adjust the suspension to 0.3N-potassium hydroxide. The tube was incubated at 37° for 1 hr to hydrolyse the RNA to oligonucleotides. The tube

was agitated intermittently throughout the period of hydrolysis. The hydrolysate was neutralised by the addition of 0.13 ml of 10N-perchloric acid, and 4.5 ml of 1N-perchloric acid were added. Any precipitate was removed by centrifugation and washed by resuspension with 5 ml of 0.5N-perchloric acid. The precipitate was repelleted and washed with a further 6 ml of 0.5N-perchloric acid. The combined supernatants (20 ml) were adjusted volumetrically to 100 ml with water and $E_{1\text{ cm}}^{260}$ read in a Unicam SP800 spectrophotometer at room temperature using a 0.1N-perchloric acid blank. In order to determine $E_{1\text{ cm}}^{260}$ for bean RNA following hydrolysis by 0.3N-KOH for 1 hr, samples of bean RNA in 0.3N-potassium hydroxide were incubated at 37° in the constant temperature cell of a Unicam SP800 spectrophotometer and the $E_{1\text{ cm}}^{260}$ followed throughout hydrolyses. There was on average a 4% rise in $E_{1\text{ cm}}^{260}$ during the hydrolysis period. Assuming an $E_{1\text{ cm}}^{260} = 24.0$ prior to hydrolysis, the $E_{1\text{ cm}}^{260}$ for 1 hr hydrolysed V. faba RNA = 24.97.

Samples in which $E_{1\text{ cm}}^{260}$ exceeded 1.0 were quantitatively diluted since the relationship between $E_{1\text{ cm}}^{260}$ and concentration was only linear for $E_{1\text{ cm}}^{260}$ below 1.0.

15. Aminoacylation of bean or yeast tRNA with $[^{14}\text{C}]$ -phenylalanine

Preliminary charging experiments

The $[^{14}\text{C}]$ phenylalanine acceptor activity of samples of bean or yeast tRNA was assayed by incubation in a system containing 0.1M-Tris-HCl, pH 7.6 at 30°; 2mM-ATP; 15mM-magnesium chloride; 10mM-dithiothreitol; 20 μ M- $[^{14}\text{C}]$ phenylalanine sp. act. 48mC/mM ; 20 μ M- $[^{12}\text{C}]$ -protein amino acid

mixture minus phenylalanine, 0.1 mg tRNA and the enzyme fraction (Bean DE52, optimum concentration having been separately determined); in a total volume of 100 μ l. The handling of these small volumes was facilitated by the use of silicised 15 ml glass centrifuge tubes in which the reaction mixture remained as a discrete drop at the bottom of the tube resulting in a quantitative mixing of the components without any losses due to wetting of the incubation tube walls. Incubation mixtures were made up using ice-cold reagents and were maintained at this temperature until incubation was initiated. Incubations were carried out in a water-bath at 30° for 20 min, with occasional agitation and were terminated by the addition of 1.0 ml ice-cold 10% (w/v) TCA.

16. Measurement of radioactivity in aminoacyl-tRNA

A. Millipore filter method

Following termination of the incubation by 10% (w/v) trichloroacetic (TCA), precipitation of the tRNA was completed by standing for at least 1 hr at 0°. The precipitate was resuspended using a "Whirlmix" agitator and quantitatively collected and washed on a 2.5 cm diameter Millipore filter under a low vacuum. The incubation tube was washed X3 with 2.5 ml of ice-cold 10% (w/v) TCA and the washings drawn through the filter. The filter and filter-holder were then washed a further three times using 2.5 ml of 5% (w/v) TCA.

This extensive washing procedure was found necessary to reduce background radioactivity to a negligible level when working with high specific activity radiochemicals and was adopted as standard procedure. Also, in those experiments

involving $[^{35}\text{S}]$ methionine, the 10% (w/v) TCA contained 0.1M- $[^{32}\text{S}]$ methionine to reduce background $[^{35}\text{S}]$ to a minimum value.

The washed filter was removed, dried under an I.R. lamp and counted in a Beckman LS200B scintillation counter, with automatic background subtraction. The counting efficiency of the scintillation counter for $[^{14}\text{C}]$ was determined using a sealed reference vial containing a known amount of n-hexadecane. $1-[^{14}\text{C}]$ dispersed in scintillation fluid. The Millipore filter method of counting radioactivity was applicable to both preliminary charging experiments and to the assay of column fractions after the chromatographic separation of tRNA^{met} species.

B. Filter paper disc assay

In order to investigate the time course of tRNA aminoacylation the incubation size was increased (1 ml) and 0.1 ml aliquots were removed at specific time intervals and pipetted onto a 2.0 cm diameter disc of Whatman 3MM chromatography paper. The disc was dried in a stream of air and subjected to the washing procedure of Mans & Novelli (1960), except that the hot (90°) TCA wash was omitted. The radioactivity present in the washed disc was measured using a scintillation counter as above.

The filter paper disc method was also applicable to the counting of $[^{14}\text{C}]$ -aminoacyl-tRNA solutions.

17. Preparation of aminoacyl-tRNA: bulk charging

tRNA was aminoacylated using the above system at a concentration of 1 mg/ml. After incubation for 20 min at 30° the reaction was terminated by shaking for 15 min at room temperature

with an equal volume of 90% (w/w) phenol. The reaction mixture was cooled in ice for 1 hr, and the phases separated by centrifugation at 1,000 x g av. The aqueous phase was removed and adjusted to 0.1M-potassium acetate by the addition of 1M-potassium acetate, pH 6.0 at 0°. The phenolic layer was re-extracted by shaking with 0.1M-potassium acetate, pH 6.0 at 0°, the phases separated, and the aqueous layer removed. To the combined aqueous layers was added 2½ volumes of 95% ethanol and precipitation of tRNA completed by standing at -20° for at least 1 hr. The pellet was collected by centrifugation and dissolved in 0.1M-potassium acetate. A further 2½ volumes of 95% ethanol were added and tRNA precipitation completed at -20° for 1 hr. The pellet was recovered by centrifugation and dried in a dessicator under vacuum. The dried aminoacyl-tRNA was stored as a lyophilised powder at -70° or dissolved in 0.01M-potassium acetate (pH 6.0 at 0°) and dialysed against 18 l of 0.01M-potassium acetate (pH 6.0 at 0°) for 18 hr at 0°. The aminoacyl-tRNA solution was stored batchwise in liquid N₂ until required.

18. Estimation of the specific activity of [¹⁴C]-aminoacyl-tRNA

The specific activity of aminoacyl-tRNA samples was expressed in terms of pmoles of amino acid esterified per mg of tRNA.

19. In vitro amino acid incorporating systems

All incubations were carried out in previously sterilised glassware and all solutions were prepared using previously autoclaved (0.7 Kg/cm² for 15 min) distilled water. Incubations

were carried out in thick-walled, siliconised test tubes as previously described (see Methods, section 15). Prior to incubation, all solutions were maintained in ice and on addition of ribosomes or microsomes were incubated in a water bath at 30°. The incubation tubes were shaken manually every few minutes and prior to the removal of aliquots for assay. Zero time controls were routinely carried out and all results corrected for the values obtained.

A. Poly(U) directed transfer system

The poly(U) directed transfer of $[^{14}\text{C}]$ -amino acids from $[^{14}\text{C}]$ -aminoacyl-tRNA to peptidyl material was assayed in a system containing 60mM-Tris/HCl, pH 7.6 at 30°; 0.2mM-GTP; 5mM-dithiothreitol; 7mM-magnesium chloride; 70mM-potassium chloride; 0.2 mg poly(U); 0.2 mg (or that concentration which promoted maximum incorporation of phenylalanine) tRNA aminoacylated with $[^{14}\text{C}]$ phenylalanine (sp. act. 48mC/mM) and 19X $[^{12}\text{C}]$ -amino acids; 0.5 mg ribosomes or microsomes as determined by $E_{1\text{ cm}}^{260}$, and bean DE52 (or other) enzyme (the optimum volume having been separately determined and being that volume which promoted maximum incorporation of phenylalanine), in a total volume of 500 μ l. Incubations were carried out at 30° for 20 min, unless otherwise stated, and incorporation of $[^{14}\text{C}]$ phenylalanine into peptidyl material was determined by either the Millipore filter or filter paper disc method. In the case of the Millipore filter method, incubation was terminated by the addition of a 10-fold excess of ice-cold 5% (w/v) TCA and the tubes were heated at 90° for 15 min in a water bath to hydrolyse remaining $[^{14}\text{C}]$ phenylalanyl-tRNA. Tubes were

cooled to 0° in ice and filtered through a 2.5 cm diameter Millipore filter and washed as previously described (see Methods, section 16A).

When the filter paper disc method of assay was used, 0.1 ml aliquots were withdrawn from the incubation tube and applied to a 2.5 cm diameter disc of Whatman 3MM filter paper. The discs were dried in a stream of air and hydrolysis and washing were carried out according to the method of Mans & Novelli (1960).

Dried Millipore filters and filter paper discs were counted in a Beckman LS 200B scintillation counter using automatic background subtraction.

B. Poly(U) directed complete system

The incorporation of [¹⁴C] phenylalanine into peptidyl material was assayed in the complete incubation system. The incubation mixture contained the following 60 mM-Tris/HCl buffer; pH 7.6 at 30°, 0.2 mM-GTP; 5 mM-dithiothreitol; 16 mM-magnesium chloride; 70 mM-potassium chloride; 4 mM-ATP; 10 mM-creatine phosphate; 30 μg phosphocreatine kinase; 20 μM- [¹⁴C] phenylalanine (sp. act. 48 mC/mM), 20 μM- [¹²C]-protein amino acid mixture minus phenylalanine; 0.2 mg-poly(U), 0.2 mg deacylated tRNA, bean DE 52 enzyme (optimum volume separately determined) and 0.5 mg ribosomes or microsomes as determined by E₁²⁶⁰ cm; in a total volume of 500 μl. Incubation was carried out at 30° for 20 min, unless otherwise stated. The [¹⁴C] phenylalanine incorporated into hot (90°) TCA insoluble material was assayed by the Millipore filter or filter paper disc methods as described above.

20. Presentation of results of [¹⁴C]-amino acid incorporation experiments

The results of all experiments, unless otherwise stated,

are presented in terms of pmoles of $[^{14}\text{C}]$ -amino acid incorporated/
mg rRNA.

21. Poly(A) directed complete system

A. Preparation of 5% (w/v) TCA - 0.25% (w/v)
sodium tungstate

The pH of 5% (w/v) TCA was adjusted using 10 N-sodium hydroxide to between pH 1.5 and 2.0. To the solution was added, with constant stirring, 10 mls of 25% (w/v) sodium tungstate. Throughout the addition of tungstate the pH of the solution was maintained between pH 1.5 and 2.0 by the simultaneous addition of 5% (w/v) TCA. The volume was adjusted to 1 l with 5% (w/v) TCA and the pH maintained at $\text{pH} > 1.5$ < 2.0 by simultaneous addition of 10N-NaOH. The solution was prepared freshly just prior to use.

B. Poly(A) directed incorporation of $[^{14}\text{C}]$
lysine into peptidyl material

The incorporation of $[^{14}\text{C}]$ lysine into material insoluble in 5% (w/v) TCA - 0.25% (w/v) sodium tungstate was assayed in a system containing 100 mM-Tris-HCl; pH 7.6 at 30°; 0.2 mM-GTP; 10 mM-GSH; 8 mM-magnesium chloride; 10 mM-creatine phosphate; 30 μ g phosphocreatine kinase; 4 mM-ATP; 20 μ M- $[^{14}\text{C}]$ lysine (sp. act 310 mC/mM); 20 μ M $[^{12}\text{C}]$ -protein amino acid mixture minus lysine; 0.2 mg Poly(A); 0.2 mg deacylated tRNA; and the optimum volume (as separately determined) of bean 105,000 x g supernatant protein as enzyme source, and 0.5 mg as determined by $E_{1\text{ cm}}^{260}$, of bean ribosomes in a total volume of 500 μ l. Incubations were carried out for 20 min at 30°, as previously described.

The incorporation of $[^{14}\text{C}]$ lysine into peptidyl material was assayed on filter paper discs. At the end of the incubation period, 0.1 ml of 2% (w/v) polylysine were added to act as a carrier, the contents of the tube were mixed and the two 0.1 ml aliquots were withdrawn and pipetted directly on to 2.0 cm diameter discs of Whatman 3MM filter paper. The discs were dried in a stream of air and washed according to the method of Bollum (1967). The dried discs were counted in a Beckman LS 200B scintillation counter with automatic background subtraction.

22. Resolution of complementary fractions involved in peptide chain elongation

A. Gel filtration on Sephadex G200

The resolution and partial purification from bean concentrated dialysed 105,000 x g supernatant of complementary fractions involved in the transfer of amino acids from aminoacyl-tRNA into peptidyl material, was carried out essentially according to the method of Moldave (1968).

The single calcium phosphate gel treatment was replaced by two treatments, the calcium phosphate gel pellets being combined and eluted as a whole.

The partially purified soluble protein was applied to a 30 x 2.4 cm Sephadex G200 column which had been previously equilibrated at 4° with a buffer containing 0.05M-Tris/HCl buffer, pH 7.6 at 4°; 0.15M-potassium chloride; 10^{-4} M-EDTA and 10^{-3} M GSH. The sample was washed in with, and the column developed by upward flow with the above buffer at a flow rate of 11.5 ml/hr. Fractions were collected at 30 min intervals and the $E_{0.5\text{ cm}}^{280}$ of the effluent was monitored using an Isco

optical unit attached to a Servoscribe potentiometric chart recorder. Elution was continued for 48 hr until the $E_{0.5\text{ cm}}^{280}$ fell to a base-line value.

B. Assay of the column fractions

The column fractions and samples taken throughout the purification procedure were assayed for protein by the method of Lowry et al. (1951), and for their ability to promote the poly(U) directed incorporation of $[^{14}\text{C}]$ phenylalanine from $[^{14}\text{C}]$ phenylalanyl-tRNA into peptidyl material using ammonium chloride washed V. faba ribosomes. The assay system contained 60 mM-Tris/HCl, pH 7.6 at 30°; 0.2 mM-GTP; 70 mM-potassium chloride; 10 mM-magnesium chloride; 4 mM-GSH; 0.2 mg poly(U), 0.2 mg yeast $[^{14}\text{C}]$ phenylalanyl-tRNA; 0.5 mg (as determined by $E_{1\text{ cm}}^{260}$) ammonium chloride washed ribosomes, and either 1 mg partially purified supernatant protein or 0.02 ml aliquot of the column fractions in a total volume of 500 μ l. No allowance was made for the K^+ present in the column fractions.

Incubations were carried out for 20 min at 30° and the incorporation of $[^{14}\text{C}]$ phenylalanine from $[^{14}\text{C}]$ phenylalanyl-tRNA into peptidyl material was determined using the Millipore filter method.

Assays were carried out at 4 mM-GSH to prevent activation of contaminating ribosomal TF_2 .

Complementary fractions were detected by assaying all other fractions in combination with fractions corresponding to peaks of aminoacyl transferring activity. Fractions were assayed as above.

C. Gel filtration on Sepharose 4B

A 2.4 x 30 cm column of Sepharose 4B was equilibrated at 4° with a buffer containing 0.05M-Tris-HCl, pH 7.5 at 4°; 0.15M-potassium chloride, 10⁻⁴M-EDTA and 10⁻³M-GSH. 10 ml of concentrated dialysed 105,000 x g bean supernatant was applied to the top of the column. When the sample had been absorbed, the column was developed with the same buffer at a flow rate of 40 ml/hr. Fractions were collected at 6 min intervals and the E_{0.5 cm}²⁸⁰ of effluent monitored using an Isco optical unit attached to a Servoscribe potentiometric recorder. Elution was continued until E_{0.5 cm}²⁸⁰ reached a base-line value.

The fractions were assayed for protein by the method of Lowry et al. (1951), and for activity in promoting the transfer of [¹⁴C] phenylalanine from [¹⁴C] phenylalanyl-tRNA into peptidyl material, as described above. The assays were carried out without correcting for K⁺ in the column fractions. Complementary fractions were detected by assaying all other fractions in combination with fractions corresponding to peaks of aminoacyl transferring activity.

D. Comparisons of different enzyme preparations and fractions are made in terms of their Specific Activity. Specific Activity = units of enzyme/mg protein. 1 unit is taken to be the amount of enzyme catalysing the incorporation of 1 pmole [¹⁴C] phenylalanine under the assay conditions described.

23. Characterisation of the products of *in vitro* system

A. Preparation of the microsomes

In an attempt to avoid possible mRNA degradation during storage, particles were prepared and used on the same day. The particles were prepared from 60 day developing cotyledons

by either the normal procedure (see Methods, section 4A), or using a rat-liver high-speed supernatant fraction (prepared according to Blo bel & Potter, 1966), reported to contain an endogenous RNase inhibitor (Roth, 1956; Blo bel & Potter, 1966; Northup et al., 1966), as the extractant.

B. Incubation procedure

The particles were incubated in the complete system described earlier (see Methods, section 19B), except that the total volume was 5 ml, poly(U) was omitted, and the radioactive amino acid was [³⁵S] methionine (26 nMoles; sp. act. 15C/mM). Incubations were carried out for 40 min at 25°. At the end of each incubation period, 0.1 ml samples were taken from each incubation, hydrolysed, filtered, washed and counted to determine the amount of incorporation.

C. Carboxymethylation of protein

To each incubation was added an equal volume of 6M-guanidinium hydrochloride in 2M-Tris buffer, pH 8.7 and 0.1 ml 2-mercaptoethanol. After 2 hr at 35°, a 2 molar excess of solid iodoacetic acid was added together with sufficient solid Tris buffer to bring the mixture to pH 7.8. The mixture was left to react in the dark at room temperature until it gave a negative nitroprusside test. It was then dialysed overnight against running tap water in the cold room and then the protein precipitated by the addition of 2 volumes of cold ethanol. After 1 hr the precipitate was collected by centrifugation and residual ethanol removed under vacuum and the precipitate resuspended in 2 ml distilled water.

D. Tryptic digestion

The carboxymethylated protein was digested with trypsin (2% trypsin/protein) at 35°, the pH being maintained at pH 8.2 by means of an autotitrator containing 0.05M-sodium hydroxide. When the digestion was complete (approximately 90 min) the digest was clarified by centrifugation and the clarified digest was then freeze-dried.

E. Fingerprinting

The freeze-dried digest was dissolved in 0.2 ml of electrophoresis buffer pH 3.5 and applied as a 1 cm streak to a 58 x 68 cm sheet of Whatman No. 3 chromatography paper. The origin was 15 cm from the top and edge of the paper and the direction of chromatography was parallel to the short axis of the paper. A marker spot of methyl orange was placed next to the sample. Descending chromatography using the solvent Butanol/Glacial Acetic acid/water/pyridine (60:15:48:40) was carried out in a closed tank until the marker spot had moved 35 cm (about 18 hr). The chromatogram was air-dried in a fume hood and a second marker spot of methyl orange was applied adjacent to the origin and the paper rechromatogrammed until the marker spot had again run 35 cm. After the chromatograms had been again thoroughly dried overnight, they were subjected to high-voltage electrophoresis, pH 3.5 in a direction perpendicular to chromatography. The sample was placed nearest the anode and electrophoresis was carried out at 2.5 kV for 60 min. The fingerprint was thoroughly dried and subjected to autoradiography.

24. Resolution of methionyl-tRNAs

A. Preparation of the formyl donor (N^{10} -formyl tetrahydrofolic acid)

The formyl donor was prepared by the method of Marcker (personal communication).

100 mg of folic acid were dissolved in 4 ml Analar formic acid and shaken at 0° in the dark for 48 hr. Excess formic acid was removed (by evaporation) in a vacuum desiccator. The resultant solid was redissolved in 0.1M-potassium acetate, pH 7.7, and N^{10} formyl folic acid was precipitated by the drop-wise addition of hydrochloric acid. Precipitation was completed at 0° and the precipitate collected by centrifugation. The N^{10} formyl folic acid was washed by resuspension in water and repelleted and the procedure repeated. The precipitate was dried in a vacuum desiccator and stored in a foil-covered tube in a vacuum desiccator at -20° . Immediately prior to use the N^{10} formyl folic acid was reduced. Approximately 1 mg of N^{10} formyl folic acid was dissolved in 1-2 ml of 0.1M-potassium acetate, pH 7; and catalytically reduced by gaseous hydrogen in the presence of a few grains of a 5% Rhodium on alumina for 90-120 min. The solution, containing N^{10} -formyl-tetrahydrofolic acid, will be referred to as the formyl donor and $100 \mu\text{l}$ were used per 1 ml incubation.

B. Chromatography of *V. faba* tRNA on DEAE-Sephadex A50

DEAE-Sephadex was washed by the method of Nishimura et al. (1967). A 1 x 50 cm column was poured and equilibrated at 0° with buffer A (0.375M-sodium chloride; 0.008M-magnesium chloride; 1mM-2-mercaptoethanol and 0.02M Tris/HCl, pH 7.5

at 0°). Approximately 20 mg deacylated V. faba tRNA (prepared as described in Methods, section 11) in Buffer A was applied to the column and eluted with a linear gradient of 250 ml Buffer A and 250 ml Buffer B (0.475M-sodium chloride; 0.016M-magnesium chloride, 1mM-2-mercaptoethanol and 0.02M Tris/HCl, pH 7.5 at 0°). The flow rate was maintained at 12 ml/hr using an LKB pump and 2.4 ml fractions were collected at 12 min intervals using an LKB fraction collector. The $E_{0.5}^{260}$ cm of the column effluent was continuously monitored using an Isco optical unit and analyser system connected to a Servoscribe potentiometric recorder. Column fractions were either assayed immediately for methionine acceptor activity or were stored at -20° until assayed. The methionine acceptor activity was determined on 50 μ l aliquots in a system containing 0.1M-Tris/HCl, pH 7.6 at 30°; 5mM-ATP; 15mM-magnesium chloride; 10mM-DTT; 70 mM-potassium chloride; 0.02mM [35 S] methionine (Sp. act. 150 mC/mM) and bean DE52 supernatant enzyme (the optimum volume having been separately determined) in a total volume of 200 μ l. The samples were incubated at 30° and the radioactivity determined by the previously described Millipore filter method.

Since comparisons were made only between different incubations performed on the same occasion, and since the tRNA content of the fractions varied, the methionine acceptor activity of the column fractions is presented in terms of counts per minute (c.p.m.).

C. Chromatography of V. faba tRNA on BD-Cellulose

Commercial BD-cellulose (Schwartz Bioresearch) was washed by agitation with 2M-NaCl and then with Buffer I (0.3M-sodium

chloride; 1mM-EDTA; 10mM-sodium acetate; pH 4.5 at 4°; 1mM 2-mercaptoethanol). A 1 x 32 cm column was poured and equilibrated with Buffer I at 4° overnight. 28 mg of deacylated bean tRNA in 4 ml of Buffer I were applied to the column and eluted with a linear gradient of 300 ml Buffer I and 300 ml of Buffer 2 (1.0M-sodium chloride; 1mM-EDTA; 10mM-sodium acetate; pH 4.5 at 4°, 1mM-mercaptoethanol). A flow rate of 12ml/hr was maintained by an LKB peristaltic pump and fractions were collected at 15 min intervals in tubes containing a concentrated solution of Tris-HCl, pH 7.6 at 4°, and magnesium chloride to bring the final pH of the fractions to pH 7.5 and final Mg²⁺ to 10mM. The column effluent was continuously monitored at 260 nm, using an Isco optical unit and analyser attached to a Servoscribe potentiometric chart recorder. The fractions were mixed by agitation immediately after collection and stored at -20° until assayed.

Fractions were assayed for methionine acceptor activity as described above for DEAE-Sephadex fractions, using [³⁵S] methionine (sp. act. 510 mC/mM).

D. Aminoacylation with [³⁵S] methionine of separated methionine accepting tRNAs

Methionine acceptor tRNAs resolved by the above methods were aminoacylated with [³⁵S] methionine (sp. act. as stated) in the presence and absence of formyl donor using bean DE52 supernatant or E. coli DE52 supernatant. 1 ml aliquots of those fractions corresponding to peaks of methionine acceptor activity were aminoacylated in a total volume of 4 ml in the previously described system. E. coli DE52 supernatant (the optimum volume having been separately determined),

replaced bean DE52 supernatant in incubations charged by E. coli enzyme. Incubations were carried out at 30° for 30 min in siliconised thick-walled test tubes. Incubation was terminated by the addition of an equal volume of ice-cold distilled water and the reaction mixture was adjusted to 0.1M-potassium acetate. An equal volume of 80% (w/w) freshly redistilled phenol was added and the tubes sealed and shaken for 15 min at 4°. The phases were separated by centrifugation at 1,000 x g av. for 30 min. The aqueous phase was removed using a Pasteur pipette, and tRNA was precipitated by the addition of 2 volumes of absolute ethanol. Precipitation was completed by standing at -20° for 1 hr. In those incubations in which the tRNA concentration was low, precipitation was completed by standing at -20° overnight. The precipitate was collected by centrifugation for 30 min at 1,000 x g av., the ethanol decanted and the pellet washed by resuspension in absolute ethanol at -20°. The pellet was collected by centrifugation and the ethanol decanted. The pellet was dissolved in the minimum volume of 0.1M-potassium acetate, pH 6.0 at 4°, and reprecipitated by 2 volumes of ethanol. The pelleted tRNA was again washed by resuspension in ethanol and the final pellet was dried in a desiccator under vacuum. The changed tRNA was stored in a vacuum desiccator at -20° until required.

25. Digestion of [³⁵S] methionyl-tRNAs with pancreatic ribonuclease

[³⁵S] methionyl-tRNA was dissolved in 50 μl of freshly prepared 0.1M-potassium acetate, pH 6.0 at 4°, and incubated at room temperature for 5 min with 10 μg of bovine pancreatic RNase A. The reaction mixture was applied as a 1 cm streak

at the origin of a sheet of Whatman 3MM chromatography paper (50 x 15 cm), and air-dried. Control spots of $[^{35}\text{S}]$ -methionine were also applied at the origin. The $[^{35}\text{S}]$ methionyl-adenosine ester and formyl- $[^{35}\text{S}]$ methionyl-adenosine ester liberated by RNase digestion, were separated by ionophoresis in pyridium acetate buffer, pH 3.5 at 3 kV for 1 hr. The ionogram was dried in air overnight and an autoradiograph prepared by exposing the ionogram to Industrial G x-ray film for 18-48 hr, depending upon the activity present.

26. Digestion of $[^{35}\text{S}]$ methionyl-tRNA with ribonuclease T₁

The $[^{35}\text{S}]$ methionyl-tRNA was dissolved in 10mM-potassium acetate pH 6.0, containing 1mM-EDTA. Ribonuclease T₁ was then added to concentration of 1/20 of the tRNA present. When the concentration of $[^{35}\text{S}]$ methionyl-tRNA was very low, 0.25 mg deacylated E. coli tRNA was added to act as carrier. The final concentration of tRNA was 1 mg/ml. The reaction mixture was incubated in siliconised glass centrifuge tubes for 20 min at 37°. After incubation the reaction mixture was freeze-dried. The dried reaction mixture was dissolved in 50 μ l of 10mM-potassium acetate and streaked on the centrally placed origin of a sheet of Whatman 3MM chromatography paper. The streaks were air-dried and the oligonucleotides separated by ionophoresis in pyridium acetate buffer pH 3.5, at 3 kV for 2½ hr. An autoradiograph was prepared as above.

27. Authentication of radioactive fragments

The identity of the $[^{35}\text{S}]$ methionyl-adenosine ester and the formyl- $[^{35}\text{S}]$ methionyl-adenosine ester obtained after

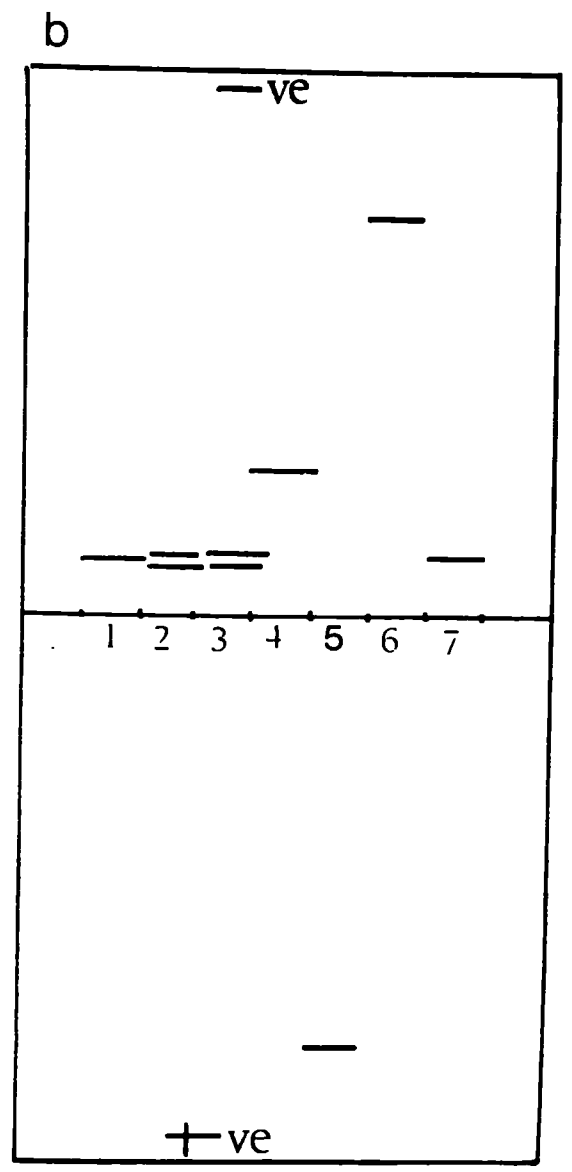
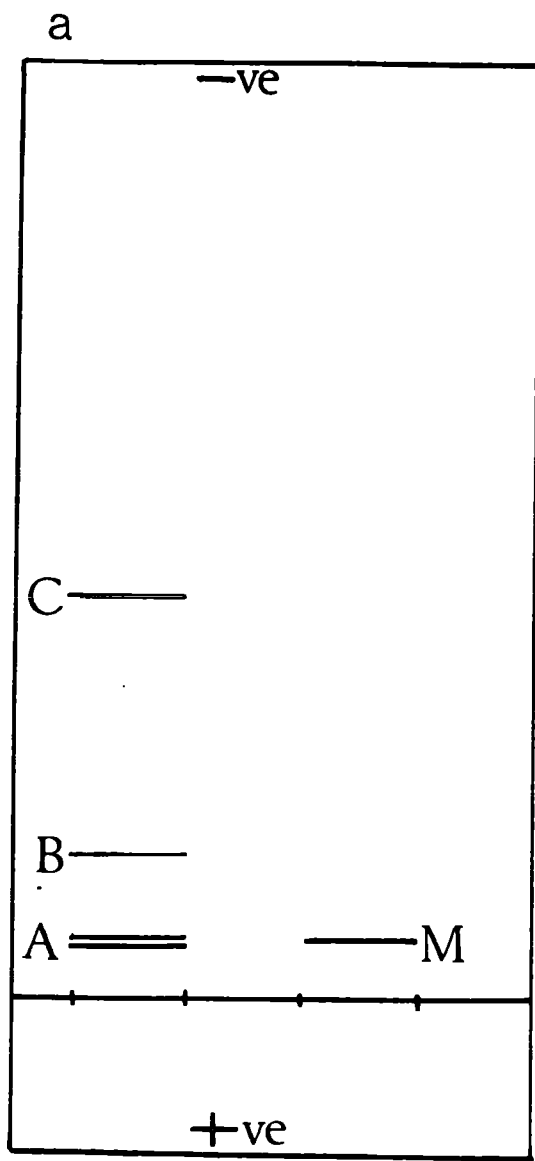
ionophoresis of pancreatic RNase digests were authenticated by elution, hydrolysis and re-electrophoresis of the radioactive spots. The radioactive spots were cut out of the ionogram and stapled to a piece of Whatman 3MM chromatograph paper. The strips were eluted with 3 ml of water in a saturated atmosphere. The eluate was dried in a desiccator under vacuum. The eluate was hydrolysed in 1 ml of 0.5M-ammonium hydroxide pH 11.0, at 37° for 1½ hr. The hydrolysates were evaporated in a desiccator under vacuum until free of any trace of ammonia and then applied as a 1 cm streak to the origin of a strip of Whatman 3MM chromatography paper and subject to ionophoresis and autoradiography as previously described. A comparison of the electrophoretic mobilities of the radioactive spots before and after alkaline hydrolysis could then be made.

Fig. 8 shows a typical autoradiograph of a pancreatic RNase digest of bean [³⁵S] methionyl-tRNA. Travelling towards the cathode were two distinct bands in addition to free methionine. Band A consisted of two discrete bands which when subject^{to}/alkaline hydrolysis retained their original mobility. Their mobility closely corresponded with that of free [³⁵S] methionine applied as a marker, suggesting their identification as free [³⁵S] methionine and an oxidation product (methionine sulphoxide). Band B appeared only in those preparations in which formylation occurred in the presence of a formyl donor and was very faint in all cases. Following alkaline hydrolysis in diluted ammonium hydroxide (pH 11.0), the substance eluted from band B, did not give methionine, but a compound which was acidic at pH 3.5 and

Fig.8. Authentication of the radioactive fragments obtained by digestion of 60 day developing bean $[^{35}\text{S}]$ met-tRNAs with bovine pancreatic RNase.

8a. Diagrammatic representation of an autoradiograph of an ionogram of pancreatic RNase digest of 60 day developing bean $[^{35}\text{S}]$ met-tRNA. The $[^{35}\text{S}]$ met-tRNA was prepared as described (Methods, section 24D) and digested with pancreatic RNase (Methods, section 25). The digest was subjected to ionophoresis for 1 hr., pH 3.5, 3kV. 'M' refers to free $[^{35}\text{S}]$ methionine applied as a marker spot.

8b. Diagrammatic representation of an autoradiograph of an ionogram (pH 3.5, 3kV, 1 hr.) of (1) $[^{35}\text{S}]$ methionine; (2) band A (Fig.8a) eluted and reelectrophoresed; (3) band A eluted and hydrolysed in dilute NH_3 (pH 11.0) at 37° for 1.5 hr.; (4) band B (Fig.8a) eluted and reelectrophoresed; (5) band B eluted and hydrolysed as for (3) above; (6) band C (Fig.8a) eluted and reelectrophoresed; (7) band C eluted and hydrolysed as for (3) above.



migrated towards the anode. This indicated that it had gained an additional negative charge after hydrolysis since before it was slightly basic. This was consistent with its identity as N-formyl-methionyl-adenosine, the aminoacyl linkage having been cleaved by hydrolysis to yield N-formyl-methionine in which the α -amino group is blocked.

Band C was present in all preparations and on subsequent elution and hydrolysis material from band C was converted to methionine. This was consistent with known alkali-lability of the aminoacyl-tRNA ester linkage and suggested that it was the methionyl-adenosine ester.

28. N-terminal analysis of $[^{35}\text{S}]$ -labelled polypeptides

$[^{35}\text{S}]$ methionine was incorporated into peptidyl material in the previously described complete system (Methods, section 19B). The total reaction volume was reduced to 100 μl , and 0.2 mg poly(U) was replaced by 60 μg poly(AUG) (1:2.5:1) or 50 μg poly(UG) (1:1). The deacylated tRNA was replaced by 7 pmoles of $[^{35}\text{S}]$ met-tRNA₁ (peak 2) or 6 pmoles $[^{35}\text{S}]$ met-tRNA₂ (peak 1) from DEAE Sephadex (1 pmole = 22,500 c.p.m.). The Mg^{2+} concentration was 4mM. After incubation at 33° for 30 min, the reaction tubes were cooled in ice and the reaction terminated by the addition of 100 μl of 0.1M-EDTA containing 10 μg of bovine pancreatic RNase A. Enzymatic digestion was allowed to proceed for 2 min at 33° and terminated by the addition of 5 ml ice cold 5% (w/v) TCA, 0.1M- $[^{32}\text{S}]$ methionine. Precipitation was completed by standing in ice for 1 hr and the precipitate pelleted at 1,00 x g av. for 30 min. The supernatant was carefully decanted, and the pellet washed by

resuspension in ice cold TCA . The precipitate was again pelleted and washed a further twice as above, and dried overnight in a desiccator under vacuum. N-terminal analysis was performed by the method of Blombäck^{et al} (1966). After extraction of the reaction mixture with n-butyl acetate the aqueous and organic phases were quantitatively transferred to scintillation vials and dried in a desiccator under vacuum. 10 ml of scintillation fluid was added and the samples counted in a Beckman LS200B scintillation counter with automatic background subtraction. Control vials in which appropriate volumes of aqueous and organic phase had been dried were also counted.

The results were expressed as % radioactivity present in the N-terminal position.

29. AUG dependent binding of $[^{35}\text{S}]$ met-tRNA species to *V. faba* ribosomes

AUG dependent binding of $[^{35}\text{S}]$ met-tRNA species to bean ribosomes was determined by the method of Nirenberg and Leder (1964). The reaction mixture contained in a total volume of 45 μl , 88mM-Tris-HCl, pH 7.6 at 25°, 55mM-potassium chloride, 2.8mM-DTT, 6.66mM-GTP, 0.25 mg bean ribosomes (as determined by $E_{1\text{ cm}}^{260}$), 0.10 units of the synthetic triplet AUG, and either 40 pmoles $[^{35}\text{S}]$ met-tRNA₁, 20 pmoles $[^{35}\text{S}]$ met-tRNA₂ or 7 pmoles $[^{35}\text{S}]$ met-tRNA₃, together with the indicated Mg^{2+} concentration. Incubations were carried out for 15 min at 25°. Reaction was terminated by the addition of 3 ml of ice-cold buffer containing 88mM-Tris-HCl, pH 7.6 at 25°, 55mM-potassium chloride, 10mM Mg^{2+} . The reaction mixture was quantitatively transferred to a Millipore filter and washed using 3 x 3 ml of ice-cold buffer. The dried Millipore filters were counted in a

Beckman LS200B scintillation counter with automatic background subtraction. The results are presented in terms of pmoles $[^{35}\text{S}]$ met-tRNA bound per incubation and are corrected for binding in the absence of AUG at the relevant Mg^{2+} concentration.

30. Release of methionyl-puromycin from *V. faba* ribosomes

The release by puromycin of AUG dependently bound $[^{35}\text{S}]$ met-tRNA from bean ribosomes was assayed using the above system. After incubation at 25° for 15 min, $5\ \mu\text{l}$ of 10mM-puromycin (neutralised to pH 7.0) added and the reaction mixture re-incubated for 15 min. The reaction was terminated by the addition of 1 ml of 0.1M-disodium hydrogen phosphate pH 8.1, and 1.5 ml of ethyl acetate (saturated with Na_2HPO_4 buffer). The phases were mixed on a Whirlimix ten times, 5 secs each, and the phases separated by centrifugation at 1,000 x g av. for 15 min at 4° , 1 ml of the organic layer was removed, dried on a hot plate and the residue counted in a Beckman LS200B scintillation counter with automatic background subtraction. The results are expressed in terms of pmoles of $[^{35}\text{S}]$ methionyl-puromycin released per incubation and are corrected for binding in the absence of AUG at the relevant Mg^{2+} concentration.

RESULTS

1. Characterisation of microsome and ribosome preparations isolated from developing and germinating seeds of *V. faba*.

A. Absorption spectra

The absorption spectra of suitable dilutions of microsome and ribosome preparations isolated from germinating and developing seeds of *Vicia faba* are shown in Figs. 9 and 10. Details of the changes in absorption maxima and minima and 260:235 nm absorption ratio are presented in Tables 2,3.

B. Sucrose density gradient centrifugation

Because of the essential similarity of the clarified microsome preparations obtained from developing and germinating seeds, the sucrose density gradient profile of microsomes isolated from developing seeds was assumed to be representative of microsome preparations. Centrifugation of 2 mg, as estimated by $E_{1\text{ cm}}^{260}$, of microsomes on a 15-45% (w/v) isokinetic sucrose gradient (Fig.11) resulted in a large broad peak of membrane-bound material which travelled furthest down the gradient followed by 5 polysome peaks, a large monosome and 2 small sub-unit peaks. The monosome peak was identified as such on the basis of its nucleic acid content. Lonsdale (1972) has shown it to contain both 25S and 18S RNA. Particles having a sedimentation rate greater than the monosome peak were assumed to be polysomes and those with a sedimentation rate less than the monosome peak were assumed to be sub-units.

Ribosomes prepared from developing beans in the presence of the non-ionic detergent Triton X100 were analysed on isokinetic gradients (Fig.12). A sharp monosome peak was resolved from the rapidly sedimenting

Fig.9. Absorption spectra of microsome and ribosome preparations from 60 day developing cotyledons of V.faba.

The preparations were resuspended in water at a suitable dilution and the absorption spectra determined using a Unicam SP 800 spectrophotometer.

- (a) Microsomal preparation.
- (b) Triton X-100 ribosomes.
- (c) NH_4Cl washed ribosomes.

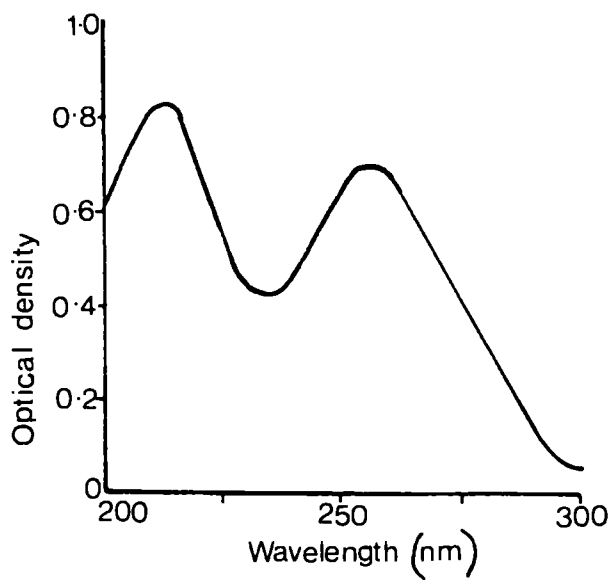
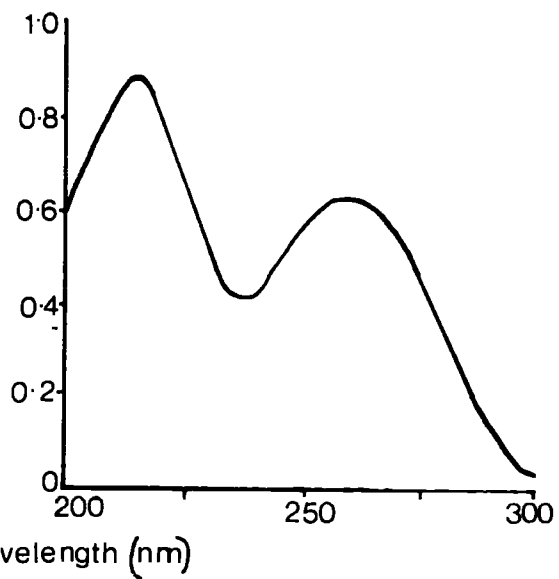
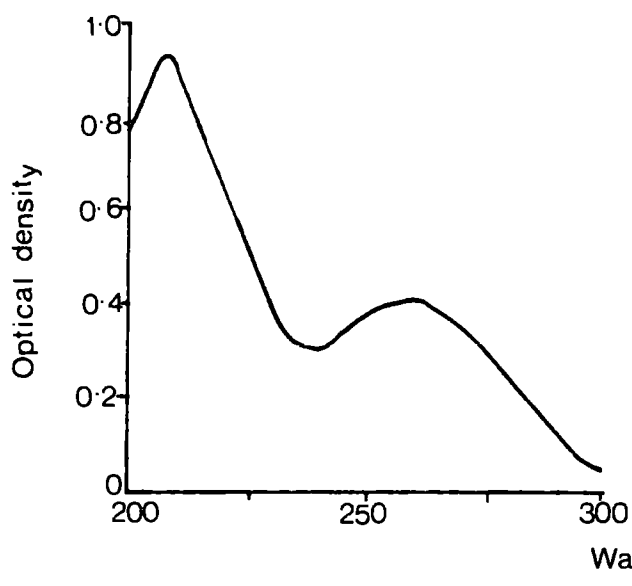


Table 2

Changes in absorption spectra and 260nm : 235nm absorption ratio of microsomes and ribosomes prepared from 60 day developing cotyledons of Vicia faba.

| Type of preparation | Absorption nm | | 260 : 235 ratio |
|--|---------------|---------|-----------------|
| | Minimum | Maximum | |
| Clarified microsomes | 238 | 258 | 1.26 |
| 4%(w/w) Triton X100 ribosomes | 236 | 257 | 1.50 |
| NH ₄ Cl washed and dialysed ribosomes | 234 | 256 | 1.56 |

The microsomal or ribosomal preparations were resuspended in water at a suitable dilution and the absorption spectrum determined using a Unicam SP800 spectrophotometer.

Fig.10. Absorption spectra of microsome and ribosome preparations from 3 day germinated seeds of V.faba.

The preparations were suspended in water at a suitable dilution and the absorption spectra determined using a Unicam SP 800 spectrophotometer.

- (a) Microsomal suspension.
- (b) Triton X-100 ribosomes.
- (c) NH_4Cl washed ribosomes.

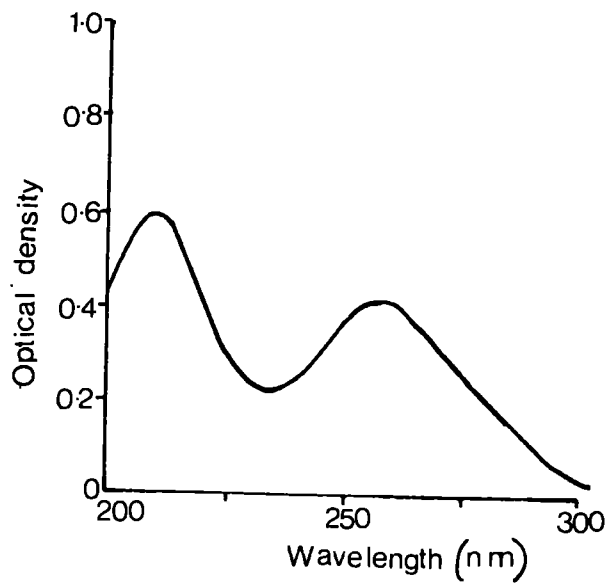
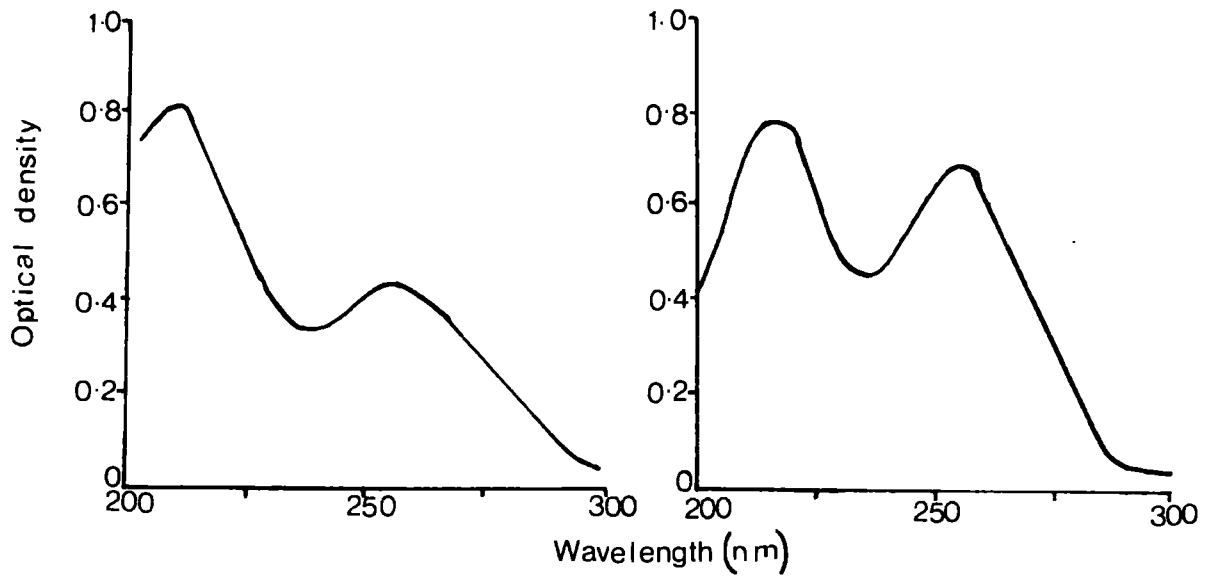


Table 3

Changes in absorption spectra and 260nm : 235nm absorption ratio of microsomes and ribosomes prepared from plumules of 3-day germinated seeds of Vicia faba.

| Type of preparation | Absorption nm | | 260 : 235 ratio |
|---|---------------|---------|-----------------|
| | Minimum | Maximum | |
| Clarified microsomes | 237 | 256 | 1.24 |
| 1% Triton X100 ribosomes | 236 | 256 | 1.48 |
| NH ₄ Cl washed and dialysed ribosomes | 235 | 254 | 1.71 |

The microsomal or ribosomal preparations were resuspended in water at a suitable dilution and the absorption spectrum determined using a Unicam SP800 spectrophotometer.

Fig.11. Sucrose gradient sedimentation profile of microsomes prepared from 60 day developing cotyledons of V.faba.

The microsomes were prepared as described in the Methods (Section 4A).

2 mg. microsomes were layered onto a gradient prepared from 6 ml 15% sucrose and 20 ml 55% sucrose, buffered in 10mM-Tris, 25mM-KCl, 1mM-MgCl₂, pH 7.6. The gradient was centrifuged at 95,000xg av. for 3 hr. in the 3 x 23 ml swing out rotor of an M.S.E. Superspeed '65' centrifuge. 'T' and 'B' indicate top and bottom of the gradient respectively.

