Cell-free protein synthesizing systems from Vicia faba (L)

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CELL-FREE PROTEIN SYNTHESIZING SYSTEMS FROM VICIA FABA (L)

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ABSTRACT

Vicia faba (L) seeds form protein as their major food reserve. The two major storage proteins, vicilin and legumin, which account for up to 20% dry weight of the mature seed are formed within a comparatively short period during seed development, and during this period, a significant part of the total metabolism of the cell is directed towards their synthesis. An active cell-free amino acid incorporating system has been isolated from developing seeds, and its conditions of activity determined in *in vitro* incubations using polyuridylic acid as a messenger. Bacterial contamination of the incubations was low. The activity of this system in amino acid incorporation is compared with other *in vitro* systems from plants.

Microsomes, enzyme fractions and tRNA were prepared from developing seeds of different ages, and the above system was used to assay changes in the amino acid incorporating activity of components from different ages of seeds, to see if any correlation could be found between the changes in the *in vivo* protein synthetic activity during seed development with the changes in the *in vitro* efficiency of the components in incorporation. Rates of *in vitro* amino acid incorporation were compared with the estimated rate of *in vivo* synthesis of the storage proteins. The changes in the

in vitro amino acid incorporating activity are discussed with reference to the anatomical changes occurring during seed development, as seen in electron micrographs of developing seeds, and in particular with reference to the changes in the free and membrane bound ribosome content. Further evidence for the role of the membrane bound ribosomes in storage protein synthesis is presented.

CONTENTS

INTRODUCTION

In the 1940's it was realized that nucleic acids played a decisive role in protein biosynthesis and since then work on the mechanism of protein synthesis has been inseparably linked with studies of the nucleic acids and their interactions with proteins. The linear arrangement of the four bases, adenine, thymine, guanine and cytosine in the deoxyribo nucleic acid* (DNA) molecule determines the arrangement of the amino acids in a given protein, through the intermediary, messenger ribonucleic acid (mRNA), which translates the DNA code and transfers it to the cytoplasm, where protein synthesis mainly occurs.

In vivo and *in vitro* studies of amino acid incorporation over the last 20 years, on a wide range of organisms have led to the acceptance of a general scheme of protein synthesis for all organisms. The first studies on *in vivo* incorporation of labelled amino acids were done by Hultin (1950) with chicks and Keller (1951) with rats. Both recorded that the liver microsomes were more highly labelled than any other fraction. When the labelled microsomes were disintegrated with the anionic detergent deoxycholate, the radioactivity was found

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* The abbreviations and conventions used throughout this thesis are those recommended in "The Biochemical Journal" (Biochem J. *116*, 12, 1970).

mainly in the "deoxycholate-insoluble fraction," which was shown by ultracentrifugal analysis and electron microscopy to consist chiefly of ribosomes. (Littlefield, Keller, Gross and Zamecnik, 1955). In vitro studies soon followed, which have proved much more useful in deciphering the genetic code, in identifying the co-factors needed for protein biosynthesis, determining the conditions of reaction and, over the last few years, identifying the individual stages of chain initiation, elongation and termination that are involved in protein biosynthesis.

Siekevitz (1952) reported the first in vitro system from rabbit liver, in which there was evidence for peptide bond formation. His system contained a microsome fraction, mitochondria with substrates to provide energy, soluble cytoplasm and a radioactive amino acid. It was later shown (Zamecnik and Keller, 1954) that the mitochondria were unnecessary and could be replaced by ATP and a source of high energy phosphate (e.g.) phosphoenol pyruvate plus pyruvic kinase to regenerate the ATP.

The first cell-free system from plants reported to incorporate amino acids was described by Stephenson, Thimann and Zamecnik (1956) using tobacco leaf extracts. Since then, active amino acid incorporating systems have been recorded from a wide range of plant material (see Mans, 1967;

Boulter, 1970, for references), and although work on protein synthesis in plants is not as far advanced as work done with bacterial and mammalian systems, there is solid evidence that the overall process is similar.

Protein biosynthesis involves the formation and stepwise elongation of peptide chains from the N-terminal amino acid (Dintzis, 1961). The order of addition of the amino acids is under strict genetic control and is determined by the sequence of bases in mRNA; each amino acid being specified by a codon consisting of three bases. The first steps in the elucidation of the genetic code were undertaken using synthetic polynucleotides of known composition, but random sequence as messengers in in vitro incorporation in Escherichia coli, but problems in determining the actual sequence of the bases in the triplets resulted in some ambiguities. The final unambiguous elucidation of the code was the result of the triplet binding experiments of Nirenberg et al. (Nirenberg, Caskey, Marshal, Brimacombe, Kellogg, Doctor, Hatfield, Levin, Rottman, Pestka, Wilcox and Anderson, 1966) and the production and use of synthetic polynucleotides of known repeating sequence in in vitro incorporation by Khorana's group. (Khorana, Buchi, Ghosh, Gupta, Jacob, Kossell, Morgan, Narang, Ohtsuka and Wells, 1966).

All 64 possible sequences of three nucleotides have been assigned to amino acids, with the exceptions of UAA, UAG and UGA which probably act as chain terminators, known as ochre, amber and opel respectively. Although the genetic code was originally determined for E.coli, work with a variety of other organisms suggests that it is probably universal.

Crick (1957, 1958) thought it unlikely that the amino acids would be capable of binding directly to the mRNA template. He postulated the existence of small "adaptor" molecules which would recognise and bind the amino acids and would interact with specific nucleotide sequences of the template mRNA. Hoagland, Zamecnik, Sharon, Lipman, Stulberger and Boyer (1957) working with in vitro rat liver protein synthesising systems showed the presence of an amino acyl RNA complex which dissociated in the presence of a microsomal suspension, with the incorporation of the amino acid into the microsomal fraction. This RNA fraction is known as transfer RNA (tRNA).

tRNA has since been purified from a wide range of sources, and there is at least one specific tRNA for each of the 20 common amino acids used in protein biosynthesis. All tRNA species are characterized by the presence of at least three distinct sites:-

(i) the ACC terminal end, which accepts the amino acid;

(ii) the anticodon, which recognises and binds to the relevant codon of the mRNA;

(iii) a complex site which is recognised by the aminoacyl tRNA synthetase enzyme specific to that species of tRNA.

The first primary structure of a tRNA species to be elucidated was alanine tRNA from yeast (Holley, Apgar, Everett, Madison, Marquisee, Merrill, Penswick and Zamir, 1965). Since then the nucleotide sequences of a wide range of tRNA species have been determined and all are very similar (see reviews, Lengyel and Söll, 1969; Zachau, 1969), reflecting not only similarities in function, but also evolutionary relationships. The most likely structure of the tRNA molecule is the clover leaf model proposed by Holley et al. (1965), and all tRNA species of known nucleotide sequence can be arranged in this form, although the nucleotide sequences in the paired regions differ (Zachau, 1969). The tertiary structure of tRNA has not yet been fully elucidated, although it is thought that the loops of the clover leaf are folded over each other, to form a compact structure, held together by further base pairs and hydrogen bonds, and from which the anticodon region and the amino acid carrying 3' terminal adenosine moiety protrude. (Cramer, Doepner, Haar, Schlimme and Seidel, 1968). It has been suggested that some of the unusual nucleotides present in tRNA, which may account for up to 20% of the nucleotides per molecule (Madison,

Everett and King, 1966) may be intimately involved in the tertiary structure of tRNA (Crick, 1966, Woese, Dugre, Kondo and Saxinger, 1966), as they occupy specific and characteristic positions in each species of tRNA.

The first step in protein biosynthesis is the activation of the amino acids and the formation of an amino acyl - tRNA complex. These reactions are catalysed by amino acyl tRNA synthetase enzymes that are specific for each tRNA species, and can be represented by the following equations:

$$\text{Enzyme} + \text{ATP} + \text{AA}' \rightleftharpoons \text{Enzyme} - \text{AA}' - \text{AMP} + \text{PPi}$$

$$\text{Enzyme} - \text{AA}' - \text{AMP} + \text{tRNA}' \rightleftharpoons \text{AA}' - \text{tRNA}' + \text{AMP} + \text{Enzyme}$$

The overall reaction is usually studied by measuring the acceptance of [^{14}C] amino acid by tRNA. The first part of the reaction can be studied independently from the second by the amino acid dependent exchange of [^{32}PPi] with ATP, or by the ATP dependent synthesis of amino acid hydroxymates (Stulberg) and Novelli, 1962). There is some evidence that ATP is bound to the enzyme before the amino acid (Allende, Allende, Gatica, Celis, Mora, Matamala, 1966; Rouget and Chapeville, 1968), and that the site for amino acid activation is different from the site for amino acyl transfer (Cassio, 1968).

The second part of the reaction was more difficult to study, until work by Allende and Allende (1964) and Norris and Berg (1964) showed that the enzyme-amino acyl-adenylate compound

could be isolated from the reaction mixtures by passing through sephadex G-50 or G-75 columns. Norris and Berg (1964) working with highly purified isoleucyl tRNA synthetase from E.coli and substrate amounts of enzyme with [^{3}H] and [^{14}C] isoleucine showed that 1 mole of enzyme bound 1 mole of isoleucyl-adenylate and the isolated enzyme - isoleucyl - adenylate compound would transfer the isoleucyl moiety to tRNA.

A wide range of amino acyl tRNA synthetase enzymes have been isolated from many different sources, and although most of the work has been done with bacterial or animal enzymes, there has been considerable progress in the work on purified plant synthetases over the last few years. Alanyl tRNA synthetase has been purified from tomato roots (Attwood and Cocking, 1965); arginyl and lysyl tRNA synthetases from Canavalia ensiformis (Fowden and Frankton, 1968); valyl, lysyl and methionyl tRNA synthetases from wheat germ (Moustafa and Lyttleton, 1963); prolyl tRNA synthetase from Phaseolus aureus and Polygonatum multiflorum (Peterson and Fowden, 1965); phenylalanyl and tyrosyl tRNA synthetases from Phaseolus aureus and phenylalanyl tRNA synthetase from Leucaena leucocephala (Smith and Fowden, 1968). Large scale isolation and purification of prolyl tRNA from Phaseolus aureus has been reported by Dunnill, Dunnill, Boddy, Houldsworth and Lilly (1967), who report a yield of 42g per

enzyme from 25Kg. seeds, representing 10.5% recovery.

The activation of amino acids and the formation of amino acyl tRNA could be considered one of the most important reactions in protein synthesis, as it is the step at which the 20 unit language of the protein is translated into the 4 unit language of the nucleic acids. There is no subsequent check on whether the tRNA is carrying the correct amino acid during the formation of peptide bonds on the ribosome. Chapeville, Lipman, von Ehrenstein, Weisblum, Ray and Benzer (1962) desulphurated cysteinyl - $tRNA^{cys.}$ to alanyl - $tRNA^{cys.}$ with Raney Nickel, and found that the alanyl groups appeared in the positions of cysteine in the protein chain. Thus, a mistake at this level, either by esterification of the wrong amino acid to tRNA, or by selection of the wrong tRNA will result in the insertion of an amino acid in a wrong position in the growing peptide chain. Depending on the protein and the position of the error, such a mistake could be fatal. The tRNA synthetases have, therefore, to recognise accurately their tRNA's and amino acids.

Mistakes are, however, known to occur in vitro, although at a low level (Bergman, Berg and Dieckman, 1961; Loftfield, 1963; Norris and Berg, 1964; Baldwin and Berg, 1966; Kondo and Woese, 1969). Isoleucyl tRNA synthetase from E.coli is capable of recognising and binding valine in vitro, but the

valyl moiety cannot be transferred from the enzyme-valyl-adenylate compound to any species of tRNA, and breaks down to enzyme, free valine and AMP in the presence of tRNA. Certain amino acid analogues, however, may be recognised by amino acyl tRNA synthetase enzymes and transferred to tRNA. Peterson and Fowden (1963) report that prolyl tRNA synthetase from Phaseolus aureus will catalyse the incorporation of azetidine -2- carboxylic acid (a lower analogue of proline) into an amino acyl compound, although the corresponding synthetases from Convalaria and Polygonatum in which this compound occurs naturally, will not. This finding would seem to be correlated with the in vivo toxicity of this compound in P.aureus, but not in Polygonatum.

The mechanism by which the amino acyl tRNA synthetase enzymes recognise their relevant species of tRNA is unknown, but it is probably complex. Schulman and Chambers (1968) suggest that the recognition site between valyl tRNA synthetase and $tRNA^{val.}$ involves the first three base pairs at the 3' hydroxyl end of the clover leaf model of tRNA as the sole recognition site of the enzyme. Staehelin, Rogg, Baguley, Gisberg and Wehrle (1968) have shown that rat liver $tRNA^{ser}$ can be charged by seryl tRNA synthetase from yeast, as well as its own enzyme, although the nucleotide sequence in the presumed recognition site differ in two out of three base pairs in rat liver and yeast $tRNA^{ser.}$

More recent work by Lagerkvist and Rymo (1970) in which an average of seven nucleotide residues were removed from the 3' hydroxyl terminus of $tRNA^{val}$ from yeast, shows that the enzyme will still form a stable compound with the tRNA, and that altered $tRNA^{val}$ was a competitive inhibitor of native $tRNA^{val}$.

The anticodon has been suggested as the recognition site for amino acyl tRNA synthetases, as it is different in all species of tRNA, but this seems unlikely as it is known that the same synthetase enzyme will charge two different tRNA's that are specific for the same amino acid, but which differ in their anticodons (Sundharadas, Katz, Söll, Konigsberg and Lengyel, 1968). Several specific changes in or near the anticodon resulting in loss of codon recognition do not affect synthetase recognition of tRNA (Thiebe and Zachau, 1968; Yoshida, Furuichi, Kaziro and Ukita, 1968).

It seems most likely that recognition of tRNA by amino acyl tRNA synthetase enzymes is a complex process, involving the tertiary structure of the tRNA. (Fresco, Adams, Ascoine, Henley and Lindahl, 1966). Changes in the physical properties of tRNA can alter its interaction with the synthetase enzymes. Cramer et al.(1968) and Schlimme, v.d. Haar, and Cramer (1969) report that temperature dependent charging of $tRNA^{ser}$ from yeast shows a sharp drop at 39^{o}, which is not due to denaturation of the enzyme which only occurs at 45^{o} and above.

Baev, Fodor, Mirzabekov, Akselrod and Kazarinova (1967) have shown that both halves of $tRNA^{ala}$ and $tRNA^{val}$ are necessary for recognition by their synthetase enzymes, so that the recognition site would seem to be composed of different parts of the tRNA molecule, brought together in an ordered structure. More recent work by Chambers (1969) has shown that the stem of the clover leaf of the tRNA molecule is intimately involved in the recognition of tRNA by its specific synthetase, and that the loops are not essential.

Whatever the nature of the amino acyl tRNA synthetase site, it seems that it has been conserved throughout evolutionary development. Anderson (1969) has shown complete cross reactivity of alanyl tRNA synthetase and $tRNA^{ala}$ from E.coli, lobster muscle, rat liver and human spleen, and for arginyl and lysyl tRNA synthetases and tRNA from E.coli and human spleen.

As discussed earlier, the ribosome is the site of both in vivo and in vitro protein synthesis. Two different size classes of ribosomes exist: 70S which occur in procaryotic organisms, mitochondria and chloroplasts; and 80S which occur in the cytoplasm of eucaryotes. All ribosomes consist of two unequal subunits, 30S and 50S in 70S ribosomes (Tissières and Watson, 1958) and 40S and 60S in 80S ribosomes (Chao, 1957). The smaller subunit contains a single molecule of 16S or 18S

RNA and about 20 different proteins, while the larger subunit contains one molecule of 23S, 25S or 28S RNA, one molecule of 5S RNA and 30-40 different proteins. (Peterman, 1964; Spirin and Gavrilova, 1969).

Whatever their source, ribosomes seem to have the same function in protein synthesis, which suggest that the general features of structure have been preserved irrespective of differences in size or chemical composition. The structure and arrangement of tRNA and protein in the ribosome has been studied in the hope that it will elucidate more precisely the role of the various fractions in amino acid incorporation. Cotter, McPhie and Gratzer (1967) working with yeast, have suggested a model for the ribosome in which the helical regions of the tRNA project from the surface of the ribosome, and the amorphous, non-helical regions with their associated proteins project into the centre of the ribosome.

Protection of about 30-35 amino acids of the growing polypeptide chain from proteolytic enzymes (Malkin and Rich, 1967) and the inaccessibility of a segment of the mRNA of about 30 nucleotides length (Takanami and Zubay, 1964; Takanami, Yan and Jukes, 1965) suggest that there is a protected region inside the ribosome where peptide bond formation and polypeptide chain elongation occur. (Redman and Sabatini, 1966). Similar conclusions have been reached from electron microscopic studies of ribosomes

from a wide range of sources (Nanninga, 1963; Bruskov and Kiselev, 1968 a and b;) and are supported by hydrodynamic measurements of the ribosome (Peterman and Pavlovec, 1969).

Cox and Bonanou (1969) have suggested that the larger ribosomal subunit is horse shoe shaped and that the smaller subunit forms a cap over it. They have suggested a model structure for the rabbit reticulocyle ribosome along similar lines to those suggested by Cotter et al.(1967) for yeast ribosomes. The helical regions of the tRNA are arranged on the inside and outside of the ribosomal subunits, and are linked by the single stranded non-helical regions. The ribosomal proteins are associated with the non-helical regions of the tRNA, and their location is determined by the nucleotide sequence of the tRNA. (Osawa, 1968; Cox and Bonanou, 1969). Alteration of 6-8 bases of the 16S RNA by nitrous acid resulted in tRNA that was unable to form a functional 30S subunit with 30S ribosomal proteins, although there was no degradation of the RNA. (Nomura, Traub and Bechman, 1968). Protein-protein interactions play an important role in maintaining the secondary structure of the tRNA and the stability of the ribosomal structure (Osawa, 1968). This can be seen in the persistance of 70S and 80S ribosomes as distinct structural entities after extensive hydrolysis of the RNA moiety by RNase. (Santer, 1963; Cox, 1969)

when all protein biosynthetic activity has been lost. The protein molecules are essential for full activity of the ribosomal subunits. Absence or alteration of one of the protein molecules can affect the function of the ribosome. (Traub, Hosokawa, Craven and Nomura, 1967; Traub and Nomura, 1968 a,b,c; Traub, Söll and Nomura, 1968, Terwaoka, Tamaki and Tanaka, 1970).

Most recent work suggests a purely structural role for the 5S tRNA. (Cox and Bonanou, 1969, Forget and Reynier, 1970).

Mangiarotti and Schlessinger (1966) and Cundecliffe (1968) have suggested that free 70S ribosomes do not occur in E.coli in vivo, and all the ribosomes exist as free 30S and 50S subunits or as parts of polysomes. 70S ribosomes are artifacts that are caused by polysome breakdown during preparation of the ribosomes. Ribosomal subunit exchange has been demonstrated in vivo (Kaempfer, Meselson and Raskas, 1968) and in vitro (Kaempfer, 1968) in E.coli and it was found that it was coupled with protein synthesis. Guthrie and Nomura (1968) have suggested that the following sequence of events occurs during protein synthesis:-

(i) 30S subunit forms a complex with mRNA and formylmethionyl tRNA in the presence of GTP and certain initiation factors.

(ii) 50S subunit becomes attached to the complex, to form a 70S ribosome, as part of a polysome, which persists during the synthesis of the protein.

(iii) Once the protein is completed, the 70S ribosome dissociates into 30S and 50S subunits.

Similar cycling of the 80S ribosomal subunits have been reported by Hogan and Korner (1968); Kabat and Rich (1969); Kaempfer (1969) and Heywood (1970). Heywood suggests that a similar sequence of events occurs in eucaryotic cells as was suggested by Guthrie and Nomura (1968).

New amino acyl residues enter the polypeptide chain attached to tRNA, so that during the process of protein synthesis, the C' end of the polypeptide chain is not free, but attached to tRNA. (Nathans and Lipmann, 1961; Takanami, 1962; Gilbert, 1963; Bretscher, 1963, 1965). Thus, the formation of every peptide bond is the result of a reaction between peptidyl tRNA and amino acyl tRNA, during which, the peptidyl tRNA is increased by one amino acyl residue, and free tRNA is released.

The simultaneous presence of peptidyl tRNA and amino acyl tRNA in the functioning ribosome suggests that there are two separate tRNA binding sites on the ribosomes and their presence has been confirmed by experimental evidence. The mRNA binding centre and the amino acyl tRNA binding site are located on the smaller subunit, and isolated 30S subunits show the same capacity for selective binding of amino acyl tRNA in the presence of a template polynucleotide as the complete ribosome. (Okamoto and Takanami, 1963; Takanami and Okamoto, 1963; Suzuka, Kaji and Kaji, 1965; Kaji, Suzuka and Kaji, 1966; Pestka and Nirenberg, 1966). The peptidyl binding site which has an increased

affinity for N-blocked amino acyl tRNA's, and which only binds amino acyl or deacylated tRNA weakly, is located on the larger subunit. (Gilbert, 1963; Bretscher and Marcker 1966; Cannon, 1967). If the functioning ribosome is dissociated into subunits, the peptidyl tRNA remains firmly bound to the larger subunit, and Schlessinger, Mangiarotti and Apirion (1967) have shown that the peptidyl tRNA is the major contributor to the stabilization of the subunit association into a complete ribosome. The peptidyl-transferase centre is also located exclusively on the 50S subunit, and is an inherent part of it. (Traut and Monro, 1964; Monro, 1967).

Translation of mRNA consists of three main phases; initiation, elongation and termination.

Most of the original work on <u>in vitro</u> amino acid incorporation was done using very high magnesium levels, where the need for "proper" chain initiation is eliminated (Eisenstadt and Lengyel, 1967). Presumably under these conditions, amino acyl tRNA may attach to both the amino acyl and peptidyl sites on the ribosomes, and peptide bond formation occur between them. Mosteller, Culp and Hardesty (1968) working with the rabbit reticulocyte system found that the magnesium optimum for [^{14}C] valine incorporation into globin was 3mM, but the magnesium optimum for the polyuridylic acid (poly-U) directed polyphenylalanine synthesis was 10mM. This could, however, be reduced to 4mM by

preincubating the ribosomes with poly-U, deacylated tRNA, KCl and reduced glutathione, i.e. to about the same magnesium optimum as globin synthesis by natural messenger. $tRNA^{phe}$ was found to be responsible for this shift in the magnesium optimum (Culp, Mosteller and Hardesty, 1968).

Most of the work on chain initiation has been done with bacterial systems, especially E.coli, and a mechanism involving formylmethionyl tRNA as the only natural chain initiating tRNA in protein biosynthesis for procaryotic organisms is now generally accepted.

Waller (1964) observed that about 40% of E.coli proteins had methionine as the N-terminal amino acid, although methionine only constituted about 2·5% of the total amino acids in protein. This was strongly suggestive of the role of methionine in polypeptide chain initiation. Clark and Marcker (1966a) reported the presence of two methionine accepting tRNA's in E.coli, which could both be charged by the same methionyl tRNA synthetase enzyme, although only one of the tRNA species could be enzymatically formylated to N-formylmethionyl tRNA (fMet - tRNA). The tRNA's are therefore designated $tRNA_F^{met}$ and $tRNA_M^{met}$. The formylation of $tRNA_F^{met}$ is catalysed by a transformylase with formyl tetrahydrofolic acid acting as the formyl donor (Marcker, 1965). The transformylase is absolutely specific for met-$tRNA_F$, and will not formylate any other amino acyl tRNA's, indicating that it recognises a structural region unique to $tRNA_F^{met}$.

The coding properties of the 2 $tRNA^{met}$ differ. Both respond to the codon AUG, but $tRNA_F^{met}$ also responds to GUG, which is also the codon for valine. It has been suggested that GUG codes for valine when it occurs intramolecularly in mRNA and for fMet-tRNA when it is terminal. fMet-tRNA is always incorporated into the N' terminal position in proteins, never internally (Clark and Marcker, 1966, a, b), and it has been suggested that this is due to its inability to form a complex with the ribosome transfer factor FII and GTP, in contrast to all other amino acyl tRNA's (Ono, Skoultchi, Klein and Lengyel, 1968).

$tRNA_F^{met}$ has been reported present in many other species of bacteria (see Marcker and Smith, 1969 for references), in the mitochondria of yeast and rat liver (Smith[illegible] and Marcker, 1968) and in the mitochondria of HeLa cells (Galper and Darnell, 1969). There is, however, no evidence for $tRNA_F^{met}$ or any similar derivatives in the cytoplasm of higher organisms, despite extensive searches by many groups, and Marcker and Smith (1969) consider that its existence here is unlikely. This indicates that two different mechanisms for the initiation of protein synthesis exist, and implies that this part of the genetic code is not universal. Chain initiation involving the use of fMet-tRNA is therefore a characteristic of the 70S ribosome only.

Very little work has been done on chain initiation on the 80S ribosome, although work by Rich, Eikenberry and Malkin (1966) and Arnstein and Rahamimoff (1968) has indicated that valyl tRNA is the chain initiator for haemoglobin synthesis. This presents an interesting parallel between the reticulocyte and E.coli systems in that the codon GUG might be acting as the initiator in both cases.

Although the actual initiator molecule may be different, it seems likely that the mechanism of chain initiation on both the 70S and 80S ribosomes is similar, in that the role of the subunits is common to both, and initiating factors and GTP are needed (Heywood, 1970).

The initiating factors can be isolated from E.coli ribosomes by washing with 1M NH_4Cl. The fractions F_1, F_2 and F_3 of Iwaski, Sabol, Wahba and Ochoa (1968) are considered to be analogous to the fractions A, B and C of Revel and Gros (1966) and Revel, Lelong, Brawerman and Gros (1968). There is some indication that F_2 may consist of several non-identical subunits (Ochoa, 1968). Heywood (1970) has isolated initiation factors from chick muscle ribosomes by washing with 1M KCl.

Ochoa (1968) reports that F_3 is needed for the ribosomal binding of mRNA, and there is some evidence that this occurs

before the F_1 and F_2 dependent binding of fMet-$tRNA_f$ to the 30S subunit (Anderson, Bretscher, Clark and Marcker, 1967; Brown and Doty, 1968).

The formation of the 30S - mRNA - fMet - tRNA initiator complex requires GTP (Kolakofsky, Dewey, Herschey and Thach, 1968) although the GTP analogue, 5-guanylmethylenediphosphonate (GMPPCP) can substitute for GTP in this reaction, indicating that the GTP is not hydrolysed. Subsequent to this, the 50S subunit is attached to the 30S initiation complex, (Kolakofsky, Ohta and Thach, 1968). The next step involves the translocation of the fMet - tRNA from the amino acyl site on the 30S subunit to the peptidyl site on the 50S subunit. This is catalysed by F_2 and involves the hydrolysis of GTP. (Kolakofsky, Dewey, Hershey and Thach 1968). The amino acyl site is free to accept another amino acyl tRNA, and chain initiation is completed.

Unformylated Met - $tRNA_f$ cannot initiate protein synthesis in E.coli (Kolakofsky and Nakamoto, 1966) and in vivo protein synthesis stops if the formylation of Met - $tRNA_f$ is inhibited. (Shih, Eisenstadt and Lengyel, 1966). This indicates the importance of the N-blocked amino acyl tRNA in chain initiation.

Nakamoto and Kolakofsky (1966) have shown that poly-U directed phenylalanine incorporation at high magnesium levels could occur without a specific initiator, but at low magnesium levels, dipeptidyl tRNA could act as a chain initiator. Lucas-Lennard

and Lipmann (1967) using Pseudomonas fluorescens reported that N-acetylphenylalanine could act as a chain initiator for polyphenylalanine synthesis. This finding has subsequently been confirmed for both 70S and 80S ribosomes. The effect of various modified tRNAs on polyphenylalanine incorporation has been studied more recently by Igarashi (1970), who reports that any N-blocked, peptidyl like $tRNA^{phe}$ will act as an initiator for the polyphenylalanine system.

Progress in the elucidation of the intermediate steps in peptide chain elongation is mainly the result of two developments:
(i) the use of simple messenger RNAs i.e. homopolynucleotides in cell-free amino acid incorporating systems,
(ii) the isolation of the amino acid transfer factors involved in the process.

It seems likely that there are three main transfer factors involved in chain elongation in procaryotic organisms, and they have been isolated from a range of different micro-organisms by several different workers. Uniformity of nomenclature has not been established yet, but the factors Ts, Tu and G isolated from E.coli and Pseudomonas fluorescens by Lucas-Lenard and Lipmann (1966) correspond to the factors S_1, S_3 and S_2 isolated from Bacillus stearothermophilus by Skoultchi, Ono, Moon and Lengyel (1968) and the factors FI_s, FI_u and FII of Ravel and

and Shorey (1969). All three protein fractions must be present for chain elongation to occur, as well as GTP, although the necessity for GTP was obscured in the early work by the use of high magnesium concentrations (Kurland, 1966; Ravel, 1967).

The presence of two transfer factors TF - 1 and TF - 2 has been reported in the reticulocyte system by Arhlinghaus, Shaeffer and Schweet (1964) and are analogous to T_1 and T_2 reported from a rat liver system by Ibuki and Moldave (1968).

Allende (1969) reports the partial purification of the transfer factors from wheat embryo, and App (1969) has isolated two factors, factor I and factor II, from the crude, high speed supernatant from rice embryos, which are required for in vitro polyphenylalanine synthesis. App suggests that factor I might be analogous to TF - 1 from reticulocytes and $FI_{(S+U)}$ from E.coli.

The last step in peptide chain initiation is the translocation of the fMet-tRNA from the amino acyl site on the 30S subunit, to the peptidyl site on the 50S subunit, leaving the amino acyl site open. The first step in peptide bond formation is the attachment of a new amino acyl tRNA to this site. Ravel and Shorey (1969) and Shorey, Ravel, Garner and Shive (1969) have proposed a scheme for the intermediate steps in the binding of amino acyl tRNA to the ribosomes which is compatible with all

the present experimental data. Their notation of FI_s, FI_u and FII for the transfer factors will be used in the following discussion.

FI_s and FI_u react with GTP in the presence of magnesium, to form an unstable protein - GTP complex, designated Complex I. The protein moiety of the complex has not been identified, although it has been suggested that FI_s has a purely catalytical function, and is not part of the complex (Ertel, Redfield, Brot and Weissbach, 1968). In the presence of Mg^{2+} and NH_4^+, Complex I reacts with amino acyl tRNA (but not deacylated tRNA or N substituted amino acyl tRNA) to form a second, more stable protein - GTP - amino acyl tRNA complex, referred to as Complex II. (Gordon, 1967, 1968, Ono et al. 1968; Ravel, Shorey, Froehner and Shive, 1968). The nature of the bonds holding Complex II are unknown.

The transfer of amino acyl tRNA from Complex II to the free amino acyl site on the ribosome in the presence of Mg^{2+} involves the hydrolysis of GTP, and results in the formation of a FI_u - GDP complex (Complex III) and inorganic phosphate is released. (McKeehan, Sepulveda, Lin and Hardesty, 1969; Ono, Skoultchi, Waterson and Lengyel, 1969 a,b). Complex III will bind to the ribosome in the presence of GMPPCP, but Complex III cannot be released, and no peptide bond formation occurs.

(Haenni and Lucas-Lenard, 1968; Skoultchi, Ono, Waterson and Lengyel, 1970). These results indicate the need for GTP cleavage before peptide bond formation.

Very little work has been done on the binding of tRNA to plant 80S ribosomes, although de Groot, Kaufman and Shafrir (1967) have demonstrated GTP dependent enzymatic binding of phenylalanyl tRNA to wheat ribosomes and Jerez, Sandoval, Allende, Henes and Ofengand (1969) have shown the formation of a GTP - protein complex from extracts of wheat embryos that will bind unblocked amino acyl tRNAs. Denatured tRNA will not react, indicating the necessity for the correct tRNA conformation, as well as the unblocked amino acyl group.

After the attachment of the amino acyl tRNA to the ribosome, peptide bond formation occurs between the carboxylic group of the terminal amino acyl residue of the peptidyl tRNA and the α amino group of the amino acyl tRNA. This reaction is catalysed by the enzyme peptidyl transferase, which is a constituent protein of the 50S subunit. (Monro, 1967; Monro and Marcker, 1967; Monro, Cerna and Marcker, 1968). Skogerson and Moldave (1968) propose that a similar situation occurs on the mammalian ribosome. GTP and the supernatant proteins do not seem to be involved in the reaction, but both monovalent and divalent cations (K^+ or NH_4^+ and Mg^{2+}) are needed, and the 3'

terminal nucleotide sequence (CCA) of both the peptidyl and amino acyl tRNA molecules seems to be involved. (Rychlik, Chladek and Zemlicka, 1963; Monro et al. 1968).

Puromycin, a structural analogue of the 3' terminal amino acyl group of tRNA (Yarmolinsky and de la Haba, 1959) acts as a competitive inhibitor of amino acyl tRNA during peptide bond formation, resulting in the formation of a peptide bond between it and the growing polypeptide chain, and the release of peptidyl puromycin from the ribosome. (Smith, Traut, Blackburn and Monro, 1965; Skogerson and Moldave, 1968; Weissbach, Redfield and Brot, 1968). The use of puromycin has, therefore, played a very important role in elucidating the mechanism of peptide bond formation as distinct from the various other stages in chain elongation. (Heintz, Salas and Schweet, 1968).

Immediately after peptide bond formation, the newly formed peptidyl tRNA is located in the amino acyl site on the ribosome and the free tRNA is in the peptidyl site. Translocation, involving the movement of the peptidyl tRNA from the amino acyl site to the peptidyl site and the movement of the ribosome along the mRNA by the length of one codon, requires enzyme factor FII and involves the hydrolysis of GTP. (Pestka 1968, 1969; Ono et al. 1969a). In the absence of translocation, no further peptide bond formation can occur (Lucas-Lenard and Haenni, 1969).

During chain elongation, the growing polypeptide chain remains linked to tRNA and bound to the mRNA-ribosome complex. After completion, the peptide chain is released from both of these bonds in the course of a composite process known as chain termination. Termination occurs when the ribosome and its attached peptidyl tRNA reach a termination codon in its progress along the messenger. Three chain terminator codons occur in E.coli, UAA, UGA and UAG, although UAA is the one used most frequently in vivo. (Garen, 1968). Most of the recent work on chain termination has been done in vitro by the sequential use of the initiator AUG and terminator trinucleotides, to first bind f[^{3}H]Met-tRNA to E.coli ribosomes and to subsequently release f[^{3}H] methionine. (Caskey, Tompkins, Scolnick, Caryk and Nirenberg, 1968).

Formylmethionine release is promoted by the presence of two release factors, R_1 and R_2, which catalyse chain termination in response to UAA or UAG and UAA or UGA respectively. (Scolnick, Tompkins, Caskey and Nirenberg, 1968). The presence of a release factor - terminator codon - ribosome intermediate, before formylmethionine release has been reported by Scolnick and Caskey (1969) who suggest that the role of the release factors may be to recognise the termination codons.

Milman, Goldstein, Scolnick and Caskey (1969) have identified another protein factor, S, which plays no part in

the termination mechanism, but stimulates the release of the polypeptide chain from the ribosome, possibly by stimulating terminator codon recognition. They suggest that S may have a purely catalytic function in chain termination similar to the role of transfer factor FIs in the binding of amino acyl tRNA to ribosomes.

Ribosomes exist in two states in the cell, free in the cytoplasm, or attached to the endoplasmic reticulum. The proportion of the two classes varies from cell to cell, or even in the same cell at different stages of its development. Cells which are actively dividing tend to contain mainly free ribosomes, whereas undividing, differentiated cells contain mainly membrance bound ribosomes. Secretory cells, e.g. liver, pancreas etc. contain a much higher proportion of membrane bound ribosomes than non-secreting cells. (Palade and Siekevitz, 1956; Henshaw, Bojarski and Hiatt, 1963; Blobel and Potter, 1967; Loeb, Howell and Tomkins, 1967) and this has led to the suggestion that the free ribosomes synthesize protein for intracellular purposes, while the membrane bound ribosomes synthesize proteins for export. (Birbeck and Mercer, 1961; Siekevitz and Palade, 1960; Campbell and Sargent, 1967).

The protein synthetic capacity of both the free and membrane bound ribosomes has been compared <u>in vivo</u> (Sellinger and Ohlsson, 1969) and <u>in vitro</u> (Redman, 1968; Priestley, Pruyn and Malt, 1969;

Takagi and Ogata, 1969) and both are found to be equally active. Priestley et al. have shown that the glycoproteins are synthesized exclusively on the membrane bound ribosomes from Kidney, and Ganoza and Williams (1969) have shown that the membrane bound ribosomes from rat liver are responsible for the synthesis of serum albumin, while the free ribosomes were synthesizing several non-serum proteins.

Studies on the changes in membrane bound and free ribosomes during the development in Vicia faba seeds have shown that these are quite separate classes of ribosomes, and there is no exchange between them while storage protein synthesis is occurring. (Payne, 1968; Payne and Boulter, 1969). A similar suggestion has been made for the free and membrane bound ribosomes from rat liver, although there is no experimental evidence for it in this tissue (Takagi and Ogata, 1969).

Although the ribosomes are the chief sites of protein synthesis in the cell, evidence has been accumulating that organelles such as the chloroplast and mitochondrion are also capable of limited protein synthesis (see Boulter, 1970 for review and references). It seems, however, that these organelles are only semi-autonomous in that they are responsible only for the synthesis of certain proteins, the rest being under genetic control, and synthesized on the cytoplasmic ribosomes (Kirk and Tilney - Bassett, 1967).

Plants, in contrast to animals, often store energy reserves in the form of protein. This situation is probably most developed in the maturation of the seeds of leguminous plants, and these seeds in the early stages of germination are completely independent of external nitrogen sources. In Vicia faba, the broad bean, storage protein may account for up to 20% of the dry weight of the mature seed (Grzesiuk, Mierzwinska and Sojka, 1962) and consists of two main protein components, vicilin and legumin. (Danielson, 1952). Most of the storage protein is synthesized in about 30 days during the development of the seeds, so that this tissue could be expected to provide material suitable for the study of protein synthesis in vitro.

