Cytoplasmic controls of storage protein synthesis in pea (Pisum sativum L.)

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Cytoplasmic Controls of Storage Protein Synthesis in Pea (*Pisum sativum* L.)

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For My Mother

ABSTRACT

The *in vitro* translation of pea storage protein subunits was investigated using four polysome driven cell-free systems, derived from wheat-germ, reticulocyte lysate, pea axes and pea cotyledons.

Re-initiation of protein synthesis occurred in each of the systems and accounted for about 25%, 34%, 44% and up to 45% of protein synthesis in the polysomes driven wheat-germ, cotyledon, axes and reticulocyte lysate systems respectively.

The polysome translation products of all the systems were very similar and vicilin 50,000 and 47,000 mol.wt. polypeptides, convicilin 70,000 mol.wt. polypeptide, and legumin precursor of 60,000 mol.wt. were shown to be synthesised using immunoprecipitation techniques and by comparing the CNBr cleavage products of *in vitro* and *in vivo* synthesised polypeptides. Modification of the vicilin 50,000 and 47,000 mol.wt. polypeptides occurred by the removal of short amino acid sequences, when microsomal membranes were present in the cell-free system, although sequestering of the synthesised polypeptides into membrane-bound vesicles could not be demonstrated. The plant derived cell-free systems were capable of a limited amount of polypeptide modification, which was absent from the reticulocyte lysate system.

cDNA: mRNA hybridisation techniques were employed in a study of the abundance and complexity of the mRNA population of pea cotyledons at three seed developmental stages, (9, 14, and 19 daf). An increase in very abundant mRNAs was noted between 9 and 14 day stages (up to 6 mRNAs), during which time rapid storage protein accumulation commences, while a progressive decrease in the number of rare RNAs was apparent during development.

Preliminary investigations into the proportion of single-copy DNA, and of nuclear poly(A)$^+$-RNA, present in the polysomal poly(A)$^+$-RNA were carried out; approximately 5% of single-copy DNA was transcribed into the polysomal poly(A)$^+$-RNA of cotyledons, (at 14 daf), and approximately 50% of nuclear poly(A)$^+$-RNA may be present in polysomal poly(A)$^+$-RNA of cotyledons at 9 daf.

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INTRODUCTION

INTRODUCTION

Seventy percent of the edible protein produced in the world is from seeds, (Spencer and Higgins, 1979), and it is legume seeds which provide a major source of protein in the diets of both people and animals, particularly in many developing countries. Legumes also play a significant role in desirable crop rotation, (PAG Bulletin, 1973), due to their nitrogen fixing root nodules.

However, although legume seeds are rich in protein, (about 25-50% total protein, compared to 7-10% for members of Gramineae, Danielsson, 1949), there is an urgent need to improve legume crops both in terms of yield and nutritional value, particularly as legume seed proteins are deficient in essential sulphur amino acids, (cysteine and methionine), Croy et al., (1980c), and Casey and Short, (1981).

A better understanding therefore of the basic processes of biosynthesis and translocation of plant and seed storage proteins could lead to more efficient screening and selection techniques for desirable legume varieties, (PAG Bulletin, 1973), and ultimately perhaps lead to the ability to manipulate the plant genome and provide more direct methods of crop improvement, (Chrispeels et al., 1979). However, Cocking, (1981), has pointed out many problems which may arise from attempting to improve crops using the newer techniques of genetic manipulation, (eg. a change in protein conformation may impair seed-fill, etc.).

In any case, before the manipulation of seed proteins can be considered to be feasible, it will be necessary to characterise them fully. Information about legume seed proteins began to be discovered at the turn of this century when Osborne and Campbell, (1898), separated seed proteins into an albumin, (water soluble) and a globulin, (water insoluble), fraction and further divided the globulin fraction into two major proteins on the basis of heat coagulation and solubility in ammonium sulphate solution. They were called legumin, which does not coagulate at 100°C, and vicilin which coagulates at 95-100°C, and is also more soluble in ammonium sulphate solution than legumin. Danielsson, (1949), characterised these 2 fractions further using ultracentrifugation and isoelectric precipitation and found that all the

species of legume seed which he tested, (34), contained a legumin fraction which sedimented at about 11-13s and had an isoelectric point of about pH 4.8, while 30 out of 34 species of legume seed also contained a vicilin fraction with a sedimentation constant between 7s and 9s, and an isoelectric point of approximately pH 5.5.

It is the globulin fraction of legume seed proteins which constitutes the storage proteins of the legume. These proteins fulfill the definition of storage proteins, (Derbyshire et al., 1976), that is they constitute more than 5% of the seed protein, are unique to seed, (Millerd, 1975), and are synthesised at one stage of plant development, stored, and then used at a later, more metabollically active stage, (ie they provide a nitrogen source on germination, as demonstrated in the case of peas by Basha and Beevers, 1975, and Thomson et al., 1978).

Considerable research has been carried out using several of the main legume species important to man, eg. soyabean, (Glycine max L.), Beachy et al., (1978), Hill and Breidenbach, (1974); French bean, (Phaseolus vulgaris L.), Hallet al., (1972 and 1978), Sun et al., (1975 and 1978), Bollini and Chrispeels, (1979); Common bean, (Vicia faba L.), Wheeler and Boulter, (1966), Payne and Boulter, (1969), Bailey and Boulter, (1970), Wright and Boulter , (1972); Lupinus angustifolius, Blagrove and Gillespie, (1975); Pea, (Pisum sativum L.), Beevers and Poulson, (1972), Millerd and Spencer, (1974), Evans et al., (1979), Croy et al., (1979, 1980a, b and c), Gatehouse et al., (1980 and 1981), Higgins and Spencer, (1977, 1980 and 1981), Hurkman and Beevers, (1980); and Castor Bean (Ricinus communis), Roberts and Lord, (1981).

The structure of legumin and vicilin has been at least partly elucidated for some of these species and the overall similarity between closely related species (eg. Vicia faba and Pisum sativum, Croy et al., 1979) shown. The differences in structure of the storage protein subunits between varieties may give an idea of the type and degree of changes which the plant will tolerate before affecting the viability of the seed, which after all is produced to benefit the plant, not man, Millerd, (1975). More sophisticated isolation and identification techniques have, however, revealed a degree of microheterogeneity within

each of the storage protein types, (Wright and Boulter, 1974 , Thomson et al., 1978, Casey, 1979, Krishna et al., 1979, Croy et al., 1980a and b, Spencer and Higgins, 1979, Casey and Short, 1981).

The storage protein of pea seeds have been studied by various groups in some detail. Beevers and Poulson, (1972), estimated that 25% of pea cotyledons is protein and that 80% of the protein is globulin, which is in agreement with the figure (80%) obtained by Raacke, (1957).

Legumin is the primary protein in the globulin fraction of most pea seeds, (although some varieties have been reported where this is not so), Raacke, (1957). It has been shown to be a hexameric protein with a mol.wt. of 360,000 to 400,000, each monomer consisting of a 40,000 (acidic) and a 20,000 (basic) mol.wt. subunit linked by disulphide bonds, (Croy et al., 1979). Both subunits of legumin isolated from mature seeds have been shown to be heterogeneous with regard to charge and mol.wt., (Krishna et al., 1979). The level of glycosylation of the subunits is very low, Basha and Beevers, (1976), Casey, (1979), Gatehouse et al., (1980) and Hurkman and Beevers, (1980). Each 60,000 mol.wt. subunit contains 7 cysteine and 4 methionine residues and legumin is therefore of nutritional importance, Croy et al., (1980c). Recent research involving cloning of legumin genes (Croy et al., 1982), confirms that a 60,000 mol.wt. precursor is coded for in pea cotyledon mRNA and that the 20,000 subunit is coded for at the 3' end of the mRNA. The probable presence of 4 single copy legumin genes in genomic DNA was demonstrated.

The vicilin fraction, which is usually present at between 25-75% of the level of legumin in the mature seed, is of a heterogeneous subunit composition (Croy et al., 1980b), and has recently been shown to composed of 2 proteins, (Croy et al., 1980c). Convicilin is a protein of about 290,000 mol.wt. and probably consists of 4 subunits each of 71,000 mol.wt. This protein has a limited amount of heterogeneity and the level of glycosylation is insignificant. Convicilin is immunologically related to the other protein in the vicilin fraction, namely vicilin. Native vicilin in mature seeds has a mol.wt. of 180,000-200,000, (Derbyshire et al., 1976), and is glycosylated, (Basha and Beevers, 1976); it is composed of heterogeneous subunits, the major ones being 50,000 and 33,000 mol.wt., with a minor 17,000 mol.wt. subunit, (Gatehouse et

al., 1981, Thomson et al., 1978), and an uncharacterised 65,000 mol.wt. subunit.

Developing cotyledons contain a 47,000 mol.wt. subunit which is not apparent at maturity and is thought, (from results of pulse chase experiments), to be a precursor to the 33,000 mol.wt. subunit, (Gatehouse et al., 1981). Mature vicilin does not contain any methionine or cysteine and therefore is nutritionally poor, however, convicilin does contain 1 cysteine and 1 methionine residue per subunit.

The relative levels of synthesis of the storage proteins changes during seed development, (Beevers and Poulson, 1972); the onset of accumulation of the proteins is sequential first vicilin, legumin and then convicilin, (Higgins and Spencer, 1979, Millerd and Spencer, 1974, Croy et al., 1980c).

The accumulation of storage proteins begins about a third of the way through the developmental cycle of a legume seed, (Derbyshire et al., 1976), and has been correlated with several morphological changes in the cotyledon cell, (reviewed by Dure, 1975). The first phase of the seed development is one of rapid cell division, this is accompanied by endoreduplication of the DNA, up to 64c, the function of which is unknown, but does not lead to a specific increase in storage protein mRNA, (Dure, 1975, Millerd and Spencer, 1974, Spencer and Higgins, 1979). There is also rapid RNA synthesis which has been shown principally to be of tRNA and ribosomal RNA, which is necessary to increase the protein translation machinery needed during the next developmental phase, (Millerd, 1975, Spencer and Higgins, 1979). These processes then decline and the second phase of development is characterised by cell expansion and a massive increase in accumulation of storage proteins and other storage reserves. There is proliferation of the rough endoplasmic reticulum (RER), (Bain and Mercer, 1966, Opik, 1968, Briarty et al., 1969, Boulter et al., 1972, Chrispeels et al., 1979), and an increase in the ratio of polysomes to monosomes in the cotyledon cells, (Beevers and Poulson, 1972, Briarty et al., 1969). There is also a decrease in vacuole size, and protein bodies start to develop; these are single-membrane-bound organelles, about 2μm diameter,

in which storage protein is deposited, (Briarty et al., 1969, Varner and Schidlovsky, 1963, Opik, 1968, Graham and Gunning, 1970, Thomson et al., 1978, Baumgartner et al., 1980).

The origin of protein bodies is a much debated point, as is the route of storage protein translocation between synthesis and deposition. EM studies which show the elaboration of RER at the onset of storage protein synthesis, have led to the suggestion that storage proteins are synthesised on RER, (Baumgartner et al., 1980, Briarty et al., 1969, Bailey et al., 1970) and are transported to protein bodies either via smooth ER and dictyosomes, as discussed by Chrispeels et al., (1979) and Harris, (1979), in a manner similar to that of animal secretory proteins, (Caro and Palade, 1964, Jamieson and Palade, 1965 and 1967), or alternatively via vesicles arising from the ER itself. The protein bodies are thought by some to originate from division of the vacuole, Bain and Mercer, (1966), Opik, (1968), rather than to be of plastid origin; it is possible that this is the initial source of protein bodies, and when these are filled protein bodies are derived from plastids.

The final phase of seed development is characterised by a decline in protein synthesis, loss of RER and a decrease in the ratio of polysomes to monosomes, (indicating a decrease in translatable mRNA), as dessication of the seed proceeds.

The research described above into the structure and localisation of storage proteins provided a background for investigations into the mechanisms of plant storage protein synthesis, (reviewed by Allende, 1969, Zalik and Jones, 1973, and Boulter, 1970).

The discovery of cell-free protein synthesising systems, which were capable of translating exogeneously supplied mRNA to produce identifiable polypeptides, described in the reviews by Millerd, (1975), Higgins and Spencer, (1979), and Hall, (1979), coupled with the techniques to extract polysomes and fairly pure mRNA, (Taylor 1979, and Hall, 1979, give a review of this aspect), provided an indispensable tool for investigating in detail the mechanisms of protein biosynthesis on the ribosome, eg. initiation, translation of mRNA and the role of ribosomes and tRNA,

(Yarwood et al., 1971), and termination of protein synthesis, (Mans, 1967, Boulter, 1970, Boulter et al., 1972, and Zalik and Jones, 1973, have reviewed the large amount of information accumulated about these processes).

The development of the wheat-germ system by Marcus and co-workers provided an economical, plant derived cell-free system, which was used in conjunction with the bacterial and animal systems that were initially developed.

Seed storage proteins provide an excellent system for studying the regulation and control of the synthesis of specific polypeptides, as a high proportion of the protein synthesising activity is concentrated, at a specific stage of development, within a specific organ, into the production of a few major polypeptides, (Thomson et al., 1978, Dure, 1975, Millerd, 1975, Hall, 1979).

The methods of control over the onset of storage protein accumulation have not yet been elucidated. Control mechanisms could be effective at several points between the stage of transcription of heteronuclear RNA, (hnRNA), by DNA dependent RNA polymerase, from the genomic DNA, to that of translation of mRNA in the cytoplasm, (see discussion in the review by Lodish, 1976). Possible sites of control are 1) at transcription, by regulation of RNA polymerases, or by blocking of the gene, eg. by histones (Hall, 1979), until a 'signal' for synthesis of a specific mRNA is received; 2) splicing of the hnRNA could be a point of control, also processing of the hnRNA, eg. polyadenylation, methylation and capping, (with 7 methyl guanosine), may be necessary before functional mRNA is released, (review by Lewin, 1975, Abelson, 1979, Mans et al., 1979); 3) transport of nuclear mRNA to the cytoplasm could be controlled; and 4) turnover of mRNA may be regulated; 5) cytoplasmic mRNA may be stored, (ie be unavailable for translation), some mRNA is believed to be synthesised during the mature phase of embryogenesis of seeds and stored during dessication, to be used in very early stages of germination, (Payne, 1976, Galau and Dure, 1981), this may, however, be a specialised case as there is little evidence for the presence of other untranslated mRNA in eukaryotic cytoplasm, (Lodish, 1976, Hall, 1979); 6) translation

of the mRNA could be controlled by the rate of initiation of protein synthesis, or by the presence, (or absence), of isoaccepting tRNAs, or by the absence of a promotor, or the presence of an inhibitor, (as in the synthesis of globin, which is regulated by the presence of haemin, in the reticulocyte cells, Ochoa and de Haro, 1979); 7) turnover of polypeptides could also be regulated, (though evidence from in vitro and in vivo studies show this is unlikely in storage protein synthesis, Higgins and Spencer, 1979). Post-translational modifications of the polypeptides may be necessary and may affect the synthesis and accumulation of further polypeptides.