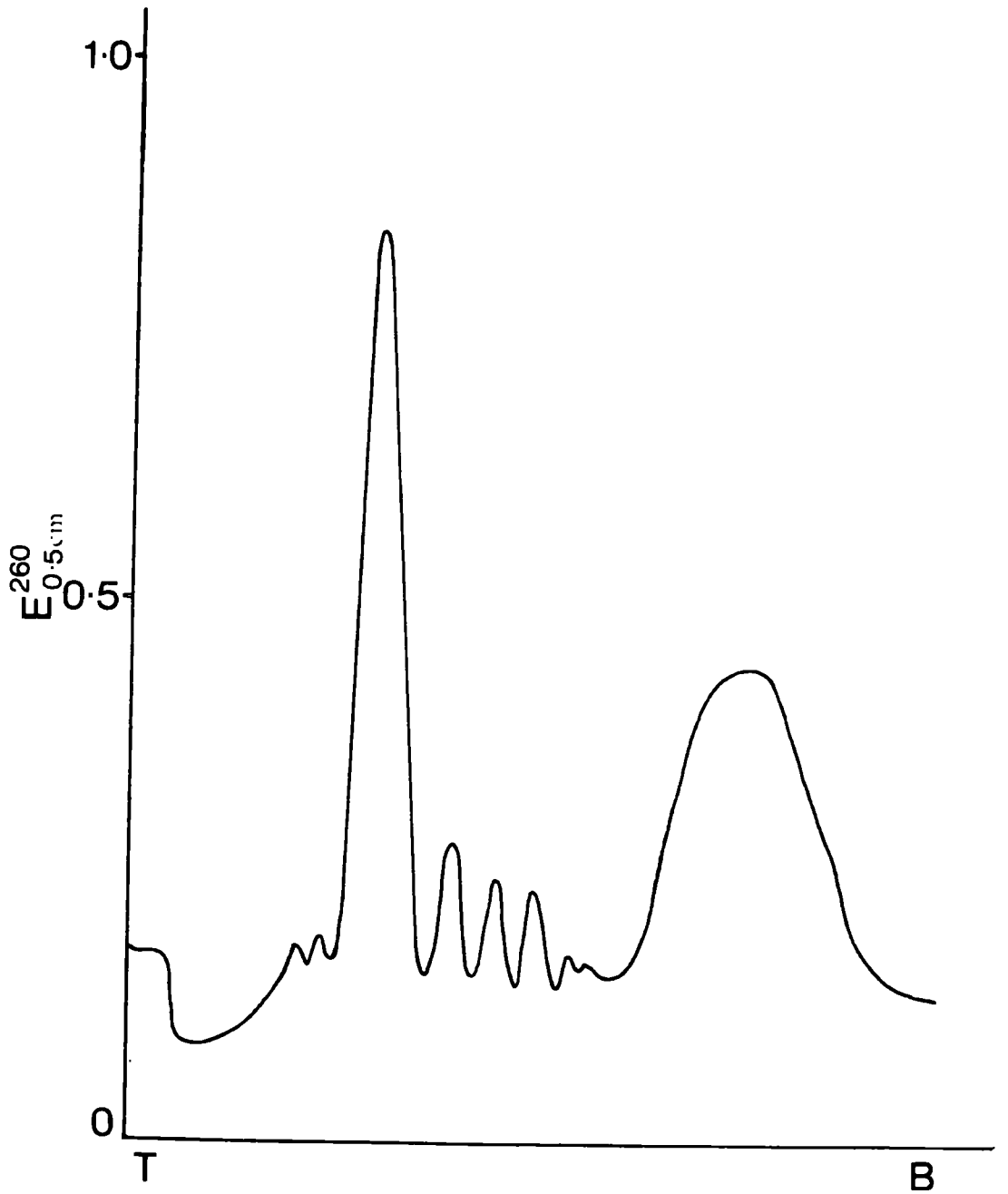
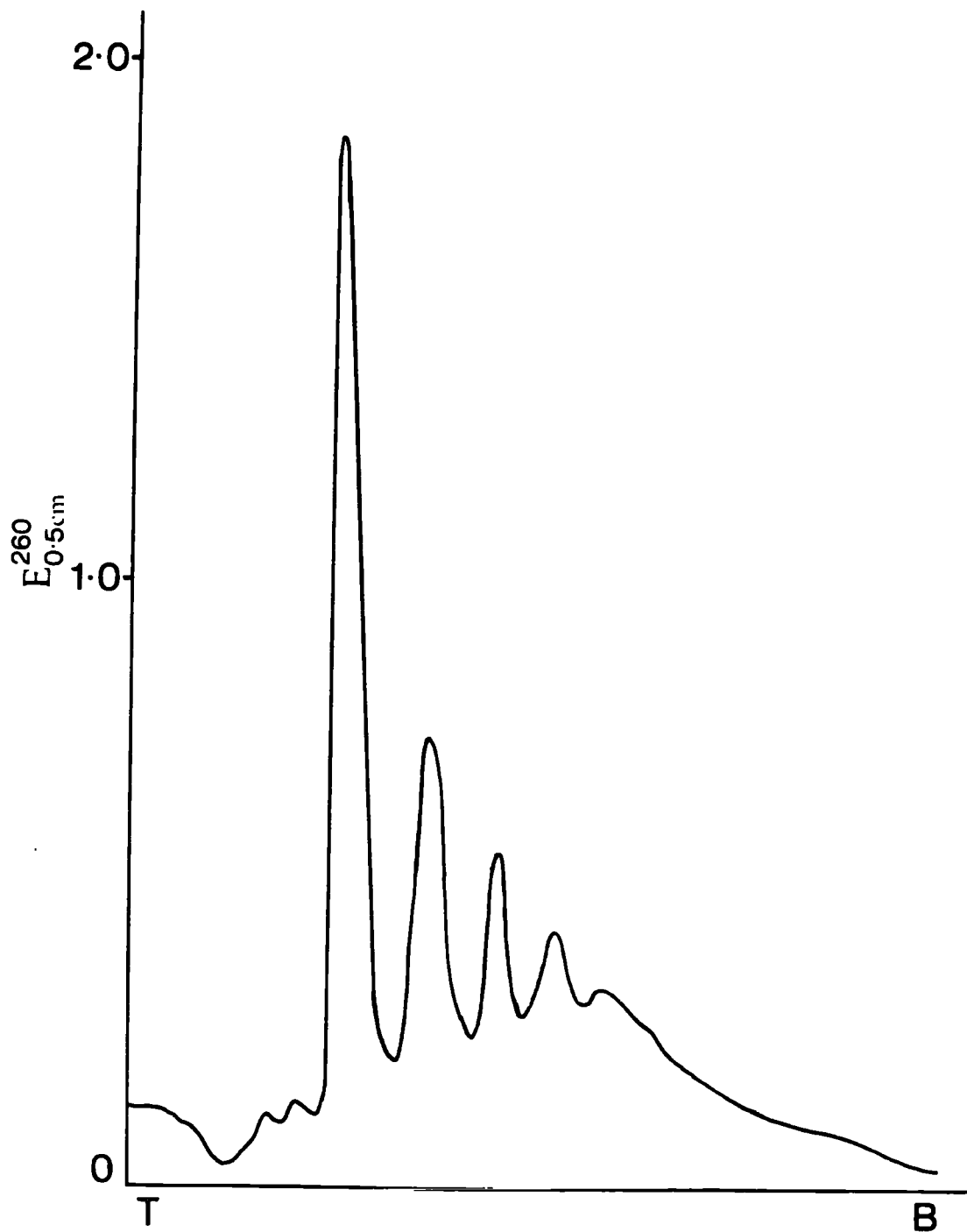


Fig.12. Sucrose gradient sedimentation profile of

**Triton X-100 ribosomes prepared from 60 day
developing cotyledons of V.faba.**

**The ribosomes were prepared as described in the
(Methods, section 4D.**

**One mg of ribosomes were layered onto a gradient
prepared from 10 ml 8% sucrose and 20 ml 30%
sucrose, buffered as in Fig.11. The gradient was
centrifuged at 65,000xg av. for 2 hr in the 3 x 23 ml
rotor of an M.S.E. Superspeed '65' centrifuge.
'T' and 'B' are top and bottom of the gradient
respectively.**



polysomes and less rapidly sedimenting subunits. Ribosome preparations prepared using 4%^(w/w) Triton X100 showed a greater proportion of polysome material than microsomal preparations from the same material. Up to four distinct polysome peaks were resolved. The proportion of sub-units remained essentially the same in both microsome and ribosome preparations. There was, however, in ribosome preparations no evidence of the diffuse peak attributable in microsome preparations to membrane-bound material.

C. RNA content

The % RNA in a variety of microsome and ribosome preparations as determined by the modified Schmidt Thannhausen procedure is shown in Table 4.

D. Analysis in the Model E analytical ultracentrifuge

Microsome and ribosome preparations from 3 day germinated beans were analysed in the Model E ultracentrifuge and the results presented in Fig. 13a. The upper section of the photographs shows the behaviour of a microsome preparation, the lower section a ribosome suspension prepared in the presence of 0.5%^(w/v) sodium deoxycholate. The microsome preparation showed four distinct peaks of sedimenting material and there is evidence of a very small, rapidly sedimenting peak for which no accurate S value could be determined. The 0.5%^(w/v) DOC ribosome preparation also showed evidence of four major sedimenting fractions essentially similar to those of the microsome preparation, and there was also evidence of an additional peak of material sedimenting at a slower rate. No accurate S value could be assigned to this peak because of the diffuse nature and small amplitude of the peak. This material must be assumed to represent only a minor

Table 4

% RNA in ribosome and microsome preparations as determined by the modified Schmidt Thannhauser procedure.

| Type of preparation | % RNA | |
|------------------------|------------------------------|------------------------------|
| | 3 day germinating bean | 60 day developing bean |
| Unclarified microsomes | 35.5 | 12.7 |
| Clarified microsomes | 39.5 | 39.5 |
| Triton X100 ribosomes | 43.4 | 43.5 |

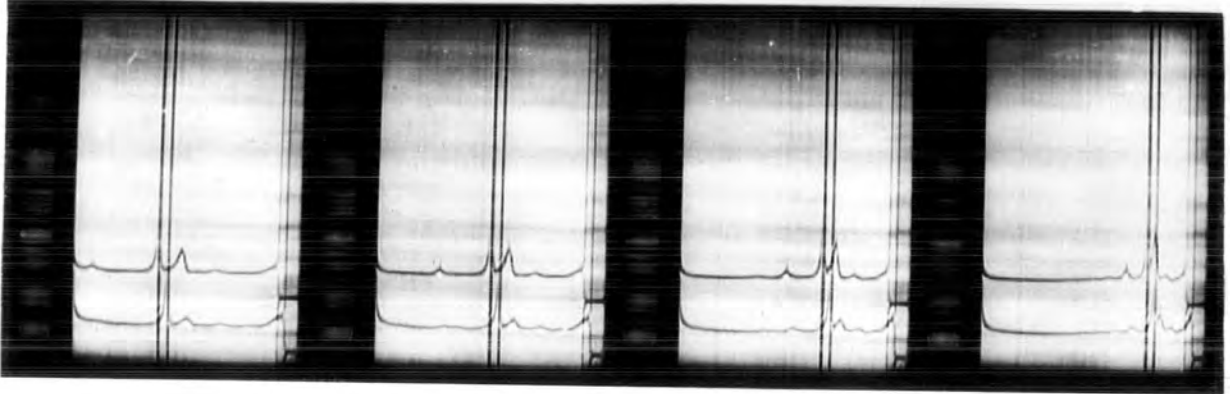
The % RNA was determined using the modified Schmidt Thannhauser procedure as described in the Methods, Section 14c.

Fig.13a. Schlieren profiles of a microsome and a ribosome preparation from 3 day germinated seeds of V.faba. Preparations (10 mg/ml) were centrifuged at 20° in a 12mm, 4° cell at 52,000 rev./min. Photographs were taken at 2 min intervals using a 3 sec exposure. The bar angle was 70° .

"A" is a microsome preparation.

"B" is a 0.5% (W/V) DOC ribosome preparation.

A
B



component of the ribosome preparation.

Fig. 13b shows the sedimentation behaviour of a 1%^(w/w) Triton X 100 ribosome preparation from 3 day germinated beans. As well as the four major sedimenting fractions common to all three preparations there was some evidence of two additional more rapidly sedimenting peaks. All the preparations used contained approximately 10 mg as determined by $E_{1\text{ cm}}^{260}$, of microsomes or ribosomes/ml. S values were calculated for each preparation and are presented in Table 5.

In order to assign accurate S values to each fraction the effect of concentration upon S value was determined. The four major fractions present in all three preparations were assumed to represent the same ribosome species in each case therefore the variation in S values obtained was assumed to represent concentration differences in the preparations and experimental variation. The concentration dependence of S value was determined for the 0.5%^(w/v) DOC ribosome preparation and the results shown in Fig. 14. The graphs were extrapolated to zero concentration to obtain S^0 , the sedimentation coefficient at zero concentration. The values obtained were assumed to represent the sedimentation coefficients of the major components of microsome and ribosome preparations from 3 day germinated bean. Peak 3 was equated with the monosome peak, whilst peaks 1 and 2, sedimenting at a slower rate, assumed to represent ribosome sub-units. The most rapidly sedimenting peak, was assumed to be a dimer consisting of two monomeric ribosomes. Assuming the ribosome to be a globular structure, the theoretical S value for a dimer (2n) composed of 2 x n monomers was calculated using the formula:

Fig.13b. Schlieren profile of a 1%^(w/w)Triton X-100 ribosome preparation from 3 day germinated seeds of V.faba. Details of cell size, and centrifugation parameters are given in the legend to Fig. 13a.

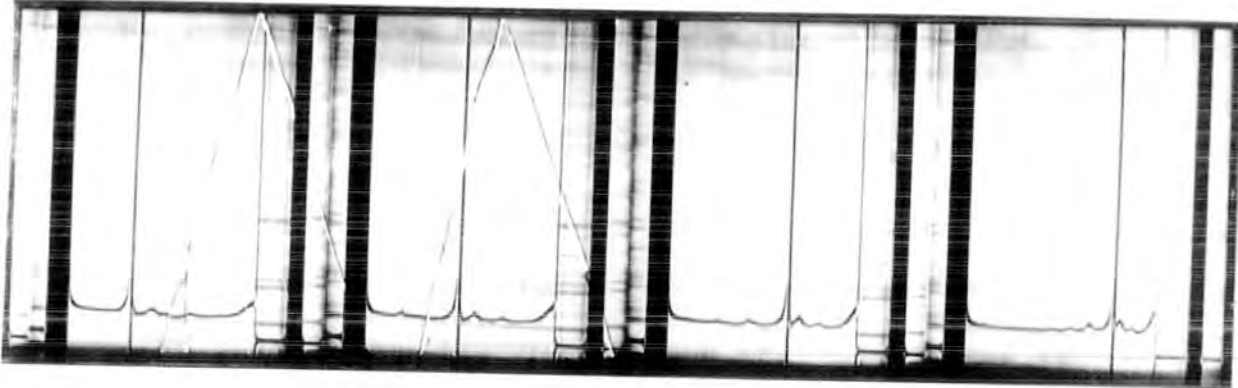


Table 5

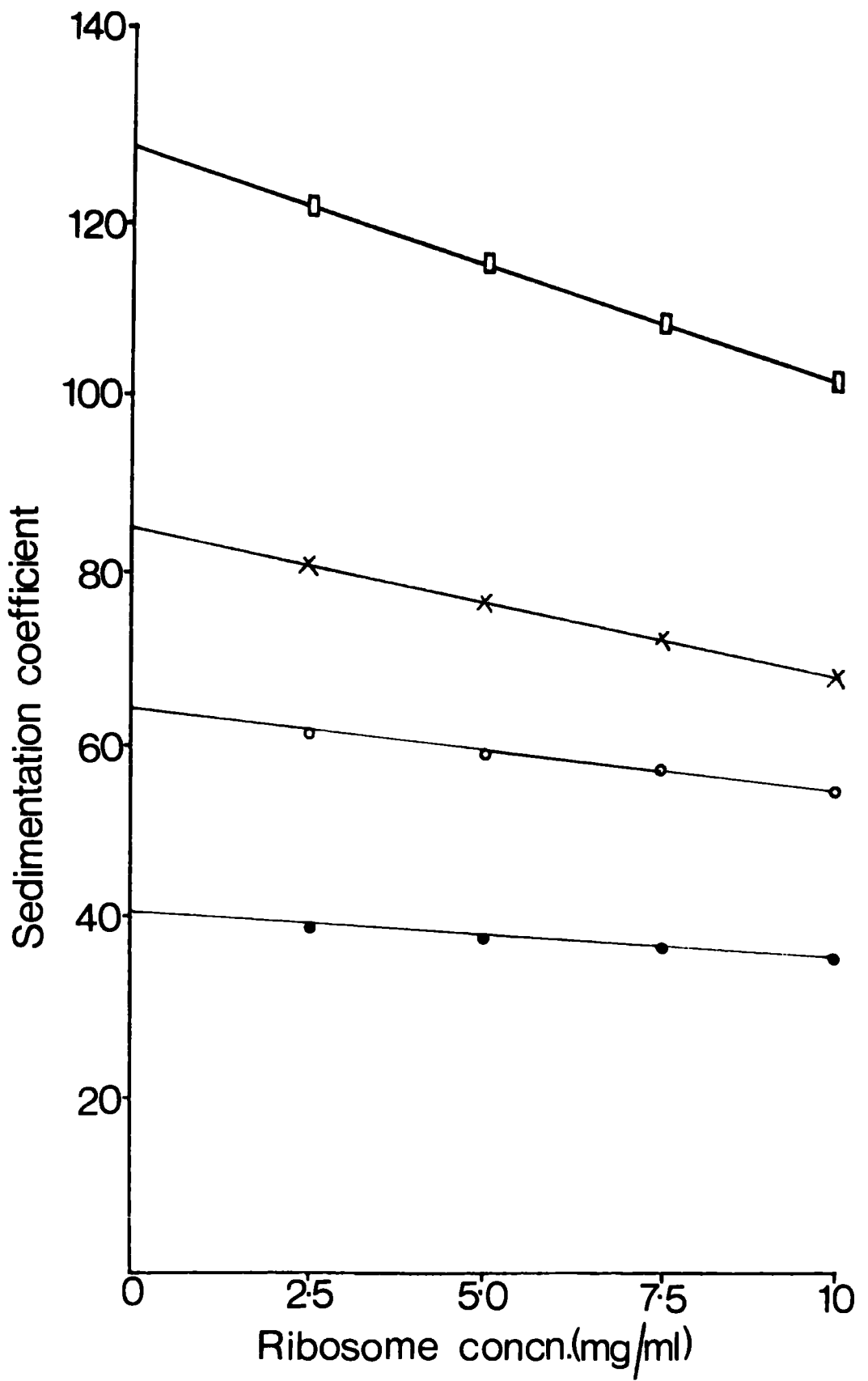
Sedimentation coefficient(s) of the major fractions of microsome and ribosome preparations from 3 day germinated seeds of Vicia faba.

| Type of preparation and concentration in mg/ml | Direction of sedimentation → | | | |
|---|---------------------------------|--------|--------|--------|
| | Peak 1 | Peak 2 | Peak 3 | Peak 4 |
| Microsome (11.5 mg/ml) | 38 | 58 | 69 | 106 |
| 0.5% ^(w/v) DOC ribosomes (10.0 mg/ml) | 37 | 56 | 69 | 103 |
| 1% ^(w/w) Triton X100 ribosomes (11.3 mg/ml) | 40 | 61 | 71 | 110 |

Parameters of centrifugation as detailed in the legend to Fig. 13.

Fig.14. The effect of ribosome concentration upon sedimentation coefficient. The 0.5% (w/v)DOC ribosome preparation was diluted to the required concentration and sedimentation coefficient determinations were carried out as described in the legend to Fig.13a.

| | S^0 (sedimentation coefficient at zero concentration) |
|------------------|---|
| ● ————— ● Peak 1 | 41 |
| ○ ————— ○ Peak 2 | 64 |
| × ————— × Peak 3 | 85 |
| □ ————— □ Peak 4 | 129 |



$$\frac{S_2}{S_1} = \left[\frac{M^2}{M_1} \right]^{0.61} \quad S_2 = \left[\frac{M^2}{M_1} \right]^{0.61} \times S_1$$

where S = sedimentation coefficient, M = molecular weight

Assuming S_1 to be 85.0 and $M^2/M_1 = 2$, the theoretical 'S' value for a dimer composed of 2 monomer ribosomes was calculated to be 129. Peak 4 was therefore identified as a polysome containing two ribosomes.

2. Counting of $[^{14}\text{C}]$ and $[^{35}\text{S}]$ compounds

The counting error, 2σ, was automatically determined by the scintillation counter for each sample counted. The value is related to the activity of the sample and decreases rapidly as the counting time increased. All data presented in this thesis is based on a 10 min sample count, the counting error being between ±3% and ±5%, however samples of low activity i.e. 200 c.p.m. were counted for 30 min. The background level remained approximately 50-60 c.p.m/sample with a counting error of ±15%. Since the efficiency of the scintillation counter for both $[^{14}\text{C}]$ and $[^{35}\text{S}]$ compounds was in excess of 85% the values obtained were not corrected for efficiency.

3. Interchangeability of the components of the poly U-dependent amino acid incorporating system

The major components of the amino acid incorporating systems (microsomes/ribosomes, tRNA and enzyme fraction) prepared from developing or germinating seeds of Vicia faba(L) were to a considerable extent interchangeable. Table 6 shows the values routinely obtained in the complete and transfer system with the various types of microsome

Table 6

Incorporation obtained in the poly(u) dependent complete and transfer systems with microsomes and ribosomes isolated from developing and germinated seeds of Vicia faba (L)

| Type of preparation | Incorporation (pmoles phenylalanine /mg rRNA in complete system) | Incorporation (pmoles phenylalanine/ mg rRNA in transfer system) |
|--|---|---|
| <u>60 day developing bean</u> | | |
| unwashed microsomes | 530.00 | 74.50 |
| microsomes washed by resuspension in resuspending medium | - | 55.30 |
| 4% (^w / _w) Triton X100 ribosomes | - | 71.8 |
| NH ₄ Cl washed ribosomes | - | 19.20 |
| <u>3 day germinated bean</u> | | |
| unwashed microsomes , | 422.0 | 165.7 |
| microsomes washed by resuspension in resuspending medium | - | 156.5 |
| 1% (^w / _w) Triton X100 ribosomes | 530.0 | 157.0 |
| NH ₄ Cl washed ribosomes | 44.6 | 81.20 |

The complete and transfer systems are described in the Methods. In all cases 0.2mg of yeast tRNA were used in conjunction with the optimum volume of bean dialysed enzyme.

or ribosome preparations. Microsomes from either type of material had approximate equivalent activity in the complete system. However in the transfer system the microsomes or ribosomes prepared from germinating seeds were more active than those obtained from 60 day developing seeds.

Table 7 indicates that tRNA and DE52 enzyme fraction prepared from developing and germinating beans were completely interchangeable with no significant variation in activity. tRNA and enzyme fractions were, however, routinely prepared from 60 day developing seeds because of the greater availability of material and the ease of handling. Both tRNA and enzyme fractions were stored for up to 1 year in liquid nitrogen without loss of activity.

Bean DE52 enzyme fraction catalysed the aminoacylation of commercially obtained yeast tRNA with only a 20% decrease in activity as calculated using the supplied specifications for levels of aminoacylation with the homologous enzyme. The Mg^{2+} optimum for aminoacylation of bean tRNA with DE52 supernatant was 10mM (Payne, 1970), for yeast tRNA the optimum was displaced to 16mM.

The deacylated yeast tRNA substituted for bean tRNA in the complete system with a resultant increase in activity. Using bean tRNA levels of 300-350 pmoles phenylalanine incorporated/mg rRNA were obtained, in the complete system whilst levels of 400-550 p moles phenylalanine incorporated/mg rRNA were obtained using yeast tRNA. From charging experiments ratio of the specific

Table 7

Interchangeability of tRNA and DE52 enzyme fraction from germinated and developing bean in the Poly(U) dependent complete system.

| | | | | |
|---|-------|-------|-------|-------|
| 1%(^w / _w) Triton X100 ribosomes | G | G | G | G |
| DE52 enzyme | G | G | D | D |
| Deacylated tRNA | G | D | G | D |
| <hr/> | | | | |
| Incorporation (pMoles phenylalanine/mgrRNA) | 491.0 | 504.8 | 477.0 | 518.2 |
| Incorporation as % of complete germinated system | 100% | 102% | 97% | 105% |

The complete system is described in the Methods, and contained 0.2mg of deacylated tRNA and the optimum volume (as separately determined) of DE52 enzyme fraction in a total volume of 500 μ l. In the table G indicates that the component was derived from 3 day germinated beans, and D from 60 day developing cotyledons of V. faba.

activity (pmoles phenylalanine/mg tRNA) of bean: yeast tRNA was shown to be 29.5:38.0. It seems likely, therefore, that the difference in incorporation in the complete system may be largely attributable to differences in specific activity of the tRNA used. Substitution of bean tRNA by yeast tRNA resulted in a displacement of the Mg^{2+} optimum for complete amino acid incorporating system from 10 mM to 16 mM.

Aminoacylated yeast tRNA could substitute for aminoacylated bean tRNA in the transfer system, with an increase in activity which reflected the greater specific activity with respect to phenylalanine of the yeast tRNA. The Mg^{2+} optimum for the transfer system was independent of tRNA source and was 7 mM.

Ribosomes, tRNA and 40/70 enzyme fraction prepared from rabbit reticulocytes could substitute for the appropriate bean component with varying degrees of efficiency. Reticulocyte ribosomes and tRNA could replace bean Triton X100 ribosomes and bean tRNA in both the complete and transfer systems without significant variation in the levels of activity. However substitution of bean dialysed supernatant by reticulocyte 40/70 enzyme fraction was associated with a significant reproducible increase in activity in both the complete and transfer system. Table 8 shows the effect of substitution of bean dialysed enzyme by reticulocyte 40/70 enzyme fraction using microsomes or ribosomes prepared by a variety of methods. The increase was most pronounced with 0.5% (^{w/w}) DOC ribosomes and NH_4Cl ribosomes.

Table 8

Interchangeability of bean dialysed enzyme fraction and reticulocyte 40/70 enzyme fraction in the Poly(U) dependent complete system using microsomes or ribosomes isolated from 3 day germinated bean.

| Method of preparation of microsomes or ribosomes | Incorporation (pmoles phenyl-alanine/mgrRNA) with bean dialysed enzyme | Incorporation (pmoles phenyl-alanine/mgrRNA) with reticulocyte 40/70 enzyme fraction | Incorporation by reticulocyte 40/70 enzyme as a % of that obtained with bean enzyme |
|--|--|--|---|
| Unwashed microsomes | 512.0 | 1032.0 | 201% |
| 1% ^{w/w} Triton X100 ribosomes | 570.0 | 1008.0 | 176% |
| 0.5% ^{w/v} DOC ribosomes | 16.20 | 162.5 | 1002% |
| NH ₄ Cl ribosomes | 44.50 | 246.5 | 548% |

The complete system is given in the Methods. All microsomes or ribosomes were prepared from 3 day germinated beans and in all cases 0.2mg deacylated yeast tRNA, was used. In both cases the optimum volume of enzyme, as separately determined, was used.

4. Comparison of the efficiency in poly(U)-directed amino acid incorporation of microsomes and ribosomes prepared by various methods.

Table 6 shows the values routinely obtained in the complete and transfer system with the various types of micromes and ribosomes. There was no significant difference in activity between unwashed and washed micromes and Triton X100 ribosomes from the same tissue when compared in the same system, using the same enzyme and tRNA source. However Table 8 indicates that the liberation of ribosomes from the endoplasmic reticulum by treatment of the post mitochondrial supernatant with 0.5%^(w/v)DOC resulted in a significant decrease in activity. A similar decrease in incorporation was observed in the transfer system when 0.5%^(w/v)DOC ribosomes were used. Treatment of ribosomes with 0.5M-ammonium chloride in an attempt to remove the factors involved in peptide chain elongation was associated with a concomitant decrease in incorporation in both the complete and transfer systems (Table 6).

When reticulocyte 40/70 enzyme fraction was used as the enzyme fraction with microsomes or Triton X100 ribosomes prepared from 3 day germinated bean there was an approximate 100% increase in amino acid incorporation in the poly(U) dependent complete system as shown in Table 8. Substitution of bean enzyme by reticulocyte 40/70 enzyme resulted in approximately 500% and 1000% increase in incorporation with NH_4Cl and 0.5%^(w/v) DOC ribosomes respectively.

Table 9 shows the effect of the method of ribosomes preparation on the dependence of incorporation upon added enzyme, in the transfer system. Washing of the microsomes by repelleting

Table 9

Effect of method of ribosome preparation upon the enzyme dependence of Poly(U) dependent amino acid incorporation in the transfer system.

| Method of microsome or ribosome preparation | Incorporation (pmoles phenylalanine/mgrRNA) in the transfer system with bean dialysed enzyme | Incorporation (pmoles phenylalanine/mgrRNA) in the transfer system in the absence of enzyme | Incorporation in the absence of enzyme as a % incorporation in the presence of enzyme |
|--|--|---|---|
| Unwashed microsomes | 165.7 | 11.10 | 6.65% |
| Microsomes washed by resuspension from resuspending medium | 156.5 | 1.53 | 1% |
| 1% ^{w/w} Triton X100 ribosomes | 157.0 | 2.160 | 1.38% |
| NH ₄ Cl ribosomes | 25.40 | 0.64 | 2.52 |

The transfer system is described in the Methods. All microsomes or ribosomes were prepared from 3 day germinated beans and in all cases 0.3mg of deacylated yeast tRNA was used, the volume of enzyme employed was determined separately and was that volume promoting maximum incorporation of phenylalanine.

from resuspending medium resulted in a considerable decrease in the incorporation obtained in the absence of added enzyme. Liberation of ribosomes from the ER by treatment with 1%^(w/w) Triton X100 resulted in a similar increase in the dependence upon added enzyme. Washing of the 1%^(w/w) Triton X100 ribosomes with 0.5M-ammonium chloride resulted in a considerable decrease in incorporation in the presence of enzyme as well as in the absence of added enzyme. When the incorporation in the absence of enzyme is expressed as a % of that obtained in its presence the decreased incorporation in the absence of enzyme is no longer obvious. Values for incorporation by NH_4Cl ribosomes in the absence of enzyme were often not significantly greater than the zero time control value.

5. Characterisation of the *in vitro* aminoacid incorporating systems

A. tRNA aminoacylation system

This system was used in the preliminary assay of aminoacylation of yeast and bean tRNA, and in the bulk preparation of aminoacyl-tRNA.

Using yeast tRNA, levels of 450pmoles phenylalanine/mg tRNA were obtained in preliminary charging experiments, however in bulk charging experiments levels of 380pmoles phenylalanine/mg tRNA were routinely obtained. Table 10 indicates that the major loss in radioactivity occurred during the initial ethanol precipitation involved in recovery of the aminoacylated tRNA from the incubation mixture.

Table 10

Loss of $[^{14}\text{C}]$ phenylalanine from $[^{14}\text{C}]$ phenylalanyl-tRNA during recovery from the aminoacylation incubation mixture.

| Sample removed at end of aminoacylation period (pmoles phenylalanine/mg tRNA) | Samples removed after first ethanol precipitation (pmoles phenylalanine/mg tRNA) | Sample removed after second ethanol precipitation (pmoles phenylalanine/mg tRNA) |
|---|--|--|
| 474.2 | 367.2 | 341.3 |
| % loss of radio-activity | 22.5% | 28.0% |

100mg of yeast tRNA were aminoacylated with $[^{14}\text{C}]$ phenylalanine as described in the Methods. 2x0.1ml samples were removed at the end of the above stages and 5.0ml 5%^(w/v) TCA ice cold TCA were added. After standing for 1 hr in an ice bath the precipitate was collected on a Millipore filter and washed using 3 x 5 ml of ice cold 5%^(w/v) TCA and counted as described in the Methods. The tRNA concentration was determined from the $E_{1\text{cm}}^{260}$ using a further 0.1ml sample.

The effect of temperature on aminoacylation of yeast tRNA with phenylalanine using bean DE52 supernatant as enzyme fraction is shown in Fig.15. There was a rapid increase in aminoacylation as the temperature increased from 0°-20°. Between 20-30° there was a linear, but very slight, increase in aminoacylation with increasing temperature. Beyond 30° the degree of aminoacylation decreased with increasing temperature. At 30° more than 60% of the observed aminoacylation occurred within the first 10 min. of incubation, and the aminoacylation was complete within 20 min (Fig.16).

The effect of magnesium concentration on aminoacylation is shown in Fig.17. The degree of aminoacylation of yeast tRNA with phenylalanine increased with increasing (Mg^{2+}), reaching a maximum at 16mM. Thereafter, increasing the concentration to 20mM had no further effect. Such a result is in striking contrast to that of Payne (1970). Using a bean 105,000xg av. supernatant as enzyme source, this author found a sharp magnesium optimum for aminoacylation of tRNA extracted from 80 day developing beans at 10mM. With a second optima at 20mM (between the two optima there was a minimum at 15mM).

The degree of aminoacylation increased with increasing enzyme volume up to a maximum level, beyond which addition of further enzyme had no significant effect upon the amount of aminoacylation obtained (Fig.18). That there was no significant decrease in aminoacylation with addition of enzyme in excess of the amount required for maximum incorporation optimum indicates the relative absence from the enzyme preparation of ribonucleases and esterases.

Fig.15. Effect of temperature on the aminoacylation of yeast tRNA with $[^{14}\text{C}]$ phenylalanine (48mC/mM). The tRNA aminoacylation system is described in Methods 15 and contained 0.1mg yeast tRNA and the optimum volume (as separately determined) of DE52 supernatant in a total volume of 100 μ l. The incubations were maintained for 20 min at the stated temperature.

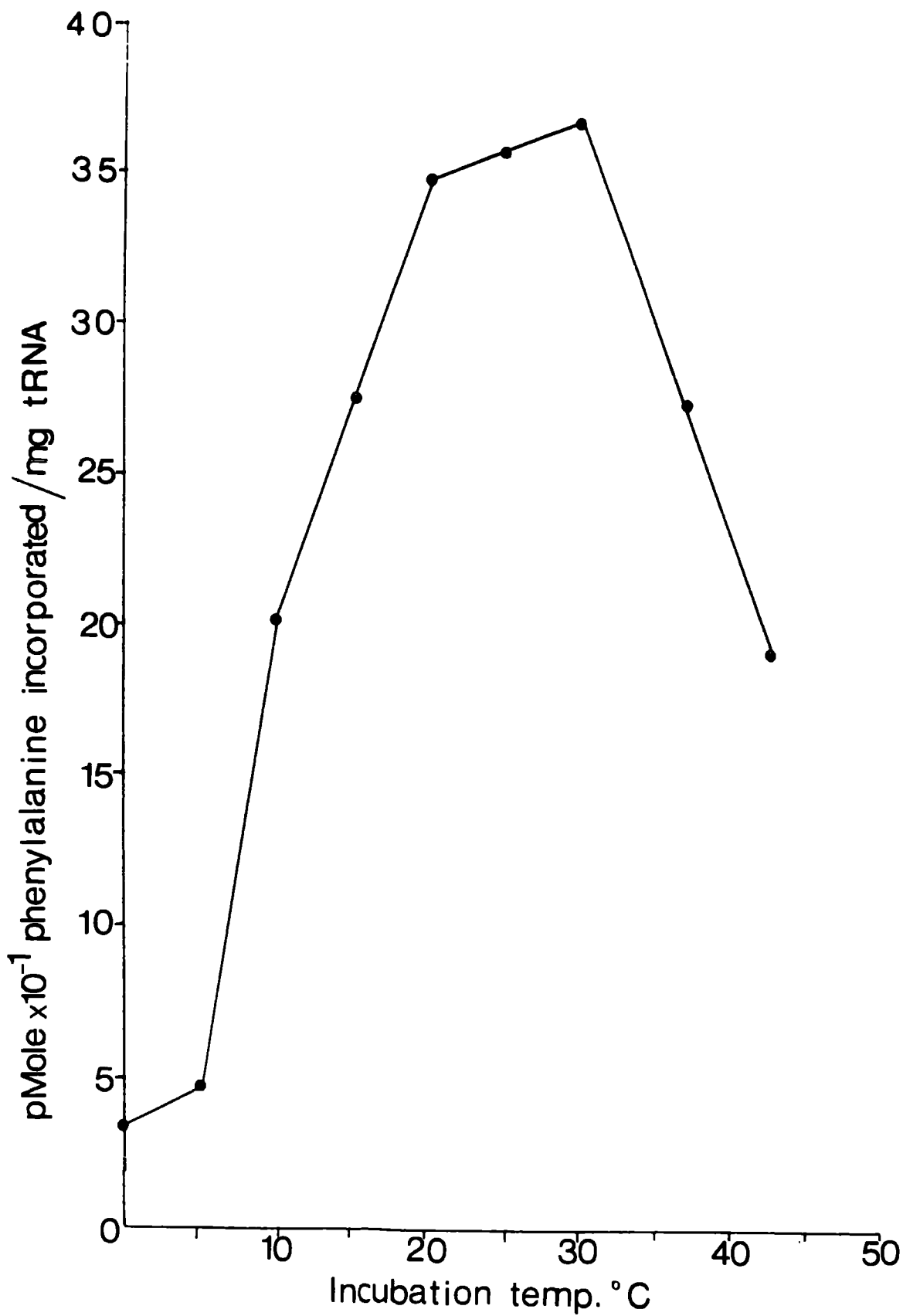


Fig.16. The time course of aminoacylation of yeast tRNA with $[^{14}\text{C}]$ phenylalanine. The tRNA aminoacylation system is described in Method 15 and contained 0.1mg tRNA and the optimum volume (as separately determined) of bean DE52 supernatant in a total volume of $100\ \mu\text{l}$. Incubations were maintained at 30° for the stated time interval.

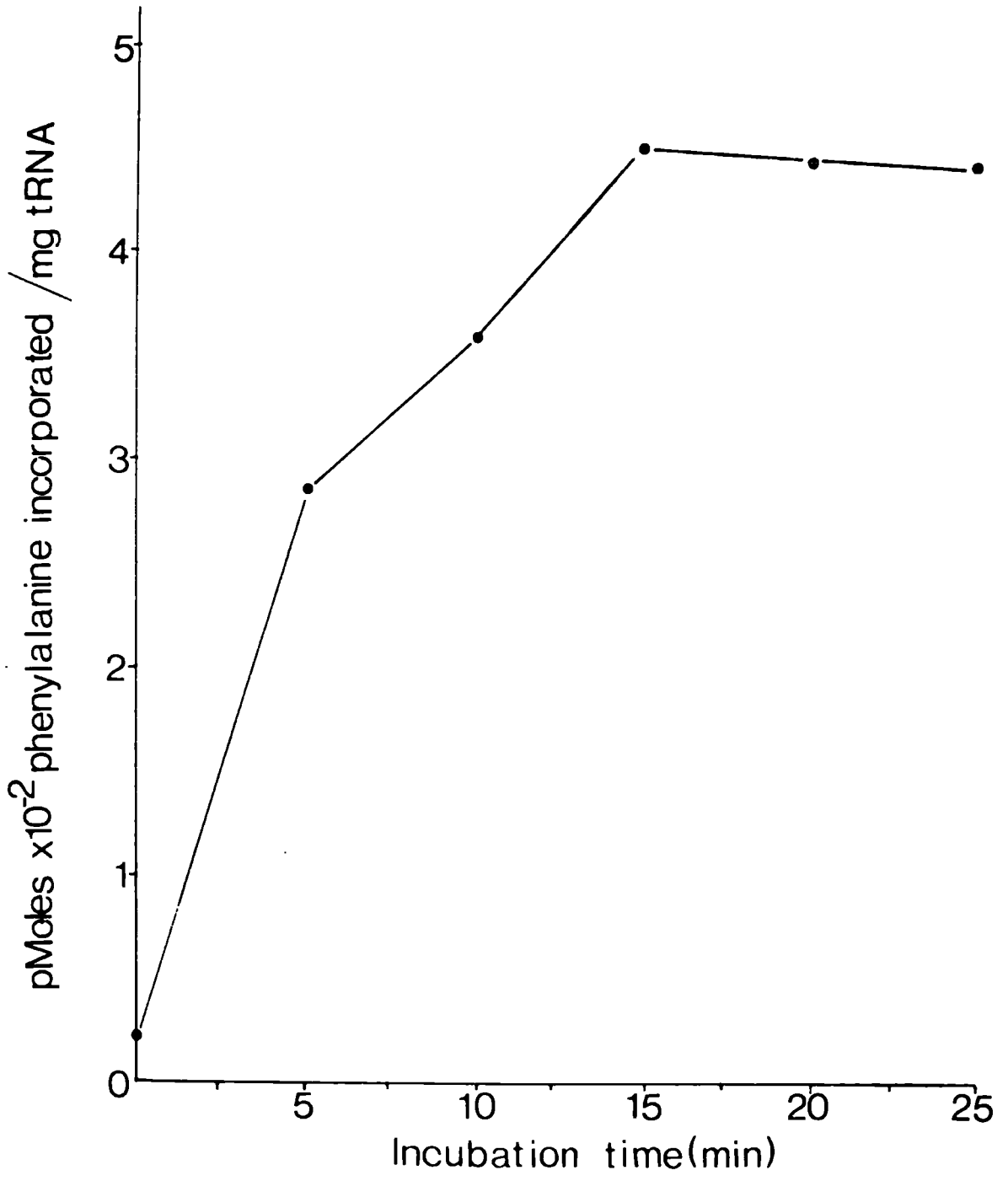


Fig.17. The effect of magnesium concentration upon the aminoacylation of yeast tRNA by $\left[^{14}\text{C}\right]$ phenylalanine (48mC/mM). The tRNA aminoacylation system is described in the methods (section 15) and contained 0.1mg yeast tRNA and the optimum volume of bean DE52 supernatant in a total volume of 100 μ l.

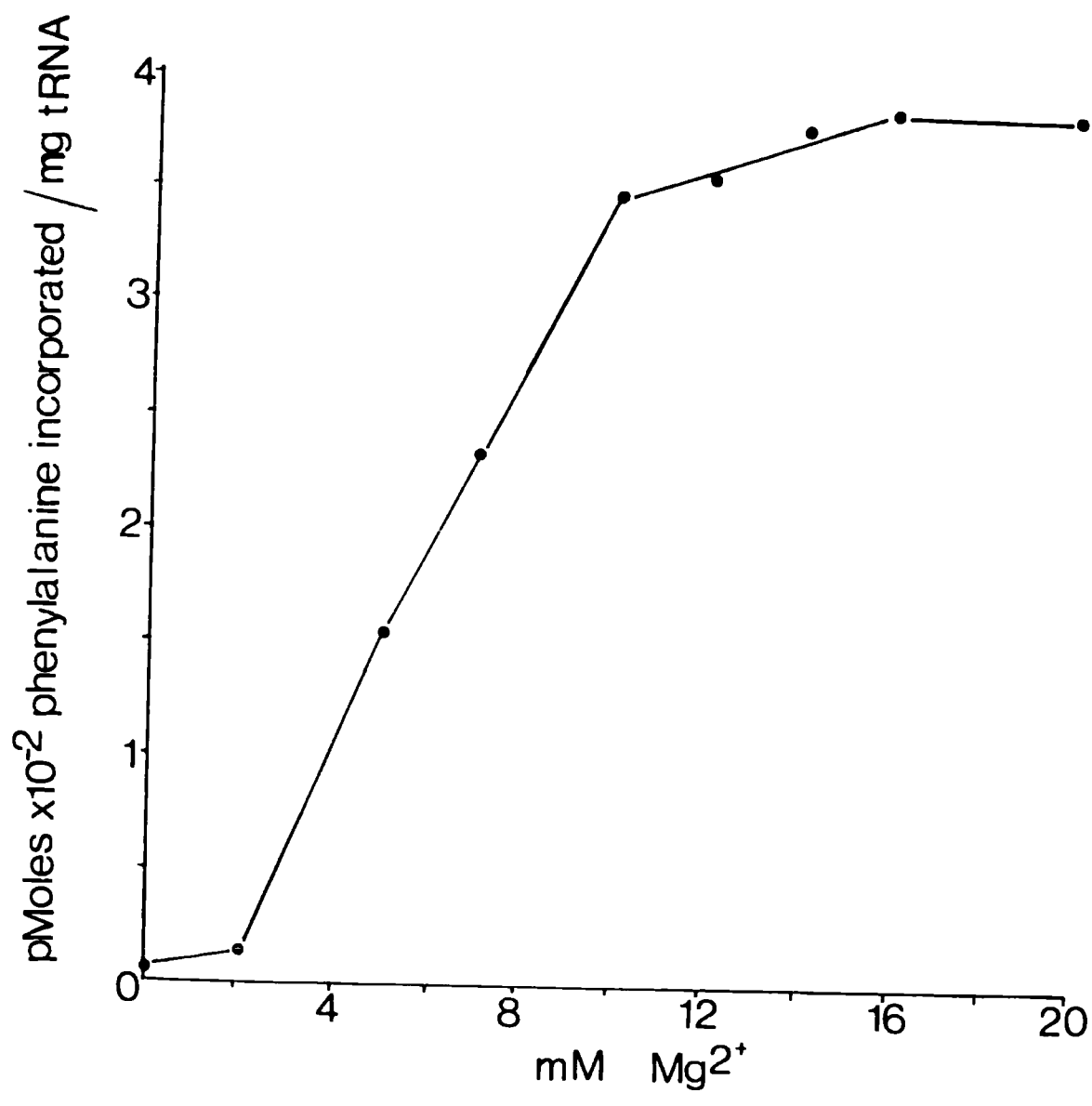
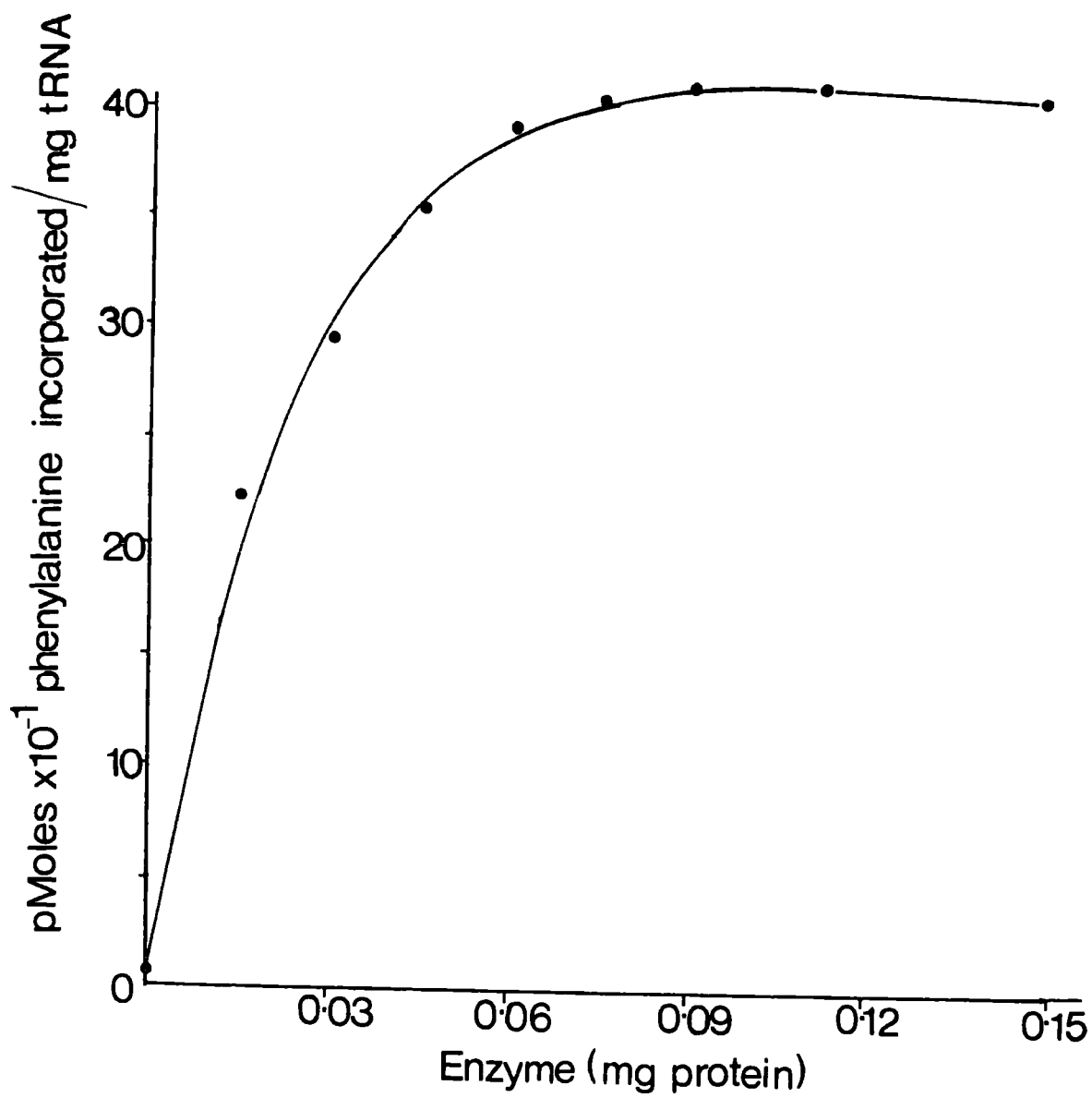


Fig.18. Effect of V. faba DE52 supernatant enzyme on aminoacylation of yeast tRNA with [¹⁴C]phenylalanine(48mC/mM). The aminoacylation system is described in Methods, Section 15.



The concentration of the DE-52 supernatant was usually approximately 15mg/ml, concentrations of approximately 1mg DE52 enzyme/ml incubation promoted maximum aminoacylation.

Both aminoacylated and deacylated tRNA stored in liquid nitrogen remained active for periods in excess of 1 year. Repeated freezing and thawing had no effect upon the activity of either aminoacylated or deacylated tRNA.

B. The poly(U)-dependent complete system

In the presence of microsomes or ribosomes, deacylated tRNA and enzyme fraction containing all those enzymes required for aminoacylation of tRNA, and peptide chain elongation and translocation, and a suitable informational template, free amino acids can be incorporated into hot (90°) TCA insoluble (peptidyl) material.