Storage protein accumulates within discrete organelles in the cytoplasm, the protein bodies, which are bounded by a lipoprotein membrane and appear to originate by subdivision of the cell vacuole (Briarty, Coult and Boulter, 1969). Similar structures have been reported in Phaseolus vulgaris (Opik, 1968); in cotton (Englemann, 1966); in Soybean (Tombs, 1967); in Pisum sativum (Bain and Mercer, 1966) and in wheat (Graham, Jennings, Morton, Palk and Raison, 1962; Graham, Morton and Raison, 1963; Jennings, Morton and Palk, 1963).

Morton and Raison (1963) isolated ribosomes (76S), tRNA and tRNA synthetase enzymes from wheat protein bodies and have shown

that the protein bodies are capable of incorporating amino acids into several protein components that are separable by starch gel electrophoresis (Morton and Raison, 1964). They suggest that the protein bodies are complex structures that contain all the components necessary for protein synthesis.

Protein bodies isolated from developing V. faba seeds by Wheeler (1965) were found to incorporate [^{14}C] leucine over a prolonged period. It was, however, impossible to demonstrate any significant incorporation of [^{14}C] leucine into peptidyl material when the protein bodies were prepared under aseptic conditions. These results have been confirmed by Yarwood (1968) and are in agreement with results obtained by Wilson (1966) using sterile preparations of protein bodies from maize. No trace of ribosomes or any of the other components involved in protein biosynthesis have been found in protein bodies from V. faba. It would therefore seem, that these are incapable of protein biosynthesis and that all the storage protein is synthesized on the cytoplasmic ribosomes.

An amino acid incorporating system has been isolated from the cotyledons of developing V.faba seeds, and has been characterized by incubation. As developing material is limited to a few months of the year, material prepared from the plumules of germinating seeds was used when developing material was

unavailable. The major components of protein synthesis have been isolated from developing seeds at different stages during their development, to see if changes in *in vivo* protein synthetic activity during the development of the bean seeds are paralleled by *in vitro* changes in the protein synthetic efficiency of the various components.

The particulate fraction will be referred to as "microsomes" or the "microsomal fraction", as no detergent was used during isolation to remove membranous material. The preparations, therefore, contain both free and membrane bound ribosomes and polysomes.

MATERIALS AND METHODS

1. Biological material

A. Developing seeds

Seeds of Vicia faba (L) var. triple white were obtained from the Tyneside Seed Company, Gateshead, and plants were grown at the University of Durham Science Laboratories. Seeds were sown in pots during February in heated greenhouses and the young plants planted out of doors at the end of March. Sowings after this date were made directly in the open.

The flowers were observed daily and were labelled on the first day that they were fully open. Pods were collected immediately before use. At each selected age, pods of approximately the same size were collected, and the material was further standardised by selecting seeds of about the same size from each age of pod.

The age of material given in the text refers to the number of days the pods had developed after the flower was fully open. It was assumed that pollination occurred on the day that the flowers opened, and previous work (Wheeler, 1965) has shown that this method gave material which was, on the average, of the same age.

B. Germinating seeds

Method I

Dry seeds of V.faba were imbibed in running tap water for 24 hours and were surface sterilized by shaking in 10% (w/v) calcium hypochlorite solution, containing 3·5% available chlorine, for three minutes. The hypochlorite solution was decanted and the seeds were rinsed in several changes of sterile tap water until most of the odour of chlorine had gone. The seeds were germinated in the dark at room temperature for four days in covered, sterile trays lined with absorbent paper towelling well moistened with sterile tap water. After this time, the plumules had emerged from beneath the testas and were about 5 mm. long. A strict examination for infection was maintained and contaminated seeds were rejected.

Method II

Seeds were imbibed and surface sterilized as for Method I. They were planted in moist vermiculite in seed boxes which were kept in the dark under an intermittant overhead water spray for 6 - 8 days.

2. Chemicals and reagents

Except for those listed below, chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and were of

analytical grade when available.

Adenosine monophosphate 2'(3') mixed isomers Adenosine 5' triphosphate, disodium salt Guanosine 5' triphosphate, sodium salt Creatine phosphokinase Phosphocreatine Polyridylic Acid, potassium salt Trizma base, Analytic grade	from Sigma Chemical Company Ltd., London
L - Gluthathione (Reduced) 2,5 - Diphenyloxazole (P.P.O.) 1,4bis(2-(5- phenyloxazolyl)) benzene (P.O.P.O.P.)	from Koch-Light Laboratories, Colnbrook, Bucks.
L-Amino-Acids "A" grade	from California Corporation for Biochemical Research, Los Angeles, U.S.A.
Orcinol	from Hopkin Williams Ltd., Chadwell Heath, Essex.
Oxoid Nutrient Broth No. 2	from Oxoid Ltd., London
Davis Standard Agar	from Davis Gelatine Ltd., Warwick.

Millipore filters Cat. HAW P00010 from Millipore Corporation, Bedford, Mass.

Radioactive chemicals.

[1-^{14}C] phenylalanine specific activity 48 mCi/mM

[U-^{14}C] Amino-acid mixture specific activity 52 mCi/m Atom carbon

from the Radiochemical Centre, Amersham, Bucks.

3. Preparation of reagents and solutions

Solutions for Lowry's Folin method of protein determination

Working solution (made up fresh before each set of determinations)

2% Na_2CO_3 (anhydrous) in 0·1N NaOH	50 ml
1% $CuSO_4$ ($5H_2O$)	0·5 ml
2% sodium potassium tartrate	0·5 ml

Folin reagent 1N Folin and Ciocalteu's phenol reagent.

Solutions for the orcinol determination of RNA

1% orcinol solution	10 ml
Concentrated Hydrochloric Acid (11·4N)	40 ml
10% $FeCl_3$ ($6H_2O$)	1·0 ml

Scintillation fluid

P.P.O.	4·5 g
P.O.P.O.P.	0·1 g
Analar Toluene	to 1 l

Nutrient agar

Oxoid nutrient broth No. 2	25 g
Davis standard agar	10 g
Distilled water	to 1 l

Quarter strength Ringer's solution

Sodium chloride	2·25 g
Potassium chloride	0·105 g
Calcium chloride	0·12 g
Sodium bicarbonate	0·05 g
Distilled water	to 1 l

pH 7·0.

Extractant for microsome isolation

0·45M	Sucrose
0·005M	Magnesium chloride
0·016M	Potassium chloride
0·05M	Tris-HCl buffer, pH 7·6 at 0°

Resuspension medium for microsomes

0·0001M	Magnesium chloride
0·001M	Potassium chloride
0·01M	Tris - HCl buffer pH 7·6 at 0°

Extractant for tRNA

0·003M Magnesium chloride

0·024M Potassium chloride

0·1M Tris-HCl buffer pH 7·6 at room temperature

Solutions required for polyacrylamide gel electrophoresis

Acrylamide and bisacrylamide were recrystallized by the method of Loening (1967).

2-5% stock acrylamide solution

Recrystallized acrylamide	15 g
Recrystallized bisacrylamide	0·75 g
Distilled water	to 100 ml

5-7·5% stock acrylamide solution

Recrystallized acrylamide	15 g
Recrystallized bisacrylamide	0·375 g
Distilled water	to 100 ml

Stock E buffer

0·15M	NaH_2PO_4
0·005M	disodium EDTA
0·18M	Tris-acetic acid buffer pH 7·6 at room temperature

Preparation of gels	2·6%	5%
2-5% stock acrylamide	5·0 ml	-
5-7·5% stock acrylamide	-	5·0 ml
E buffer	5·8 ml	3·0 ml
Distilled water	17·8 ml	6·7 ml

The solutions were degased under vacuum for about 20 sec. 0·025 ml Temed (N.N.N'.N' tetramethylethylenediamine) and 0·25 ml freshly prepared 10% ammonium persulphate solution were added.

4. Determination of protein

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using Bovine Serum Albumin as the standard, over the range 0-1000 µg. BSA ml. standard solution. $E^{500}_{1cm.}$ was measured on the Unicam SP800 Spectrophotometer (fig. 1).

5. Determination of RNA by the Orcinol method

RNA was determined by the method of Markham (1955) as described by Campbell and Sargent (1967). Adenosine monophosphate (0-50 µg. ml. standard solution) was used as the standard solution, and corrected for RNA content assuming a purine: pyrimidine ratio of 1:0·83 for V.faba (Yarwood, 1968). $E^{670}_{1cm.}$ was measured on the Unicam SP800 spectrophotometer (fig. 2).

6. Estimation of RNA from optical density at 256 mµ

The concentration of microsomal suspensions and solutions of tRNA were determined from $E^{256}_{1cm.}$ of suitable dilutions, assuming an optical density of 11·3 for a 1 mg./ml. suspension of microsomes (T'so and Vinograd, 1961) and an optical density of 24·0 for a 1 mg./ml. solution of tRNA (Yarwood 1968).

Fig.1. Calibration graph for the determination of protein by the method of Lowry et al. (1952).

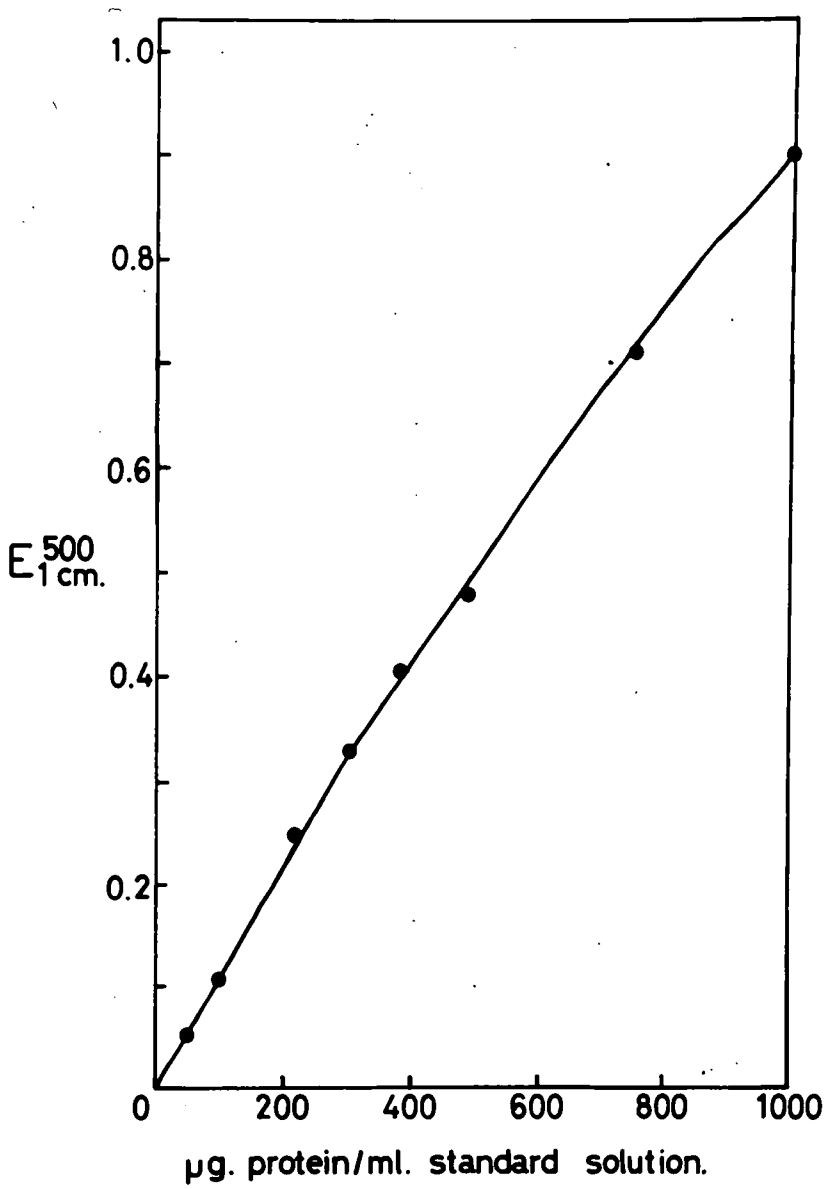
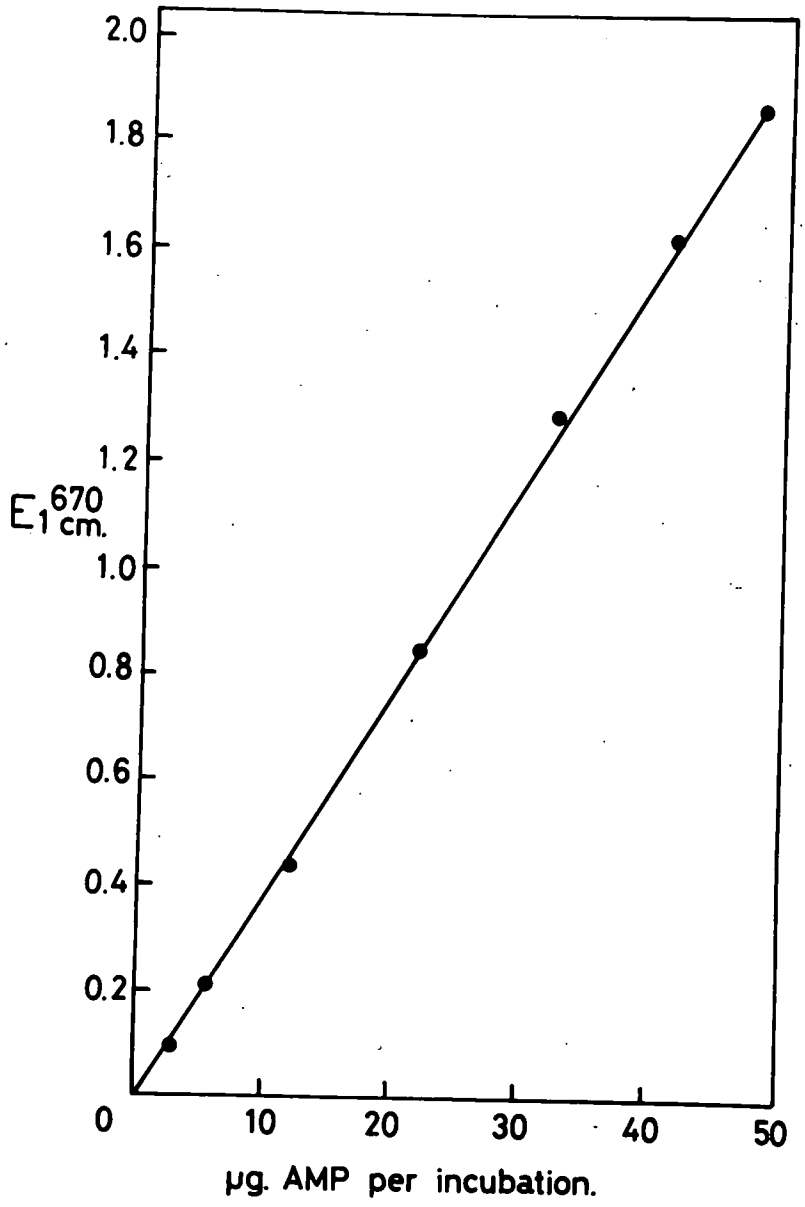


Fig.2. Calibration graph for the determination of RNA by the Orcinol Method of Markham (1955)



7. Measurement of radioactivity in hot (90°) trichloroacetic acid insoluble (peptidyl) material

Method I

Incorporation of [^{14}C] amino acids into peptidyl material was determined by the filter paper disc method of Mans and Novelli (1960, 1961). 0·1 ml aliquots of the incubation mixture were pipetted onto 2·2 cm diameter discs cut from Whatman 3 M M chromatography paper. The discs were dried under an infra-red heat lamp and washed by the method of Mans and Novelli (1961).

The discs were placed in the scintillation vials with 10 ml scintillation fluid, and the radioactivity present in the washed discs was measured using a Beckman liquid scintillation counter, model LS-200B at an average counting efficiency for [^{14}C] compounds in full window of approximately 90%. The samples were counted twice for 5 min. The position of the disc in the scintillation fluid and increasing volumes of scintillation fluid above 3 ml had no effect of the level of counts obtained (Mans and Novelli 1961). Radioactivity increased linearly with increasing amounts of labelled protein (Mans and Novelli 1961) and with increasing volumes of radioactive solution applied to the discs, up to 0·2 ml (Yarwood 1968).

A vial containing a blank disc and 10 ml scintillation fluid was always included among the samples counted, so that counts due to background radiation and other sources such as "noise" could be measured.

Method II

Incubations were stopped by the addition of 5% (w/v) trichloroacetic acid (TCA) and were heated at 90° for 15 min. The tubes were cooled to 0° and the TCA insoluble precipitate was collected onto a millipore filter, washed with 5%(w/v) TCA and allowed to dry. The radioactivity on the millipore filter was measured as for Method I.

8. Measurement of [^{14}C] amino acyl transfer RNA

Incorporation of [^{14}C] amino acids into amino acyl tRNA was measured as for Method I, but omitting the hot TCA wash.

9. Presentation of the results of [^{14}C] amino acid incorporation studies

In experiments using a single [^{14}C] amino acid, the results are presented as μμmole [^{14}C] amino acid incorporated per mg. microsomes or per mg. microsomal RNA. In the experiments using the [^{14}C] amino acid mixture, the results are presented as c.p.m./mg. microsomes or per mg. microsomal RNA.

10. Presentation of the results of charging tRNA

The results of charging tRNA are expressed as c.p.m. per mg. tRNA.

11. Counting of bacteria

Aliquots of amino acid incorporation incubations were checked for bacterial contamination. Serial dilutions were made in quarter strength Ringer's solution and 0·1 ml samples were plated onto Nutrient agar plates. Triplicate plates were made of each dilution. The plates were incubated at 37° for two days before the colonies were counted.

12. Isolation of microsomes from the cotyledons of developing Vicia faba seeds

Glassware used in the preparation of microsomes was autoclaved before use. Any appartus that could not be autoclaved was rinsed out with ethanol and allowed to dry. All solutions were made with sterile distilled water, and precautions were taken throughout to reduce the risk of microbial contamination as much as possible. All procedures were carried out at 2 - 4°.

Bean seeds were harvested as described previously, testas removed using sterile scapels and forceps and approximately 100 g. cotyledons ground in a pestle and mortar with sterile, acid washed sand, using 1·5 ml. microsomal extractant per g. starting material. The resultant brei was centrifuged at 4000 x g. for 5 min. and the supernatant decanted through four layers of sterile gauze. The pellet was reground with a further 0·5 ml. extractant per g. starting material and centrifuged as before. The supernatants were combined

and the pellet composed of cell debris and sand, discarded.

The supernatant was centrifuged at 20,000 xg. for 15 min. The post-mitochondinal supernantant was decanted through sterile gauze and recentrifuged at 105,000 xg. for two hours to pellet the ribonucleoprotein particles.

The supernatant was decanted through sterile gauze and stored at -20° until required. The centrifuge tubes were inverted over filter paper to drain off any remaining fluid, the sides wiped carefully with clean tissue and the surface of the pellet washed with 2-3 ml. resuspension medium. The ribonucleoprotein particles were resuspended in the resuspension medium at a concentration of approximately 20 mg/ml. microsomes as determined by $E^{256}_{1cm.}$, and were stored in 0·5 ml. aliquots at -70°.

The yield of microsomes depended on the age of the cotyledons and varied from 1-3 mg. microsomes g. starting material, or 0·4-0·7 mg. mircrosomal RNA g. starting material.

13. Isolation of microsomes from the plumules of germinating bean seeds

The seeds were germinated by Method I. The plumules were carefully removed, frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar under liquid nitrogen. The powder was resuspended in 2-3 vol. extractant, transferred to rimless pyrex boiling tubes and homogenized with 10 strokes of a tephlon

homogenizer with a tube clearance of approximately 0·3 mm. The homogenates were pooled and the ribonucleoprotein particles were isolated by the method described above.

The yield was approximately 4 mg. microsomes g. starting material.

14. Preparation of enzyme fractions

The enzyme preparations used were the high speed supernatant fractions after pelleting of the ribonucleoprotein particles at 105,000 xg. for two hours.

15. Extraction of transfer Ribonucleic Acid from developing Vicia faba seeds

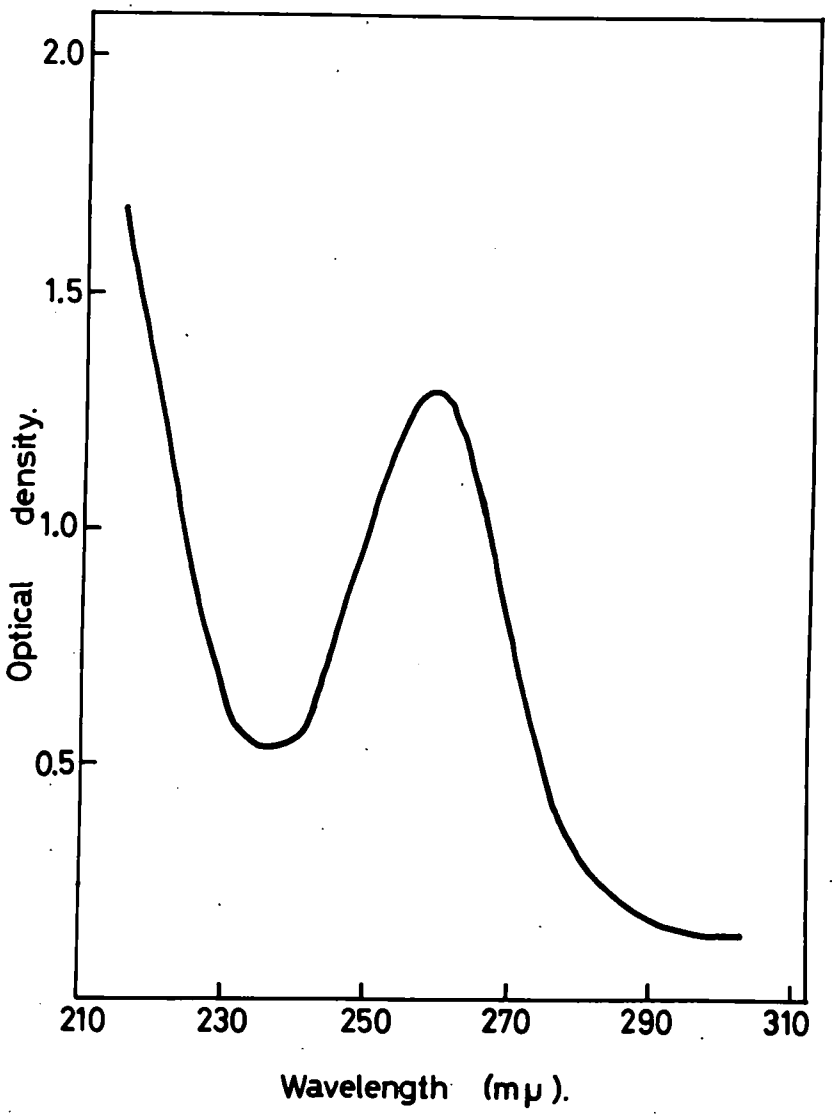
The pods were harvested as described previously, and the intact seeds (cotyledons and testas) collected.

The seeds were blended in a Townsend and Mercer top drive macerator for 90 sec. in 1 vol. tRNA extractant: 2 vol. 90% (v/v) phenol (1·5 vol./g. seeds). The homogenate was stirred for 60 min. in an ethanol-ice bath with an overhead stirrer and the aqueous and phenol phases were separated by centrifuging at 2,380 xg. for 40 min. The aqueous phase was removed and recentrifuged at 23,000 xg. for 40 min. to complete separation of the phases. The aqueous phase was collected, adjusted to 0·1 M potassium acetate using 1M potassium acetate pH 6·0 at room temperature and 2 vol.

absolute ethanol were added to precipitate the nucleic acids. After standing at -20° for at least 1 hr, the precipitate was recovered by centrifuging at 2,380 xg. for 15 min. and the ethanol was discarded. The precipitate was redissolved in 1·8 M Tris - HCl buffer, pH 8·9 at room temperature and incubated at 37° for 45 min. to remove any attached amino acids (Mosteller, Culp and Hardesty 1967). The solution was adjusted to 0·1M potassium acetate and 2 vol. absolute ethanol were added. After standing at -20° for one hour, the nucleic acid precipitate was collected by centrifugation, as above. The pellet was resuspended in 1M sodium chloride, and the low molecular weight species of RNA were extracted by incubating in an ice-bath for 15 min, shaking manually at regular intervals. The insoluble, high molecular weight RNAs were pelleted at 2,380 xg. for 30 min. and discarded. The supernatant was collected, the RNA precipitated with 2 vol. absolute ethanol for one hour and collected by centrifuging at 4,000 xg. for 15 min. The ethanol was decanted and excess ethanol removed from the inside of the centrifuge tubes with absorbent paper towelling. The pellet was redissolved in a minimal volume of 0·05M Tris-HCl buffer pH 7·6 at room temperature and recentrifuged at 4,000 xg. for 20 min. to remove any traces of insoluble material.

A sample was removed to determine the tRNA concentration by $E^{265}_{1cm.}$ on the Unicam SP800 spectrophotometer (fig. 3). A 5 ml. aliquot was removed and stored at -70° to compare the activity of the tRNA

Fig.3. Absorption spectrum of tRNA



tRNA was dissolved in 0·05M Tris-HCl buffer, pH 7·6 at room temperature, and the absorption spectrum of a suitable dilution was determined in water.

before and after chromatography. The remainder was stored at -20° until it was chromatographed (usually overnight).

The yield of tRNA before chromatography was up to 4 mg./g. starting material, depending on the age of the seeds.

16. Extraction of transfer Ribonucleic Acid from germinating seeds of Vicia faba

V.faba seeds were germinated as in Method II. The plumules were harvested after eight days and the tRNA extracted as above. The yield of tRNA before chromatography was approximately 0·8 - 1·0 mg./g. starting material.

17. Preparation of DE52 column for chromatography of tRNA

30 g. Whatman's preswollen DE52 cellulose and 180 ml. 0·05M Tris-HCl buffer pH 7·6 at room temperature were stirred rapidly in a beaker and the pH adjusted to 7·6 with 5N HCl. The DE52 was allowed to settle, the supernatant discarded, further Tris-HCl buffer was added and the pH checked. The procedure was repeated and the suspension poured into a measuring cylinder. When the DE52 had settled, the supernatant was decanted, 0·5 vol. 0·05 M Tris-HCl buffer pH 7·6 added, and the suspension was poured into a 1·5 cm diameter column. After 60 min. the excess Tris was run out of the column to compact the DE52 and further Tris buffer was added to the top of the column, which was ready for use.

18. Purification of transfer Ribonucleic Acid by DE52 cellulose chromatography

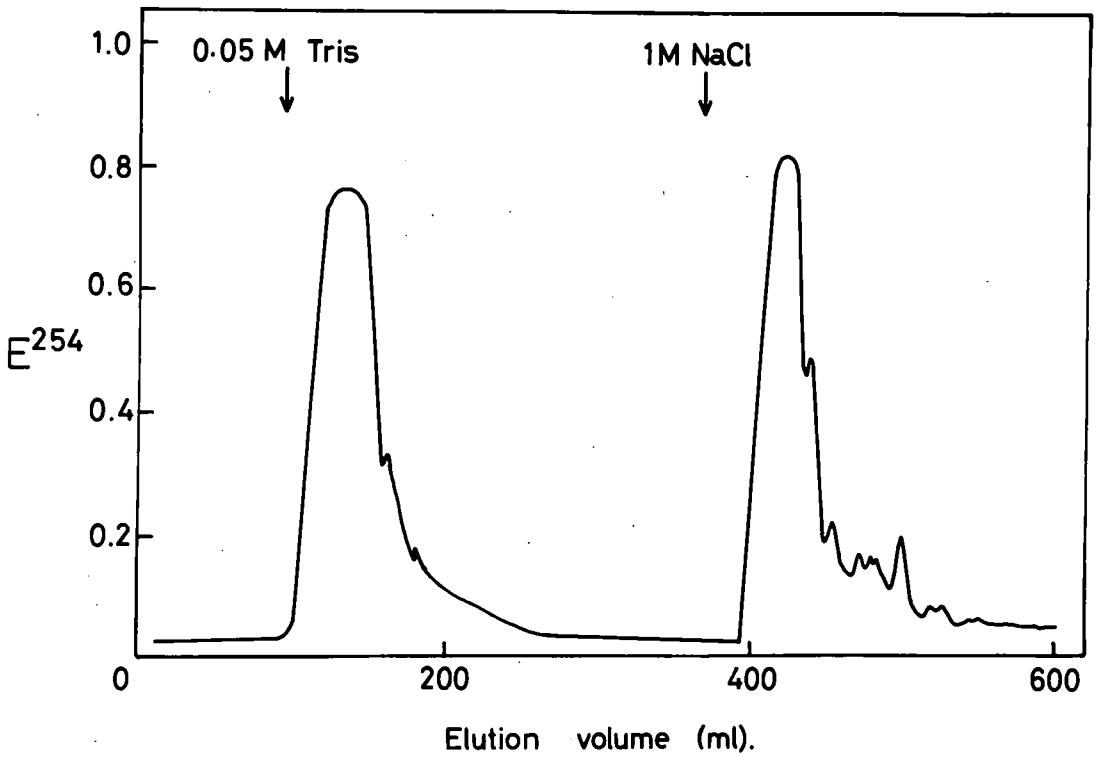
Chromatography was carried out in the cold room at 2 - 4° using the general method of Smith and Fowden (1968).

tRNA in 0·05M Tris-HCl buffer pH 7·6 was absorbed onto a 15 x 15 cm. DE52 column previously equilibrated with 0·05 M Tris-HCl buffer as described above. The column was connected to an Isco model 222 ultraviolet analyser and a servoscribe chart recorder set to run at 3 cm./hr. and 20 min.

The column was washed through with 0·05M Tris-HCl buffer at 7·6, which removed most of the extraneous material, causing an increase in the 254 mμ absorbency of the eluate. When the base line had returned to its original position, the tRNA was eluted from the column with 1M NaCl in 0·05M Tris-HCl buffer pH 7·6 (fig. 4). All the eluate showing absorption at 254 mμ was collected.

The eluate was adjusted to 0·1M with respect to potassium acetate, 2 vol. absolute ethanol were added and the tRNA precipitated overnight at -20°. The tRNA was collected by centrifuging at 4,000 x g. for 15 min., the ethanol was decanted and all traces of ethanol were removed from the pellet under vacuum. The pellet was redissolved in the minimal vol. 0·05M Tris-HCl buffer pH 7·6 and stored in 0·5 ml aliquots at -70°. A small sample was removed to determine the tRNA concentration from $E^{256}_{1cm.}$

Fig.4. Chromatography of tRNA on DE52 Cellulose



tRNA prepared from developing seeds was loaded on 15 x 1·5 cm column of DE52 cellulose in 0·05M Tris-HCl buffer, pH 7·6. Extraneous material was eluted with 0·05M Tris and tRNA was eluted with 1M NaCl.

19. Preparation of charged transfer RNA

tRNA prepared from 60 day old developing bean seeds was charged with [^{14}C] phenylalanine and 19[^{12}C] amino acids by the method of Ravel, Mosteller and Hardesty (1966) except that the tRNA was incubated at 25° for 30 min., the "40/70 ammonium sulphate fraction" was replaced by the 105,000 x g. supernatant fraction from 60 day seeds and final chromatography on Sephadex G25 was omitted.

The tRNA charged to a level of 4,500 c.p.m./mg. tRNA.

20. Characterization of tRNA by polyacrylamide gel electrophoresis

tRNA preparations from developing V.faba seeds of different ages before and after chromatography, were run on 2·6% and 5% gels by the method of Loening (1967). Electrophoresis was carried out at room temperature in E buffer diluted 5 fold in distilled water and containing 2 g./l. sodium dodecyl sulphate. Gels were pre-electrophoresed at 5m A/tube and 8v/cm. length of gel for at least one hour. 10 µl. of 1 mg/ml. tRNA solution in E buffer containing 5% (w/v) sucrose and 0·2% (w/v) sodium dodecyl sulphate were loaded onto the gels. 2·6% gels were run for 60 min. and 5% gels for 90 min.

The gels were soaked in the electrophoresis buffer for about one hour before they were scanned. Most of the non-RNA, ultra violet absorbing material was lost during this time, giving a clearer base line, although there was little diffusion of 4S and

5S RNA. The gels were scanned using a Joyce-Loebl chromoscan at 265 mμ using a 265 mμ interference filter with a medium pressure mercury lamp and a light beam 50 - 150 μ x 1-2 mm. The instrument was set to expand the scanned length of the gel by the factor 3.

The area beneath the traces was determined using a Technicon Integrator Calcular, Model AAG.

21. Assay of amino acyl acceptor capacity of transfer RNA

Amino acyl acceptor capacity of the tRNA preparations and the amino acyl synthetase activity of the high speed supernatant enzyme fractions was measured in a system containing the following in μmoles per ml.: Tris-HCl buffer pH 7·6 at 25°, 100; $MgCl_2$, 10; reduced glutathione (GSH), 20; ATP, 2; [^{14}C] phenylalanine (48 mCi/mM), 0·02; with 1 mg. tRNA/ml, and the volume of enzyme promoting maximum charging of tRNA (usually 0·06 ml/ml. incubation).

Incubations were carried out at 25°, and after 20 min., 0·1 ml. aliquots were removed onto filter paper discs which were washed and counted as described earlier.

22. Cell-free amino acid incorporating systems

Thin walled, narrow bore test tubes were used for all incubations. Incubations were made using solutions at 0° and were maintained at this temperature in an ice-bath, until the subcellular particles were added, when they were transferred to a constant temperature bath at 25°. Incubation tubes were shaken manually every few minutes, and prior to aliquots being removed for assay.

A. The Complete System

The complete incubation mixture contained the following in µmole/ml. incubation: Tris-HCl buffer, pH7·8 at 25°, 60; $MgCl_2$, 10; KCl, 70; GSH, 10; GTP, 0·2; ATP, 4; creatine phosphate, 10; [^{14}C] phenylalanine, 0·02; 19x[^{12}C] amino acid mixture, 0·02; also per ml. incubation: Poly-U, 0·2 mg; tRNA, 0·4 mg; microsomes, 1 mg. as determined by $E^{256}_{1cm.}$ and the volume of enzyme giving maximum incorporation as determined by concentration curves (which was usually 0·06 ml/ml. incubation).

2 x 0·1 ml. aliquots were removed after 20 and 40 min. incubation onto filter paper discs, which were washed and counted by Method I.

B. The Transfer System

The transfer incubations contained the following in µmoles/ml. incubation: Tris-HCl buffer, pH 7·6 at 25°, 60; $MgCl_2$, 10; KCl, 70; GSH, 10; GTP, 0·02; also per ml. incubation: Poly-U, 0·2 mg; tRNA previously charged with [^{14}C] phenylalanine and 19 x [^{12}C] amino acids, 0·4 mg; enzyme, 0·06 ml; and microsomes, 1 mg. as determined by $E^{256}_{1cm.}$

The amount of incorporation after 20 min. was measured on millipore filters by Method II.

RESULTS

1. Counting of [^{14}C] compounds

The 2 σ counting error, which represents the 95% confidence limit, was automatically determined for every sample. This value is related to the activity of the sample (fig. 5) and decreases rapidly as the counting time increased. (fig. 6).

All results were obtained using a counting time of 5 min. The 2 σ counting error was not greater than 10% and usually lay between ± 3% to ± 5%, except for the background determinations which were approximately 50-60 c.p.m./sample, with a 2σ counting error of ± 15%

2. Interchangeability of the components of amino acid incorporation from developing and germinating seeds

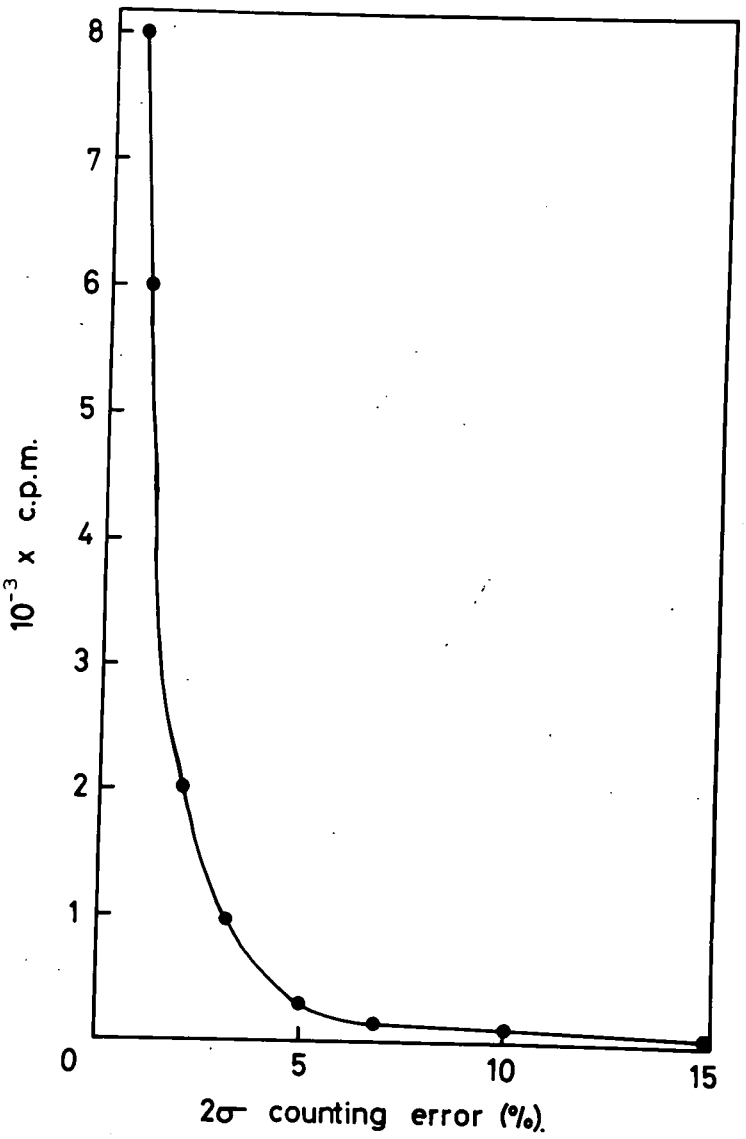
The three major components of the amino acid incorporating system (microsomes, enzymes and tRNA) prepared from germinating and developing seeds were interchangeable, with less than 10% loss in activity. Systems from germinating and developing seeds had similar poly-U, Mg^{2+}, pH and temperature requirements. Components from developing seeds were, however, more active in amino acid incorporation than those from germinating material.