Although the mechanisms which control the onset of storage protein accumulation have not yet been characterised, general opinion favours transcriptional or post-transcriptional control to be the primary mechanism in tissues such as plant seeds, with translational or post-translational controls playing a secondary role, (Meinke et al., 1981, Goldberg et al., 1981, Hall, 1979); this conclusion has been reached as a result of experiments demonstrating the abundance and complexity of mRNA, using DNA:RNA hybridisation techniques, in different tissues and at different developmental stages, and using in vitro methods to translate the very abundant messages. Several research workers have summarised their work in 'Genome Organization and Expression in Plants', (Leaver, 1980), (eg. Goldberg, Larkins et al., Hall et al., Bishop et al., Dure et al.,). The abundance and complexity of a mRNA population can be calculated from the kinetics of the hybridisation of an excess of driver mRNA to labelled cDNA, (Bishop et al., 1974). The mRNA population is often divided into 3 abundance classes, a very abundant class containing a few species of mRNA which are each present in a large number of copies per cell, (up to tens of thousands), an abundant class of several hundred to several thousand different sequences in several hundred copies each per cell, and a rare class of mRNA consisting of many thousands of different sequences, but only represented by a few copies per cell, this third class represents most of the genetic diversity of the cell.

A change in complexity and in abundance levels of mRNA as seen during the development of tissues such as seed cotyledons, the presence of a few

very abundant mRNAs when an increase in synthesis of a specific set of proteins occurs, and a decrease in this mRNA class when protein accumulation declines, has been demonstrated, in soyabean, Goldberg, (1981), and in cottonseed, Galau and Dure, (1981); these mRNAs are predicted to be coding for these specific proteins.

This study aimed to investigate the in vitro synthesis of pea storage proteins and to examine some cytoplasmic, co- and post- translational controls involved in the modification of the protein subunits. Evans et al., (1979), had demonstrated the synthesis of pea storage proteins in the wheat-germ and reticulocyte lysate systems. This work was continued, and the similarity of in vitro and in vivo synthesised polypeptides demonstrated using immunoprecipitation and CNBr cleavage of the polypeptides and SDS PAGE. The effect of the presence of membranes, (as microsomes and protein bodies) in the cell-free system was also investigated.

Polypeptides synthesised, from polysomes, in the conventional cell-free systems, (ie wheat-germ and reticulocyte lysate), were compared to those synthesised in homologous systems, derived from pea axes and pea cotyledons, in order to examine the possibility of 'pea specific factors' which would modify the storage protein polypeptides, especially legumin which was synthesised in a precursor form in vitro. The amount of re-initiation of protein synthesis on polysomes, in the 4 cell-free systems was also investigated.

In order to attempt to correlate the rapid increase in storage protein synthesis with an increase in polysomal mRNA, specifying these proteins, the change in mRNA population at 3 developmental stages, (9, 14 and 19 days after flowering), was followed using 2 approaches. Firstly the in vitro synthesised translation products of polysomal poly(A)$^+$-RNA were examined by SDS PAGE. Secondly the complexity and abundance of the mRNA population at each of the 3 stages was studied using complementary DNA: mRNA hybridisation techniques. Preliminary experiments to demonstrate the amount of single-copy DNA and nuclear poly(A)$^+$-RNA present in polysomal poly(A)$^+$-RNA were carried out. However, due to lack of time and problems in obtaining relatively large quantities of material, these are to be regarded as preliminary experiments.

MATERIALS

Materials

Chemicals

Radioactive chemicals were obtained from the Radio-chemical Centre, Amersham, U.K., as follows:-

L-[^{35}S] methionine, 800-1200 Ci/mmol; L-[4,5^{3}H]leucine, 188 Ci/mmol; [^{3}H]acetic anhydride, 500 mCi/mmol; deoxy[5-^{3}H]cytidine 5'-triphosphate, ([^{3}H]dCTP)18 Ci/mmol; N-acetyl-D-[1-^{3}H]glucosamine, 11 Ci/mmol; D-[2-^{3}H]mannose, 12 Ci/mmol deoxycytidine 5'-[α-^{32}P]triphosphate ([^{32}P]dCTP),>350 Ci/mmol.

The enzymes used were from a variety of sources; proteinase K (fungal) was from British Drug House Chemicals Ltd., Dorset, U.K.; avian myeloblastosis virus (AMV) reverse transcriptase was obtained from the Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Bethesda, MD, USA; S_1 nuclease (from Aspergillus oryzae) was from Boehringer Mannheim Corp., Lewes, East Sussex, U.K.

Actinomycin D and Miracloth were obtained from Calbiochem, La Jolla, CA, USA, and oligo(dT)$_{10}$ and oligo dT-cellulose, grade T-3, were from Collaborative Research Inc., Waltham, MA, USA. Chloramphenicol, (D(-)threo-2,2-Dichloro-N[-hydroxy-(hydroxymethyl)-p-nitrophenethyl]acetamide), aurintricarboxylic acid (ATA), protein A-sepharose CL-4B, and calf thymus DNA were from Sigma London Chemical Co., Poole, Dorset, U.K. Rabbit globin mRNA was purchased from Uniscience Ltd., Cambridge, U.K. Repelcote was obtained from Hopkin and Williams, Romford, Essex, U.K. Whatman glass fibre discs (GF/C) and DEAE cellulose paper and Drummond microcaps were from A. and J. Beveridge Ltd., Edinburgh, U.K.

The X-ray film used for fluorography was either Kodak X-Omat or Fuji RX.

All other chemicals were obtained from BDH Chemicals (see above) and were of AnalaR grade or the best available.

Biological Materials

The wheat-germ which was used to make the cell-free extract was from Niblack's Foods Inc., Rochester, NY, USA. Pea seeds of the variety Feltham First (Sutton Seeds, Ltd., Reading, Berks., U.K.) were used routinely, but other varieties ie Kelvedon Wonder, Little Marvel and Mangetout (Sutton Seeds Ltd.) were also used.

The term 'native' legumin, or vicilin, refers to the storage protein subunits isolated from mature seeds and used as unlabelled markers for gel electrophoresis, while the term 'standard' legumin, or vicilin, refers to native storage protein subunits which have been tritiated for use as markers for radioactive samples.

1. METHODS

1-1 The Preparation of Cell-free Protein Synthesising Systems

The following preparations were made using autoclaved solutions; if buffers contained a high concentration of sucrose, the sucrose and the buffer were autoclaved separately. Glassware was baked overnight at 170°C, and other apparatus e.g. polycarbonate centrifuge tubes, was autoclaved prior to use. Extraction procedures were carried out at 2-4°C and the mixing of the *in vitro* protein synthesising assay components was carried out on ice. Radioactive amino acids used in these assays were L-[^{35}S]-methionine and L-[4,5-^{3}H]leucine; these were both stored in 10μl aliquots under liquid nitrogen. The [^{3}H]leucine was dissolved in ethanol and it was necessary to dry the required amount on a vacuum line and redissolve it in an appropriate volume of sterile distilled water; the [^{35}S] methionine was already dissolved in water.

1-1-1 Wheat-germ Cell-free System

The wheat-germ system was prepared using the method of Gordon and Payne (1976), which was based on that of Roberts and Paterson (1973), except that the wheat-germ extract was not preincubated, as the endogenous template activity of the wheat-germ was low.

The extract was made by grinding 3g of wheat-germ with 3g of pasteur pipette tips (2-3mm long) for 2 min in a mortar, and then adding 14 ml of extraction buffer (20mM HEPES/KOH pH 7.6, 100 mM KCl, 1mM Mg acetate, 2mM $CaCl_2$ and 6mM 2mercaptoethanol (2ME)) in 2 instalments. The homogenate was centrifuged at 30,000g$_{av}$ for 12 min and the supernatant removed, avoiding the floating fatty layer. A fine Sephadex G25 column, about 20cm high, was packed in a 60ml sterile syringe using Sephadex which had been soaked overnight in column buffer (20mM HEPES/KOH pH 7.6, 100mM KCl, 3mM Mg acetate and 6mM 2ME). The column was washed with 2 volumes of column buffer and then the supernatant from the wheat-germ S30 extract applied, and run through, followed by column buffer. The fast-moving, buff-coloured, turbid region was collected in small tubes (about 15 drops/tube), and the most concentrated fractions (judged by eye) were pooled to give approximately 4.5 ml of extract. This extract was stored as beads of about 40μl under liquid nitrogen.

The in vitro translation of polysome and poly $(A)^+$-RNA templates in the system was described by Evans et al. (1979), using a method based on Gordon and Payne (1976). In a total assay volume of 10μl, there was 5μl of wheat-germ extract+creatine phosphokinase (CPK) at 40μg/ml extract, 5-10μCi of labelled aminoacid in a 1 μl volume, 50-100μg of polysomes or 1-2μg of poly$(A)^+$-RNA in a 2μl volume, 80mM KCl, 3mM Mg acetate, 32μM spermine, 50μM each of 19 unlabelled L-amino acids, 2mM dithiothreitol (DTT), 20μM GTP, 1mM ATP and 8mM creatine phosphate. The assay was incubated at 30^{o}C for 1 h. Samples removed at specific times during a time course of a polysome driven assay were electrophoresed on a polyacrylamide gel.

The optimal conditions for the translation of microsomes (50-100 μg/10μl assay) in the system were determined by measuring the level of incorporation of labelled amino acid into polypeptides at different concentrations of KCl, Mg acetate, spermine and creatine phosphokinase (CPK).

1-1-2 Reticulocyte Lysate System

The reticulocyte lysate system was prepared essentially by the methods of Pelham and Jackson (1976), from a rabbit reticulocyte lysate (supplemented with creatine kinase 50μg/ml and haemin, 25μM), which was treated with $CaCl_2$ and Ca^{2+} dependent micrococcal nuclease to eliminate the endogenous protein synthesis; the nuclease was then inactivated by chelating the Ca^{2+} with an excess of ethylene glycol-bis(2-aminoethyether)-N,N'-tetraacetic acid (EGTA). The translation system was stored as beads (35μl) under liquid N_2.

The in vitro translation system was used with polysome and poly$(A)^+$-RNA templates as described by Croy et al. (1980) ie in a 22μl assay volume there was 16μl of reticulocyte lysate translation system, 2 μl of template (80-100μg polysomes, 1-2μg poly$(A)^+$-RNA), 1μl of radioactive amino acid, 9mM creatine phosphate, 100mM KCl, 45μM of each of 19 unlabelled amino acids and 0.5mM $MgCl_2$ in the polysome system or 0.25 mM $MgCl_2$ in the poly $(A)^+$-RNA system. The assays were routinely incubated at 30^{o}C for 1h. In some experiments the time course of the polysome driven system was established and products were

electrophoresed on polyacrylamide gels. The optimal conditions for translation with microsomal templates were those established by Croy et al. (1980) for polysomes.

1-1-3 Pea Axes System

Axes were aseptically removed from partly dried seeds (about 25 daf) and an extract made following the method of Peumans et al. (1980).

1.5 to 2ml of axes were ground with 10ml of filter sterilised buffer (20mM HEPES/KOH pH 7.8, 120mM KCl, 2mM Mg acetate, 6mM 2ME). A further 9ml of buffer was added and the homogenate was centrifuged at 10,000$g_{av.}$ for 4 min. The supernatant was carefully removed and 1ml retained as a sample of total extract. A column was prepared in a 60ml syringe from 15g of fine Sephadex G25 which had been swollen by soaking in 200ml of extraction buffer overnight at 4^{o}C. The column was washed with 2 volumes of extraction buffer, and then the supernatant was layered onto the column and allowed to run through, followed by extraction buffer. Fractions were collected from the buff-coloured portion which could easily be seen travelling down the column. Approximately 15 drops were collected per tube, and the 10 most concentrated fractions (judged by eye) were pooled, and then stored as beads under liquid nitrogen. The 'total extract' was also stored as beads under liquid nitrogen.

The *in vitro* protein synthesising system used was based on that of the wheat-germ system, (see above), *ie* 5μl extract, 2μl of template (50-100μg of polysomes or microsomes), 5-10μCi of radioactive aminoacid, 19 unlabelled aminoacids, DTT, ATP, GTP, creatine phosphate, as in the wheat-germ system, and KCl, Mg acetate, spermine and CPK in a total volume of 10μl; and incubated at 30^{o}C.

The optimal concentrations of KCl, Mg acetate, spermine and CPK were determined for the system, when driven by polysomes and microsomes, by measuring the percentage incorporation of labelled aminoacid into polypeptides when the system contained different concentrations of the component being measured. A time course of incorporation of the labelled amino acid into polypeptides was also determined by removing

samples from a large volume assay, at certain time intervals, part of the sample was used to determine the percentage incorporation and the remainder was electrophoresed on a polyacrylamide gel.

1-1-4 Pea Cotyledon System

A pea cotyledon extract was made using the method described by Beevers and Poulson (1972).

60g of 14 day old cotyledons were ground in a mortar with a total of 30ml of buffer (0.25M sucrose in 20mM Tris pH 7.6, 10mM $MgCl_2$, 15mM NaCl and 5mM 2ME.), and then homogenised in a Willem's polytron for 10s at speed 2. The homogenate was filtered through 1 layer of Miracloth and then centrifuged at 20,000g_{av} for 15 min. A small volume (about 0.5ml) of this extract was retained as 'total extract', the remainder was centrifuged at 140,000g_{av} for 90min. The supernatant, (ie the 'purified system') and the 'total extract' were stored as beads, of approximately 50μl, under liquid nitrogen.

The optical density (O.D.), at 260nm, of the purified system and the 'total extract' was measured and the approximate amount of polysomes present calculated (using the formula 10μg/ml RNA has an absorbance of 0.1 at 260nm).

The in vitro protein synthesising assay was based on that of the wheat-germ system (see above). A 10μl assay volume was used which contained 5μl extract, 5-10μCi of labelled amino acid, 19 unlabelled amino acids DTT, ATP, GTP, creatine phosphate as in the wheat-germ system, and KCl, Mg-acetate, spermine and CPK; incubation was at 30^{o}C for 60 min. The optimal concentrations of KCl, Mg acetate, spermine and CPK were determined for the system when polysomes were the 'template' by measuring the percentage incorporation of labelled amino acid into polypeptides when the system contained different concentrations of the relevant component. The effect of varying volumes of extract in the system was also determined. A time course of the percentage incorporation of labelled amino acid into polypeptide was measured by removing samples from an assay at specific time intervals.