In this work the incorporation of [¹⁴C] phenylalanine in response to the synthetic messenger, poly(U) was characterized using material prepared from developing and germinating beans. The basic characteristics of the system were the same with material prepared from developing or germinating beans and the only significant difference noted was in the levels of incorporation obtained (Table 6).

Table 11 shows the requirements of the system using microsomes from germinated seeds. There was an almost complete dependence upon added messenger, Poly(U). Levels of 300-500 pmoles phenylalanine incorporated/mg rRNA were routinely obtained in the presence of Poly(U), in its absence levels of 10-25 pmoles phenylalanine/mg rRNA were obtained.

Table 11

Dependence of incorporation of $[^{14}\text{C}]$ phenylalanine in the Poly(U) dependent complete system upon added components.

| Incubation | Incorporation (pmoles phenylalanine/ mg rRNA) | % complete |
|---------------------------------------|---|------------|
| complete system | 358 | 100 |
| complete-Poly(U) | 23 | 6 |
| complete-GTP | 242 | 67 |
| complete-ATP | 61 | 17 |
| complete-ATP generating system | 177 | 49 |
| complete-ATP-ATP generating system | 3 | 1 |
| complete-DTT | 209 | 58 |
| complete-enzyme | 21 | 6 |
| complete-tRNA | 33 | 9 |
| complete-microsomes | 1 | < 1 |

The complete system used was as described in the Methods and contained 0.5mg microsomes isolated from 3 day germinated beans, 0.2mg deacylated yeast tRNA and the optimum volume (as separately determined) of bean dialysed enzyme in a total volume of 500 μ l.

The effect of pH on the Poly(U) dependent incorporation of phenylalanine is presented in Fig.19. The sharp pH optimum at pH7.6 was followed by a decrease in incorporation with increasing pH up to pH8.6 at which point the incorporation had fallen to 75% of its value at the pH optimum. An increase of 0.6 pH units above the optimum of pH7.6 was accompanied by a 17% decrease in activity whereas decreasing the pH 0.6 units below the optimum resulted in an 86% fall in activity.

The rate of Poly(U)-dependent phenylalanine incorporation was strongly temperature dependent (Fig.20.) The time course of phenylalanine incorporation (Fig.21) at 30° shows a linear increase in incorporation with time up to 40 min at 30°, whereas at 37° the initial rate of incorporation was greater but the maximum incorporation was lower and was attained over a period of 30 min. At both 30° and 37° incorporation was virtually complete after 40 min and further incubation for periods of up to 120 min did not result in a significant increase in incorporation indicating the relative absence of significant amounts of bacteria from the preparations (Mans and Novelli, 1964).

Figure 22 shows the dependence of incorporation upon added magnesium. Incorporation increased with increasing magnesium to reach a maximum between 16-18mM, addition of further magnesium up to 20mM was accompanied by a decrease in incorporation.

As shown in Table 11, maximum levels of phenylalanine incorporation were dependent upon the addition of GTP and ATP. The incorporation obtained was also dependent upon the addition of enzyme. In most cases dialysed supernatant or DE52 supernatant

Fig. 19. Effect of pH upon the Poly(U) dependent incorporation of [14 C]phenylalanine in the complete system. The complete system is described in Methods, Section 19B and contained 0.5mg microsomes prepared from 3 day germinated seeds of V. faba and 0.2mg deac ylated yeast tRNA and the optimum volume of dialysed bean supernatant in a total volume of 500 μ l.

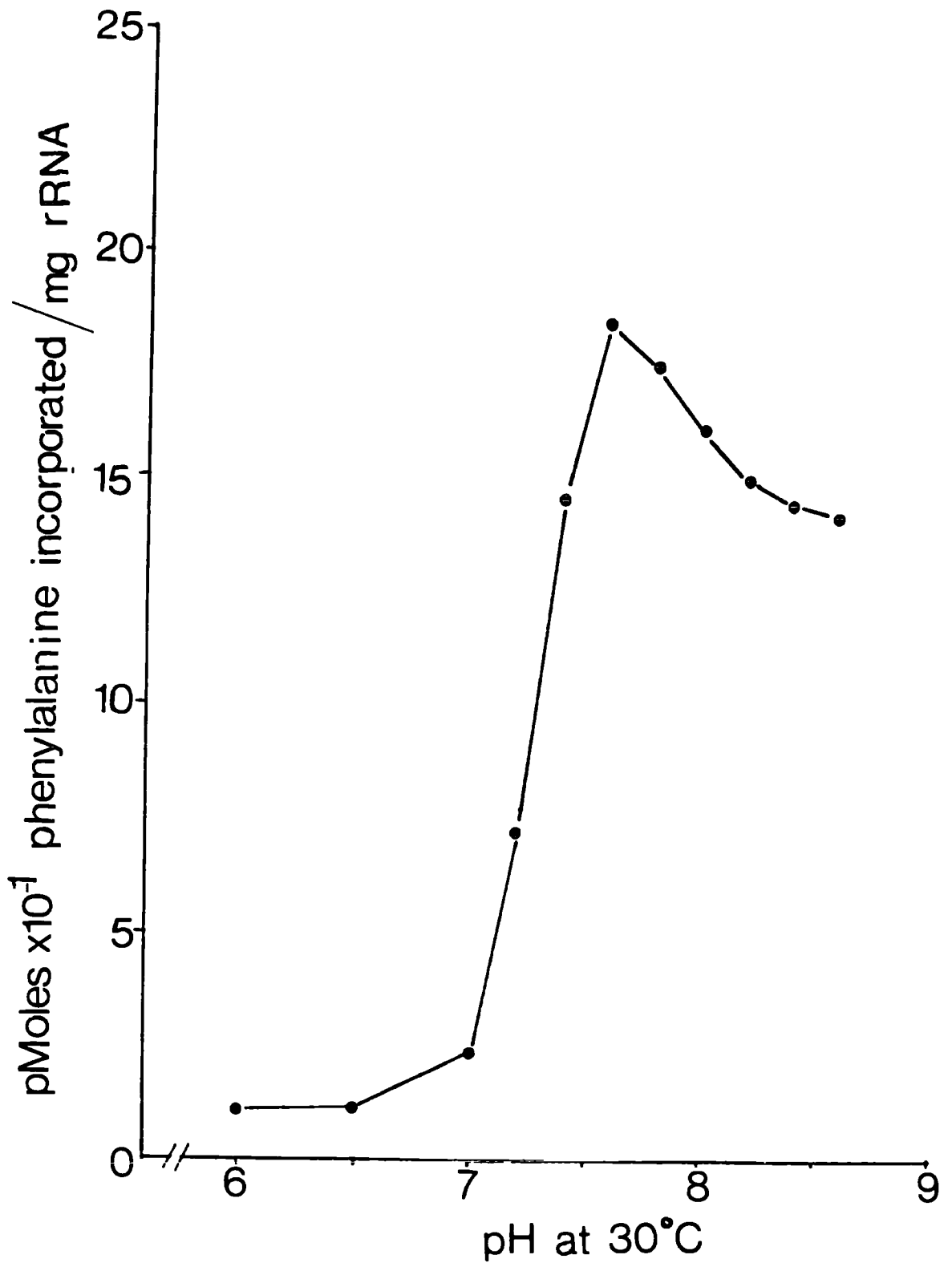


Fig. 20. Effect of temperature on the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalanine in the complete system. The complete system is described in Methods, Section 19B and contained 0.5mg microsomes prepared from 3 day germinated beans, 0.2mg deacylated yeast tRNA and the optimum volume as separately determined of bean dialysed DE52 enzyme in a total volume of 500 μ l. Incubations were maintained at the stated temperature for 20 min.

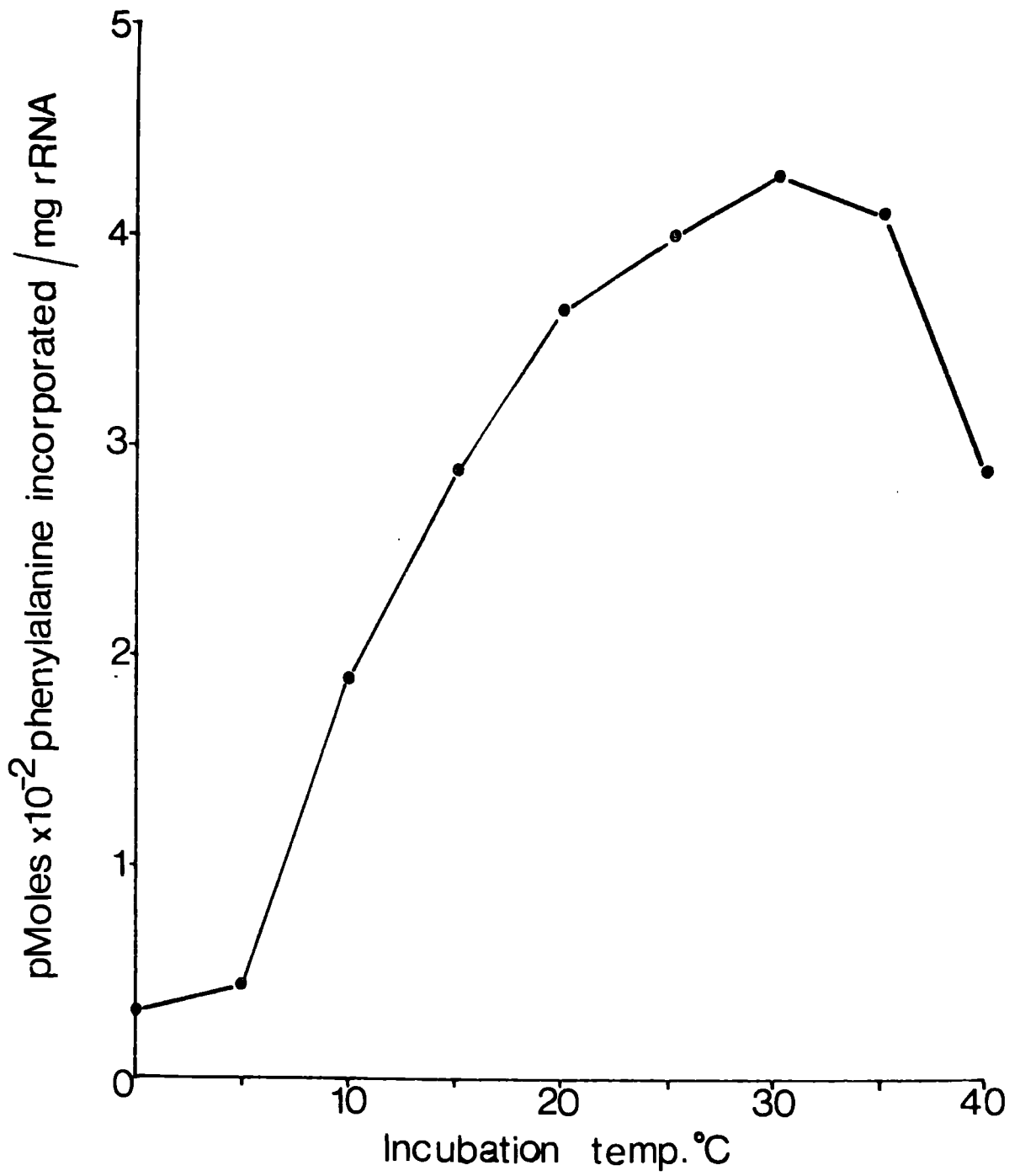


Fig.21. The time course for incorporation of $[^{14}\text{C}]$ phenylalanine in the Poly(U) dependent complete system. The complete system is described in Methods, Section 19B and contained 0.5mg of microsomes from 3 day germinated seeds of V. faba, 0.2mg deacylated yeast tRNA and the optimum volume of bean dialysed enzyme in a total volume of 500 μ l.

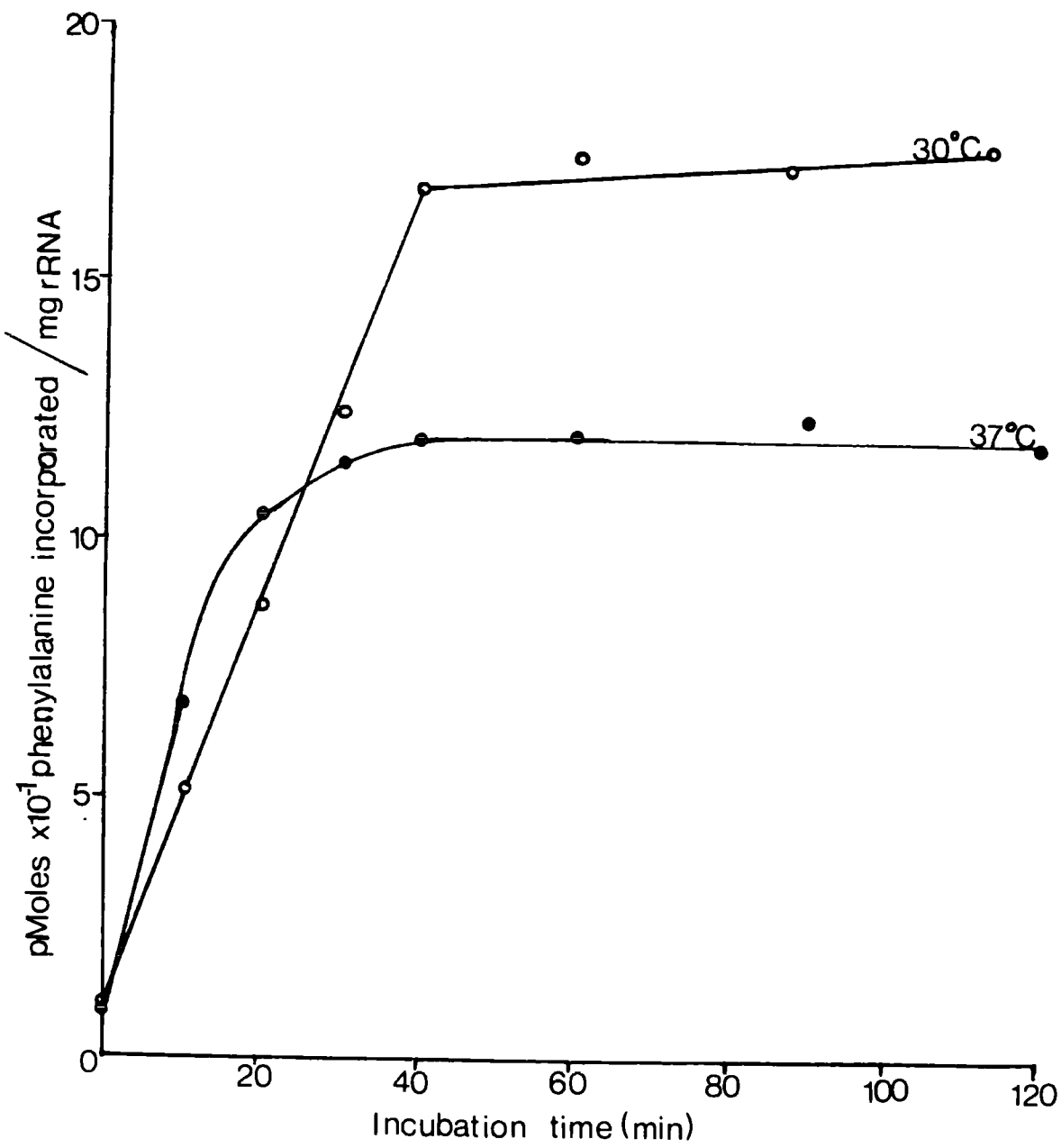
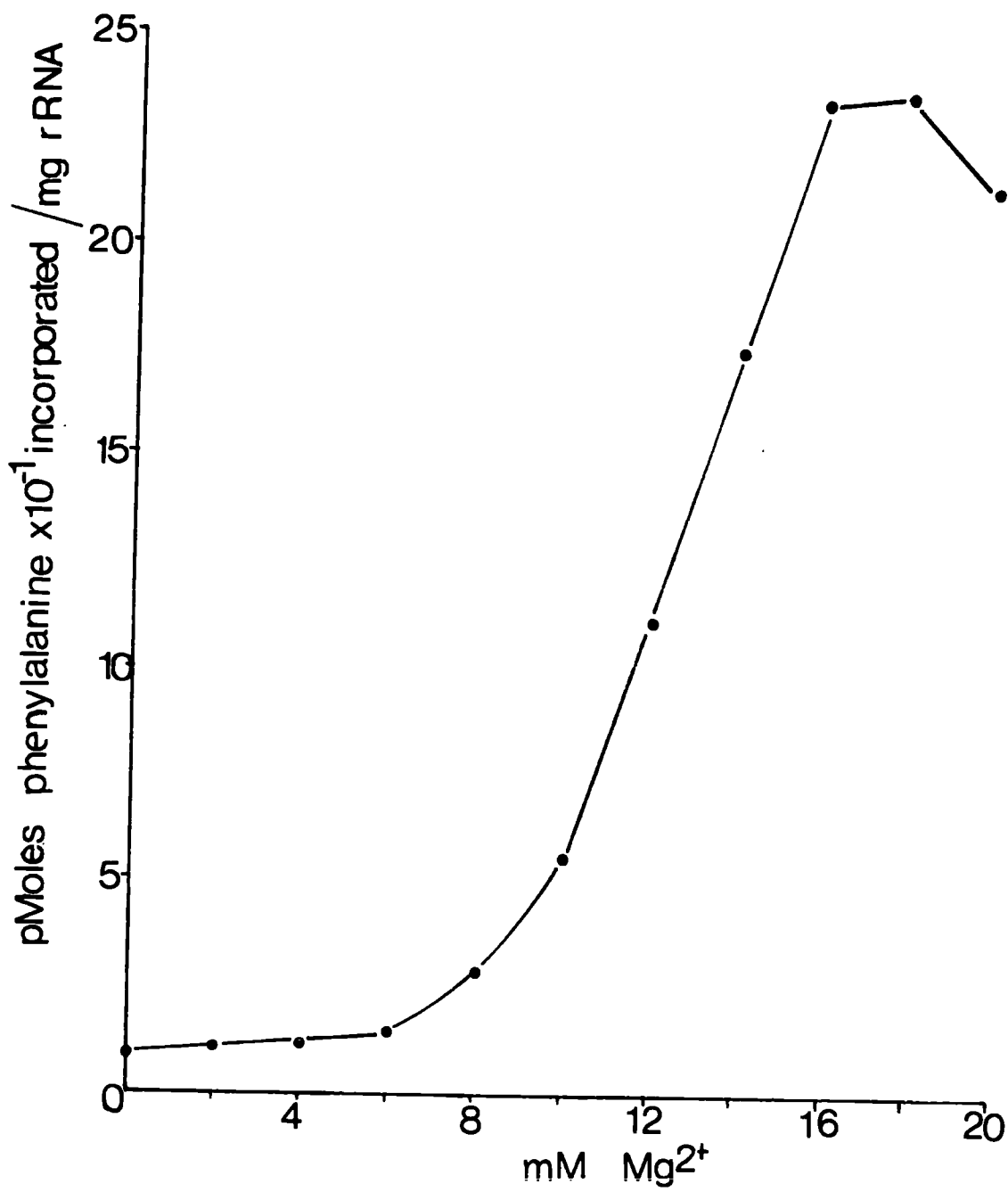


Fig.22. Effect of magnesium concentration on the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalaine in the complete system. The complete system is described in Methods, Section 19B.



was used as enzyme source. The amount of enzyme used was that volume which resulted in the maximum amount of incorporation and was routinely in the region of 1 mg protein/1 ml incubation. Table 11 also shows that there is considerable dependence upon added tRNA, however dependence upon added tRNA was always less than upon added enzyme. There was no significant incorporation in the absence of microsomes or ribosomes.

C. Poly(U) dependent transfer system

The transfer of phenylalanine from aminoacyl linkage in [^{14}C]phenylalanyl-tRNA into peptide bond in polyphenylalanine was measured in the Poly(U) directed transfer system. (Tables 12 and 13)

Incorporation of [^{14}C]phenylalanine into peptide was strongly dependent upon the presence of the synthetic messenger, Poly(U). In the absence of Poly(U) only low but significant and reproducible levels of incorporation were obtained, these are attributed to natural messenger RNA attached to the microsomes or ribosomes. In the absence of microsomes or ribosomes there was no significant incorporation of phenylalanine.

In the absence of added enzyme the incorporation by bean microsomes was decreased to 6% of that obtained in the complete transfer system. However using 1% Triton X100 ribosomes the level of incorporation in the absence of added enzyme fell to 2% of that obtained with the complete system. Using either bean microsomes or ribosomes only partial dependence upon added GTP and DTT could be demonstrated, and in each case the level of dependence was closely similar.

Table 12

Dependence of Poly(U) directed transfer system upon added cofactors using bean microsomes.

| Component | Incorporation (pmoles phenylalanine/ mgrRNA) | % complete transfer system |
|-----------------------------|--|-------------------------------|
| complete transfer system | 143 | |
| complete-enzyme | 8.7 | 6 |
| complete-Poly(U) | 5.9 | 4 |
| complete-GTP | 73.7 | 52 |
| complete-DTT | 17.5 | 12 |
| complete-microsomes | 1.2 | 1 |

The transfer system contained 0.5 mg of microsomes from 3 day germinated beans, 0.2mg yeast [^{14}C]-phe-tRNA (311 pmoles/mgRNA) and the optimum volume (as separately determined) of bean dialysed enzyme. Incubation was carried out at 30^o for 20 min.

Table 13

Dependence of the Poly(U) directed transfer system upon added cofactors using bean ribosomes.

| Component | Incorporation (pmoles phenylalanine/ mg rRNA | % complete transfer system |
|-----------------------------|--|-------------------------------|
| complete transfer system | 141.0 | |
| complete-enzyme | 2.44 | 2% |
| complete-Poly(U) | 4.21 | 3% |
| complete-GTP | 81.00 | 57% |
| complete-DTT | 18.40 | 13% |
| complete-ribosomes | 0.92 | 1% |

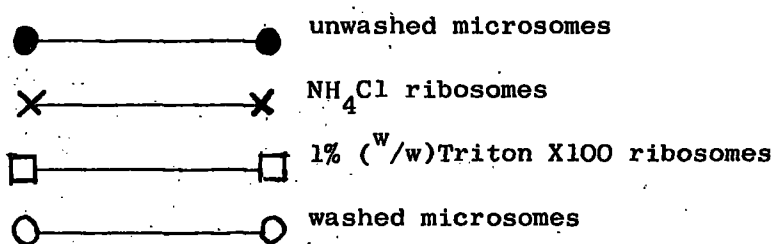
The transfer system contained 0.5mg of 3 day germinated bean Triton X100 ribosomes, 0.2mg of yeast $[^{14}\text{C}]$ -phe-tRNA (311 pmoles/mg tRNA) and the optimum volume (as separately determined) of bean dialysed enzyme. The conditions of incubation were as described in the Methods.

The transfer system was strongly dependent upon added magnesium, incorporation increasing with increasing levels of magnesium to reach a sharp optimum at 7mM. The addition of further magnesium up to 20mM being accompanied by an approximately 88% decrease in phenylalanine incorporation. Figure 23 shows the effect of magnesium concentrations upon incorporation with various types of microsomes or ribosome preparations prepared from 3 day germinated seeds, the shape of the curve is basically similar in each case, and the magnesium optimum for all four types of preparation was 7mM.

Incorporation of phenylalanine in the Poly(U) dependent system exhibited a sharp temperature optimum at 30° (Fig.24). Figure 25 shows the time courses for incorporation at 25°, 30° and 37°. At 25° and 30° incubation continued throughout the 60 min assay period. In each case the high initial linear rate of incorporation was maintained for approximately 20 min after which there was a gradual decline in the rate of incorporation. The highest level of incorporation was achieved at 30°. At 37° however the initial rate of incorporation was faster but was maintained for only 5 minutes and incorporation was virtually complete within 20 min and the maximum incorporation obtained was 50% lower than that obtained at 30°.

Incorporation of phenylalanine was dependent upon the concentration of [¹⁴C]phenylalanyl-tRNA added to the incubation (Fig.26), increasing to a maximum at approximately 0.20 mg [¹⁴C]phe-tRNA/500 μ l incubation. This amount of [¹⁴C]phe-tRNA was therefore routinely added to the transfer system rather than adding a constant

Fig.23. Effect of magnesium concentration on the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalanine in the transfer system. The transfer system contained 0.2mg yeast $[^{14}\text{C}]$ Phe-tRNA (sp.act. 342pmoles/mgtrRNA) and 0.5mg of the stated type of microsomes or ribosomes from 3 day germinated beans, and the optimum volume (as separately determined) of bean dialysed supernatant.



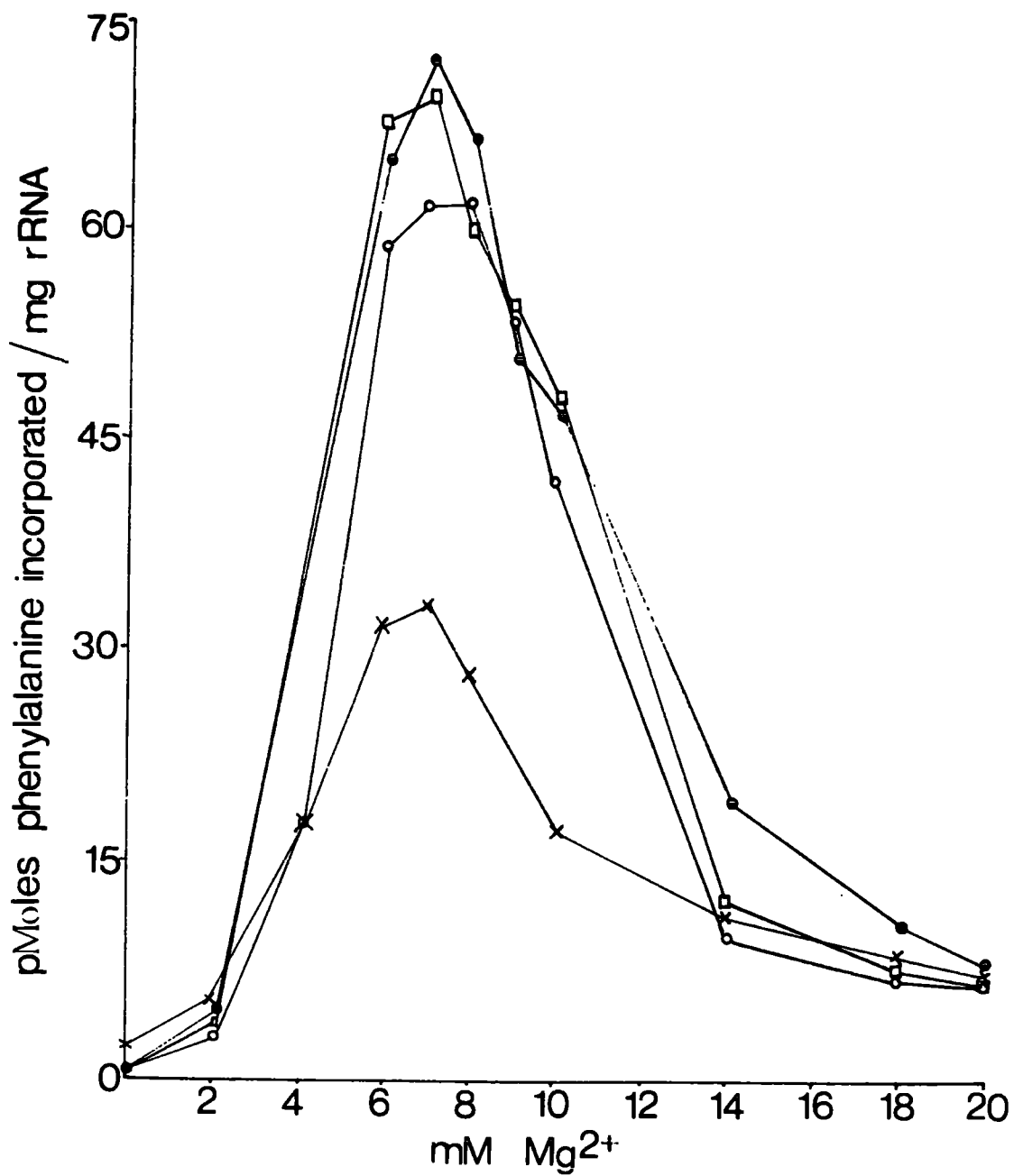


Fig.24. Effect of temperature upon the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalaine in the transfer system. The transfer system is described in Methods, Section 19A and contained 0.5mg of NH_4Cl ribosomes prepared from 3 day germinated beans, 0.2mg yeast $[^{14}\text{C}]$ Phe-tRNA (sp.act.382pMoles/mgRNA) and the optimum volume (as separately determined) of G25 supernatant prepared from 60 day developing beans. Incubations were maintained at the stated temperature for 20 min.

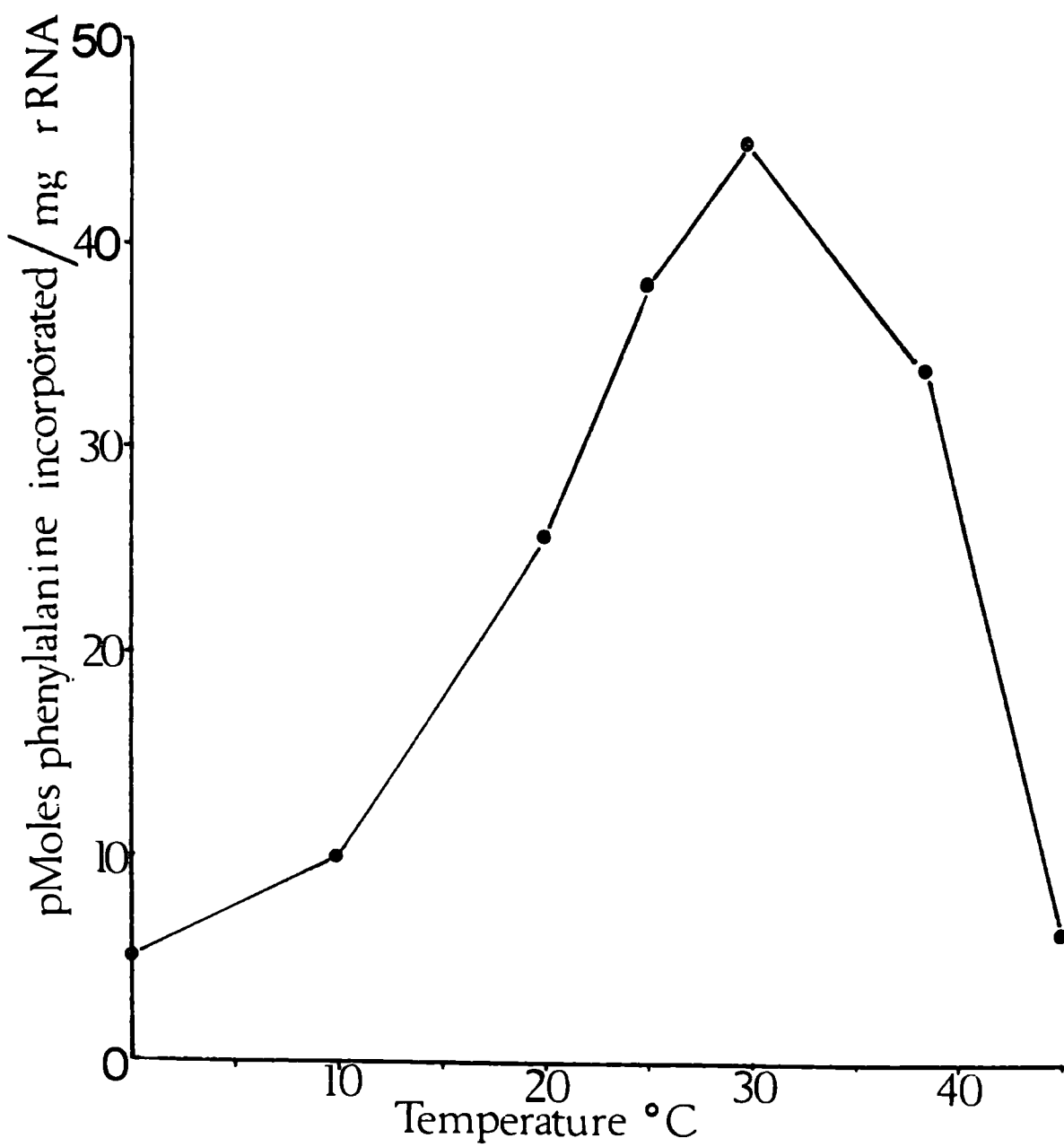


Fig.25. Time course for the Poly(U) dependent transfer system at different temperatures. The transfer system is described in Methods, Section 19A and contained 0.5mg of NH_4Cl ribosomes, 0.2mg of yeast $[\text{}^{14}\text{C}]$ Phe-tRNA (sp.act.382pMoles/mg tRNA) and the optimum volume (as separately determined of G25 supernatant).

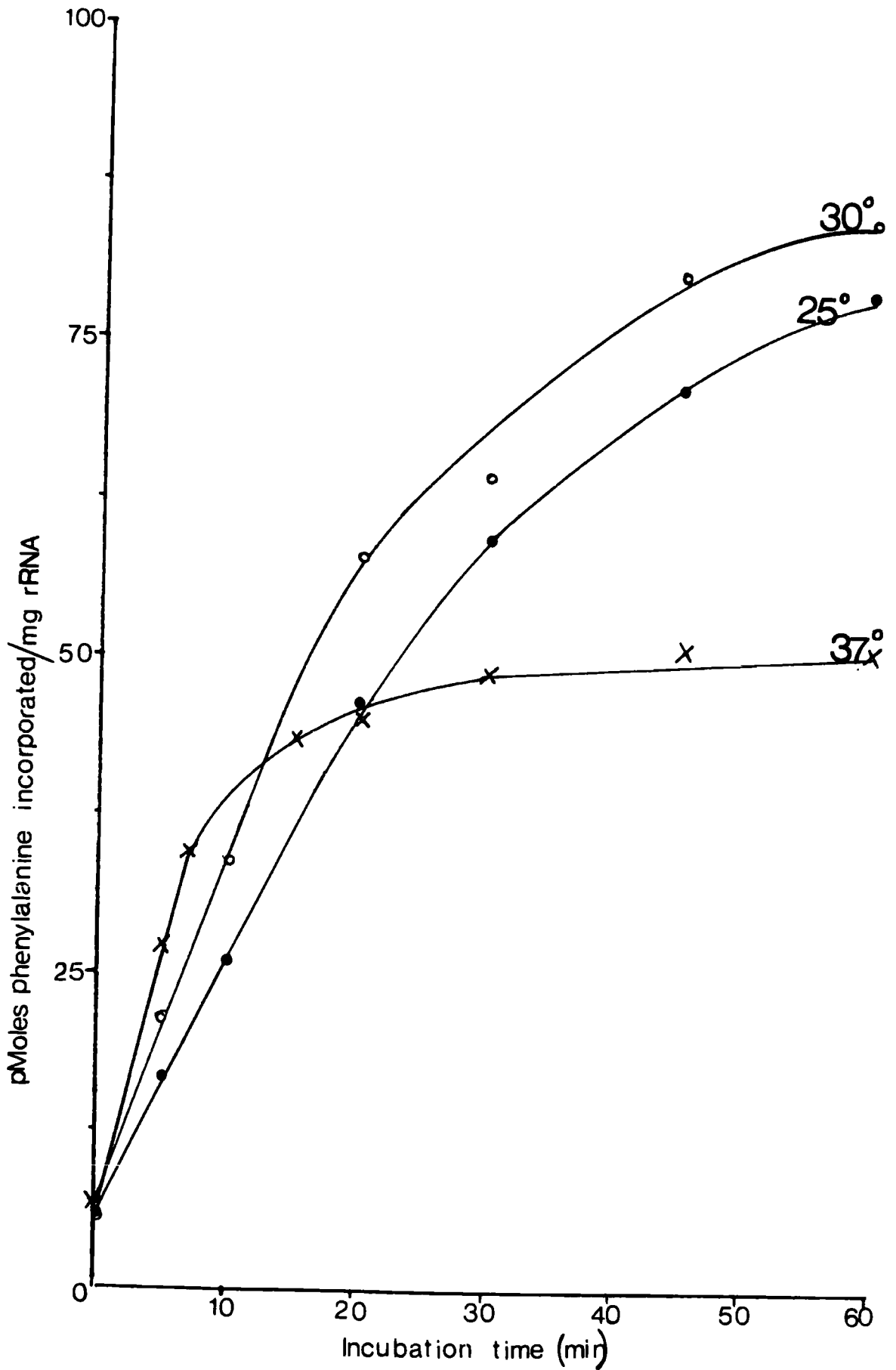
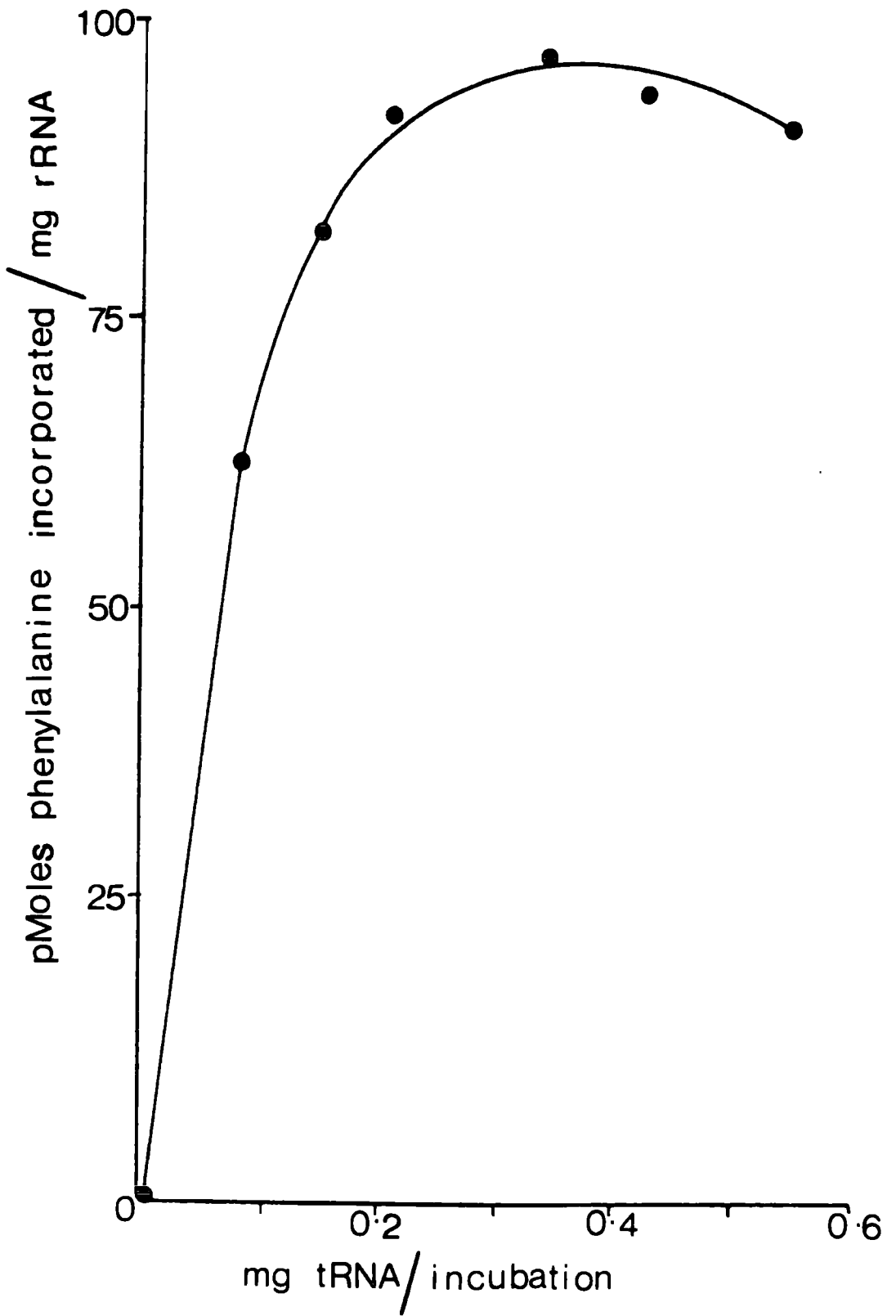


Fig.26. Effect of $[^{14}\text{C}]$ Phe-tRNA concentration upon the incorporation of $[^{14}\text{C}]$ phenylalanine in the Poly(U) dependent transfer system. The transfer system is described in Methods, Section 19A, and contained the stated amount of yeast $[^{14}\text{C}]$ Phe-tRNA (sp.act.382pmoles/mgtrNA).



number of counts of [^{14}C]phenylalanine since it appears likely that the optimum incubation conditions as determined and described above prevail only for a specific tRNA concentration rather than for a specific [^{14}C]phenylalanine concentration. It seems likely that a variation in the amount of tRNA added to the system would result in an alteration in the parameters of the system.

There was a sharp pH optimum at pH7.6, increase or decrease in pH beyond this point resulted in a decrease in incorporation (Fig.27). The system was however more sensitive to decreasing pH, a fall of 0.6 pH units from the optimum at pH7.6 resulted in a 88% decrease in incorporation, whilst an increase of 0.6pH units was accompanied by only a 33% decrease in activity.

For each batch of enzyme used, the concentration of enzyme necessary for maximum incorporation was determined, values in the region of 1mg protein/1ml incubation were routinely obtained for dialysed supernatant.

6. Comparison of the efficiency of various enzyme preparations in the Poly(U) dependent transfer system

Table 14 shows a comparison of the efficiencies of various enzyme preparations in the Poly(U) dependent transfer system using 1% Triton X100 ribosomes prepared from 3 day germinated beans. The activity of the crude 105,000xg av. supernatant was increased by 350% by dialysis and removal of precipitated globulins. Following dialysis however, the concentration of the supernatant was so low as to necessitate its concentration by ultrafiltration, this was therefore adopted as a routine procedure.

Fig.27. Effect of pH on the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalanine in the transfer system. The transfer system is described in Methods, Section 19A and contained 0.5mg of microsomes prepared from 3 day germinated beans, 0.2mg yeast $[^{14}\text{C}]$ Phe-tRNA (sp.act.382pmoles/mgtrNA) and the optimum volume of bean dialysed supernatant in a total volume of 500 μl .

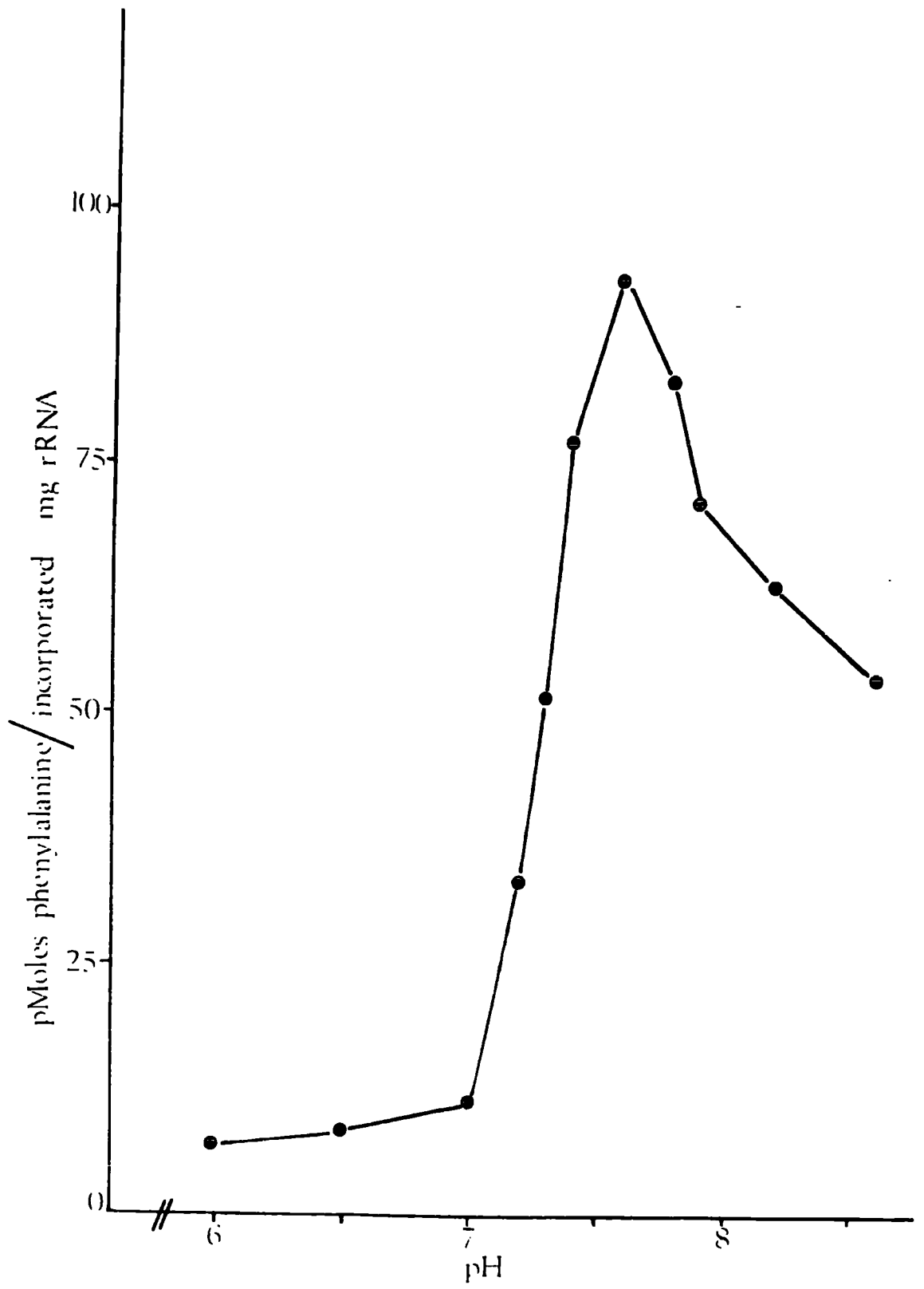


Table 14

Efficiencies of various enzyme preparations in the Poly(U)-dependent transfer system.

| Enzyme preparation | Incorporation (pmoles $[^{14}\text{C}]$ phenylalanine/ mg rRNA) |
|---|--|
| Crude 105,000xg av. supernatant | 44.8 |
| Dialysed 105,000xg av. supernatant | 146.0 |
| Concentrated dialysed supernatant | 157.0 |
| G ₂₅ supernatant | 134.2 |
| DE52 supernatant | 171.8 |
| 70%(NH ₄) ₂ SO ₄ fraction | 103.7 |
| DE52 supernatant after ultrafiltration | 164.7 |

The efficiencies of various enzyme preparations was compared in the Poly(U)-dependent transfer system containing 0.5mg of 3 day germinated bean Triton X100 ribosomes, 0.2mg of $[^{14}\text{C}]$ -phe tRNA (sp.act 311 pmoles/mg tRNA) and the optimum volume (as separately determined) of the stated enzyme.

The activity of enzyme prepared in this manner was equivalent to that prepared by dialysis of the concentrated protein solution which drained from inclined flasks of 105,000xg av. supernatant.

Removal of contaminating small molecules by chromatography on Sephadex G₂₅ and of tRNA by chromatography on DEAE-cellulose DE52 resulted in 300% and 380% increase in activity respectively. Concentration of DE52 supernatant by precipitation at 70% ammonium sulphate resulted in a 40% decrease in activity compared to the original DE52 supernatant.

7. Incorporation of [¹⁴C]lysine in the Poly(A) directed complete system

Table 15 shows a comparison of the Poly(U) directed incorporation of [¹⁴C]phenylalanine into polyphenylalanine and the Poly(A) directed incorporation of [¹⁴C]lysine into polylysine. In both the reticulocyte and bean complete systems the addition of Poly(U) resulted in a greater than ten fold increase in incorporation over the Poly(U) level. However in both the reticulocyte and bean complete systems there was no significant difference in the levels of incorporation of [¹⁴C]lysine in the presence or absence of Poly(A). However the results obtained with each system were reproducible. Since the levels of incorporation obtained with the reticulocyte and bean system were not identical it seems unlikely that they represent only [¹⁴C]lysine which remained on the filter paper after the washing procedure.

8. Resolution of the complementary fractions involved in peptide chain elongation

A. Chromatography on Sephadex G200

The method used was essentially that of Moldave (1968).

Table 15

Comparison of Poly(U) directed incorporation of phenylalanine and Poly(A) directed incorporation of [^{14}C] lysine in the complete system with reticulocyte ribosomes and microsomes prepared from 60 day developing beans.

| Incubation conditions | Incorporation (pmoles phenylalanine/ mg rRNA) | Incorporation (pmoles lysine/ mg rRNA) |
|-----------------------------------|---|--|
| Reticulocyte ribosomes-Poly(U) | 56.4 | |
| Reticulocyte ribosomes+Poly(U) | 611.0 | |
| Reticulocyte ribosomes-Poly(A) | | 214.0 |
| Reticulocyte ribosomes+Poly(A) | | 198 |
| Bean microsomes-Poly(U) | 20.0 | |
| Bean microsomes+Poly(U) | 215.7 | |
| Bean microsomes-Poly(A) | | 130.0 |
| Bean microsomes+Poly(A) | | 126.0 |

Duplicate determinations were carried out in all cases and the average values presented. The Poly(A) complete system is described in the methods and contained 0.5mg of reticulocyte ribosomes or bean microsomes, 0.2mg of deacylated reticulocyte or bean (germinating) tRNA, and the optimum volume (as separately determined) of reticulocyte 40/70 enzyme or bean 105,000xg av. supernatant. The sp.act. of the [^{14}C] lysine used was 100mC/mM.

Samples were taken at each step in the purification procedure and assayed for their ability to promote Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalanine from $[^{14}\text{C}]$ phe-tRNA into peptidyl material, using ammonium chloride washed bean ribosomes. In each case approximately 1 mg of protein, as determined by the Lowry Folin estimation, was used. The specific activity of the protein fraction increased throughout the purification procedure and the specific activity of the 25-60% ammonium sulphate fraction applied to the G200 column was almost twice that of the initial supernatant (Table 16).

A single peak of 280nm absorbing material was eluted from the column. When 0.2ml aliquots of the fractions were assayed individually for their ability to promote Poly(U) dependent incorporation, a single peak of activity extending from fractions 16-55 was found. ^(Fig.28) This protein fraction eluted ahead of the main peak of effluent protein. 0.1ml samples of fraction 25 were assayed in combination with other fractions in order to resolve any protein fractions having a complementary effect in peptide chain elongation. The fractions 25-65 were simultaneously reassayed individually.