3. Characterization of the cell-free amino acid incorporating systems

A. The complete system

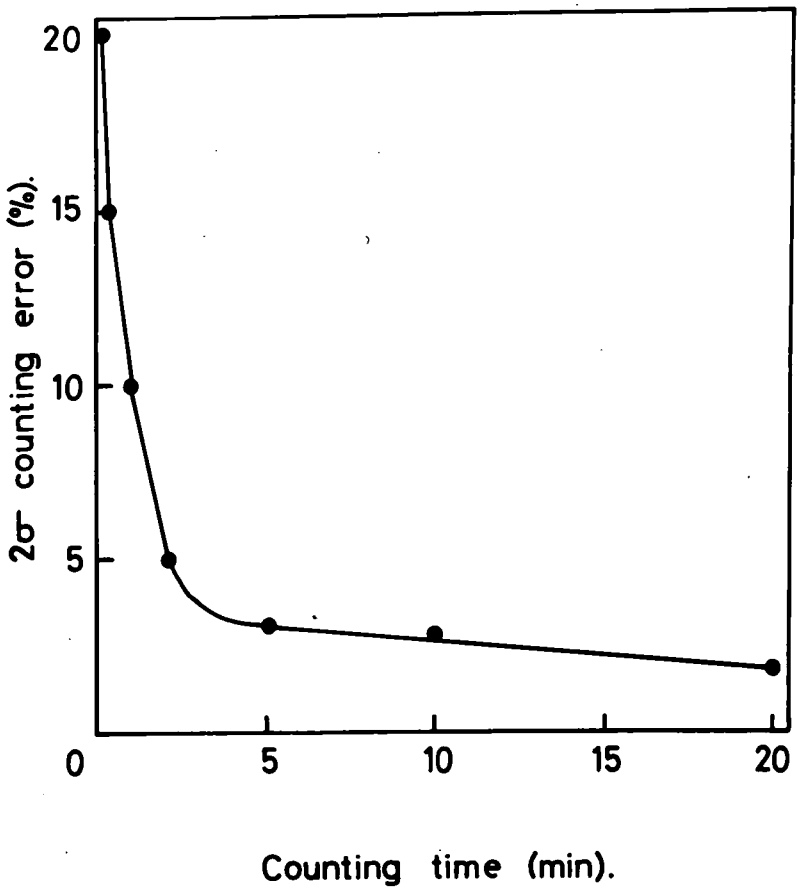
Free amino acids can be incorporated into hot (90^{o})

Fig.5. Changes in the 2 σ counting error with increasing radioactivity of the sample



Filter paper discs to which different amounts of charged tRNA were added were counted for 5 min. 5 counts were made for each disc.

Fig.6. Changes in the 2 σ counting error with counting time



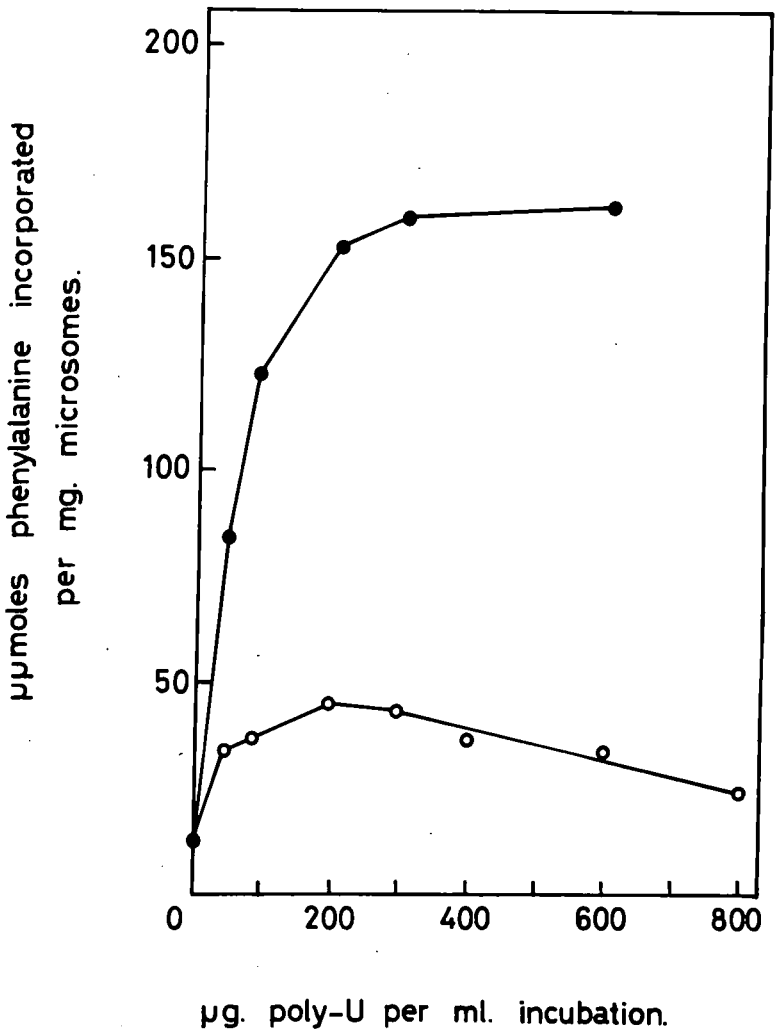
A filter paper disc of radioactivity approximately 900 c.p.m. was counted for different lengths of time. The results are the average of 5 separate counts.

trichloroacetic acid insoluble (peptidyl) material in the presence of deacylated tRNA, microsomes and enzymes. In the system used in this work, incorporation was measured using [^{14}C] phenylalanine in the presence and absence of the artificial messenger poly-U, which promotes polyphenylalanine synthesis. The results presented in fig. 7 show that phenylalanine incorporation was dependent upon the addition of poly-U. and maximum incorporation occurred at a poly-U concentration of 200 µg./ml. incubation. Levels of 100-150 µµmoles phenylalanine incorporated per mg. microsomes after 20 min. were routinely recorded. In the absence of poly-U. phenylalanine incorporation varied between 10-20 µµmoles phenylalanine per mg. microsomes after 20 min. Further addition of poly-U. after 20 min. incubation did not result in increased phenylalanine incorporation (Table 1).

Poly-U. directed phenylalanine incorporation was strongly influenced by temperature (fig. 8), maximum incorporation occurred at 25° with 20 min. incubations, although the optimum temperature decreased with increasing length of incubation to 15° with 120 min. incubations. Temperature had little effect on incorporation in the absence of Poly-U.

The effect of temperature on the time courses of phenylalanine incorporation is shown in fig. 9. Poly-U. directed incorporation was linear over 120 min. at 15°, whereas at 25° it was linear

Fig.7. Effect of poly-U on [^{14}C] phenylalanine incorporation in the complete system



The complete system is described in Methods and contained microsomes and enzyme fraction (0·06 ml./ml. incubation) from 60 day seeds

—●—● tRNA from 40 day seeds

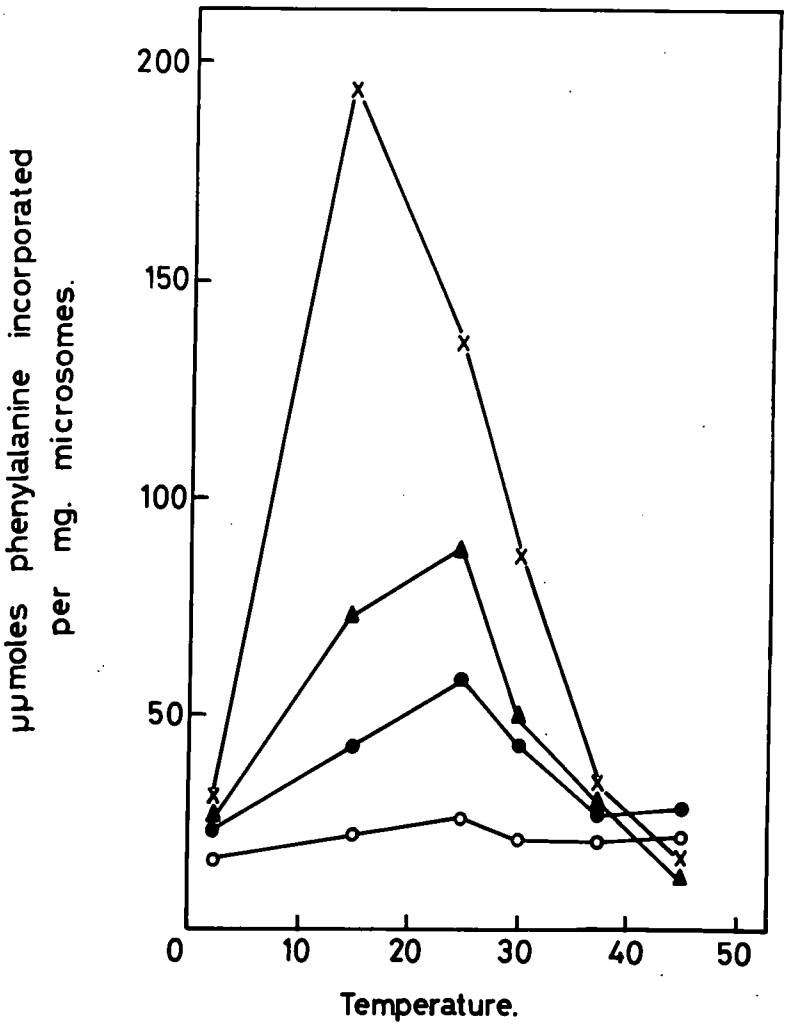
—○—○— tRNA from 80 day seeds

Table 1. Effect of adding further poly-U on Phenylalanine incorporation

Incubation	µµmoles phenylalanine incorporated per mg. microsomes		% increase in incorporation between 20-40 min. incubation
	After 20min.	After 40min.	
(1) Control (No additions)	136	223	49
(2) 100 µg. poly-U added after 20 min.	135	218	48
(3) Control, 0·05 ml. water added after 20 min.	130	211	48

0·5 ml. incubations contained 100 µg. poly-U; microsomes and enzyme (0·03 ml.) from 60 day cotyledons and tRNA from 30 day seeds. After 20 min. incubation, 2 x 0·1 ml. aliquots were removed for assay and a further 100 µg poly-U (in 0·05 ml.) added as indicated.

Fig.8. The effect of temperature on amino acid incorporation in the complete system

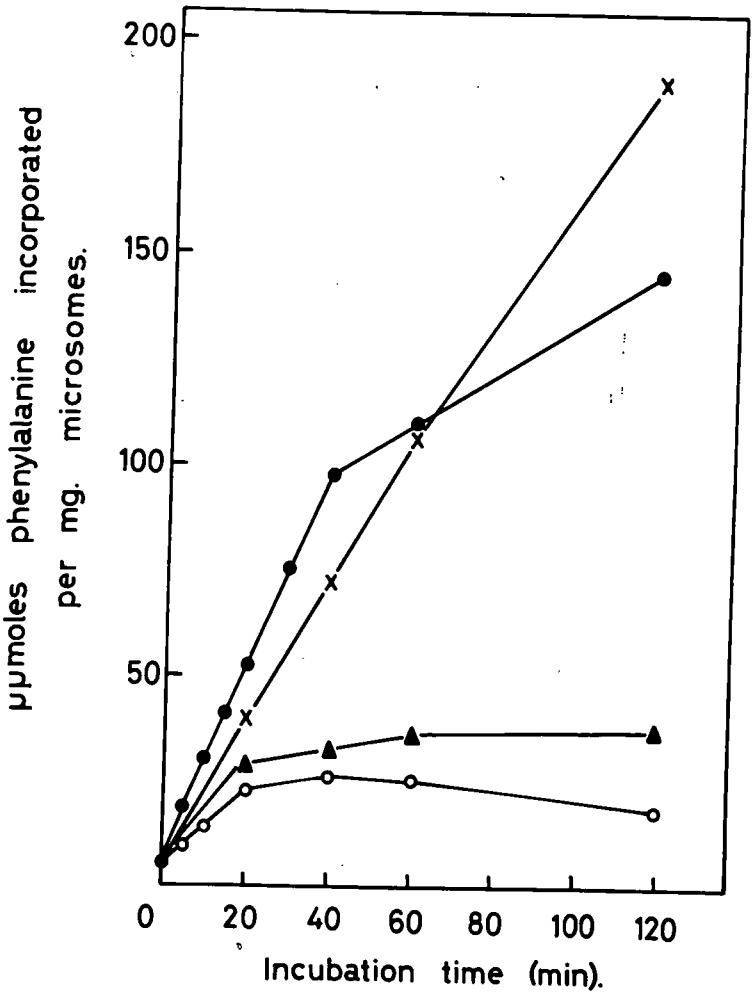


Incubations contained 8mM Mg^{2+}; ~~microsomes~~, enzyme fraction and tRNA from 60 day developing seeds

O—O— Incubations minus poly-U. Samples removed after 20 min. incubation

In incubations containing poly-U, 2 x 0·1 ml. samples were removed after 20 min. (●—●—); 40 min. (▲—▲—) and 120 min. (x—x—) incubation for assay.

Fig.9. Time curves for [^{14}C] phenylalanine incorporation at different temperatures



Incubations contained 8mM Mg^{2+} and microsomes, enzyme fraction and tRNA from 60 day developing seeds.

O—O— Incubations minus poly-U at 25°

x—x— Incubated with poly-U at 15°

●—●— Incubated with poly-U at 25°

▲—▲— Incubated with poly-U at 37°

over 40 min. and then tailed off. At 37°, or in the absence of poly-U, incorporation was completed within 20 min.

The effect of pH on phenylalanine incorporation is shown in fig. 10, and the effect of magnesium concentration in fig. 11.

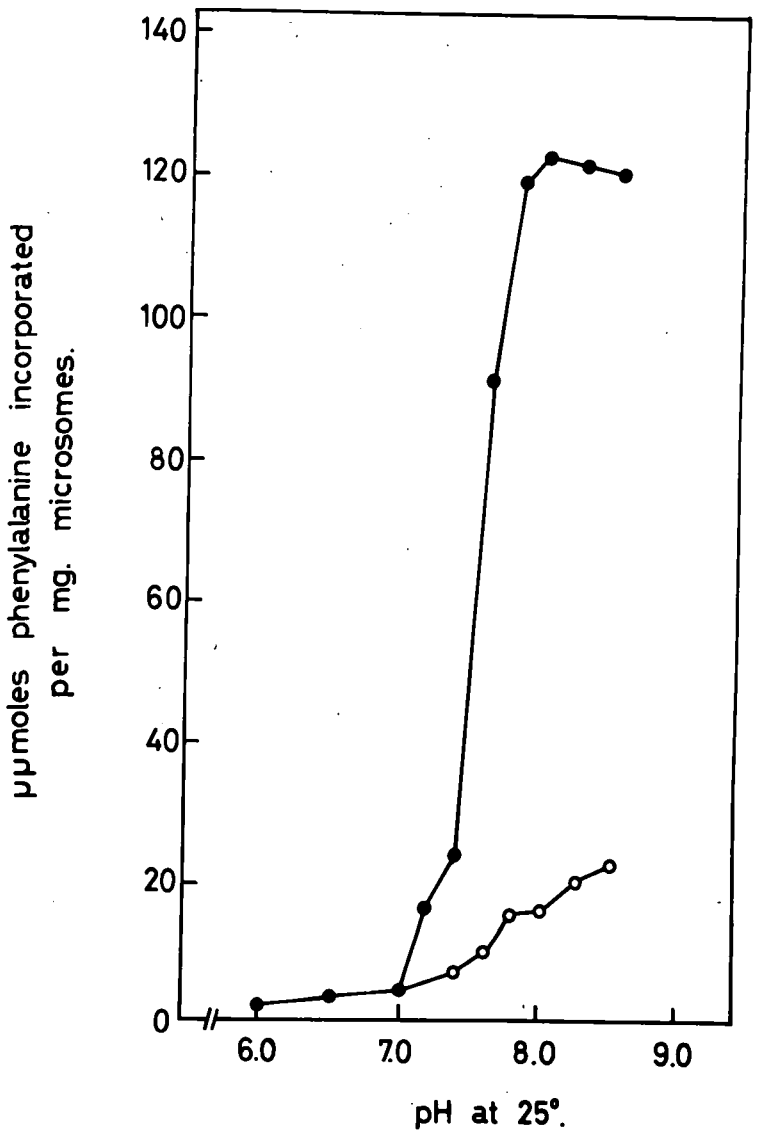
As the enzyme fraction used in all the in vitro amino acid incorporation work was the general enzyme preparation remaining after the microsomes had been sedimented, concentration curves were done for each of the enzyme fractions to determine the volume of enzyme which stimulated maximum phenylalanine incorporation. This volume, which was usually 0·06 ml./ml. incubation (fig. 12) was used in all subsequent work, unless otherwise stated.

A tRNA concentration of 0·4 mg/ml. incubation stimulated maximum phenylalanine synthesis in this system (fig. 13).

Phenylalanine incorporation was dependent upon the addition of microsomes, enzyme, tRNA, ATP and the ATP generating system (Table 2). Addition of ATP and the ATP generating system after 60 min. incubation resulted in a 40% increase in [^{14}C] phenylalanine incorporation over a further 20 min. incubation (fig. 14). The system showed only slight dependence upon GTP.

Bacterial contamination in the incubations was of the order of 3×10^3 colonies/ml. incubation to 2×10^4 colonies/ml. incubation, as determined by plating on Nutrient agar.

Fig.10. Effect of pH on amino acid incorporation in the Complete System

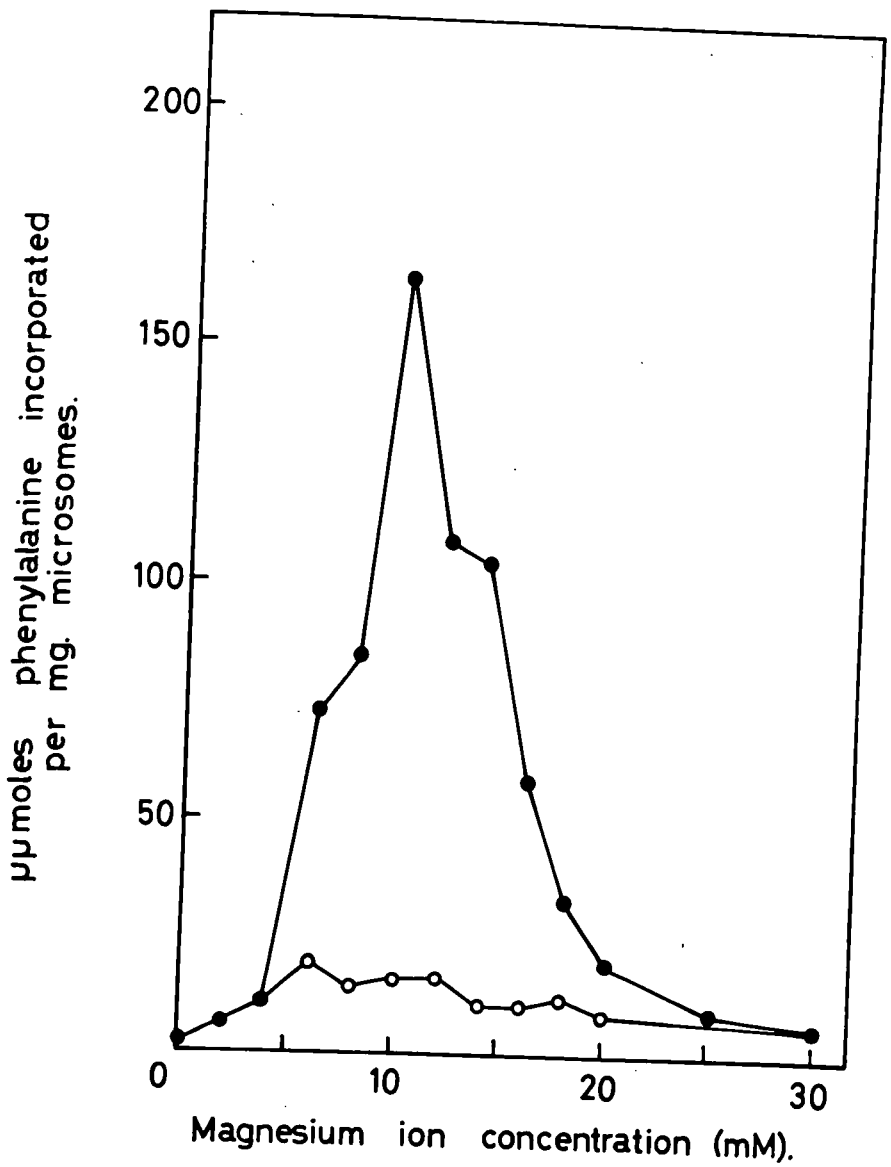


Incubations contained microsomes and enzyme fraction (0·06 ml./ml. incubation) from 60 day cotyledons and tRNA from 7 day germinated seeds.

●—● Complete

O—O minus poly-U

Fig.11. Effect of Magnesium concentration on [^{14}C] phenylalanine incorporation in the Complete System

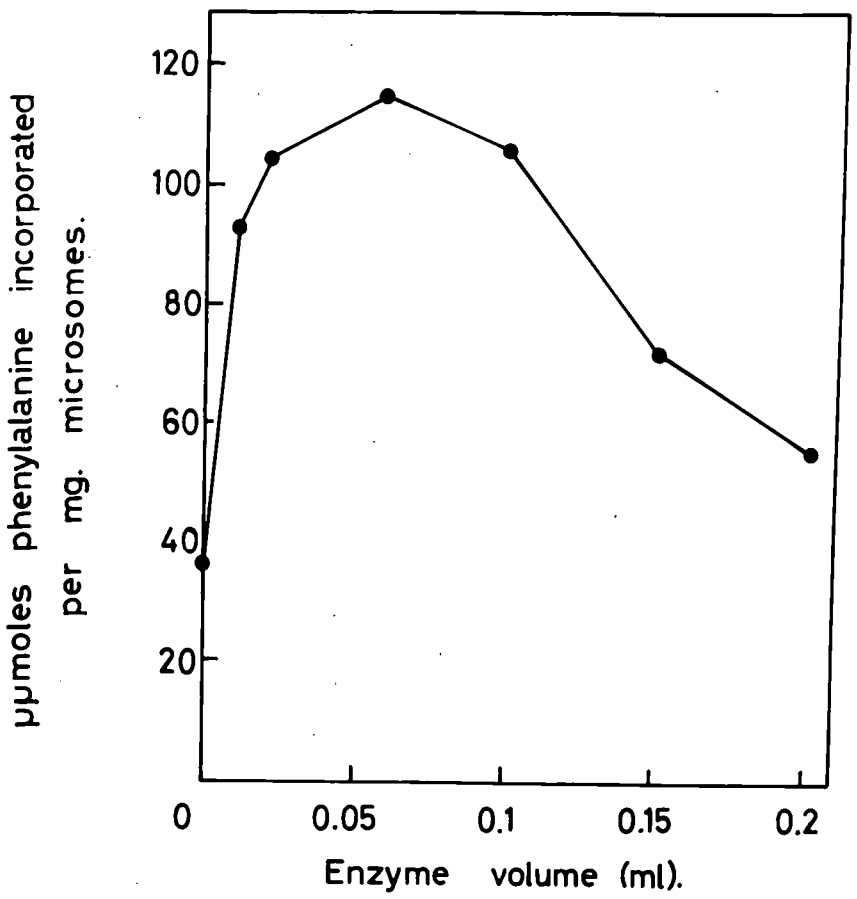


Incubations contained microsomes and enzyme fraction from 60 day cotyledons and tRNA from 50 day seeds.

●—● Complete

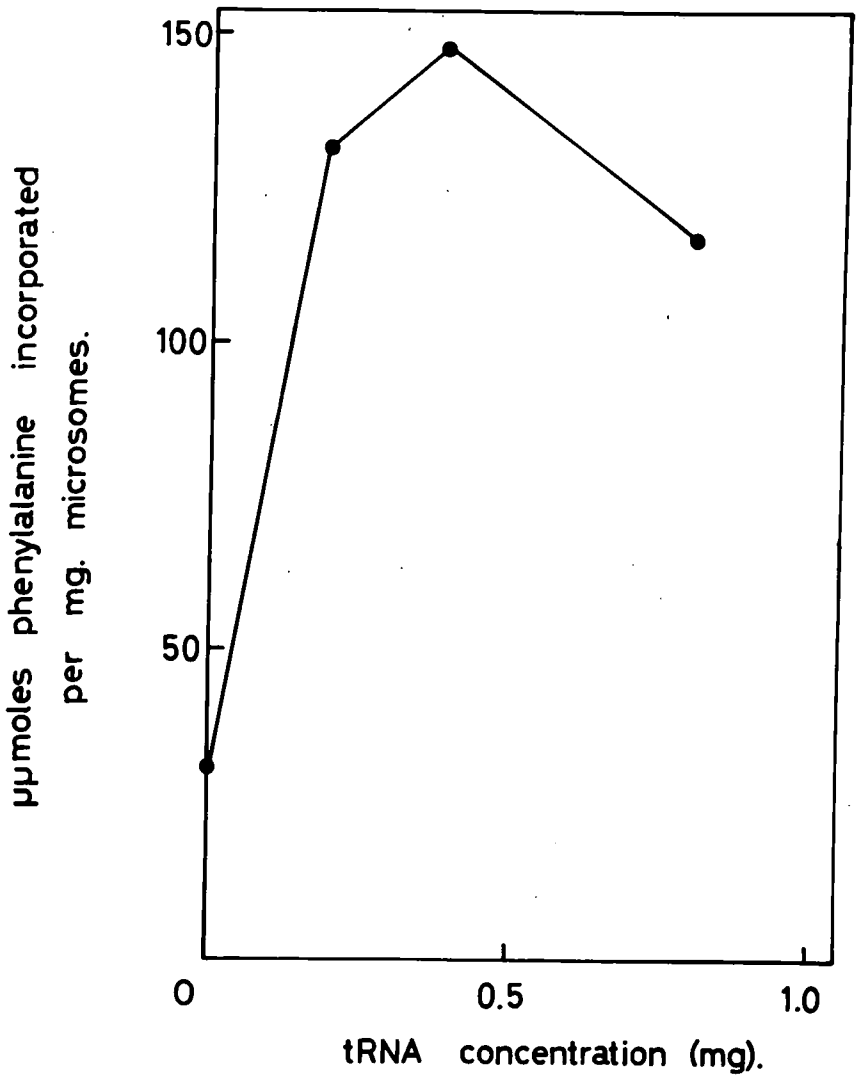
○—○ minus poly-U

Fig.12. Effect of enzyme concentration on [^{14}C] phenylalanine incorporation in the Complete System



Incubations contained microsomes, enzyme fraction and tRNA from 60 day developing bean seeds

Fig.13. Effect of tRNA concentration on [^{14}C] phenylalanine incorporation in the complete system



Incubations contained microsomes, enzyme fraction and tRNA from 60 day developing bean seeds.

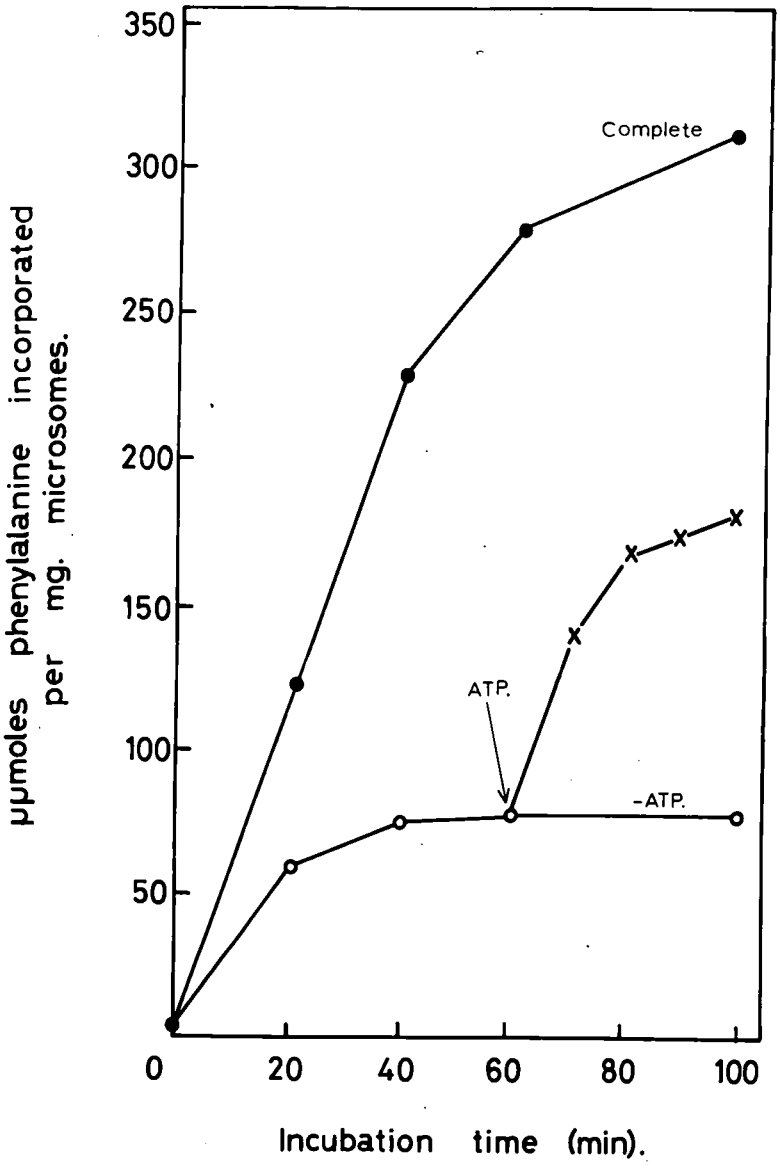
Table 2. Characteristics of [^{14}C] phenylalanine incorporation in the complete system

Incubations	c.p.m./sample	µµmoles phenylalanine incorporated/mg. microsomes	% complete
Complete + Poly-U	1300	133	
Complete - Poly-U	182	18	
-GTP + Poly-U	1224	124	90
-GTP - Poly-U	165	17	
-ATP + Poly-U	574	59	40
-ATP - Poly-U	76	8	
-Enzyme + Poly-U	354	36	27
-tRNA + Poly-U	305	30	23
-Enzyme - tRNA + Poly-U	38	3	2
-Microsomes + Poly-U	50	-	4

The complete incubation mixture was described in methods, and contained microsomes and enzyme from 60 day cotyledons and tRNA from 30 day seeds.

2 x 0·1 ml. aliquots were removed for assay after 20 min. incubation.

Fig.14. Effect of the omission of ATP and the ATP generating system on poly-U directed phenylalanine incorporation



Legend as for Table 2.

2 x 0·1 ml. aliquots were removed at the times shown

Table 3. Composition of 14[U-^{14}C] amino acid mixture

A 14[U-^{14}C] amino acid mixture (code CFB. 104) was purchased from the Radiochemical Centre, Amersham, which was prepared from individual amino acids isolated from Chlorella, and purified by ion-exchange and paper chromatography.

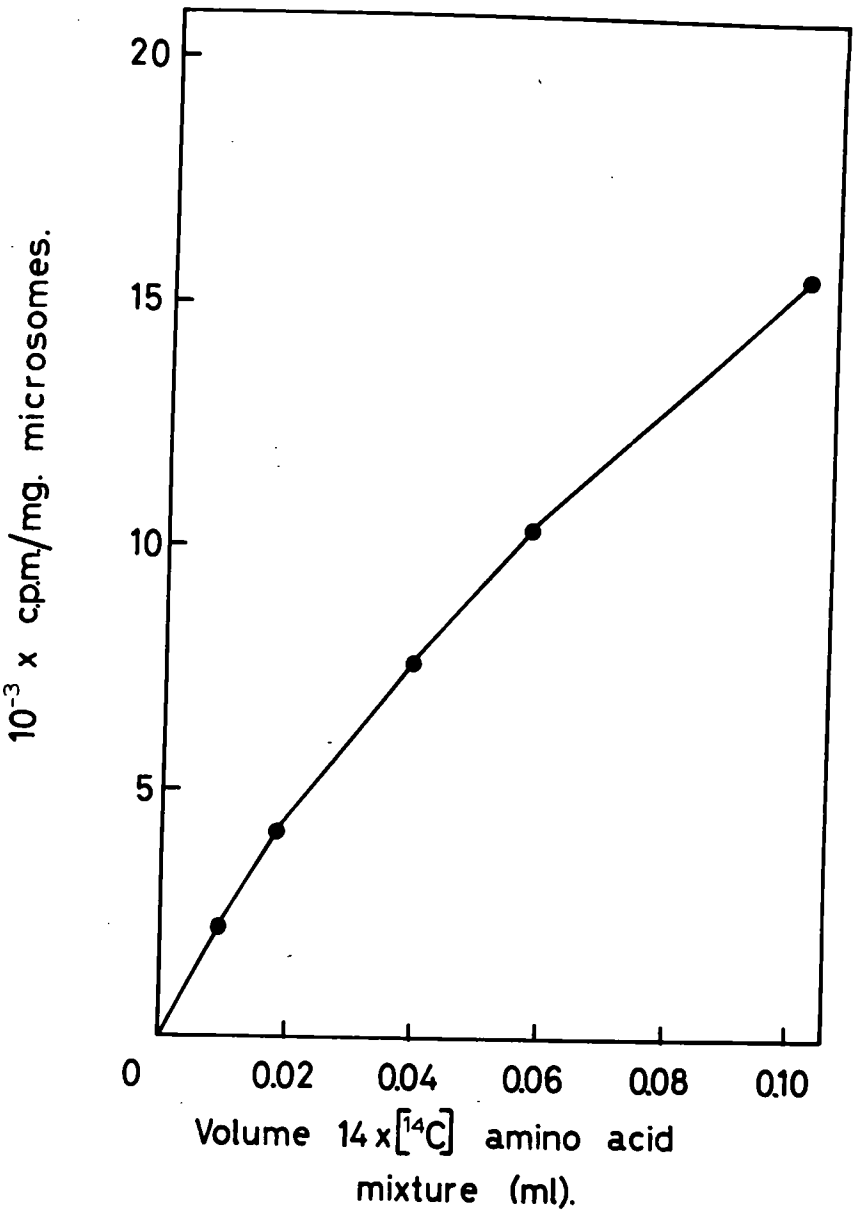
Composition by activity:-

Alanine	10%	Lysine	5·5%
Arginine hydrochloride	6·5%	Phenylalanine	7·0%
Aspartic acid	9·0%	Proline	6·0%
Glutamic acid	12·5%	Serine	5·0%
Glycine	5·0%	Threonine	6·0%
Leucine	12·0%	Tyrosine	3·5%
Isoleucine	5·0%	Valine	7·0%

Specific activity 52 mCi/Atom carbon.

Radioactive concentration 16 ml/mCi.

Fig.15. Concentration curve for amino acid incorporation using the 14[U-^{14}C] amino acid mixture



Incubations as in Methods, except that [^{14}C] phenylalanine was replaced by the amino acid mixture.

Microsomes and enzyme fraction from 60 day cotyledons and tRNA from 40 day seeds were used.

Microsomes and tRNA were stored at -70°, and retained their activity over at least nine months storage. Enzyme fractions were stored at -20° and were still active after 15 months storage. Freezing and thawing did not affect phenylalanine incorporation, although the microsome preparations were usually discarded once they had been thawed. Incorporation of [^{14}C] phenylalanine in incubations containing microsomes and enzyme from 60 day cotyledons and tRNA from 40 day seeds varied between 145-160 µµmoles per mg. microsomes in 20 min., in a wide range of assays carried out between October 1969 and February 1970.