1-1-5 Extended Incubations of the Cell-free Systems

When cell-free systems were incubated for more than 3h chloramphenicol (CAP), at 110μg/ml and at 30μg/ml, was added to prevent protein synthesis by prokaryotic contaminants (Pestka, 1971b). Subsequently, CAP was used at 30μg/ml as this concentration was found to be effective.

1-2 Preparation of Templates

Pea seeds were germinated and grown as described by Evans et al. (1979); unless otherwise stated the variety of seed used was Feltham First. The testa and embryos were aseptically removed from the harvested seeds, and the cotyledons were stored either under liquid nitrogen or at -70°C.

Procedures were normally carried out at 2-4°C, glassware was heat sterilised and all other equipment, and buffers, were autoclaved.

The following buffers were used in the extraction procedures.

A:- 0.2M Tris-HCl, 0.2M sucrose, 60mM KCl, 30mM $MgCl_2$, 1mM Dithiothreitol (DTT), pH 8.5 at 2°C.

B:- 40mM Tris-HCl, 20mM KCl, 10mM $MgCl_2$, pH 8.5 at 2°C.

C:- 20%(w/v) Triton X-100, 0.25M sucrose, 10mM DTT.

HEPES buffer:- 20mM HEPES, 100mM KCl, 3mM Mg acetate, pH 7.6.

1-2-1 'Total' Polysomes

'Total' polysomes (ie free polysomes and polysomes released from membranes), were extracted from pea cotyledons using the method of Evans et al. (1979), which was based on that of Larkins and Davies (1975). The procedure involved the centrifugation of a post-mitochondrial supernatant through a dense sucrose cushion, in order to preferentially pellet polysomes.

Frozen cotyledons were homogenised (1:3 w/v) in buffer A with a Willem's polytron for 3s at speed 8. Then 0.1 volume buffer C was added, and the homogenate was filtered through 1 layer of Miracloth. Routinely 44g of cotyledons were homogenised in batches of 2g each, and the homogenate pooled; this gave 8 pellets of polysomes.

The homogenate was centrifuged at $500g_{av.}$ for 10 min, and then the supernatant was recentrifuged at $29,000g_{av.}$ for 10 min. This second, post-mitochondrial supernatant was carefully layered over a 10ml pad of sucrose (700mg/ml buffer B), and centrifuged at 95,000g av for 2h. The polysomal pellet produced was washed 2 times with sterile distilled waterand either frozen and stored at $-70^{o}C$, or resuspended in about 100µl of HEPES buffer and the concentration of polysomes estimated by measuring the $O.D._{260}$ of 2µl diluted to 1ml with sterile distilled water (using the factor 1mg/ml polysomes has an absorbance of 10 units at 260nm). The remainder of the polysome suspension was stored in 10µl aliquots under liquid nitrogen. 'Total' polysomes of Vicia faba were also extracted using this procedure.

1-2-2 'Free' Polysomes

Free polysomes (ie not membrane bound polysomes) were extracted using the same procedures as those for the extraction of 'total' polysomes except that no Triton X-100(ie buffer C) was added after the homogenisation of the cotyledons. The cotyledons were homogenised using an osterizer (John Oster MFG. Co., Cyclotrol eight) at 'medium' setting for 2 min, this procedure was used in all extractions where membranes were to be kept intact; the polytron possibly emits ultrasonic waves which would disrupt membranes.

1-2-3 Microsomes

Microsomes were extracted from 14 day old cotyledons using the method of Cameron-Mills et al. (1978), with slight modifications. This method involved flotation of a suspension of the $29,000g_{av.}$ pellet in a discontinuous sucrose gradient.

A 29,000$g_{av.}$ pellet was produced from a pea cotyledon homogenate using the procedures described under the extraction of polysomes. The pellet was resuspended in 9 ml of 2.26M sucrose in buffer B; this suspension was overlaid with 6ml portions of 1.75M, 1.50M and 0.20M sucrose, each in buffer B, and centrifuged in a swing out rotor at 96,000g av. for 16h at 2°C. The two bands which formed, one at the 1.75-2.26M sucrose interface (band I) and one at the 1.5-1.75M sucrose interface (band II) were removed using a syringe. The 2 samples were further purified by diluting with an equal volume of buffer B (to lower the density of the suspension) and centrifuging through a sucrose cushion, of 1.3M sucrose in buffer B, at 165,000 gav. for 1h.

The pellets were washed 2 times with sterile distilled water and either stored at -70°C, or resuspended in 50µl HEPES pH 7.6. The $O.D._{260}$ of 1-2µl of microsomes, diluted to 1ml with distilled water, was measured and the concentration of polysomes present with the membranes was estimated, the remainder of the sample was stored in 10µl aliquots under liquid nitrogen. An electron micrograph was taken of a sample of microsomes. Routinely 12g of cotyledons were used; this gave one pellet each of band I and band II microsomes.

1-2-4 'Released' Polysomes

Polysomes were released from microsomes using an adaptation of a method from Larkins and Davies (1975).

A microsomal pellet was resuspended in 2ml of buffer A and 0.1ml of Triton X-100 in buffer A was added to bring the concentration of Triton X-100 to 2% (v/v). After mixing thoroughly, this suspension was layered over a 4ml sucrose cushion (700mg/ml buffer B), then centrifuged at 95,000gav for 2h. The pellet was washed 2 times with sterile distilled water and dissolved in 50µl HEPES, pH 7.6. The $O.D._{260}$ of 2-5µl samples, made up to 1ml with distilled water, was measured and the amount of ribosomes present calculated. The remainder of the samples was stored in 10µl aliquots under liquid nitrogen.

1-2-5 'Stripped' Microsomes

Ribosomes were stripped from microsome membranes following a method of Cameron-Mills and Ingversen, (1978).

A microsomal pellet was resuspended in 2ml of buffer (40mM Tris-HCl, 20mM KCl, pH 8.5) made 30mM with respect to EDTA and incubated at 0°C for 30 min. 0.5ml samples were layered onto a 2 step gradient consisting of 1.5ml of 0.3M sucrose over 0.73M sucrose (in the suspension buffer), and centrifuged at 100,000 gav for 1h. Each pellet was resuspended in 30µl of HEPES pH 7.6, the $O.D._{260}$ measured of 2-5µl samples, as previously, and the remainder of the sample stored in 10µl aliquots under liquid nitrogen.

1-2-6 Poly(A)$^+$ - RNA

Poly(A)$^+$-RNA was isolated from total polysomes using the method described by Evans et al.,(1980), which was based on that of Krystosek et al.,(1975). This method involved removing the protein from the polysome sample and purifying the poly(A)$^+$-RNA by attaching it to an oligo-dT-cellulose column, washing off the impurities, and then eluting the poly (A)$^+$-RNA, with buffer of low ionic strength.

An oligo dT-cellulose column, water jacketed (25°C), was packed, using about 1g of cellulose, giving a column about 5cm high. The buffers used with the column were degassed to prevent bubbles appearing in the column. The column was washed with sterile distilled water, then 10mM Tris, 1mM EDTA, 0.1% sodium dodecylsulphate (SDS) pH 7.4 for 15 min, then with 0.4M NaCl, 10mM Tris, 0.1% SDS, pH 7.4 before use. The column was attached to a UV absorbance monitor (ISCO UA-5) and fractions were monitored at 254 nm as they were eluted from the column. Samples and buffers were loaded onto the column using a pump.

Polysomal pellets (see 1(a)) were resuspended in 0.5% (w/v) SDS, 50mM Tris, 30mM KCl, pH 7.4 containing 2mg of Proteinase K, in a 5ml volume, and incubated at room temperature for 2h. The sample was then

made 0.4M with respect to NaCl, 0.1% with respect to SDS and 10mM with respect to Tris, and loaded onto the prepared oligo-dT-cellulose column. The column was washed with 100ml of 0.4M NaCl, 10mM Tris, 0.1% SDS, pH 7.4, to remove (poly(A)$^+$-RNA,) ribosomal RNA etc., and then the poly(A)$^+$-RNA was eluted with salt-free buffer (10mM Tris, 1mM EDTA 0.1% SDS, pH 7.4). This fraction (about 10ml) was heated at 65^{o}C for 10 min and then plunged into ice. It was made 0.4M with respect to NaCl, by adding the appropriate volume of 0.5M NaCl, 10mM Tris, 0.1% SDS pH 7.4 buffer, and reloaded onto the regenerated oligo-dT-cellulose column, washed and eluted as previously. The sample was made 0.2M with respect to sodium acetate at pH 5.6, and precipitated with 3 volumes of ethanol at -20^{o}C overnight. The precipitate was centrifuged at 25,000g$_{av}$ at 0^{o}C for 30 min, washed 3 times with cold 80% ethanol and dried on a vacuum line. The poly (A)$^+$-RNA was then dissolved in 20-50 μl of sterile H_2O and the concentration estimated by measuring the O.D.$_{260}$ of 1-5μl made up to 1 ml with distilled water using the equation 1mg/ml RNA has an absorbance of 25 units at 260nm. The remainder of the sample was stored under liquid nitrogen in 10μl aliquots.

1-2-7 Sucrose Gradient Analysis of the 'Templates'

Profiles of the different 'templates' were obtained by fractionating the samples on sucrose gradients using the method of Evans et al., (1979). A sample of the template, containing approximately 400μg RNA, was made up to 1ml with buffer B + 1% Triton X-100, layered onto a 21 ml linear 15-60% (w/v) sucrose gradient (the sucrose was in buffer B) and centrifuged at 95,000gav for 2h at 2^{o}C in a 3x23ml swing-out rotor. The gradients were expelled from the centrifuge tubes with 70% (w/v) sucrose in buffer B and were scanned at 254 nm using an ISCO Model 640 fractionator equipped with a UA-5 absorbance monitor.

1-3 Protein Body and Albumin Extraction

1-3-1 Protein Bodies

A crude sample of protein bodies was extracted from 19 day old cotyledons using the method of Croy (1977). The procedures were carried out at 2-4°C; 5g of cotyledons were homogenised in 50ml buffer (0.5M sucrose in 0.05M $NaPO_4$ pH 7.6) for 1 to 2 min using an osterizer set at low speed. The homogenate was centrifuged at 300g for 5 min, the starch pellet discarded, and the supernatant centrifuged at 10,000g for 10 min. Each pellet was resuspended in 1ml of buffer and layered onto a step sucrose gradient, consisting of 5ml 90% (w/v) sucrose, 8ml 70% (w/v) sucrose and 8 ml 30% (w/v) sucrose, each in 0.05M $NaPO_4$, pH 7.6, and centrifuged at 60,000g_{av} for 2h in an 8 x 23ml swing-out rotor. The protein body layer, which was at the 70-90% sucrose interface was removed with a syringe. The protein concentration of the sample was estimated by measuring the O.D. at 280nm, (1mg/ml storage protein has an $O.D._{280}$ of approximately 0.8).

Reduced and unreduced samples of protein bodies containing 15-30µg of protein were electrophoresed on a polyacrylamide gel and stained with Coomassie Brilliant Blue R250 (Coomassie Blue).

Samples of varying amounts of protein bodies were added to cell-free translation assays; translation products were also incubated with varying amounts of protein bodies for different lengths of time (1h-18h) at 30°C. Some incubations were altered to pH 5 by the addition of 4M Na Acetate pH 5, as the pH optimum for enzymes associated with protein bodies is pH 4-5 and the assay systems and extraction buffers are at pH 7.6

1-3-2 Albumin Extraction

Albumins were extracted from 14 day old cotyledons using one of two methods. The first was a direct method. 1g of cotyledons were finely chopped with a razor blade, into 2 ml of buffer, (33mM Na acetate, pH 4.8 + 1% Triton X-100) and incubated at 4°C for 75 min, stirring occasionally. The mixture was spun in an haematocrit (Hawksley micro-

haematocrit centrifuge) for 10 min, (12,000g) the supernatant, which contained albumins was decanted and adjusted to pH 7.6 with 1M K_2HPO_4. The second method used was indirect. 5g of cotyledons were chopped into 5 ml of buffer (50mM Tris pH 7.5, 0.2M NaCl + 1% Triton X-100) and ground to a paste using a pessle and mortar. A further 15 ml of buffer was added and the sample was centrifuged at 30,000g for 20 min. The supernatant was dialysed overnight, at 4°C, against 33mM Na acetate pH 4.8 to precipitate the globulins, and then centrifuged as above. The globulin was freeze dried, while the supernatant was dialysed overnight against H_2O, to precipitate the albumins, and then centrifuged, as above, and the precipitate freeze dried. Another sample was extracted using the same procedure except that no Triton X-100 was added to the extraction buffer.

Aliquots of the samples were electrophoresed on a polyacrylamide gel which was then stained with Coomassie Blue.

Different amounts of albumin, extracted using the indirect method minus Triton X-100 were added to the reticulocyte assay, and the translation products examined by electrophoresis and fluorography. The albumin extract was also added to the reticulocyte translation products at a final concentration of 1mg/ml, and incubated at 30°C for different lengths of time.

1-4 Labelling Standard Proteins

Legumin and vicilin were labelled with tritium by acylation with [^{3}H] acetic anhydride using the method of Fraenkel-Conrat H., (1957).

The protein preparations (gift of Dr. J. Gatehouse) were dissolved in sterile distilled water (10mg in 100µl), 100µl of saturated Na acetate was added, to retard the hydrolysis of the acetic anhydride, and the mixture cooled on an ice bath. 5µl of [^{3}H] acetic anhydride, containing 25mCi, was diluted to a 20µl volume with cold acetic anhydride (1mg/µl), and 10µl was added to the protein solution, in 2µl aliquots. The mixture was incubated for 1 h at

0°C, and then dialysed against sterile distilled water for about 2 days at 4°C. The protein was freeze dried, and then redissolved in 200µl of 1% (w/v) SDS. Samples of these standard proteins were pipetted onto GFC filter papers , counted, and the remainder stored in 15µl aliquots under liquid nitrogen.

1-5 Immunoprecipitation of Storage Proteins from Cell-Free Translation Products

Antibodies to vicilin and legumin of pea and of Vicia faba were prepared by Dr. J. Gatehouse using methods described in Evans et al., (1979)

Immunoprecipitation of vicilin and legumin subunits from translation products was carried out using a double antibody method, Croy et al. (1980a); 1µl (1-2µg) of affinity purified Immunoglobulin G (IgG) was added to 20µl of translation product and incubated at 30°C for 1 h. Some protein A sepharose was preswollen with HEPES pH 7.6, washed in the same buffer and the volume adjusted so that 10µl of slurry was equivalent to about 0.13 mg of IgG. The translation products plus IgG were mixed with 10µl of slurry, and incubated at 30°C for 1-2h, then 50µl (approximately 0.2 mg) of preimmune IgG (ie nonspecific) was added and the mixture incubated for 1h at 30°C. The sepharose was pelleted by centrifugation in a haematocrit ($12{,}000g_{av}$) for 5 min, and was washed 4-5 times with buffer (0.2M Tris HCl, 0.5M NaCl, pH 8, 0.05% azide), before being taken up in 30-40µl of SDS sample buffer, ready for electrophoresis.