Despite indications of three peaks of activity (Fig.29) there was no evidence of any complementation between the various fractions since in no case was the activity of any combination of fractions greater than that of fraction 25 alone. When fractions 25-65 were reassayed individually on this second occasion the shape of the peak obtained was essentially similar to that obtained initially and there was no significant decrease in the activity of the protein fractions.

Table 16

Changes in the activity of 60 day developing bean concentrated dialysed supernatant during the steps involved in the resolution of the enzymes involved in peptide chain elongation.

| Step in the Purification procedure | pmoles phenlyalanine incorporated/ mg rRNA/mg enzyme protein in Poly(U) dependent transfer system (Sp.act.) |
|--|---|
| Initial concentrated dialysed supernatant | 26.0 |
| pH5.2 supernatant neutralised to pH7.0 | 31.9 |
| G ₂₅ effluent | 40.7 |
| Ca ₃ (PO ₄) ₂ effluent | 41.2 |
| 25-65%(NH ₄) ₂ SO ₄ fraction | 53.6 |

The modified Poly(U) dependent system used to assay activity of bean concentrated dialysed supernatant during the steps involved in the resolution of the enzymes involved in peptide chain elongation is described in the Methods. In each case approximately 1mg of protein/500 μ l incubation was used, 0.2mg yeast ^[rd]phe-tRNA (sp.act.335 pmoles/mg tRNA) and 0.5mg ammonium chloride ribosomes prepared from 60 day developing bean. In the absence of added enzyme, a background level of 2.94 pmoles phenylalanine were incorporated per mg rRNA.

Fig.28. Chromatography on Sephadex G200 of partially purified V. faba soluble protein. 120mg of partially purified protein (25-65%) $(\text{NH}_4)_2\text{SO}_4$ fraction (Methods, Section 22A), in a volume of 8 ml were chromatographed on a 30x2.4cm column of sephadex G200. Fractions of approximately 5.75 ml were collected and the ability of fractions to promote the Poly(U) dependent incorporation of $[^{14}\text{C}]$ phenylalanine from $[^{14}\text{C}]$ Phe-tRNA into peptidyl material was assayed using NH_4Cl washed ribosomes prepared from 60 day developing cotyledons of V. faba. 0.2ml aliquots of the various fractions were incubated with 0.5mg of ribosomes and 0.2mg yeast $[^{14}\text{C}]$ Phe-tRNA (sp.act 335pmoles/mgTRNA) in the modified transfer system (Methods, Section 22B) The background level of incorporation in the absence of added enzyme is indicated

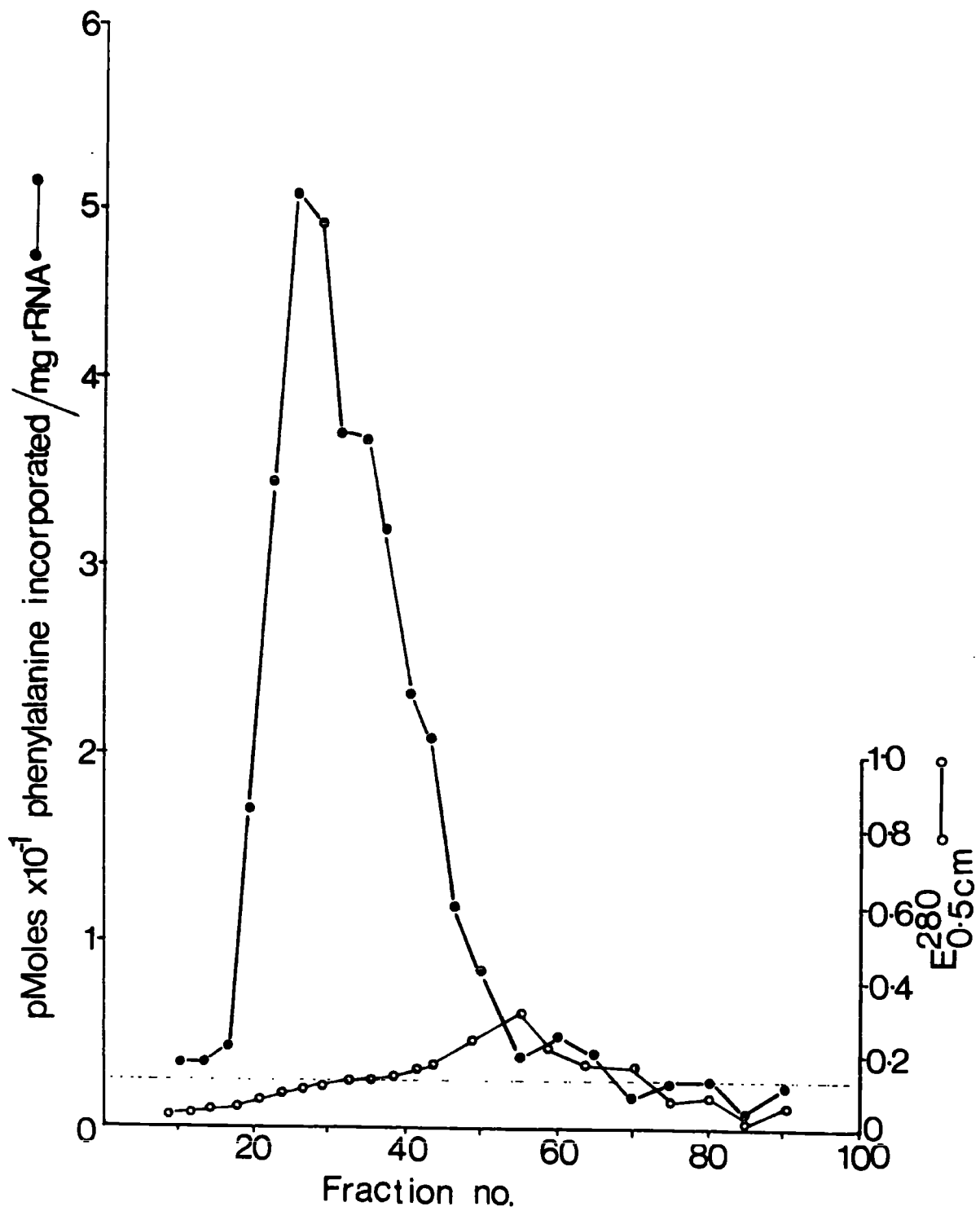
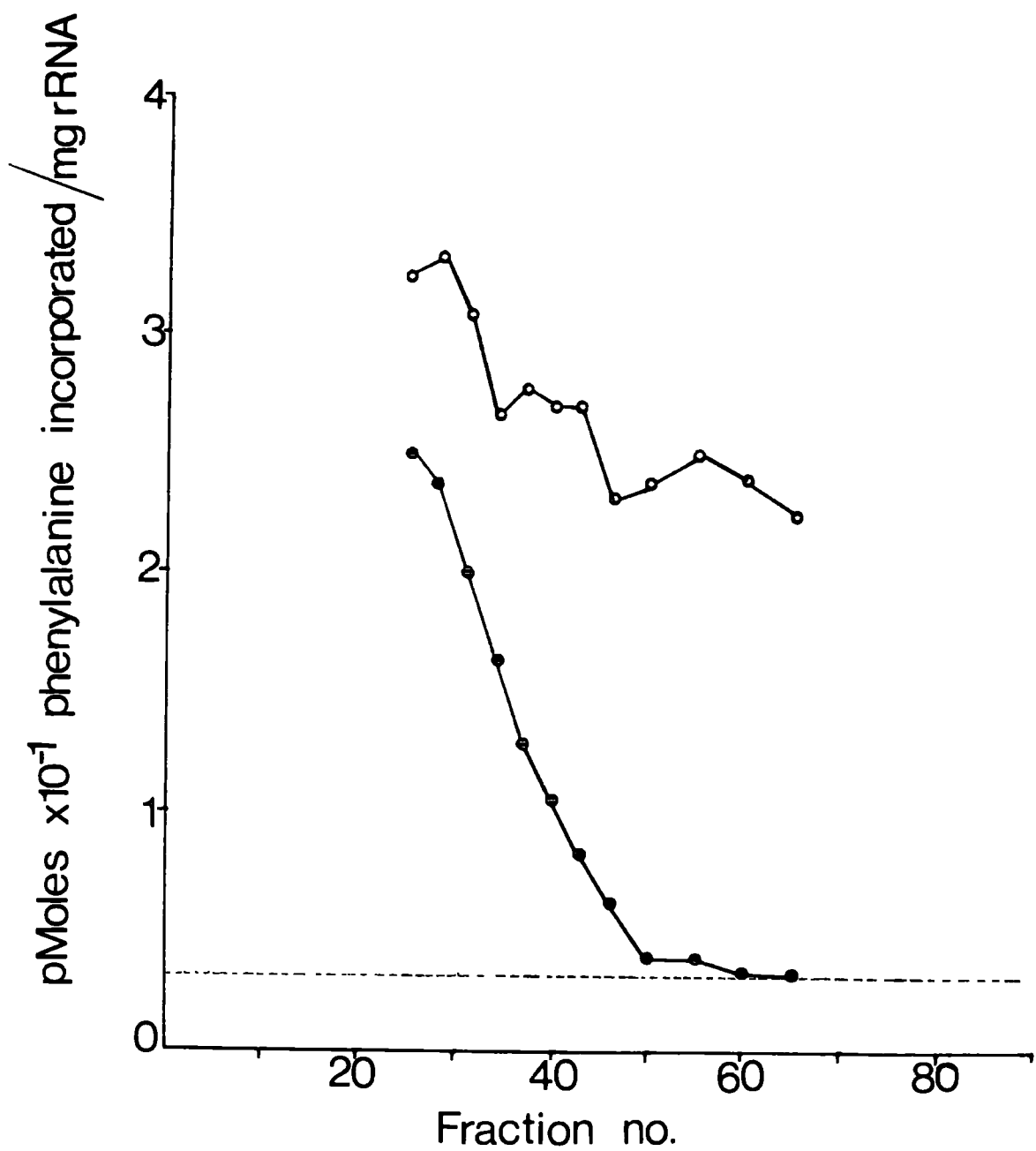


Fig. 29. Complementary effect of G200 fraction 25 upon aminoacyl transfer by fractions 28-65. The complementary effect of 0.1ml of fraction 25 upon aminoacyl transfer of the fractions 25-68 was measured in the modified transfer system (Methods, section 22B) containing 0.5mg NH₄Cl washed ribosomes prepared for 60 day developing beans, 0.2mg yeast [¹⁴C] Phe-tRNA (sp. act. 335pmoles/mgtRNA) and 0.1ml of the stated fraction. Fractions were 25-65 and were assayed individually in the above described system.

- 0.1ml Fractions 25-65 assayed individually
- 0.1ml Fraction 25 in combination with 0.1ml of indicated fraction.

The background level of incorporation in the absence of added enzyme is indicated



Between each set of assays the fractions were stored at -20° . When the fractions were thawed on the third occasion there was a complete loss of activity.

The experiment was repeated on a further occasion with a similar lack of resolution.

B. Chromatography on Sepharose 4B

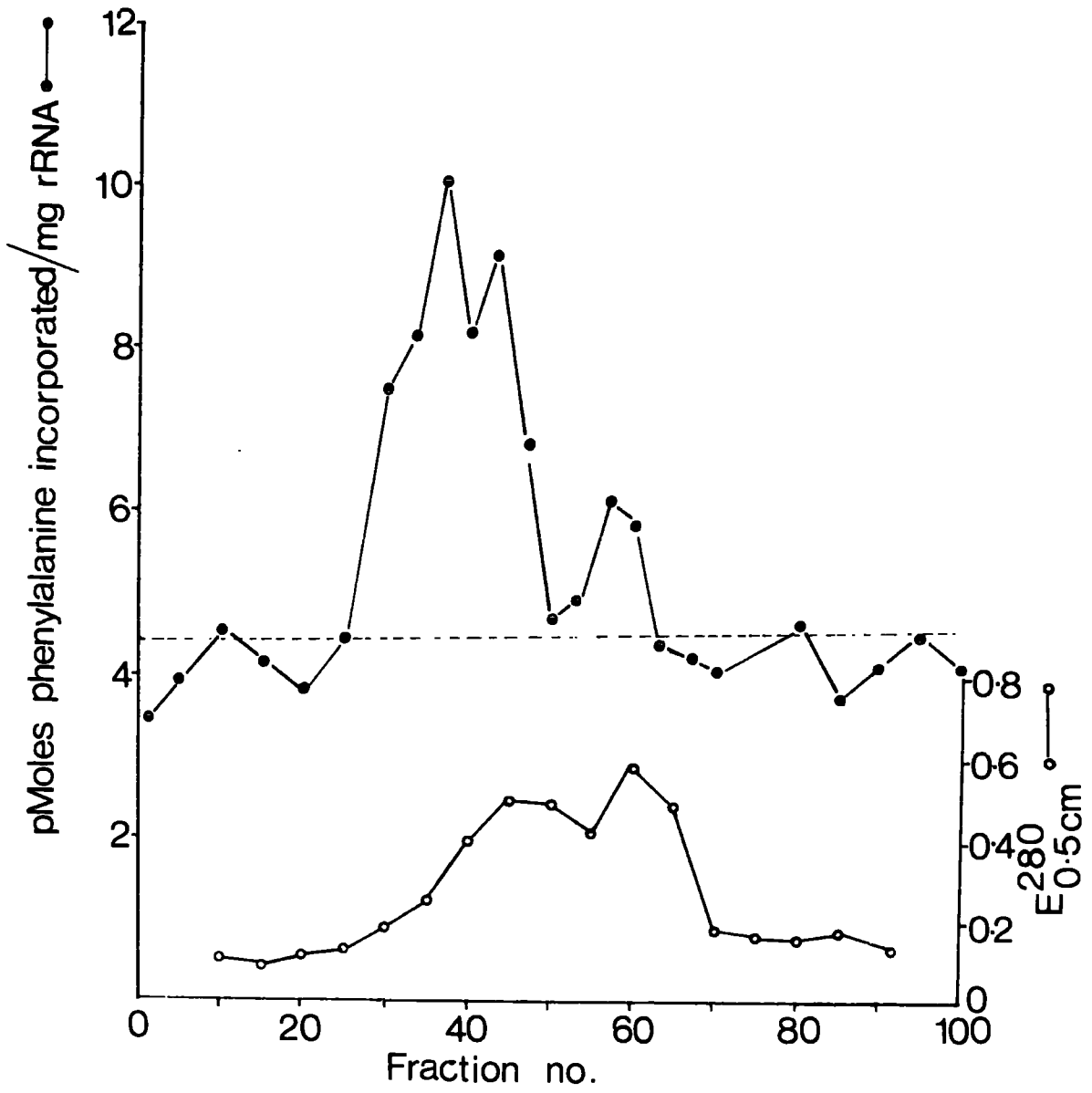
10ml of concentrated dialysed 105,000xg av. bean supernatant were chromatographed on a Sepharose 4B column, at a flow rate of 40ml/hr as described in the methods. Fractions were collected at 6 min intervals. Two poorly resolved peaks of 280nm absorbing material were eluted for the column (Fig.30.)

0.1ml samples of the fractions were assayed for their ability to promote the poly(U) dependent incorporation of [14 C]phenylalanine from [14 C]phe-tRNA into peptide material using ammonium chloride x 2 washed ribosomes.

Two distinct peaks of transfer activity were obtained, a major peak extending from fractions 25-50, which appeared to represent two incompletely resolved peaks of activity, and a minor peak extending from fraction 50-63.

0.1ml of fraction 37, corresponding to the peak of aminoacyl transfer activity was assayed in combination with 0.1ml of fractions 40-67 in order to detect any complementation between the various fractions. Simultaneously fractions 37-67 were reassayed individually. When fractions 37-67 were assayed individually on this second occasion the peak of activity was found to be displaced, the activity of fraction 37 having decreased relative to its former value, the

Fig.30. Chromatography of concentrated dialysed bean 105,000xg supernatant on Sepharose 4B. 10ml of concentrated dialysed 105,000xg supernatant were chromatographed on a 2.4x30cm column of Sepharose 4B (Methods, Section 22c) and the column fractions assayed in the modified transfer system (Methods, Section 22B). The background level of incorporation in the absence of added enzyme is indicated



activity of fraction 40 remained essentially unchanged whilst the activity of fraction 43 had increased by approximately 15%. Fraction 43 now represented the peak of transfer activity. There was no indication of the minor peak extending from fraction 50-63 which was evident in the initial set of assays and the values obtained for these fractions were equivalent to those of the minus enzyme background control.

When fractions 40-67 were assayed in combination with fraction 37, 2 peaks of aminoacyl transfer activity were detected (Fig.31). The peak extending from fractions 40-53 coincided with the second part of the double peak obtained in the original assay (Fig.30) but because of the difference in activities obtained on the second assay, it appeared as a distinct peak.

A novel peak extending from fractions 53-63 was obtained, this peak coincided with the position of the second peak obtained in the initial assays which, when assayed on the second occasion, was no longer apparent. It may be assumed that this peak represents a complementary effect of fraction 37 upon the transfer activity of the fractions 53-63 since combination of fraction 37 with these fractions results in a 100% increase in transfer activity.

The fractions were stored at -20° between successive sets of assays (performed on consecutive days) but on thawing for a third occasion there was a complete loss of transfer activity.

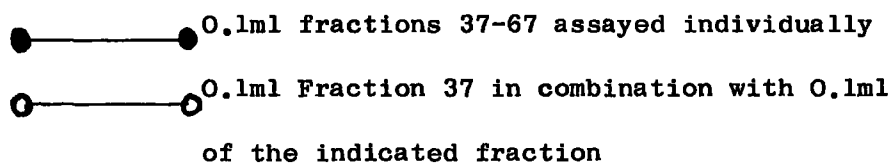
9. Characterisation of the product of *in vitro* system

A. Levels of incorporation

Table 17 shows the level of incorporation achieved by the normal microsomes and microsomes prepared in the presence of rat

Fig. 31. Complementary effect of Sepharose 4B fraction 37 upon aminoacyl transfer by fractions 40-67.

The complementary effect of fraction 37 upon aminoacyl transfer by fractions 40-67 was assayed in the modified transfer system described in the legend to Fig. 29.



The background level of incorporation in the absence of added enzyme is indicated

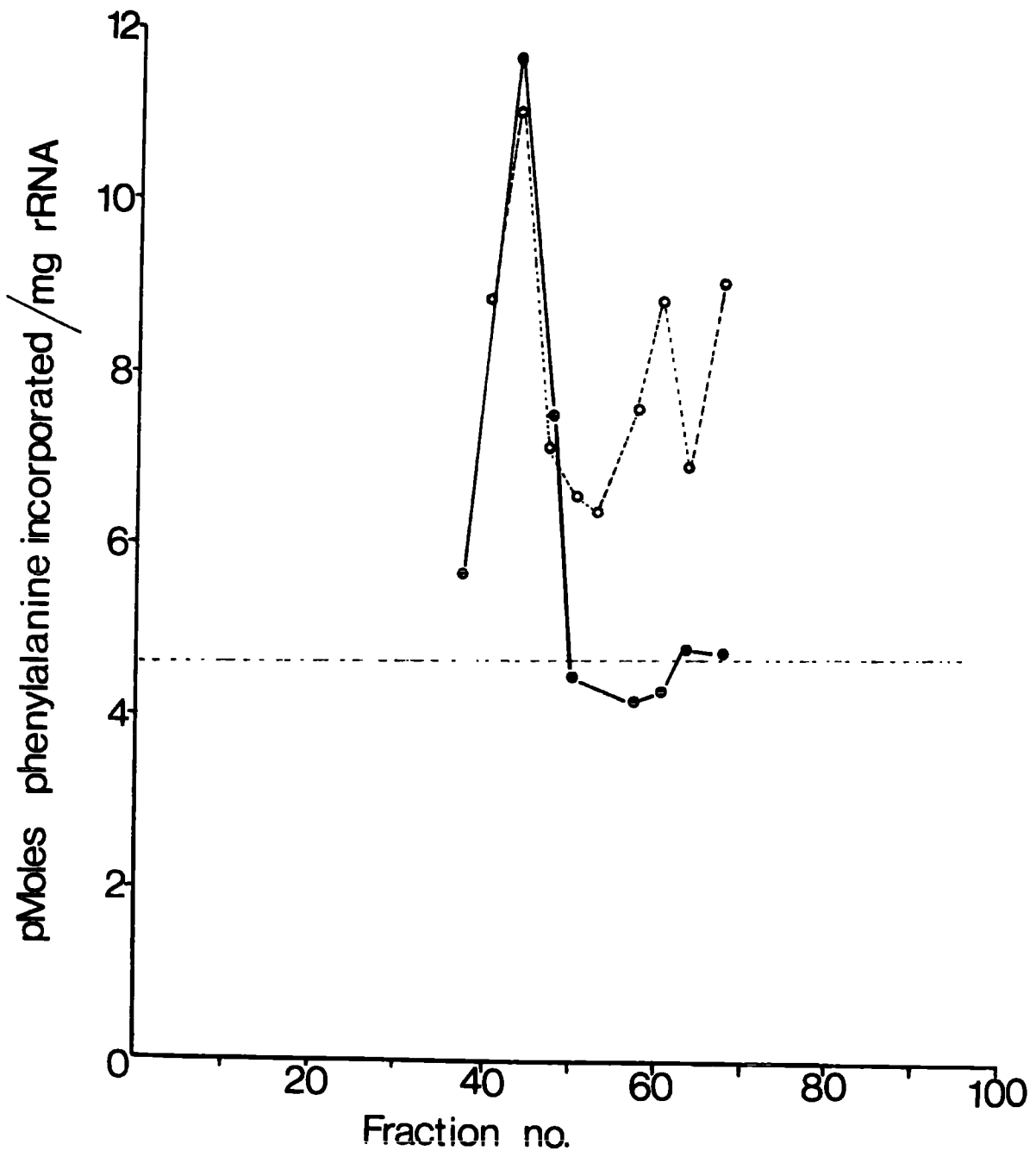


Table 17

Incorporation of [^{35}S]methionine in response to endogenous mRNA by normal microsomes from 60 day developing seeds of V. faba and microsomes prepared in the presence of rat liver high speed supernatant.

Normal microsomes
(cpm/5ml incubation)

Microsomes prepared
in presence of rat
liver s/n.
(cpm/5ml incubation)

1,152,750

938,100

The complete system is described in the methods and contained 5 mg of microsomes, 2 mg of deacylated tRNA prepared from 60 day developing bean and the optimum volume (as separately determined) of V. faba concentrated, dialysed enzyme fraction. Since [^{35}S]methionine incorporation directed by endogenous mRNA was measured, Poly(U) was omitted, and a [^{12}C]-amino acid mixture - methionine replaced the [^{12}C]-amino acid mixture - phenylalanine. Incubation was carried out at 25° for 40 min.

liver high speed supernatant. The activity of the normal microsome preparations was 18% greater than those prepared in the presence of rat liver high speed supernatant.

B. Tryptic Fingerprint

The tryptic fingerprint is shown in Fig.32. Despite a considerable degree of streaking a number of distinct spots could be identified. For purposes of comparison the overlay to fig.32 shows a map of the ninhydrin positive tryptic fingerprints of CM-legumin. (Bailey and Boulter, 1970). The overlay is positioned so that the neutral peptides of Bailey and Boulter are aligned with the area of most dense radioactivity in the carboxymethylated (CM-) in vitro product. Two of the [³⁵S]methionyl peptides of the digested CM-in vitro product ran to approximately the same position as two of the [³⁵S]methionyl peptides obtained on tryptic digestion of purified CM-^[35]S-legumin.

10. Resolution of methionyl tRNA's

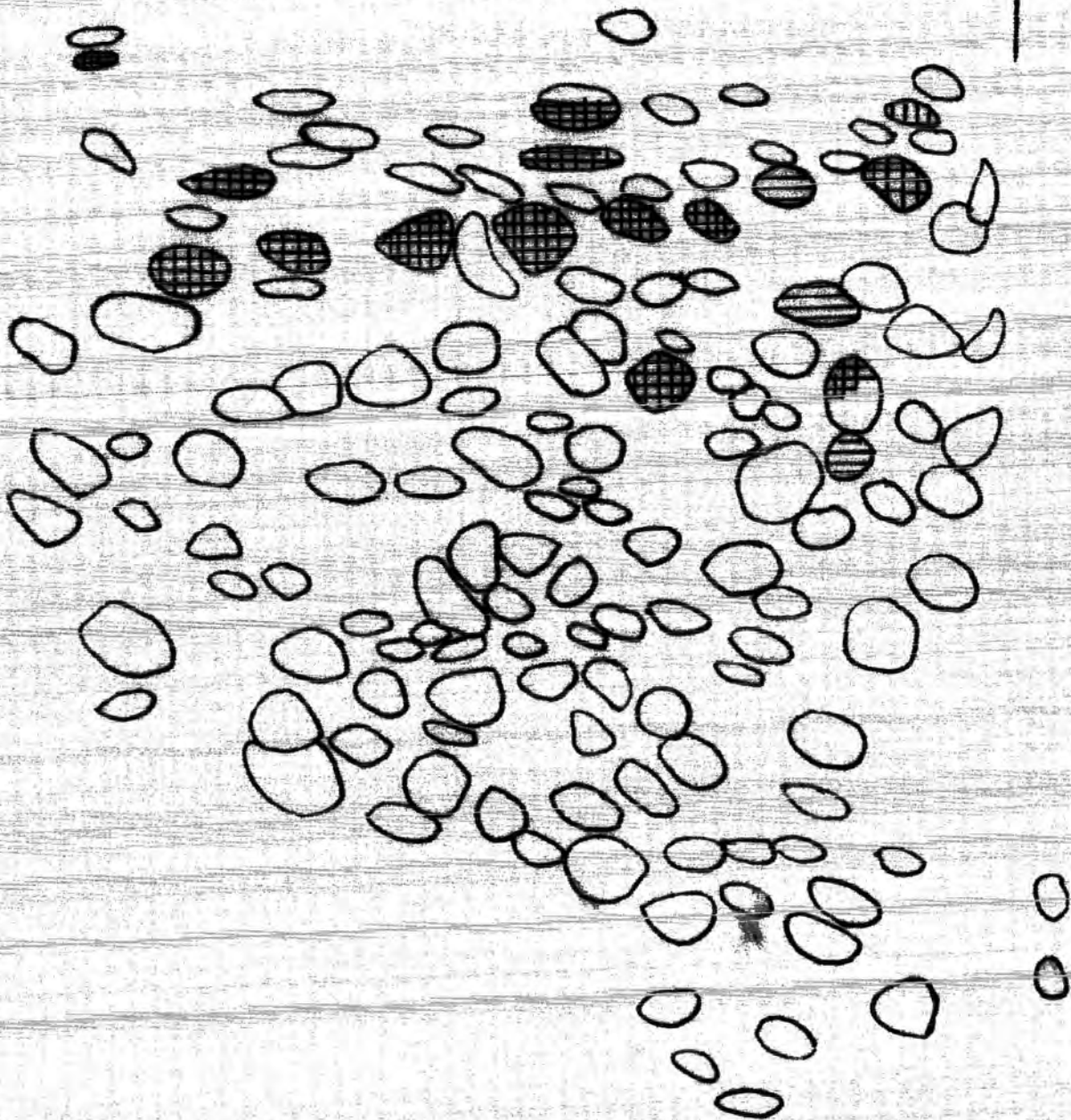
A. Chromatography on DEAE-Sephadex A50

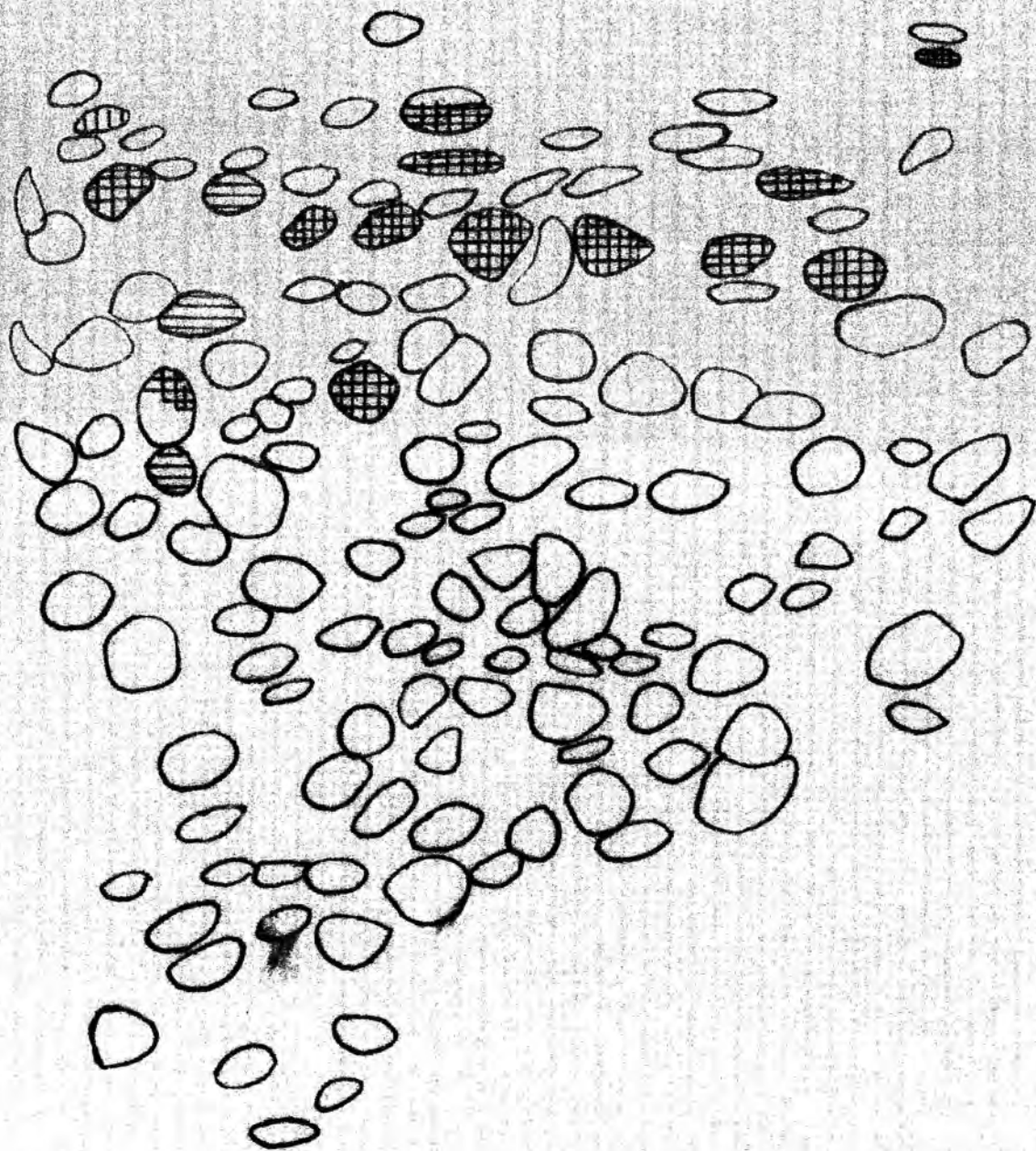
18mg of 60 day developing bean deacylated tRNA in buffer A (0.375M-NaCl, 0.005M-MgCl₂, 1mM-mercaptoethanol and 0.02M-Tris-HCl pH7.5) were applied to a 1 x 50 cm column of DEAE-Sephadex A50 and eluted using a linear gradient of 250ml buffer A and 250 buffer B (0.475M-NaCl, 0.016M-MgCl₂, 1mM-mercaptoethanol, 0.02M-Tris-HCl pH7.5). The elution profile, as determined by continuous monitoring at 260nm, and the methionine acceptor capacity of selected fractions of tRNA after chromatography are shown in Fig.33. Two distinct peaks of 260nm absorbing material were eluted. The first peak to elute was a well defined peak eluting between fractions 10-25, the second peak was a broad diffuse peak extending from

Fig. 32. Autoradiograph of a tryptic fingerprint of the $[^{35}\text{S}]$ -labelled CM-in vitro product.

Microsomes prepared from 60 day developing cotyledons of V.faba were incubated in the complete system (Methods, section 19B) containing 26 nMoles $[^{35}\text{S}]$ methionine (15C/mM) in a total volume of 5.0ml. Carboxymethylation was carried out as described (Methods, section 23C) and the CM-in vitro product was digested with trypsin and fingerprinted as described (Methods, sections 23D & E). The overlay is a diagrammatic representation of the ninhydrin positive tryptic peptides of CM-legumin, positioned so that the neutral peptides are aligned with the area of most dense radioactivity in the CM-in vitro product. The overlay also indicates the sulphur containing peptides (horizontal hatching) obtained from tryptic digests of carboxymethylated $[^{35}\text{S}]$ -legumin. Vertical hatching indicates radioactive peptides from digests of $[^{14}\text{C}]$ carboxymethylated-legumin; cross-hatched peptides were therefore radioactive in both digests. The origin is at the top right hand corner and is indicated — .

The overlay is reproduced from Bailey and Boulter (1970) with permission.







fractions 25-75. When selected fractions were aminoacylated using 60 day developing bean DE52 supernatant as enzyme source, two incompletely resolved peaks of methionine acceptor activity were obtained eluting at 0.39M and 0.40M-sodium chloride and extending from fractions 25-63. The peaks of methionine acceptor activity will be referred to as Peak 1 and Peak 2 in order of elution. Both peaks were aminoacylated to approximately the same extent by bean DE52 supernatant.

This type of separation was routinely obtained when fractions were assayed using 60 day developing bean DE52 supernatant as the enzyme source, however during the development of this technique E.coli DE52 supernatant was used as a source of enzymes and separations of the type as shown in Fig.34 were obtained. The difference in elution profile of the tRNA may be ascribed to differences in the conditions under which separation was performed and to the absence of 2-mercaptoethanol from the buffers. Two peaks of methionine acceptor activity were obtained. The methionine acceptor activity obtained was approximately 40% of that obtained using bean supernatant under the conditions described in the legend to Fig.33. The first peak eluting at 0.408M-NaCl was equated with the Peak 2 eluting at 0.40M under the previously described conditions and the novel peak eluting at 0.425M-NaCl probably represents a met-tRNA species not previously resolved. However the extremely low degree of aminoacylation with E.coli DE52 supernatant precluded any further investigation of this peak.

Fig.33. Chromatography of V. faba tRNA on DEAE-sephadex A50.

Methionine acceptor capacity assayed using bean DE52 enzyme. 20mg of deacylated 60 day developing bean tRNA (prepared as described in Methods, Section 11) were chromatographed on a 1x50 cm column of DEAE-sephadex A50 and the methionine acceptor capacity assayed using bean DE52 supernatant enzyme as described in Methods, Section 24B.

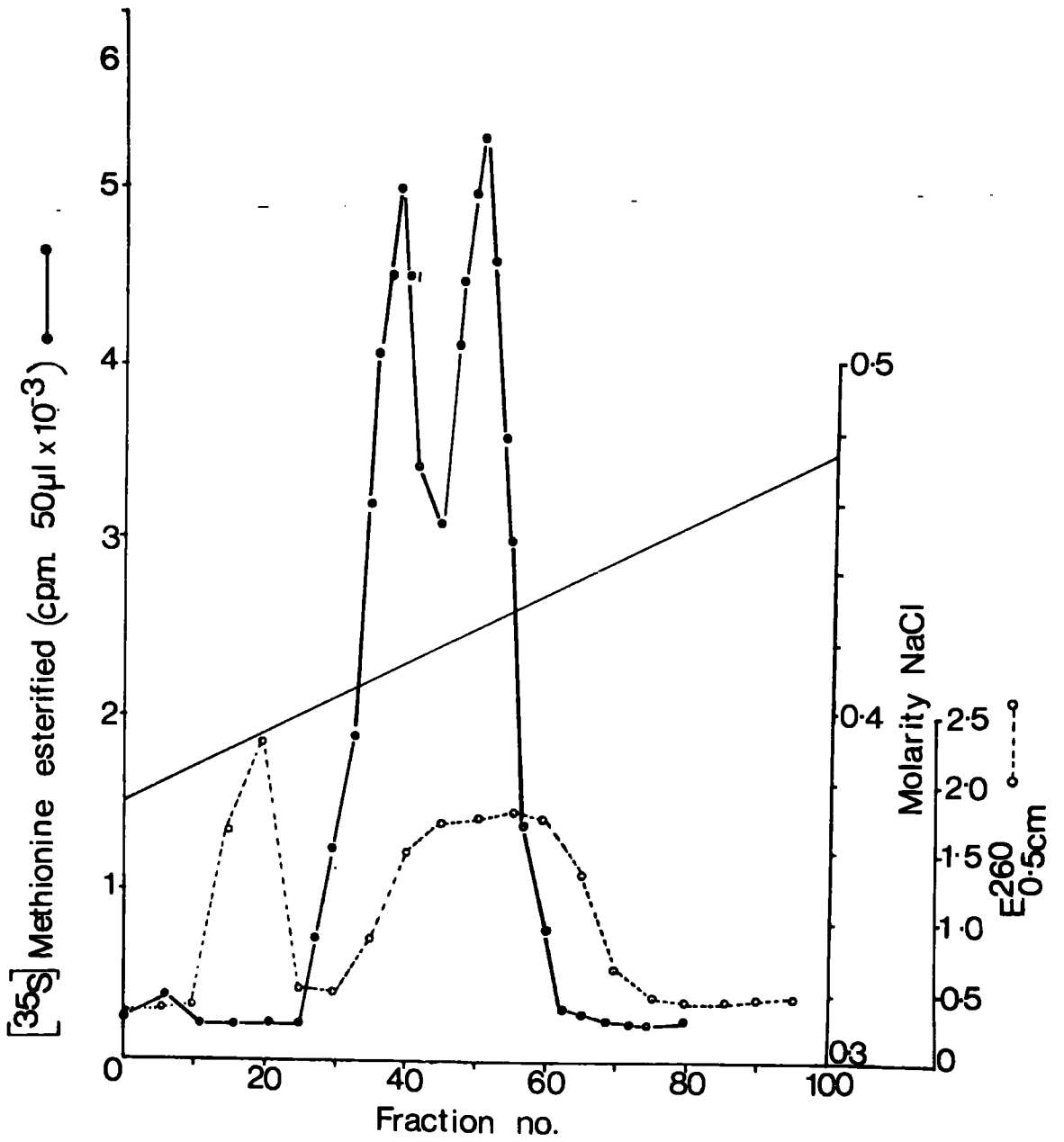
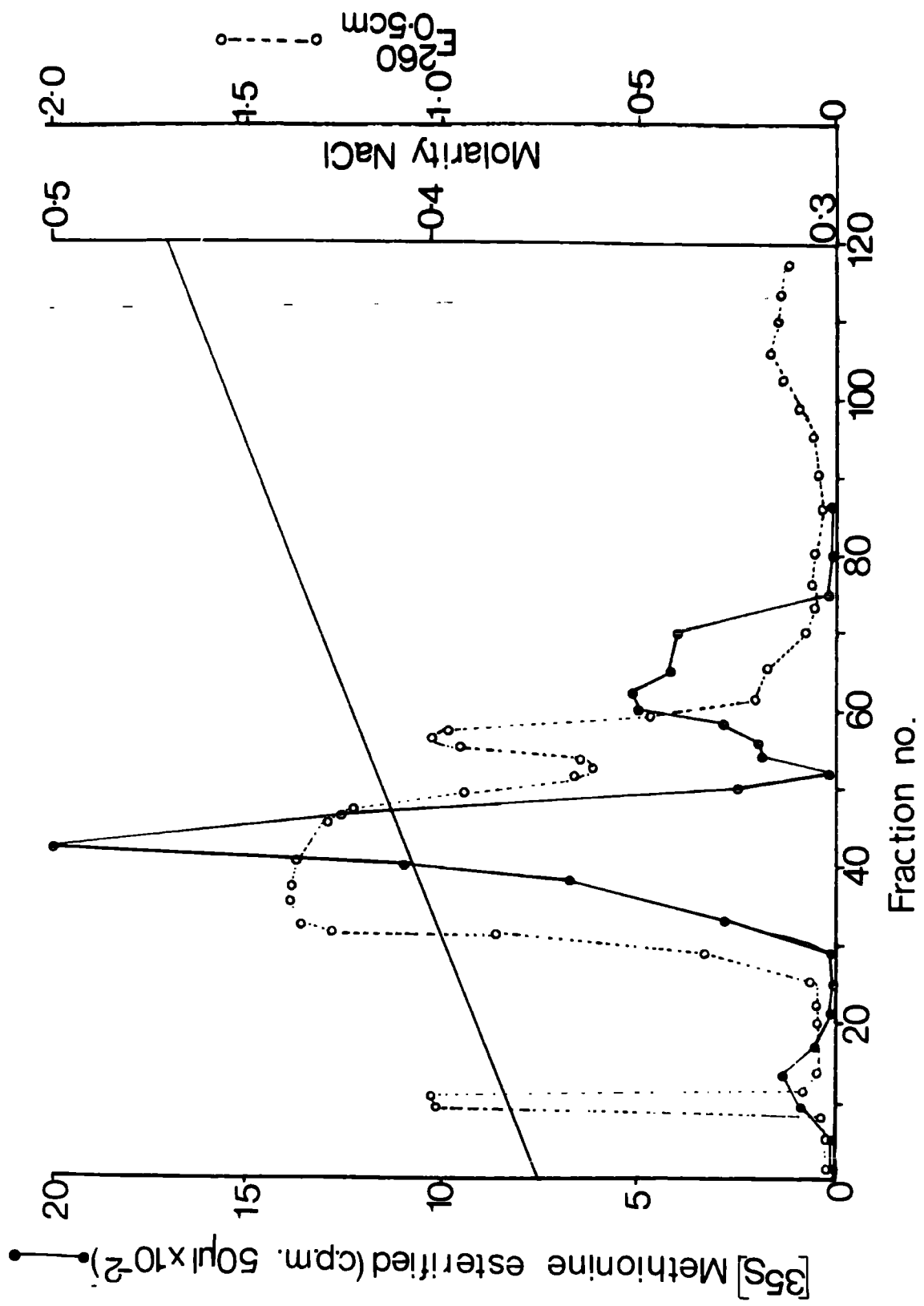


Fig. 34. Chromatography of V. faba tRNA on DEAE-Sephadex A50.

Methionine acceptor capacity assayed using E. coli DE52 enzyme. Chromatography was carried out as described in Methods, Section 24B except that the 1mM-2-mercaptoethanol was omitted from either the columns equilibration or elution buffers and chromatography was carried out at 20°. The methionine acceptor capacity of the fractions was assayed as described in Methods, Section 24B using E. coli DE52 supernatant enzyme.



B. Chromatography on BD-cellulose

28 mg of bean deacylated tRNA were chromatographed on a 1 x 32cm column of BD-cellulose. Five poorly resolved major peaks of 260nm absorbing material were eluted (Fig.34b). When the fractions were assayed for methionine acceptor capacity using bean DE52 supernatant two major and one minor peak of activity were obtained. In keeping with the nomenclature used by the wheat germ workers (Tarrago et al., 1970; Leis and Keller, 1970; Marcus, et al., 1970), these are referred to as $\text{tRNA}_1^{\text{met}}$, $\text{tRNA}_2^{\text{met}}$ and $\text{tRNA}_3^{\text{met}}$, in order of their elution from the column. The $\text{tRNA}_1^{\text{met}}$ eluted at 0.55M-sodium chloride, between fractions 63-78 in a well defined peak, whilst $\text{tRNA}_2^{\text{met}}$ eluted at 0.68M-sodium chloride, between fractions 103-115 and was incompletely resolved from $\text{tRNA}_3^{\text{met}}$ which eluted at 0.73M-sodium chloride extending from fractions 115-128. The relative methionine acceptor capacities using bean DE52 supernatant were in the order $\text{tRNA}_1^{\text{met}} > \text{tRNA}_2^{\text{met}} > \text{tRNA}_3^{\text{met}}$.

C. Effect of enzyme source on the degree of aminoacylation of the separated column fractions

Fractions corresponding to peaks of methionine acceptor activity were aminoacylated using 60 day developing bean or E.coli DE52 supernatant in the presence or absence of formyl donor. Only in the case of DEAE-Sephadex peak 2 was there a significant alteration in the degree of aminoacylation by bean enzyme in the presence of the formyl donor. Addition of the formyl donor resulted in a 30% decrease in the acceptor capacity of peak 2. Using the fractions separated by either method, only one of the major fractions ($\text{tRNA}_1^{\text{met}}$ and DEAE-Sephadex peak 2) was

Fig. 34b. Chromatography of 60 day developing bean tRNA on BD-cellulose. 28mg of deacylated tRNA were chromatographed on a 1x32cm column of BD-cellulose and the column fractions assayed for methionine acceptor activity using bean DE52 supernatant enzyme as described in Methods, Section 24B.

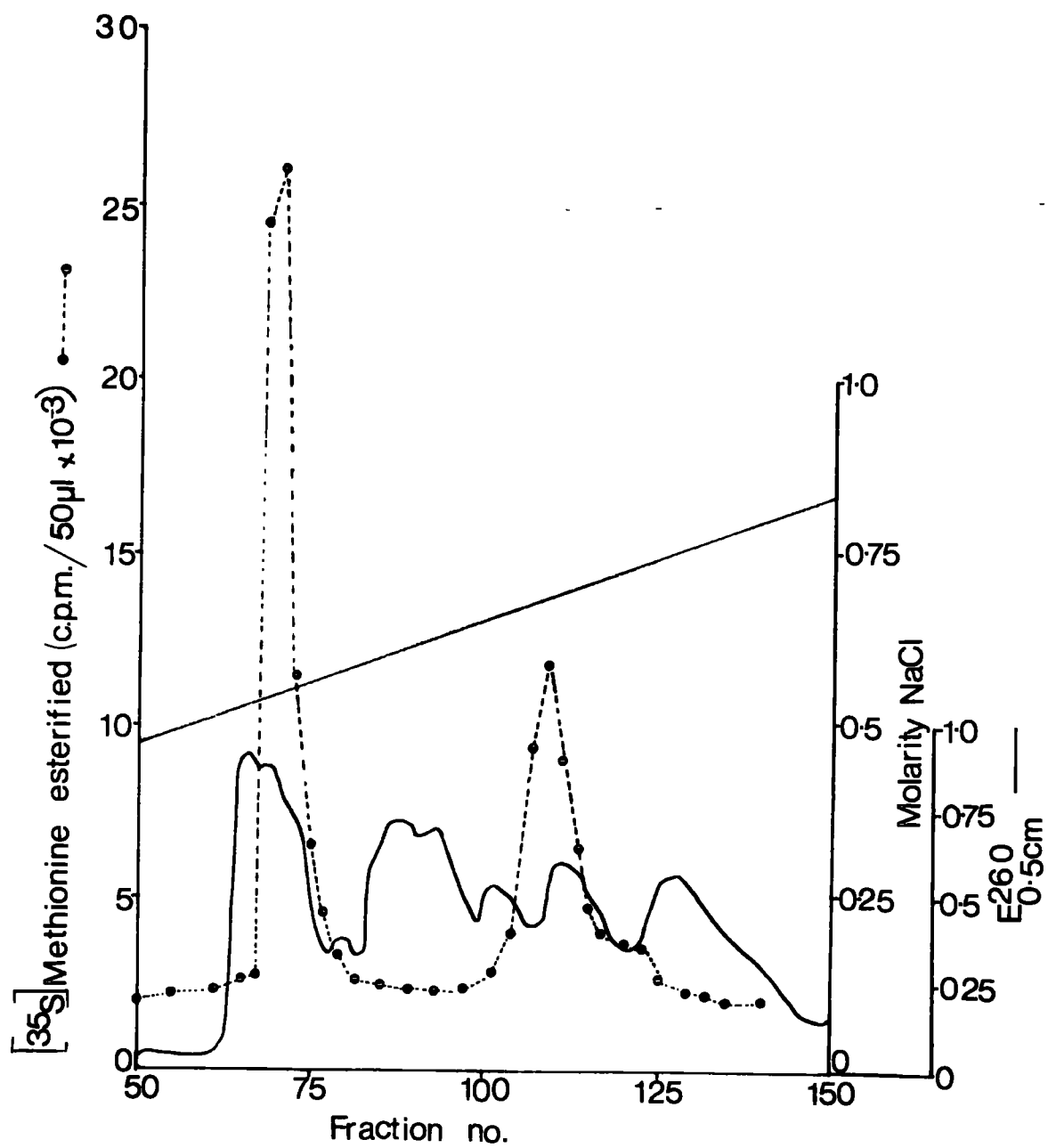


Table 18

Effect of enzyme source and formyl donor upon the degree of aminoacylation of the separated column fractions

| | BD-cellulose | | | DEAE-sephadex | | <u>E. coli</u> c.p.m./0.01mg |
|---------------------------|----------------------------------|----------------------------------|----------------------------------|---------------|--------|---------------------------------|
| | trNA ₁ ^{met} | trNA ₂ ^{met} | trNA ₃ ^{met} | Peak 1 | Peak 2 | |
| Bean Enz | 15564 | 7510 | 2875 | 19620 | 19421 | 4976 |
| Bean Enz + F.D. | 16345 | 7885 | 2315 | 18650 | 13460 | 6372 |
| <u>E. coli</u> Enz | 14275 | - | 3245 | 2005 | 16496 | 8892 |
| <u>E. coli</u> Enz + F.D. | 14921 | - | 3415 | 2145 | 22264 | 11310 |

Fractions were assayed as described in Section 23B methods, except that where appropriate bean enzyme was replaced by the optimum volume (as separately determined) of E. coli DE52 supernatant and 20 μ g of formyl donor (F.D.) was added. The results are expressed as c.p.m./esterified/0.05 ml column fraction. Sp.act. of the [³⁵S]methionine was 510mC/mM.

aminoacylated with E.coli enzyme in either the presence or absence of a formyl donor. Only in the case of DEAE-Sephadex peak 2 did addition of the formyl donor result in a significant change in the degree of aminoacylation by E.coli DE52. In the presence of the formyl donor there was a 28% increase in the methionine acceptor activity of DEAE-Sephadex peak 2. Control samples of unfractionated commercially obtained E.coli tRNA were aminoacylated with bean or E.coli DE52 supernatant, with or without formyl donor. Addition of the formyl donor resulted in 28% and 27% increases respectively in the degree of methionine acceptor activity.