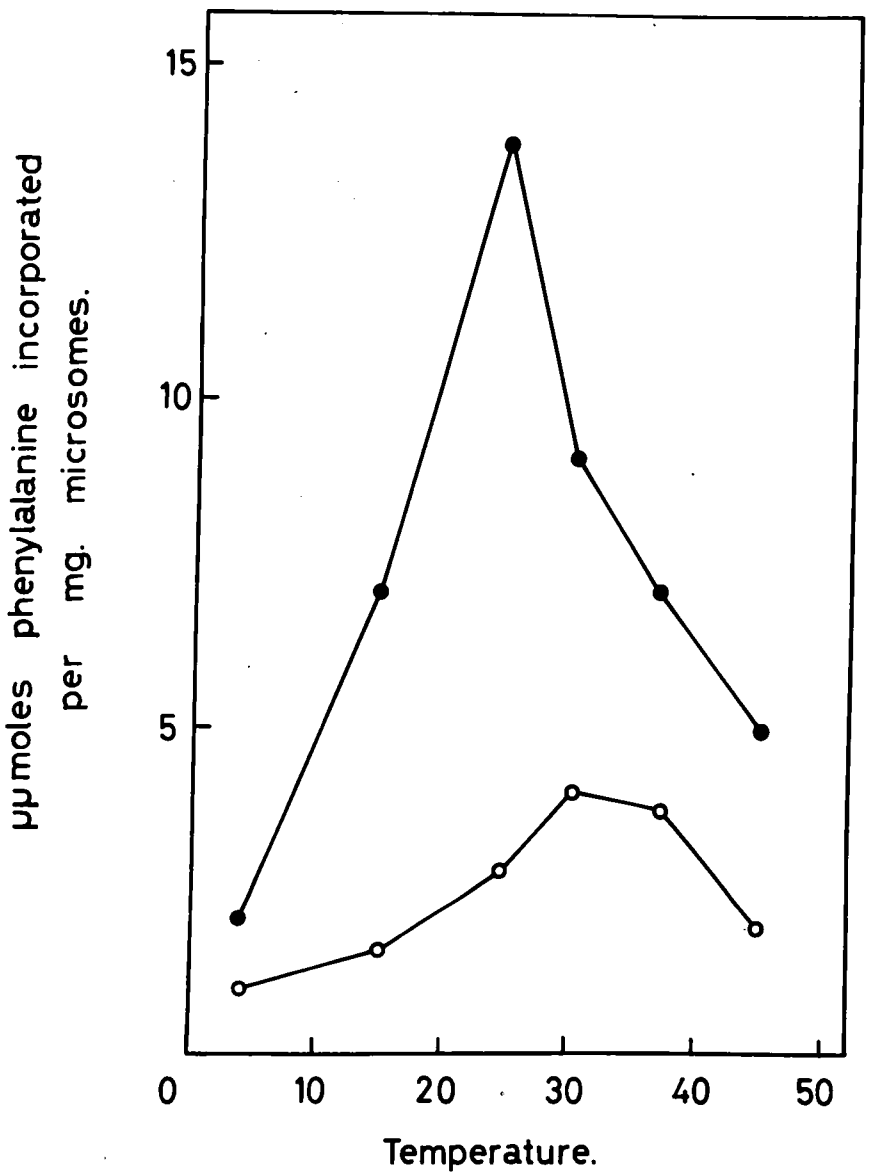
Total incorporation of a wide range of amino acids was studied using a [U-^{14}C] amino acid mixture (Table 3). Incorporation in the absence of poly-U was proportional to the volume of the amino acid mixture included in the incubations (fig. 15).

B. The Transfer System

Incorporation of amino acids into peptidyl form from pre-charged tRNA, in the presence of microsomes and enzyme was measured in the transfer system.

Phenylalanine incorporation was dependent upon poly-U; maximum incorporation being 20 µµmoles/mg. microsomes in the presence of poly-U and 2-3 µµmoles/mg. microsomes in the absence of poly-U after 20 min.

Fig.16. Effect of temperature on amino acid incorporation in the Transfer system



Incubations as in Methods, containing microsomes and enzyme fraction from 60 day cotyledons, and tRNA from 60 day seeds charged with [^{14}C] phenylalanine and 19x[^{12}C] amino acids to a level of 4,500 c.p.m./mg. tRNA.

Table 4. Effect of co-factors on [^{14}C] phenylalanine incorporation by the transfer system

Incubation	c.p.m./incubation	µµmoles phenylalanine incorporated/mg. microsomes	% complete
Complete + Poly-U	470	9	
Complete - Poly-U	53	1	
-GTP + Poly-U	379	7	80
-Enzyme + Poly-U	278	5	58
-Microsomes + Poly-U	110	-	23

Incubations contained microsomes from 60 day cotyledons, enzyme from 80 day cotyledons and tRNA from 60 day seeds pre-charged with [^{14}C] phenylalanine and 19 x [^{12}C] amino acids to a level of 4,500 c.p.m./mg. tRNA.

Incorporation was influenced by temperature (fig. 16) and had the same pH and Mg^{2+} requirements as the complete system. Incorporation was dependent upon the addition of microsomes and enzyme, although it was only partially dependent upon GTP (Table 4).

C. Charging of tRNA

This system was designed to assay the amino acyl acceptor capacity of tRNA and the amino acyl synthetase activity of the enzyme fractions.

Certain tRNA preparations could be charged to levels of 30,000 cpm/mg. tRNA with [^{14}C] phenylalanine, although average values were around 20,000 - 25,000 cpm/mg. tRNA. Radioactivity was, however, lost during re-extraction of charged tRNA from the incubations, during the preparation of pre-charged tRNA to be used in the transfer system (Table 5).

The effect of temperature on charging of tRNA with phenylalanine is shown in fig. 17. Charging was less temperature sensitive than amino acid incorporation into peptidyl form.

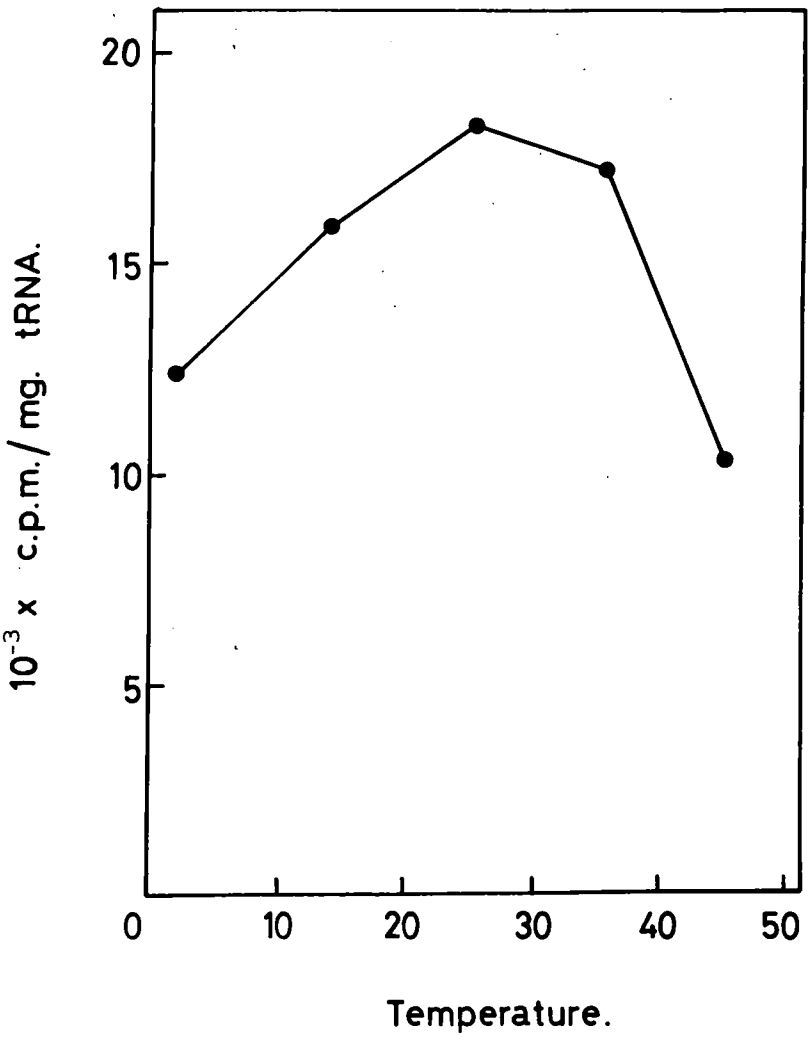
Virtually all charging occurred within 10 min. and prolonging the incubation period resulted only in a slight increase in charging (fig. 18). Magnesium concentration had little effect on the charging of tRNA with phenylalanine (fig. 19). Maximum charging occurred at a magnesium concentration of 10mM, rising

Table 5. Loss in radioactivity of charged tRNA during phenol re-extraction from the incubation mixture

Samples removed at end of charging period c.p.m./mg. tRNA	Samples removed after first ethanol precipitation c.p.m./mg. tRNA	Samples removed after second ethanol precipitation c.p.m./mg. tRNA
5,194	2,789	1,576
5,234	3,370	2,199
% loss in radioactivity		
	46	70
	46	58

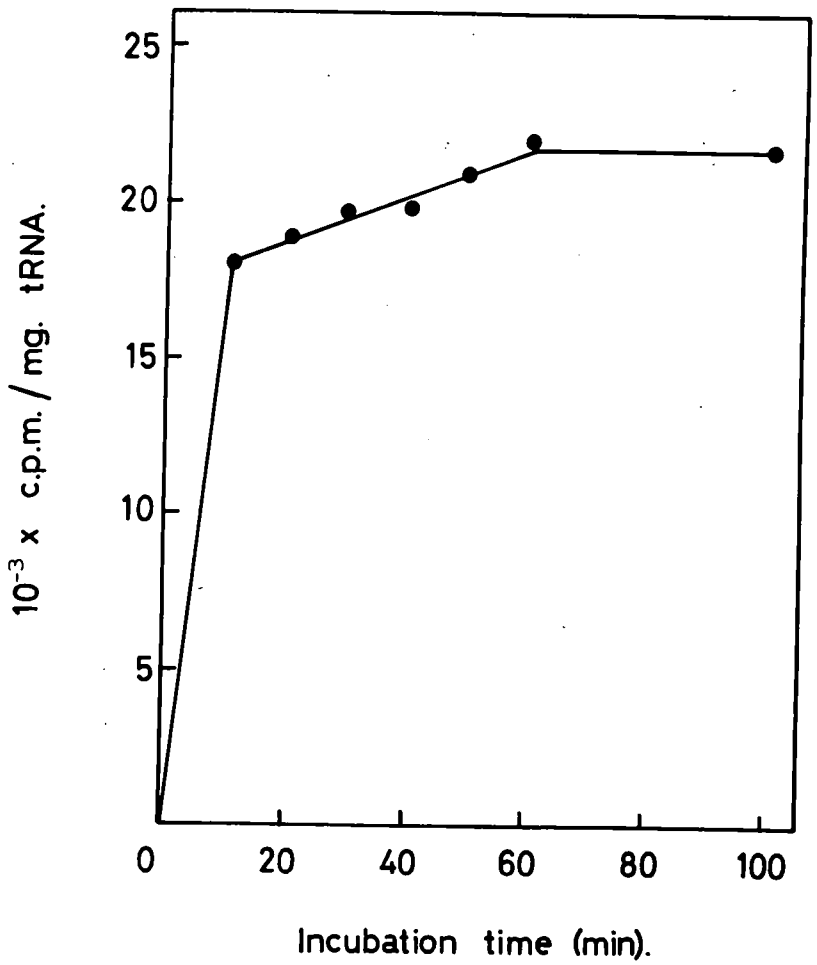
2 ml. incubations were set up as in methods and incubated for 30 min, using tRNA from 80 day cotyledons before chromatography. The tRNA was re-extracted by the method of Ravel et. al. (1966). 4 x 0·1 ml. samples were removed at the above stages in the re-extraction procedure, onto filter paper discs which were washed and counted as described in methods.

Fig.17. Effect of temperature on charging of tRNA with [^{14}C] phenylalanine



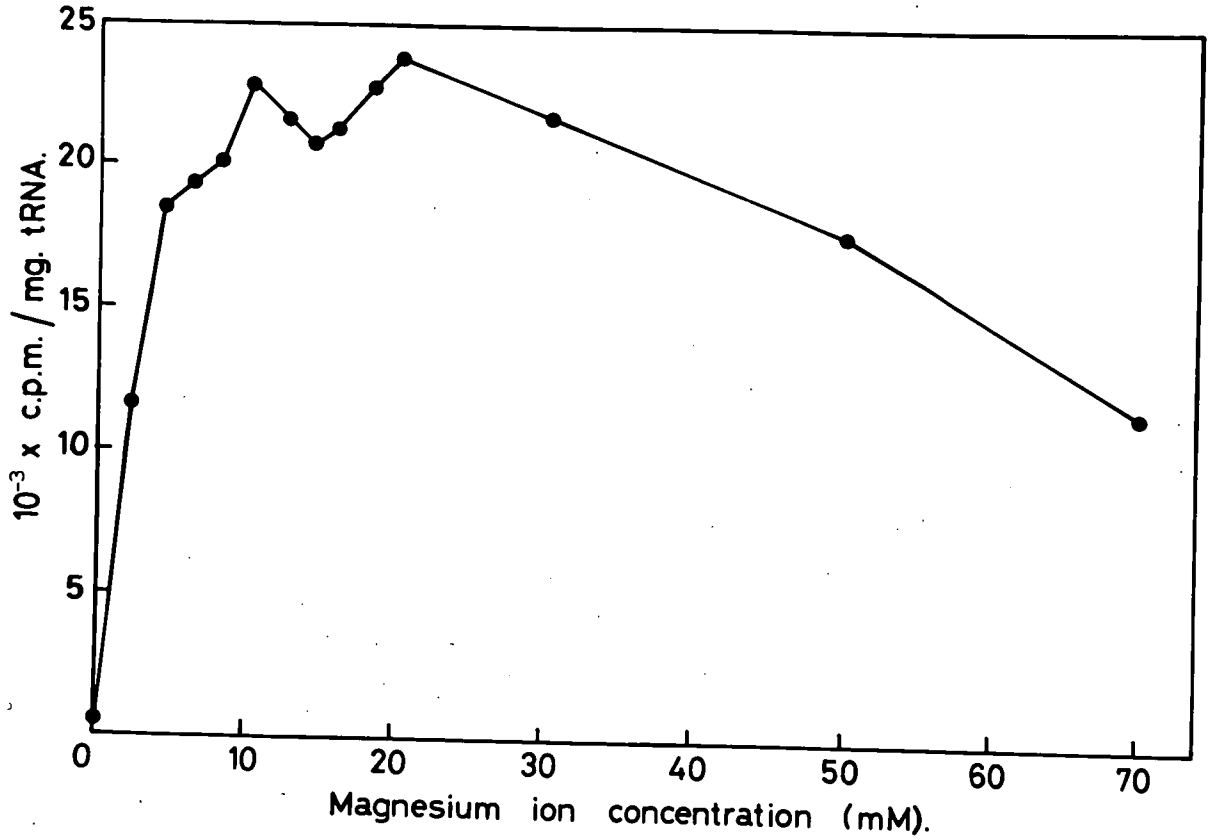
Incubations contained tRNA from 80 day seeds and enzyme fraction from 70 day cotyledons.
Each value is the average of 4 determinations.

Fig.18. Time curve for charging of tRNA with [^{14}C] phenylalanine



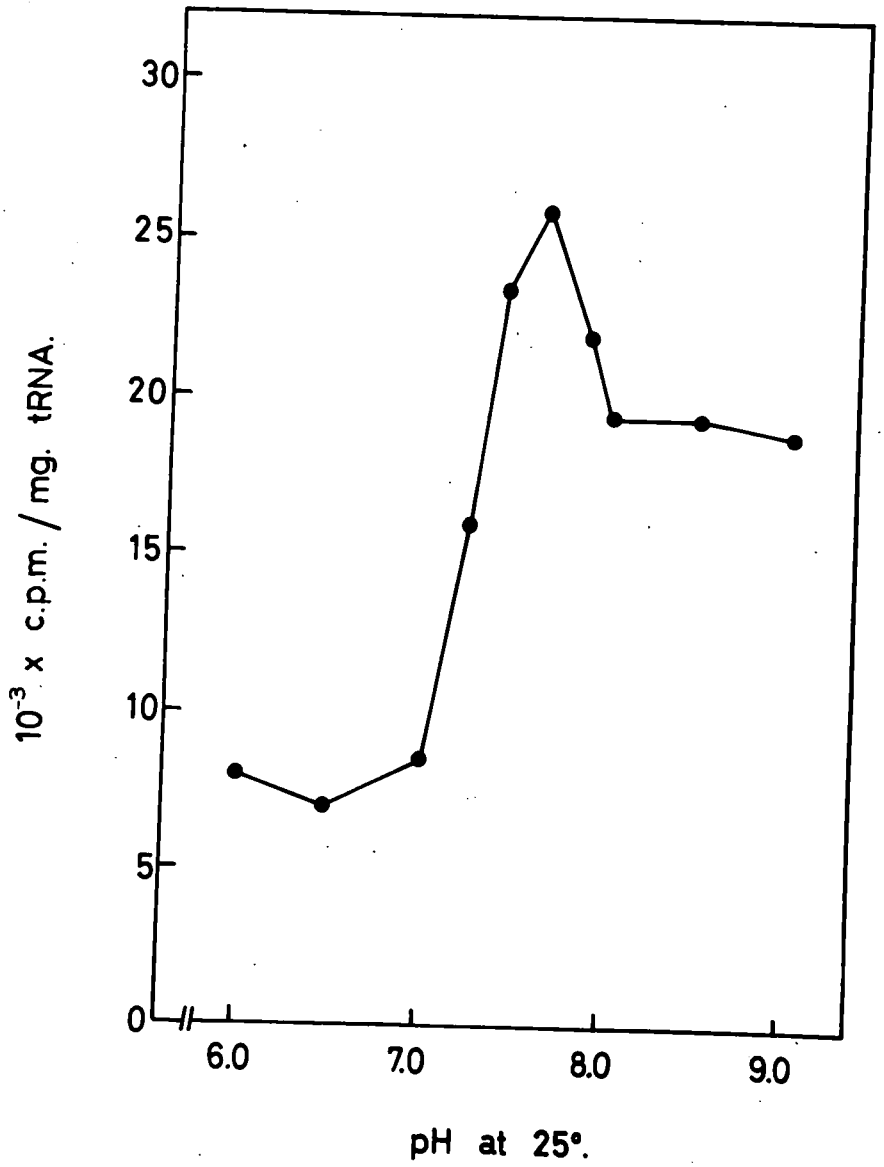
Incubations contained tRNA from 80 day seeds and enzyme fraction from 70 day cotyledons.
Each value is the average of 4 determinations.

Fig.19. Effect of Magnesium concentration on charging of tRNA with [^{14}C] phenylalanine



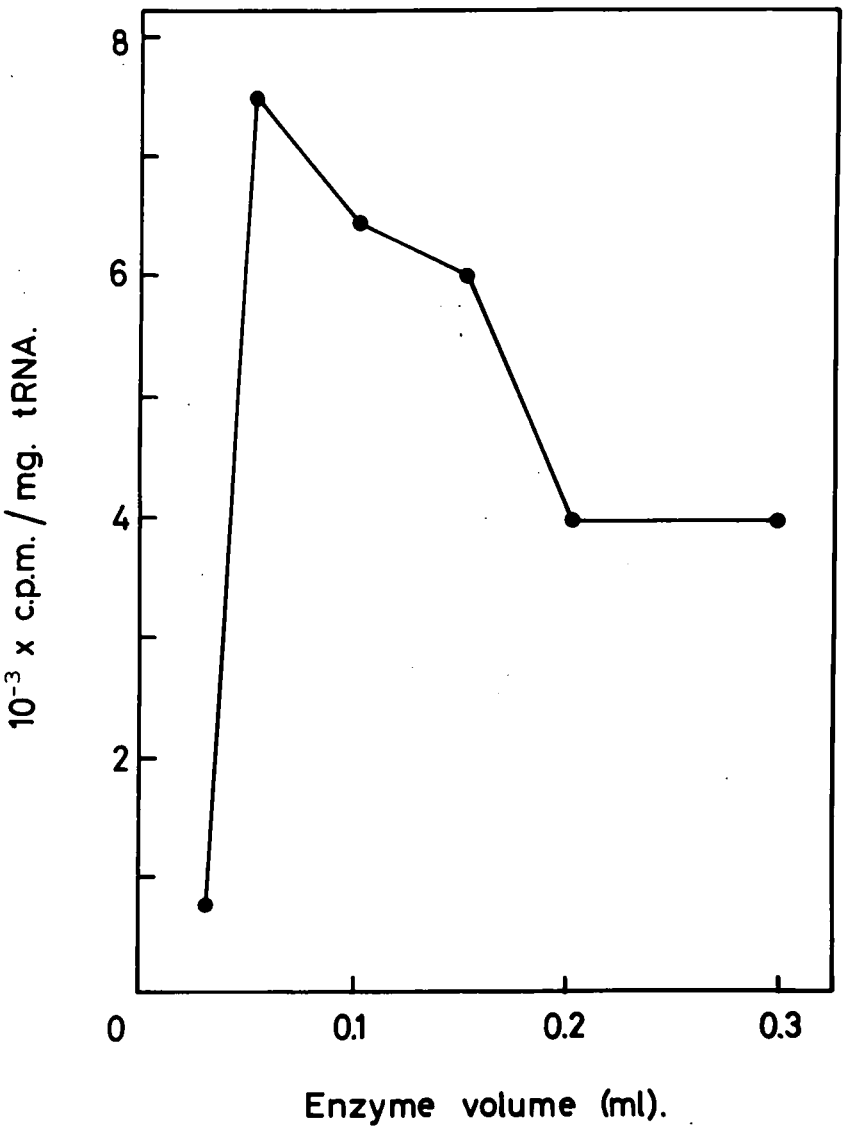
Incubations contained tRNA from 80 day seeds and enzyme fraction from 70 day cotyledons. Each value is the average of 4 determinations.

Fig.20. Effect of pH on charging of tRNA with [^{14}C] phenylalanine



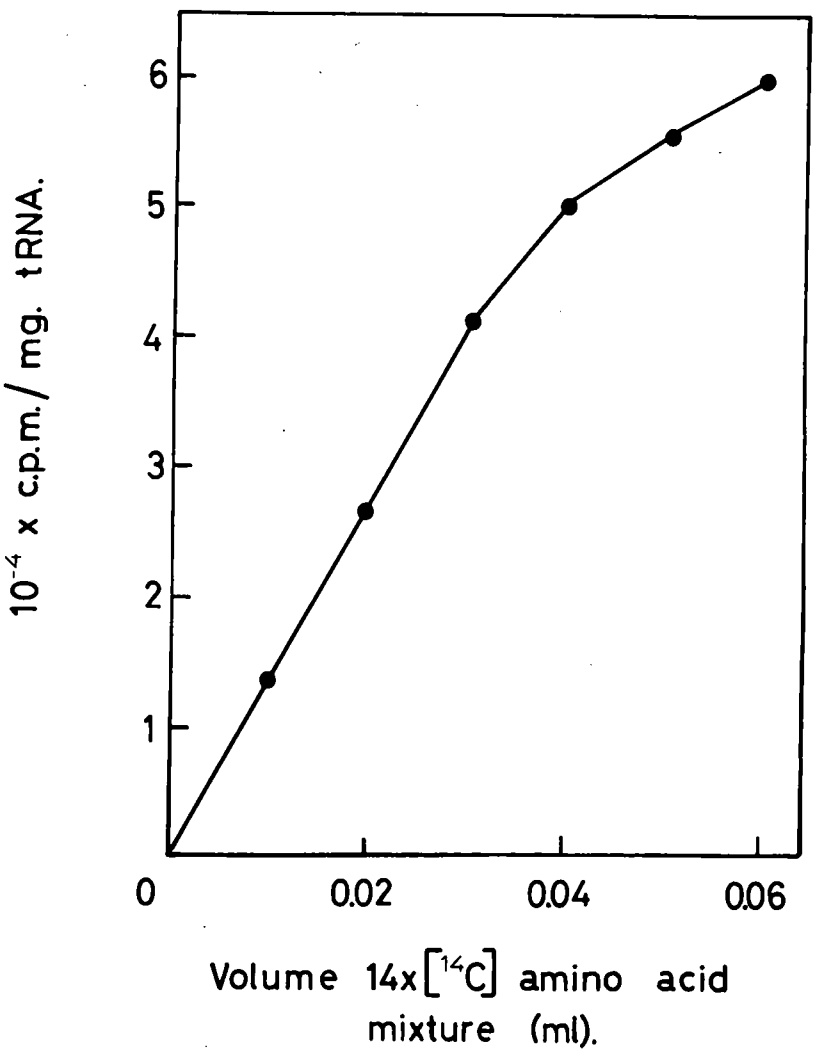
Incubations contained tRNA and enzyme fraction from 70 day seeds.
The results are the average of 4 determinations.

Fig.21. Effect of enzyme concentration on charging of tRNA with [^{14}C] phenylalanine



Incubations contained tRNA prepared from 10 day germinated bean seeds and enzyme fraction from 60 day cotyledons. The results are the average of 4 determinations.

Fig.22. Effect of adding different volumes of 14x[U-^{14}C] amino acid mixture on charging of tRNA



Incubations as in Methods, except that [^{14}C] phenylalanine was replaced by 14x[U-^{14}C] amino acid mixture.

tRNA and enzyme fraction from 60 day seeds were used.
The results are the average of 4 determinations.

Table 6. Effect of the omission of enzyme and tRNA on charging of tRNA with 14[U-^{14}C] amino acid mixture

Incubation	After normal washing procedure		After 90°, 5% (w/v) TCA wash	
	c.p.m./sample	c.p.m./mg.tRNA	c.p.m./sample	c.p.m./mg.tRNA
Complete	1,825	48,000	58	1,520
- Enzyme	95	2,500	50	1,300
- tRNA	118	-	30	-

Legend as for fig. 22.

After 20 min. incubation, 4 x 0 1 ml. samples were removed onto filter paper discs. 2 discs were washed by the normal washing procedure (Methods - 8). The other 2 discs were subjected to the full washing procedure of Mans and Novelli (1961) including 90°, 5% (w/v) TCA wash for 15 min.

to a second optima at 20mM. There was, however, a very sharp pH optimum at 7·6 at 25° for charging of tRNA with phenylalanine (fig. 20). Maximum charging of tRNA occurred with an enzyme concentration of 0·06 ml/ml. incubation (fig. 21).

Total amino acyl acceptor capacity of the tRNA or the total amino acyl synthetase activity of the enzyme preparations was determined using the 14[U-^{14}C] amino acid mixture in Table 3. The level of charging was proportional to the volume of amino acid mixture added (fig. 22) and was dependent upon the addition of tRNA and enzyme. The amino acyl tRNA complexes were 95% hydrolysed by 5% (w/v) TCA at 90°. (Table 6).

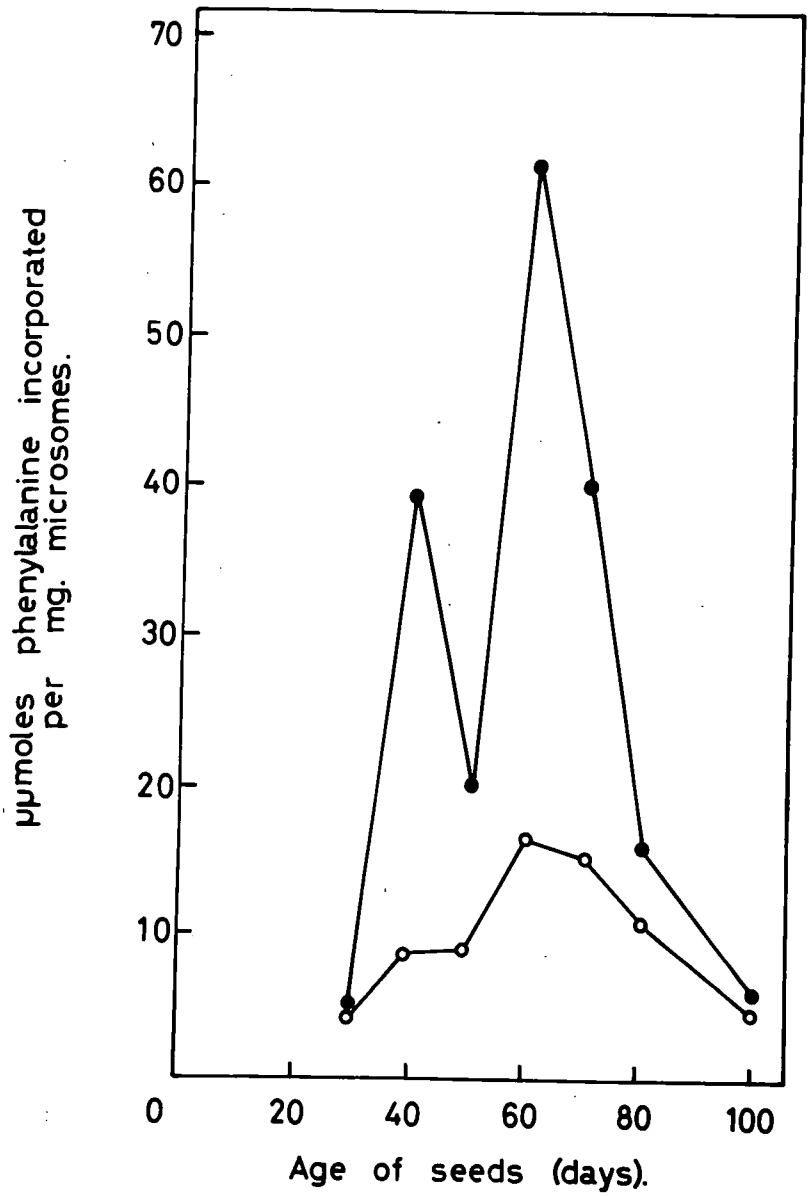
tRNA stored at -70° remained active over at least nine months storage, and repeated thawing and freezing did not affect its activity.

4. Changes in the *in vitro* amino acid incorporating activity of components from developing *Vicia faba* seeds of different ages.

A. Overall changes in *in vitro* incorporation by components from developing seeds of different ages.

Microsomes, enzyme fractions and tRNA were prepared from developing seeds of flowers that opened during the second week of June, 1969. The development of this group of seeds was followed throughout the rest of the season. Microsomes, enzyme

Fig.23. Overall changes in the in vitro amino acid incorporating activity during development of Vicia faba seeds - Based on microsome content as determined by E^{256}_{1cm}

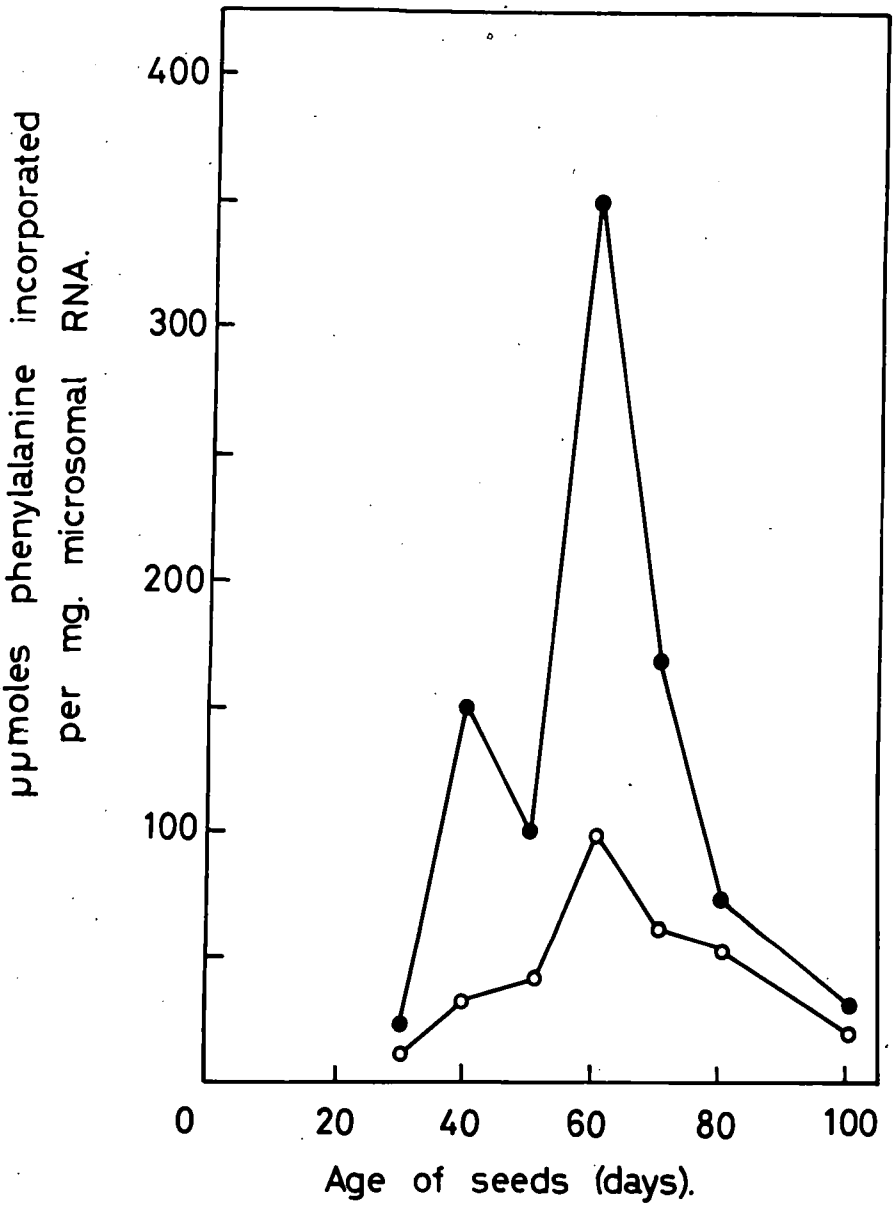


Incubated as in Methods.
Results are corrected for 0·4 mg. tRNA per ml. incubation.

●—● Incubations containing poly-U

O—O— Incubations minus poly-U

Fig.24. Overall changes in the *in vitro* amino acid incorporating activity during development of *Vicia faba* seeds - Based on RNA content of microsome preparations, determined by the Orcinol reaction



Incubated as in Methods.
Results are corrected for 0·4 mg. tRNA per ml. incubation.

●—● Incubations containing poly-U

O—O— Incubations minus poly-U

and tRNA were prepared from seeds 30, 40, 50, 60, 70, 80 and 100 days after flowering. After 100 days, the seeds were mature and had dried out.

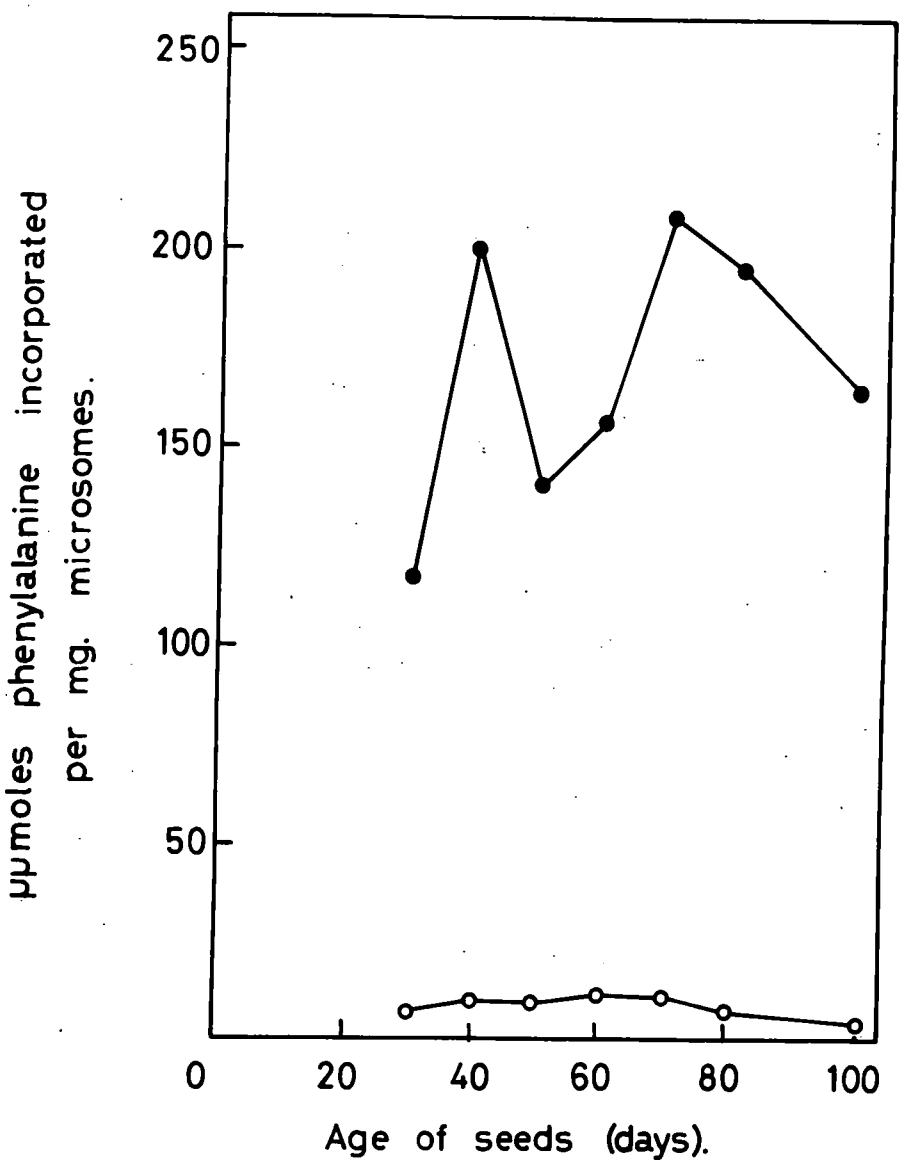
The overall changes in <u>in vitro</u> incorporating activity during development were measured by incubating all the components prepared from the same age of seed material together in a complete system and comparing the activity at the different ages with each other. Thus, incubations of 30 day material contained all components from 30 day seeds etc.

The results are shown in fig. 23 and 24 and were calculated on the basis of microsome content of the preparations (from $E^{256}_{1cm.}$) and also on their actual RNA content, as determined by the orcinol reaction.

Phenylalanine incorporation in the absence of poly-U reflects the activity of natural messenger and functional polysome aggregates in the incubations, and was greatest in preparations from 60 day seeds.