1-6 The Measurement of Protein Synthesis

1-6-1 The Incorporation of Radioactive Label into Polypeptides

The incorporation of radioactive amino acids into polypeptides was measured by counting tricarboxylic acid (TCA) precipitated samples in a liquid scintillation counter (Mans and Novelli, 1961). Samples of translation products (1-5μl) were pipetted onto glass fibre filter paper discs, dried and washed in 10% cold TCA for 30 min (200ml TCA/40discs), then for 15 min in 5% TCA, 10 min in 5% boiling TCA, then 2x5 min in 5% TCA at room temperature. The discs were then rinsed in ethanol and dried. Total counts present in the original sample were measured by counting unwashed discs. The discs were submerged in scintillation fluid (Toluene: Triton X-100, 2:1, 5g 2,5-diphenyl oxazole (PPO)/l toluene), and counted for at least 5 min using a Packard scintillation counter. A more sensitive scintillation fluid PPO 3g/l, 1,4-Di [2- (5-phenyloxazolyl)]-benzene (POPOP) 300mg/l in toluene was used for samples containing low counts (ie less than 1000 cpm/μl).

1-6-2 SDS - Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide (17%(w/v)) slab-gels (SDS-PAG) were made using a modified Laemmli method (1970).

The acrylamide solutions and Tris-HCl buffers were degassed before use; the ammonium persulphate was dissolved immediately before use.

Main gel:-

22.5 ml 1M Tris HCl pH 8.8
34.5 ml main gel acrylamide (12g acrylamide+54mg NN'methylene-bis-acrylamide in 40ml H_2O)
1.5 ml ammonium persulphate (20mg/ml)
0.6 ml 10%(w/v) SDS
20 μl N,N,N'N' -Tetramethylethylenediamine(TEMED)

The main gel was poured between the glass plates to about 1cm from the top, and overlaid with distilled water while setting.

Stacking gel:- 2.5 ml 1M Tris HCl pH 6.8
14.8 ml H_2O
2.0 ml stacking gel acrylamide (0.6g acrylamide + 8.6mg bis acrylamide in 2 ml H_2O)
0.5 ml ammonium persulphate (20mg/ml)
0.2 ml 10%(w/v) SDS
10 µl TEMED

The top of the main gel was rinsed with stacking gel and then the stacking gel poured into the remaining space and the well-former positioned.

The samples were mixed with either reducing or non-reducing sample buffer (ie buffer with or without 2ME).

Sample buffer:- 3 x stock:- 12.05 ml H_2O
6.25 ml 1M Tris HCl pH 6.8
10.00 ml glycerol
5.00 ml 2ME
2.00 g SDS

The samples were held in a boiling water bath for 3 min, and then loaded onto the gel using a syringe. Routinely 250,000 cpm of translation products were loaded onto a track, while about 50,000-100,000 cpm of immunoprecipitates and 25,000 cpm standard proteins were loaded per track; these levels of radioactivity required 2-3 days exposure to presensitised film to show polypeptide band patterns. Unlabelled protein samples were loaded onto the gel using about 30µg protein per track.

The electrophoresis buffer used contained 14.1g glycine, 30g Tris, 10g SDS, pH 8.3 in a litre; a few drops of bromophenol blue were added to the upper gel tank, to act as a marker. Electrophoresis was carried out at 15mA until the samples had entered the stacking gel and then at 25mA until the bromophenol blue was at the base of the main gel (about 6h).

1-6-3 Staining the Protein Subunits

Gels were stained with Coomassie Blue by immersion in 0.025% Coomassie Blue, 7% glacial acetic acid, 30% methanol overnight, destained in 7% glacial acetic acid, 30% methanol, and then dried under vacuum between 2 layers of cellophane using a gel dryer (GSD-4, Pharmacia).

1-6-4 Fluorography

Gels on which radioactive samples had been separated were washed in 10% TCA, 30% methanol, to fix the polypeptide bands, and then processed for fluorography using the method of Bonner and Laskey, (1974). The gels were washed for 30 min in dimethyl sulphoxide (DMSO), and then for a further 30 min in fresh DMSO, to expel any water present in the gels, then they were washed for 3h in DMSO + PPO (300g/l). The PPO was precipitated in the gels by washing in 30% methanol for 1h, the presence of methanol prevented the gels swelling but did not appear to affect the amount of PPO precipitated in the gels. The gels were dried under vacuum between 2 layers of cellophane using a heated gel dryer, (Bio-Rad 224 Gel Slab Dryer).

The dried gels were attached to glass plates and exposed to presensitised film as in the method of Laskey and Mills,(1975). The film, which was laid on a white surface, was exposed to a flash from an electronic flash gun (covered with an orange filter and Whatmann filter paper), which was held 95cm above the film. The film and gel were sandwiched together between glass plates, with the 'flashed' side of the film facing the gel, and stored at -70^{o}C for the required time.

The film was developed in Ilford phenisol for 5 min, fixed in Kodafix for 5 min, and washed for 1h in running water.

1-7 Cyanogen Bromide Cleavage of Polypeptides

Storage proteins extracted from cotyledons (ie 'native' proteins) and *in vitro* synthesised, immunoprecipitated, polypeptides were cleaved using cyanogen bromide (CNBr), following the method of Gross, (1967) as described by Croy et al., (1980 (a)).

The sample, (either protein (1.0mg), extracted from mature seeds, or tritiated immunoprecipitate) was dissolved in a minimum volume (300 µl) of 70% formic acid and 8mg CNBr crystals were added. The mixture was incubated in the dark at room temperature for 24h and then dried on a vacuum line. The precipitate was dissolved in 250µl of sample buffer (see 6b) and electrophoresed on an SDS-PAG and either processed for fluorography, or the protein bands stained.

Translation products labelled with [^{35}S] methionine were also treated with CNBr, as above.

1-8 Tryptic Digestion of Polypeptides

The partial digestion of 'native' storage proteins, and of *in vitro* translation products was carried out as described by Croy et al., (1980).

100µl of trypsin coupled to sepharose 4B (gift of Dr. J. Gatehouse, 4mg trypsin ml^{-1} gel) was added to 2mg of storage protein in a total volume of 1ml. This mixture was incubated at room temperature for 1h at pH 8.0, and then centrifuged at 10,000g for 5 min to pellet the sepharose. The supernatant was freeze dried and redissolved in sample buffer for SDS-PAGE, Carrier legumin or vicilin (20µl of 2mg/ml) was added to 30µl of translation product, then 12µl of the sepharose bound trypsin was added and the mixture incubated at room temperature, or between 0°C and 4°C for 1h. Immunoprecipitates from the supernatant were prepared for SDS-PAGE and fluorography.

1-9 The Inhibition of Initiation in the Cell-free Systems Using Aurintricarboxylic Acid

The effect of the concentration of aurintricarboxylic acid, (ATA) on cell-free protein synthesis, was measured by adding ATA in a 1µl volume to the incubation 'mix', before the cell-free extract or the 'template' was added, to give final concentrations of ATA from 10^{-6}M to 10^{-1}M. The system was incubated and sampled, as described above, to determine the level of incorporation of labelled amino acid into polypeptides. The inhibition of protein synthesis due to the presence of ATA was calculated using:-

$$\% \text{ inhibition} = \frac{\text{incorporation in Xcpm/µl} - \text{incorporation in Ycpm/µl} \times 100}{\text{incorporation in Xcpm/µl}}$$

where X = relevant control (no ATA) and Y = sample containing ATA.

Samples of assays, which were inhibited by 50% or more, were subjected to SDS PAGE and fluorographs taken of the gels.

The effect was determined of preincubation of the cell-free system, either at 0°C or at 30°C, before the addition of ATA. The concentration of ATA which caused about 50% inhibition of protein synthesis was used in these assays. The ATA was added as a 1µl volume to the assay after different lengths of time of preincubation, from 0-15 min, the assays were then incubated at 30°C for a further 45 min, and were sampled as described previously.

1-10 Examination of the Poly(A)$^+$-RNA Population Composition

1-10-1 Synthesis of Single Stranded cDNA

Single stranded complementary DNA (cDNA) was synthesised from poly $(A)^+$-RNA using modifications of the method of Evans et al., (1980). Apparatus was siliconised (with Repelcote) and sterilised.

The final reaction volume of 50μl contained 50μM [^{3}H]dCTP (about 50μCi), 50mM Tris-HCl, pH 8.3 at 37°C, 20mM DTT, 6mM $MgCl_2$, 60mM NaCl, 0.5mM each of 3 unlabelled dNTP, 2.7μg actinomycin D, 40mM sodium pyrophosphate, 300μg oligo $(dT)_{10}$, 50 units of avian myeloblastosis virus (AMV) reverse transcriptase and 1-2.5μg poly(A)$^+$-RNA. This mixture was incubated at 37°C for 30 min, then placed in a boiling water bath for 3 min. The mixture was made 10mM with respect to EDTA and brought to 0.3N NaOH by the addition of 1N NaOH, then incubated for 2h at room temperature in order to digest the mRNA. The mixture was brought to pH7 by the addition of 10% acetic acid and protein removed by extraction with phenol-chloroform (1:1 v/v). The cDNA sample was then purified by separation on a Sephadex G-50 column (packed in a 2ml syringe) using 3M NaCl, 0.5M Tris pH 7.5 as the column buffer. The [^{3}H]cDNA peak and the [^{3}H]dCTP peak were determined by measuring the counts per minute in each fraction from the column; the two peaks were clearly separated. The [^{3}H]cDNA fractions were pooled and ethanol precipitated with 0.1 volume 2M NH_4 acetate and 3 volumes of ethanol at -20°C, overnight. The precipitate was centrifuged at 12,000g$_{av}$ for 20 min, washed twice with 80% ethanol and dried under vacuum. The [^{3}H]cDNA sample was then dissolved in 20μl of sterile distilled water.

1-10-2 Hybridisation Methods

The hybridisation of [^{3}H]cDNA to poly(A)$^+$-RNA was carried out using the method of Goldberg, (1978), to determine the proportion of [^{3}H]cDNA which was hybridised to poly(A)$^+$-RNA at specific Rot values. The Rot value depends on the concentration of poly(A)$^+$-RNA in moles l^{-1}, which must be in excess of the [^{3}H]cDNA, the length of time of the incubation (in seconds) and the salt concentration factor Britten et al., (1974), the higher the salt concentration the faster the reaction rate. The excess of poly(A)$^+$-RNA over [^{3}H]cDNA is necessary to make the reaction a pseudo-first order reaction, ie the change in poly(A)$^+$-RNA concentration due to hybridisation with [^{3}H]cDNA must be negligible so that the reaction can be assumed to be unaffected by the change. The rate of hybridisation can then be assumed to be inversely proportional to the base sequence complexity of the poly(A)$^+$-RNA itself (Monahan et al., 1976).

[^{3}H]cDNA was diluted to the required concentration with hybridisation buffer, 30mM Pipes, pH 6.7, 0.1mM EDTA, 0.1% SDS and NaCl; 3 different concentrations of NaCl were routinely used, 0.045M, 0.18M, 0.75M, these altered the hybridisation rates by a factor of 0.0133, 1.0, and 5.82 respectively, Britten et al.(1974). The [^{3}H]cDNA was mixed with the required concentration of poly(A)$^+$-RNA, which was also diluted in hybridisation buffer, so that an excess of at least a 1000 to 1 poly(A)$^+$-RNA: [^{3}H]cDNA, measured in moles, was present. However, under some circumstances (very low Rot values) the excess was considerably lower (100:1), and in these cases controls were used to check that the reaction rate was not affected. The samples were overlaid with paraffin, which had been equilibrated in the presence of excess hybridisation buffer of the appropriate salt concentration at the correct reaction temperature, to prevent the evaporation of the samples. The samples were placed in a water bath at 98-100^{o}C for 5 min, to dissociate any double strands among the polynucleotides, and then were incubated at the required temperature. The higher salt concentration buffer (0.75M) required an incubation temperature of 68^{o}C, while the lower concentrations required a 60^{o}C incubation, Britten et al.,(1974).

Aliquots of 2-5μl of the samples were removed at time intervals and were pipetted into 110μl of cold S_1, nuclease buffer, (0.25M NaCl, 1mM Zn acetate, 30mM Na acetate, pH 4.5). The single nucleotide strands (ie not hybridised) were digested with S_1 nuclease using the method of Maxwell et al., (1978). Denatured calf thymus DNA (20 μg/ml of sample) was added to the sample as a carrier, and the sample was halved. To one half of the sample 3μl of S_1 nuclease (750 units) was added, while 3μl of S_1 nuclease buffer was added to the other half, both samples were incubated at 37^{o}C for 80 min. Each sample was spotted onto DEAE cellulose paper, dried and then washed 4 times in 16 min in 0.5M $NaPO_4$ buffer, 2 times for 1 min each in distilled water and finally in 95% ethanol, and dried. The amount of radioactivity on the papers was counted and the proportion of [^{3}H]cDNA which was hybridised to poly(A)$^+$-RNA was calculated by comparing the counts remaining in the digested sample (double strands) and the counts in the undigested sample (single and double strands). The amount of self hybridisaton of the [^{3}H]cDNA was measured by carrying out the hybridisation procedures in the absence of poly(A)$^+$-RNA, and the final results were corrected by this factor.

When small samples were to be hybridised, 2μl of the [^{3}H]cDNA and poly(A)$^+$-RNA mix were sealed into 5μl siliconised, sterile microcaps, held at 98-100^{o}C for 5 min and then incubated at the correct temperature. At the required time the microcap was quickly wiped and dipped into liquid air, and then the contents were emptied into S_1 nuclease buffer and the analysis continued as above.

Hybridisation curves (proportion of [^{3}H]cDNA hybridised plotted against Rot values) were accumulated for homologous hybridisations (ie poly(A)$^+$-RNA: cDNA made from that poly(A)$^+$-RNA from 9 day, 14 day and 19 day old cotyledons), and for heterologous hybridisations between poly(A)$^+$-RNA from 9 day and 14 day old cotyledons.

The best fit curve was determined by a least squares fit programme (supplied by Dr. I.M. Evans, based on the programme of Pearson et al., 1976) and subsequent calculations (see Goldberg et al., 1981) were based on that data.

[^{32}P] end labelled single-copy DNA from pea leaf was prepared by Dr. I.M. Evans. This was hybridised to an excess of poly(A)$^+$-RNA, extracted from 14 day old cotyledons, at different Rot values, (as above), and a hybridisation curve was drawn.