11. Identification of methionine accepting tRNA species

A. Digestion of [³⁵S]methionyl-tRNA's with pancreatic ribonuclease

(i) Unfractionated 60 day developing bean [³⁵S]methionyl-tRNA

100 μ g of 60 day developing bean tRNA were aminoacylated with [³⁵S]methionine (sp.act.530mG/mM) in the presence of formyl donor using 60 day developing bean DE52 supernatant as an enzyme source. As a control a similar quantity of commercially obtained unfractionated deacylated E.coli tRNA was aminoacylated under identical conditions using E.coli DE52 supernatant. Following digestion by bovine pancreatic RN'ase and subsequent electrophoretic separation of the aminoacyl-adenylates an autoradiograph (Fig 35) was prepared. In the case of E.coli [³⁵S]methionyl-tRNA the major radioactive fragment was identified as [³⁵S]methionyl-adenosine. There was also present substantial amounts of a fragment identified as N-formyl-^{[35}S]methionyl-adenosine as well as traces of free methionine.

Fig.35. Autoradiograph of an ionogram of bovine pancreatic RNase

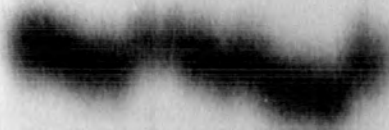
A digest of unfractionated $[^{35}\text{S}]$ met-tRNA from E. coli and 60 day developing bean. Unfractionated tRNA's were aminoacylated by $[^{35}\text{S}]$ methionine (sp.act.6.3C/mM) by homologous DE52 supernatant in the presence of formyl donor. After re-isolation the $[^{35}\text{S}]$ met-tRNA's were digested with pancreatic RNase ($10\mu\text{g}$) and applied to Whatman 3MM paper and electrophoresed at pH3.5, 3kV, 1hr. 1, Digest of unfractionated E. coli $[^{35}\text{S}]$ met-tRNA; 2, digest of 60 day developing bean $[^{35}\text{S}]$ met-tRNA.

X = free methionine

Y = N-formyl-methionyl-adenosine

Z = methionyl-adenosine

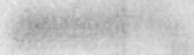
Z



← METHIONYL-ADENOSINE.

(

Y



← N-FORMYL-METHIONYL ADENOSINE.

X

← Free met.

1.

2. ← ORIGIN.

Digestion of bean $[^{35}\text{S}]$ methionyl-tRNA also yielded methionyl-adenosine as the major fragment but with only a slight trace of N-formyl-methionyl-adenosine. Free methionine was again also present in trace amounts.

(ii) $[^{35}\text{S}]$ methionyl tRNA's resolved by DEAE-Sephadex chromatography

DEAE-Sephadex fractions corresponding to peaks of methionine acceptor activity were aminoacylated with $[^{35}\text{S}]$ methionine (sp.act. 279C/mM) using E.coli DE52 and 60 day developing bean DE52 supernatants in the presence of formyl donor and the $[^{35}\text{S}]$ methionyl-tRNA digested by pancreatic RN'ase. Separation by ionophoresis and subsequent autoradiography gave the results shown in Fig.36). The identification of the radioactive fragments was authenticated by comparison with those obtained using unfractionated $[^{35}\text{S}]$ methionyl-tRNA prepared and digested simultaneously under identical conditions and, as previously described, by elution, hydrolysis and re-electrophoresis. In the case of the E.coli tRNA aminoacylated by either bean or E.coli supernatant, spots corresponding to methionyl-adenosine and N-formyl-methionyl-adenosine were, as expected, obtained in addition to free methionine.

The fraction corresponding to DEAE-Sephadex peak 1 when aminoacylated by E.coli supernatant showed in addition to free methionine, indications of spots corresponding to both methionyl-adenosine and N-formyl-methionyl-adenosine, however, in view of the very high specific activity of the $[^{35}\text{S}]$ methionine it must be pointed out that these represent components present in almost insignificant proportions. When peak 1 was charged by bean supernatant a strong spot identified as methionyl-adenosine was obtained as well as

Fig.36. Autoradiograph of an ionogram of bovine pancreatic

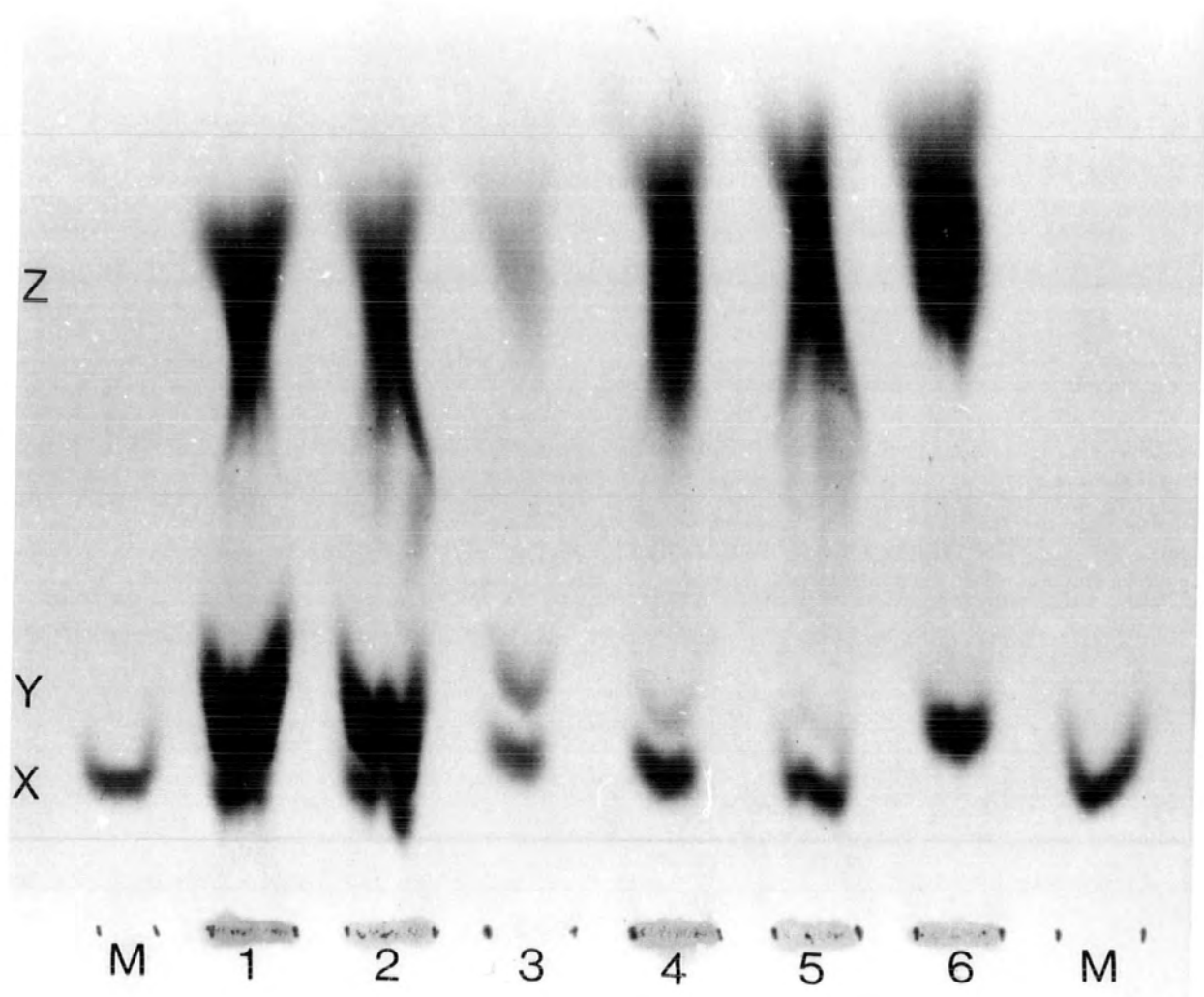
RNase A digests of unfractionated E. coli [³⁵S]met-tRNA and DEAE-sephadex peak 1 and peak 2 [³⁵S]met-tRNA's. Aminoacylation with [³⁵S]methionine (sp.act.27C/mM) was carried out in the presence of formyl donor as described in Methods, Sections 24D. After re-isolation the [³⁵S]met-tRNA's were digested with bovine pancreatic RNase A as described in Methods, Section 25 and electrophoresed at pH3.5, 3kV, 1hr. 1. Digest of E. coli tRNA aminoacylated by E. coli DE52 supernatant; 2, digest of E. coli tRNA aminoacylated by bean DE52 supernatant; 3, Peak 1 tRNA aminoacylated as for 1; 4, Peak 1 tRNA aminoacylated as for 2; 5, peak 2 tRNA aminoacylated as for 1; 6, peak 2 aminoacylated as for 2.

M = free methionine applied as a standard

X = free methionine

Y = N-formyl-methionyl-adenosine

Z = methionyl-adenosine



slight indications of an N-formyl-methionyl-adenosine residue. This is consistent with the data of Table 18 showing a low degree of aminoacylation of peak 1 by E.coli supernatant.

When peak 2 was aminoacylated in the presence of E.coli supernatant a strong spot corresponding to methionyl-adenosine was obtained. In addition to free methionine there was some indication of a fragment identified as N-formyl-methionyl-adenosine. Using bean enzyme only the methionyl-adenosine and methionine spots were identifiable.

(iii) [³⁵S]methionyl-tRNA's resolved by chromatography on BD cellulose

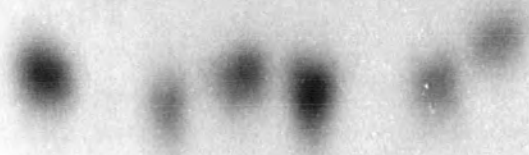
Figure 37 shows the autoradiograph obtained after ionophoresis of pancreatic RN'ase digests of BD cellulose fractions corresponding to [³⁵S]methionyl-tRNA₁, tRNA₂ and tRNA₃ which had been aminoacylated in the presence or absence of formyl donor using 60 day bean DE52 supernatant. E.coli [³⁵S]methionyl-tRNA was used as a control. In both the presence and absence of formyl donor E.coli [³⁵S]methionyl-tRNA gave spots corresponding to methionyl-adenosine and N-formyl-methionyl-adenosine however in the presence of the formyl donor there was a considerably greater proportion of the N-formyl-methionyl-adenosine.

When tRNA₁ was aminoacylated in either the presence or absence of formyl donor only one spot corresponding to methionyl-adenosine was identified. Similarly, for tRNA₂, there was no indication of any spot corresponding to N-formyl-methionyl-adenosine. The degree of aminoacylation of tRNA₃ was so low that no spots could be identified on the figure shown. However other autoradiographs

Fig.37. Autoradiograph of an ionogram of pancreatic RNase digest of unfractionated *E. coli* [³⁵S]met-tRNA, [³⁵S]met-tRNA₁^{met}, [³⁵S]met-tRNA₂^{met} and [³⁵S]met-tRNA₃^{met}. Aminoacylation by [³⁵S]methionine (sp.act 510mC/mM) was carried out as described in Methods, Section 24D. and the re-isolated [³⁵S]met-tRNA's were digested by bovine pancreatic RNase A as described in Method, Section 25. 1,tRNA₁^{met} aminoacylated by 60 day developing bean DE52 supernatant in the absence of added formyl donor; 2,tRNA₃^{met} aminoacylated as for 1;3,tRNA₂^{met} aminoacylated as for 1;4,unfractionated *E. coli* tRNA aminoacylated as for 1;5,tRNA₁^{met} aminoacylated by bean DE52 supernatant in the presence of added formyl donor; 6,tRNA₃^{met} aminoacylated as for 5; 7,tRNA₂^{met} aminoacylated as for 5; 8, unfractionated *E. coli* tRNA aminoacylated as for 5; M= free methionine applied as a standard

X = methionine
Y = N-formyl-methionyl adenosine
Z = methionyl adenosine

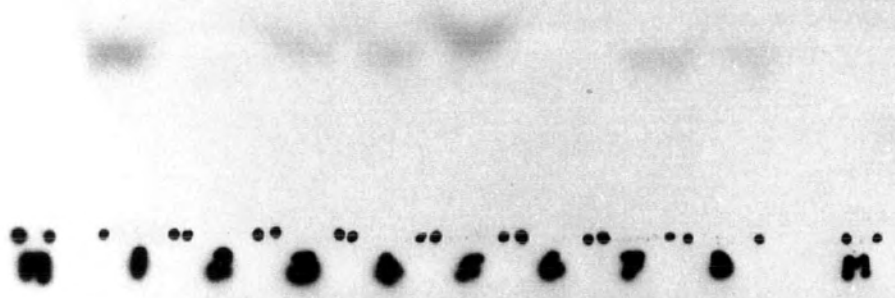
Z



Y



X



prepared using high specific activity (27C/mM) methionine revealed traces of an N-formyl-methionyl-adenosine spot.

B. Digestion of $[^{35}\text{S}]$ methionyl tRNA's with T_1 ribonuclease

(i) DEAE-Sephadex peaks 1 and 2

Fractions corresponding to DEAE-Sephadex peak 1 and peak 2 and unfractionated deacylated E.coli tRNA were aminoacylated with $[^{35}\text{S}]$ methionine (sp.act. 14C/mM) in the presence or absence of formyl donor using 60 day bean DE52 supernatant or E.coli DE52 supernatant. The $[^{35}\text{S}]$ methionyl-tRNA's were digested by T_1 ribonuclease and the resultant oligonucleotides separated by ionophoresis.

Figure 38 is an autoradiograph of an ionophoretogram of the $[^{35}\text{S}]$ methionyl-tRNA's corresponding to DEAE-Sephadex and Peaks 1 and 2/ shows that these differ in nucleotide composition from the corresponding E.coli tRNA's. In addition to spots corresponding to methionine and methionine sulphoxide, three labelled oligonucleotides were identified in T_1 digests of E.coli unfractionated $[^{35}\text{S}]$ methionyl-tRNA. These are identified as corresponding to the met-tRNA_M(X), met-tRNA_F(Y) and f-met-tRNA_F(Z) fragments.

Peak 1 tRNA was not significantly aminoacylated by E.coli DE52 in either the presence or absence of formyl donor and no radioactive fragments were detectable. When peak 1 tRNA was aminoacylated in the presence of bean DE52 supernatant, only one labelled fragment, in addition to methionine and methionine sulphoxide was identifiable. The labelled oligonucleotide did not move as a discrete spot but streaked badly towards the positive electrode. The mobility was

Fig.38. Autoradiograph of an ionogram of a T_1 ribonuclease digest of E. coli and bean $[^{35}\text{S}]$ met-tRNA's. Unfractionated E. coli tRNA and DEAE-sephadex , Peak 1 and Peak 2, were aminoacylated with $[^{35}\text{S}]$ methionine (sp.act.14C/mM) as described in Method, Section 24D, after re-isolation the $[^{35}\text{S}]$ met-tRNA's were digested with T_1 ribonuclease as described in Methods, Sections 26.

a, aminoacylated by 60 day developing bean DE52 supernatant in the presence of added formyl donor, b, as for a in the absence of formyl donor; c, aminoacylated by E. coli DE52 supernatant in the presence of formyl donor; d, as for c in the absence of formyl donor. Peak 1 was not aminoacylated under conditions c and d and only one sample was electrophoresed.

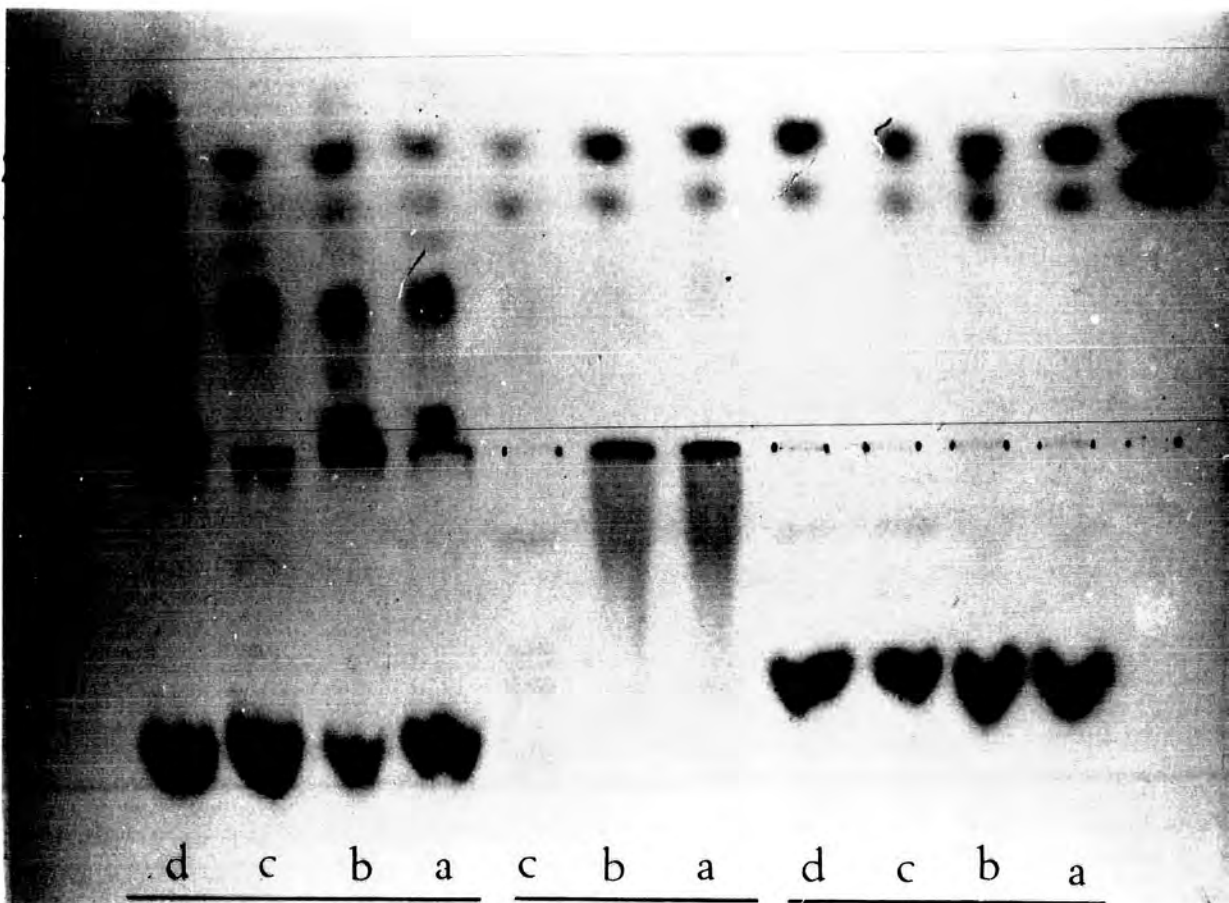
X = $[^{35}\text{S}]$ met-tRNA_M fragment

Y = $[^{35}\text{S}]$ met-tRNA_F fragment

Z = f- $[^{35}\text{S}]$ met-tRNA_F fragment

M_1 , M_2 are methionine sulphoxide and methionine respectively.

M
M



d c b a

E.coli

c b a

Peak 1

d c b a

Peak 2

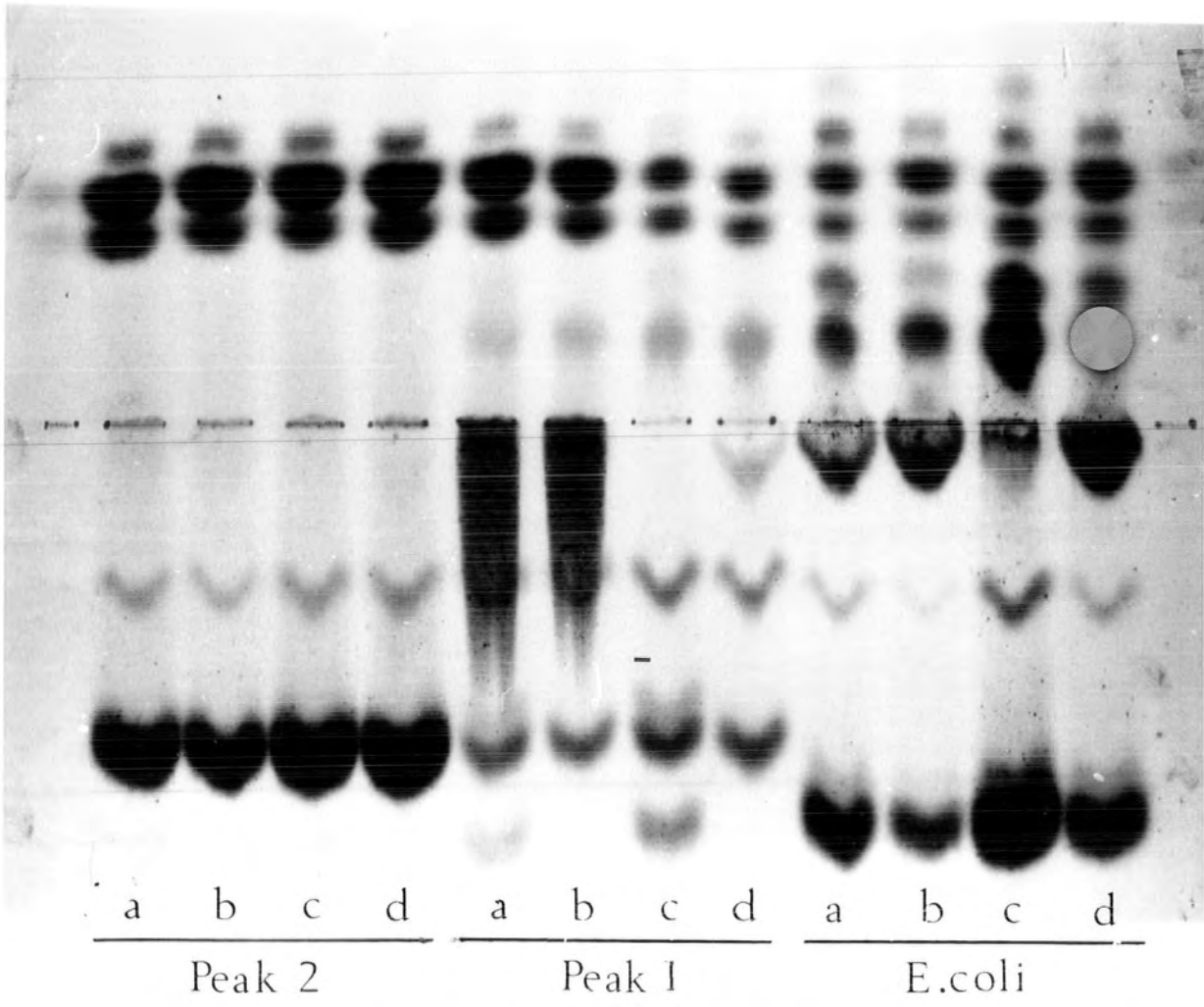
quite discrete from that of either peak 2 or E.coli $[^{35}\text{S}]$ met-oligonucleotides. The $[^{35}\text{S}]$ methionyl-oligonucleotide from peak 2 ran towards the anode as a discrete spot. Its mobility was greater than that of the corresponding fragment from E.coli met-tRNA_F being almost the same as that from f-met-tRNA_F. Only in the case of E.coli tRNA was there any significant difference between samples aminoacylated in the presence and absence of formyl donor.

Figure 39 shows an autoradiograph prepared under similar conditions to that shown in Fig.38. However the specific activity of the $[^{35}\text{S}]$ methionine used in aminoacylation was increased to 27C/mM. The results are identical to those shown in Fig.38, but in this case, there is evidence of more than one $[^{35}\text{S}]$ met-oligonucleotide derived from peak 1 tRNA. When peak 1 tRNA was aminoacylated by bean DE52 supernatant in either the presence or absence of the formyl donor a major fragment was obtained which streaked towards the anode. This fragment was absent when E.coli DE52 s/n was used as the enzyme source. However in all four samples there was evidence of a fragment with identical mobility to that of the E.coli met-tRNA_M fragment, when aminoacylation took place in the presence of formyl donor there is evidence of a fragment corresponding to the E.coli f-met-tRNA_F fragment. When aminoacylation took place in the presence of E.coli enzyme without formyl donor there is evidence of a fragment corresponding to the E.coli met-tRNA_F fragment. There is also present a fragment with mobility identical to that of peak 2 tRNA indicating in this case incomplete resolution of the three tRNA species. It is unlikely that these additional fragments with mobilities identical to E.coli

Fig.39. As for Fig.38. Aminoacylation was carried out using

$[^{35}\text{S}]$ methionine (sp.act.27C/mM) and samples of Peak 1

aminoacylated under conditions C and d were electrophoresed.



tRNA fragments are derived from contaminating E.coli DE52 supernatant since an equal volume of supernatant was used in the preparation of peak 2 tRNA samples and in this case there is no evidence of such fragments. It must be stressed that because of the very high specific activity of the $[^{35}\text{S}]$ methionine (27C/mM) used, these spots represent fragments present in extremely low proportions.

(ii) tRNA₁, tRNA₂, tRNA₃ as resolved by BD-cellulose

Fractions corresponding to tRNA₁^{met}, tRNA₂^{met}, and tRNA₃^{met} were aminoacylated with $[^{35}\text{S}]$ methionine (510mC/mM) using 60 day developing bean DE52 supernatant in the presence or absence of formyl donor. The $[^{35}\text{S}]$ methionyl-tRNA's were digested with T₁ ribonuclease and the resultant oligonucleotides were separated by ionophoresis. Figure 40 shows the resultant ionophoretogram. Unfractionated E.coli $[^{35}\text{S}]$ methionyl-tRNA, prepared and digested under identical conditions was used as a marker.

The results are essentially similar to those obtained with peak 1 and peak 2 as resolved by DEAE-Sephadex. tRNA₃ was not aminoacylated to a sufficient extent to produce discernible spots after autoradiography.

$[^{35}\text{S}]$ met-oligonucleotides identified as derived from the met-tRNA_M, met-tRNA_F, and f-met-tRNA_F species (X, Y and Z respectively) were obtained with E.coli tRNA. In the absence of formyl donor the intensity of the spot corresponding to the met-tRNA_F-derived fragment was greater than in the presence of the formyl donor whilst the reverse was true of the fragment from f-met-tRNA_F. $[^{35}\text{S}]$ met-oligonucleotide derived from tRNA₁ ran as a discrete spot having a mobility almost the same as that of $[^{35}\text{S}]$ -oligonucleotide from E.coli

Fig. 40. Autoradiograph of an ionogram of T_1 ribonuclease digests of unfractionated E. coli $[^{35}\text{S}]$ met-tRNA and bean $[^{35}\text{S}]$ met-tRNAs resolved by chromatography on BD-cellulose. Aminoacylation by bean DE52 supernatant $[^{35}\text{S}]$ methionine (sp.act. 14C/mM) and digestion were carried out as detailed in the legend to Fig.38.

1, tRNA₁^{met} aminoacylated in the absence of added formyl donor;
2, tRNA₃^{met} aminoacylated as for 1; 3, tRNA₂^{met} aminoacylated as for 1; 4, E. coli tRNA aminoacylated as for 1; 5, tRNA₁^{met} aminoacylated in the presence of formyl donor; 6, tRNA₃^{met} aminoacylated as for 5; 7, tRNA₂^{met} aminoacylated as for 5; 8, E. coli tRNA aminoacylated as for 5. M_1, M_2 = methionine sulphoxide and methionine respectively.

$X = [^{35}\text{S}]$ met-tRNA_M fragment

$Y = [^{35}\text{S}]$ met-tRNA_F fragment

$Z = f- [^{35}\text{S}]$ met-tRNA_F fragment

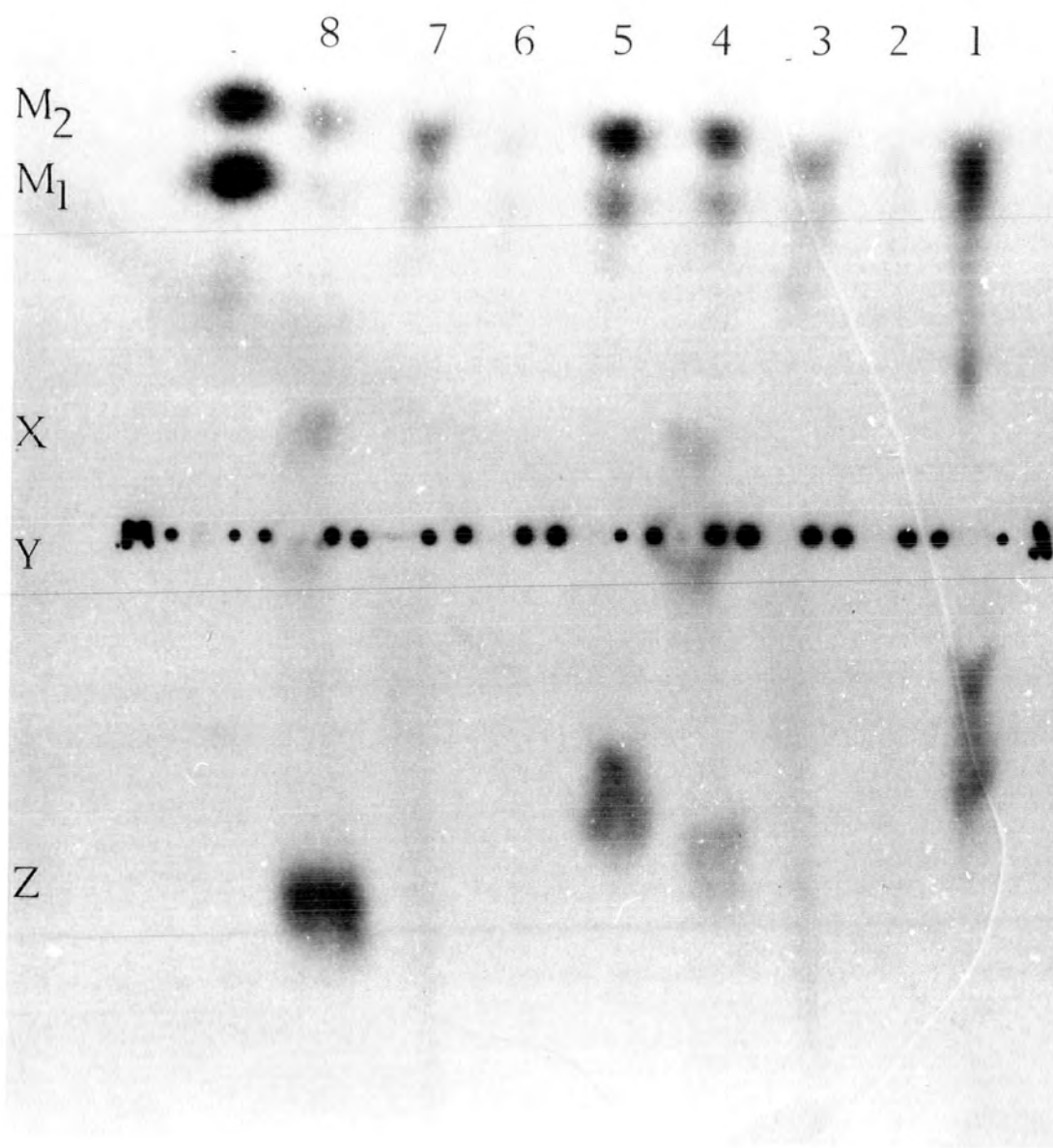


Table 19N-terminal analysis of $[^{35}\text{S}]$ methionine polypeptides

| Messenger | % Methionine in N-terminus | |
|------------|----------------------------|-----------------------|
| | met-tRNA ₁ | met-tRNA ₂ |
| Endogenous | 69% | 13% |
| Poly(AUG) | 59% | 14% |
| Poly(UG) | 82% | 20% |

Percentage incorporation into N-terminal position. The complete amino acid incorporating system has been described in Methods; it also contained in a total vol. of 100 μ l, 60 μ g poly(AUG) or 50 μ g Poly(UG), together with 7 pmoles met-tRNA₁ or 6 pmoles met-tRNA₂ from DEAE-Sephadex (1 pmole = 22500 c.p.m.) $[\text{Mg}^{2+}]$ was 4mM. After 30 min., 10 μ g pancreatic RNase in 100 μ l of 100mM EDTA was added and incubated for 2 min. The reaction was terminated by the addition of 5 ml ice cold 5% (w/v) TCA, 0.1 M Methionine and the precipitation washed x 3 with the same solution. N terminal analysis was by the method of Blombäck et al., (1966).

F-met-tRNA_F. Whilst the [³⁵S] met-oligonucleotide from tRNA₂, like that from peak 1 from DEAE-Sephadex, streaked towards the anode. Peak 1 and 2 DEAE-Sephadex correspond to tRNA₂^{met} and tRNA₁^{met} respectively from BD-cellulose, yielding identical fragments with identical electrophoretic behaviour. In no case was there evidence of additional fragments with mobility related to those of the E.coli [³⁵S] met-oligonucleotides.

12. N-terminal analysis of [³⁵S] methionine polypeptides (Table 19).

Methionine labelled polypeptides synthesised in response to the synthetic messengers, Poly(AUG) Poly(UG) and to endogenous messenger, using [³⁵S] met-tRNA₁^{met} and [³⁵S] met-tRNA₂^{met} were analysed for % methionine in the N-terminal position. In all cases met-tRNA₁^{met} (i.e. peak 2 from DEAE-Sephadex) was more effective in donating [³⁵S] methionine into the N-terminal position than was [³⁵S] met-tRNA₂^{met} (DEAE-Sephadex Peak 1). Using [³⁵S]-met-tRNA₃ no significant results could be obtained because of the extremely low proportion of this met-tRNA species in the bean tRNA preparations and its low degree of aminoacylation.

13. AUG dependent binding of [³⁵S] met-tRNA species to bean ribosomes

The ability of [³⁵S] met-tRNA's to bind to bean ribosomes in response to the synthetic triplet AUG was assayed. Table 20 shows that met-tRNA₁ and met-tRNA₃ were bound at low Mg²⁺ concentrations (i.e. < 10mM), whereas 20mM Mg²⁺ was required for significant binding of met-tRNA₂.

Table 20

AUG dependent binding of $[^{35}\text{S}]$ met-tRNA species to bean ribosomes

| mM Mg^{2+} | 2.2 | 4.4 | 6.6 | 11.0 | 20.0 |
|---------------------------------------|------|------|------|------|------|
| met-tRNA ₁ pmoles bound | 0.05 | 0.15 | 0.19 | 0.17 | 0.76 |
| met-tRNA ₂ pmoles bound | - | - | - | 0.04 | 0.23 |
| met-tRNA ₃ pmoles bound | 0.06 | 0.05 | 0.08 | 0.10 | 0.13 |

The AUG dependent binding of $[^{35}\text{S}]$ met-tRNA to ribosomes extracted from 3 day germinated beans was determined by the method of Nirenberg and Leder (1964) as described in Section 28, Methods. met-tRNA species were resolved by chromatography on B.D. cellulose and aminoacylated with $[^{35}\text{S}]$ methionine (sp.act.510mC/mM) using 60 day developing bean DE52 supernatant. All figures have been corrected for binding in the absence of AUG at the relevant Mg^{2+} concentration.

14. Release of methionyl-puromycin from bean ribosomes AUG dependent

The reaction between puromycin and $[^{35}\text{S}]$ met-tRNA bound in response to the triplet AUG, was used to distinguish between binding at the initiation (peptidyl) and aminoacyl-tRNA sites (Bretscher and Marker, 1966) on bean ribosomes. The results in Table 21 in comparison those in Table 20 show that only methionine bound as $[^{35}\text{S}]$ met-tRNA₁ or as $[^{35}\text{S}]$ met-tRNA₃ is released as methionyl-puromycin. $[^{35}\text{S}]$ met-tRNA₂ bound in response AUG at high magnesium concentration was not released by incubation with puromycin. The additional molecules of the $[^{35}\text{S}]$ met-tRNA₃ bound at high magnesium were not released by puromycin.

Table 21

The release by puromycin of $[^{35}\text{S}]$ met-tRNA bound to bean ribosomes in response AUG.

| mM Mg^{2+} | 2.2 | 4.4 | 6.6 | 11.0 | 20.0 |
|---------------------------------------|------|------|------|------|------|
| met-tRNA ₁ pmoles released | 0.08 | 0.14 | 0.12 | 0.18 | 0.21 |
| met-tRNA ₂ pmoles released | - | - | - | - | - |
| met-tRNA ₃ pmoles released | 0.03 | 0.04 | 0.05 | 0.04 | 0.05 |

The release of AUG dependently bound $[^{35}\text{S}]$ met-tRNA as $[^{35}\text{S}]$ methionyl-puromycin was assayed in the system described in Section 29, Methods. All figures have been corrected for release in absence of AUG at the relevant Mg^{2+} concentration.

DISCUSSION

During protein synthesis, either in vivo or in vitro, a complex series of reactions occur involving a particulate component (microsome, ribosome or polysome), tRNA, and a variety of enzymes which result in the polymerisation of amino acids into a polypeptide. The reaction between these basic components is facilitated by a number of soluble factors which act in several different ways. In order to understand the interactions between the components involved in protein synthesis it is essential that the components themselves be fully characterised. Because components derived from different sources differ in their degree of reactivity, no absolute criterion of reactivity can be established, e.g. purified tRNA preparations from different sources may exhibit widely different specific activities. However, such components do exhibit strong physical similarities, especially in the case of particles and tRNA and it is therefore possible to check the purity or homogeneity of a preparation by comparison with a series of criteria. However it must be stressed that such criteria of purity give absolutely no guide to the in vitro activity of such preparations. Kúcan (1966) has demonstrated that a single lesion in rRNA within a ribosome is sufficient to render it inactive in protein synthesis but would have no effect on the physical characteristics of such a particle.

The particulate component of the protein synthesising machinery has been isolated from a number of sources by a variety of methods. All these methods involve rupture of the cells to expose the cytoplasmic matrix and separation of the ribosomal particles from the remaining particulate and soluble material by centrifugation.

As discussed in the Introduction the type of particulate preparation is dictated by the type of problem under investigation. In those cells in which the bulk of the protein is known to be synthesised upon membrane-bound ribosomes it would appear logical to use a preparative procedure in which this intimate spatial relationship was maintained. However as has been stressed in the Introduction the endoplasmic reticulum is a highly heterogeneous component associated with which are a host of degradative activities. It is for this reason that many workers prefer to sacrifice the spatial relationship of membrane and ribosome.

The simplest criterion of the purity of a preparation of microsomes or ribosomes is the shape of its ultraviolet absorption spectrum. The RNA component of such preparations absorbs maximally at 260 nm whilst the protein absorbs more strongly at shorter wave lengths, it follows therefore that the less extraneous protein present the higher the ratio of absorption at 260 nm: 235 nm will be. From the data presented in Tables 2 and 3 it can be seen that the removal of the particles from the endoplasmic reticulum by the non ionic detergent, Triton X100 results in a displacement of both the absorption maxima and minima towards shorter wave lengths and a concomitant increase in the ratio 260 nm: 235 nm. Assuming a particle with 50% RNA: 50% protein to have a ratio of 260 nm: 235 nm of 1.67, the ratio of 1.26 and 1.24 in the case of microsomes from developing cotyledons and the plumules of germinated seeds respectively, indicates that in both cases there is a considerable degree of contamination associated with such preparations. (Peterman, 1964). The ratio of 1.50 and 1.48 in the case of Triton X100 ribosomes from

the two types of material indicates approximately 20% extraneous protein (i.e. a ratio of % RNA: % protein of 40:60) attached to the particles (Peterman, 1964). On the basis of its effect upon the 260 nm: 235 nm ratio, washing of the particles with 0.5M - ammonium chloride and subsequent dialysis appears to considerably reduce the amount of extra protein attached to ribosomes prepared from germinated seeds whilst having no effect upon those from developing material. It is unlikely that this is in fact so, there appears to be no reason to assume that the ribosomes extracted by Triton X100 from the two sources should in any way differ from one another. Assuming that all membrane material has been completely solubilised, the remaining proteins attached to the ribosome should be those intimately involved with the function of the ribosome in protein synthesis, and ammonium chloride should be equally capable of removing such proteins from both types of preparation. Payne (1968) has demonstrated that the majority of the ribosomes in the 60 day developing bean are membrane-bound, and furthermore these represent a discrete type of particle which does not exchange with the free cytoplasmic ribosomes. The possibility therefore exists that some protein associated with the attachment of these particles to the membrane remains firmly attached to the ribosome and is not removed by the ammonium chloride treatment. A ratio of 260 nm: 235 nm of 1.71 would indicate an RNA content in excess of 50%, this would not appear unreasonable in view of the results of Yarwood (1968) who found a ratio of RNA : protein of 1:1 for microsomes from the plumules of germinated beans.

One of the most frequently used criteria for the purity of a ribosomal preparation is the RNA content, estimated either from its absorption spectrum or determined chemically. Ribosomes from plant sources have been shown to consist of 40 - 60% RNA and 60 - 40% protein and the results obtained here from the chemical characterisation of the microsomal and ribosomal preparations are in agreement with these values (Table 4). The value of 12.7% RNA obtained for unclarified microsomes isolated from 60 day developing bean correlates well with that obtained by Payne (1970) using the Orcinol method (as described by Campbell and Sargent, 1967). Such a low value is highly indicative of a considerable degree of contamination. That such contaminating material is present can be seen from the effect on the %RNA value of a single low speed centrifugation used to clarify such preparations. After such a clarification the % RNA obtained was identical to that obtained with clarified microsomes from germinated material. The microsomes prepared from germinated seeds were similarly clarified by a low speed centrifugation however the slight change in % RNA attests to the much lower degree of contamination of such material by fragments of membrane etc. When the microsomes prepared from developing bean are pelleted and resuspended, a turbid green precipitate rapidly settles out, removal of this material by centrifugation and examination of its U.V. absorption spectrum reveals a considerable degree of U.V. absorbing material. The absorption spectrum of this material is relatively linear between 230 nm and 300 nm and shows neither the maximum at 260 nm associated with RNA or the maximum at 280 nm attributable to protein. It absorbs strongly at wavelengths below 230 nm, suggesting it is

probably a complex mixture of disrupted membrane material. The value for RNA in Triton X100 ribosomes from the two sources correlates with the U.V. absorption data which predicts a ratio of % RNA:% protein of 40:60. It is significant that approximately equal values are obtained for clarified microsomes and ribosomes from the two classes of material, suggesting that at least chemically there is no significant difference between the ribosomes from the two sources.

Analysis on sucrose density gradients of microsomes and Triton X100 ribosomes prepared from 60 day developing cotyledons revealed essentially similar profiles. The major difference being the presence in the microsome preparation of a broad band of rapidly sedimenting material. This was identified as membrane-bound material and its absence from the ribosome preparation indicates the efficiency of Triton X100 in solubilising membrane material. The disappearance of the membrane-bound material/ and concomitant increase in the amount of polysomes in preparations made in the presence of Triton X100, suggests that the membrane-bound material consists to a considerable extent of membrane-bound polysomes rather than single ribosomes or subunits. Essentially similar gradient profiles have been reported for microsome and ribosome preparations from Vicia faba (L) by Lonsdale (1972) and Payne (1970) and from leaf tissue by Pearson (1969) and Pearson and Wareing (1970).

The particle preparations applied to these gradients were analysed immediately after preparation and prior to storage at -70° . As a matter of convenience particle preparations were stored for considerable periods at -70° or -196° prior to use. Lonsdale (1972)

has shown that the polysome distribution remains unaffected by a single freezing at -70° but that refreezing on a second occasion results in considerable breakdown of polysomes. The major breakdown product was identified as ribosomal subunits. It is for this reason that particle preparations were divided into small batches and used only once after freezing.

The identification of a particular component on a sucrose gradient was based on that of Lonsdale (1972). The most positive method of identification of a sedimenting fraction is on the basis of its sedimentation coefficient at zero concentration, S° . The sedimentation coefficient of 129S for the most rapidly sedimenting component of these particle preparations is in agreement with its identification as a dimer composed of 2, 85S monosomes (Table 5). The value of 85S correlates well the value of 82S calculated by Payne (1968) and Yarwood (1968) using sucrose density gradient analysis of Vicia faba microsomes. The sedimentation coefficients of the less rapidly sedimenting peaks strongly support their identification as ribosomal subunits (Fig.13). The absence of significant amounts of polysome material from these sedimentation profiles may be explained by the mode of handling of the particle preparations. Prior to examination the preparations were frozen overnight at -70° following which they were maintained in ice for a period of 6 hours. Such degradation is not unexpected in view of the known lability of polysomes and Lonsdale (1972) has shown that considerable degradation of Vicia faba polysomes results when preparations are maintained at 0° . Although it is commonplace to refer to eukaryotic cytoplasmic ribosomes as 80S, as has been previously stressed, this is purely a

broad distinction based on functional similarities exhibited by ribosomes with high sedimentation coefficients. Values of 80 - 83S have been obtained for cytoplasmic ribosomes of cabbage, clover, ryegrass, tobacco, spinach and kidney beans (Lyttleton, 1960; 1962; Clark et al., 1964; Spenser, 1965; Boardman et al., 1965; Odintsova et al., 1967). The linear dependence of the sedimentation coefficient upon concentration indicates that there is not a significant amount of interaction between ribosomes and other material present in the preparation (Hess and Lagg, 1963).

Characterisation of the particle preparations on sucrose density gradients and in the analytical ultracentrifuge revealed a considerable proportion of the ribosomes were present as free ribosomes. In view of the results of Legon et al. (1973) and Bishop (1966) implicating the 40S ribosomal subunit in initiation in eukaryotic systems, and of the existence of subunits in particulate preparations, it may be pertinent to discuss the significance of the single ribosome (monosome) pool demonstrated in the above preparations. The role of the monosome, be it free or membrane-bound, in eukaryotic systems remains controversial. There is now evidence of the existence in eukaryotes of a subunit cycle similar to that described for the prokaryotic organisms. It is clear that pools of subunits, monosomes and polysomes exist in eukaryotic systems (Girard et al., 1965; Ristow and Kohler, 1966; Bishop, 1966; Joklik and Becker, 1965; Lonsdale, 1972). Using long term labelling experiments Hogan and Korner (1968) demonstrated that free subunits are in equilibrium with those in polysomes whilst Kaempfer (1969) has demonstrated that yeast ribosome undergo extensive subunit exchange during growth, all of which are suggestive

of the existence of a ribosome subunit cycle essentially similar to that described for prokaryotes. Jacobs - Lorena and Baglioni (1970) present evidence for the existence of such a subunit cycle. These authors have shown that when native He La ribosomal subunits, labelled in the rRNA, are added to an active cell-free rabbit reticulocyte system they enter into polysomes and are subsequently converted to ribosomes. Incubation of the ribosomal subunits in a cell-free system from which polysomes have been removed failed to promote conversion to ribosomes, the conversion of subunits into polysomes and finally into ribosome was shown to be dependent upon active protein synthesis. Kaempfer (1969) presents similar evidence for the existence of such a cycle in the yeast Candida krusei. There may be a significant difference in the cycle in eukaryotes since the free ribosome pool does not equilibrate rapidly with the added subunits or with freshly run off ribosomes (Jolik and Becker, 1965; Jacobs - Lorena and Baglioni, 1970; Howard et al., 1970). These authors report that the rate of entry into polysomes is slower than might be expected if all reinitiations were carried out by the free subunits added. Jacobs-Lorena and Baglioni (1970) suggest that upon termination the ribosomes released from the polysome are dissociated and the subunits so produced are used preferentially in reinitiation. These authors explain such preferential utilisation on the basis of a conformational restraint of mRNA such that ribosomes released at the 3' end are maintained in close proximity to the 5' initiation site. Howards et al. (1970) suggest that speculations upon mRNA conformation are unnecessary and calculate, on the basis of mRNA size and subunit concentrations, that statistically reattachment at a subunit should be strongly favoured over exchange with the subunit pool.

As well as the evidence for the existence of a subunit cycle most of the necessary factors have been isolated from eukaryotes. The initiation factors, so intimately associated with the existence of such a cycle, have been isolated from the ribosomes of rabbit reticulocytes. (Pritchard et al., 1970) and a dissociation factor equivalent to DF (F_3) or anti-association factor has been isolated by Petre (1970) from Saccharomyces cerevisiae and by Kaempfer and Kaufman (1972) from rabbit reticulocytes. Kyner and Levin (1972) have isolated from mouse fibroblast ribosomes a reassociation factor (RF) which promotes the reassociation of 40S and 60S ribosomal subunits. Reassociation is independent of mRNA and aminoacyl - tRNA but is enhanced by GMPPCP and inhibited by aurintricarboxylic acid. A similar factor has been described by Wettenhall et al. (1971) from rat liver.

Given that a subunit cycle does exist in eukaryotes, the origin and function of the free monosome is still controversial. The demonstration by Adamson et al. (1972) that in rabbit reticulocytes the free monosome pool does not exchange with either the ribosomes in polysomes or with the free ribosome pool strongly suggests that the free ribosome peak observed in particulate preparations represents not ribosomes which have just completed translation and have not yet dissociated but an inert pool whereby subunits can be shunted off and maintained in an inactive form until required. During low rates of protein synthesis, when the return of single ribosomes to the active ribosomal pool is not favoured, subunits newly released from polysomes would be expected to recycle preferentially. Such preferential recycling has been demonstrated in the reticulocyte cell-free system (Adamson et al., 1969). In the case of E.coli

ribosomes there is evidence that the single ribosomes can be distinguished by physical and functional criteria. Kaempfer (1970) assigns an S value of 65S to free ribosomes compared to a value of 70S for monosomes obtained by RNase digestion of a polysome extract. Single ribosomes, unlike polysomes are dissociated by RNase into subunits. Ron et al. (1968) and Kelley and Schaechter (1969) have shown that single ribosomes require a higher Mg^{2+} ion concentration to prevent their dissociation than do polysomal ribosomes.

Having concluded that the free ribosome peak represents an inert pool whereby subunits are stored under conditions favouring low rates of protein synthesis it must then be assumed that the particles engaged in vivo in rounds of translation are the native subunits and the polysomes. It must be with caution however that such conclusions are extrapolated to an in vitro situation where unlike that in vivo, translation of a synthetic message is occurring in an unphysiologically high Mg^{2+} concentrations. It has been demonstrated that under conditions of high magnesium the need for 'proper' chain initiation is eliminated (Eisenstadt and Lengyel, 1967) and it is likely that association of the synthetic messenger, Poly(U), with the ribosome template can occur in the absence of dissociation into subunits and under such conditions the free ribosomes may become involved in translation.