Phenylalanine incorporation in the presence of poly-U, reflects this activity as well as incorporation by subunits and monosomes in the incubations, that are inactive unless a messenger is added. These results reflect the potential rather than the actual amino acid incorporating activity of the incubations. There were two peaks of poly-U directed phenylalanine incorporating activity as

Fig.25. Changes in the in vitro efficiency of microsomes prepared from different ages of developing V.faba seeds in amino acid incorporation - Based on Microsome content as determined by $E^{256}_{1cm.}$

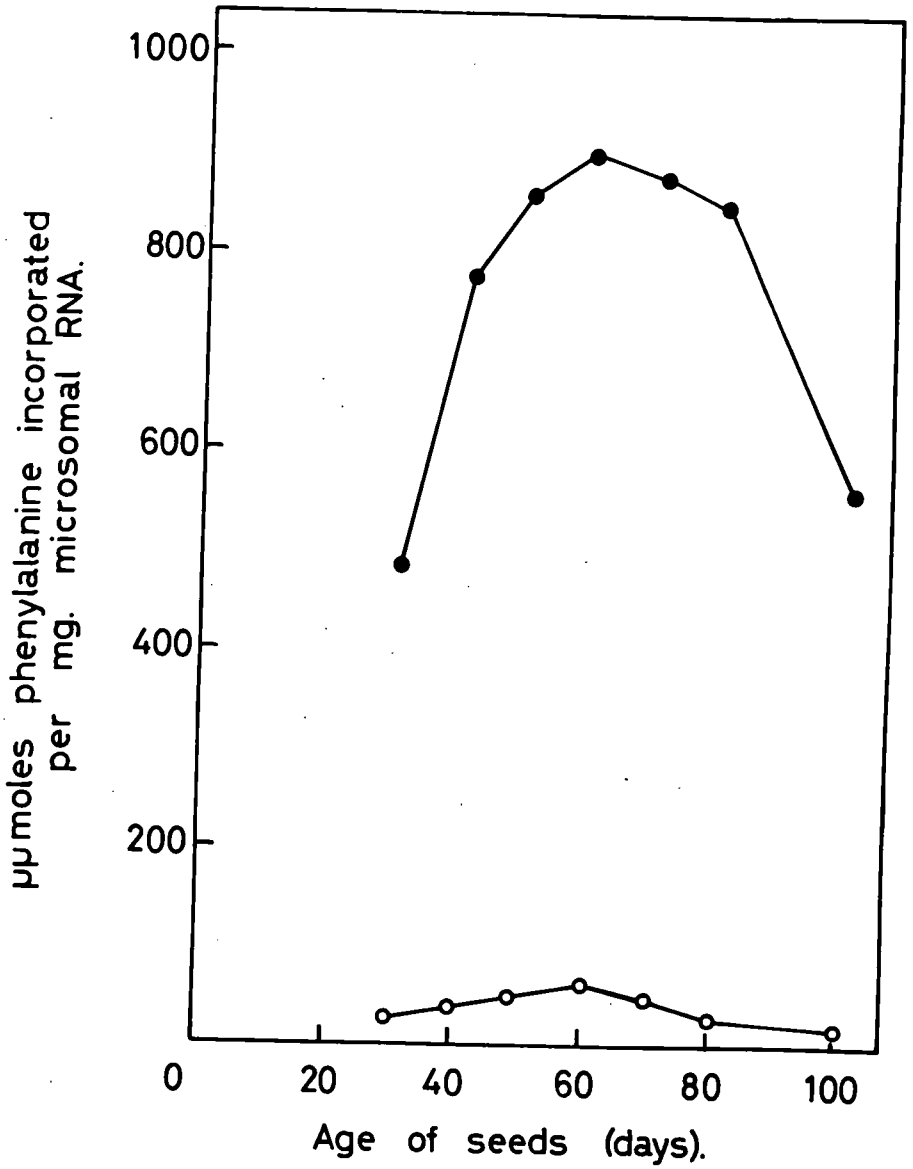


Incubations contained enzyme fraction from 60 day cotyledons and tRNA from 40 day seeds

●—● plus poly-U

○—○ minus poly-U

Fig.26. Changes in the in vitro efficiency of microsome prepared from developing V.faba seeds of different ages in amino acid incorporation - Based on RNA content of microsomes as determined by the Orcinol method.



Incubated as in Methods.
Results are corrected for 0·4 mg. tRNA per ml. incubation.

●—●— Incubations containing poly-U

○—○— Incubations minus poly-U

the seeds developed; the first with material from 40 day seeds, rising to a second peak with material from 60 day seeds. This pattern occurred whether the results were calculated on the basis of microsome content, or on the actual RNA content of the microsome preparations. There was very little activity with material from 30 or 100 day seeds, even when an artifical messenger was added to the system.

B. Changes in the activity of the microsomes prepared from developing seeds of different ages

The changes in the in vitro activity of the microsomes in amino acid incorporation during the development of the bean seeds were measured by incubating the various microsome preparations with standard tRNA and enzyme fractions in the complete system. Enzyme from 60 day cotyledons and tRNA from 40 day seeds were selected for the standards.

Phenylalanine incorporation in the absence of added messenger was very low in all the microsome preparations.

Poly-U directed incorporation of phenylalanine when calculated per mg. microsomes as determined by $E^{256}_{1cm.}$ showed a double peaked curve of activity as the seeds matured (fig. 25). These results were similar to those obtained with the 1968 harvest.

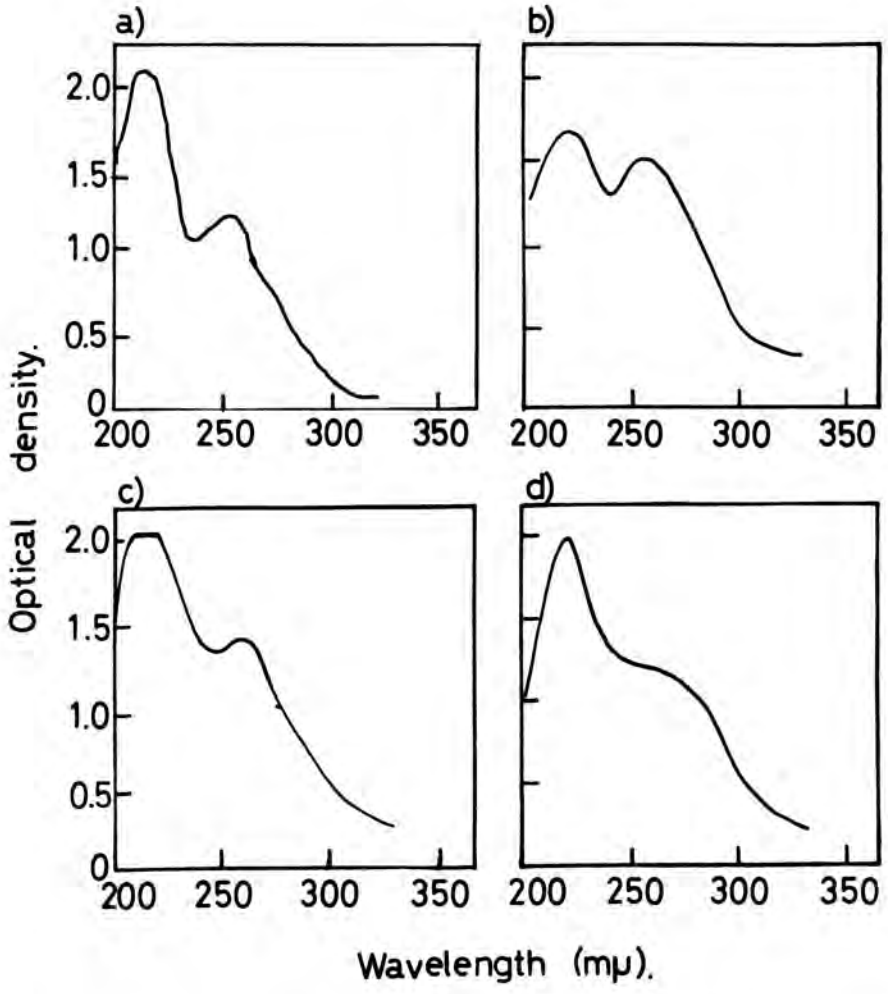
In fig. 26, the activity of the microsome preparations in amino acid incorporation has been recalculated on their RNA content,

Table 7. Changes in the RNA : Protein ratio of microsomes prepared from developing V.faba seeds of different ages

Age of cotyledons from which microsomes were prepared	RNA concentration (mg./ml.)	Protein concentration (mg./ml.)	RNA : Protein ratio
30	5·25	8·0	1·5
40	6·0	13·6	2·3
50	3·75	13·6	3·6
60	3·5	17·6	5·0
70	3·5	16·0	4·7
80	5·0	20·4	4·1
100	3·6	16·0	4·4

RNA and protein were determined as described in Methods.

Fig.27. Absorption spectra of Microsome preparations from developing V.faba seeds



a) Microsomes prepared from cotyledons of 30 day seeds

b) Microsomes prepared from cotyledons of 40 day seeds

c) Microsomes prepared from cotyledons of 50 day seeds

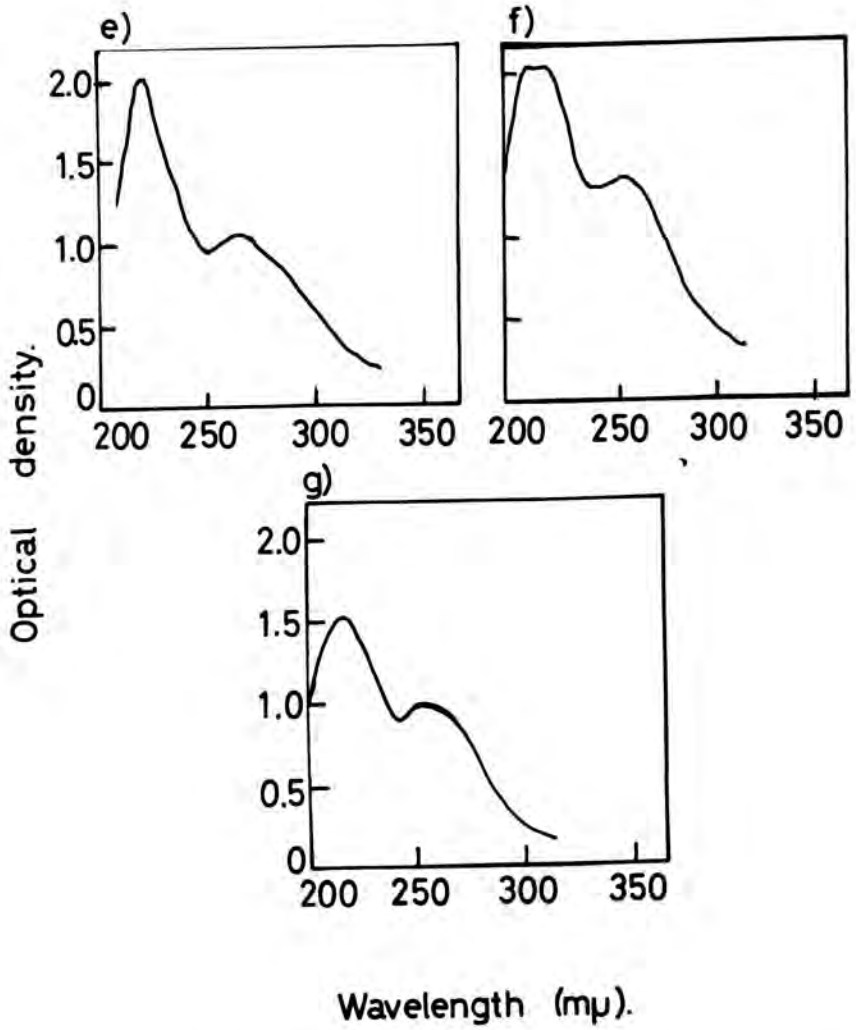
d) Microsomes prepared from cotyledons of 60 day seeds

Fig.27 (contd.)

e) Microsomes prepared from cotyledons of 70 day seeds

f) Microsomes prepared from cotyledons of 80 day seeds

g) Microsomes prepared from cotyledons of 100 day seeds



Aliquots of the Microsomal suspensions were diluted 200 fold in distilled water.

Absorption spectra were determined using the Hilger and Watts Ultrascan recording spectrophotometer H999 Mark II.

Table 8. Changes in absorption spectra and 260 : 235 absorption ratio of microsomes prepared from different ages of V.faba cotyledons

Age of Cotyledons (days)	Absorption (mμ)		260:235 ratio
	Minimum	Maximum	
30	238	256	1·17
40	240	256	1·09
50	243	256	0·94
60	248	256	0·81
70	246	256	0·85
80	242	256	0·89
100	242	256	0·84

Legend as for fig. 27

as determined by the orcinol method, and the results show that phenylalanine incorporation increased with increasing age of the developing bean seeds up to a maximum at 60 days after flowering.

Microsomes from 30 and 100 day cotyledons were capable of considerable incorporation in the presence of added messenger.

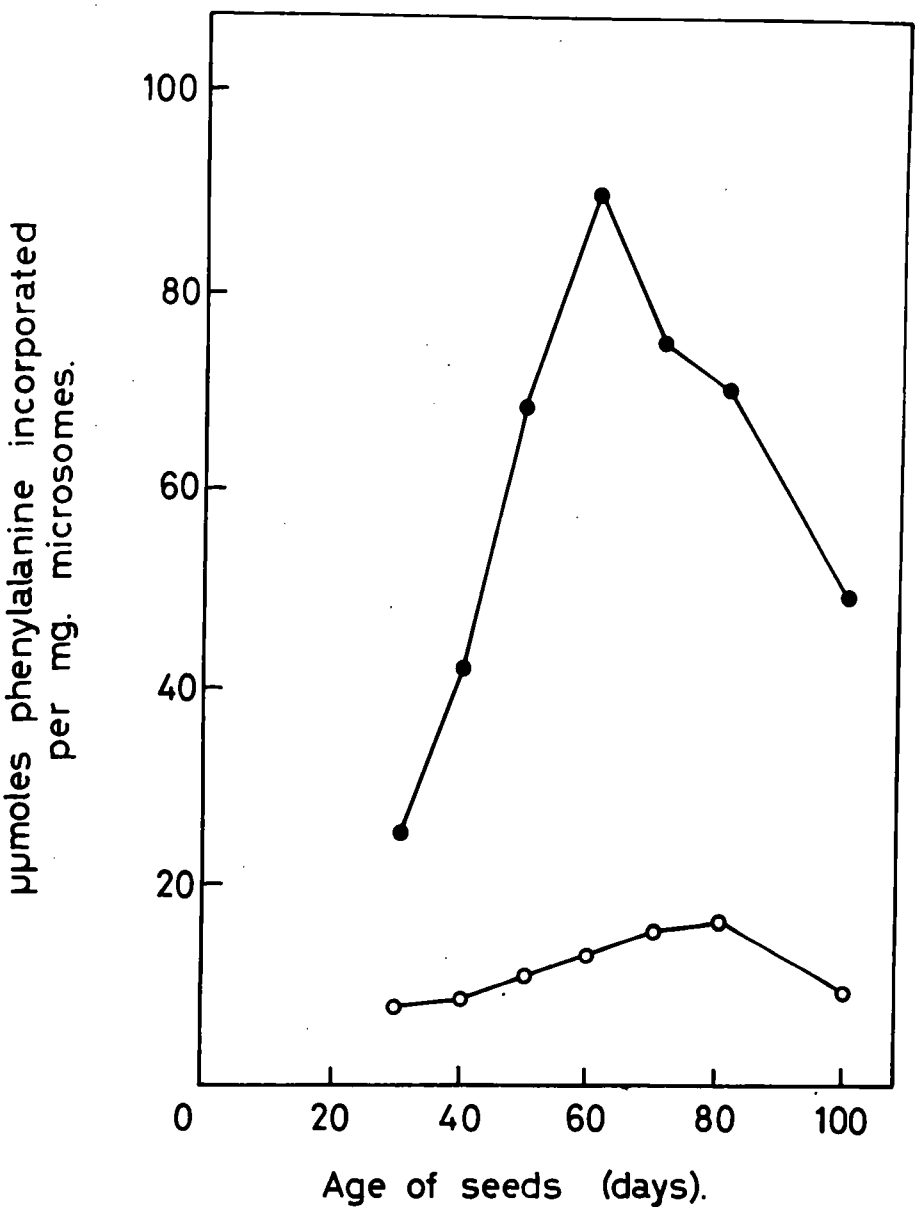
C. Changes in the RNA: Protein ratio of microsomes prepared from different ages of developing bean seeds

The changes in the RNA: Protein ratio of microsomes prepared from cotyledons at different stages of their development are shown in Table 7. The increasing protein content of the microsome preparations influenced their absorption spectra, (fig.27), resulting in a decreased 260:235 absorption ratio and displacement of the wavelength of minimum absorption (Table 8).

D. Changes in the in vitro activity of enzyme fraction from developing seeds of different ages

Total amino acid incorporating activity of the enzyme fractions from different ages of developing seeds was measured in the complete system, using standard microsomes and tRNA. The concentration of enzyme promoting maximum phenylalanine incorporation had been determined previously, and this concentration was used. (0·02 ml./ml. incubation for enzyme fractions from 30 and 40 day cotyledons, and 0·06 ml./ml. incubation for enzyme from 50, 60, 70, 80 and 100 day cotyledons).

Fig.28. Activity of enzyme fractions prepared from developing V.faba seeds of different ages in in vitro amino acid incorporation

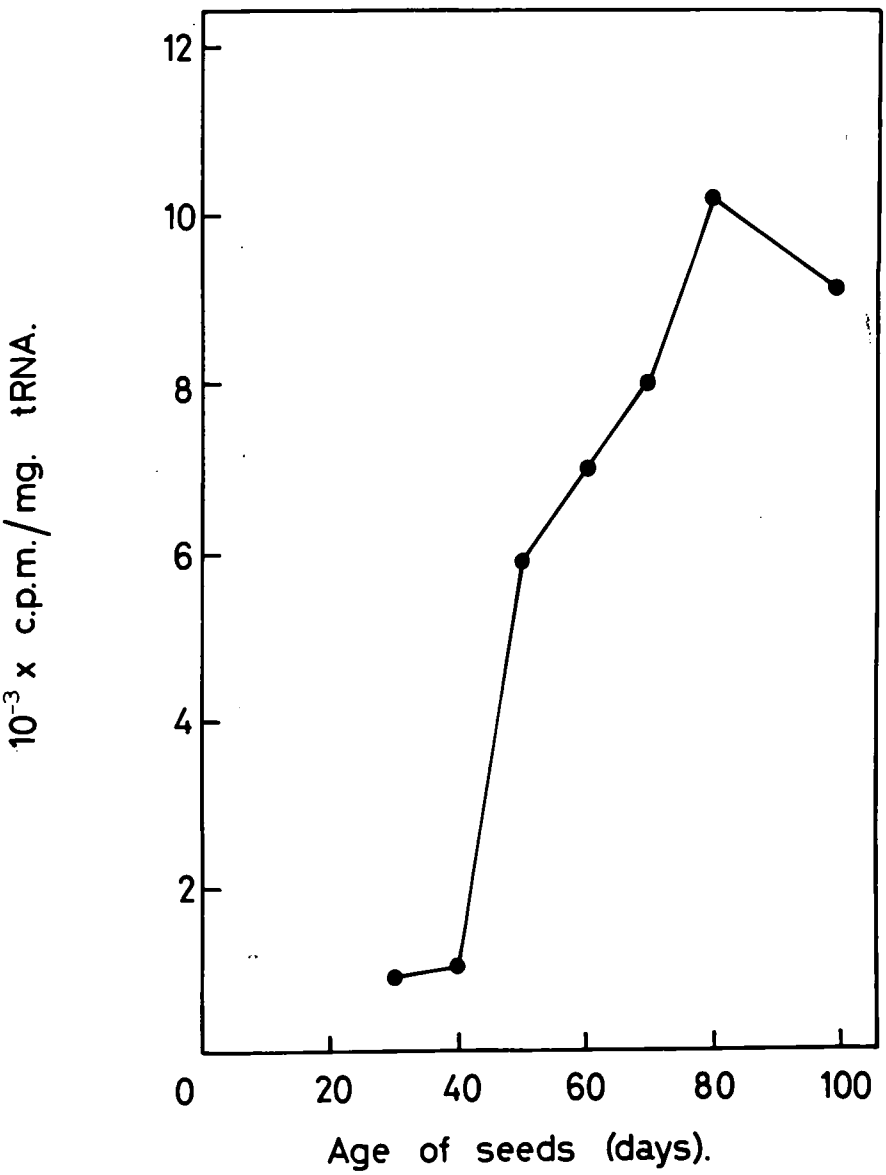


Complete incubations as in Methods containing microsomes from 60 day cotyledons and tRNA from 40 day seeds.

●—● plus poly-U

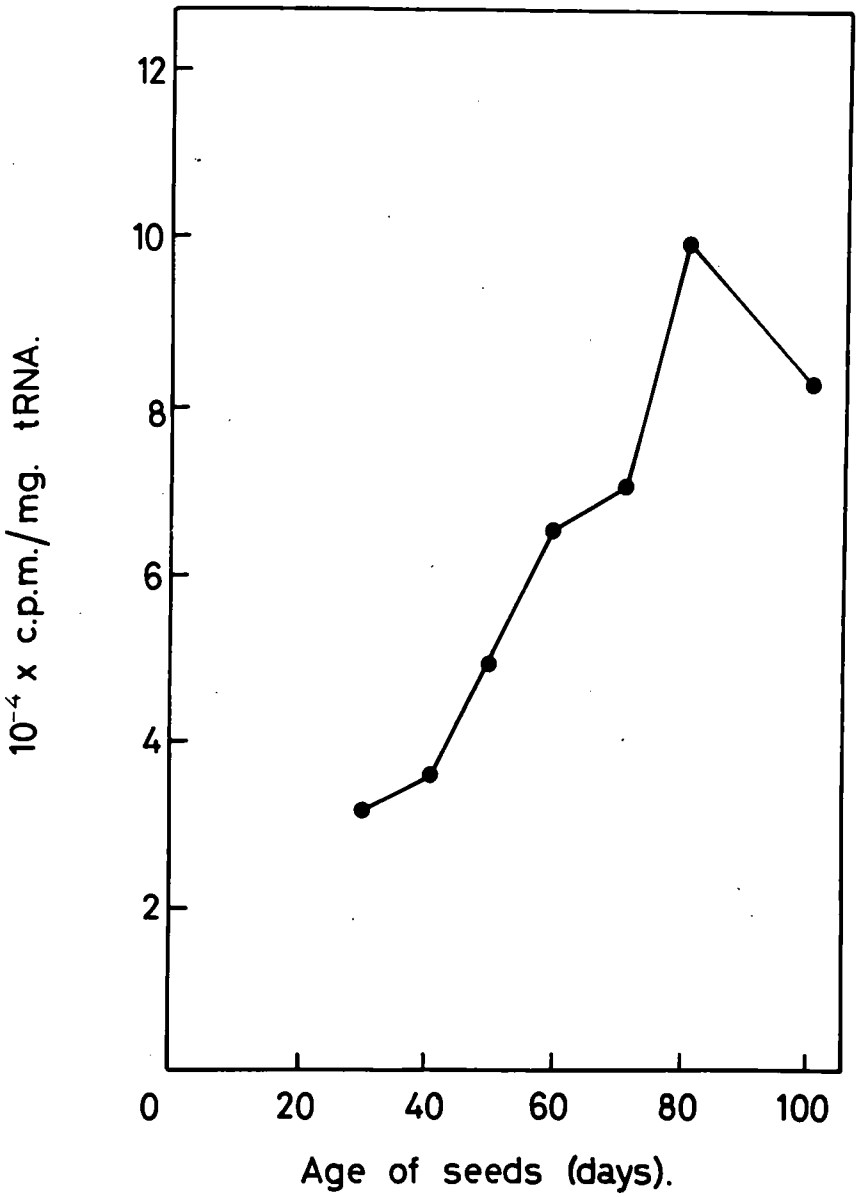
o—o minus poly-U

Fig.29. Phenylalanyl synthetase activity of enzyme fractions from developing seeds of different ages



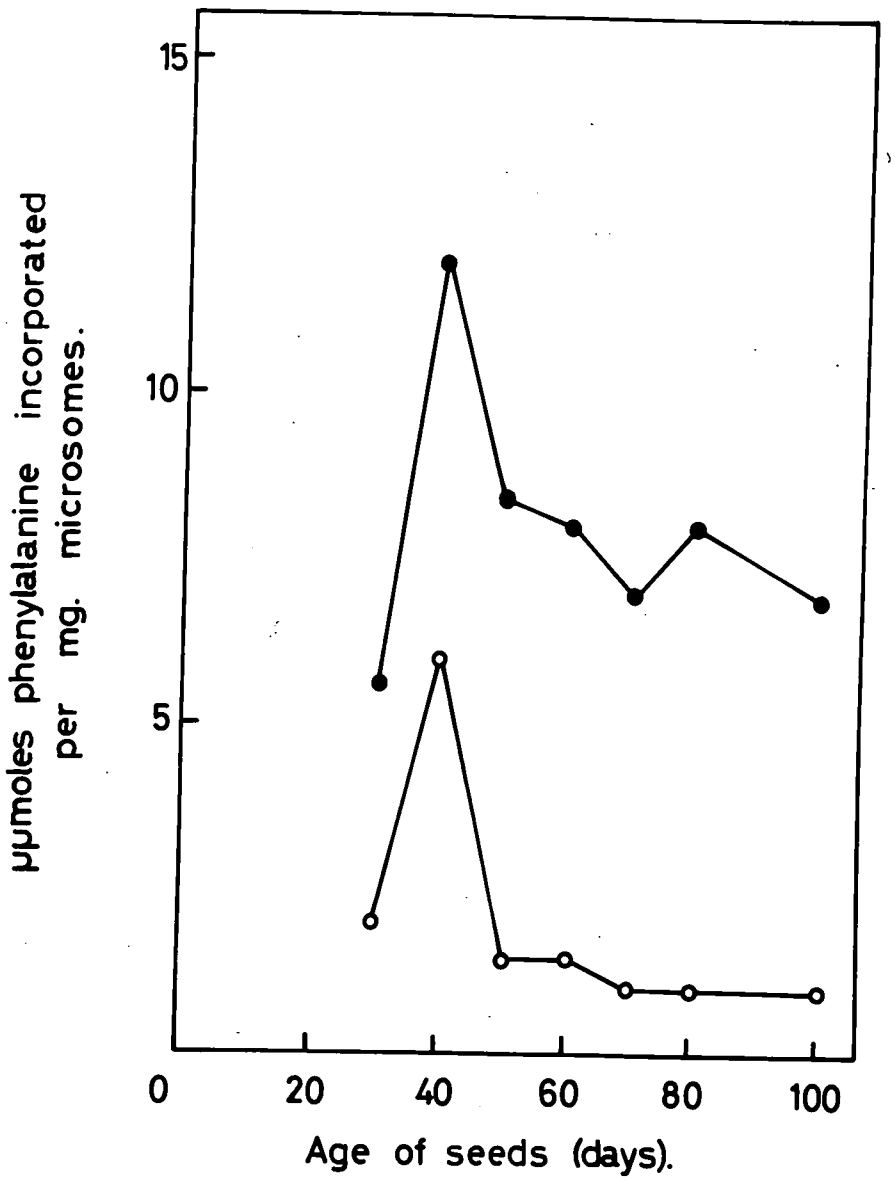
Charging incubations as in Methods, containing tRNA from 100 day seeds
Results are the average of 4 determinations.

Fig.30. Total amino acyl synthetase activity of enzyme fractions from developing seeds of different ages



Legend as for Fig.29, except that [^{14}C] phenylalanine was replaced by 14x[U-^{14}C] amino acid mixture.

Fig.31. Activity of enzyme fractions from developing seeds of different ages in the Transfer system



Incubations as in Methods, containing microsomes from 60 day cotyledons and tRNA from 60 day seeds charged with [^{14}C] phenylalanine and 19x[^{12}C] amino acids to a level of 4,500 c.p.m./mg. tRNA.

●—● Plus poly-U

O—O— Minus poly-U

Table 9. Protein concentration of enzyme fractions prepared from developing seeds of different ages

Age of seeds	Protein concentration (mg./ml.)	Protein concentration promoting maximum phenylalanine incorporation (µg./ml. incubation)
30	8.2	160
40	10.2	200
50	13.4	800
60	18.4	1,100
70	18.8	1,130
80	20.0	1,200
100	15.4	920

Protein concentration was determined on aliquots of the enzyme fractions as described in Methods.

The results are shown in fig. 28. The enzyme fraction from 60 day cotyledons was the most active in [^{14}C] phenylalanine incorporation in a poly-U directed system, and enzyme from 80 day cotyledons was most active in incorporation in the absence of poly-U. (A similar pattern of activity was obtained with material from the 1968 harvest).

Phenylalanyl and total amino acyl synthetase activity of the enzyme fractions was measured using [^{14}C] phenylalanine and the 14[U-^{14}C] amino acid mixture in the tRNA charging assays, using standard tRNA. The results are presented in fig. 29 and 30. The amino acyl synthetase activity of the enzyme fractions increased during seed development to a maximum with material from 80 day cotyledons.

Phenylalanyl charged tRNA was used in the transfer system to measure the transferase activity of the various enzyme fractions, using standard microsomes. The results are shown in fig. 31 and it can be seen that enzyme fractions from 40 day cotyledons were most active in this system.

The protein concentration of the enzyme fractions is shown in Table 9.

E. The effect of DE52 chromatography on the activity of tRNA in amino acid incorporation

DE52 chromatography improved the amino acyl acceptor capacity of tRNA (Table 10) and its activity in *in vitro* amino acid

Table 10. Effect of DE 52 chromatography on phenylalanyl acceptor capacity of tRNA

Source of tRNA	c.p.m./mg. tRNA		%increase in Activity
	Before chromatography	After chromatography	
30 day seeds	1,436	12,440	88
60 day seeds	19,068	36,220	34
100 day seeds	4,322	13,835	45

Incubated as in Methods, using enzyme from 70 day cotyledons.

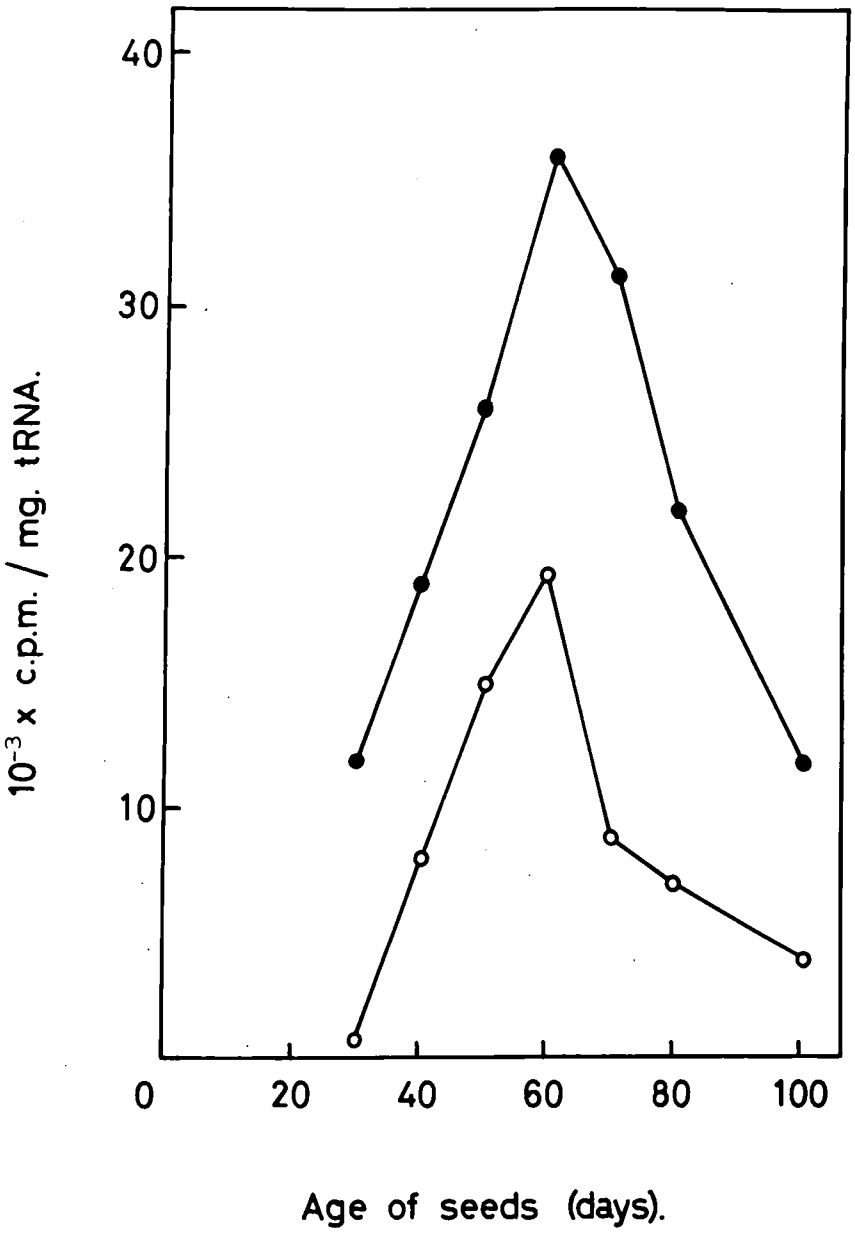
3 x 0·1 ml. samples were removed from assay after 20 min.

Table 11. Effect of DE52 chromatography on the activity of tRNA in [^{14}C] phenylalanine incorporation

Source of tRNA	µµmoles phenylalanine incorporated/mg microsomes		%increase in activity
	Before chromatography	After chromatography	
30 day seeds	81	161	50
40 day seeds	108	164	30
60 day seeds	65	82	22

Incubations contained microsomes and enzyme from 60 day cotyledons. 2 x 0·1 ml. samples were removed for assay after 20 min.

Fig.32. Changes in phenylalanyl acceptor capacity of tRNA prepared from developing seeds of different ages - the effect of DE52 chromatography on charging capacity

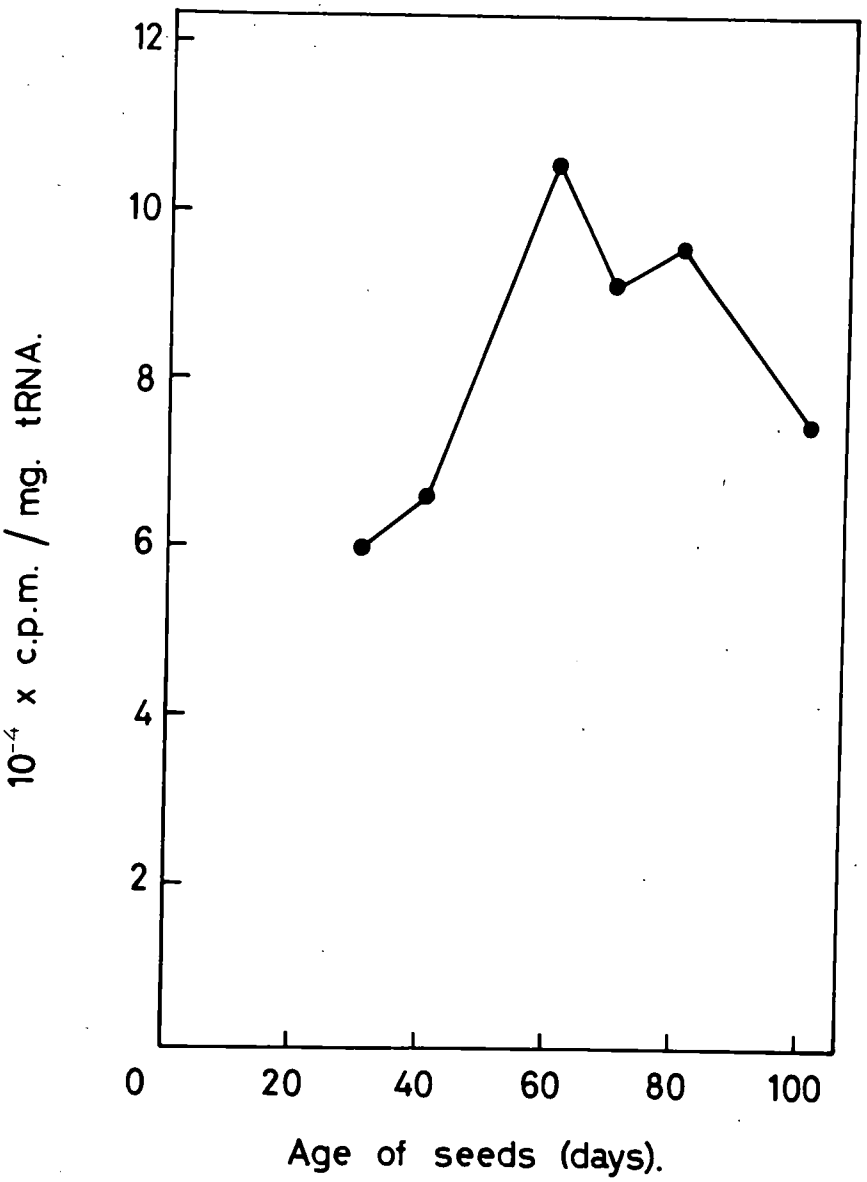


Incubations as in Methods, containing enzyme from 70 day cotyledons

O—O— tRNA before chromatography

●—● tRNA after chromatography

Fig.33. Changes in the total amino acyl acceptor capacity of tRNA prepared from developing seeds of different ages.



Incubations as in Methods, except that [^{14}C] phenylalanine was replaced by 14x[U-^{14}C] amino acid mixture.

Enzyme from 60 day cotyledons and tRNA after DE52 chromatography were used.

incorporation in the complete system (Table 11).

F. Changes in the amino acyl acceptor capacity of tRNA during seed development

Changes in the phenylalanyl and total amino acyl acceptor capacity of tRNA prepared from developing seeds of different ages were measured in the charging assay system with [^{14}C] phenylalanine and the 14[U-^{14}C] amino acid mixture.