Poly(A)$^+$-RNA isolated from nuclei of 9 day old cotyledons by Dr. I.M. Evans was also used as a template for the manufacture of [^{3}H]cDNA. The [^{3}H]cDNA was hybridised to an excess of poly(A)$^+$-RNA from 9 day old cotyledons at different Rot values, in order to obtain a hybridisation curve.

2. RESULTS

2-1 The Characterisation of In vitro Cell-free Systems and the Analysis of Their Translation Products

'Total' polysomes were translated in the wheat-germ, reticulocyte lysate, pea axes and pea cotyledon systems, and the efficiency of polypeptide synthesis, measured as the percentage incorporation of the radioactive amino acid into TCA precipitable polypeptides, was determined. The translation products were examined by electrophoresis on polyacrylamide gels and by immunoprecipitation. Identification of a subunit depended on these criteria but the mol.wt. values are 'apparent' mol.wts., using the gel procedure.

2-1-1(a) The Wheat-germ and Reticulocyte Lysate Systems

The translation of 'total' polysomes in both these systems gave an incorporation of 12-40% of radioactive amino acid into polypeptides, the variation being due to different batches of template. The optimum length of time for the incubation of the wheat-germ system was 45 min, but for the reticulocyte lysate system was 60 min. The length of the incubation (between 15 min and 3h) did not appear to qualitatively affect the translation products.

The major translation products of both systems were subunits of storage proteins, legumin (60,000 mol.wt.) and vicilin (50,000 and 47,000 mol.wt.), as shown by electrophoretic mobility and immunoprecipitation, (Fig. 1 and 2). The legumin 60,000 mol.wt. polypeptide did not reduce to give 40,000 and 20,000 mol.wt. components in the presence of 2ME, although the native subunit did. The reticulocyte products appeared to contain fewer lower mol.wt. bands than did the wheat-germ products.

Another difference between the translation products of the two systems was in the vicilin subunits, those synthesised in the wheat-germ system were single polypeptide bands, while those synthesised in the reticulocyte lysate system consisted of doublets, (ie two polypeptides with a difference in mol.wt. of approximately 1,000), when labelled with [^{3}H]leucine, but appeared as single bands when the labelled amino acid was [^{35}S] methionine. The single polypeptide produced in the wheat-germ system coincided with the lower band in each of the two doublets produced in the reticulocyte lysate system.

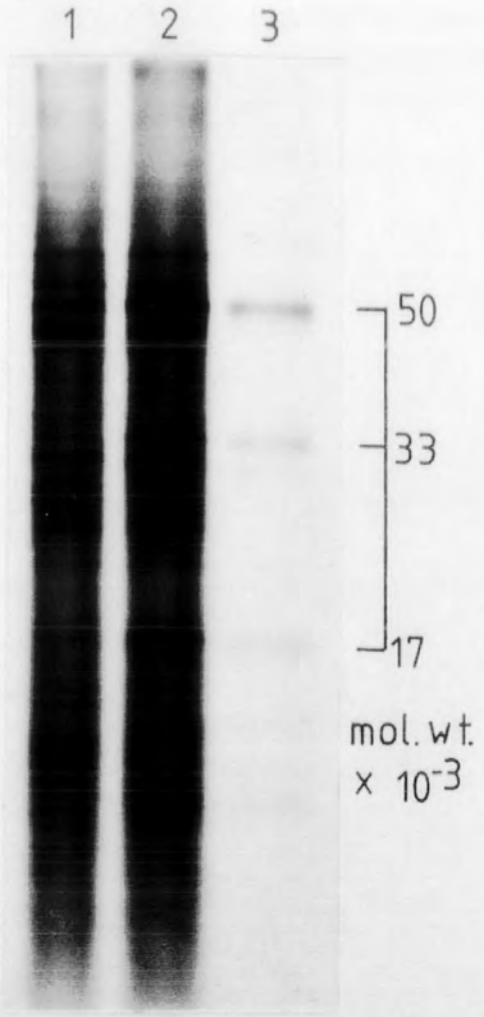


Fig. 1. The Translation Products of a Polysome Driven Wheat-germ Cell-free System

Track 1, wheat-germ translation products: track 2, wheat-germ translation products + vicilin standard: track 3, vicilin standard. Polypeptides which are coincident with the 50,000 mol.wt. vicilin standard are synthesised. A faint band is also coincident with the 33,000 and the 17,000 mol.wt. standard polypeptides.

The translation products were labelled with [^{35}S] methionine.

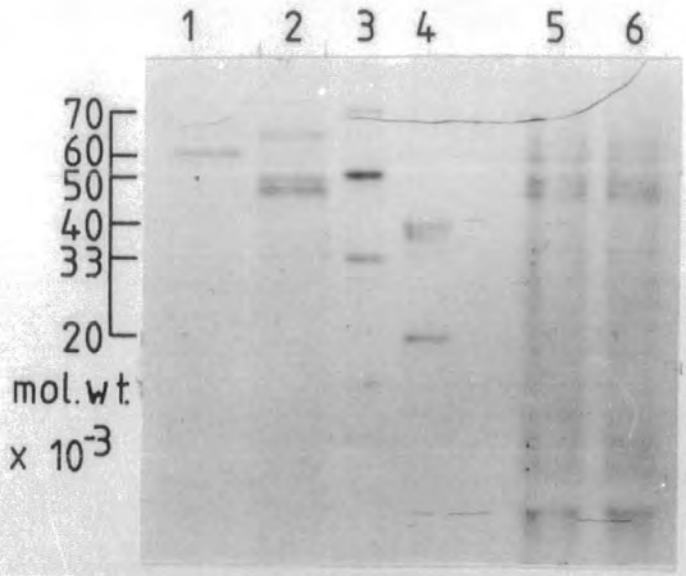


Fig. 2. The Translation Products of a Polysome Driven Reticulocyte Lysate System

Tracks 1 and 2 legumin and vicilin immunoprecipitated from translation products: track 3, standard vicilin: track 4, standard legumin: tracks 5 and 6, total translation products. The vicilin 50,000 and 47,000 mol.wt. subunits of translation products are doublets. All samples were in reducing buffer (see Methods), the 60,000 mol.wt. legumin subunit synthesised *in vitro* was unaffected, however, the standard legumin dissociated forming a 40,000 and a 20,000 mol.wt. polypeptide. The translation products were labelled with [^{3}H] leucine

2-1-1(b) The Pea Axes Cell-free System

When 'total' polysomes and [^{3}H] leucine were added to the axes system, otherwise unsupplemented, about 50% of the added counts were incorporated into polypeptides. The results presented in Fig. 3 show the optimal conditions for the polysome driven system; the optimisation of the system increased the incorporation of labelled amino acids to between 50 and 90%. The total extract from the axes was also capable of protein synthesis when polysomes were the template; these polypeptides, and the translation products of the purified system were similar to those obtained using 'total' polysomes in a wheat-germ system, (Fig 4A).

The 50,000 and 47,000 mol.wt. subunits were immunoprecipitated from both polysome and microsome translation products, but the 60,000 mol.wt. legumin subunit was only immunoprecipitated from the polysome translation products (Fig.4B), and it too, was in a form which was not reduced in the presence of 2ME.

2-1-1(c) The Pea Cotyledon System

The measurement of the O.D.$_{260}$ of the total extract and the purified system showed that they contained approximately 2.5µg/µl and 0.9µg/µl polysomal RNA respectively. However, when the total extract and the purified system were incubated without an exogenous template, the percentage incorporation of radioactive amino acid into polypeptides was only 0.1%. The addition of 14 day polysomes and [^{3}H] leucine to the purified system resulted in about 7% of the counts present being incorporated into polypeptides; the efficiency of the system was increased by optimisation (Fig. 5), which resulted in between 8 and 16% incorporation of the labelled amino acid. Various volumes of cell-free extract were incubated with other components of the system, but this had little effect on the levels of incorporation.

Microsomes had a very low protein synthesising capacity in the system, compared to polysomes; less than 3% incorporation of counts into polypeptides was measured.

Fig. 3. Optimisation of the Pea Axes System.

The affect of the change in concentration of A, KCl; B, Mg acetate with 75mM KCl; C, spermine with 1.5mM Mg acetate and 75mM KCl; D, CPK with 60μM spermine, 1.5mM Mg acetate and 75mM KCl; on the incorporation of [^{3}H] leucine into TCA precipitable polypeptides in the polysome driven cell-free system. E shows a time course for the incorporation of [^{3}H] leucine into polypeptides under optimised conditions. ie 75mM KCl, 1.5mM Mg acetate, 60μM spermine and 0.08μg/10μl CPK, with 100μg of polysomes incubated at 30°C.

Fig.3

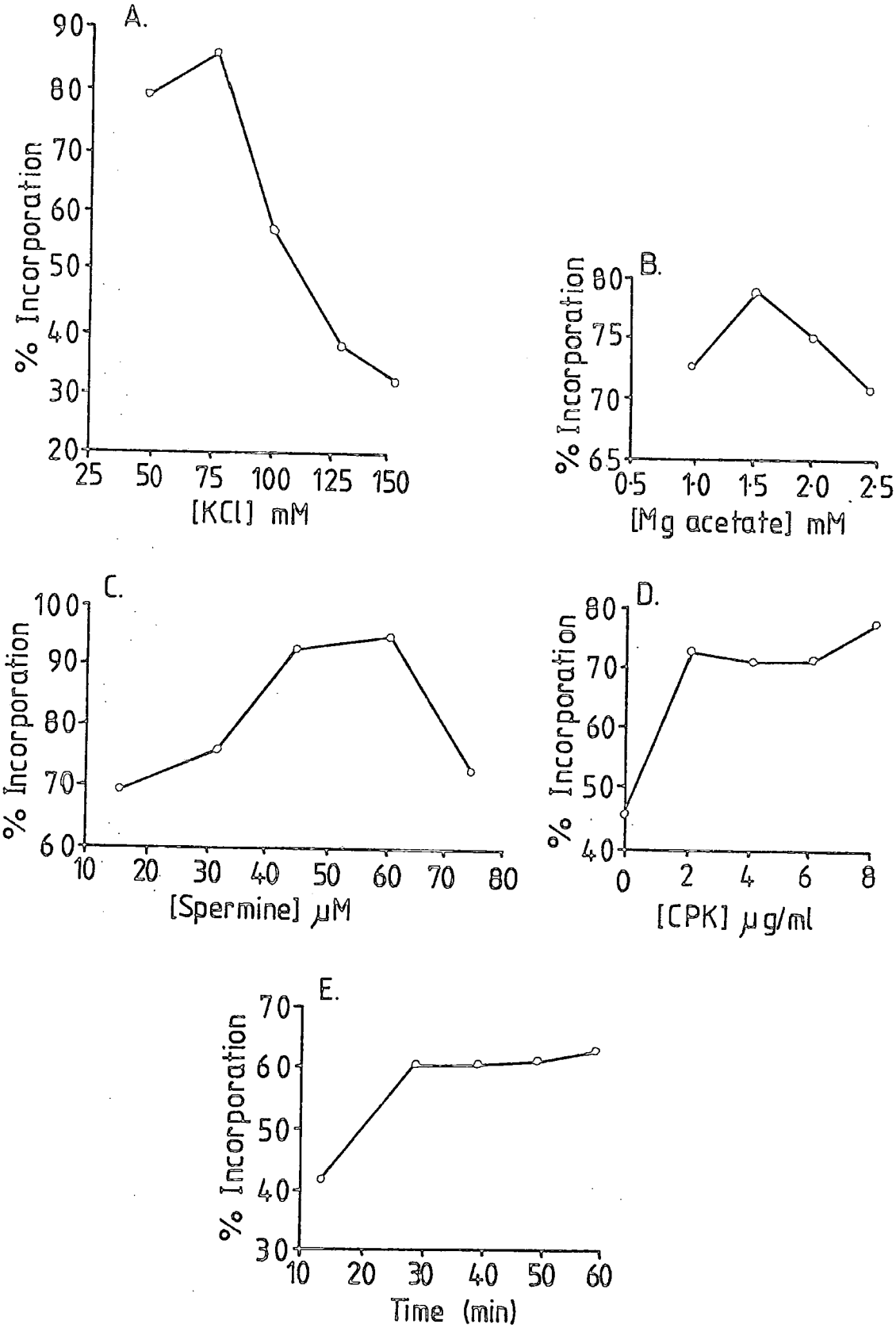

A.
% Incorporation
90
80
70
60
50
40
30
20
25
50
75
100
125
150
[KCl] mM
B.
% Incorporation
80
75
70
65
0·5
1·0
1·5
2·0
2·5
[Mg acetate] mM
C.
% Incorporation
100
90
80
70
60
10
20
30
40
50
60
70
80
[Spermine] µM
D.
% Incorporation
80
70
60
50
40
0
2
4
6
8
[CPK] µg/ml
E.
% Incorporation
70
60
50
40
30
10
20
30
40
50
60
Time (min)

Fig. 4A. A Comparison of Products from a Polysome Driven Axes System, and Products from a Polysome Driven Wheat-germ System

Track 1, vicilin standard; track 2, wheat-germ products; track 3, axes system (purified) products; track 4, products of a total axes extract, tracks 5 and 6 legumin standards. Samples in tracks 1-5 were reduced. The translation products were labelled with [^{3}H] leucine. The products of the 3 systems were very similar except for slight differences in the 30,000 mol.wt. region.

Fig. 4B. Immunoprecipitation of Storage Protein Subunits from Translation Products of the Pea Axes System

Track 1, immunoprecipitate of polysome products with anti-legumin IgG; track 2, immunoprecipitate of polysome products with antivicilin IgG; track 3, microsome products; track 4, vicilin polypeptides immunoprecipitated from microsome products; track 5, vicilin standard. The translation products were labelled with [^{3}H] leucine. The tracks were from the same gel.