Finally the possibility must not be ignored that the in vitro distribution of particles seen in the analytical ultracentrifuge and in sucrose density gradients in no way reflects the in vivo distribution. The preponderance of free ribosomes observed may have resulted from the association of native subunits as a result of the

extraction procedure, or may represent free ribosomes released by degradation of polysomes. Such free ribosomes tend not to dissociate because of the presence of peptidyl-tRNA which stabilises the interaction between the large and small subunit (Spirin and Gavilova, 1969). Vournakis and Rich (1972) have distinguished between free and polysome ribosomes (i.e. those released by degradation of translating polysomes) on the basis of sedimentation coefficient. Free ribosomes sedimented at 78S in comparison to polysome ribosomes which sedimented at 85S. This value is in agreement with the value obtained for V. faba ribosomes and would suggest that the large monosome peak in part represents ribosomes liberated from degraded polysomes.

The adaptor molecule, tRNA, is routinely prepared by many workers from the high speed supernatant remaining after the particles have been pelleted. The method involves precipitation of the tRNA at pH5 and separation from contaminating protein by treatment with water saturated phenol (Ravel et al., 1966). Such a method is, however, applicable only to those tissues in which there is little RNase activity (e.g. reticulocytes). In order to obtain preparations of tRNA having high amino acid acceptor capacity it is essential that nuclease activity be reduced to a minimum. This can be achieved by homogenisation of the tissue directly in the presence of water-saturated phenol and buffered extractant. The effect of the phenol is to inhibit the action of endogenous nucleases as well as bringing about deproteinisation of the tissue. If the phenol extraction is delayed until after the pelleting of subcellular particles considerable nuclease activity results. Such a method is commonly

used with plant material which is characterised by significant amounts of endogeneous nucleases (Allende, 1969; Glitz and Dekker, 1963; Anderson and Cherry, 1969). Yarwood (1968) and Payne (1970) used the direct phenol extraction technique for the isolation of crude tRNA preparations from developing cotyledons of Vicia faba (L) Using polyacrylamide gel electrophoresis, Payne (1970) has shown that tRNA prepared from similar tissue by an identical method is highly contaminated with 25S and 18S and 5S rRNA. Chromatography on DE52 removed all of the high molecular weight rRNA's and most of the 5S rRNA, leaving an almost homogeneous preparation of 4S RNA (i.e. tRNA).

tRNA is a relatively complex molecule which depends for its activity upon the maintenance of a strictly defined tertiary structure. It can however be degraded to a certain extent without any significant alteration in its physical characteristics. Care must be taken therefore in equating the physical 'purity' of tRNA with its functional activity. Similarly the degree of aminoacylation of a tRNA preparation is no indication of its total ability to donate the amino acid into protein. Nishimura and Novelli (1964) have shown that tRNA's of E. coli have different susceptibilities to RNase T₁ and that some of the partially digested tRNA's still accept amino acids but do not transfer them into protein. There are several reported examples of the reconstitution of fragments of tRNA molecules which are capable of accepting an amino acid. (Oda et al., 1969; Imura et al., 1969).

Allende (1969) outlined three objectives of in vitro amino acid - incorporating systems:

(1) The determination of the relative activities of the protein synthesising mechanisms of tissues under specific conditions.

(2) the study of the properties of the components involved in the process of protein synthesis.

(3) the achievement of the synthesis of a specific protein. In order to achieve the first objective it is essential to use a system which interferes as little as possible with the components and approximates as closely as possible to the in vivo situation. By definition such systems should be directed by an endogenous mRNA. Parisi and Ciferri (1966) have characterised such a system from castor bean seedlings. The activity of such systems is usually low and they are extremely susceptible to nucleases.

In order to study the properties of the components involved in the process of protein synthesis, a simplified system using highly purified components and the best attainable incorporation is desirable. In order to completely understand the properties and interactions of all the components involved one single system may be inadequate. It is essentially through the use of simplified Poly(U) dependent system from E.coli similar to that described by Nishizuka and Lipmann (1966b) that the mechanism of polypeptide chain elongation has been elucidated. This system, however, provided little relevant information on the mechanism of polypeptide chain initiation under physiological conditions. It was only with the advent of systems directed by natural messengers (e.g. bacteriophage RNA) that this problem was resolved.

The first two objectives have been readily achieved using cell-free amino acid incorporating systems. Data for plant material

is limited, this seems to reflect the number of groups involved rather than any inherent characteristic of the material.

Until recently the third objective has proved singularly elusive; the integrity of a system developed to synthesise a specific protein must be carefully maintained. There are now several firmly substantiated reports in the literature of the synthesis of specific proteins by in vitro systems from animal and bacterial sources. (Lockard and Lingrel, 1969; Laycock and Hunt, 1969; Matthews et al., 1971; Stavnezer and Huang, 1971; Berns et al. /1972). To date there has been no unequivocal demonstration of the synthesis of a specific protein in a plant amino acid - incorporating system. Schwartz et al. (1965) synthesised a phage coat protein in a Euglena gracilis chloroplastic system directed by f_2 bacteriophage RNA, Sela and Kaesberg (1969) using tobacco chloroplast ribosomes directed by tobacco mosaic viral-RNA claim to have synthesised the viral coat protein. Klein et al. (1972) have synthesised a viral coat protein in a wheat embryo system directed by satellite tobacco necrosis viral-RNA. It is significant that in all these cases the product was not a plant protein. Duffus (1967) reports that a microsomal cell-free system from the aleurone layers of germinating barley is capable of synthesis of α - amylase. In view of the requirements of this system it is more likely that the increase in α - amylase reported results not from de novo synthesis but from activation of a precursor molecule.

Hoagland (1960) has pointed out that the following criteria should be fulfilled if incorporation of a radioactive amino acid is to be equated with protein synthesis.

(i) Incorporation of the L-amino acid should be irreversible once the amino acid has entered the protein. The total amount of radioactive amino acid in the protein should not subsequently be reduced by continued incubation in the presence of an excess of the same cold amino acid.

(ii) Incorporation should be dependent upon the addition of a metabolic energy source.

(iii) Incorporation of the radioactive amino acid should be shown to be in a true peptide linkage in the protein, as seen by its appearance in identifiable peptides upon partial hydrolysis of the protein.

(iv) The amino acid should be located internally within the peptide and not merely in the terminal position.

(v) The amino acid should appear in a single specific isolatable protein of the cell of origin.

The above criteria have seldom all been satisfied in any one particular system and only recently has there been unequivocal demonstration of the latter point.

One of the major problems associated with the use of in vitro amino acid - incorporating systems, especially those in which incorporation is determined as a hot trichloroacetic acid insoluble precipitate, is that of attributing the observed incorporation to the endogenous or supplied mRNA and not to bacterial contamination. This problem becomes particularly acute when working with plant material which may be contaminated with micro-organisms from the surface of the plant. Preparations from animal sources are less likely to be subject to contamination if they are taken from internal organs. Yarwood (1968) has shown that the majority of contaminating

micro-organisms present in homogenates of V. faba cotyledons are sedimented along with the mitochondria by centrifugation at 20,000 x g av. for 30 min.

Many workers have prescribed criteria for the recognition of microbial participation in amino acid - incorporating systems (App and Jagendorf, 1964; Mans and Novelli, 1964; Hall and Cocking, 1966; Allende, 1969; Boulter, 1970; Beevers and Poulson, 1972). In general the criteria are: a dependence upon an exogenously supplied source of energy; a short time course of incorporation; and a sharp Mg^{2+} optimum.

Boulter (1970) states that most cell-free systems are unstable and that amino acid incorporation is completed within 20-30 min or less. Beevers and Poulson (1972) reach a similar conclusion but state that the cause of the limitation of reaction (in the case of Poly(U) direction) is not clear. Allende (1969) cautions that the components of most cell-free systems rapidly become limiting and that if incorporation continues at a good rate after 1.5-2 hrs bacterial contamination should be suspected. Although this appears generally to be a valid criterion several examples have been described of systems in which incorporation continued for such periods of time in the absence of 'significant' amounts of bacteria (Ellis and MacDonald, 1967; Payne, 1970).

The definition of a 'significant' amount of bacteria is necessarily vague and must be made with respect to a specific system. Davis and Cocking (1967) suggest that in incubations with less than 10^3 bacteria/ml, incorporation due to bacteria is negligible. In contrast App and Jagendorf (1964) reported that there was no

linear relationship between levels of bacterial contamination and amino acid incorporation by chloroplast preparations. Davis and Cocking (1967) suggest that different micro-organisms vary greatly in their ability to incorporate amino acids under the conditions of in vitro incubations. Caution must always be exercised in extrapolating from a bacterial count on a plating medium to activity under the in vitro condition. Bacteria vary enormously in their ability to produce colonies on any one medium, and bacterial assays are subject to variations in plating time, temperature and constituents of medium. In order to be at all meaningful the conditions of plating must simulate those of the in vitro incubation monitored.

The most reliable diagnostic tool for the recognition of bacterial participation involves a combination of (a) kinetic time course of incubation, (b) dependence upon added components. A consideration of the kinetics of incorporation should be highly indicative of unwanted contamination. Mans and Novelli (1964) describe the typical biphasic curve of bacterial contamination in which there is little incorporation for the first 60 min compared with a subsequent rapid increase in activity. This is in comparison to that obtained with ribosomal incorporation which proceeds linearly over the first 20 - 60 min and then falls off as the substrate becomes depleted and products accumulate. The time courses for incorporation of $[^{14}\text{C}]$ phenylalanine (Figs. 21 and 25,) are therefore ribosomal time courses rather than bacterial.

Since wild type bacteria (the most likely contaminants in such incubations) have minimal requirements, the greater the dependence of incorporation upon added substances the less likely incorporation

is to be due to microbial contamination. A consideration of the dependence upon added cofactors of both the complete and transfer systems (Tables 11, 12 and 13) indicates that incorporation is unlikely to be due to bacteria. In order to demonstrate the requirements of a particular system it is essential that the ribosome (or microsomes), tRNA and enzyme source are free of contaminating components, for example, contaminating GTP in the enzyme preparation may be supplying the GTP of an apparently non GTP-dependent system. In order to diagnose microbial contamination it is not sufficient to omit one component, a systematic omission of a permutation of components is required before interpretation in terms of incorporation by micro-organisms can be eliminated. Since bacterial contamination in *V. faba* systems has been previously found to be $3 \times 10^3 - 2 \times 10^4$ bacteria/ml (Yarwood, 1968, Payne, 1970) routine plating of aliquots of incubation mixture was not necessary, however in many cases controls experiments were included from which essential components had been omitted and where feasible time courses were constructed.

A portion of the work described here was an attempt to refine a previously used microsomal system (Yarwood, 1968) by purification of the components involved. This system incorporated [^{14}C] phenylalanine into a hot trichloroacetic acid insoluble precipitate (polyphenylalanine) in response to the synthetic messenger Poly(U). The system required the presence of tRNA aminoacylated with [^{14}C] phenylalanine and the remaining 19 [^{12}C]-amino acids and was dependent upon ATP and an ATP generating system (phosphocreatine and phosphocreatine kinase). Amino acid incorporation systems tend to be classified as one of two types: Complete systems in which tRNA is aminoacylated and transferred into peptidyl linkage and the resultant incorporation of

radioactive amino acid into peptidyl form only is assayed (often in the form of hot TCA insoluble precipitate). During the assay method any $[^{14}\text{C}]$ -aminoacyl-tRNA is hydrolysed during the hot acid treatment and here aminoacylation per se is not measured. The so called transfer system measures the transfer of radioactive amino acid from radioactively labelled aminoacyl - tRNA into peptidyl form. The incorporation of amino acid is assayed in a similar manner to that for the complete system, any residual aminoacylated - tRNA being hydrolysed by the hot TCA. The transfer system requires the addition of labelled aminoacyl-tRNA but is independent of ATP or an ATP generating system, there being no requirement for ATP in the steps proceeding tRNA aminoacylation. In order to study the steps involved in the transfer of amino acid from aminoacyl-tRNA into peptide linkage the transfer system is preferable to the complete system since transfer can be studied in the absence of aminoacylation. The aminoacylation of tRNA can be studied in a separate system from which ribosomes and ATP are omitted and in which aminoacylation is measured by the incorporation of radioactive label into a cold TCA insoluble precipitate. The incubation conditions with respect to ionic concentration of these three systems are closely similar and in discussing the dependence of such systems upon added components it is convenient to discuss all three together.

Two basic methods exist for the collection and washing of the TCA insoluble precipitates obtained in such systems. Mans and Novelli (1960) describe a method whereby aliquots of incubation mixture are pipetted on to 2 cm discs of Whatman 3 MM filter paper and air dried. These discs can then be washed with TCA (cold or hot), dried and counted.

However such a treatment involves considerable time expenditure. If discs are to be treated differently, this procedure is cumbersome. The preferable method is that of Nirenberg and Leder (1964) in which the precipitate is filtered, under slight vacuum, through a nitrocellulose Millipore filter of the correct pore size. The precipitate is retained in the pores of the filter and can be washed rapidly. An obvious advantage is that samples can be handled much faster, (the delay between incubation and washing can be less than 1 min). Such a procedure allows different incubations to be washed using different methods. A variety of labile complexes can be retained on such filters which would merely be washed out of filter paper disc. (Witness the use of such filters in investigation of the complexes formed by the bacterial elongation factors T_s , T_u and G, Lucas-Lenard and Lipmann, 1971). Unpublished work from this laboratory has shown that there is no significant difference between identical incubations assayed by the filter paper and Millipore filter technique and hence all the data presented here has been obtained using the latter method. The conditions described by Yarwood (1968) for counting of filter paper discs have been shown to be equally applicable to the Millipore filter discs.

A considerable amount of the data presented here relates to the Poly(U) dependent transfer of $[^{14}\text{C}]$ phenylalanine from yeast $[^{14}\text{C}]$ phenylalanyl-tRNA into peptide linkage in a heterologous system using ribosomes and enzyme prepared from V. faba. The choice of a commercial yeast preparation in preference to the homologous bean tRNA was dictated by the relative scarcity of bean tRNA. Although bean tRNA can be prepared throughout the year from the plumules of germinated beans, this process is extremely time consuming since each plumule

must be dissected individually, otherwise high levels of RNase are encountered and yields of tRNA are low. The developing bean was available for only a limited time, since only field grown material readily fruits and the quantity of tRNA that could be prepared was insufficient for both the experiments involving the characterisation of the amino acid - incorporating systems and those involving resolution of methionyl-tRNA. It seemed, therefore, preferable to conserve bean tRNA for the latter experiments where a heterologous tRNA would not have sufficed.

Yeast tRNA was consistently found to be acylated with $[^{14}\text{C}]$ phenylalanine to a higher degree than was bean tRNA using the same enzyme under identical conditions. From charging experiments the ratio of the specific activity (pmoles/mg tRNA) with respect to $[^{14}\text{C}]$ phenylalanine of bean: yeast tRNA was found to be 29.5:38.0. This is in striking contrast to the results of Liddell (1972) who found a ratio of approximately 188:56. The difference most probably reflects the difference in the enzyme source. Liddell (1972) used a high speed supernatant fraction from Phaseolus aureus as the source of aminoacyl synthetases in contrast to the V. faba preparation used in this work. Liddell investigated the ability of a P. aureus supernatant to catalyse the aminoacylation by six different amino acids of four independent tRNA's and found that for the amino acids tested the acceptor capacities were, on average, in the order germinated V. faba > developing V. faba > P. aureus > yeast tRNA. Data of this type suggests that the difference in amino acid acceptor capacity results not only from variations in the amount of the different tRNA species in a given sample, but may, to considerable extent reflect the integrity of the preparation itself. It seems unlikely that the

acceptor capacity of yeast tRNA for all six amino acids should be inherently less than that of a Vicia faba preparation. There is a considerable degree of variation among the reported levels of aminoacylation by $[^{14}\text{C}]$ phenylalanine of V. faba tRNA preparations prepared simultaneously from the same material under identical conditions by independent investigators (witness the results of Yarwood, 1968; Payne, 1970; Liddell, 1972). In the absence of any absolute value for the acceptor capacity of a particular tRNA preparation it must be concluded that the variation represents not variation in absolute acceptor capacity but variation in the tRNA and enzyme preparations used. Although the aminoacyl synthetases have been demonstrated to exhibit species specificity it would be unwise to evoke such differences as accounting for the above results, since such lack of interchangeability is usually evidence between tRNA from prokaryotic and eukaryotic sources (Hayashi, 1966; Lagerkvist and Waldenström, 1964). Yarwood (1968) has shown complete interchangeability of crude synthetase preparations from V. faba and rabbit reticulocyte. Although a number of workers have recorded lower acylation in heterologous than homologous systems (Moustafa, 1966; Loftfield and Eigner, 1963), it should be stressed that these represent comparisons rather than absolute values.

It would be, perhaps, more pertinent to consider not the degree of interchangeability of the synthetase and tRNA preparations from yeast and V. faba but the extent to which the heterologous tRNA can donate the amino acid into peptide linkage. The ratio of the efficiency of bean:yeast tRNA in donating the accepted amino acid strongly suggests that such differences reflect only differences in the specific activity of the $[^{14}\text{C}]$ phenylalanine tRNA preparations, and the heterologous

system appears to function as efficiently as the homologous. Such an argument is, however, only applicable to the Poly(U) directed system at 'high' (7mM) magnesium concentration, where the need for physiological initiation is bypassed. Substitution of heterologous tRNA under those conditions where 'proper' initiation was required (i.e. low magnesium, in the presence of a messenger RNA carrying initiation codons) may produce a completely artefactual situation with no resemblance to that in vivo.

Prior to its use in the complete system and as a preliminary to the preparation of $[^{14}\text{C}]$ -aminoacyl-tRNA for use in the transfer system, the acceptor capacity of small samples of tRNA was assayed. It was routinely the case that the degree of aminoacylation obtained in a small preliminary incubation was never achieved when the procedure was repeated on a preparative scale, (Table 10). The loss of radioactivity was associated with the reprecipitation of the tRNA following its recovery from the phenol-denatured incubation mixture and at the final washing by reprecipitation. The loss cannot be explained by a loss of tRNA at each step since this would only reduce the yield and not the specific activity of the preparation. Since the fall in aminoacylation cannot be correlated with the length of time for which the aminoacyl-tRNA was maintained as an ethanolic precipitate it is unlikely that the hydrolysis occurs at this step. It seems most likely that the hydrolysis occurs at 0° in 0.1M-potassium acetate prior to reprecipitation or during the phenol deproteinisation of the incubation mixture at room temperature. A similar phenomenon has been reported by Payne, (1968).

The complete system may be regarded as a synthesis of the aminoacylation and transfer systems since both reactions are occurring simultaneously and it may be argued that the optimum conditions for the complete system represent a compromise between those of the aminoacylation and transfer system. It must be assumed that when the parameters governing the aminoacylation reaction are matched by those governing the transfer reaction, that maximum incorporation will be achieved. If however the parameters governing the two reactions are widely divergent then incorporation will be dependent upon a compromise between such parameters. For example, the optimal temperature for both aminoacylation of tRNA^{phe} and for the transfer of tRNA^{phe} into peptide linkage (Figs. 15 and 24) was identical and is reflected in an identical optimum temperature for the complete system. A mutual concession is exemplified by the dissimilar response of the reactions of aminoacylation and transfer to changes in Mg²⁺ (Figs. 17, 22 and 23).

An essential component of all protein synthesising systems is some form of mRNA molecule. mRNA constitutes only a small proportion of all the RNA molecules being synthesised at any time and although it is known to be associated with actively translating ribosomes, polysomes, it has proved to be very elusive, especially from plant materials. Ideally a polysome preparation should require no supplementation with exogenous mRNA, this has rarely proved however to be so. Payne (1970) has demonstrated the almost complete dependence of a *V. faba* amino acid incorporating system upon exogenous Poly(U). mRNA is extremely labile and its extraction from plant polysomes preparations has not proved feasible. There are however, reports of a mRNA isolated from reticulocytes polysomes, however it is only when such mRNA's are shown

to produce a characterisable product that their identity as mRNA is substantiated. Labrie (1969) reports the isolation from animal reticulocyte polysomes of an RNA fraction having the properties of mRNA. Drach and Lingrel (1966) found a reticulocyte RNA fraction stimulated amino acid incorporation in an E. coli system. Characterisation of the product revealed, however, that it was not globin but a bacterial protein. Pemberton et al. (1972) report the isolation and characterisation from duck^c reticulocyte polysomes of the haemoglobin mRNA. This 10S RNA molecule directs the synthesis of the α II chain of duck haemoglobin in a rabbit reticulocyte cell-free system. Similar RNA molecules directing the synthesis of identifiable proteins have been isolated by a number of workers (Laycock and Hunt, 1969; Lockard and Lingrel, 1969; Heywood, 1969; Stavnezer and Huang, 1971; Mathews et al., 1971). It is however notable that there are as yet no confirmed reports of the isolation of a plant mRNA capable of directing the synthesis of a characterisable plant protein. Lonsdale (1972) failed to extract the globulin mRNA from the polysomes of developing cotyledons of V. faba. Even in the presence of RNase inhibitors, there was no evidence of an undegraded RNA species with characteristics expected of such a molecule.

The use of bacteriophage RNA's in bacterial systems has become commonplace (Lodish, 1968; Coolsma and Haselkorn, 1969); in such systems the product has been characterised as viral specific proteins.

A number of workers report the use of plant viral mRNA in bacterial systems (Voorma et al., 1965; Von Ravensway Claasen et al. 1967; Albrecht et al., 1969), however the products lack any resemblance

to or show limited similarity to the authentic coat protein. A mammalian system has now been described which synthesises viral specific polypeptides in response to adenovirus mRNA (Coffier et al., 1971).

Boulter (1970) has observed that the establishment of a natural messenger system is necessary before the understanding of the mechanism of protein synthesis on plant ribosomes is complete. Since plant mRNA's are not a viable proposition, then RNA's derived from viruses which infect plants are regarded as a possible substitute. Liddell (1972) has characterised an in vitro system from P. aureus directed by Turnip Yellow Mosaic virus - RNA (TYMV - RNA), however in view of the host specificity exhibited by TYMV the validity of the TYMV-RNA as a model of a P.aureus mRNA is doubtful.

The use of viral mRNA to elucidate aspects of the mechanism of protein synthesis on eukaryotic ribosomes, although feasible, is not without its difficulties. As has been described in the Introduction, the introduction of a bacteriophage RNA into a bacterial system results in a cessation of the synthesis of the normal host proteins and a preferential association of the bacterial ribosomes with the 'phage RNA. The initiation factors have been implicated in this mRNA specificity. Since the change from host-directed to phage-directed protein synthesis is sensitive to chloramphenicol, it implies that synthesis of new and specific proteins is a prerequisite for the change in mRNA specificity. It seems that in this manner viral mRNA's may be regarded as competitively superior to normal mRNA's and we must therefore ask how far these viral RNA's are in fact comparable to normal mRNA's.

Although the addition of viral mRNA's to plant systems may be attractive, it is uncertain how successful it may prove. A considerable body of evidence suggests that one of the initiation factors is messenger specific. These messenger specific factors are thought to be present on the ribosome and may be involved in initiation of protein synthesis by binding the mRNA's to the ribosome. It would seem therefore that the use of plant viral mRNA's will be most successful in cell-free systems prepared from plants susceptible to the particular viral infection. Because of the presence of a tRNA^{Val} structure within its single RNA chain, TYMV can be amino-acylated by valine. In view of the absence of such structures from normal eukaryotic mRNA's the applicability of viral RNA's as models of cytoplasmic RNA's remains uncertain.

In the absence of a suitable natural mRNA recourse must be made to a synthetic messenger, such as homo - and heteropolynucleotides. In order to interact with the ribosome and perform its template role a template polynucleotide should not possess a perfect secondary structure or a large fraction of stable co-operative helical regions (Spirin and Gavrilova, 1969). Thus, neither the usual native DNA's nor double - stranded viral RNA's nor helical complexes of single polyribonucleotides of the type of the Poly(A)-Poly(U) complex, as well as synthetic polynucleotides with an especially large G content, possess template activity and are generally incapable of combining with ribosomes (Takanami and Okamoto, 1963 a,b; Nirenberg and Matthaei, 1961b; Singer, et al., 1963; Nirenberg et al., 1963). In order to associate with the ribosome, the polynucleotide must possess single-stranded helical regions, and it is important that the 5'-terminal

nucleotide should not be included within the helix. It has been suggested (Spirin and Gavrilova, 1969) that provided the ribosome can attach to a 5'-nonhelical end of a polynucleotide that the translating ribosome can uncoil short helical regions of the polynucleotide during the process of translation.

The most widely used synthetic template has been Poly(U). It is the use of Poly(U) which has elucidated most of the steps involved in peptide chain elongation. However, Poly(U) is limited in the extent to which it is applicable to problems of initiation and termination. Both of these functions are the result of specific codons, other than UUU, which are absent from Poly(U).

It is now accepted that initiation in vivo involves the specific binding, at low magnesium and in the presence of specific initiation factors, of an initiator tRNA to an initiation codon (AUG, or less probably GUG). Although Lucas-Lenard and Lipmann (1967) have demonstrated a specific initiation mechanism operative with Poly(U) at low magnesium which involves the binding of an initiating aminoacyl - tRNA such as N-acetyl-phenylalanyl-tRNA, this is now recognised as a completely artefactual situation and has little relevance to the process in vivo. App and Barton (1965) report that streptomycin inhibits the in vitro Poly(U) - directed incorporation of phenylalanine by rice embryo ribosomes, whereas the polysome incorporation, using endogenous messenger, was considerably more streptomycin resistant suggesting that caution should be exercised in accepting Poly(U) as an authentic model for natural rice mRNA.

Moore (1966), studying the binding of Poly(U) to E.coli ribosomes found that the formation of polysomes depends on the ratio of Poly(U):

ribosomes. When ribosomes were present in molar excess over Poly(U), polysome complexes were formed, but when Poly(U) was in excess it complexed with single ribosomes. In the present work Poly(U) has been added in excess, hence the Poly(U) would be expected to be associated with the monosome peak. Using isokinetic gradients it has in fact been demonstrated that the radioactivity present in a Vicia faba Poly(U) - dependent microsomal amino acid incorporating system is associated with the monosome fraction. (Payne et al., 1971).

A lag phase is reported to be characteristic of Poly(U) dependent systems (Nakamoto et al., 1963; Allende et al., 1964; Nishizuka and Lipmann, 1966b). However Figs. 21 and 25 reveal the complete absence of a lag phase in either the Poly(U) dependent complete or transfer systems. The absence of such a lag phase may be easily explained on the basis that insufficient samples were assayed at different times in the initial phase of reaction, thus by graphically connecting the point at zero time to that of the first sampling interval, an apparently linear response was obtained. Similar results have been reported for other V. faba amino acid incorporating systems (Yarwood, 1968; Payne, 1970). Both these workers found the incorporation proceeded linearly after 5 min of incubation, suggesting that the duration of the lag phase in these systems was less than 5 min.

A lag phase is a feature of the in vitro systems in which the template polynucleotide lacks a specific initiating codon, it is precisely the initiation step which, in such systems is rate limiting. That initiation does occur is evident from the activity of such systems, but in the absence of a precise initiation mechanism the process becomes

random and the statistical probability of such an event becomes so low as to become rate-limiting. Since the process of initiation involves the entry of a specific methionyl-tRNA (or an analogue of it) into the peptidyl (P) site, be it directly or via the A site, it has been suggested that since the usual aminoacyl - tRNA's are not retained in this site, it is the frequency of this rare event which is rate-limiting. Nakamoto and Kolakofsky (1966) obviated the lag phase in a Poly(U) system by exogenous supplementation of a dipeptidyl - tRNA, an analogue, of the initiator tRNA.

The absence of any significant lag phase in the aminoacylation reaction (Fig.16) may be due solely to the frequency of sampling but there is no apparent reason why such a lag phase of the type described for the amino acid incorporating systems, should exist. Given the presence of enzyme (i.e. synthetase) and substrates there should be no significant delay in the instigation of reaction. However, Loftfield (1972) has noted that the addition of substrates to the synthetase is a random process dependent only upon the concentration and K_d 's of the substrates, and under conditions where all parameters are optimal the lag phase will be reduced to a minimum.

The absence of any significant microbial contribution to incorporation measured in either aminoacylation, transfer or complete system, is suggested by the linearity of the incorporation over the first 30 min (15 in the case of aminoacylation) and the subsequent levelling off of incorporation over periods of up to 120 min. The initial linearity and subsequent levelling off in the rate of incorporation in all three systems indicates that some change in the incubation condition has occurred such that the incorporation of radioactivity is no longer favoured. Three possible alternatives exist

to explain this phenomenon (a) exhaustion of substrates;
(b) accumulation of inhibitory components; (c) an inherent
instability in the components of the incubation following incubation
for extended periods of time at the temperature of incubation.
The most significant contribution to the decrease in activity is
probably the depletion of the components of incubation mixture,
Coleman (1969) using a 'dynamic' system, in which the concentrations
of ATP, GTP and amino acids were maintained essentially constant,
attributed a decrease in activity after 20 min to the depletion
of the polysome supply. Since ⁱⁿthe 'dynamic' system low mol.wt.
degradation products were continually removed from the incubation
mixture by the occlusion within the pores of the gel, it is unlikely
that the tailing off in activity observed in such systems is
significantly due to the accumulation of degradation compounds.
The fact that the instability of the Poly(U) dependent amino-acid
incorporating system during the period of incubation is a function
of the temperature is indicated by the form of the time course at
different temperatures (Figs. 21 and 25). Although at 30°, the
optimum temperature for Poly(U) dependent incorporation, the initial
rate of reaction was lower than that at 37°, the incorporation
continued in a linear fashion for a longer period and a higher level
of incorporation was achieved suggesting that at temperatures above
30° the falling off in incorporation results not only from depletion
of the substrates but from an inherent instability of the system at
this temperature. If the system was as stable at 37° as at 30°, an
equal level of incorporation should have been attained but within a
shorter time interval. It would seem justifiable therefore, to assume
that the levelling off at 30° results predominantly from a depletion

of the components of the incubation mixture whilst at temperatures above the optimum instability as a result ^{of} degradative changes become significant. Although both aminoacylation and Poly(U) dependent amino acid incorporation exhibited temperature optima (Figs. 15, 20, 24) of 30°, there is a subtle distinction between the temperature dependence of these systems. In all three systems there was very little activity below 5° and incubation at temperatures above 30° resulted in a decrease in the total activity of the system. However the activities of the aminoacylation and complete systems were relatively independent of temperature between 20° and 30°. At 20° the activities of the aminoacylation and complete systems were respectively 82.5% and 90% of their value at 30°, whilst the transfer system exhibited a greater degree of temperature dependence, achieving at 20° only 55% of the activity obtained at 30°. The activity of the complete system appears, in this instance, to be limited by the aminoacylation reaction rather than the transfer reaction.

The temperature dependent increase in the velocity of an enzyme reaction may be mediated by a variety of factors, such as an effect on the rate of formation of the enzyme-substrate complex; an effect on pH due to an alteration of the pKa of one or more components, or to a change in the pH of the buffer. The complexity of the systems used excludes any prediction of the precise steps which are temperature dependent. However the form of the time course curves at varying temperatures are precisely those predicted by Dixon and Webb (1958) for a system in which an increase in the rate of reaction at increased temperature is counterbalanced by decreased stability.

Since the binding of poly(U) to ribosomes at high magnesium concentration occurs non-enzymatically (Spirin and Gavrilova, 1969) it would appear to be relatively temperature insensitive. Krahn and Paranchynch (1970) have shown that the binding of Poly(U) to E. coli ribosomes occurs more efficiently at the critical temperature at which the ordered helical state is converted to a random coil. In the absence of significant nuclease activity the affinity of Poly(U) for E. coli ribosomes is greater at 10⁰, more than five times greater than at 37⁰. Since the binding of Poly(U) was completed in 30 sec the authors conclude that at incubation temperature above 30⁰ binding at Poly(U) to ribosomes is not a rate limiting parameter for temperature optima of polypeptide synthesis.

In Poly(U) dependent systems a factor which may contribute to the temperature optimum is the statistically random insertion within the P site, of the first phenylalanyl-tRNA molecule. McLaughlin et al. (1966) have recorded that the formation of a ternary complex involving ribosome-template-aminoacyl-tRNA stabilises the association of the ribosome and template. This reaction has been shown to be favoured by increasing temperature, at 37⁰ the binding of aminoacyl-tRNA is double that at 24⁰, at 15⁰ it is many times lower than at 24⁰, whilst at temperatures of 0-5⁰ such binding is difficult to observe. After the insertion of the first aminoacyl-tRNA into the P site the remaining reactions of protein synthesis are enzymatic and therefore show considerable temperature dependence.

The counterbalancing instability introduced into the systems by increasing temperature results from an inactivation of the components at temperatures in excess of some optimum. The temperature stability of the various labile components involved in the system will

vary and the optimum temperature for incorporation reflects the sum total of all the temperature sensitive reactions involved.

The integrity of the ribosomes is maintained by a variety of weak forces and is profoundly temperature dependent. Bodley (1969) reported denaturation of E. coli ribosomes with increasing temperature, whilst numerous investigators have implicated mRNA breakdown at increased temperatures as one of the causes of instability in their in vitro systems (Schiebel et al., 1969; Byfield and Scherbaum, 1966; 1967a, b). Mangiantini et al. (1965) and Friedman (1968) have suggested that the optimum temperature of amino acid incorporation reflects the temperature at which an organism lives and that the thermostability of the ribosome may be a rate-limiting factor in determining the upper growth temperature of an organism.

It is unlikely that ribosomal thermostability alone is the prime determinant of the optimum temperature of amino acid incorporation systems. Payne (1970) working with essential similar systems to those described here, but using developing V. faba tRNA, consistently found temperature optima of 25^o, suggesting that to some extent the thermostability of such systems is a function of the tRNA source.

Sarin et al. (1966) have demonstrated several changes in the physical properties of the tRNA between 35-40^o, these changes are attributed to an unfolding of the tertiary structure and it is likely that the sharp drop in aminoacylation exhibited at temperatures above 30^o was due to conformational changes in the tRNA molecule. The temperature at which tRNA denaturation occurs is highly variable and Novelli (1967) concluded that different tRNA's can be denatured under a variety of conditions.

The instability of the system must also to an extent reflect the instability of the many enzymes involved. The temperature instability of wheat embryo transfer factors has been described by Allende (1969). Incubation at 37° for 10 min resulted in a 90% decrease in activity. Despite some protection offered by sulphhydryl compounds, incubation for 10 min in the presence of glutathione resulted in a 75% fall in activity.

The aminoacylation of yeast tRNA^{phe} has been shown by Liddell (1972) to be independent of pH over the range pH7.4-8.9. Since similar results have been obtained by other workers the effect of pH upon the aminoacylation reaction was not intensively investigated. Innumerable workers have reported that the pH optima of the aminoacyl-tRNA synthetases lie towards the alkaline side of neutrality (Mans, 1967). The aminoacyl-tRNA ester linkage becomes labile at high pH, hence the practice of carrying out deacylation of pH8.9 in solutions of high ionic strength (2M-Tris).

There was a striking similarity between the pH dependence of the complete and transfer systems. Since the aminoacylation of tRNA^{phe} is relatively independent of pH in the range pH7.4 - 8.9, the decrease in activity of both systems with increasing pH would suggest that this is due to an effect of pH on reactions proceeding the aminoacylation.

A pH optimum is characteristic of most enzyme reactions and may be due to (a) a true reversible effect on the velocity of reaction, (b) an effect of the pH on the affinity of the enzyme for its substrate, (c) an effect of pH on the stability of the enzyme, which may become irreversibly destroyed on one or both sides of the optimum. Since

the system studied may be regarded as a multi-enzyme system it is difficult to determine exactly which enzymes are sensitive to pH in the region employed and in what way pH changes effect the activity of the enzyme. Since the pH curves are not symmetrical it is unlikely that the optimum reflects the activity of a single enzyme which exists in only one active state. In such a case the ionisation of two particular groups in the active centre will largely determine the activity, namely those which ionise first as the pH moves away from the optimum on the acid and alkaline sides respectively. McKeehan and Hardesty (1969) report that purified rabbit reticulocyte TFI (i.e. binding enzyme) exhibited a broad pH optimum between pH6 and pH10, suggesting the involvement of functional groups with pKa's of approximately 5.5 and 9.5. Maden and Monro (1968) found that the activity of E. coli peptidyl-transferase was maximal over the range pH8.5 - 9.5 and from pH8.5 there was a rapid fall off in rate. It is most probable that the pH optimum observed in both complete and transfer systems reflects not the optimum for a single enzyme but represents a mutual concession among all the enzymes involved in peptide chain elongation.

The pH optimum of 7.6 reported here is in striking conflict to a report by Heredia and Halvorson (1966). Using a transfer system from the yeast hybrid Saccharomyces fragilis~~X~~Saccharomyces dobzanskii these workers found a pH optimum at pH6.5 at 20°. These workers attribute the low pH optimum to an instability of yeast phenylanyl-tRNA at alkaline pH. Sarin and Zamecnick (1964) have similarly reported that at pH7.5 and 35°, a rapid spontaneous deacylation of

phenylalanyl-tRNA occurs which is accelerated by Tris. The difference in pH stability of the yeast tRNA used in this study must be attributed to specific differences. The tRNA used being isolated from the yeast S. cerevisiae. Although the pH stability of the aminoacyl linkage was not investigated in this system it is most unlikely, in view of the pH dependence of both complete and transfer systems, that the yeast tRNA used here was unstable in the region of pH7.5.

The aminoacylation, complete and transfer systems exhibited a marked dependence upon added Mg^{2+} (Figs. 17, 22 and 23). In all cases the Mg^{2+} concentration of the incubation is the concentration of exogenously supplied magnesium and is not corrected for Mg^{2+} present in the particle or enzyme preparations which was assumed to be negligible, in comparison. The striking difference between the Mg^{2+} dependence of the complete and transfer systems represents a further example of a concession between the aminoacylation and transfer reactions. Since the Mg^{2+} requirement of the transfer system is much lower than that of the complete system it can be concluded that the additional Mg^{2+} required by the complete system is involved in the aminoacylation reactions and the reactions proceeding aminoacylation have a lower requirement for Mg^{2+} . The pronounced optimum exhibited at 7mM for the transfer system contrasts with the much less sharp optimum for aminoacylation and complete systems. In the case of the transfer reactions Mg^{2+} concentrations above 14mM are inhibitory, whereas addition of magnesium beyond an optimum value (up to 20mM) did not significantly inhibit either the aminoacylation or complete

systems. Allende (1969) reports a similar reduction in Mg^{2+} requirement between complete and transfer systems from wheat embryo. Igarashi and Paranchynch (1967) record a reduction in the magnesium requirement between E. coli Poly(U) directed complete and transfer systems. These authors investigated Mg^{2+} dependence in the range 2-50mM for the complete system and found that Mg^{2+} concentrations in excess of 20mM were inhibitory. Since aminoacylation was independent of Mg^{2+} in the range 8-24mM, it is likely that the inhibition of the complete system above 20mM results from an inhibition of the transfer reaction at such concentrations.

Figure 23 indicates that the magnesium requirement for the transfer reaction was not influenced significantly by the type of particle preparation used, suggesting that the contaminating Mg^{2+} present in such preparations is either (i) negligible, or (ii) identical. Since both microsomal and ammonium chloride preparations have the same optimum it is likely that the only significantly Mg^{2+} associated with the particle preparations is that intimately involved in maintaining the structural integrity of the ribosome.

Using a V. faba tRNA preparation Liddell (1972) and Payne (1970) report a Mg^{2+} optimum of 10mM for the V. faba complete system indicating that the Mg^{2+} requirements of aminoacylation are to some extent dependent upon the tRNA source. Boulter (1970) summarising the Mg^{2+} requirement of 10 plant systems concludes that the various Mg^{2+} optima reported are in part a reflection of the different Mg^{2+} levels used in the preparation of ribosomes and enzyme fraction and subsequent storage since ribosomes are known to absorb Mg^{2+} during isolation and storage.

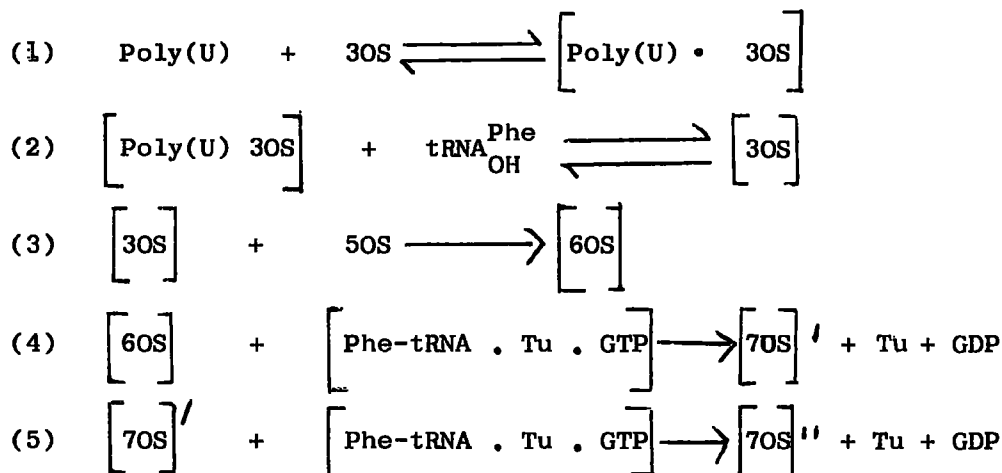
As with all enzyme utilising ATP, the aminoacyl synthetases require Mg^{2+} (Loftfield, 1972). ATP takes part in the aminoacylation reactions in the form of its magnesium chelate. Loftfield suggests that the occasionally observed inhibition of the ATP:PPi exchange reaction by Mg^{2+} results from decreased concentrations of $MgP_2O_7^{2-}$ and increased concentrations of $Mg_2P_2O_7$. In some instances the Mg^{2+} requirement for aminoacylation can be replaced by spermine (Takeda and Igarashi, 1970).

Magnesium has been shown to be involved in maintaining the integrity of the ribosome (Tissieres and Watson, 1958), and in binding mRNA to the ribosome (Moore and Asano, 1966). Ravel and Shorey (1969) have described the stabilisation of the Tu-GTP-aminoacyl-tRNA complex by Mg^{2+} . In the absence of Mg^{2+} , GTP and aminoacyl-tRNA are shown to dissociate from the complex, hence the participation of Mg^{2+} in aminoacyl-tRNA binding. Ayuso and Heredia (1968) failed to detect a Mg^{2+} optimum for enzymatic binding of phenylalanyl-tRNA to yeast ribosomes, but binding below 10mM was dependent upon a supernatant factor and GTP whilst this dependence was largely overcome by increasing the magnesium concentration to 20mM. A similar result has been demonstrated in this laboratory, (unpublished data) using V. faba enzyme fractions. Lin et al., (1969) report a sharp magnesium optimum of 8mM for the GTPase activity of rabbit reticulocyte TF1.

Levels of approximately 10mM Mg^{2+} are commonly reported for the transfer and complete systems for eukaryotes whilst prokaryotic systems require approximately 15-20mM. These levels of Mg^{2+} are generally referred to as 'high' when compared to those required for the translation of natural mRNA molecules containing specific

initiator codons. Under conditions of 'high' magnesium initiation proceeds via a non physiological mechanism. (Lengyel and Söll, 1969). The 'high' magnesium levels in systems utilising synthetic messages has been shown to be required only at the initiation step for rapid passage through the lag phase and once translation is proceeding the Mg^{2+} concentration can be reduced to 7-10mM in prokaryotic systems (Revel and Hiatt, 1965; Nakamoto and Kolakofsky, 1966). The requirement for 'high' magnesium has been implicated in the stabilisation of the ternary complex of ribosome - template - aminoacyl - tRNA and in increasing the affinity of the P site for the aminoacyl - tRNA. Thus the probability of an appropriate neighbouring arrangement of the two aminoacyl - tRNA's on the ribosome is increased and the first peptide bond can be formed. Analogues of the initiator tRNA can serve as chain initiators under conditions of low magnesium. Igarashi (1970) has demonstrated that peptidyl-tRNA^{Phe} can initiate Poly(U) dependent amino acid incorporation at 5mM in an E. coli system. This author also showed that the addition of modified aminoacyl-tRNA's at 'high' magnesium to such a system was inhibitory. Nakamoto and Kalakofsky (1966) obviated the lag phase in a Poly(U) system by the use of the diphenylalanyl-tRNA, whilst/^{Lucas-}Lenard and Lipmann (1967) showed that N-acetylphenylalanyl-tRNA had a similar effect. A shift in the magnesium optimum for phenylalanine polymerisation from 7mM to 4mM has been achieved by pre-incubation of V. faba ribosomes and Poly(U) in the presence of deacylated tRNA (Yarwood et al., 1970). A similar result has been reported by Mosteller et al. (1968) for a rabbit reticulocyte system. Such results have been explained by Schreder and Noll

(1970) on the basis of what is postulated to be a primitive initiation mechanism. These authors suggest that under conditions of high magnesium, in the presence of deacylated tRNA, initiation proceeds by a series of events represented as



In this case, initiation is considered as a special case of chain elongation and must start with a translocation, with deacylated tRNA occupying the P site of the 50S subunit. For mRNA lacking an initiation triplet, AUG, the initiation complex is formed by interaction of the 30S subunit, the 5' terminal codon, (UUU in the case of Poly(U)), and the cognate deacylated tRNA. Addition of the 50S subunit results in the formation of a 60S particle. The deacylated tRNA stabilises the interaction between the mRNA and 30S subunit and by suitable conformational changes aligns the 50S particle with the 30S particle to form the 60S complex. In the following step (e.g. 4) the GTP dependent binding of the binding complex Phe-tRNA . Tu . GTP catalytically converts the 60S complex into a 70S complex. The addition of the final Phe-tRNA molecule necessary for peptide bond formation, is postulated by these authors to be into a third ribosomal site, such that translocation occurs before peptide bond formation and advances the

two Phe-tRNA molecules into the reactive P and A sites. These workers present substantial evidence in favour of such a reaction and it is most probable that a reaction sequence of this type is involved in initiation at "high" Mg^{2+} . The observed 'shift' in Mg^{2+} optimum following pre-incubation in both the V. faba and reticulocytes systems is readily explained on the basis of such initiation occurring at high magnesium during the pre-incubation which then allows translation to proceed at low Mg^{2+} .

Only a partial GTP dependence could be demonstrated for both the complete and transfer systems (Tables 11, 12). Using similar systems Liddell (1972) and Payne (1970) report no significant dependence upon GTP, whilst Allende (1969) using wheat embryo systems reports a 64% and 90% dependence in the complete and transfer systems. In view of the absolute requirement for GTP in aminoacyl-tRNA binding and in translocation, the relative independence of this system is merely a reflection of the degree of contaminating GTP present in the particle and enzyme fractions. Allende (1969) reports the GTP independence of apparently sterile chloroplast and mitochondrial amino acid incorporating systems.

In view of the absolute necessity for ATP in the aminoacylation reaction and its lack of function in the transfer reaction, the ATP dependence of these systems was not investigated. The complete system showed a considerable dependence upon added ATP since in the absence of ATP and ATP generating system the activity fell to 1% of its value in the complete system. However the 17% activity obtained in the absence of ATP but in the presence of the ATP generating system implies that the ATP regenerating system was, in

part, able to supply the systems need for ATP. Such a phenomenon has been reported by a variety of workers (Van K mmen, 1967, Allende, 1969, Liddell, 1972). Liddell (1972) using a Poly(U) dependent system from P. aureus found only partial dependence upon ATP and an ATP generating system with V. faba and P. aureus tRNA's whilst a complete dependence was demonstrable with yeast tRNA. The author concludes this was due to contaminating ATP in the tRNA preparations. Step-henson and Zamecnik (1961) present data consistent with such a suggestion. These workers demonstrated that ATP co-precipitated with tRNA during ethanolic precipitation and could be detected by the ratio of absorption at 260nm:280nm. A ratio of greater than 2 being diagnostic of contaminating ATP. This can be removed by dialysis.

The high energy compound, creatine phosphate is included along with the enzyme, phosphocreatine kinase (ATP-creatine transphosphorylase) as an ATP generating system. During activation AMP is formed which is phosphorylated to yield ATP. An anomaly exists here since AMP is not a substrate for phosphocreatine kinase. It is likely that some form of myokinase activity is involved in a transphosphorylation reaction between ATP and AMP to form two molecules of ADP which can accept a phosphate group from creatine phosphate.

The dependence of the transfer system on the presence of a sulphydryl compound (DTT) was almost complete whilst the complete system showed a 42%^{dependence} upon DTT. In view of the requirement of the synthetase enzymes for - SH groups the marked difference in DTT dependence is inexplicable. It may be that aminoacylation is less sensitive to the omission of sulphydryl compounds. The transfer

factors and the peptidyl-transferase have a well documented requirement for - SH groups. The requirement for sulphhydryl compounds in both aminoacylation and amino acid incorporating systems is basically to buffer the essential - SH groups of the enzymes against changes resulting from auto-oxidation reactions. Early work performed in the course of this investigation used 2-mercaptoethanol or reduced glutathione (GSH) as the sulphhydryl reagent, however in view of the unpredictability of such compounds, the more powerful sulphhydryl compound, dithiothreitol was used. Skogerson and Moldave (1968) report the stimulating effect of DTT upon TF1 and TF2 activity.

The small, but nevertheless significant, incorporation obtained in the complete system, in the absence of added tRNA and enzyme using V. faba microsomes is indicative of some contamination present in the microsome preparations. It is significant that the contamination by tRNA was routinely found to be greater than by enzyme.

When an in vitro system is characterised it is necessary that certain parameters be arbitrarily fixed and the other parameters varied to find the optimum conditions relevant to those fixed parameters. Such fixed parameters in the amino acid incorporating systems described were the ribosome and tRNA concentrations. The ribosome concentration was fixed at 1mg ribosomes/ml, the concentration being determined on the basis of $E_{1\text{cm}}^{260}$. The tRNA concentration of the complete system was fixed at 0.4mg deacylated tRNA/ml incubation and the other parameters varied to yield maximum amino acid incorporation under these conditions. Similarly for the transfer system, Fig.26 indicates that under the conditions described maximum incorporation was promoted by the addition of approximately 0.4mg/ml of $[^{14}\text{C}]$ phenylalanyl-tRNA.