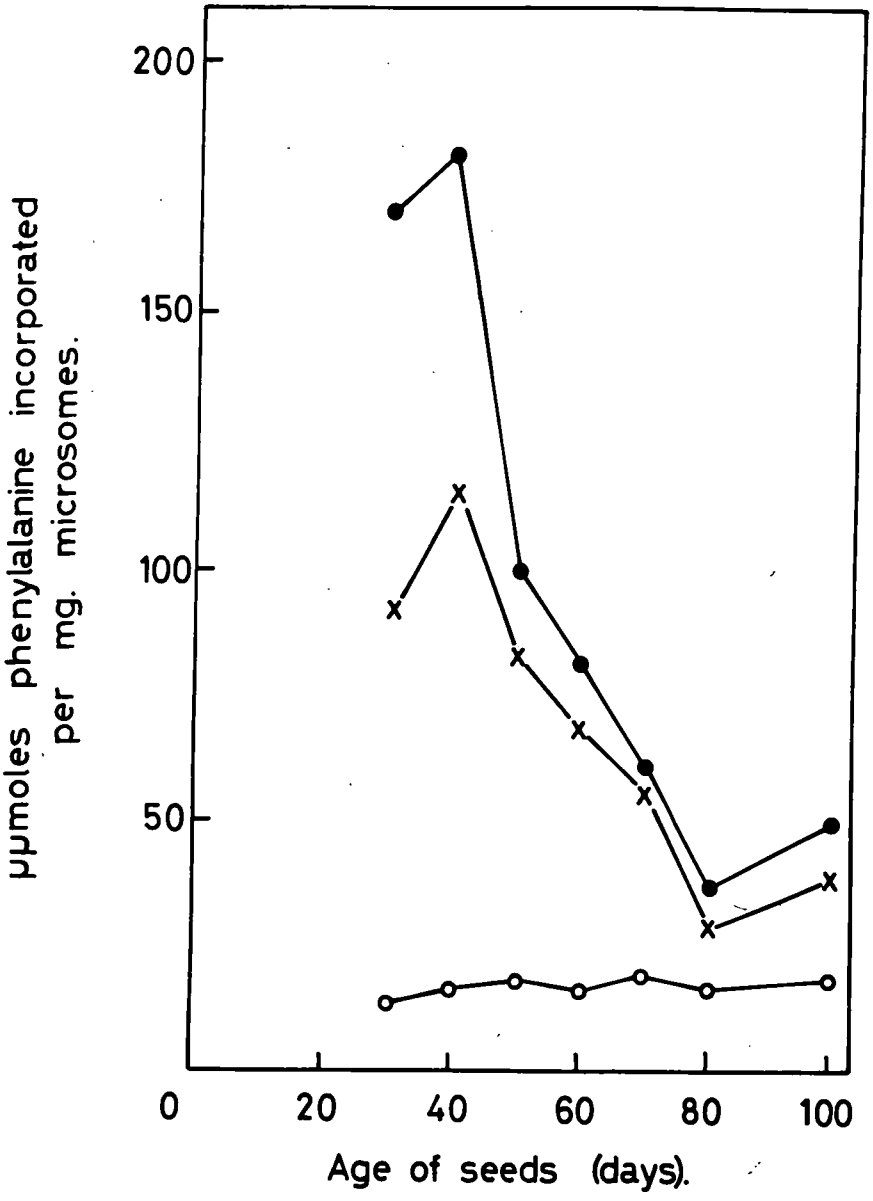
Fig. 32 shows the changes in phenylalanine acceptor capacity of tRNA prepared from developing seeds of different ages, and compares activity before and after chromatography. These results were independent of the enzyme fraction used and a similar pattern was obtained using enzyme from 40 day cotyledons. Although chromatography improved the amino acyl acceptor capacity of the tRNA, it did not alter the pattern of activity of tRNAs from different ages of seeds.

Changes in the total amino acyl acceptor capacity of the tRNA measured with the 14[U-^{14}C] amino acid mixture followed the same pattern as phenylalanyl acceptor capacity (fig. 33); tRNA from 60 day seeds being the most active.

G. Changes in the activity of tRNA from developing seeds of different ages in in vitro amino acid incorporation

The activity of tRNA prepared from different ages of developing seeds in in vitro incorporation was measured in the

Fig.34. Activity of tRNA prepared from different ages of developing seeds in phenylalanine incorporation in the Complete System - the effect of DE52 chromatography on activity



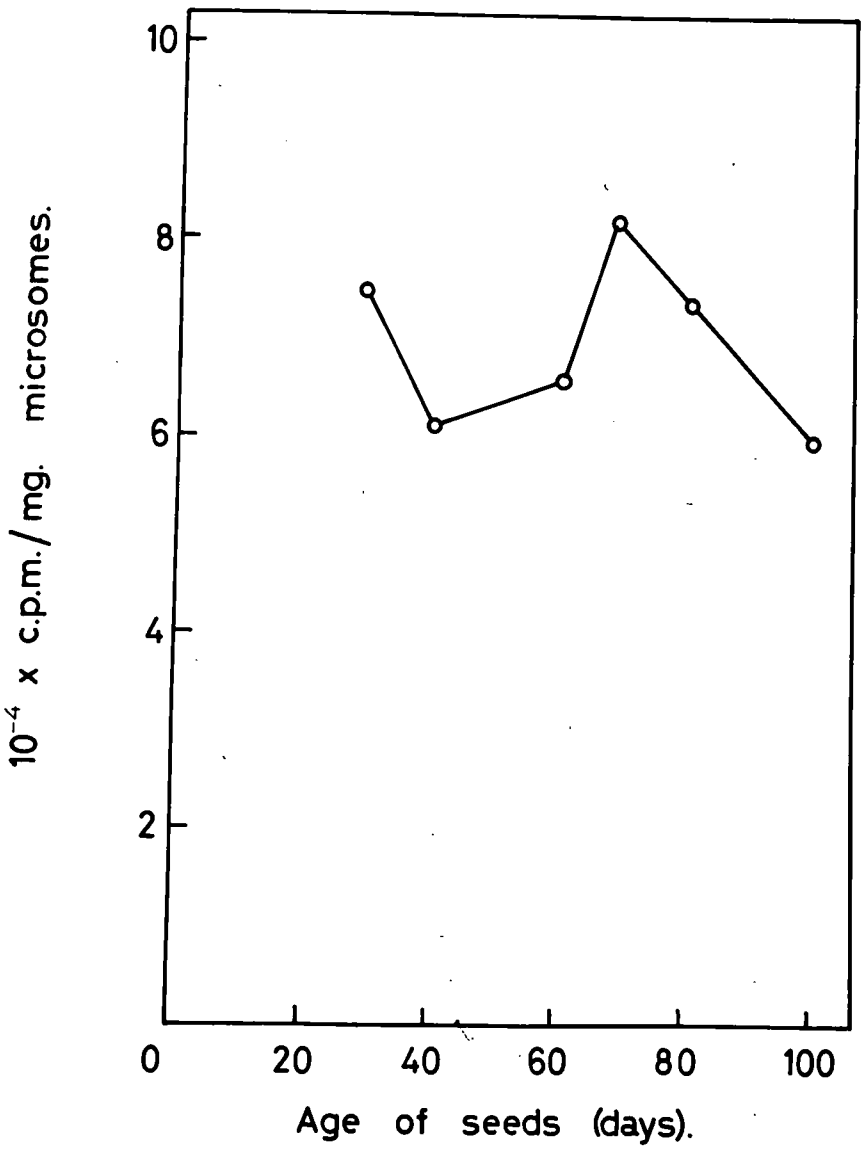
Incubated as in Methods, using microsomes and enzyme from 60 day cotyledons. Results corrected for 0·4 mg. tRNA per ml. incubation.

x—x— tRNA before chromatography; plus poly-U

●—●— tRNA after chromatography; plus poly-U

○—○— tRNA after chromatography; minus poly-U

Fig.35. Activity of tRNA prepared from different ages of developing seeds in amino acid incorporation using 14x[U-^{14}C] amino acid mixture



Incubations contained microsomes and enzyme from 60 day cotyledons, and [^{14}C] phenylalanine was replaced by 14x[U-^{14}C] amino acid mixture and poly-U was omitted.

complete system using standard tRNA and enzyme.

The results for [^{14}C] phenylalanine incorporation are presented in fig. 34. DE52 chromatography improved the activity of tRNA in amino acid incorporation, although the overall changes in the pattern of activity during seed development were not altered; tRNA from the younger seeds being the most active in poly-U directed phenylalanine incorporation. In the absence of poly-U there was very little difference in the activity of the various tRNA preparations in [^{14}C] phenylalanine incorporation, or in total amino acid incorporation using the 14[U-^{14}C] amino acid mixture (fig. 35), although tRNA from 80 day seeds was slightly more active.

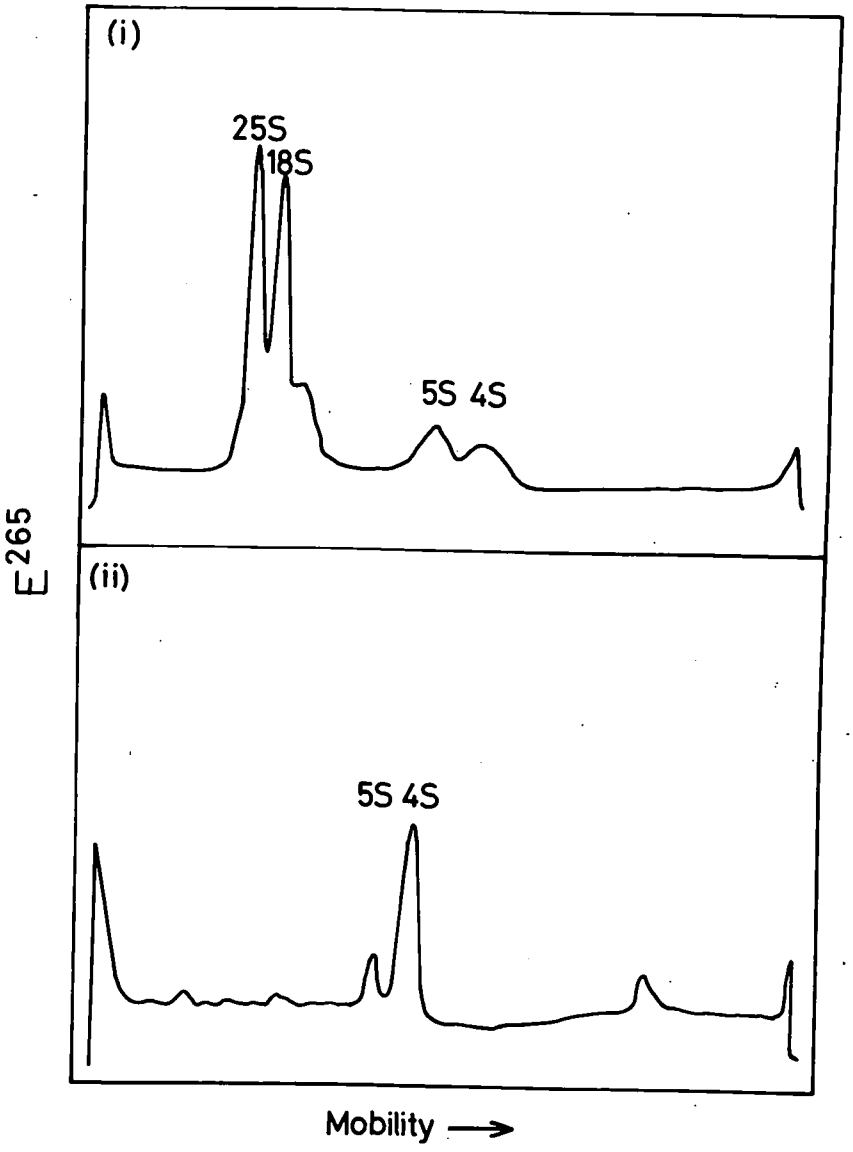
H. Characterization of tRNA preparations by polyacrylamide gel electrophoresis

Samples of tRNA before and after DE52 chromatography, prepared from different ages of developing bean seeds were run on 2.6% and 5% gels respectively, as described in Methods. The values of 25S, 18S, 5S and 4S (Loening, 1967) assigned to the various RNA fractions were used for easy identification of the RNA components, and do not imply accurately measured sedimentation co-efficients or mobilities.

The traces of the tRNA preparations run on polyacrylamide gels are shown in fig. 36. It can be seen that before

Fig.36. Polyacrylamide gel electrophoresis of tRNA prepared from developing V.faba seeds of different ages

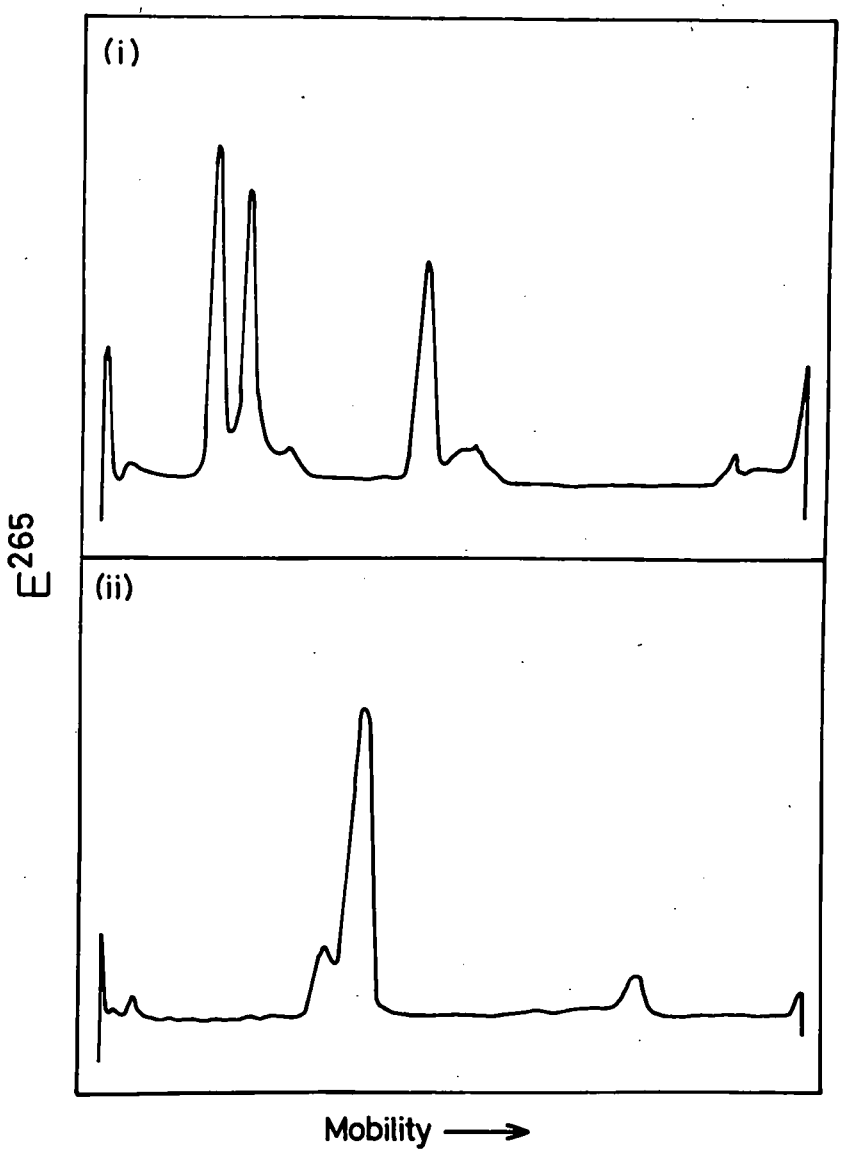
a) tRNA from 30 day seeds



(i) tRNA before chromatography run on 2·6% gels

(ii) tRNA after chromatography run on 5% gels, as described in Methods.

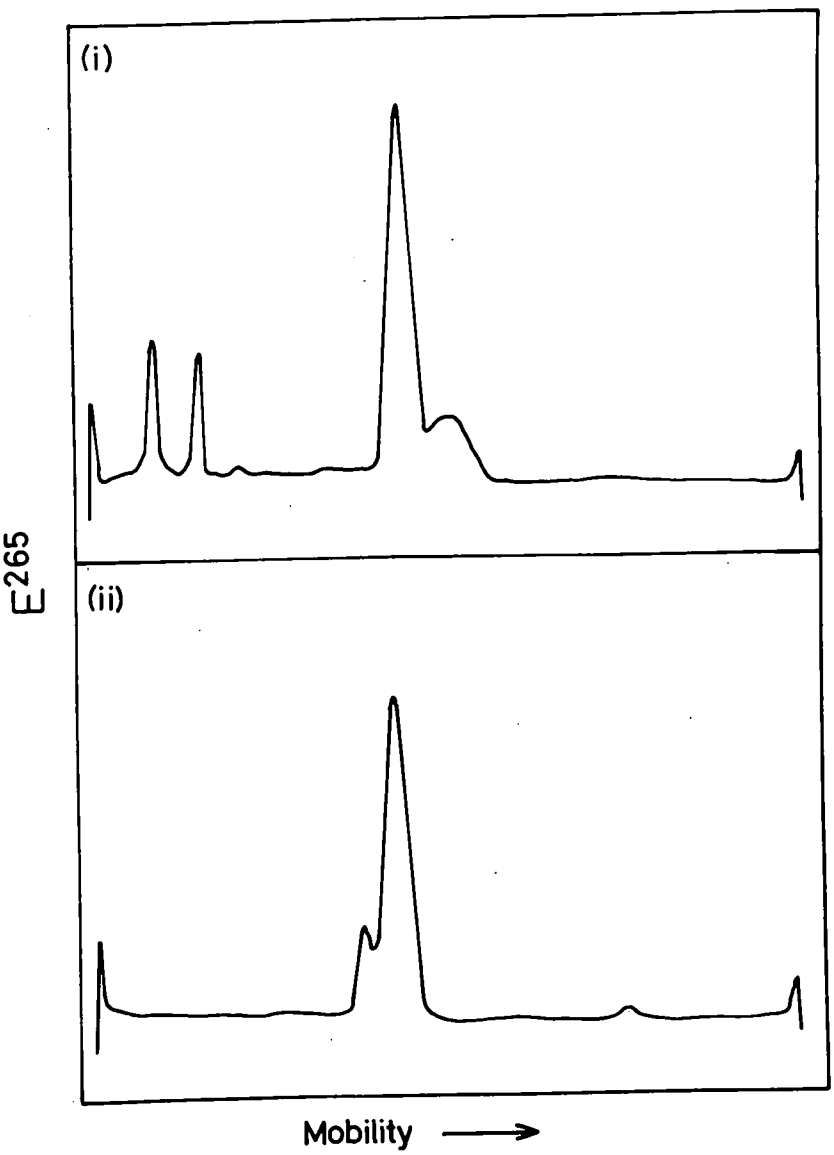
Fig.36. (cont). b) tRNA prepared from 40 day seeds



(i) tRNA before chromatography run on 2·6% gel

(ii) tRNA after chromatography, run on 5% gel.

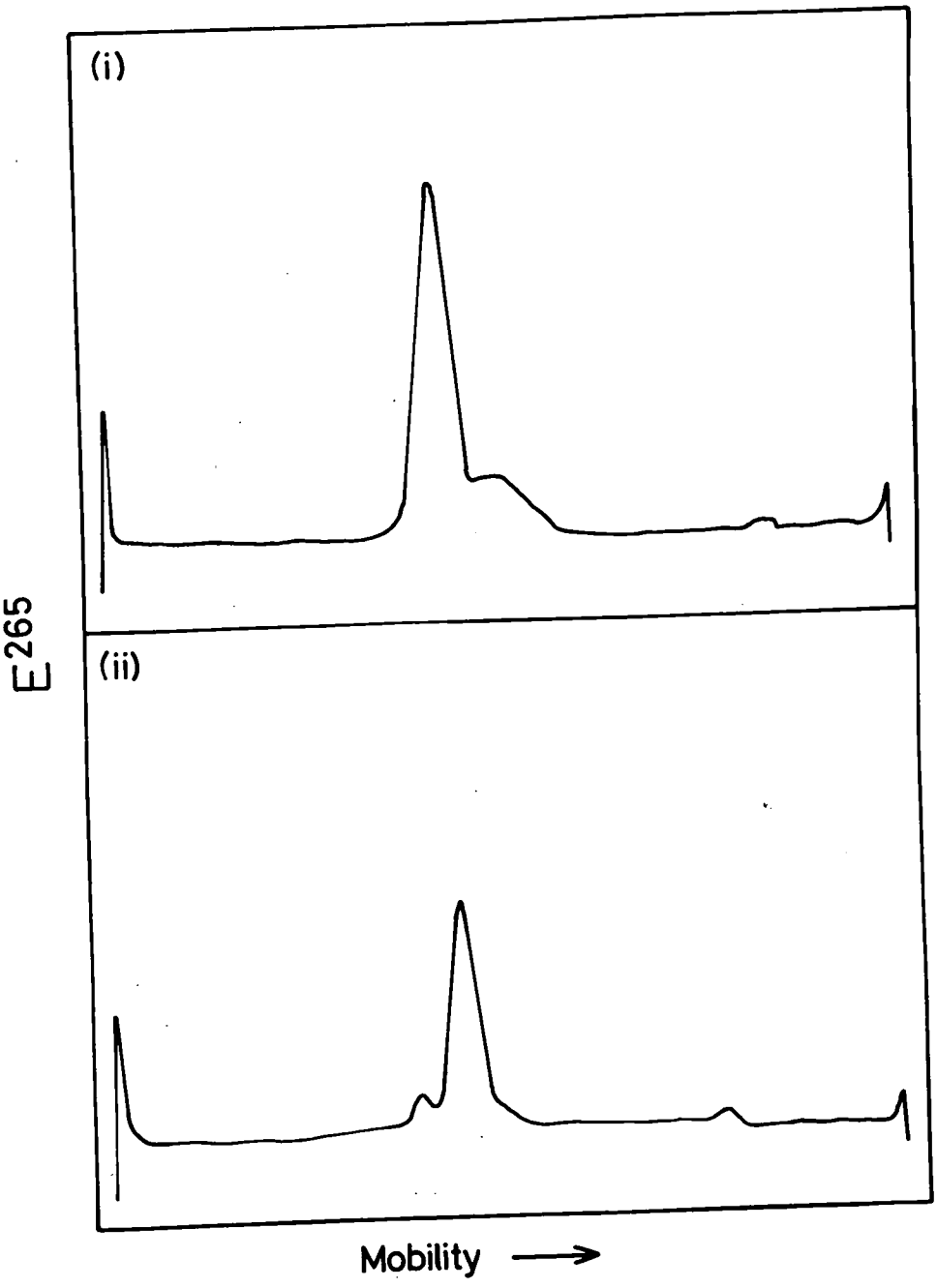
Fig.36. (cont) c) tRNA prepared from 50 day seeds



(i) tRNA before chromatography run on 2·6% gel

(ii) tRNA after chromatography run on 5% gel

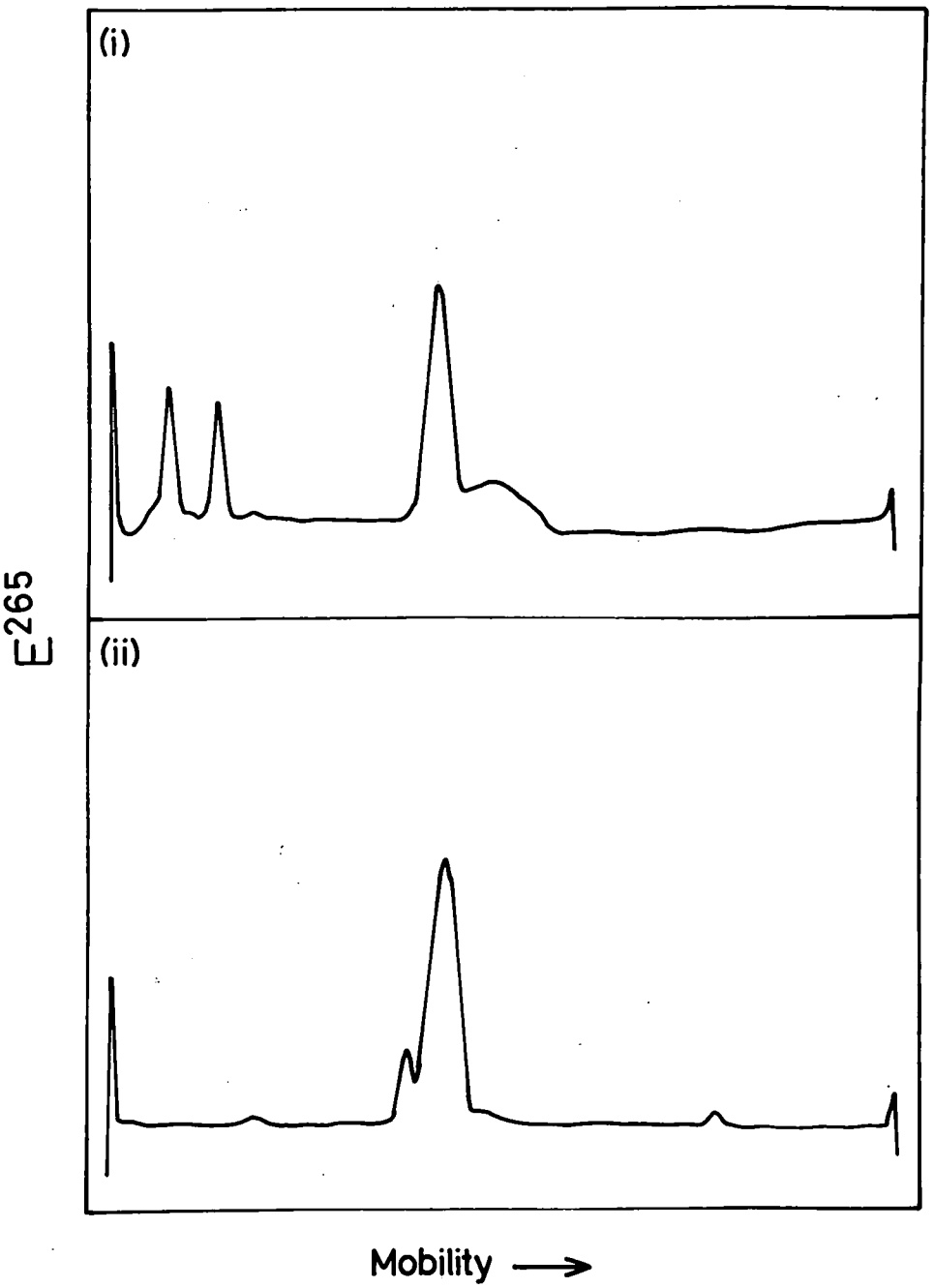
Fig.36. (cont) d) tRNA from 60 day seeds



(i) tRNA before chromatography run on 2·6% gels

(ii) tRNA after chromatography run on 5% gels

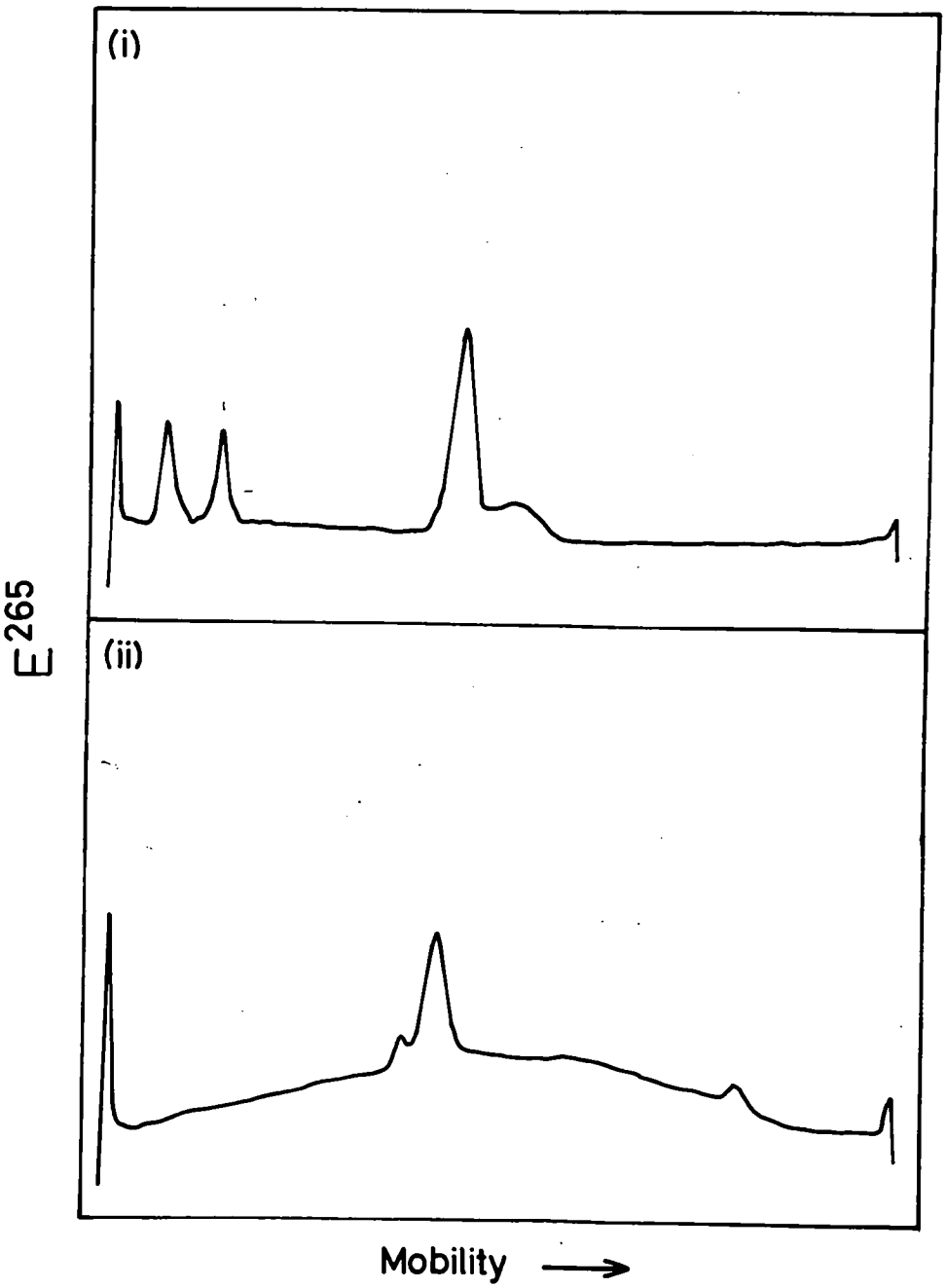
Fig.36. (cont) e) tRNA from 70 day seeds



(i) tRNA before chromatography run on 2•6% gels

(ii) tRNA after chromatography run on 5% gels

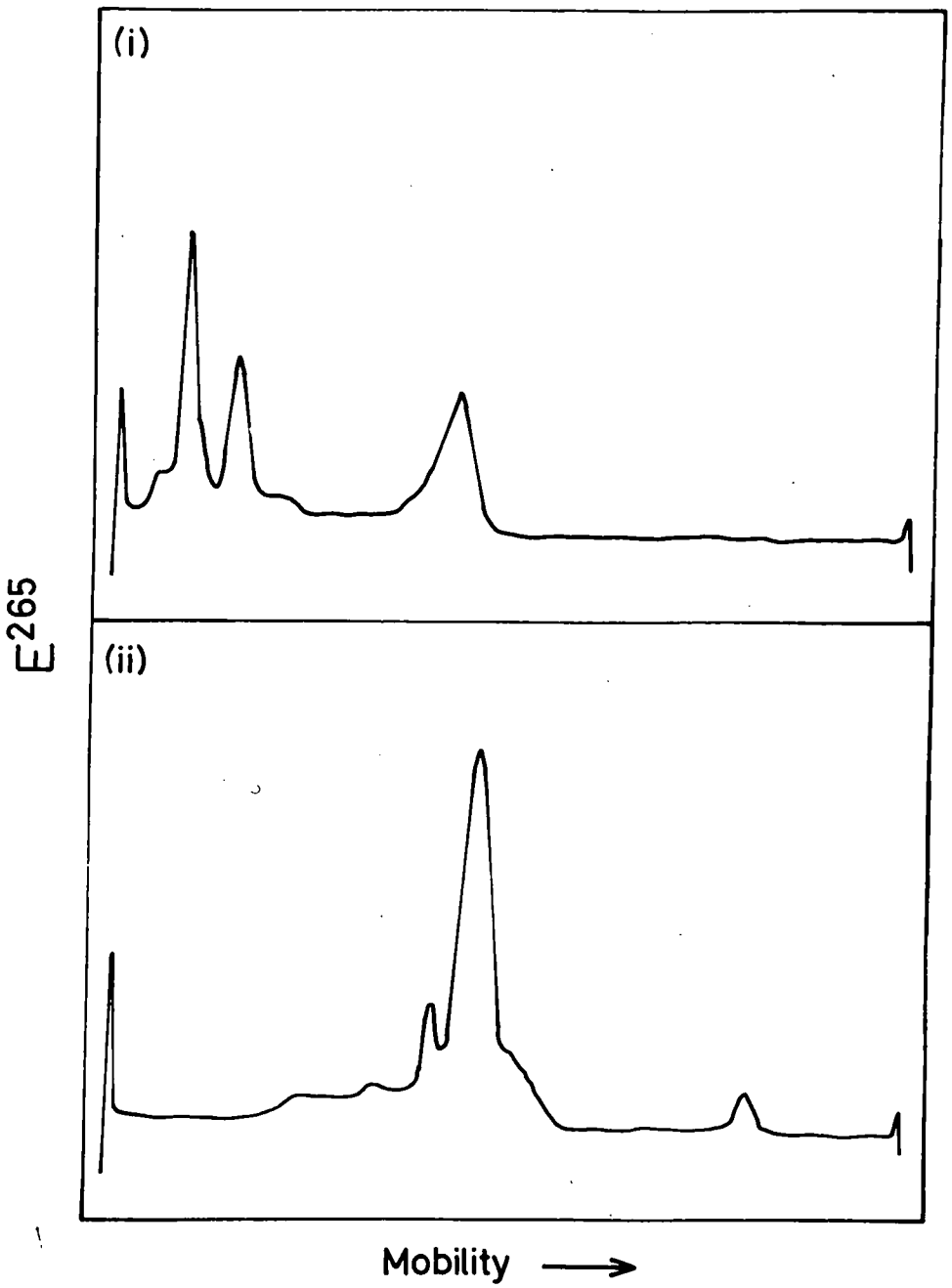
Fig.36. (cont) f) tRNA from 80 day seeds



(i) tRNA before chromatography run on 2·6% gels

(ii) tRNA after chromatography run on 5% gels

Fig.36.(cont) g) tRNA from 100 day seeds



(i) tRNA before chromatography run on 2•6% gels

(ii) tRNA after chromatography run on 5% gels

Table 12. Percentage composition of tRNA preparations as determined by polyacrylamide gel electrophoresis

(a) tRNA preparations before DE52 chromatography

Source of tRNA	4S	5S	18S	25S	Ratio 4S : Total RNA
30 day seeds	9	9	19	20	4.9
40 day seeds	7	25	17	25	10.3
50 day seeds	19	48	6	8	4.3
60 day seeds	23	65	-	-	3.9
70 day seeds	11	35	19	12	6.2
80 day seeds	8	41	9	13	9.2
100 day seeds	-	21	17	21	-

(b) tRNA preparations after DE52 chromatography

Source of tRNA	4S	5S	Ratio 4S : 5S RNA
30 day seeds	26	6	0.23
40 day seeds	53	10	0.19
50 day seeds	59	11	0.19
60 day seeds	53	9	0.17
70 day seeds	60	15	0.25
80 day seeds	24	9	0.37
100 day seeds	29	13	0.45

Legend for Table 12.

tRNA samples were run on polyacrylamide gels as described in methods. Areas under the trace and the individual RNA peaks were determined using the Technicon Integrator Calculator Model AAG. 4 determinations were made for each reading. The area of the individual peaks was determined as a percentage of the total area.

Table 13. 4S RNA content of tRNA preparations from different ages of developing bean seeds, determined by polyacrylanude gel electrophoresis

(a) tRNA before chromatography

Source of tRNA	Amount RNA loaded on gels (µg./ml.)	Amount 4S RNA present on gel (µg./ml.)	Concentration 4S RNA in tRNA (µg./ml.)	Concentration tRNA as determined by $E^{256}_{1cm.}$
30 day seeds	10	0·95	0·36	3·83
40 day seeds	10	0·74	0·15	2·1
50 day seeds	32	6·06	0·61	3·2
60 day seeds	18	0·41	0·21	9·0
70 day seeds	10	0·11	1·98	18·0
80 day seeds	9·6	0·76	0·47	7·76
100 day seeds	9·9	-	-	6·3

(b) tRNA after chromatography

Source of tRNA	Amount RNA loaded on gels (µg./ml.)	Amount 4S RNA present on gel (µg./ml.)	Concentration 4S RNA in tRNA (µg./ml.)	Concentration tRNA as determined by $E^{256}_{1cm.}$
30 day seeds	10·1	2.66	0·77	2·93
40 day seeds	9·9	5·22	1·67	3·16
50 day seeds	21·6	12·68	1·27	2·16
60 day seeds	10·2	5·44	5·44	10·16
70 day seeds	10·1	6·03	5·43	9·1
80 day seeds	10	2·45	1·57	6·33
100 day seeds	9·5	4·62	2·61	5·63

Legend as for Table 12.