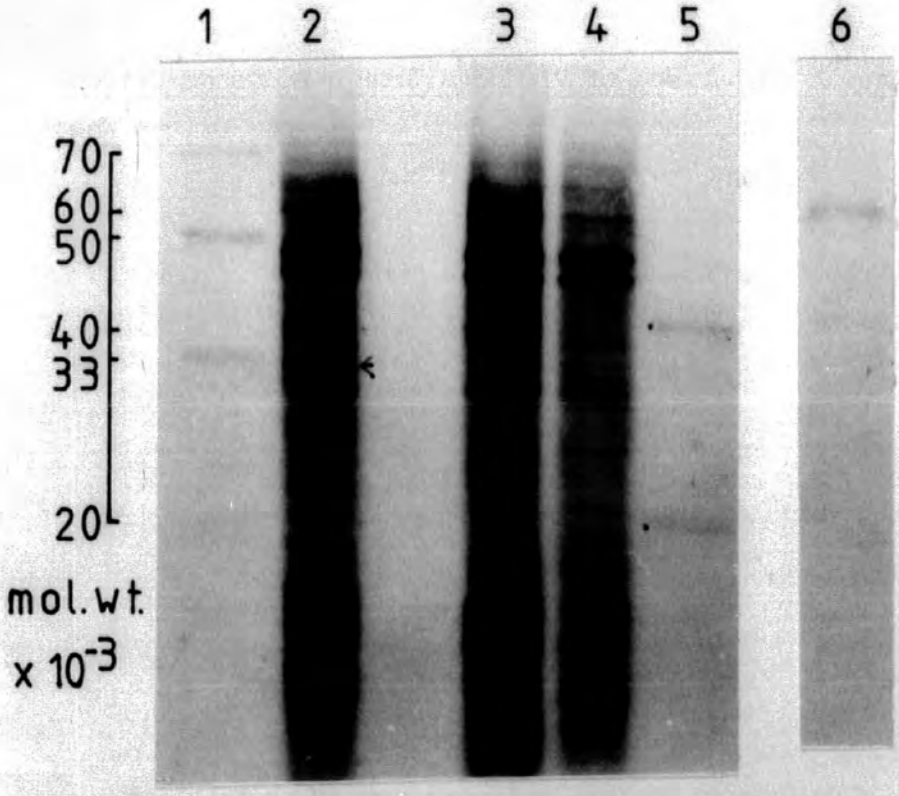


Fig. 4A.

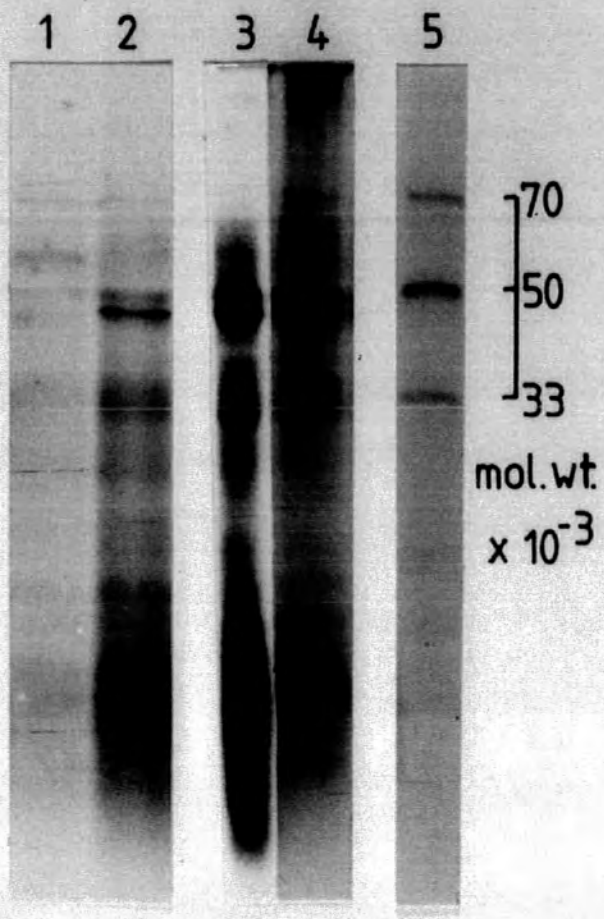
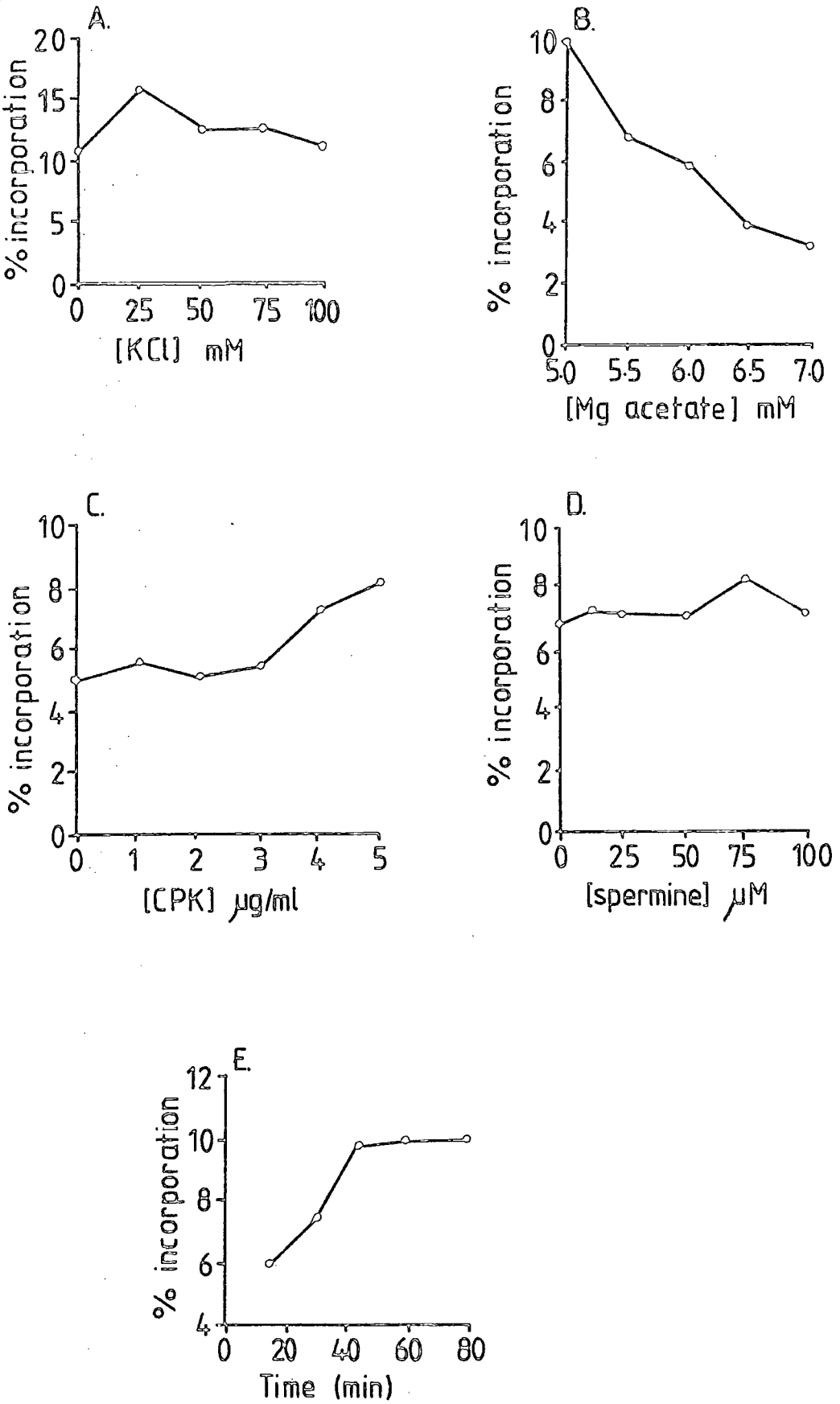


Fig. 4B

Fig. 5. Optimisation of the Pea Cotyledon System.

The percentage incorporation of [^{3}H] leucine into TCA precipitable polypeptides using polysomes as the 'template'. Varying concentrations of A, KCl; B, Mg acetate with 25mM KCl; C, CPK, with 25mM KCl and 5mM Mg acetate; D, spermine with 5µg of CPK/ml and KCl and Mg acetate as for C. E shows a time course for the optimised system ie 5mM Mg acetate, 5µg CPK/ml, 25mM KCl, and 75µM spermine. The optimum incubation time was 60 min.

Fig.5


A.
% incorporation
20
15
10
5
0
0 25 50 75 100
[KCl] mM
B.
% incorporation
10
8
6
4
2
0
5.0 5.5 6.0 6.5 7.0
[Mg acetate] mM
C.
% incorporation
10
8
6
4
2
0
0 1 2 3 4 5
[CPK] µg/ml
D.
% incorporation
10
8
6
4
2
0
0 25 50 75 100
[spermine] µM
E.
% incorporation
12
10
8
6
4
0 20 40 60 80
Time (min)

A fluorograph of polysome translation products of both the purified system and the total extract is shown in Fig. 6; the polypeptide patterns were very similar to that of the translation products of the polysome driven axes system, except that the 47,000 mol.wt. band appeared to be a doublet. The 50,000 and 47,000 mol.wt. subunits of vicilin and the 60,000 mol.wt. legumin subunit were identified by immunoprecipitation of the polysome translation products.

2-1-2 Cyanogen Bromide Cleavage of Legumin and Vicilin

A comparison was made of CNBr cleavage products of 'native' storage proteins and of *in vitro* synthesised storage protein subunits.

The cleavage of 'native' legumin, by cyanogen bromide, produced 15,000 and 18,000 mol.wt. polypeptides in both reduced and unreduced samples; but traces of the original legumin subunits remained. The cleavage products of *in vitro* translated legumin (labelled with [^{3}H] leucine) also contained a 15,000 mol.wt. polypeptide, (Fig. 7, tracks 1-4).

The 50,000 and 33,000 mol.wt. subunits of 'native' vicilin, and the 50,000 and 47,000 mol.wt. polypeptides of *in vitro* translated vicilin (labelled with [^{3}H] leucine) were not visibly affected by treatment with CNBr; however, the 70,000 mol.wt. subunit, was cleaved, and a 16,000 mol.wt. polypeptide appeared in both samples (Fig. 7, tracks 6-9). Several polypeptides between 30,000 and 60,000 mol.wt. also appeared in the cleavage products of 'native' vicilin.

In vitro synthesised legumin and vicilin, which were labelled with [^{35}S] methionine, produced no visible polypeptides after treatment with CNBr.

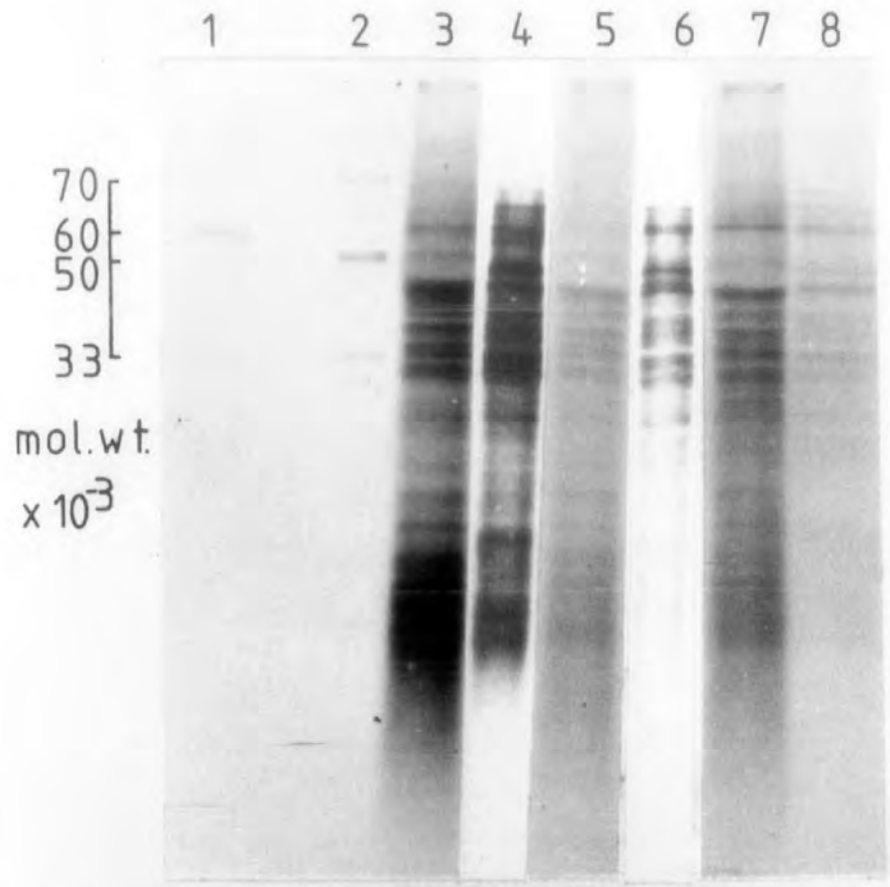


Fig. 6. A Comparison of Translation Products from a Pea Cotyledon System and a Pea Axes System

Track 1, legumin standard; track 2, vicilin standard; tracks 3 and 4, products of microsomes and polysomes in the axes system; tracks 5 and 6, products of microsomes and polysomes in the pea cotyledon system; tracks 7 and 8, products of microsomes and polysomes in the 'total' cotyledon extract. The samples in tracks 2-8 contained reducing buffer. The translation products were labelled with [^{3}H] leucine.

Protein synthesis was low in the microsome driven cotyledon systems.

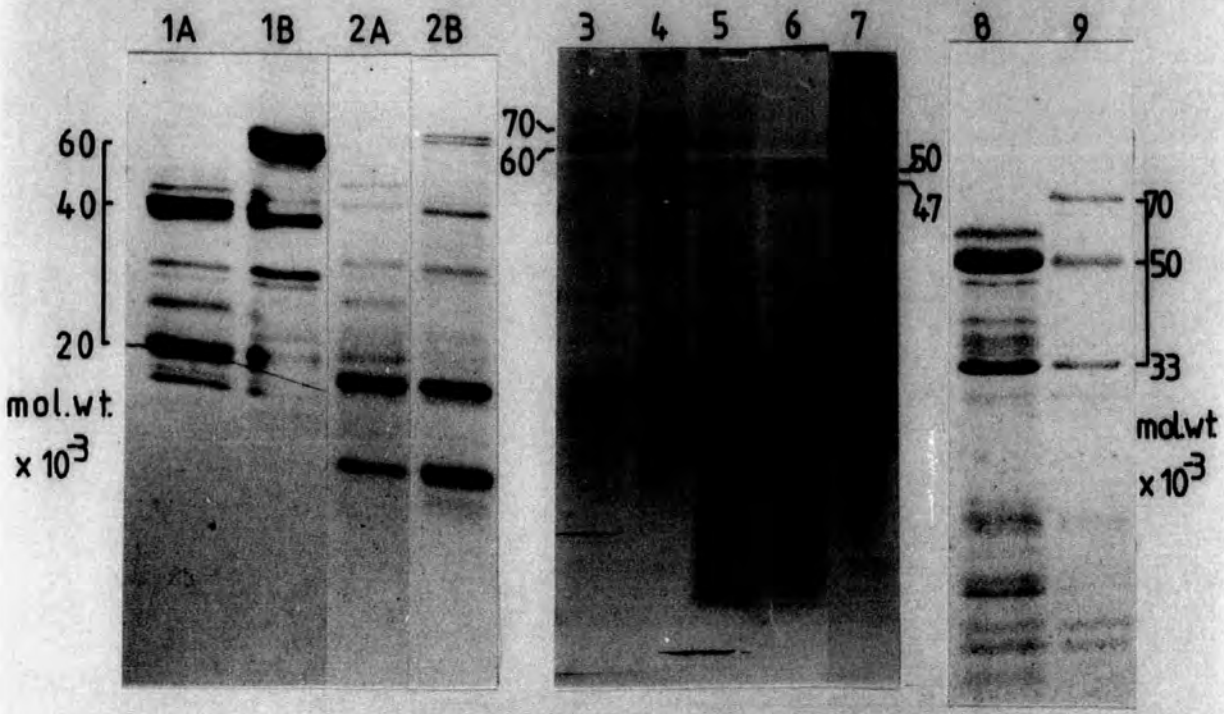


Fig. 7. Cyanogen Bromide Cleavage of Storage Protein Subunits

Track 1 native legumin, (A) reduced, (B) unreduced; track 2, CNBr treated native legumin (A) reduced, (B) unreduced; tracks 3 and 4, legumin and vicilin immunoprecipitated from translation products (7); tracks 5 and 6 as 3 and 4 then treated with CNBr; track 7, total reticulocyte lysate translation products (using polysomes); tracks 8 and 9 native vicilin, treated with CNBr and untreated.