The slight decrease in incorporation in the presence of excess aminoacyl-tRNA probably results from sub-optimal conditions following a variation in the incubation composition.

The dependence of aminoacylation upon added enzyme fraction was almost complete, (Fig.18) indicating the lack of contaminating synthetases in the yeast tRNA preparation. Addition of excess enzyme did not result in decreased incorporation suggesting that under the conditions of aminoacylation there was no significant nuclease activity. The absence of significant protease activity under the conditions employed in the transfer and complete systems can be seen from figures 21 and 25, since prolonged incubation did not result in a decreased recovery of incorporated label. Although the protein concentration of the DE52 enzyme was routinely determined it was not found to be a reliable guide to the volume required for maximum aminoacylation or amino acid incorporation. Enzyme concentration curves were prepared for all enzyme fractions, and the volume promoting maximum amino acid incorporation was used in all subsequent work.

The complete and transfer systems both showed a complete dependence upon the synthetic messenger, Poly(U) hence it was routinely added to incubations.

It is customary to express the activity of in vitro systems in terms of pMoles of radioactivity incorporated/mg rRNA. In the absence of any standard incubation time it becomes difficult to compare the activities of various systems. The maximum level of incorporation achieved in the complete system was approximately

500 pMoles phenylalanine/mgrRNA in a 20 min incubation. This is in accordance with values reported by Payne (1970) and compares favourably with levels of 160 and 100 pMoles phenylalanine/mgrRNA achieved in wheat and carrot root ribosomal systems (Allende and Bravo, 1966; Leaver and Key, 1967).

The lower activity of the transfer system (Table 6) probably reflects the fact that the concentration of phenylalanyl-tRNA soon becomes limiting. In those incubations in which maximum incorporation was achieved (approx. 150 pMoles phenylalanine/mgrRNA) 80% of the radioactive counts added as $\left[^{14}\text{C}\right]$ phenylalanyl-tRNA were recovered in the hot TCA insoluble precipitate. The considerably greater levels achieved in the complete system result from the regeneration of aminoacyl-tRNA during the incubation.

Although comparisons of the level of incorporation achieved by in vitro systems may be useful they are relatively meaningless since the protein synthetic activities of the tissues from which they were derived vary greater. It is, therefore, of more relevance to compare the rates achieved in vitro with the in vivo rates. Payne (1968) has ⁰calculated that, on average, the in vivo rate of storage protein synthesis in the developing cotyledon of V. faba is approximately $112/\mu\text{g.protein/mgrRNA/hr}$. Poly(U) dependent incorporation of phenylalanine in V. faba complete system using a bean enzyme fraction was 500 pMoles/mgrRNA/20min. This was equivalent to about $0.25/\mu\text{g}$ phenylalanine incorporated/mgrRNA/hr. Indicating that the in vitro is well below 1% of the in vivo rate.

The low levels of incorporation in in vitro amino acid incorporating systems undoubtedly results from massive degradative

changes associated with the removal of extremely labile components from a precise spatial relationship within the cell and their mixing together in hostile conditions in the test tube. The introduction of less drastic extraction techniques will no doubt facilitate the preparation of in vitro systems with considerably enhanced rates of reaction. The rabbit reticulocyte lysate system used by Legon et al. (1973) synthesised protein at a rate comparable with that in vivo for a period of up to 1 hr, attesting to the extreme mildness of such an extraction technique. Unfortunately the presence of relatively impermeable cell walls limits the application of such a technique to the multicellular plants.

For reasons previously described it was most convenient to use yeast tRNA in the amino acid incorporating systems described here, and as also explained, it was completely interchangeable with its V. faba counterpart with a difference in activity which was a reflection only of the difference in specific activities of the two preparations with respect to phenylalanine acceptor capacity.

Since the enzyme fraction required for amino acid incorporation was routinely obtained from developing beans, and the ribosomes were obtained from either developing or germinated beans it was necessary to verify the interchangeability of these components. The data presented in Table 7 demonstrates the complete interchangeability of tRNA and enzyme fraction from developing and germinated beans with Triton X100 ribosomes from germinated beans. Yarwood (1968) demonstrated a similar interchangeability of the components of the V. faba and rabbit reticulocyte amino acid incorporating systems.

The interchangeability of the components of prokaryotic and eukaryotic amino acid incorporating systems has been reviewed by Ciferri and Parisi (1970), these authors conclude that there is almost complete interchangeability of components from systems with the same basic ribosome type (i.e. 70S or 80S). In view of which the complete interchangeability of the developing and germinated tRNA is unremarkable. Payne (1970) has shown that the in vitro activity of ribosome, tRNA and enzyme fractions from developing cotyledons of V. faba is dependent upon the age of the seed. In all three cases, preparations from 60 day beans showed maximum activity. This was correlated with the maximum rate of storage protein formation in vivo. In view of such a finding, a discrepancy might have been expected between the activity of preparations from developing and germinated beans. The lack of difference in the activity of the tRNA suggests that there are no significant changes in the activity of tRNA^{Phe} species between germination and subsequent seed development. Since only Poly(U) dependent phenylalanine incorporation was measured it would be unwise to assume that the whole spectrum of tRNA species remains unaltered. The approximately identical acceptor capacity of tRNA prepared from the two sources is a further indication that no dramatic change in the tRNA^{Phe} species occurs between germination and fruition.

The almost identical activity of the enzyme fraction from developing and germinated beans may be artefactual. Unlike the high speed supernatant fraction used by Payne (1970) the enzyme preparation used here has been passed through a DEAE-cellulose column to remove

tRNA. In all cases the volume of enzyme promoting maximum incorporation was used. This may have masked differences in the amounts of the enzymes involved in protein synthesis in the supernatant which would have been revealed if a specific amount of protein had been added to the incubation. It is likely that changes in the specific activities of the enzymes involved would have been revealed had the enzyme fractions been resolved into the various enzymes required for protein synthesis.

A comparison of the activities of various types of particles from germinated and developing seeds in the Poly(U) dependent complete and transfer systems (Table 6) is again indicative of the fact that the activity of the complete system more strongly reflects the aminoacylation than the transfer functions involved. The greater activity of particle preparations from germinated beans than from developing beans in the transfer system is independent of the method of ribosome preparation. There is no simple unequivocal explanation of the difference in activities. The fact that the difference in activity is independent of preparative method would seem to suggest that it is a true function of the ribosomes rather than an artefact of preparative methods. More relevant data may have been obtained from a comparison of the activities of both types of preparation with endogenous mRNA. In the absence of appreciable levels of incorporation directed by endogenous mRNA, it would have been pertinent to fractionate preparations actively engaged in Poly(U) dependent peptide synthesis on sucrose density gradients in order to investigate which components of the preparation were involved. It is likely that in the preparations from germinated

beans there is a higher proportion of free 80S type ribosomes to which Poly(U) can bind than in preparations from developing beans where a significant proportion of the particles may contain mRNA (intact or degraded) and are thus unable to form a complex with Poly(U). If such was the explanation one might expect significantly greater levels of incorporation in response to endogenous mRNA. Data from this laboratory (unpublished) suggests this may be so but the levels of incorporation achieved in each case are so low as to be almost insignificant.

One of the aims of this work, was as described previously, to refine an existing crude microsomal system in order to study the mechanism of interaction of the components involved in protein synthesis. Prior to the resolution of the transfer factors present in the supernatant it was necessary to establish a well defined ribosomal system in which incorporation was completely dependent upon added enzymes. At the same time it was desirable to achieve a system in which appreciable levels of incorporation were obtained. A transfer system is the ideal system in which to study the interaction of the transfer enzymes and to routinely assay fractions obtained during the course of their purification. In view of the heterogeneity of the E.R. it was felt that a microsomal system was not the ideal system for such work, despite the almost complete dependence upon added enzyme shown by washed microsomes from 3 day germinated beans. (Table 9). The increase in enzyme dependence, in the absence of a significant decrease in activity, of the Triton X100 ribosomes suggests that this procedure had removed some of the contaminating transfer factors associated with the ribosome without appreciably affecting ribosomal integrity. The almost identical response of

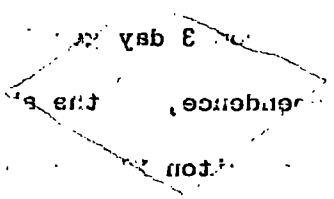
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unwashed microsomes and Triton X100 ribosomes to rabbit reticulocyte 40/70 enzyme indicates the functional similarity of these two preparations.

Many investigators have reported the use of the bile salt, sodium deoxycholate (DOC) in solubilising microsomal membranes (see Von der Decken, 1967). Payne (1968) reports the complete solubilisation of V. faba microsomal membranes at 0.5%^(w/v)DOC. However because of the drastic fall in activity following the use of this detergent it was not adopted as a routine procedure. Lonsdale (1972) has attributed the loss of activity following DOC treatment to ribosomal denaturation. The destruction of ribosomes by DOC has been previously noted by Burka (1967) and Golub and Clegg (1969), and is attributed to denaturation and loss of ribosomal proteins.

Washing of Triton X100 ribosomes, to remove transfer factors with 0.5M-ammonium chloride was also accompanied by a considerable decrease in activity, since this procedure has, however, been shown to remove transfer factors from rat liver ribosomes without significantly affecting ribosome integrity (Moldave and Skogerson, 1967) it was used as a routine procedure prior to the assay of transfer factor containing fractions. Use of this procedure reduced the absolute incorporation in ^{the} absence of added enzyme to a low level but because of the low total activity of such preparations the % dependence upon added enzyme was less than for other preparative methods.

The data in Table 8 demonstrating the differential response of the various preparations to the addition of reticulocyte 40/70 enzyme fraction suggests that some factor removed by the ammonium chloride and DOC procedures, and essential for the activity of the system, is present in sub-optimal amounts in the bean dialysed enzyme fraction, hence the stimulation by 40/70 enzyme. If this factor is regarded as sub-optimal (or unstable) in the bean enzyme, then the less pronounced enhancement by reticulocyte 40/70 enzyme of the activity of microsomal or Triton X100 preparations suggests that this factor must be present on ribosomes prepared by these methods. Its apparent presence on these ribosomes may be indicative of its stabilisation when associated with the ribosome. For reasons to be discussed later it is suggested that this factor which is limiting in the bean enzyme preparation and which is removed from the ribosome during ammonium chloride and DOC washing, is the binding enzyme which is involved in the enzymatic binding of the aminoacyl-tRNA to the A site on the ribosome.

The V. faba amino acid incorporating system described by Yarwood (1968) used a 105,000 x g. supernatant as a source of the enzymes involved in protein synthesis. Using this enzyme only low levels of aminoacylation could be achieved, and it was necessary to use reticulocyte 40/70 enzyme fraction to aminoacylate the bean tRNA. Incorporation of a -SH reagent into the ribosome extractant, and subsequent dialysis of the supernatant against a buffer, containing -SH reagent and removal of precipitated globulin resulted in a considerable increase in activity. This may have resulted from the removal of large quantities of sucrose and other small molecules. It is noteworthy that dialysis resulted in the

elimination of a considerable number of ninhydrin positive substances (as determined by thin layer chromatography), suggesting its effect in reducing the endogenous amino acid content of the supernatant. Dialysis of the supernatant resulted in a 350% increase in activity in the transfer system indicating that diffusible substances present in the supernatant had also been inhibitory to the transfer reactions.

Passage of the supernatant through a G25 column had a similar effect upon the activities of the enzymes involved in both aminoacylation and transfer reactions, again implicating the removal of small molecules in increasing the activity of these enzymes.

In order to study the transfer reactions, and more especially to assay the resolution of methionyl-tRNA's a supernatant free of contaminating tRNA was required. This was achieved by passage of dialysed or G25 supernatant through a DEAE-cellulose column. The activity of this preparation remained essentially the same as that of dialysed and G25 supernatants (Table 14) but attempts to aminoacylate the supernatant in the absence of added tRNA were unsuccessful, indicating the complete absence of contaminating tRNA.

Passage of the supernatant through DEAE-cellulose often resulted in considerable dilution and the volume of the supernatant required for maximum aminoacylation or transfer was often inconveniently large. It was necessary to increase the concentration of this supernatant, by one of two readily available methods. Concentration by ultrafiltration resulted in no significant decrease in activity whilst concentration by precipitation with ammonium

sulphate, despite considerable care in its execution, was routinely accompanied by a 40% decrease in the activity of the supernatant (Table 14). It must be assumed that this was due to some degree of denaturation of the proteins. Precipitation by ammonium sulphate has been routinely adapted to effect a partial purification and concentration of the enzymes involved in aminoacylation and transfer reactions from rabbit reticulocytes without significant loss of activity (Lin, et al., 1966) attested once more to the extreme lability of the V. faba transfer factors.

Perhaps it would not be superfluous to point out that the interchangeability of the reticulocyte and V. faba enzyme fractions (Table 8) at least suggests that the genetic code used by both rabbits and beans is essentially similar, offering further confirmation of the universality of the genetic code. It should be stressed, however, that the factors have only been demonstrated to be interchangeable in Poly(U) dependent systems. The final proof that both organisms utilise the same code will be demonstration, in a complete reticulocyte system, of the synthesis of a bean protein in response to bean mRNA and vice versa, the synthesis by a complete bean system, of the rabbit haemoglobin in response to the haemoglobin mRNA. The latter possibility should not prove unfeasible in view of the recent isolation of haemoglobin/^{mRNA} from a variety of sources.

In view of the known limitations of Poly(U) as an ideal mRNA, it was decided to investigate the use of Poly(A) as a synthetic messenger. The complete lack of Poly(A) dependent incorporation in both bean and reticulocyte systems under the conditions of the incubations suggests that Poly(A) was unable to act as a synthetic

mRNA in these systems (Table 15). It is noteworthy that the lack of activity could not be attributed to inactivity of the microsome preparation since both systems showed a considerable activity with a Poly(U) template. It may be argued that the lack of activity results from incomplete precipitation of the resultant Poly-L- ^{14}C lysine. This seems unlikely in view of the presence of added carrier poly-L- ^{12}C lysine which was precipitated by the 5% TCA containing 0.25% sodium tungstate at pH2 (Sela and Katchalski, 1959). This precipitant is known to precipitate polycationic peptides. In contrast to the average size of the polypeptide product directed with Poly(U) (about 30 residues, Gilbert, 1963b), the oligolysine peptides produced in an E. coli Poly(A) directed system varies between about 3 and 10 residues. Peptides of this size are precipitated by TCA/tungstate. Bretscher (1964) has demonstrated that the abundance of dilysine obtained in the E. coli Poly(A) directed system is not due to digestion of the polylysine with a trypsin-like enzyme present in the bacterial extract, since most of the polylysine is attached to tRNA. The reason for the difference in size of the polypeptide products with Poly(U) and Poly(A) is unclear; however, the complex of ribosome, Poly(A) and polylysyl-tRNA appears to be much less stable than the corresponding ribosome, Poly(U), polyphenylalanyl-tRNA complex (Bretscher et al., 1965; Ganoza and Nakamoto, 1966).

Using a rat liver complete system, Gardner et al. (1962) demonstrated the Poly(A) dependent incorporation of lysine into a TCA/tungstate insoluble precipitate. The level of Poly(A) needed to promote maximum incorporation was of the order of $160\mu\text{g/ml}$, however even at $1,200\mu\text{g/ml}$ significant incorporation was still obtained.

In view of such findings it is unlikely that the Poly(A) concentration used here (400 μ g/ml) was either limiting for or inhibitory to incorporation.

A similar lack of Poly(A) dependent incorporation has been reported by Basilio et al. (1966). Using a wheat germ system these workers failed to detect Poly(A) dependent incorporation over a wide range of magnesium concentrations and incubation conditions. Basilio et al. suggest that the lack of Poly(A) directed incorporation reflects the inability of Poly(A) to bind to wheat germ ribosomes. Spirin and Gavrilova (1969) suggest that the binding of mRNA to the ribosome involves in addition to a binding centre formed by a protein component, a degree of interaction between the template polynucleotide and rRNA. The demonstration of Poly(A) dependent incorporation in both the E. coli and rat liver systems indicates that these ribosomes must be capable of binding Poly(A). Spirin and Gavrilova point out that the complex, ribosome-mRNA, is stabilised by the introduction of the appropriate aminoacyl-tRNA. The introduction of lysyl-tRNA into the system of ribosomes and Poly(A) has been shown to make the binding of ribosomes to Poly(A) even somewhat stronger than the binding of the ribosome to Poly(U) in the presence or absence of phenylalanyl-tRNA (McLaughlin et al., 1966). It seems likely that the lack of Poly(A) directed incorporation may reflect an inability of plant ribosomes to participate in the formation of a Poly(A)-ribosome complex. The possibility must not be excluded that such a complex could be formed under other incubation conditions.

Poly(U) dependent amino acid transfer from aminoacyl-tRNA into peptide linkage has been shown to be dependent upon GTP and two soluble aminoacyl transfer factors in both mammalian and bacterial systems (Arlinghaus, et al., 1964; Gasior and Moldave, 1965; Lucas-Lenard and Lipmann, 1966; Skoultchi et al., 1968; Felicetti and Lipmann, 1968).

A preliminary attempt was made to demonstrate the presence in V. faba supernatant of two complementary fractions required for aminoacyl transfer. Since the separation of two such complementary fractions from rat liver was well documented (Moldave, 1968), the application of such methods to the resolution of the V. faba transfer factors was investigated. It was assumed that the plant factors would resemble more closely those of the mammalian than the bacterial system and would therefore be most probably resolved by methods similar to those applied to the rat liver system. Basically the technique involves the column chromatographic resolution of a partially purified protein fraction and the detection of complementary fractions using washed ribosomes.

Although the transfer factors are usually regarded as supernatant in origin, they are associated with both microsomes and ribosomes. In order to demonstrate complementation between resolved factors it is essential that the ribosomes used in the assay methods are essentially free of contaminating transfer factors. Moldave and Skogerson (1967) have outlined a technique for the removal of contaminating factors involving DOC and ammonium chloride washing procedures. Crude ribosomes are prepared by extraction of microsomes with DOC solutions and purified by several washes in

solutions containing high Mg^{2+} (1 x 0.05M and 4 x 0.01M Mg^{2+}).

The criterion used for ribosomal purification being the absence from the ribosomes of aminoacyl-transferring enzymes. Residual TF2 although difficult to remove from the ribosome, can be removed by treatment with 0.5M-ammonium chloride in the presence of 0.01M Mg^{2+} .

Treatment of V. faba microsomes with DOC leads to complete loss of activity as a result of high endogenous RNase activity associated with the microsomal membranes. In order to circumvent this loss of activity due to RNase, the ribosomes were liberated from the E.R. by Triton X100 treatment of the post-mitochondrial supernatant. Liberation of ribosomes at this step was not accompanied by loss in activity (Tables 6 and 9). Contaminating transfer factors were removed from washed Triton X100 ribosomes by treatment with 0.5M-ammonium chloride, as described in the Methods. Residual ammonium chloride being removed by dialysis. This wash procedure was accompanied by a considerable decrease in the activity of the preparations and addition of supernatant failed to restore the activity to its previous value. (Table 14). The extremely low incorporation obtained with such particles in the absence of added enzyme probably reflects the absence of contaminating transfer factors from ammonium chloride washed ribosomes. Richter and Klink (1967) describe a wash procedure for the preparation of transfer factor-free ribosomes from yeast, involving a treatment with 0.8% (w/v) DOC followed by centrifugation through a discontinuous sucrose density gradient. These ribosomes are completely dependent upon added enzyme but have a considerably decreased activity in polymerisation in the presence of added supernatant. The addition of spermine resulted in increased activity but the activity of the particles

remained 100% lower than that of unwashed particles.

The supernatant from ammonium chloride wash solution would seem an ideal source of transfer factors, however dialysis of this wash fluid and quantitative addition to ammonium chloride washed ribosomes failed to promote significant amino acid incorporation.

The procedure of Moldave (1968) was followed, the only variation introduced being the addition of 10^{-3} M glutathione to the Sephadex-G200 equilibration and elution buffers. In view of the known lability of the transfer factors in the absence of -SH compounds, it was felt that the inclusion of such in the buffer may help to protect the factors from auto-oxidative denaturation. Table 16 indicates the increasing specific activities of the transfer factors during the preliminary purification procedures prior to chromatography on G200. The increase in the specific activity of the factors during these preliminary procedures indicates that no resolution had occurred prior to G200 chromatography. Fractions were assayed in a modified transfer system in which the GSH concentration was 4mM to prevent activation of ribosomal TF2.

The single peak of transfer activity obtained when G200 fractions were assayed for their ability to promote Poly(U) dependent incorporation of phenylalanine with ammonium chloride washed ribosomes in the modified transfer system suggested that there had been no resolution of the factors. However, as pointed out by Moldave, (1968) TF2 is a frequent contaminant of ribosomal preparations and the possibility existed that this peak of activity represented incorporation promoted by a combination of ribosomal TF2 and the resolved TF1. When a partially purified rat liver transfer

factor preparation is applied to a G200 column and assayed as described the aminoacyl transferring activities of the individual column fractions is extremely low. There are, however, very slight indications of two peaks of activity. When aliquots from the region of the first peak are assayed in combination with the remaining fractions, a peak^{of} transferring activity is found coincident with the position of the second small peak, and vice versa. Aliquots from each of the peak fractions therefore complement one another indicating the resolution of the two factors. TF1 is shown to elute first followed by, but almost completely separated from, TF2.

Assuming such a situation to be the case with the V. faba transfer factors, the peak of aminoacyl transferring activity obtained when the fractions were assayed individually (Fig.28) is in the correct position, and is consistent with the complementation of resolved TF1 by ribosomal TF2. In an attempt to verify that such was in fact the same, the fraction (25) corresponding to the peak of transfer activity was assayed in combination with the remaining fractions. The complete lack of complementation of the type obtained in the rat liver system indicates that under the conditions of chromatography there had been no resolution of the V. faba transfer factors. A 0.2ml aliquot was used to assay fractions individually whilst on the occasion of the second assay 0.1ml aliquots of the fractions was assayed individually and in combination with the 0.1ml of the peak fraction. The almost quantitative halving of the activity obtained when fractions were assayed individually on the second occasion indicates a linear dependence of incorporation upon concentration and the stability of the factors to at least one cycle of freezing and thawing.

Superficially Fig.29 may be thought to reveal 3 peaks of aminoacyl transfer activity. When fractions 28-65 were assayed in combination with the peak fraction, however, the resultant activities were not significantly greater than when fraction 25 was assayed alone.

It may be significant that, unlike that used by Moldave, the equilibration and elution buffer contained glutathione; it seems unlikely that the inclusion of this -SH reagent could influence the separation of the factors. TF1 from rat liver exists in multiple forms, but they all appear to be aggregates of TF1, their mol. wt. ranging from 100,000 to greater than 300,000 (Schneir and Moldave, 1968), the TF2 from rat liver has a mol. wt. of between 60,000-65,000. Assuming the V. faba factors to have molecular weights in this range, their separation purely on the basis of molecular weight differences would have been anticipated on Sephadex-G200. McKeehan and Hardesty (1969) report the dissociation of TF1 from rabbit reticulocyte into three identical subunits of mol. wt. 62,000. Chromatography on Sephadex G200 would not resolve completely such subunits from TF2. It is significant, however, that such subunits are enzymatically inactive.

The lack of demonstrable complementation may have resulted from the use of limiting amounts of one of the fractions. Using essentially the same purification procedure adopted here Moldave (1968) indicates that the ratio of the concentrations of resolved TF1 to TF2 required for demonstrable complementation was approximately 1:5. The presence of extremely low concentrations of one of the factors following chromatographic resolutions may explain the lack of complementation. To this end it would have been useful to assay for complementation using varying concentrations

of each column fraction.

If it is assumed that TF2 is likely to be present as a ribosomal contaminant, then the peak of incorporation obtained when the fractions were assayed initially could be due to complementation between resolved TF1 and ribosomal TF2. In the absence of contaminating TF1 no complementation between resolved TF2 and ribosomal TF1 would be seen and hence the absence of a second peak. Since only fractions 25-68 were assayed for complementation, the possibility cannot be excluded that the two factors had been completely resolved and that TF2 activity eluted beyond fraction 68 and was not therefore demonstrated.

In view of the similarity in mol.wt. of the transfer factors from rat liver and reticulocytes, it seems likely that those of V. faba will be approximately similar. Assuming mol.wts of approximately 100,000 and 60,000 for the V. faba TF1 and TF2 respectively, they should be separated on a Sepharose 4B gel (exclusion limits 1×10^4 — 3×10^6).

Since the demonstration of complementation between two transfer factors constituted only a preliminary investigation prior to their resolution, the initial purification steps were abandoned and the behaviour of concentrated dialysed supernatant on Sepharose 4B was investigated.

Two major fractions of transfer activity were obtained when the column fractions were assayed individually (Fig.30). The relative activities of the fractions are consistent with the suggestion that the peak eluting first represented activity due to

resolved TF1, since TF1 is easier to remove from ribosomes than TF2 therefore the contamination due to TF2 would be expected to be greater than TF1.

When aliquots of fractions 40-67 were assayed in combination with aliquots of fraction 37, complementation was seen to occur between fraction 37 and fractions 50-67 (Fig.31). Between each set of assays (a period of 24 hr) the fractions were stored frozen at -20° . The complete absence of the second peak (i.e. fractions 50-67) on the occasion of the second assay indicates the lability of some component present to freezing and rethawing. However the increase in transfer activity observed when these fractions were assayed in combination with fraction 37 suggests that these fractions were complemented by fraction 37. It is noteworthy that the activity of these fractions in combination with fraction 37 was significantly greater than that due to fraction 37 alone, indicating that the increase in activity resulted from a complementation between the two factors and was not simply due to fraction 37 alone. The presence of an actual peak of activity between fractions 52-63 is further evidence that activity resulted from a degree of interaction between fractions. If the activity of the combination assays represented only activity due to fraction 37 it is probable that the value obtained would have been identical in all cases.

Because of the complete lack of activity of fractions 50-67 when the fractions were thawed on the second occasion the possible complementary effect of fraction 60, upon the activity of fractions 25-50 was not investigated. Assuming the mol.wts. of the V. faba

transfer factors were in the same range as those of their rat liver counterparts, the peak eluting first from Sepharose 4B would be expected to be due to TF1 activity, whilst that eluting second would be analogous to the rat liver TF2.

Despite the demonstration of complementation between the two protein fractions, certain facts require an explanation. When the column fractions were initially assayed individually maximum activity was obtained with fraction 37, this was assumed therefore to represent a peak of eluant protein which when assayed in combination with ammonium chloride ribosomes exhibited transfer activity, whilst the subsidiary peak at fraction 43 was assumed to represent either experimental variation and a shoulder of activity. On the occasion of the second assay, the activity of fraction 37 had significantly decreased whilst that of 40 and 47 had remained approximately constant and the fraction showing maximum transfer activity was displaced to fraction 43. This resulted in the absence of the initial peak of activity, and the appearance of a new peak at fraction 43. Since fractions 25-33 were not reassayed on this second occasion it is impossible to attribute the displacement of the peak unequivocally to a change in the activity of the fractions constituting this portion of the peak but the possibility at least warrants an explanation. The form of the first peak suggests it may represent two incompletely resolved components, the change in the form of the peak may have resulted from the instability of the component eluting first. As has been described previously there is certain justification for the assumption that the peak eluting first represents the binding enzyme TF1 whilst TF2 type

activity elutes after this. The bacterial binding enzyme (T) has been resolved by chromatography on Sephadex G200 into sub-fractions, Tu which elutes first and is unstable and Ts which elutes later and is more stable (Lucas-Lenard and Lipmann, 1966). The possibility cannot be ignored that the essentially double nature of the peak eluting first results from the presence of two components both having transfer activity with ammonium chloride ribosomes and varying in their stability to freezing and thawing.

When E. coli transfer factors are resolved on DEAE-Sephadex two major peaks of activity are obtained (Lucas-Lenard and Lipmann, 1966). The double peak eluting first results from the incomplete separation of Ts, from a mixture of Ts+Tu. In view of the different separation processes involved it is difficult to compare the resolution of the V. faba enzymes with that of the bacterial counterparts. It is most likely however that the resolution of Ts and Tu on G200 would approximate most closely to the situation on G200. The possibility cannot be eliminated therefore that the change in activities of the fractions results from the differential stability of two incompletely resolved components. Evidence consistent with such an explanation is however limited. The resolution of the bacterial factor T is well documented but the eukaryotic binding enzyme TF1 has not been resolved into active subfractions (Lucas-Lenard and Lipmann, 1971).

A further possibility is that the initial double peak represents the incomplete separation of the dimer/monomer form of a single transfer factor. In such a case the dimer would elute first followed by the monomer, hence the double peak and the

dissociation of the dimer following freezing and thawing would result in the absence of the first peak on subsequent reassay. Only when the monomeric form of the enzyme was present in limiting amounts, would an increase in the proportion of the monomeric form be accompanied by a concomitant increase in the activity of the second portion of the peak. TF1 of rat liver has been shown to exist in multiple forms, all of which possess enzymatic activity (Schneir and Moldave, 1968). Since the mol.wt. of eukaryotic TF1 is approximately 100,000 a considerably greater degree of resolution of dimer/monomer would have been expected.

A further explanation of the change in activities of the peak eluting first from Sepharose 4B is possible in terms of the existence of two discrete sets of transfer factors. It is possible that two sets of transfer enzymes exist in V. faba one derived from the cytoplasm and specific for 80S ribosomes, and another derived from organelles and specific for 70S ribosome. Krisko, et al. (1969) have shown that bacterial factor T can substitute for TF1 in the binding of aminoacyl-tRNA to eukaryotic ribosomes, and in polymerisation when supplemented with cytoplasmic eukaryotic TF2. Unlike TF1 eukaryotic cytoplasmic TF2 cannot be replaced by bacterial G factor. Thus assuming that V. faba contains 2 sets of transfer factors and accepting the above interchangeability of T but not G with TF1 and TF2 respectively on 80S ribosomes, and also the probable contamination of the ribosome preparation by TF2, a situation may exist whereby the double peak eluting first represents unresolved T and TF1 activities. In this case polymerisation would result from complementation of both T and TF1 with ribosomal TF2. The second peak to elute must

therefore represent a complementation between eukaryotic type TF2 and ribosomal TF1. In view of the inability of prokaryotic factor G to substitute for factor TF2 on 80S ribosomes, this component would not be demonstrable in the assays involving 80S type ribosomes. The difference in the activity of the double peak on the second assay must be postulated to result from the instability of one of the components.

Data reported by Ciferri and Parisi (1970) may be relevant to such an argument. These workers present evidence for the existence of two discrete sets of polymerising enzymes in cell-free extracts from the non-photosynthetic alga Prototheca zopfi, one set is active on ribosomes of the 70S type whilst the other is active on those of the 80S type. The two G type activities could be resolved from one another and from the T type activity by chromatography on DEAE-Sephadex, whilst the two T type activities were not resolved.

Yarwood et al. (1970) have presented data consistent with the proposed identification of the complementary fractions. V. faba supernatant was chromatographed on sepharose 4B under approximately identical conditions. The fractions were then assayed for complementation with partially purified reticulocyte TF1 (binding enzyme) and TF2 fractions. The peak eluting first complemented reticulocyte TF2 whilst the peak eluting second was complemented by reticulocyte TF1 suggesting the identification of the first peak as a V. faba binding activity whilst the second peak must represent V. faba translocase activity. The order of elution was thus identical to that proposed above. In addition the peak eluting

first was shown to promote the Poly(U) dependent enzymatic binding of [^{14}C]phenylalanine to reticulocyte sodium fluoride, DOC reticulocyte ribosomes (i.e. free of transfer factors), this is in agreement with its proposed identity as the V. faba binding enzyme.

At this point it may be useful to review the evidence for the resolution of binding and translocase activities in other higher plants. Allende (1969) reports a partial purification of the wheat embryo transfer factors by ammonium sulphate precipitation followed by DEAE-cellulose chromatography. Using stepwise elution a single peak of transfer activity was demonstrated, attempts to resolve the activity into several complementary components were completely unsuccessful. The author attributes this lack of success to the extreme lability of these factors, and demonstrates a 100% loss of transfer activity following incubation of partially purified wheat embryo transfer factors at 37° for 12 min. Some slight protection was observed in the presence of 0.02M-glutathione.

Legocki and Marcus (1970) report the complete resolution and partial purification of the transfer factors from wheat germ. The factors were resolved from one another by chromatography on a DEAE-cellulose column using stepwise elution. These workers do not however report the procedure whereby the chromatographic resolution of the factors was monitored. The fraction eluting first was shown to catalyse a GTP and Poly(U) dependent binding of phenylalanyl-tRNA to wheat germ ribosomes. The second factor facilitated the formation of peptidyl-puromycin from nonenzymatically bound phenylalanyl-tRNA suggesting that this factor is a translocase,

presumably transferring the nonenzymatically bound phenylalanyl-tRNA from a position non reactive with puromycin (A site) to a reactive position (P site). In view of the sigmoid relationship between phenylalanine polymerised and TF1 concentration at low levels of the factor, the authors suggest the possibility of complementary subfractions or a cooperative type of function. Attempts to obtain such subfractions were completely unsuccessful.

Using a rice embryo system, App (1969) has demonstrated the dependence of Poly(U) directed phenylalanine incorporation upon two soluble components.

It can be concluded that the overall mechanism of peptide chain elongation in plants is undoubtedly the same as in bacterial and mammalian cells but that the enzymes involved are less well characterised.

In view of the limited success of other workers to resolve the enzymes involved in peptide chain elongation from higher plant sources it would seem justifiable to persevere with this particular aspect of the work described here. Since the use of ammonium chloride washed ribosomes necessitates a considerable expenditure of time prior to the assay of column fractions it may be possible to use Triton X100 ribosomes which have been washed by resuspension from resuspending medium. The particles should be almost completely dependent upon added enzyme (Table 9) and may be sufficiently free of transfer factors to enable their use in the routine assay of column fractions. The use of low -SH concentration in the incubations (4mM) should prevent the activation of contaminating ribosomal TF2 (Moldave, 1969).

The extreme lability of the partially resolved fractions may be overcome by the addition of glycerol to the column fractions. McKeehan and Hardy (1969) have demonstrated that the binding enzyme from rabbit reticulocyte was stabilised by the inclusion of 50% glycerol in the standard buffer.

Although work presented here demonstrates complementation between two partially resolved fractions in peptide chain elongation the enzymes involved have yet to be unequivocally identified. The simplest method by which this may be achieved would involve the demonstration that one of the enzymes brought about a GTP dependent enzymatic binding to V. faba ribosomes and the identification of the bound product as phenylalanyl-tRNA. The complementary fractions may also be identified in a manner described by Yarwood et al. (1970) using partially purified binding enzyme and translocase from other sources, preferably from wheat embryo. An enzyme responsible for translocation of the peptidyl-tRNA from A to P site may be identified by its ability to form a puromycin peptide from previously bound phenylalanyl-tRNA (Legocki and Marcus, 1970), and its inhibition by fusidic acid (Kaziro et al., 1969) and diphtheria toxin and NAD (Goor and Pappenheimer, 1967).

The techniques used above for resolution of the complementary fractions both involved a separation on the basis of molecular weight differences. The use of an ion exchange resin such as DEAE or TEAE-cellulose, coupled with stepwise or gradient elution may be useful in resolving the two enzymes.

One of the criteria which Hoagland (1960) suggested should be fulfilled if incorporation of a radioactive amino acid is to be equated with protein synthesis was that the amino acid should appear in a single specific isolatable protein of the cell of origin. There has, as yet, been no unequivocal demonstration of a synthesis of a plant protein directed by a plant mRNA, by an amino acid incorporating system derived from a plant source.

Two basic approaches exist for the demonstration of protein synthesis in amino acid incorporating systems. Firstly, use can be made of a isolated purified mRNA which directs the synthesis of a specific protein from the tissue of origin. The isolation of undegraded mRNA molecules from plant materials has not yet been described. The second approach involves the use of endogenous mRNA present in polysome preparations. This approach is limited by the availability of undegraded polysome preparations. In order to characterise the product of such polysome systems it is necessary to choose a system in which a considerable proportion of the amino acids incorporated are into a single well characterised protein. The ideal tissue for such an approach is therefore a highly specialised tissue in which the synthesis of protein is directed towards the production of a single major product. It is for this reason that the developing cotyledon of V. faba would appear to be an ideal material for the establishment of a polysome system directed by endogenous mRNA, the major product of which should be the relatively well characterised storage globulins, vicilin and legumin.

In V. faba, storage protein may account for up to 20% of the dry weight of the mature seed (Grzesuik et al., 1962) and consists of two major protein components, vicilin and legumin (Danielson, 1952). The majority of the storage globulin is synthesised in about 30 days during the development of the seeds. Polysomes extracted from developing cotyledons during the period of storage globulin synthesis should contain the endogenous mRNA directing the synthesis of vicilin and legumin. Storage protein accumulates within discrete organelles in the cytoplasm, protein bodies, which are bounded by a lipoprotein membrane and appear to originate by subdivision of the cell vacuole (Briarty et al., 1969). It has been unequivocally demonstrated (Yarwood, 1968) that protein synthesis does not occur in protein bodies, nor do they contain any of the components involved in protein synthesis. It has been demonstrated (Bailey et al., 1970) that synthesis of storage globulin occurs on cytoplasmic ribosomes. The maturation of V. faba seeds takes about 100-120 days, during which time characteristic anatomical and biochemical changes are occurring in the seeds. Three main phases can be recognised in the development of the cotyledon (Briarty, et al., 1969).

(i) Phase of rapid cell division lasting from days 1-25 after flowering, followed by cell expansion from days 25-40. During this period the major organs of the embryo are formed.

(ii) This is followed by a period of intense synthetic activity, when the storage proteins of the seed accumulate. This occurs between days 45-70.

(iii) After completion of the synthesis of the storage products, the seed enters the final period of maturation and dehydration prior

to dormancy. Payne (1970) has described the anatomical changes in the cells of the developing cotyledon during its development. Prior to the initiation of storage globulin synthesis, the cells contain mainly free ribosomes, with little endoplasmic reticulum. By 50 days, there has been a tremendous proliferation of endoplasmic reticulum and membrane-bound ribosomes predominate over free ribosomes. Payne (1968) has shown that the membrane-bound ribosomes represent a new and discrete class of particles and have not arisen by the attachment of pre-existing free ribosomes to the newly formed endoplasmic reticulum. Storage protein synthesis begins around day 45 and traces of protein can be distinguished around the periphery of the protein bodies. By 60 days, storage protein is being rapidly synthesised and the protein bodies are being filled up. Storage protein synthesis is complete by about 80 days and dehydration is beginning to occur. Associated with dehydration is the breakdown of the endoplasmic reticulum and release of the membrane-bound ribosomes. In the mature dehydrated seed there is hardly any trace of E.R. and ribosomes are found free in the cytoplasm. It is evident that striking changes are occurring in the ribosome population which can be correlated with specific developmental changes. Payne (1968) and Payne and Boulter (1969) separated microsomes prepared from different ages of developing V. faba seeds on discontinuous sucrose gradients into free and membrane-bound material and determined the ratio of free:membrane-bound rRNA. They found that changes in the ratio of free:membrane-bound ribosomes correlated with in vivo changes in ribosome distribution. The ribosome content of the microsomal preparation, therefore does appear to reflect the in vivo distribution of ribosomes.

Since synthesis of storage globulin is paralleled by an increase in the proportion of membrane-bound ribosomes the inference is that synthesis of storage globulin occurs on membrane-bound ribosomes. This is consistent with the idea that it is not utilised in situ but must be transported to the protein bodies.

In order to achieve the synthesis of storage globulin in a cell-free system the ideal system would appear to be a microsomal preparation from 60 day developing V. faba in which the polysome profile was maintained as close as possible to the in vivo situation. In these preparations a high proportion of the polysomes should contain the endogenous storage globulin mRNA's. Payne (1970) investigating the relative synthetic ability of the components involved in protein synthesis prepared at different stages during the development of the cotyledon, has shown that activity due to endogenous mRNA was greatest in microsome preparations from 60 day seeds. Although synthesis of storage globulin probably occurs on membrane-bound polysomes, the role of the membrane remains unclear. The intimate spatial relationship may be necessitated by the association of mRNA with the membrane or it may be dictated by the necessity for a precise vectorial release of the nascent polypeptide into the lumen of the membrane (Redman and Sabatini, 1966). The further probability that the association of the mRNA with the membrane may offer a protection against RNase action cannot be excluded. In order to maintain a high proportion of undegraded polysomes it is necessary to reduce nuclease activity to its low level. To this end microsomes were prepared in the presence of rat liver high speed supernatant. Although rat liver supernatant may inhibit a rat liver nuclease(s) there appears to be no evidence of a similar effect of such an

inhibitor upon bean nucleases since no significant difference in levels of incorporation was observed in the presence or absence of the inhibitor (Table 17).

The simplest method of characterising the product of in vitro protein synthesis is to compare the tryptic fingerprint of the product with that of the purified protein. An identical fingerprint of the in vitro and in vivo product is good confirmation of the identity of the two proteins. Although the storage globulin of V. faba contains two proteins, vicilin and legumin, the latter is the predominant component, comprising about 75% of the total globulin synthesised in vivo. Assuming the two proteins are synthesised in the same proportion in vitro, the major product of the cell-free system would be expected to be legumin.

Legumin has a molecular weight of approximately 330,000-410,000 and is composed of three types of polypeptides. The molecular weight of the three polypeptides are 56,000, 42,000 and 23,000 and they are present in molar ratios of 1:3:6. Bailey and Boulter (1970) report the presence of 140 ninhydrin-positive peptides following tryptic digestion of carboxymethylated-legumin (CM-legumin). If $[^{14}\text{C}]$ -amino acid mixture had been used to label the 'in vitro' product difficulties could have arisen in comparing the tryptic map because of the great number of peptides. It was decided therefore to label with $[^{35}\text{S}]$ methionine since there are only three methionine containing tryptic peptides in CM-legumin (Bailey and Boulter, 1970). Since vicilin contains very little sulphur, it was assumed that the fingerprint would not be obscured by small amounts of vicilin synthesised simultaneously. The fingerprint of the

[³⁵S] labelled CM- in vitro product (Fig.32) revealed considerably more than the expected three [³⁵S] methionine-containing tryptic peptides predicted by Bailey and Boulter (1970). The streaked nature of the chromatogram results probably from the sticky nature of the digest applied to the paper which was highly heterogeneous containing not only peptides derived from trypsin digestion of the CM-in vitro product but also CM-derivatives and arising from digestion of the supernatant/ribosomal proteins. During the preparation of the fingerprint the peptides were separated by chromatography in one direction and electrophoresis in the other. During the electrophoretic separation of the peptides, not only do the charged peptides move by virtue of their charge but also migration of the neutral peptides occurs from the origin in an ascending direction by electroendosmosis which results in a displacement of these peptides. This movement depends probably to some extent upon the conditions of electrophoresis and makes comparison of the results with those of Bailey and Boulter difficult. These workers found that 8 of the 16 [³⁵S]- containing tryptic peptides migrated with the neutral peptides in a linear fashion towards the cathode and formed a line of radioactive spots at 90° to the direction of electrophoresis. This area contained the most dense distribution of radioactivity. If these spots are used as a reference point and aligned with the most dense streak of radioactivity obtained in the fingerprint of the [³⁵S] labelled CM-in vitro product as arranged in Fig.32 then comparison of the fingerprints is possible. This assumes that this streaked line represents the position reached by electroendosmosis by the neutral peptides; it must be stressed that this is merely an assumption.

A strong spot is noted moving towards the anode, this must represent a very acidic residue, this is completely absent from the tryptic fingerprint of CM-legumin and its source is completely unknown. When the supposedly neutral peptides are aligned as in Fig.32 there is an almost complete correspondence between the two major areas of $[^{35}\text{S}]$ methionine distribution and two of the methionine containing peptides of the CM-legumin. It is perhaps significant that the areas containing the greatest amounts of radioactivity should be coincident with two methionine containing peptides. If it was assumed that these represent the same methionine containing peptides as the homologous spots on the fingerprint of CM-legumin, then the absence of the third methionine containing peptide suggests that all three constituent polypeptides of legumin were not synthesised by the in vitro system. There is however a radioactive peptide with a mobility closely similar to that of the third methionine containing peptide in CM-legumin. It is possible that this may represent the same or part of the same peptide.

There are a number of spots which have no equivalent on the CM-legumin fingerprint and must represent novel peptides.

If under the conditions of incubation the constituent polypeptides of legumin were not synthesised in their entirety but fragments of these polypeptides were formed then novel tryptic peptides would be formed with completely new mobilities. The synthesis of fragments of legumin polypeptides may account for the presence of more than three $[^{35}\text{S}]$ -labelled peptides.

Bailey and Boulter (1970) report that the N-terminal residues of the constituent polypeptides of legumin are leucine, glycine and threonine. Additional $[^{35}\text{S}]$ methionine containing peptides may be expected if the initiation of legumin by a methionine residue was followed by cleavage of the N-terminal methionine residue (and possibly other terminal amino acids) prior to the completion of the polypeptide chains. In this case new $[^{35}\text{S}]$ methionine peptides would be detected which were not present in digests of pure CM-legumin.

A similar situation would obtain if the N-terminal methionine was not cleaved until the completed polypeptides were released from the ribosome. In this case polypeptides still attached to the ribosome may contain an additional methionine containing peptide.

The presence of extra peptides may indicate that polypeptides other than the constituent polypeptides of legumin are being synthesised. It is possible that the membrane-bound ribosomes, in addition to the synthesis of globulin, are actively synthesising new membrane material and the additional peptides may be membrane-specific peptides. Bailey *et al.*, (1970) using a *V. faba* cotyledon slice system to follow the synthesis and intracellular transport of globulin showed that radioactivity was initially associated with the endoplasmic reticulum and was later found in the protein bodies. However, some residual activity could not be chased from the endoplasmic reticulum suggesting that some of the incorporated radioactivity remains associated with it. The possibility should not be excluded that such residual activity represents the synthesis of new membrane material.

The presence in the microsome preparation of an active nuclease would result in degradation of the polysome preparation. The presence of additional spots may result from the translation of fragments of the legumin mRNA thus yielding new peptides.

In addition to the spots corresponding to the three methionyl peptides there is a fairly close correspondence between several of the other spots and spots present on the tryptic fingerprint of $[^{14}\text{C}]$ M-legumin (i.e. cystine containing peptides) (Bailey and Boulter, 1970). Since the $[^{35}\text{S}]$ methionine used was almost free of $[^{35}\text{S}]$ cysteine, there should be no label in peptides other than those containing $[^{35}\text{S}]$ methionine. The close similarity between some of the cystine-containing tryptic peptides of $[^{14}\text{C}]$ M-legumin and some of the peptides obtained after digestion of the $[^{35}\text{S}]$ -labelled in vitro product suggests that possibly there has been some conversion of $[^{35}\text{S}]$ methionine to $[^{35}\text{S}]$ cysteine. In view of the trans-sulphuration whereby methionine sulphur is transferred to cysteine (Simmonds, et al., 1953) such an explanation appears quite feasible. To summarise, although the synthesis of legumin by the V. faba amino acid incorporating system could not be unequivocally demonstrated there appears to be some degree of similarity between the tryptic peptides of the in vivo and in vitro products.

In the method adopted the in vitro product was not separated from the remainder of the incubation components prior to carboxymethylation. In future attempts at such a characterisation it may be profitable to separate the $[^{35}\text{S}]$ legumin by the addition of a high concentration of carrier $[^{32}\text{S}]$ legumin and purify it prior to carboxymethylation.

Such an approach would perhaps yield better fingerprints but would identify only the legumin product and would disregard any other possible products. Since in this case a much greater concentration of trypsin digested legumin would be applied to the chromatogram it may prove possible not only to locate $[^{35}\text{S}]$ methionine containing peptides by autoradiography but to detect cystine and methionine spots by the use of plationic-iodide reagent (Easley, 1965).

A further possible approach to the identification of the product of in vitro protein synthesis as legumin may be devised based on the use of antilegumin to specifically precipitate polysomes engaged in the synthesis of radioactively labelled legumin polypeptides.

Although the developing cotyledon of V. faba theoretically should present an ideal system for the characterisation of the product of a plant cell-free amino acid incorporating system, it appears that the advantages of such a system are offset to a considerable extent by the complexity of the storage protein synthesised in vivo and the high endogenous nuclease content of the tissue (Lonsdale, 1972).

The second part of the work described here was an attempt to investigate the mechanism of polypeptide chain elongation on the 80S ribosomes of V. faba.

Initiation of the synthesis of polypeptide chains involves N-formylmethionyl-tRNA(f-met-tRNA_F) in bacterial systems (Marcker and Sanger, 1964; Webster et al., 1966; and Adams and Capecchi, 1966), and in mitochondria and chloroplasts of eukaryotes (Smith and Marcker, 1968; Marcker and Smith, 1969; Bianchetti et al., 1971;

Galper and Darnell, 1969; Schwartz et al., 1967). It has therefore been proposed that this tRNA species is the universal initiator of protein synthesis on 70S ribosomes (Smith and Marcker, 1968; Marcker and Smith, 1969). Despite numerous attempts to identify an f-met-tRNA_F species in the cytoplasm of eukaryotes this molecule has not been demonstrated. Cytoplasmic tRNA preparations from mammals (Caskey et al., 1967; Smith and Marcker, 1970; RajBandary and Ghosh, 1969), yeast (Takeishi, et al., 1968) and higher plants (Allende, 1969) contain two major methionyl-accepting tRNA's. Although an active transformylase enzyme has not been detected in the cytoplasm of mammals or yeast, one of the tRNA^{met} species is formylated by E. coli transformylase and the product is indistinguishable from E. coli f-met-tRNA_F and can bind to E. coli ribosomes in the presence of AUG with the same requirements and efficiency as E. coli f-met-tRNA_F. (Marcker and Smith, 1969). The formylatable liver met-tRNA also has a high affinity for the initiator site on E. coli ribosomes as measured by the puromycin release assay (Caskey, et al., 1968). In addition, Takeishi et al. (1968) have shown that yeast f-met-tRNA can substitute for bacterial f-met-tRNA_F as an initiator in an E. coli cell-free system programmed with f₂RNA. Thus it appears that many of the unique structural features of f-met-tRNA_F required for its action as a chain initiator have been retained in one of the met-tRNA species from the cytoplasm of mammals and yeast since enzymatically formylated f-met-tRNA from these organisms appears to be indistinguishable from E. coli f-met-tRNA_F in its action in the E. coli cell-free system. Such a similarity is indeed striking in view of the difference in the primary structure of tRNA_F^{met} and the corresponding species from yeast and mammals (Marcker and Smith, 1969).