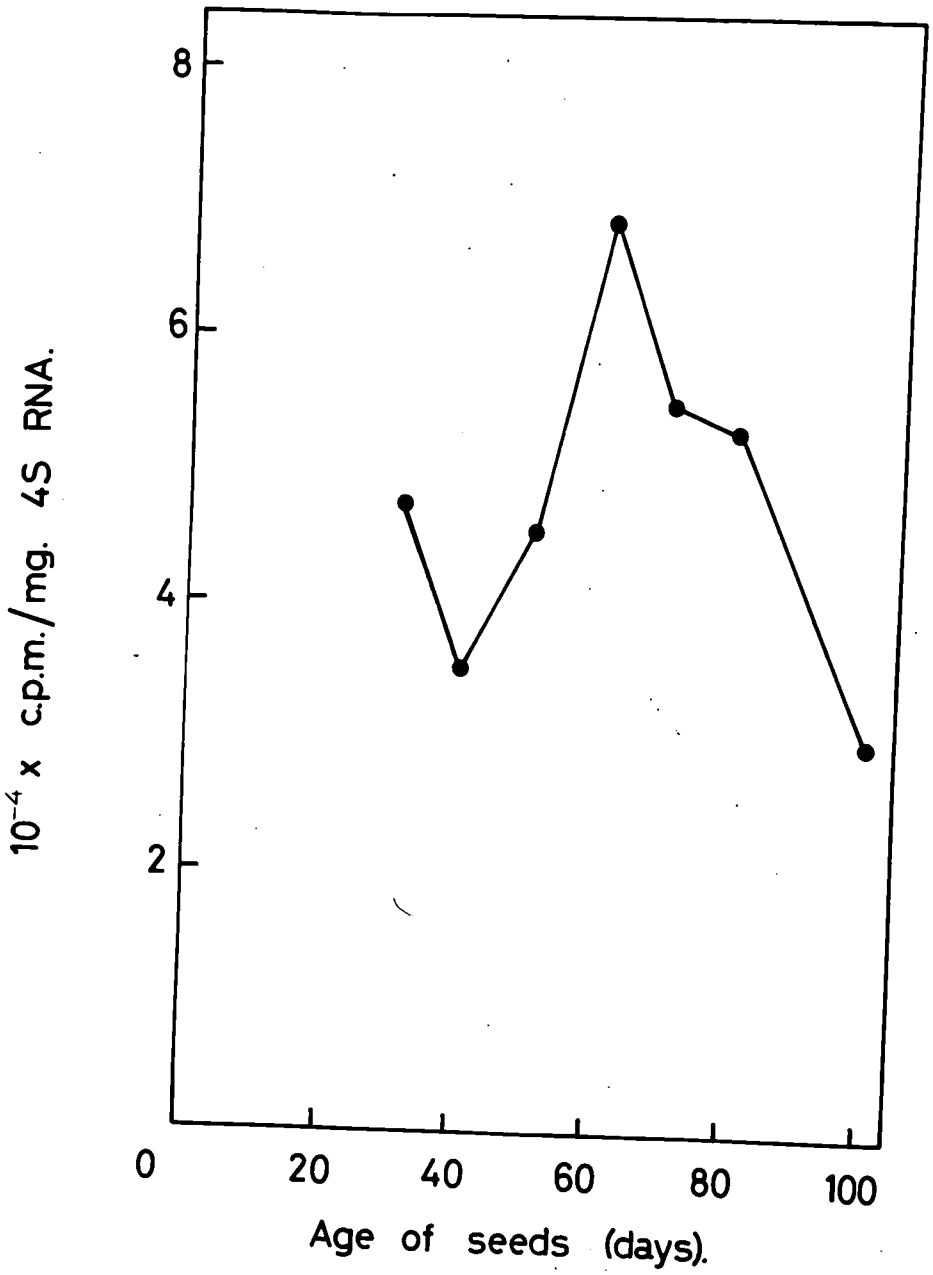
Amounts of 4SRNA were determined from area 4S peak to total area.

chromatography, the tRNA preparations (except that from 60 day seeds) contained a high proportion of contaminating tRNAs (25S, 18S and 5S). Chromatography on DE52 cellulose removed the high molecular weight tRNAs and most of the low molecular weight 5S tRNA. In these preparations, an extra small peak appeared, running in front of the main 4S peak, and had a molecular weight of approximately 880, as determined by plotting mobility against log molecular weight. (Bishop, Claybrook and Spiegelman, 1967; Loening, 1967), corresponding to about 3 nucleotides.

The total area beneath the traces and the areas of the RNA peaks were determined, and the percentage of the various RNA fractions to total area, and the ratio of 4S : total RNA is shown in Table 12. Although the ratio of 4S : 5S RNA in tRNA preparations after DE52 chromatography was similar (except in tRNA prepared from 80 and 100 day seeds, which contained comparatively less 4S RNA) the actual concentration of 4S RNA in the preparations varied considerably (Table 13) and consequently the amounts of 4S added to the charging and incorporation incubations differed. The results of the charging (fig. 32) and the incorporation (fig. 34) experiments using tRNA from different ages of developing seeds, have been recalculated on the amount of 4S RNA added to the incubations, and the results are shown in fig. 37 and 38.

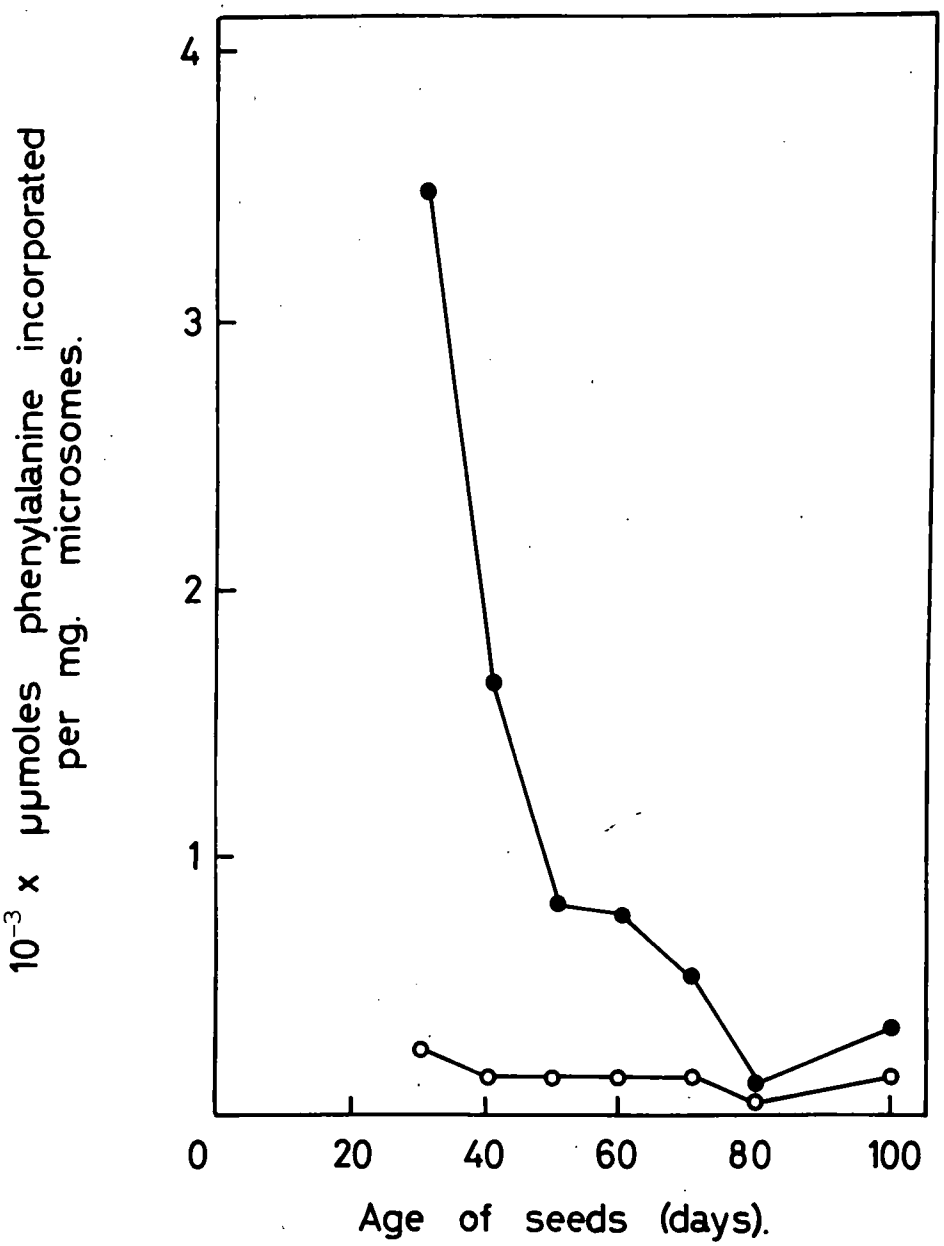
tRNA from 60 day seeds still showed maximum amino acyl acceptor

Fig.37. Changes in phenylalanyl acceptor capacity of tRNA prepared from developing seeds of different ages - Results calculated using actual 4S RNA content as determined by electrophoresis on 5% gels



Incubated as in methods using enzyme fraction from 70 day cotyledons and tRNA after chromatography

Fig.38. Activity of tRNA prepared from different ages of developing seeds in [^{14}C] phenylalanine incorporation in the Complete system - Results recalculated using actual 4S RNA content as determined by electrophoresis on 5% gels



Incubated as in Methods, using microsomes and enzyme fraction from 60 day cotyledons, and tRNA after chromatography. Results corrected per mg. 4S RNA

●—● plus poly-U

○—○ minus poly-U

capacity per mg. 4S RNA, although tRNA from 30 day seeds showed greater activity than previously. tRNA prepared from 30 day seeds was the most active in amino acid incorporation, and activity of the tRNA preparations fell off rapidly with increasing age of the seeds.

DISCUSSION

Cell-free amino acid incorporating systems have been derived from a wide range of plant tissues, especially young meristematic tissue, or seeds in which large amounts of protein are formed as storage products. Work on protein synthesis in plants, however, is not as far advanced as corresponding work on bacterial or animal systems, because studies of *in vitro* amino acid incorporation did not begin until much later, and fewer people are working with plant systems.

With few exceptions, all *in vitro* amino acid incorporating systems contain a particulate component (microsomes, ribosomes or polysomes) and a soluble enzyme source (the high speed supernatant fraction, or a fraction partially purified by $(NH_4)_2\ SO_4$ precipitation, or iso-electric precipitation at pH5). Depending on the level of purification of the supernatant enzyme fraction, tRNA may also be needed. The soluble fraction is also supplemented with GTP, ATP and an enzymatic regenerating system, a monovalent cation (NH_4^+ or K^+), $MgCl_2$, a sulphydryl compound (GSH, Mercaptoethanol or dithiothreitol) to buffer auto-oxidation reactions and is buffered for changes in hydrogen ion concentration with Tris-HCl buffer. A mixture of radioactively labelled amino acids, or a single labelled amino acid with or without the 19 other unlabelled amino acids is used as the substrate for

polypeptide synthesis. A requirement for the 19 unlabelled amino acids may not be demonstrable as they may be present as contaminants in the particle and enzyme preparations. Allende and Bravo (1966) suggest that it may be necessary to pass the enzyme fraction through a Sephadex G-25 column to remove contaminating amino acids.

mRNA is also needed for amino acid incorporation and this requirement can be supplied endogenously in the ribosome preparations. mRNA is, however, very labile and easily destroyed during ribosome extraction, and monosomes tend to predominate over polysomes in the preparations (Cundecliffe, 1968). This is particularly true for plant material, where the extraction techniques have to be harsh to break down the cell walls, and there is also considerable breakdown of mRNA and polysomal aggregates by RNase present in the supernatant.

A messenger is, therefore, usually supplied exogenously to the system, either in the form of a synthetic messenger e.g. poly-U etc., or by the addition of RNA with the properties or characteristics of a messenger, e.g. Viral RNA (Albrecht, Hoogendam, Rozenboom, Verhoef, Voorma and Bosch, 1969; Sela and Kaesberg, 1969).

In the cell-free amino acid incorporating system from V.faba seeds, there was very little endogenous mRNA activity associated with the microsomal preparations, and incorporation

was dependent upon the addition of an artificial messenger (fig. 7). Poly-U was therefore, used in all the incubations characterizing the conditions of reaction of this system.

As yet, net protein synthesis has not been shown conclusively in plant systems. An early report by Webster (1959) of net protein synthesis in a particulate system from pea seedlings has not been confirmed either by Webster or other workers. In fact, the very high rates of protein synthesis originally recorded by Webster were probably due to bacterial contamination. Hoagland (1960) has suggested that the following criteria should be met 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 the 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 - ATP and the ATP generating system.

(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 within the peptide chain, and not solely in the terminal position.

(v) The amino acid should appear in a single specific, isolatable protein of the cell of origin, e.g. Haemoglobin in the rabbit reticulocyte system (Bishop, Favelukes, Schweet and Russell, 1961).

All the above criteria have seldom been satisfied in any one investigation, and in particular, the last point has been shown in very few systems.

That the observed incorporation in any system is directly due to the ribosomes can be determined from the dependence of incorporation upon the addition of ATP and Mg^{2+}, and its sensitivity to added RNase.

A major problem in any in vitro studies on amino acid incorporation is the extent to which the observed incorporation is the result of contaminating micro-organisms. This is particularly important in work with plant systems, where preparations of the sub-cellular components are likely to be contaminated with micro-organisms from the surface of the plant. Preparations from animal sources are less likely to be highly contaminated as the tissues used in the preparation of the sub-cellular components are located within the body of the animal and are not subject to surface contamination. Work by Yarwood (1968) using V.faba, however, has shown that most of the bacteria are sedimented with the mitochondria during the preparation of ribonucleoprotein particles.

Except with very high concentrations of bacteria (greater than 10^7 bacteria/ml.), the time curves of bacterial incorporation are quite different from those of ribosomal incorporation. The bacterial curve is biphasic; there is very little incorporation in the first 60 min-incubation, and this is followed by a period of rapidly accelerating incorporation. Mans and Novelli (1964) found that the final burst of bacterial incorporation could be eliminated by the addition of 300 units of Penicillin and 500 µg. Streptomycin to the incubation. True ribosomal incorporation proceeds linearly over the first 20-60 min. incubation, depending on the source of material and the conditions of incubation, when it begins to tail off, due to exhaustion of substrates and product accumulation. The time curve for *in vitro* incorporation of [^{14}C] phenylalanine by the *V.faba* system (fig. 9) is, therefore, a ribosomal time course.

Bacterial incorporation of amino acids is independent of added GTP or ATP and the energy generating system. Although the *in vitro* system from *V.faba* showed only partial dependence upon GTP (Table 2), this was probably due to high levels of contaminating GTP in the enzyme fraction, although the use of high Mg^{2+} concentration in the incubations (10mM) could have masked the GTP requirement, as a similar situation has been found in micro-organisms. (Kurland, 1966, Ravel, 1967).

A certain amount of endogenous ATP was present in the enzyme fractions (fig. 14), but amino acid incorporation was dependent upon ATP and the ATP generating system, and addition during the course of incubation resulted in increased incorporation.

Davies and Cocking (1967) suggest that in incubations with less than 10^3 bacteria/ml., incorporation due to bacteria is negligible. App and Jagendorf (1964) however, reported that there was no linear relationship between levels of bacterial contamination and amino acid incorporation by chloroplast preparations. Davies and Cocking (1967) also suggest that different micro-organisms differ greatly in their ability to incorporate amino acids in in vitro incubations. Assays of bacterial contamination are, moreover, subject to variation in that the bacterial count recorded depends to some extent on the medium used for plating out aliquots of the incubation, the temperature the plates are incubated at, and the length of time allowed before the colonies are counted. Bacterial counts of the incubations by themselves are not reliable indicators of the degree of bacterial contamination unless they are supported by evidence from the time curves of amino acid incorporation and its dependence on added ATP.

In the V.faba system described in this thesis, the low bacterial count per incubation (3×10^3 to 2×10^4 bacteria/ml.), the nature of the time curves of amino acid incorporation and the dependence of the system on added ATP indicates that the ribosomes

were responsible for most of the observed incorporation, and not bacteria.

Two types of system have been used to study amino acid incorporation by components from V.faba seeds; the "complete" system, measuring total amino acid incorporation from charging tRNA with amino acids, binding of amino acyl tRNA to the ribosomes and transfer of the amino acyl moiety to the growing polypeptide chain; and the "transfer" system, in which the tRNA is precharged with the radioactive amino acid and amino acyl tRNA is added to the incubations. In these incubations, ATP and the ATP generating system have been omitted. The "transfer" system has not been greatly used in this thesis, because of difficulty in obtaining tRNA that was sufficiently highly charged with [^{14}C] amino acid.

Amino acid incorporation by V.faba components in both types of system had similar requirements to those from many other sources. Although incorporation was dependent upon the addition of microsomes, a certain amount of incorporation was recorded in the absence of enzyme and tRNA (Tables 2 and 4), due to the presence of contaminating enzyme in the microsome preparations, and tRNA in the enzyme fractions and microsome preparations.

No attempt was made to purify the high speed supernatant enzyme fraction, and it was found that protein concentration

varied with the source of material and was an unreliable guide to the activity of the enzyme fractions in amino acid incorporation. (Table 9). Enzyme concentration curves were done for all the high speed supernatant fractions, and the volume of enzyme promoting maximum amino acid incorporation was used in all subsequent work, rather than a predetermined protein concentration per ml. incubation. During the preparation of the microsomal and enzyme fractions, 2 vol. extractant were used per g. weight of starting material, so that it is hardly surprizing that the volume of enzyme promoting maximum amino acid incorporation should be similar in a wide range of enzyme fractions prepared from different sources of material on different occasions. Increasing concentrations of the enzyme fractions were inhibitory to phenylalanine incorporation (fig. 12) probably due to the presence of endogenous non-radioactive phenylalanine in the enzyme fractions, which, with the higher concentration of enzymes could isotopically dilute the [^{14}C] phenylalanine. Alternatively, the inhibition could be due to increasing concentrations of proteases and RNase in the enzyme fraction. With the enzyme concentration regularly used in <u>in vitro</u> amino acid incorporation, there was no evidence of decreased incorporation over a prolonged incubation period (fig. 9) indicating that there was little proteolytic activity in the incubations.

Magnesium is needed in all in vitro studies to maintain the integrity of the ribosomes (Tissières and Watson, 1958), for binding the messenger to the ribosomes (Moore and Asano, 1966) and for binding amino acyl tRNA to the ribosomes (Ravel and Shorey, 1969). Amino acid incorporating systems from different plant sources have differing magnesium optima, varying from 5mM in systems from the cytoplasmic ribosomes of tobacco leaves (Boardman, Franki and Wildman, 1966), peanut cotyledons (Marcus and Feeley, 1966) and pea epicotyls (Davies and Maclachlan, 1969) to 20mM in a system from castor bean embryo (Parisi and Ciferri, 1966), although magnesium levels between 8-10 mM are most frequently used. The different magnesium optima probably reflect the different magnesium levels used in the preparation and storage of the ribosomes. Ribosomes are known to adsorb magnesium and high levels of magnesium in the extractant during ribosome preparation allow preparations to be resuspended and stored at lower magnesium levels, without loss of activity. The type and concentration of some of the components in the incubation mixture may influence the magnesium optimum.

Poly-U directed incorporation by a V.faba cell-free system showed a sharp magnesium optimum at 10mM, although the optimum for incorporation by endogenous messenger was lower at 6mM (fig.11). Similar results were reported by Mosteller, Culp and Hardesty (1968) for amino acid incorporation by a system from rabbit

reticulocytes. Unpublished work by Boulter and Yarwood has shown that the magnesium optimum for phenylalanine incorporation by V.faba microsomes can be reduced by pre-incubating the microsomes with poly-U in a system similar to that reported by Mosteller et al.(1968).

The pH optima for amino acid incorporation (fig. 10) and charging of tRNA with [^{14}C] phenylalanine (fig. 20) were similar to those recorded for all other plant systems. Unpublished work from this laboratory, however, has shown slight differences in the pH optima for charging of tRNA with other amino acids.

A wide range of incubation temperatures from 25° to 37° have been used to study amino acid incorporation in cell-free systems from plants. Experiments were, therefore, carried out to determine the optimum temperature for in vitro phenylalanine incorporation by components from developing and germinating V.faba seeds (figs. 8, 9 and 11). Similar time curves for amino acid incorporation were obtained by Bamji and Jagendorf (1966) for wheat chloroplasts although the time of incubation was much shorter.

The effect of temperature on amino acid incorporation is complex and reflects the effect of two opposing reactions, there is the simple chemical effect of increased incorporation with increasing temperature, but this is counterbalanced by the decreased stability of ribosomes and mRNA at higher temperatures

and heat denaturation of the enzymes. This effect can be seen in the different time curves for phenylalanine incorporation at different temperatures (fig. 9). At 15° there was a slow steady rate of amino acid incorporation, but with comparatively little loss of stability of the ribosomes. Consequently the time curve was linear over the 120 min. incubation. At 37°, however, thermal denaturation of the ribosomes was occurring much more rapidly, and net phenylalanine incorporation ceased after 20 min. An incubation length of 20 min. was selected for all *in vitro* studies at 25°, as this period fell well within the initial rapid rate of incorporation, and was a true reflection of the initial rate. This is important when comparing the rates of amino acid incorporating activity of different preparations, as it is only the initial rates that are meaningful.

Unlike amino acid incorporation, charging of tRNA with phenylalanine was more-or-less independent of temperature between 15-37° (fig. 17), although it was slightly higher at 25° than at 37°. Similar results have been reported by Thiebe and Zachau (1969) for charging of yeast and wheat $tRNA^{phe}$. This would suggest that it is the ribosomes, or the ribosome catalysed reactions that are sensitive to temperature.

Peterman (1964) reported that the association and dissociation of partially purified Jensen sarcoma ribosomes may be influenced by temperature, and thermal denaturation of *E.coli* ribosomes at

The activity of *in vitro* systems from plants in amino acid incorporation has been expressed in various ways by different workers, although μμmoles label incorporated/mg. RNA using extinction at 260 mμ is the most common. Problems however, arise when comparing the activity of different plant systems in that different incubation times have been used. Levels of 100-150 μμmoles phenylalanine incorporated/mg. microsomes in 20 min. were routinely recorded in the complete system in the presence of poly-U, by developing *V.faba* microsomes. This corresponds to levels of 200-300 μμmoles phenylalanine incorporated per mg. microsomal RNA (assuming that RNA forms 50% of the microsomes). The phenylalanine is presumably incorporated into polyphenylalanine, although this has not been isolated and identified.

These levels of activity compare very favourably with levels of poly-U directed phenylalanine incorporation reported for other plant systems. Allende and Bravo (1966) reported incorporation of 160 μμmoles phenylalanine/mg. RNA by wheat ribosomes in 45 min. Leaver and Key (1967) reported that ribosomes from carrot root incorporated up to 100 μμmoles phenylalanine/mg. RNA in 30 min. Incorporation in other plant systems is lower than this.

The transfer system is less active than the complete system in amino acid incorporation; 20-25 μμmoles being incorporated/mg.

RNA after 20 min. Allende and Bravo (1966) reported similar values in their wheat embryo system. The low activity of V.faba components in the transfer system was probably due to the amount of phenylalanine added to the incubation being limiting, as the tRNA used was only charged to a level of 4,500 c.p.m./mg., so that no more than 20 µµmoles of phenylalanine were being added per incubation. In the complete system, phenylalanine is added in excess, and the tRNA is being continually charged.

Although the tRNA seemed to charge well in the incubations, up to 50% of the radioactivity was lost during the re-extraction of the charged tRNA (Table 5). This suggests that the extraction procedure used here may be too severe, and a different technique for the re-extraction of charged tRNA may be needed. Alternatively, a percentage of the amino acids could be binding to the tRNA in a non-amino acyl linkage during the incubation, although this is unlikely, as the washing procedure used to prepare samples for counting was designed to remove these amino acids.

Amino acid incorporation in the absence of added messenger varies considerably in different plant systems, depending on the amount of endogenous mRNA and functional polysome aggregates present in the microsomal preparations. Values of from 8 µµmoles phenylalanine incorporated/mg. RNA in 40 min. by tobacco leaf ribosomes (van Kammen, 1967) up to 50 µµmoles phenylalanine

incorporated/mg. RNA in 45 min. for wheat embryo ribosomes (Allende and Bravo, 1966) have been reported. Levels of between 10-20 μμmoles phenylalanine/mg. microsomes have been obtained for V.faba microsomes in 20 min., corresponding to values of between 20-40 μμmoles/mg. RNA. Incorporation in the absence of exogenous messenger is probably just due to the completion of polypeptide chains rather than the initiation of new chains.

Rates of amino acid incorporation in the absence of added messenger are influenced by RNase present in the incubations (usually in the enzyme fraction) and also by the method of preparation of the ribosomes. Improved techniques of ribosomal extraction should lead to better preservation of mRNA and polysomal aggregates, resulting in better rates of amino acid incorporation, and removing the need for added messenger.

Graebe and Novelli (1966) reported an improved method for the extraction of ribosomes from wheat endosperm tissue, in which a Gaulin Homogenizer was used to homogenize the tissue, and the volume of the 30,000 xg. supernatant was reduced by a Sephadex G-25 treatment similar to that reported by Flodin, Gelotte and Porath (1960). Using this method, rates of [^{14}C] leucine incorporation were increased from 6 to 70 μμmoles/mg. ribosomal protein in 30 min.

The second part of the work involved in this thesis was the investigation of changes in the *in vitro* amino acid incorporating ability of subcellular components from different ages of developing broad bean seeds, to see if these could be correlated with changes in the *in vivo* protein synthetic activity of developing seeds. Synthesis of the storage protein of the seed is limited to a relatively short period during its development, but for this period, a significant part of the total metabolism of the seed is directed towards the synthesis of the two storage proteins, vicilin and legumin. The maturation of *V.faba* seeds takes about 100-120 days, and characteristic anatomical and biochemical changes are occurring in the seeds during this period, which can be divided into three main phases on this basis (Briarty, Coult and Boulter, 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.

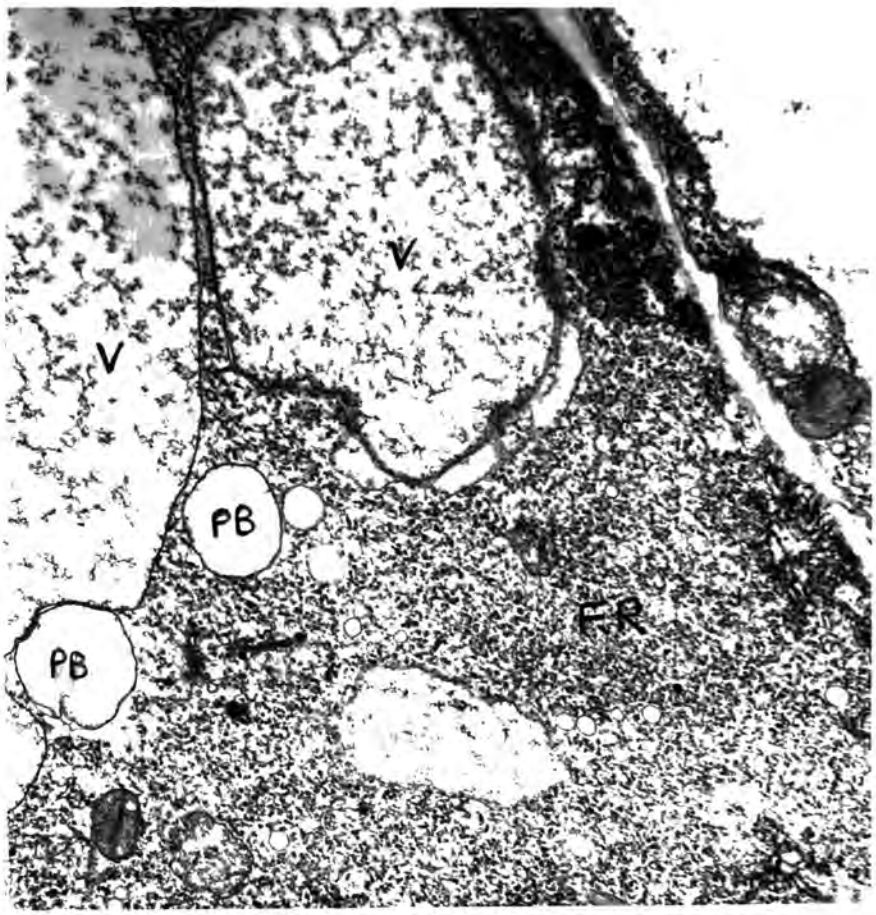
(ii) This is followed by a period of intense synthetic activity, when the storage proteins of the seed accumulate. In Durham, most of the storage protein is formed between days 45-70.

(iii)After completion of the synthesis of the storage products, the seed enters the final period of maturation and dehydration leading to dormancy.

Studies of electron micrographs made from developing bean seeds from the 1969 harvest show that considerable changes are occurring in the seeds, which are correlated with the three main periods of seed development (fig. 39). The cotyledon cells from 30 day seeds contain abundant cytoplasm, with many free ribosomes, and large empty protein bodies. After 40 days development, the cell organelles are fully differentiated, and the endoplasmic reticulum with its associated membrane bound ribosomes is beginning to appear. By 50 days, there has been a tremendous proliferation of the rough endoplasmic reticulum, and membrane-bound ribosomes predominate over free ribosomes. Storage protein synthesis is just beginning, and traces of protein can be distinguished as electron dense areas around the periphery of the protein bodies. By 60 days, storage protein is being synthesized rapidly, the protein bodies are filling up quickly, and are full by 80 days, when storage protein synthesis has ceased. The seeds have begun to dehydrate by this stage, and associated with this is the breakdown of the endoplasmic reticulum, releasing the membrane bound ribosomes. When the seed is fully mature and dehydrated (100-120 days after flowering) there is hardly any trace of endoplasmic reticulum and free ribosomes predominate.

Fig. 39 Electron micrographs of cotyledons of developing Vicia faba seeds.

a) Section through cotyledon of 30 day seeds.



1μ.

F.R. = free ribosomes; M.B.R. = Membrane bound ribosomes; P.B. = protein body; P = protein being deposited in protein body; V = cell vacuole.

Fig. 39 (cont.)

b) Section through cotyledon of 50 day seeds.

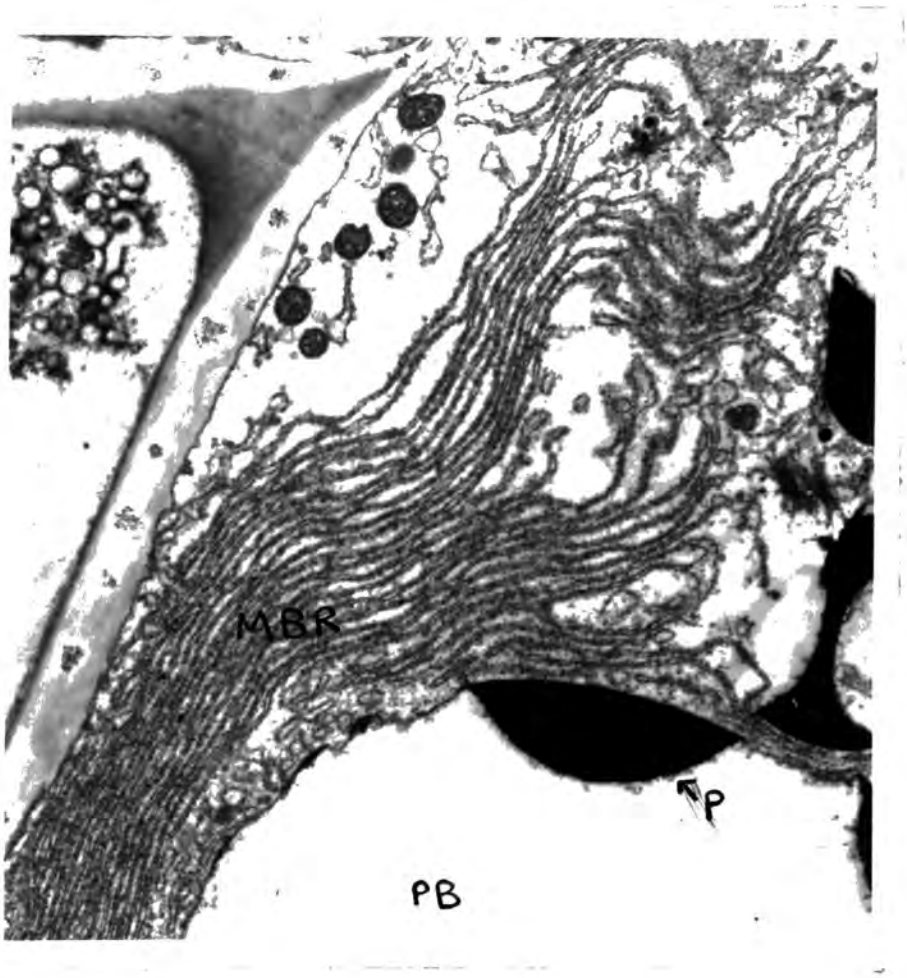
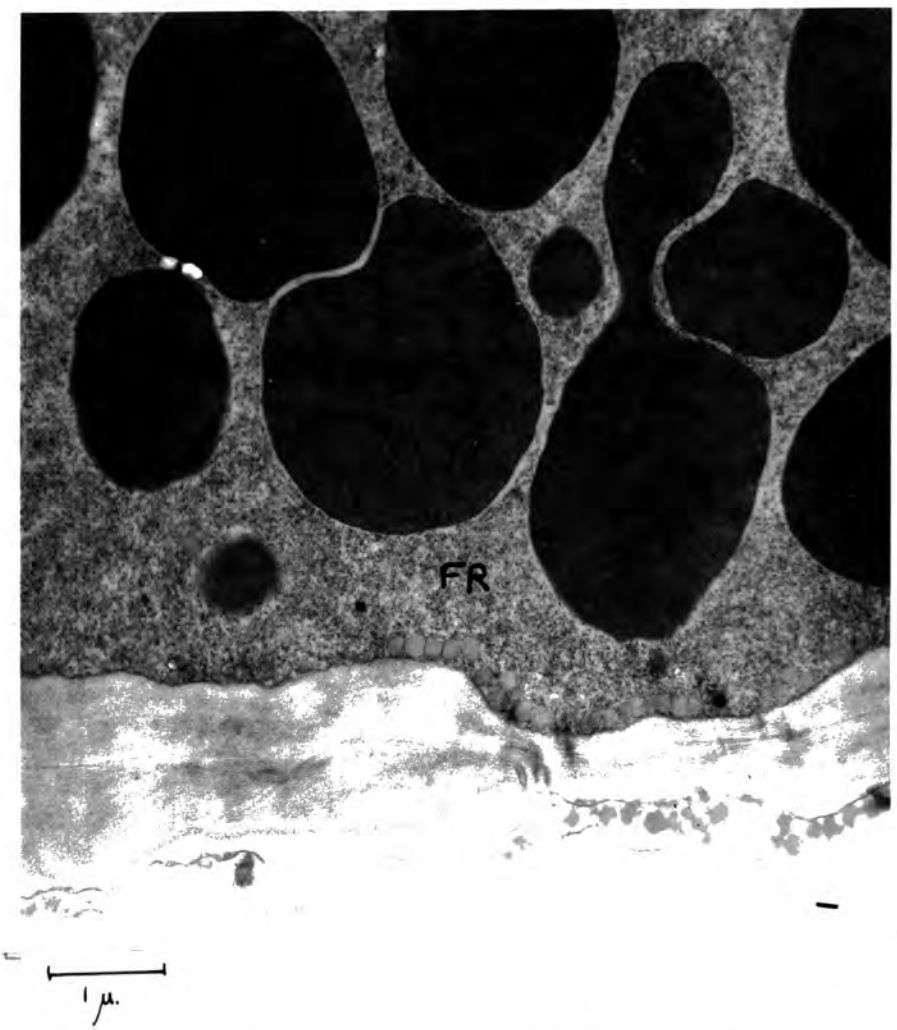


Fig. 39 (cont.)

c) Section through cotyledon of mature seeds.



Preparation of samples and electron microscopy by A. Cobb and R. Swincoe.

Striking changes are, therefore, occurring in the ribosome content of the cotyledon cells, which seem to be associated with the changes in the *in vivo* protein synthetic activity of developing seeds, which can be divided into two distinct stages:

(i) Structural protein synthesis, e.g. the formation of the cell organelles, and in particular, the formation of the endoplasmic reticulum and associated membrane bound ribosomes, prior to the onset of storage protein synthesis. This stage lasts from about 1-40 days after flowering, and corresponds to the period when the free ribosomes are most abundant.

(ii) Storage protein synthesis, when the membrane bound ribosomes are most abundant.

These stages, with their associated changes in the free to membrane bound ribosome ratios, and in particular the correlation between membrane bound ribosomes and storage protein synthesis, will be discussed more fully later with reference to the *in vitro* amino acid incorporating activity of components prepared from developing bean seeds of different ages.

Similar patterns of development have been found in other leguminous plants by Bain and Mercer (1966) working with *Pisum arvense* and Opik (1968) working with *Phaseolus vulgaris*.

It should be emphasized that the dates quoted are not absolute, and there may be a slight variation of a few days in

the onset of storage protein synthesis, depending upon the position of the bean pod on the plant, time at which flowering occurs during the growing season, and environmental conditions prevailing during the growing season, as the bean plants are grown in the open and are subject to any fluctuations in the weather. In the studies on amino acid incorporation by components of bean seeds of different ages, material was prepared from seeds that had opened during the middle of the growing season, and development of a group of seeds that had been fertilized within one week of each other was followed, to try and minimize these variations as much as possible. Although the overall changes in the in vitro amino acid incorporating activity of various components are the same from one season to the next, the precise dating of when these changes occur may vary. Results from the 1969 harvest are discussed here, although a similar pattern of activity was obtained with material from the 1968 harvest.

Electron micrographs of developing bean cotyledons indicate that the changes in the proportion of free and membrane bound ribosomes at the onset of storage protein synthesis are important in vivo. Microsomal preparations therefore have been used for studies on the changes in in vitro amino acid incorporation during seed development, as they contain both free and membrane bound ribosomes. 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 to membrane bound ribosomes from the ratio of free to membrane bound tRNA. They found that the ratio of free to membrane bound ribosomes in their preparations changed as the bean seeds matured, and the ratio reflected the proportion of free and membrane bound ribosomes in the cotyledons, as seen from electron micrographs. The ribosome content of the microsomal preparations does, therefore, seem to reflect the in vivo distribution of ribosomes.