Tracks 1, 2, 8 and 9 were from P.A.G. stained with Coomassie Blue, tracks 3-7 were from a fluorograph of [^{3}H] leucine labelled translation products.

2-1-3 Tryptic Digestion of Legumin and Vicilin

The 60,000 mol.wt. polypeptide, in unreduced samples, and the 40,000 mol.wt. polypeptide, in reduced samples, of 'native' legumin were completely digested by trypsin, and polypeptides of mol.wt. 55,000, 37,000 and 34,000 were produced. When in vitro translated legumin was digested the 60,000 mol.wt. polypeptide also disappeared the products contained several polypeptides, some of mol. wt. 55,000, 50,000 and 37,000, (Fig. 8A, tracks 1-4).

Tryptic digestion of 'native' vicilin produced 2 major polypeptides of mol.wt. 25,000 and 17,000. A 25,000 mol.wt. polypeptide was also a product of the digestion of in vitro translated vicilin, (Fig. 8B, tracks 1-4).

The presence of membranes as either microsomes, or as 'stripped' microsomes, did not afford any protection to the translation products against tryptic digestion, even when the digestion was performed at 0-4^{o}C.

2-1-4 Incorporation of [^{3}H] glucosamine and [^{3}H] mannose into In vitro Translation Products

When either [^{3}H] glucosamine or [^{3}H] mannose were added to the wheat-germ system, driven by either polysomes or microsomes, there was no significant incorporation of the labelled compound into polypeptide translation products.

2-1-5(a) A Comparison of Translation Products from Poly(A)$^{+}$-RNA from Cotyledons of Different Developmental Stages

Translation products of poly(A)$^{+}$-RNA extracted from cotyledons of 3 different developmental stages, were very similar. A fluorograph of the translation products separated on a polyacrylamide gel showed

Fig. 8. Tryptic Digestion of Storage Protein Subunits.

A. Legumin. Tracks 1 and 2 native legumin untreated, and digested with trypsin; tracks 3 and 4, *in vitro* synthesised legumin digested with trypsin, and untreated.

B. Vicilin. Tracks 1 and 2, native vicilin, untreated and digested with trypsin; tracks 3 and 4, *in vitro* synthesised vicilin polypeptides, digested with vicilin and untreated.

Tracks 1 and 2 (of both A and B) are drawings of a Coomassie Blue stained P.A.G.s, while tracks 3 and 4 are drawings of fluorographs of polypeptides immunoprecipitated from [^{3}H] leucine labelled reticulocyte lysate translation products.

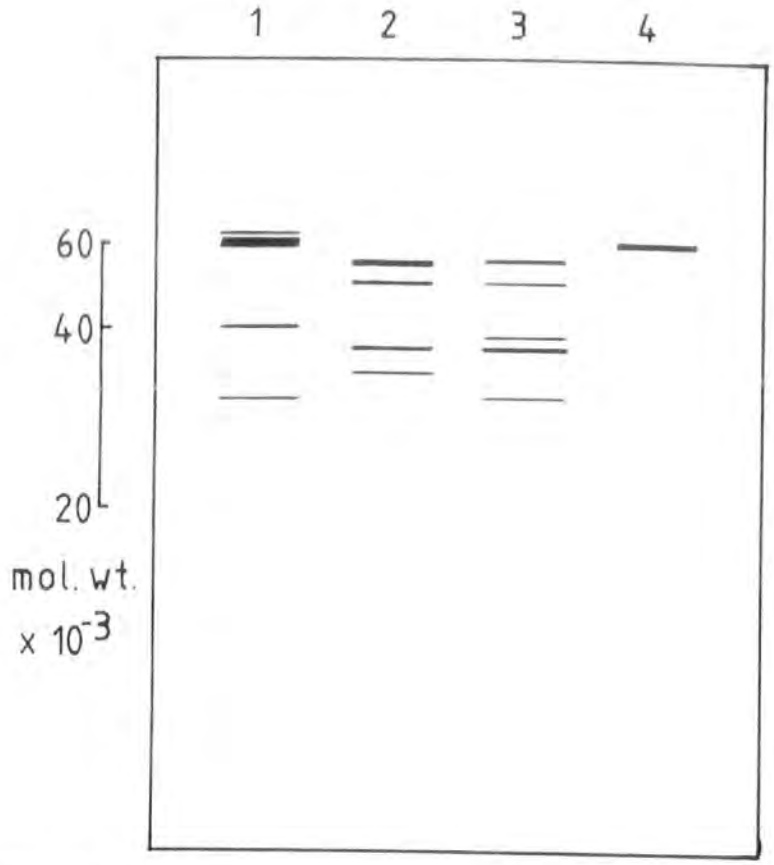


Fig. 8A

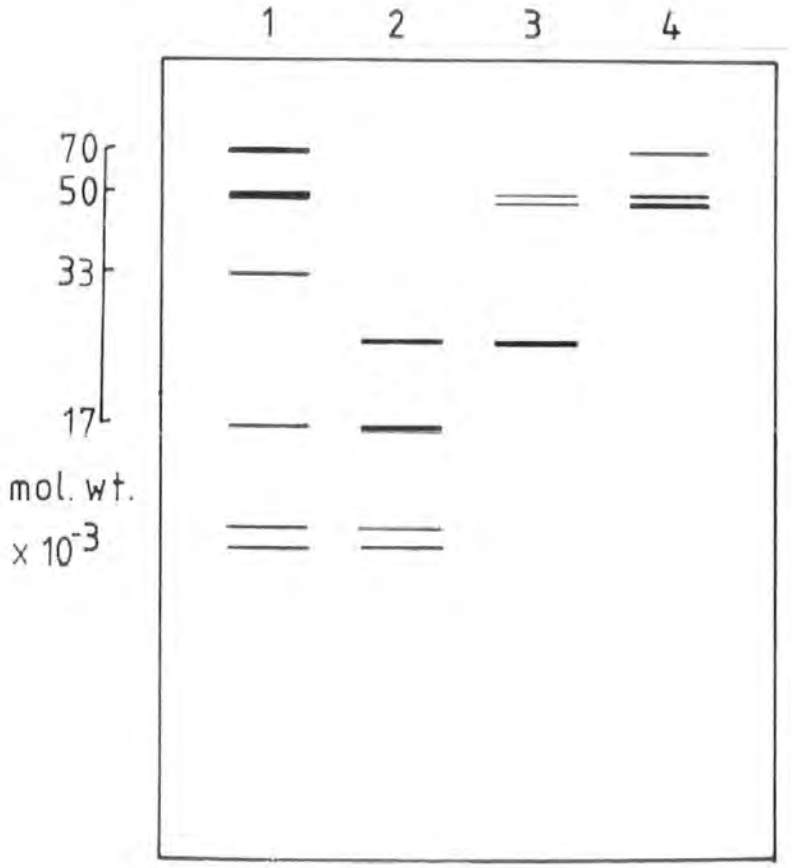


Fig. 8B

the differences between them to be in the intensity of the polypeptide bands rather than in the absence or presence of a band. The major polypeptides produced from the 9 day and 14 day poly(A)$^+$-RNA were the 50,000 and 47,000 mol.wt. polypeptides; a 69,000 and a 67,000 mol.wt. polypeptide were also synthesised in large amounts in the 14 day system. A number of polypeptide bands, which appeared to be of equal intensity, were synthesised from 19 day poly(A)$^+$-RNA, these included 70,000, 60,000, 50,000 and 47,000 mol.wt. polypeptides, (Fig. 9).

However, immunoprecipitates of translation products of polysomes from 14 day and 19 day cotyledons showed an absence of vicilin 50,000 and 47,000 mol.wt. polypeptides in the 19 day products, which were present in 14 day products. The legumin 60,000 mol.wt. subunit was present in immunoprecipitates of both samples.

2-1-5(b) A Comparison of Translation Products of Polysomes Isolated from Different Pea Varieties

The translation products of polysomes from four varieties of pea were very similar, except that the Kelvedon Wonder variety were relatively low in 60,000 mol.wt. polypeptide, (Fig. 10). The percentage incorporation of [^{35}S] methionine into polypeptides varied slightly; for Feltham First it was 12-40%, for Kelvedon Wonder, 32%, for Little Marvel, 29% and for Mangetout 37%, the endogenous incorporation was less than 1%.

2-1-5(c) Cell-free Translation Products of Polysomes from Vicia faba Cotyledons

Legumin immunoprecipitates from translation products of both 20 day and 47 day Vicia faba polysomes were polypeptides of mol.wts. 25,000 and 60,000. The major product of the 20 day polysomes was the 25,000 mol.wt. polypeptide, while the 60,000 mol.wt. polypeptide was the major product of the 47 day polysomes (Fig.11A). The 60,000 mol.wt. subunit, like that of P.sativum, was not reduced to 40,000 and

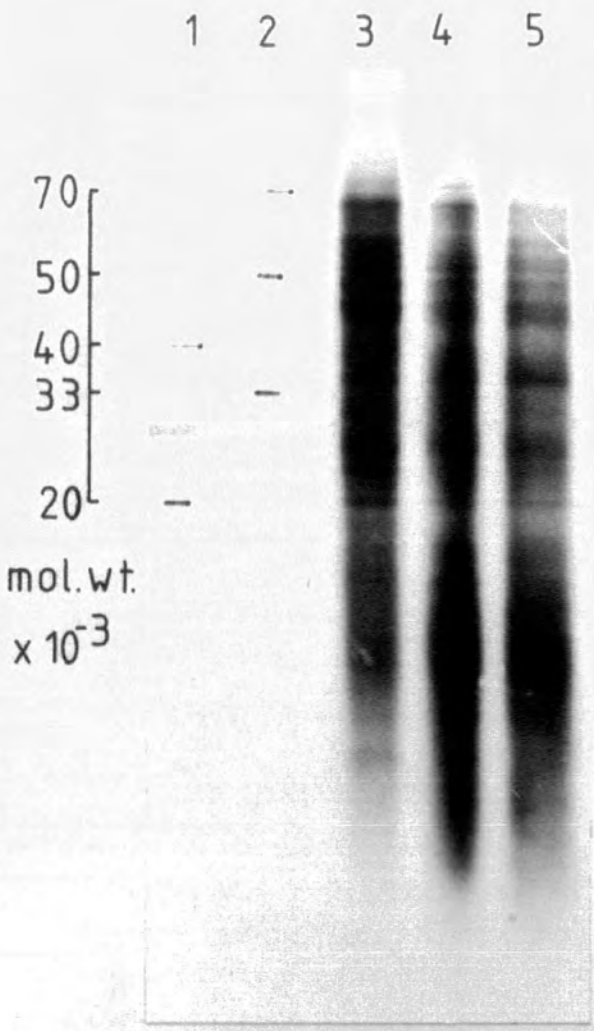


Fig. 10. A Comparison of Translation Products from Different Pea Varieties

14 day polysomes isolated from cotyledons of different pea varieties were translated in a wheat-germ cell-free system using [^{35}S] methionine as the labelled amino acid.

Tracks 1 and 2, position of reduced legumin and vicilin standards; track 3,4 and 5 translation products of polysomes from Feltham First, Kelvedon Wonder and Little Marvel cotyledons.

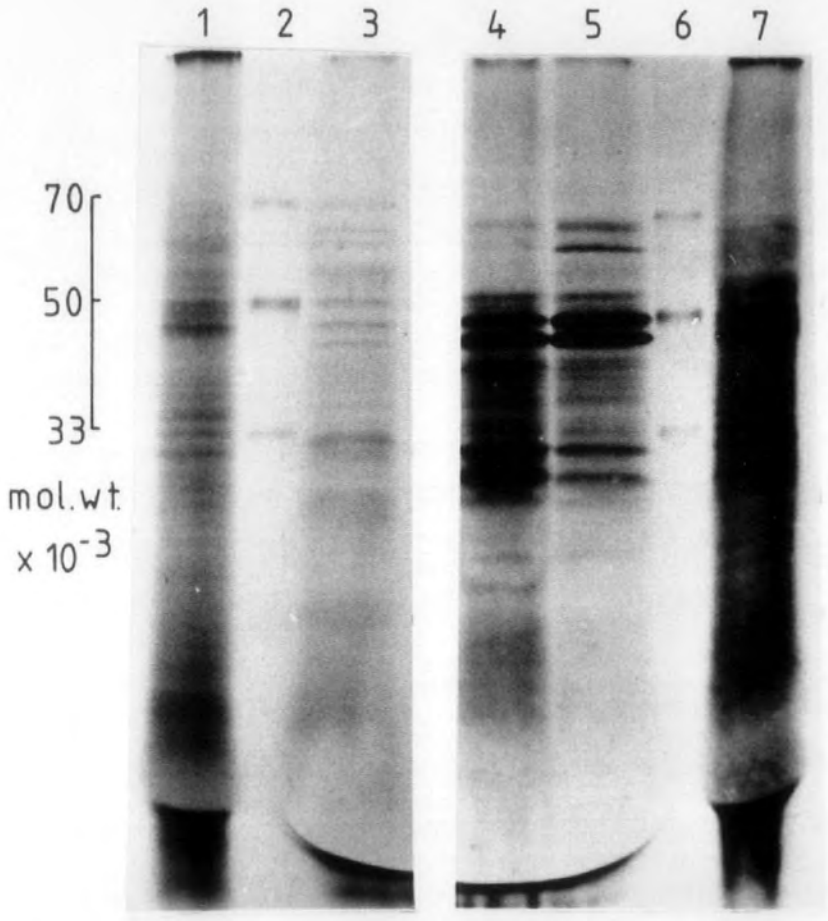


Fig. 9. A Comparison of Translation Products of Poly(A)$^+$-RNA from Cotyledons of Different Developmental Stages

Tracks 1 and 7, translation products of 'total' polysomes from 14 day old cotyledons; tracks 2 and 6, standard vicilin; tracks 3, 4 and 5 translation products of poly(A)$^+$-RNA from 19, 9 and 14 day old cotyledons.