In spite of the above evidence for the presence of a cytoplasmic formylatable met-tRNA species in mammals and yeast, there was little data available for higher plants.

A series of preliminary experiments were carried out by Dr. A. Yarwood in 1968 in conjunction with Dr. K. Marcker (M.R.C. Laboratory of Molecular Biology, Cambridge) to investigate whether a formylatable met-tRNA species was present in V. faba unfractionated cytoplasmic tRNA preparations. A crude V. faba tRNA preparation was aminoacylated with [³⁵S]methionine in the presence and absence of formyl donor using both E. coli DE52 supernatant and a crude V. faba 105,000xg av. supernatant essentially as described in the Methods (Section 23D). In a control experiment, an unfractionated E. coli tRNA sample was aminoacylated under identical conditions. Following digestion with bovine pancreatic RNase, the radioactive fragments were separated by ionophoresis essentially as described on the legend to Fig. 35. Only when E. coli tRNA was aminoacylated by E. coli enzyme was there evidence of a fragment equivalent to N-formyl-methionyl-adenosine. When aminoacylation was carried out using V. faba enzyme only the methionyl-adenosine fragment was obtained indicating the absence of an active transformylase enzyme in the V. faba 105,000xg av. supernatant. Aminoacylation of V. faba tRNA by E. coli or V. faba supernatant in the presence of absence of formyl donor failed to reveal the existence of a formylatable met-tRNA species (Yarwood, unpublished observations).

The preparations used in this preliminary investigation were extremely crude. The tRNA preparation had not been purified by chromatography on DEAE-cellulose and has subsequently been demonstrated

to contain considerable amounts of 25S, 18S and 5S rRNA (Payne, 1970). The enzyme fraction was a crude undialysed 105,000xg av. supernatant. In view of the higher degree of purification of these components routinely used in these laboratories subsequent to 1968 it was decided to repeat these earlier experiments. The work presented in this thesis was carried out using components purified by the procedures described and discussed previously.

The above procedure was repeated using a preparation of 60 day developing bean tRNA purified by chromatography on DEAE-cellulose to remove contaminating rRNAs and V. faba enzyme fraction purified by dialysis and chromatography on DEAE-cellulose. In this case (Fig.35) as well as the major radioactive fragment, identified as methionyl-adenosine, the presence of a slight trace of N-formyl-methionyl-adenosine, suggested the presence in V. faba supernatant of a transformylase enzyme and of a formylatable met-tRNA species. Comparison of the intensity of the N-formyl-methionyl-adenosine from E. coli and bean indicated that in V. faba the formylatable met-tRNA species was present in very low amounts. However, in view of the apparent absence of an active transformylase enzyme from the V. faba enzyme preparations used in the preliminary (1968) experiments, the possibility could not be excluded that the V. faba transformylase was labile. The presence of traces of free methionine results probably from inadequacies in the washing procedure and from deacylation of the aminoacylated tRNA during the washing and digestion steps.

In view of this demonstration of a formylatable V. faba methionyl-tRNA it was decided to resolve the methionyl-tRNA species and to

investigate the possible interchangeability of a formylatable V. faba met-tRNA with the E. coli f-met-tRNA_F in an E. coli cell-free system.

In the absence of the apparatus for separation based on countercurrent distribution techniques, chromatographic separation techniques were adopted. In view of the unpredictability of the V. faba transformylase activity, an E. coli DE52 supernatant was used to assay the separation of V. faba met-tRNA on DEAE-Sephadex A50. One major and one minor peak (Fig.34) of methionine acceptor activity were routinely obtained. In view of the extremely low degree of aminoacylation of the second peak, and of the demonstration of two major methionine accepting species in other organisms, it was concluded that one of two situations obtained. Either the met-tRNAs were not resolved by this technique or although resolved, one met-tRNA species remained undetected since it was not aminoacylated by E. coli enzyme reflecting some degree^{of} species specificity on the part of the E. coli met-tRNA synthetase. The minor peak of met-tRNA obtained when V. faba tRNA was resolved on DEAE-Sephadex may represent the resolution of a met-tRNA which originates from chloroplasts or mitochondria. The low degree of aminoacylation of this fraction was attributed not to an inherent low acceptor capacity but to the low concentration present in the column fractions.

When V. faba DE52 supernatant was used to assay the aminoacylation of the column fractions, two major peaks of methionine acceptor activity were identified (Fig.33) these were assumed to represent two major methionine accepting tRNA species and are referred to as Peak 1 and Peak 2 in order of elution. The lack of significant

aminoacylation by $[^{35}\text{S}]$ methionine of Peak 1 by E. coli enzyme in the presence or absence of formyl donor (Table 18) suggests that this peak was undetected in those assays using E. coli enzyme (Fig.34). The major peak of methionine acceptor capacity identified in assays using E. coli enzyme can be equated with Peak 2 since only this peak shows a significant aminoacylation by E. coli supernatant. The major peak detected by E. coli supernatant eluted at 0.408M NaCl, whilst peak 2 eluted at 0.40M NaCl suggesting they represent the same met-tRNA species. The minor peak obtained when E. coli enzyme was used to assay the column fractions remained undetected when V. faba supernatant was used. This must reflect a lack of resolution under the conditions of separation and chromatography rather than incompatibility between this met-tRNA species and methionyl-tRNA synthetases present in the V. faba enzyme preparation since a minor met-tRNA was resolved on BD-cellulose which was aminoacylated using V. faba supernatant. The absence of this particular met-tRNA species from those DEAE-Sephadex fractions assayed by V. faba enzyme may reflect only a variation in the amount of this particular tRNA species in the unfractionated tRNA preparation. Takeishi et al., (1968) report the separation on DEAE-Sephadex of two yeast methionine tRNA species. The tRNA^{met} to elute first can be formylated by E. coli supernatant and is aminoacylated to an equal degree by E. coli and yeast synthetases whilst tRNA^{met} eluting second is not formylated and is only partially charged by E. coli enzyme under identical conditions to those used to charge met-tRNA.

The demonstration of two major met-tRNA species separable by chromatography on DEAE-Sephadex gives no indication, however, of the properties and specificities of the two tRNAs or the homogeneity of the two fractions. In order to investigate the homogeneity and

possible formylation of two separated methionine accepting fractions they were aminoacylated with $[^{35}\text{S}]$ methionine under a variety of conditions and the $[^{35}\text{S}]$ met-tRNAs were digested by bovine pancreatic ribonuclease or T_1 ribonuclease and the resultant radioactive fragments separated by ionophoresis. In all such experiments unfractionated E. coli $[^{35}\text{S}]$ met-tRNA was used as a control and the mobility of the V. faba fragments compared to those of the E. coli fragments.

The presence of radioactive fragments identified as methionyl-adenosine and N-formyl-methionyl-adenosine (Fig.36) on autoradiographs of pancreatic RNase digests of Peak 1 aminoacylated by $[^{35}\text{S}]$ methionine in the presence of E. coli or V. faba enzyme and formyl donor indicates that this peak contains met-tRNA species which can be formylated by either E. coli or V. faba enzyme. This is indicative of the presence of an active transformylase in the V. faba supernatant. In view of the high specific activity of the $[^{35}\text{S}]$ methionine (27C/mM) used it should be pointed out that the low intensity of the N-formyl-methionyl-adenosine fragment indicates that this component was present in only very slight amounts.

Although peak 2 material showed slight indications of an N-formyl-methionyl-adenosine fragment when aminoacylated by E. coli enzyme, it was absent when aminoacylation was carried out using V. faba enzyme. It cannot be argued that the N-formyl-methionyl-adenosine fragment is derived from contaminating E. coli tRNA present in the E. coli enzyme fraction since this has been freed of tRNA by chromatography on DEAE-cellulose. The presence of a trace amount

of N-formyl-methionyl adenosine when peak 1 was aminoacylated by V. faba enzyme suggests that either both peaks of methionine acceptor activity represent distinct but formylatable met-tRNA species, or that both represent different major met-tRNA species which are unformylatable but that both are heterogeneous and contaminated by traces of a third unresolved met-tRNA species which can be formylated by both E. coli and V. faba enzyme. In view of the striking difference in the degree of aminoacylation of the two fractions by E. coli enzyme (Table 18) the latter explanation seems the more likely although the possibility cannot be excluded that the conditions for aminoacylation were suboptimal for formylation of a bean formylatable met-tRNA. Although diagnostic of the presence of a formylatable met-tRNA species, pancreatic RNase digestion is limited in its ability to distinguish between different unformylatable met-tRNAs since in each case the radioactive fragments obtained (i.e. methionyl-adenosine and free methionine) have identical electrophoretic mobilities and cannot be resolved.

In order to resolve the two possibilities above, the $\left[\begin{smallmatrix} 35 \\ S \end{smallmatrix} \right]$ methionyl-tRNAs were digested with T_1 ribonuclease and the fragments examined. Herbert et al., (1964) have shown during ribonuclease T_1 digestion of aminoacyl-tRNA, hydrolysis of the aminoacyl-tRNA bond is negligible. Since T_1 ribonuclease has a high specificity for guanylic residues (Sato and Egami, 1957) digestion of aminoacyl-tRNA with this enzyme yields aminoacyl oligonucleotides of various lengths depending upon the distance of the first guanylic acid residue from the amino acid acceptor end of the tRNA. If $\left[\begin{smallmatrix} 35 \\ S \end{smallmatrix} \right]$ met-tRNA species differ in either the presence of a formyl group or in the primary sequence between the first guanylic acid residue and the -CCA-met

terminus then they will yield different $[^{35}\text{S}]$ met-oligonucleotides with distinct electrophoretic mobilities. Hence digestion with T_1 RNase can distinguish between distinct unformylated $[^{35}\text{S}]$ met-tRNAs which yielded identical methionyl-adenosine fragments on pancreatic RNase digestion. Comparison of the $[^{35}\text{S}]$ met-oligonucleotides (Fig.38) obtained from Peak 1 and 2 when aminoacylation was carried out with $14\text{C}/\text{mM}$ $[^{35}\text{S}]$ methionine in the presence of V. faba enzyme (and also E. coli in the case of peak 2) revealed that two distinctly different fragments were obtained. Not only do the fragments differ in electrophoretic mobility but also in electrophoretic behaviour. The streaking of Peak 1 $[^{35}\text{S}]$ met-oligonucleotide suggests that the $[^{35}\text{S}]$ oligonucleotide obtained is relatively large indicating that there is no guanylic acid residue close to the 3' OH end of this molecule. The fact that the major $[^{35}\text{S}]$ met-tRNA species from peak 1 and peak 2 yield such strikingly different $[^{35}\text{S}]$ oligonucleotides indicates also that the two represent different met-tRNAs having different nucleotide sequences near the amino acid acceptor end. Both are distinct from the major E. coli $[^{35}\text{S}]$ met-oligonucleotides indicating that these represent distinct V. faba met-tRNA's. Smith and Marcker (1970) report the separation of the $[^{35}\text{S}]$ met-oligonucleotides obtained by pancreatic digestion of separated $[^{35}\text{S}]$ met-tRNAs from ascites tumour cells. The $[^{35}\text{S}]$ met-oligonucleotides obtained from the two met tRNA species exhibited very different mobilities, that of met-tRNA_M^{*} (the met-tRNA species which donates methionine internally) moved towards the cathode whilst that derived from met-tRNA_F^{*} (the formylatable species which acts as initiator tRNA when unformylated) moved towards the anode.

It is striking that both bean $[^{35}\text{S}]$ met-oligonucleotides moved towards the anode. The fact that ascites met-tRNA_F and V. faba Peak 2 (identified as the V. faba initiator tRNA) $[^{35}\text{S}]$ met-oligonucleotides both moved towards the anode is perhaps significant of some degree of similarity in the primary sequence of these two met-tRNA species. The f- $[^{35}\text{S}]$ met_F-oligonucleotide obtained on T₁ digestion of E. coli initiator tRNA, f-met-tRNA_F, (Fig.38) also moved towards the anode. Smith and Marcker (1970) report that separated met-tRNAs from mouse liver and yeast yielded similar digestion patterns. It is significant that in yeast, mouse liver and ascites tumour cells, the met-tRNA_F^{*} fragments had identical mobilities suggesting that the terminal sequence of all these initiator tRNAs were identical, but as pointed out by RajBhandary and Ghosh (1969) they were distinct from that of the E. coli met-tRNA_F fragment. In the absence of uniformity of digestion conditions it is not possible to compare directly the electrophoretic behaviour of the $[^{35}\text{S}]$ met-oligonucleotides obtained by T₁ digestion of V. faba met-tRNA's with those of yeast etc but the Peak 2 $[^{35}\text{S}]$ met-oligonucleotide^{is} more closely similar to that of the met-tRNA_F^{*} species from these eukaryotes than is that obtained from Peak 1. Smith and Marcker (1970) report that the $[^{35}\text{S}]$ met_m-oligonucleotide from ascites tumour cells moved towards the cathode with a mobility just less than that of free methionine and methionine sulphoxide. Fig.38 shows^a similar mobility for the E. coli $[^{35}\text{S}]$ met_M-oligonucleotide. It is noteworthy that the V. faba peak 1 met-tRNA species (later identified as met-tRNA_M) exhibited a considerably different electrophoretic behaviour, moving as a streak towards the anode. This is indicative not only of different nucleotide sequence

near the amino acid acceptor end but also of a different overall charge on $[^{35}\text{S}]_{\text{met}}$ -oligonucleotides.

When essentially the same procedure was carried out using 27C/mM $[^{35}\text{S}]_{\text{methionine}}$, not only were the fragments described above obtained but also additional fragments present in small amounts in Peak 1 were identified (Fig.39). The presence of a fragment with mobility identical to the E. coli $[^{35}\text{S}]_{\text{met}_F}$ oligonucleotide when aminoacylation was carried out using E. coli enzyme, and further the presence of an additional fragment identical to E. coli f- $[^{35}\text{S}]_{\text{met}_F}$ -oligonucleotide when aminoacylation by E. coli enzyme was carried out in the presence of formyl donor suggests that this fragment results from small amounts of an E. coli like $\text{trNA}_F^{\text{met}}$ in Peak 1. Since aminoacylation by both bacterial and bean enzymes revealed slight indications of an E. coli like $[^{35}\text{S}]_{\text{met}_M}$ -oligonucleotide fragment it is likely that these represent unresolved trNA^{met} species derived from mitochondria and chloroplasts and having identical coding properties and electrophoretic behaviour to E. coli trNA^{met} species. The presence of slight amounts of peak 2 met-tRNA attests further to the heterogeneity of Peak 1. The absence of additional fragments when Peak 2 was aminoacylated using 27C/mM $[^{35}\text{S}]_{\text{methionine}}$ indicates that this fraction was homogenous with respect to trNA^{met} . The lack of contaminating E. coli like trNA^{met} species is further evidence of the difference between the E. coli like trNA^{met} species and that V. faba trNA^{met} species identified (for reasons discussed later) as involved in polypeptide chain initiation.

In view of the incomplete resolution of the two major tRNA^{met} species on DEAE-Sephadex, it was decided to investigate the resolution upon BD-cellulose. Esterification of the hydroxyl group of DEAE-cellulose by benzoyl residues leads to a product with altered physical properties. BD-cellulose is an ion-exchange with an increased affinity for lipophilic and aromatic groups. Chromatography of crude V. faba tRNA^{met} on BD-cellulose yielded 3 peaks, designated $\text{tRNA}_1^{\text{met}}$, $\text{tRNA}_2^{\text{met}}$, $\text{tRNA}_3^{\text{met}}$ in order of elution. This is in keeping with the nomenclature of the wheat germ workers (Tarrago et al., 1970; Leis and Keller, 1970; Marcus et al., 1970b). Tarrago et al., (1970) report the separation on BD-cellulose of two major met-tRNA species from wheat embryo, the two peaks were not as completely resolved as the corresponding V. faba species. There was some slight indication of a third minor peak as obtained with V. faba. An essentially similar separation is reported by Leis and Keller (1970). These workers showed that wheat germ contained three chromatographically distinct met-tRNA's. The two major species eluting at approximately 0.56M-NaCl and 0.65M-NaCl respectively were less completely resolved from one another, than the corresponding species from V. faba. Although both V. faba and wheat germ met-tRNA's were eluted at approximately the same NaCl molarity, the apparent lack of resolution of the wheat germ met-tRNAs reflects only the difference in fraction size. In the case of V. faba 3 ml fractions were collected whilst Tarrago et al. collected 7 ml fractions, hence the apparent lack of resolution. In both cases the $\text{tRNA}_3^{\text{met}}$ eluted at approximately the same molarity and was incompletely resolved from $\text{tRNA}_2^{\text{met}}$. Takeishi et al., (1968) report the elution at 0.57M-NaCl and 0.72M-NaCl

approximately from BD-cellulose of the 2 major tRNA^{met} species from yeast. Smith and Marcker (1970) report the resolution of two methionine tRNA species from ascites tumour cells on BD-cellulose. It may be pertinent that in this case the fractions elute at approximately 0.5M-NaCl and 0.58M respectively, the fraction eluting first was identified as the met-tRNA_F^{*} species. It is perhaps noteworthy that the tRNA^{met} species from the plant sources appear to elute at closely similar sodium chloride molarities, whilst the ascites tumour cell tRNA^{met} species elute at considerably different molarities.

Although both V. faba tRNA₁^{met}, and tRNA₂^{met} were aminoacylated by bean enzyme, the degree of aminoacylation of tRNA₁^{met} was almost twice that of tRNA₂^{met}. In contrast only tRNA₁^{met} was aminoacylated by E. coli enzyme suggesting that this fraction can be equated with peak 2 from DEAE-sephadex. A similar situation has been confirmed by the various workers using wheat germ tRNA, all report that the tRNA₁^{met} was aminoacylated with approximately equal efficiency by both homologous and heterologous enzymes, whilst tRNA₂^{met} was aminoacylated only by the homologous enzyme. (Ghosh, et al., 1971, Leis and Keller, 1970; Tarrago et al., 1970)

Autoradiographs of bovine pancreatic digests of $\left[\begin{smallmatrix} 35 \\ S \end{smallmatrix} \right] \text{met-tRNA}_1^{\text{met}}$, $\left[\begin{smallmatrix} 35 \\ S \end{smallmatrix} \right] \text{met-tRNA}_2^{\text{met}}$ (Fig.37) failed to reveal any indication of an N-formyl-methionyl-adenosine fragment suggesting that neither of the major tRNA^{met} species are formylated by bean transformylase. Under the conditions described in the legend to Fig.37 the degree of aminoacylation tRNA₃^{met} was so low that no radioactive fragments

could be identified. The use of $[^{35}\text{S}]$ methionine of higher specific activity (27C/mM) revealed traces of N-formyl-methionyl-adenosine suggesting that this minor tRNA can be formylated by both homologous and heterologous enzyme. This species however is present in such exceedingly low concentrations that it must be assumed to represent a contaminating tRNA^{met} derived from organelles. It could be argued that since the steps in initiation occur relatively infrequently compared to those involved in chain elongation the amount of specific initiation tRNA required is considerably lower than the amount of $\text{tRNA}_M^{\text{met}}$ hence it may be present in low amounts. In the bacterial system, however it is known that the proportions of $\text{tRNA}_F^{\text{met}} : \text{tRNA}_M^{\text{met}}$ are approximately 60:40, suggesting that this is not in fact the case.

The autoradiographs of the T_1 digests of $[^{35}\text{S}]$ met-tRNA₁^{met}, $[^{35}\text{S}]$ met-tRNA₂^{met} revealed essentially similar fragments to those obtained from similar digests of Peak 2 and Peak 1 respectively from DEAE-Sephadex. In this instance however, there was no evidence of additional E. coli like fragments, nor cross contamination between the two tRNA^{met} species indicating the homogeneity of the fractions with respect to methionine acceptor capacity. The absence of significant contamination by E. coli like fragments probably results from the resolution of the organelle tRNA^{met} species as a distinct third peak. In view of the nature of the T_1 fragments, and of the aminoacylation by both heterologous and homologous enzyme, $\text{tRNA}_1^{\text{met}}$ is equated with that tRNA^{met} species which elutes in Peak 2 from DEAE-Sephadex, whilst $\text{tRNA}_2^{\text{met}}$ is equated with Peak 1 from DEAE-Sephadex.

In summary it may be stated that, in accordance with situation in wheat germ (Leis and Keller, 1970; Tarrago et al., 1970; Ghosh et al., 1971; Marcus et al., 1970b) and rabbit liver (Bhaduri et al., 1970) there exist in V. faba two major methionyl-tRNA's only one of which can be aminoacylated by E. coli enzyme. Neither of the major tRNA^{met} species can be formylated by homologous or heterologous enzyme and initiation cannot therefore involve a formylated met-tRNA species.

The characteristic of an initiator tRNA is that it should donate its amino acid into the N-terminal position of the nascent polypeptide in response to specific initiator codons. As will be discussed more fully later, this amino acid does not always coincide with the N-terminal amino acid of the released polypeptide since the N-terminal residue(s) may be enzymatically cleaved following or during the completion of the polypeptide. In order to ascertain which of the two major tRNA^{met} species donated its methionine residue N-terminally, these species were incubated at 4mM Mg²⁺ in a complete amino acid incorporating system directed by the synthetic messengers Poly(AUG) or Poly(UG) and by endogenous mRNA present in the V. faba microsome preparation and the percentage incorporation into the N-terminal position of the resultant polypeptides measured. At low Mg²⁺ the initiator tRNA is assumed to donate methionine N-terminally in response to the triplets AUG and GUG, whilst tRNA_M^{met} will donate methionine internally in response to the triplet AUG only. The triplet GUG, at all positions other than the 5' terminus of a translatable sequence of mRNA, codes for valine. In E. coli f-met-tRNA_F has been shown to donate its methionine specifically into the N-terminal position and does not donate methionine in response to internal AUG codons (Clark and Marcker, 1966a,b).

It would be expected, therefore, that methionine donated by the initiator tRNA in response to both Poly(AUG) and Poly(UG) would be almost exclusively N-terminal, whilst that donated by tRNA_M^{met} in response to Poly(AUG) would be exclusively internal, and no methionine should be donated internally in response to Poly(UG).

N-terminal analysis of the polypeptide products of incorporation directed by endogenous messengers Poly(AUG) and Poly(UG), (Table 19) shows that met-tRNA₁^{met} is much more effective than met-tRNA₂ at donating methionine into the N-terminal position. Although the results suggest that met-tRNA₁ donates methionine into the N-terminal position, the inconclusive nature of the results warrants explanation. The incorporation obtained was extremely low, being of the order of 500cpm/100 μ l incubation with met-tRNA₂ and 120cpm/100 μ l incubation with met-tRNA₁ and variations of only a few counts lead to significant variations in the % methionine found N-terminally. Marcus et al, 1970b using wheat embryo system programmed by tobacco mosaic virus RNA (TMV-RNA) found that 57% of the methionine donated by the initiator tRNA occurred in the N-terminal position of the synthesised polypeptides. These authors attribute the low value to a non-quantitative recovery by the fluorodinitrobenzene procedure of N-terminal amino acids. Since the results presented here were obtained using a modified Edman et al technique (Blombäck, 1966) it is unlikely that such an explanation is applicable here. It is however possible in view of the extremely low concentrations involved that significant errors result from the incomplete extraction of the reaction mixture following the Edman degradation. Bhaduri et al. (1970) found that 95% of the radio-activity donated by rabbit liver met-tRNA_F in a rabbit reticulocyte

system directed by endogenous mRNA was released after the first cycle of Edman degradation indicating its location at the N-terminus of the polypeptide products. In this instance the radioactive degradation product was located by chromatography. It is possible that such a method yields a more quantitative recovery of N-terminal $[^{35}\text{S}]$ methionine. In cell-free system derived from cytoplasmic extracts of cultured human (KB) cells infected with human adenovirus type 2(Ad2) synthesising eight Ad2 structural polypeptides, it has been demonstrated that 60% of the $[^{35}\text{S}]$ methionine donated from yeast met-tRNA_F is incorporated into the N-terminal position (Coffier *et al.*, 1971). Since $[^{35}\text{S}]$ methionine donated by $[^{35}\text{S}]$ met-tRNA₁ was found predominantly in the N-terminal position and that donated by $[^{35}\text{S}]$ met-tRNA₂ tended to occur internally in polypeptide chains it can be suggested that tRNA₁^{met} is the initiator tRNA whilst tRNA₂^{met} is equivalent to the tRNA_M^{met} which donates methionine internally in response to AUG.

One of the methods used in deciphering the genetic code was the so called 'triplet binding assay'. This method was based on the fact that particular ribonucleotides promote the binding of different aminoacyl-tRNAs to ribosomes. This binding can be conveniently assayed since free aminoacyl-tRNAs are not retained on nitrocellulose membrane filters, whereas those whose binding to ribosomes is promoted by the proper codon are retained. (Nirenberg and Leder 1964). One of the discriminating features of the bacterial initiation system is the binding of the f-met-tRNA to the ribosomes. At low Mg²⁺ concentration the reaction is specific for f-met-tRNA, and is dependent upon GTP and initiation factors

(Allende and Weissbach, 1967; Anderson et al., 1967; Leder and Nau, 1967; Ohta et al., 1967; Hille et al., 1967). Neither the unformylated nor any other aminoacyl-tRNA will bind to ribosomes in such conditions. Thus an AUG promoted GTP dependent binding at low Mg^{2+} is diagnostic of the initiating tRNA species.

Table 20 shows that only $[^{35}S]$ met-tRNA₁ and $[^{35}S]$ met-tRNA₃ were significantly bound at low Mg^{2+} concentrations, whereas 20mM Mg^{2+} was required for significant binding of $[^{35}S]$ met-tRNA₂. The binding of the initiator tRNA to ribosomes at low Mg^{2+} is known to be independent of the binding enzyme (TF₁) but is dependent upon the presence of the initiation factors. Since the particle preparation employed in this assay was a washed microsomal suspension it was assumed that these factors remained associated with the ribosomes and no additional initiation factors were added. A preliminary series of binding assays carried out in the presence of DE52 supernatant indicated that the effect of added DE52 supernatant was negligible, suggesting that AUG dependent binding of the tRNA₁^{met} and tRNA₃^{met} to V. faba ribosomes was independent of supernatant binding enzyme. It cannot however be concluded that it was completely independent of binding enzyme since this would be present as a ribosomal contaminant on the washed microsomes. The results do seem, however, to indicate the presence upon V. faba ribosomes of initiation factors. A similar AUG dependent binding at low Mg^{2+} by tRNA₁^{met} and tRNA₃^{met} has been reported for wheat germ ribosomes (Beis and Keller, 1970; Tarrago, et al., 1970; Ghosh, et al., 1971). A striking correlation is evident between the numerical values obtained here and those obtained, under similar conditions by Tarrago et al. A similar AUG dependent binding of met-tRNA_F^{*} to

reticulocyte ribosomes has been reported by Shafritz and Anderson (1970). This binding was dependent upon the initiation factors M_1 and M_2 .

Legon et al., (1973) have suggested that the initial step in eukaryotic initiation is the interaction of the 40S subunit with the initiator tRNA in the absence of mRNA. In view of this result the relevance of the AUG dependent binding at low Mg^{2+} of the initiator tRNA may seem doubtful. It should however be stressed that the microsome preparations used in this assay would contain a significant proportion of subunits (Figs. 11, 12) and would also have associated with them the initiation factor required for the dissociation of ribosomes into subunits capable of new rounds of initiation.

Although the demonstration of the AUG dependent binding at low Mg^{2+} is suggestive of the role of $tRNA_1^{met}$ as a natural initiator tRNA in the V. faba cell-free system the relevance of such data to the in vivo situation is uncertain. The possibility cannot be disregarded that this binding may represent only a vestigial activity retained by the eukaryotic $tRNA^{met}$ species. It should also be stressed that the product of the AUG dependent binding was not characterised. It was assumed that the bound $[^{35}S]_{met-tRNA_1}$ or $[^{35}S]_{met-tRNA_3}$ were not engaged in peptide bond formation, however this could have been checked by the incorporation of a hot TCA precipitation step. Lack of $[^{35}S]_{met-tRNA}$ necessitated the omission of this duplicate set of incubations.

The reaction of the AUG dependently bound $[^{35}\text{S}]$ met-tRNA was used to distinguish between binding at the initiation (peptidyl) and aminoacyl-tRNA sites (Bretscher and Marcker, 1966). Table 21 shows that only methionine bound as $[^{35}\text{S}]$ met-tRNA₁ or as $[^{35}\text{S}]$ met-tRNA₃ was released as methionyl puromycin, indicating that these tRNA^{met} species had bound at the peptidyl site. In contrast $[^{35}\text{S}]$ met-tRNA₂ and the additional molecules of the other $[^{35}\text{S}]$ met-tRNAs bound at high Mg²⁺ concentration, were not released on treatment with puromycin indicating binding at the aminoacyl site. Such data is further evidence implicating tRNA₁^{met} as a cytoplasmic initiating tRNA equivalent to the tRNA_{F*}^{met} of the mammalian systems.

In summary it may be concluded that developing seeds of *V. faba* contain two major and one minor tRNA^{met} species. Only one of the major species (tRNA₁^{met}) can be aminoacylated by *E. coli* enzyme and neither can be formylated. The minor species (tRNA₃^{met}) is aminoacylated and formylated by both *V. faba* and *E. coli* enzyme. Results of AUG dependent binding, release of methionyl-puromycin, and N-terminal analysis of the products of endogenous mRNA, Poly(AUG) and Poly(UG) directed incorporation all implicate tRNA₁^{met} in protein chain initiation on the cytoplasmic 80S ribosome. In view of its low concentration, formylation by both homologous and heterologous enzymes, AUG dependent binding and release of methionyl puromycin, tRNA₃^{met} is tentatively ascribed the role of initiator tRNA on 70S ribosomes in the chloroplasts and mitochondria. tRNA₂^{met} probably donates methionine internally in response to internal AUG triplets.

Essentially identical conclusions have been reached by the wheat

germ workers who identify $\text{trNA}_1^{\text{met}}$ as the cytoplasmic initiator on wheat germ 80S ribosomes. The almost identical behaviour of the $\text{trNA}_1^{\text{met}}$ species from V. faba and wheat germ on chromatography, AUG dependent binding, methionyl puromycin release suggests a close functional similarity between the two molecules.

It appears therefore that initiation, under the conditions investigated, in higher plants does not involve an N-blocked amino group. Although both wheat and bean appear to contain an active transformylase, the initiating tRNA does not appear to be formylatable. The source of the transformylase enzyme demonstrated in both V. faba and wheat germ enzyme preparations remains uncertain. The possibility exists that it is derived from organelles or that despite its redundancy, it is still retained as an evolutionary vestige in the cytoplasm of these higher plants.

Two trNA^{met} species have been demonstrated in yeast (Takeishi et al., 1968; RajBhandary and Ghosh, 1969) and in a number of mammalian systems (Caskey, et al., 1967; Smith and Marcker, 1970), one of which is formylatable (designated $\text{trNA}_{\text{F}^*}^{\text{met}}$), the other cannot be formylated and is designated trNA_{M}^* . Data from a variety of workers suggests that unformylated $\text{trNA}_{\text{F}^*}^{\text{met}}$ functions as a chain initiator in eukaryotes when either synthetic polymers or natural mRNA's are used as templates (Smith and Marcker, 1970; Brown and Smith, 1970; Houseman et al., 1970). In a mouse ascites tumour system, $\text{met-trNA}_{\text{F}^*}^{\text{met}}$ appears to donate its methionine moiety into the N-terminal position, in response to Poly(UG) or polymers such as AUG(U)_n, which begin with specific initiation codons. Thus $\text{trNA}_{\text{F}^*}^{\text{met}}$ can be equated with $\text{trNA}_1^{\text{met}}$

as the initiating tRNA. It is interesting that the properties of the yeast initiator tRNA are more closely similar to those of the mammalian than the plant initiator tRNA.

The evidence available seems to indicate that a special interaction occurs between met-tRNA_i (the generalised eukaryotic initiator tRNA), AUG and the 80S ribosome. This reaction may differ from that in the bacterial system in the sequence of addition of mRNA, but is probably in all other respects identical and results in the 'proper' initiation of polypeptide chain synthesis. In the bacterial system, an AUG codon at or near the end of the mRNA signifies f-met-tRNA_F and not met-tRNA_M. If however, as has been suggested by Legon et al., (1973) the first step in eukaryotic initiation is the interaction of the 40S subunit with met-tRNA_i; in the absence of mRNA then the selection of the met-tRNA_i; must be some function of the ribosome and its associated initiation factors rather than of a ribosome - mRNA complex - initiation factor complex. It would appear that in this situation the presence of a met-tRNA_i on the 40S subunit synchronises the binding of the mRNA in the correct reading frame, rather than vice versa as in the bacterial system. Schreier and Staehelin (1973a) have studied the participation of four eukaryotic initiation factors (IF-E_{1,2,3} and 4) in the initiation steps. IF-E₂ and IF-E₃ and GTP are absolutely required for the initial binding of met-tRNA_i to 40S ribosome subunits in the absence of mRNA, but only IF-E₂ is needed for the same initiation complex formation in the presence of artificial template Poly(AUG) suggesting IF-E₃ is required for binding of natural mRNA. IF-E₃ from a variety of sources is characterised by its large mass, having a sedimentation rate of about 17S. IF-E₂ was shown to form a GTP dependent complex with met-tRNA_i,

suggesting it is involved in binding met-tRNA₁ to the ribosome. In the absence of mRNA met-tRNA₁ binding to 40S subunits was just as efficient with IF-E₂ and IF-E₃ suggesting that IF-E₃ promotes the template independent binding of initiator tRNA₁, presumably complexed with IF-E₂ and GTP to 40S subunits. met-tRNA_M is not recognised or bound by these initiation factors. It is suggested that IF-E₃ combines initially with a 40S subunit to direct the binding of met-tRNA₁. Subsequently, at least the bulk of IF-E₃ dissociates from the initiation complex. It is postulated that a subcomponent of IF-E₃ is reversibly split off and remains on the met-tRNA₁.40S complex as a separate unit to help in the subsequent binding of natural mRNA. IF-E₃ may be heterogeneous with regard to such a component and thereby determine specificity of mRNA selection.

In both the bacterial and eukaryotic system, the initiator tRNA donates its methionine specifically into the N-terminal position of the nascent polypeptide and never interanly. One of the specific properties of the bacterial initiator tRNA is its inability to form a ternary complex with the transfer or elongation factor Tu and GTP (Ono et al., 1968; Skoultchi, et al., 1969). This property is essential for its function as a polypeptide chain initiator since it prevents entry into the ribosomal A site during the elongation process and consequently blocks its incorporation into the internal positions of the polypeptide chain. Tarrago et al. (1970) have demonstrated that the initiator tRNA from wheat resembles met-tRNA_F from E. coli in its inability to interact with the binding enzyme from wheat germ. It also fails to complex with the bacterial factor

Tu. Met-tRNA_M from wheat is similar to met-tRNA_M from E. coli in that it forms the ternary complex with the enzyme from both organisms. Since the binding enzyme from V. faba has not been purified such an interaction could not be investigated. The lack of promotion of AUG dependent binding of V. faba met-tRNA₁ and met-tRNA₃ at low Mg²⁺ by DE52 supernatant may indicate, however, the inability of the analogous V. faba enzyme to promote enzymatic binding of met-tRNA₁ or met-tRNA₃ to V. faba ribosomes. The inability of the initiator tRNA to form a complex with the binding enzyme and GTP has been proposed to represent the mechanism whereby the initiator tRNA is distinguished from the other aminoacyl-tRNA's. Richter and Lipmann (1970) however, have demonstrated that both formylatable and non-formylatable met-tRNAs of yeast bind to the yeast binding factor T₁. This suggests that either yeast cannot use the same mechanism as E. coli for distinguishing the initiator tRNA or that some other undetected factor is involved. In strict contradiction to this Ghosh and Ghosh (1972) report the ability of met-tRNA₁ from wheat germ to donate its methionine internally into growing polypeptides in an E. coli system. This transfer which is less efficient than met-tRNA_M is dependent upon the presence of initiation factors and f-met-tRNA_F.

Three possible models for initiation on 80S ribosomes exist.

- (1) met-tRNA₁ (without a formyl or other N-blocking group) is the only initiator and incorporates methionine into the N-terminal position of all nascent proteins. Since methionine is not a common N-terminal amino acid in eukaryotes, it must be removed again in most cases.
- (2) met-tRNA₁ may select the reading frame only and not be incorporated into protein. Such a mechanism of initiation would result in a series of steps quite discrete from those involved in bacterial initiation.

(3) a class of cytoplasmic tRNAs might exist with structural features identical to tRNA₁^{met}, these would be specifically adapted for their role as chain initiators.

The best evidence in favour of model i) is the demonstration of N-terminal methionine on nascent peptides of proteins which do not normally have a N-terminal methionine residue. The original indication of methionine as the protein initiator in eukaryotes came from the studies on initiator mutants in yeast (Stewart et al., 1969). Several mutants of isocytchrome C were isolated which had methionine as an additional amino acid at the N-terminus. It was therefore suggested that the mRNA coding for isocytchrome c carried an AUG codon that preceded the normal N-terminal threonyl codon and coded for methionine. No cytochrome c was made when the AUG codon was mutated. Further evidence for the presence of an additional methionine residue at the N-terminal of a nascent protein has been provided by Wilson and Dintzis (1970). These authors demonstrated the presence of an additional methionine residue at the N-terminus of nascent α haemoglobin chains attached to the ribosome. It is proposed that the additional methionine residue is cleaved during early stages of translation exposing the normal N-terminal residue, valine. The removal of the additional methionine N-terminal residue can occur either during translation or after the completed polypeptide has been released from the ribosome. Evidence has been provided (Jackson and Hunter, 1970) that cleavage of the N-methionine residue of nascent globin chains occurs early on in translation. Rabbit reticulocyte ribosomes were synchronised with sodium fluoride, which inhibits initiation of new polypeptide chains but does not inhibit completion of pre-existing chains. When the sodium fluoride was removed these ribosomes initiated new nascent globin chains, but elongation was prevented by the presence of high concentrations of sparsomycin. Ribosomes with attached nascent

peptides were isolated and the peptides shown to contain only methionyl-valine and methionine at high sparsomycin concentration. Since the short peptides synthesised carried methionine at the N-terminus whilst longer chains carried the valine at the N-terminus it was postulated that methionine was removed during completion of the polypeptide chains. Ribosomes carrying nascent peptide chains initiated in the presence of sparsomycin were incubated under conditions favouring elongation and release of polypeptide chains. Under these conditions valine and other amino acids were detected in the long peptides, but very little methionine. Jackson and Hunter suggest that methionine is removed from the nascent globin chains after the addition of 15-20 amino acids.

The initiator tRNA, met-tRNA_I, whether from a heterologous source such as yeast (Takeishi, et al., 1970; Housman et al., 1970) or rabbit liver (Bhaduri et al., 1970) or from the homologous source (Hunter and Jackson, 1971) donates its methionine moiety into the N-terminal position of nascent globin peptides, whereas tRNA_M donates its methionine only internally.

Wigle and Dixon (1970) have demonstrated additional methionine residue at the N-terminus of protamine synthesised by cell suspensions from trout testis; the normal N-terminal residue being proline.

Using a cell-free system from wheat embryo which synthesises viral specific peptides under the direction of TMV-RNA, Marcus et al., (1970b) have demonstrated the presence of unblocked methionine at the N-terminus of labelled peptide products of short term incubations.

There appears to be no concessions in the timing of the removal of the N-terminal methionine from those proteins where it is not the normal N-terminal residue. Caffier et al., (1971) report data consistent with the suggestion that N-terminal methionine is not necessarily cleaved while the protein is still ribosome bound. In a cell-free system from KB cells synthesising adenovirion proteins these authors found that 5% of the methionine incorporated into released proteins, both in vivo and in vitro, was present at the N-terminus. Methionine does not normally occur as an N-terminal amino acid of adenovirion proteins. It was calculated that if the synthesis of each of the eight virion polypeptides began with methionine, and the methionine was not cleaved immediately, at least 7% of the incorporated methionine in completed polypeptides should be N-terminal. The data is therefore highly suggestive of the presence of methionine as an additional N-terminal residue on released polypeptides. Similarly, Wigle and Dixon (1970) suggest that the additional N-terminal methionine residue present on newly completed protamine chains, synthesised in trout testis cells, may be removed after the completion and release from the ribosome of the polypeptide chain. Extracts of testis cells contain an enzyme activity which can cleave the bonds between methionine and the second amino acid, proline.

Rho and Gib de Busk, (1971) report the presence of an additional methionine residue at the N-terminus of short nascent peptides of proteins from mycelia of Neurospora crassa. This residue is absent from the mature proteins where glycine, alanine and serine are the major N-termini. Chatterjee et al., (1972) similarly

report the presence of methionine as the N-terminal residue of proteins synthesised in vivo in HeLa cells.

There appears to be a considerable body of evidence implicating a methionyl-tRNA species in eukaryotic protein chain initiation. It is likely that the fundamental mechanism of protein synthesis may be essentially similar in prokaryotes and eukaryotes, the significant difference being the lack of requirement for an N-blocked amino group present on the eukaryote initiator tRNA. This may reflect an evolutionary sophistication of the ribosome which has enabled it to take over the function of the formyl group and permitted this additional requirement of initiating tRNA to be dispensed with.

The evolution of an specific mechanism concerned with maintaining the fidelity of protein initiation was probably a late event in the evolution of the protein synthetic apparatus as we now know it. It is probable that primitive initiation proceeded via a mechanism resembling that proposed by Schreier and Noll (1970) which was independent of accessory initiation factors and specific initiation tRNAs. It would seem logical that such sophistications were a late addition which proceeded the evolution of specific initiation signals. This idea is supported by the pattern of degeneracy, which suggests that the initiator triplet AUG was one of the later additions to the genetic code (Crick, 1968). AUG was probably converted to this specific use, after having functioned for considerable epochs as the normal codon for methionine. There is, as yet, no convincing explanation as to the possible reason for the choice of methionine in preference

to all the other available amino acids. Perhaps when the 3-dimensional structure of the initiator tRNA is elucidated, the structural peculiarity which dictated such a choice will become evident. The assumption that the initiating mechanism was a late refinement to the archaic protein synthesising machinery would explain the status of the initiation factors. These factors are not considered as true ribosomal proteins but merely; as accessories which are bound to the ribosome. When stripped of its initiation factors the ribosome may revert both structurally and functionally to a more primitive mode of translation. It is possible that the first initiating mechanism relied solely on a terminal AUG triplet and that the evolution of untranslated 5' nucleotide sequences preceding the initiator AUG was an even later refinement ensuring increased accuracy of translation. It is likely that such structures are involved in the recognition of the mRNA by the initiation factors, such a function may implicate these regions in some form of translational control. It is probable that in the primitive protein synthesising systems, all mRNAs were translatable. Incorporation into the mRNA of some form of structure whereby translational control could be mediated would considerably reduce the amount of redundant translation. It is likely therefore that the evolution of such structures was closely paralleled by that of an initiation factor(s) which could interact only with specific pre initiator sequences to mediate a degree of translational control.

Unlike the situation in prokaryotes where a considerable degree of control of protein synthesis is exercised at the transcriptional level (Jacob and Monod, 1961) by the action of repressor proteins and sigma factors (σ) (Burgess et al., , 1969), evidence suggests that control is exercised in eukaryotes mainly at the

translational level.

The best known example of eukaryote translational control is that exhibited in Acetabularia. The behaviour of pulse-labelled RNA (Olszewska and Brachet, 1961) and of some basic proteins (Olszewska et al., 1961; Werz, 1961; De Vitry, 1965) as well as the effects of RNase (Stich and Plaut, 1958), ultraviolet irradiation (Olszewska et al., 1961) actinomycin (Brachet, et al., 1964) and puromycin (Brachet, 1963) on Acetabularia morphogenesis, have led to the suggestion that morphogenesis is probably initiated by a long-lived mRNA which is synthesised in the nucleus and accumulates at the apex of the stalk. In such a system control would be exhibited mainly at the translational level. The mechanism which such a control is mediated in Acetabularia remains obscure.

The biosynthesis of haemoglobin appears to be subject to a considerable degree of translational control. Haemoglobin is synthesised in the anucleate reticulocyte on a stable mRNA and control of its synthesis must therefore depend upon a translational rather than transcriptional mechanism. Although the precise mechanism of control is not understood, both haem and the initiation factors have been implicated in control mechanism.

Reticulocytes incubated above 25^o in the absence of haem show an abrupt reduction in the rate of globin synthesis and a disaggregation of polysomes after about 10 min, both these effects are reversible by the later additional of haem. A similar situation is observed in reticulocyte lysates, but shut off is not as readily reversed after haem supplementation. Gross and Rabinovitz (1972) postulate that the control of globin synthesis is mediated by an

inhibitor (translational repressor) of globin chain initiation that is inactivated by haem and suggest that the inhibitor is a physiological regulator. Adamson et al., (1972) suggest that endogenously formed inhibitor completes with or somehow activates one or more of the initiation factors. However Hunt et al. (1972) found that lysates seem to have a certain fixed capacity to complete several rounds of globin synthesis before shut down occurs in the absence of haem and proposes that globin synthesis stops in the absence of haem because of the exhaustion or inactivation by protein synthesis of some component in the ribosome supernatant required for initiation. Kaempfer and Kaufman (1972) suggest that the translational control of globin synthesis by haem is exerted, directly or indirectly, through the initiation factor identified as IF-M₃, the eukaryotic equivalent of the bacterial F₃. These workers found that the continued recycling of reticulocyte ribosomes in lysates at 37° required an initiation factor whose activity was rapidly lost in the absence of added haem. This factor fully maintains the polysomes, (ii) inhibits the association of 40S and 60S ribosomal subunits into polysomes, (iii) promotes the quantitative entry of added 60S subunits into polysomes, (iv) allows the accumulation of ribosomal subunits instead of single ribosomes when initiation is blocked by aurintricarboxylic acid and (v) is absolutely required for the binding of globin mRNA to ribosomes. Such functions are consistent with the properties of a factor whose function is to mediate the recycling of ribosomes through the subunit cycle. The data suggests that endogenous IF-M₃ activity is lost in the course of cell-free protein synthesis unless haem is present. In the absence of IF-M₃ all reticulocyte protein synthesis should cease since ribosomal subunits would associate and therefore initiation would be inhibited,

1F-M₃, through its haem dependence would therefore allow the coordinate regulation of synthesis of haem and of polypeptide chains, largely globin, during erythroid development.

The mechanism by which ribosomes recognise the AUG start signal at the beginning of a message is not understood. The most obvious possibilities are:

(i) the secondary structure of mRNA exposes only the starting triplet and masks internal AUG triplets,

(ii) the ribosome response to an additional signal, e.g. a specific nucleotide sequence present only near the initiating AUG triplet. The latter possibility could explain translational control if it was assumed that the signal was specific for certain classes of mRNA, and if we postulate the existence of specific protein adapters that allow ribosomes to recognise only their cognate messengers. The most likely candidate for such adaptors is the initiation factors required for the correct attachment of ribosomes to natural mRNAs. Discrimination with different bacterial F₃ fractions and bacteriophage mRNA's have been reported (see the review of Revel, et al., 1972). Groner ^{et al.} (1972) have identified a factor i, interference factor, which exists in combination with F₃ and modifies the cistron specificity of E. coli F₃.

Evidence that one of the eukaryotic initiation factors may be messenger specific is that myosin RNA from thick embryo muscle cannot function on washed reticulocyte ribosome, unless muscle initiation factors are present (Heywood, 1969). Wigle and Smith (1973) have demonstrated that the initiation factor, 1F-M₃ from ascites tumour cells can distinguish between Encephalomyocarditis viral RNA, and globin mRNA. In contrast to reports of a tissue or species specific

protein initiation factors is the report by Fuhr and Natta (1972) of a messenger specific factor within the human reticulocyte which influences the translation of endogenous mRNA's. Chemical and enzymatic characterisation suggests that this factor is a low mol. wt. (10,000) RNA or polynucleotide which possess considerable secondary structure. There seems therefore to be an increasing body of evidence implicating the initiation factors in translational control. It should be stressed that instances in which control has been shown to be mediated via the initiation factors F_3 or $1F-M_3$ are highly artefactual situations and the general applicability of such control mechanisms mediated by such factors or their specific adaptors is still uncertain.

The presence of mRNA-specific factors can only be confirmed by fractionation to obtain individual factors followed by suitable assays with mRNA under optimal conditions.

Initiation by no means is the only step in protein synthesis at which translational control can be exerted. The rate of translation can be subject to control by a variety of factors interacting at the elongation and termination step. It is however likely that the most economic control of mRNA translation is exerted at the step of protein chain initiation.

In summary and conclusion one might simply quote more fully the anonymous contributor to 'Nature' whose words appears on page ii of this thesis. 'The capacity to synthesise protein at 37°C in a dilute aqueous environment is virtually half the definition of life. It would therefore be naïve to expect the basic pathways of protein synthesis to differ greatly between organisms that are as distantly related as rabbits and bacteria. Protein synthesis

is too fundamental a property to have been left to the vagaries of parallel evolution. But on the face of things it would be equally naïve not to expect numerous variations on the basic theme to have accumulated since, for example, bacteria and eukaryotes shared a common ancestor. What is so surprising is that those anticipated idiosyncrasies are proving so few and far between'. (Nature (1970), 227, 659).

The work presented in this thesis serves well to illustrate the concept of an underlying biochemical unity in living organisms. It may also be used to strengthen the points, obvious to some, apparently less obvious to others, that higher plants are not bacteria, neither are they rabbit reticulocytes or rat livers, and that continued investigations of the biochemistry and molecular biology of this most important Kingdom are not only justifiable but immensely necessary.

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