Changes in in vivo protein synthetic activity during seed development were reproduced in vitro by incubating all the components from the same age of seed together in the complete system (fig. 24), since changes in the in vitro activity were found to parallel changes known to be occurring in vivo during seed development.

Activity due to natural messenger was greatest in in vitro incubations of material from 60 day seeds, which were known to be actively synthesizing storage protein. Rabson, Mans and Novelli (1961) have reported a similar parallel between in vitro [^{14}C] leucine incorporation by unwashed particles from maize with protein synthesis in the developing kernel. These changes in in vitro activity could be due to different natural proteins being synthesized during in vivo seed development, as proteins

containing a high percentage of phenylalanine will show up with a higher radioactive count than proteins containing less phenylalanine. Alternatively, and this seems to be most likely, the ribosomes from 60 day material may be more active in amino acid incorporation. The greater proportion of rough endoplasmic reticulum in cells from 60 day seeds may offer better protection for mRNA associated with these ribosomes against degradation by RNase during the preparation of the microsomes.

Poly-U directed phenylalanine incorporation reflects the potential rather than the actual amino acid incorporating activity of the preparations and showed a double peaked activity curve, with peaks from material prepared from 40 and 60 day seeds, which might correspond to the two main periods of *in vivo* protein synthetic activity during development, which were mentioned earlier.

Payne (1968) and Payne and Boulter (1969) have shown using [^{3}H] uridine that the membrane bound ribosomes were a separate class of ribosome from the free ribosomes, which were synthesized *de novo* before the onset of storage protein synthesis, and were not formed by free ribosomes becoming attached to the endoplasmic reticulum. The tremendous proliferation in the endoplasmic reticulum and associated membrane bound ribosomes seen in electron micrographs of developing cotyledon cells 40 days after flowering,

must be due to the activity of the existing free ribosomes and this could correspond to the first peak of activity in in vitro studies (fig. 24).

The second peak of in vitro activity covered the period when storage protein synthesis was mainly occurring in vivo, which began about 50 days after flowering, and ended by about 70 days after flowering.

Components from 80 and 100 day seeds showed little activity in in vitro phenylalanine incorporation, reflecting the fact that the seeds were entering the final period of dehydration prior to dormancy. The lack of activity could be due to loss of polysomal aggregates, decreased stability of the ribosomes, or the presence of inhibitors e.g. RNase in the enzyme fraction.

Davis (1966) has estimated that in mature seeds of Vicia faba, vicilin and legumin account for 0·0815 g. and 0·0686 g. per g. weight of seed, respectively. This corresponds to about 250 mg. storage protein per seed at maturity, which is synthesized over about 30 days, corresponding to about 340 µg. protein synthesized per hour per seed. Payne (1968) estimates that about 86-87% of the total RNA of V.faba seeds is rRNA. The RNA content of dormant seeds was found to be about 1·77 mg./g. bean meal; i.e. about 1·4 mg. rRNA/g. bean meal, or 2·5 mg. rRNA per dormant seed. Payne also estimated that 25% of the total cotyledon RNA was

hydrolysed at the onset of dehydration, and on this basis, the rRNA content of seeds prior to dehydration would be about 3·1 mg. per seed. This gives an *in vivo* rate of storage protein synthesis of approximately 112 µg. protein synthesized/mg. rRNA/hr.

Phenylalanine incorporation due to natural messenger was 100 µµmoles/mg. microsomal RNA/20 min., using components from 60 day *V.faba* seeds, corresponding to about 0·05 µg. phenylalanine incorporated/mg. microsomal RNA/hr. In these incubations, phenylalanine content only was being measured. Davis (1966) has shown that the phenylalanine content of the storage globulins is about 4·4%. Assuming that the other unlabelled amino acids are being incorporated at the same rate as phenylalanine, this would give an *in vitro* rate of 1·13 µg. protein synthesized/mg. microsomal RNA/hr., i.e. 1% of the *in vivo* rate.

Levels of amino acid incorporation recorded *in vitro* are never as high as the rates of *in vivo* protein synthesis. This is probably due to two factors, the isolation of the various components of protein synthesis, and their assay in a completely artificial system. A certain amount of degradation and loss of activity is bound to occur during the extraction of microsomes. This is particularly true for mRNA which is very labile and easily degraded during the extraction procedure. This can be seen in this system prepared from developing *V.faba* seeds, as

there is a considerable increase in phenylalanine incorporation when the artificial messenger poly-U is added. This emphasizes the need for improved extraction techniques and in particular, better protection for mRNA during ribosomal extraction.

Coleman (1969) suggests that in vitro amino acid incorporation may be less efficient than the same process in vivo, because living cells maintain a constant internal environment, which is very difficult to achieve in in vitro incubations. It is difficult to maintain constant, optimal levels of precursors and to remove unwanted products in the incubations. Substrate and product concentrations in the incubations are undergoing constant irreversible changes, and the unfavourable conditions which are gradually developing, eventually stop the reaction.

Working with an amino acid incorporating system from Bacillus amyloliquefaciens, Coleman (1969) has developed a new in vitro incorporation assay, which he hopes will overcome some of the problems inherent in the present assay method. A polysome-tRNA-enzyme mixture is eluted down a Sephadex G-25 column loaded with a radioactive amino acid mixture, GTP and ATP using a Tris, Mg^{2+}, KCl buffer. The polysome-tRNA-enzyme mixture passes round the outside of the Sephadex gel particles, which exclude molecules of molecular weight greater than 5,000, and is in continual contact with a fresh and relatively constant supply of amino acids, GTP and ATP. At the same time, the low molecular

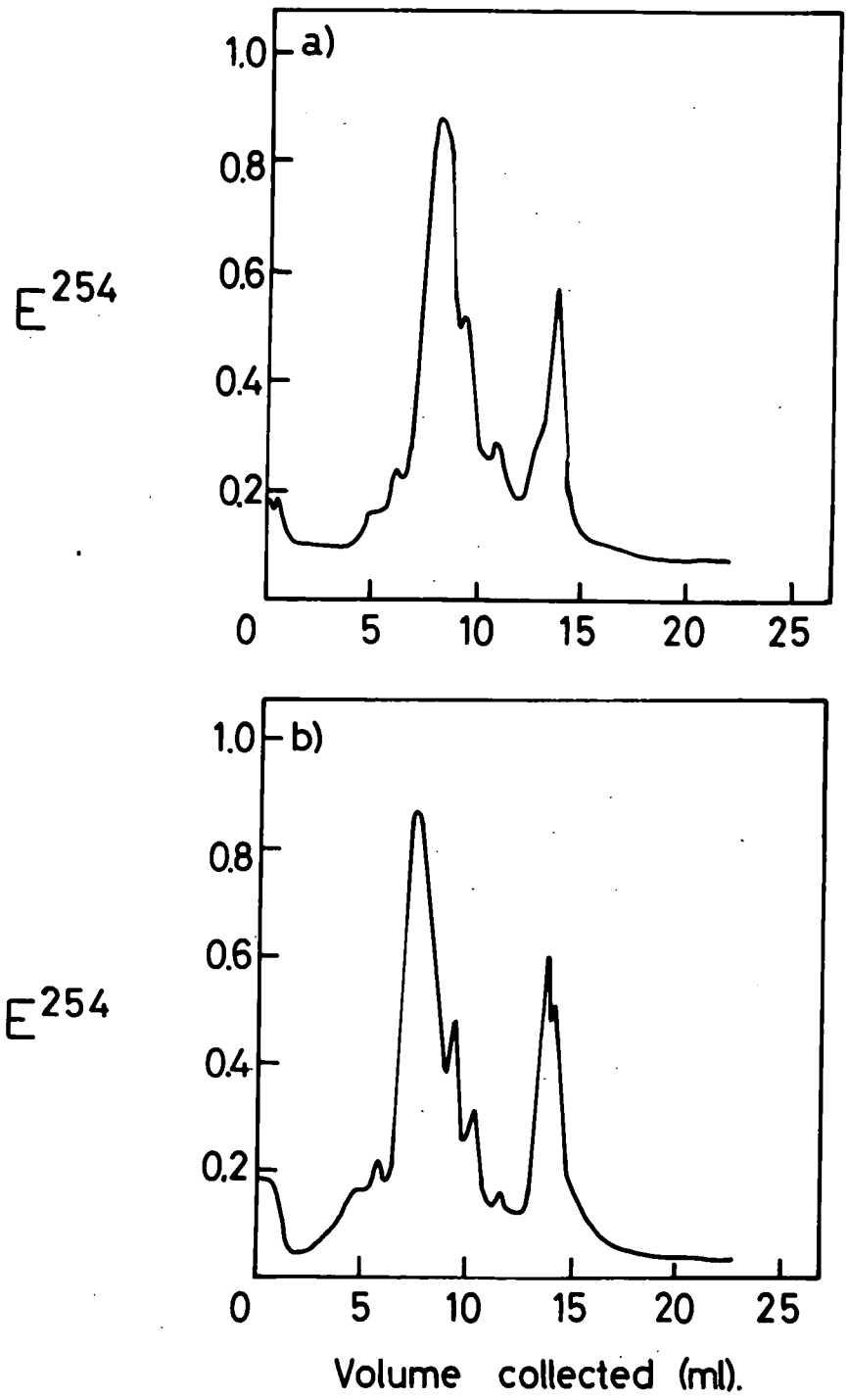
weight degradation products enter the gel particles, and therefore move down the column much more slowly than the polysome-tRNA-enzyme mixture. Coleman compared [^{14}C] amino acid incorporation into peptidyl form in a typical cell-free system from B.amyloliquefaciens, which he refers to as the "static" system, to incorporation achieved by the same components in a Sephadex column system (the "dynamic" system) under aseptic conditions. Over a 40 min. incubation period, the "dynamic" system incorporated the amino acids four times as fast as the "static" system. Although incorporation began to tail off after 20 min., Coleman suggests that this is due to exhaustion of the polysome supply, as levels of the low molecular weight precursors were not limiting.

Improved extraction techniques and the introduction of better assays for in vitro amino acid incorporation should improve the rates of observed in vitro incorporation, making them more comparable with rates observed in vivo.

Changes in the proportion and activity of free to membrane bound ribosomes are not only important in vivo but also seem to influence amino acid incorporating activity in vitro. Microsome preparations from various ages of developing bean seeds from the 1969 harvest were kindly analysed by Mrs. A. Brownrigg on isokinetic sucrose gradients, to determine the relative proportions of membrane bound material to free monosomes and polysomes, to

Fig.40. Analysis of microsomes prepared from different ages of developing V.faba seeds on isokinetic sucrose gradients

a) Microsomes prepared from cotyledons of 30 day seeds
b) Microsomes prepared from cotyledons of 40 day seeds



Legend - see p. 154

Fig.40. (cont)

c) Microsomes prepared from cotyledons of 50 day seeds
d) Microsomes prepared from cotyledons of 60 day seeds

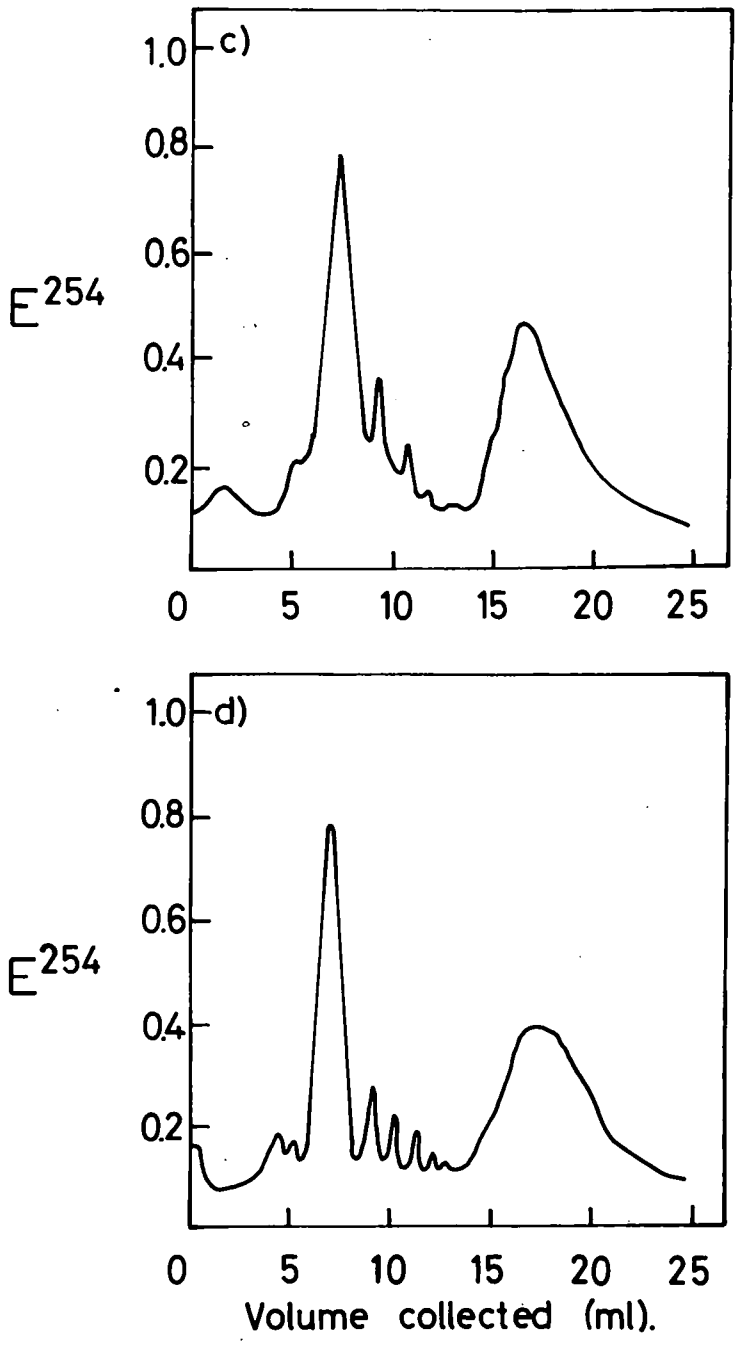


Fig.40. (comt)

e) Microsomes prepared from cotyledons of 70 day seeds
f) Microsomes prepared from cotyledons of 80 day seeds

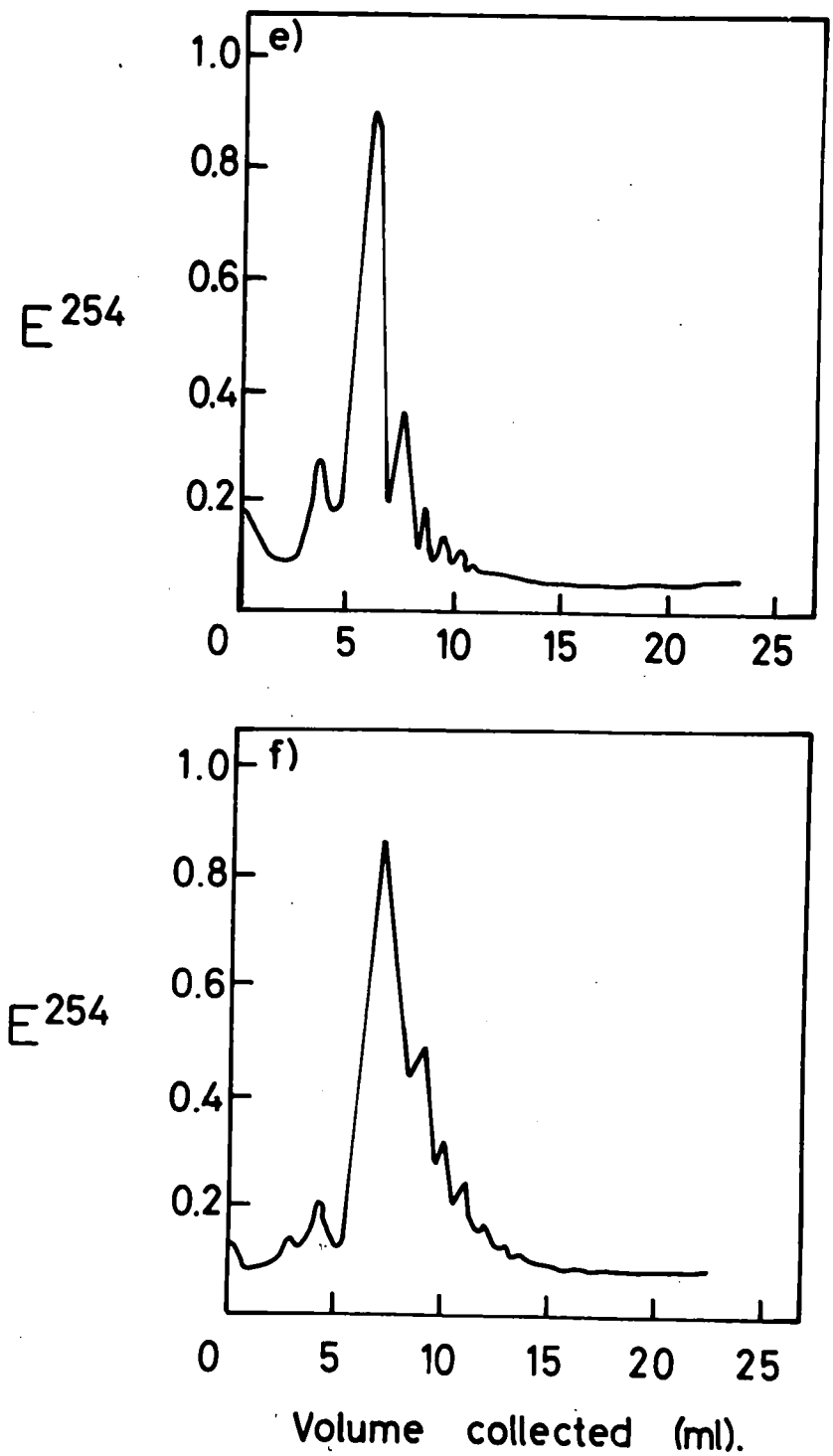
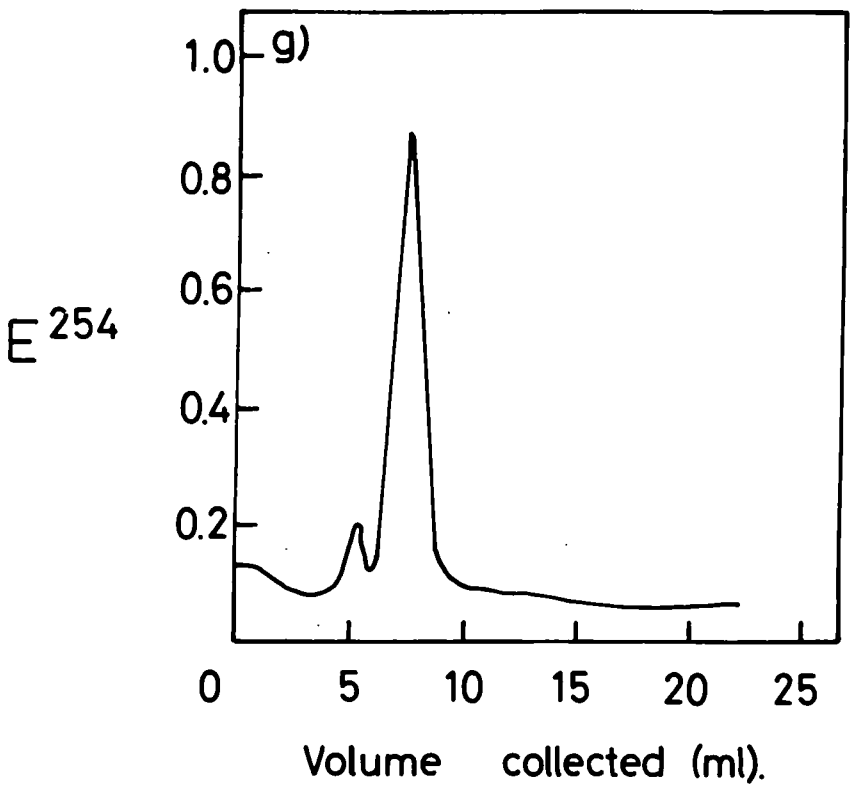


Fig.40. (cont)

g) Microsomes prepared from cotyledons of 100 day seeds



Legend for Fig.40

15% to 45% isokinetic gradients were prepared using 6 ml. 15% sucrose and 20 ml. 55% sucrose (containing 10mM Tris-HCl buffer, pH 7·6; 25mM KCl; 1mM $MgCl_2$) by the method of Henderson (1969). The gradients were standardized using ribosomes prepared from E.coli. 2 mg. of microsomal suspension was layered onto the gradient, which was centrifuged at 105,000 x g. for 3 hr. in the 3 x 23 ml. head of the M.S.E. Superspeed '65 ultracentrifuge.

E^{254} was monitored using an Isco ultraviolet analyser, Model 222, attached to a servoscribe chart recorder.

see if changes found in the in vitro activity of the microsome preparations could be related to changes in their ribosome content. The results from the sucrose gradients are shown in fig. 40.

Microsomes from 30 day cotyledons showed a major monosome peak, preceded by two small peaks, which were probably the ribosomal subunits, and there was one well defined polysome peak. A final sharp peak appeared on the gradient which might have been caused by much larger polysomes, or by small fragments of endoplasmic reticulum with a few membrane bound ribosomes attached. The peak had not migrated sufficiently far through the gradient to correspond to the typical membrane bound fraction seen in preparations from 50 and 60 day cotyledons. A similar profile was found with material from 40 day cotyledons, except that three polysome peaks could be distinguished in the free ribosomal fraction of the gradient. In preparations from 50 and 60 day cotyledons, there was a large broad peak of membrane bound material, and 4-5 polysome peaks could be distinguished in the free ribosomes. No membrane bound material was present in microsomes prepared from 70 and 80 day cotyledons, although polysomes were still present in the free ribosomal fraction. In preparations from 100 day seeds, however, all traces of polysomes were lost, and there was a single, large monosome peak, with some traces of subunits.

The ribosome content of these microsomal preparations does therefore, reflect the in vivo distribution of ribosomes, in that membrane bound ribosomes only occur in any quantity in preparations from 50 and 60 day cotyledons.

Changes in the in vitro activity of microsomes prepared from different ages of developing cotyledons were studied independently of changes in enzyme or tRNA activity, by incubating with standard tRNA and enzyme in the complete system, under optimal conditions for in vitro amino acid incorporation, so that changes in activity were not due to any of the ingredients being limiting.

Two different patterns of activity were obtained, depending on whether the results were expressed on their microsome content as determined by $E^{256}_{1cm.}$ (fig. 25), or on their RNA content as determined by the orcinol reaction (fig. 26). Protein is known to interfere with the estimation of RNA by $E^{256}_{1cm.}$, so that in preparations containing a high percentage of protein in the form of membrane bound ribosomes (e.g. those from 50 and 60 day cotyledons), extinction at 256 mμ will underestimate the microsome content. Comparison of the incorporating activity of different preparations based on values per mg. microsomes, will therefore be unreliable if much membrane bound material is present. An alternative and more reliable method is therefore needed for the expression of the results when the activity of very different

microsome preparations are to be compared. The orcinol method of RNA determination was therefore used to determine the actual RNA content of the various microsome preparations, and the amount of phenylalanine incorporation has been expressed per mg. RNA as determined by this method. Comparisons based on these figures give much truer results.

The activity of the microsome preparations from different ages of developing bean seeds increased with increasing age of seeds, to a maximum with microsomes prepared from 60 day seeds (fig. 26). This may reflect the increasing membrane bound ribosome content both in vivo in the cotyledon cells and also in the in vitro microsomal preparations, and the increasing activity of the membrane bound ribosomes in microsomes prepared from beans that are actively synthesizing storage protein in vivo. Microsome preparations from 80 and 100 day seeds were found to be active in phenylalanine incorporation in the presence of poly-U when supplied with active tRNA and enzyme fraction. This suggests that the lack of activity of these microsomal preparations when incubated with their corresponding components from 80 and 100 day seeds in the presence of poly-U was due to the presence of an inhibitor(s) or inactive tRNA or enzyme fraction.

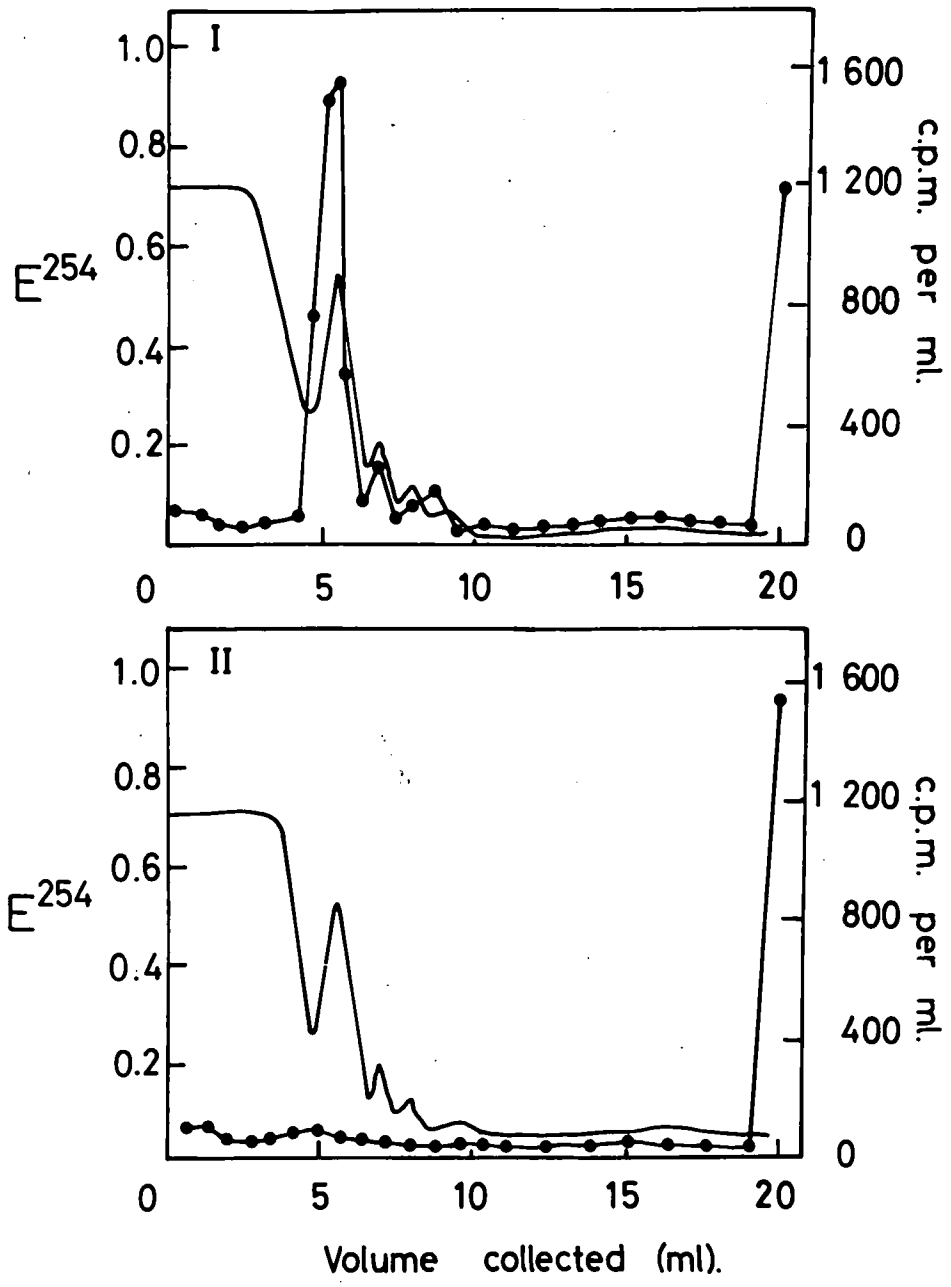
Sucrose gradient analysis of the microsomal preparations showed loss of polysomes in preparations from 100 day seeds, which would be associated with dehydration of the seed leading

to dormancy. This preparation was inactive in in vitro amino acid incorporation unless a messenger was added to the incubations. Similar results were found by Marcus and Feeley (1964) with ribosomes from dormant peanut seeds, and Barker and Rieber (1967) with ribosomes from dormant Pisum arvense seeds. Chapman and Rieber (1967) showed by electron microscopy that the inactivity of ribosomes prepared from dormant P.arvense seeds was due to lack of mRNA and polysomal aggregates.

Incubations containing microsomes from 60 day developing seeds which had been allowed to incorporate [^{14}C] phenylalanine in the presence and absence of poly-U in the complete system were analysed on sucrose gradients in collaboration with Mrs. A.Brownrigg, to identify the ribosomal fraction most active in phenylalanine incorporation. The results are shown in fig. 41. Membrane bound material sedimented through the gradient and formed a pellet at the bottom of the centrifuge tube, whereas the free ribosomal material separated into monosome and polysome peaks along the gradient.

Moore (1966) studied the binding of poly-U to 70S ribosomes and found that the formation of polysomes seemed to be controlled by the ratio of poly-U to ribosomes. When the ribosomes were present in molar excess over poly-U, polysome complexes were formed as the poly-U molecule became attached to several ribosomes.

Fig.41. Identification by sucrose gradient centrifugation of the ribosomal fraction most active in [^{14}C] phenylalanine incorporation in a microsomal preparation from cotyledons of 60 day V.faba seeds



I. Incubation containing poly-U

II. Incubation minus poly-U

—— Optical Density

●—● Radioactivity

Legend - see p.160

Legend for Fig.41

2 ml. incubations containing microsomes and enzyme fraction from 60 day cotyledons and tRNA from 40 day seeds were incubated as described in Methods, with and without poly-U. After 20 min. incubation, 0•5 ml. aliquot was removed for assay, and the remainder of the incubation was layered on to an isokinetic sucrose gradient (see Legend for Fig.40). After centrifugation, the gradient was fractionated and 0•5 ml. samples were collected in 5 ml. 5% (w/v) TCA containing 0•2 mg. Bovine Serum albumin. The samples were hydrolysed at 90^{o} for 15 min., collected on millipore filters and counted as described in Methods.

However, when poly-U was in excess polysome formation was minimal as poly-U complexed with single ribosomes, and the sedimentation profile on sucrose gradients resembled that of free ribosomes. In the in vitro incubations, poly-U was added in excess (fig. 7) and probably forms functional complexes with single ribosomes. This probably explains the large peak of radioactivity associated with the monosomes in the incubations containing poly-U (fig. 41, I). The fractions were hydrolysed in 5% TCA(w/v) at 90° for 15 min. before being assayed, so that the radioactivity was not due to the binding of amino acyl tRNA to the ribosomes without peptide bond formation. Radioactivity was also found associated with the polysomes and the membrane bound ribosomal material. Thus, both the free and membrane bound ribosomes were capable of amino acid incorporation in the presence of added synthetic messenger. In the absence of added messenger (fig. 41, II) only the membrane bound ribosomes were found to be active in amino acid incorporation, suggesting that only the membrane bound ribosomes were actively synthesizing protein in vivo in 60 day cotyledons. The free polysomes may be incorporating phenylalanine, but any activity associated with them was too low to show above the background count, and any activity associated with them would seem to be negligible compared with that of the membrane bound ribosomes.

The next major step in this work would be the isolation and amino acid analysis of the protein being formed on the membrane bound ribosomes in *in vitro* incubations, to confirm that it is either vicilin or legumin.

Further evidence for the role of the membrane bound ribosomes in storage proteins synthesis has come from the work of Bailey, Cobb and Boulter (to be published) who have traced the *in vivo* incorporation of [^{14}C] leucine into the endoplasmic reticulum of tissue slices from *Vicia faba* cotyledons actively synthesizing storage protein, and the subsequent movement of the radioactivity into the protein bodies.

Changes in the activity of the microsomal fractions from developing seeds of different ages in *in vitro* amino acid incorporation although important, are not the only factors involved in controlling the amount of *in vitro* activity. Enzyme fractions from different ages of developing seeds also show differences in ability to promote *in vitro* amino acid incorporation when incubated with standard tRNA and microsomes (fig. 28).

Enzyme fractions from 60 day cotyledons were the most active in *in vitro* amino acid incorporation, reflecting the *in vivo* situation at this stage of development, when most of the metabolic activity of the seeds is directed towards storage protein synthesis. The high speed supernant from beans of this age probably contained

more protein synthetic enzymes to total protein content, than at any other stage in seed development, and this is reflected in its increased activity in in vitro incorporation.

Ingle, Beitz and Hageman (1956) reported an increase in RNase activity during the maturation of maize seeds, and suggested that this might be significant in mRNA degradation and loss of polysomes as the seeds dehydrate and mature. Changes in RNase content and activity have not been studied during the maturation of V.faba seeds, but it is possible that the decrease in the in vitro activity of the high speed supernatant fraction from dehydrating seeds may be partly due to increasing amounts of RNase as the seed completes its development.

The changes occurring in the enzyme fraction are however, more complicated than this, as the amino acyl tRNA synthetase and transferase activities of the high speed supernatants showed different patterns of in vitro activity during seed development (fig. 29-31). These changes in activity could be due to changes in the concentration of these enzymes in the different high speed supernatant fractions, as no attempt was made to isolate or purify the synthetase and transferase enzymes. Alternatively, these changes could be due to actual changes in the activity of the enzymes, which could only be identified from precise kinetic studies with the purified enzymes. Changes like this, if they

occurred, would be very important in control of in vivo protein synthesis, and if identified might shed light on the nature of the metabolic control of seed development.

Analysis of the tRNA preparations from different ages of developing bean seeds by polyacrylamide gel electrophoresis showed that the tRNA preparations before DE52 chromatography were highly contaminated with 25S, 18S and 5S rRNA (fig. 36). All the high molecular weight rRNAs were removed by DE52 chromatography as determined by running the preparations on 2·6% gels, and electrophoresis on 5% gels showed that most of the 5S rRNA was removed. Although chromatography improved the charging capacity (fig. 32) and the activity of tRNA in amino acid incorporation (fig. 34), it did not alter the overall pattern of activity of the tRNA preparations during seed development.

The 4S content of the tRNA preparations after chromatography was determined by electrophoresis on 5% gels and the results from the charging and incorporation incubations were recalculated on this basis, rather than on total RNA content as determined by $E^{256}_{1cm.}$ (figs. 37 and 38). The observed differences in the activity of the tRNA preparations from different ages of seeds in amino acid incorporation could not, therefore, be attributed to differences in the concentration of 4S RNA in the tRNA preparations, and hence differing amounts added to the incubations, but rather

to some difference in the 4S RNA itself. Amino acid incorporating activity of the tRNA preparations was assayed under optimal conditions, using standard microsomes and enzyme fractions, so that differences in activity could not be attributed to one or other of the factors in the incubation being limiting.

In the work using poly-U as an artificial message, only the $tRNA^{phe}$ activity of the preparations was being measured, and not the total tRNA activity. It is possible that the proportion and activity of $tRNA^{phe}$ could be changing during bean seed development. Lee and Ingram (1967) reported qualitative and quantitative changes in $tRNA^{met}$ from reticulocytes of chick embryos and mature chickens, which were not caused by changes in the RNase activity of the preparations. Yang and Comb (1968) and Zeikus, Taylor and Buck (1969) reported changes in $tRNA^{lys}$ during the development of sea urchin eggs. Kaneko and Doi (1966) and Doi, Kaneko and Igarashi (1968) reported changes in $tRNA^{val}$ during sporulation of Bacillus subtilis, and Anderson and Cherry (1969) reported changes in $tRNA^{leu}$ during germination of soybean seedlings.

A measure of the phenylalanyl acceptor capacity of the tRNA preparations could be obtained from the charging incubations (fig. 32) and this gives an indication of the relative amounts of $tRNA^{phe}$ in the various preparations. It was shown using a

variety of enzymes that tRNA prepared from 60 day seeds showed maximum phenylalanine acceptor capacity (and in fact, maximum total amino acyl acceptor capacity, fig. 33). It would therefore seem unlikely that changes in the phenylalanine incorporating activity of tRNA from different ages of developing bean seeds could be attributed to changes in the $tRNA^{phe}$ content alone. Neither could the differences in activity be explained by the inability of the tRNA preparations to be aminoacylated, as all could be highly charged with [^{14}C] phenylalanine.

Slight changes in the primary structure (Gefter and Russell, 1969) or in the secondary and tertiary structure of tRNA (Adams, Lindahl and Fresco, 1967; Lindahl, Adams, Geroch and Fresco, 1967) are known to decrease the activity of tRNA species in amino acid incorporation. It is possible that similar slight changes could be present in tRNA prepared from more mature V.faba seeds, which would make it less efficient in amino acid incorporation once it had been acylated.

The development of Vicia faba seeds is a very complex process, involving great changes in the physiology and biochemistry of the seed, both at the cellular and molecular level. In this thesis, one of the aspects of seed development has been studied, that of the formation of the storage proteins. Total amino acid incorporating activity of components isolated from developing seeds

of different ages was found to parallel the changes occurring in the in vivo protein synthetic activity of the developing seeds. This indicates that definite changes were occurring in the protein synthetic machinery of the seed prior to the onset of storage protein synthesis. The most obvious change is the rapid formation of endoplasmic reticulum and increase in the proportion of membrane bound ribosomes. Evidence is accumulating in this laboratory, some of which is presented in this thesis, that the membrane bound ribosomes alone are responsible for the synthesis of the storage proteins. The mechanism of the genetic control triggering off membrane bound ribosome formation at this stage of development is obscure, but the results would indicate that simple models of transcription and translation control are inadequate, as the onset of storage protein synthesis in this instance involves co-ordinated action of several different cell organelles and membranes. Changes in the activity of the enzyme fractions and tRNA prepared from different ages of developing seeds must also be taken into account.

The termination of storage protein synthesis, and the onset of seed dehydration leading to dormancy are possibly controlled by changes in the physiological state of the seeds with increasing age; e.g. exhaustion of available amino acid pools and energy resources.

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