The templates were translated in the reticulocyte lysate system, with [^{3}H] leucine as the labelled amino acid. Tracks 1, 2 and 3 were from one fluorograph, tracks 4, 5 and 6 were from another.

Fig. 11. Translation Products of Vicia faba polysomes in the Reticulocyte Cell-free System.

A. Legumin synthesised by 20 and 47 day polysomes

Tracks 1 and 8, vicilin standards; track 2, 20 day polysome products; track 3, legumin immunoprecipitated from 2; tracks 4 and 7, reduced legumin standards; track 5, legumin immunoprecipitated from 47 day polysome products which are shown in track 6. The 25,000 mol.wt. polypeptide present in the translation products and immunoprecipitated by antilegumin IgG, has not been positively identified. The translation products were labelled with [^{35}S] methionine.

B. Vicilin synthesised by 20 and 47 day polysomes

Tracks 1 and 2, vicilin polypeptides immunoprecipitated from 20 and 47 day polysomes; track 3, standard vicilin; tracks 4 and 5, translation products of 'total' polysomes from 14 and 19 day old pea cotyledons (for comparison); tracks 6 and 7, translation products of polysomes from 20 and 47 day old Vicia faba cotyledons. The translation products were labelled with [^{3}H] leucine.

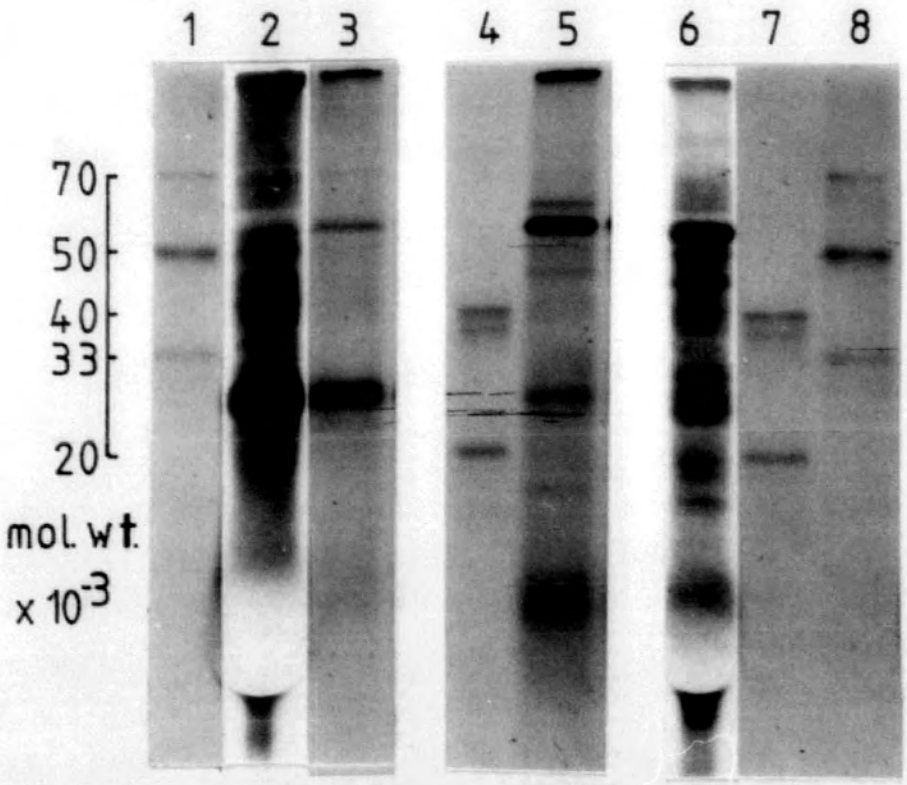


Fig. 11A.

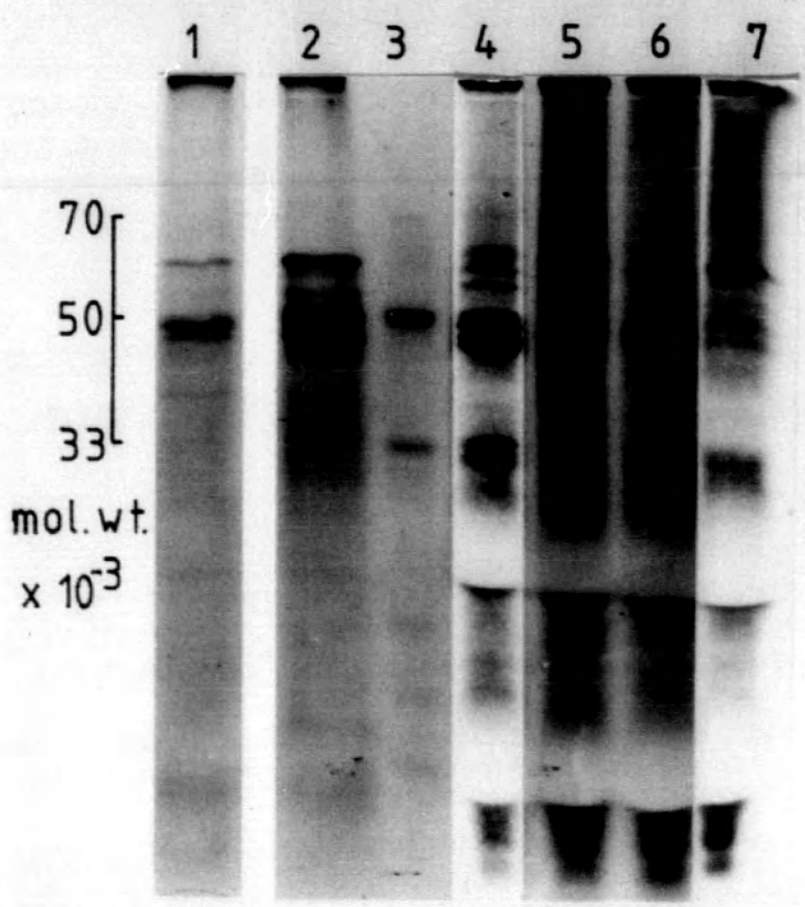


Fig. 11B.

20,000 mol.wt. subunits in the presence of 2ME, although the native form was.

The immunoprecipitated vicilin products of 20 day polysomes were 65,000, 48,000 and a minor 47,000 mol.wt. polypeptides, whereas the 47 day polysome products contained 65,000, 50,000 and 47,000 mol.wt. vicilin polypeptides, (Fig. 11B).

2-2 The Study of Initiation in Cell-free Systems

2-2-1 The Wheat-germ System

The presence of 10^{-4}M aurintricarboxylic acid during translation of poly(A)$^{+}$-RNA, in this system, caused 100% inhibition of protein synthesis, whereas synthesis in the polysome driven system was only reduced by 25%; a concentration of 5 x 10^{-3}M ATA was necessary to completely inhibit polysomal protein synthesis (Fig. 12). The degree of inhibition of protein synthesis calculated over the total incubation period of the cell-free system was similar to that calculated as the inhibition of the initial rate of protein synthesis.

Preincubation of the poly(A)$^{+}$-RNA driven system at 30^{o}C decreased the level of inhibition by ATA much more effectively than did preincubation at 0^{o}C, (Fig. 13).

The translation products of the polysomes translated in the presence of different concentrations of ATA were visualised on the fluorograph shown in Fig. 14, which shows an absence of some polypeptide bands in the 40,000 mol.wt. region at higher ATA concentrations. A slight increase in mol.wt. of the 60,000 subunit of legumin was also apparent at higher concentrations of ATA, compared to the control.

Fig. 12. A Time Course of Incorporation of [^{35}S] methionine into TCA Precipitated Counts, in the Wheat-germ Cell-free System, in the Presence of ATA.

A. Poly(A)$^{+}$-RNA driven system; ATA concentrations were (1) 10^{-4}M, (2) $5x10^{-5}$M, (3) 10^{-5}M, (4) $5x10^{-6}$M, (5) 10^{-6}M.

B. Polysome driven system; ATA concentrations were (1) 10^{-2}M, (2) $5x10^{-3}$M, (3) 10^{-3}M, (4) $5x10^{-4}$M, (5) 10^{-4}M.

In both A and B (6) was a control (ie H_2O replaced ATA); (7) and (8) were endogenous samples - and + ATA (10^{-4}M, A; 10^{-2}M B).

Fig. 13. The Effect of Preincubation of Samples, Before ATA Addition, in the Poly(A)$^{+}$-RNA Driven Wheat-germ Cell-free System.

Values show the inhibition by 5×10^{-5}M ATA of incorporation of [^{35}S] methionine into TCA precipitated counts, during a 40 min incubation. Preincubation was at (a) 30^{o}C or (b) 0^{o}C. (Initiation processes are slowed down at 0^{o}C compared to 30^{o}C.)

Fig.12A

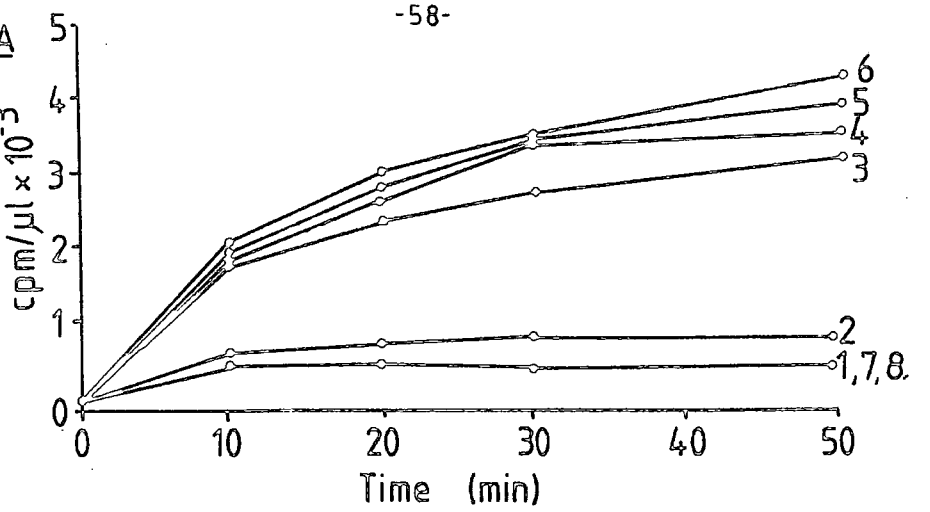


Fig.12B

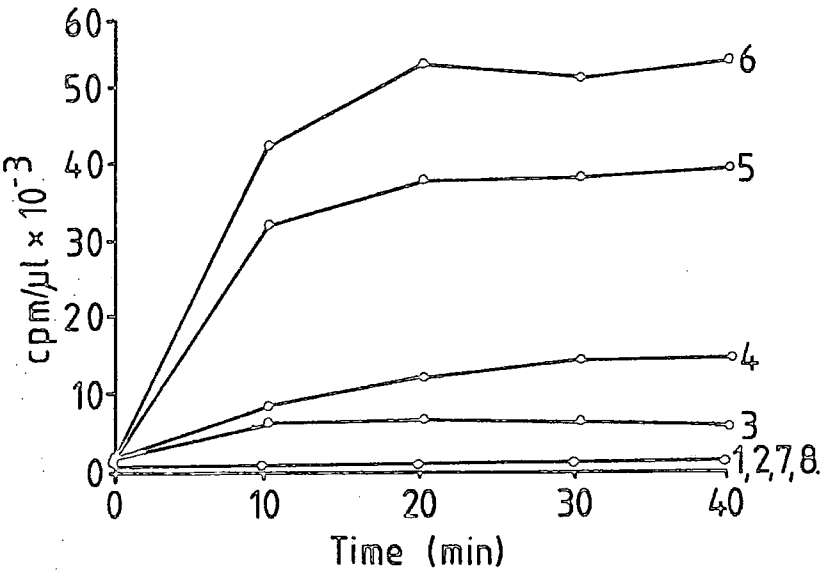
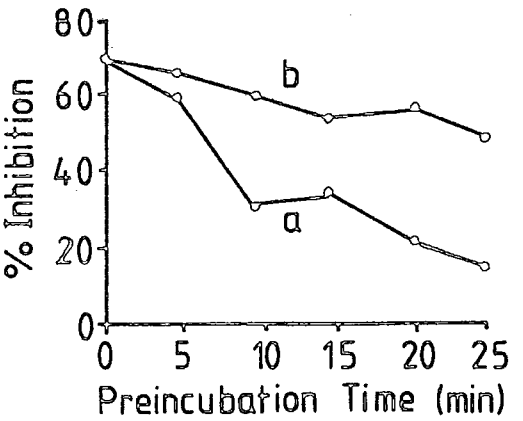


Fig.13



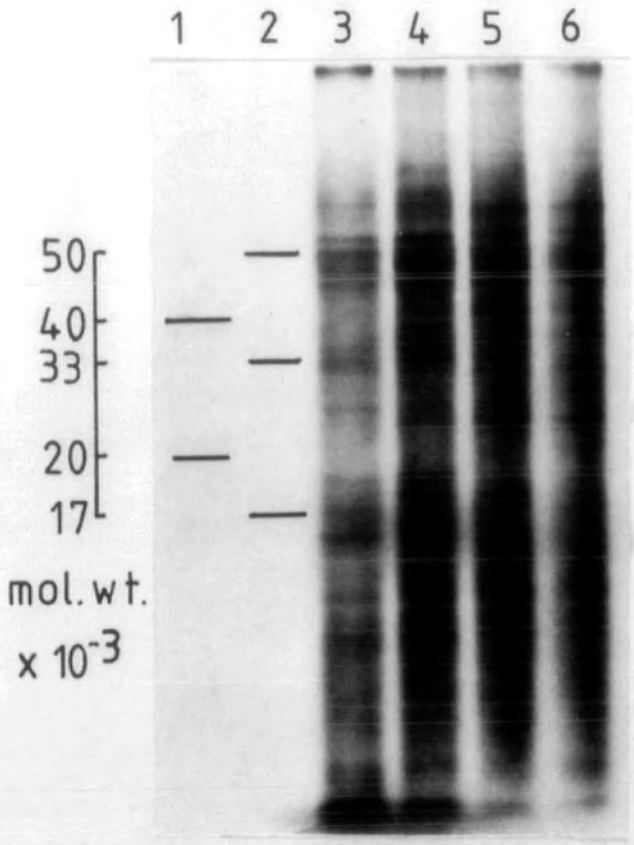


Fig. 14. Translation Products of 14 day Polysomes in the Wheat-germ System, in the Presence of ATA.

Track 1, standard legumin (reduced); track 2, standard vicilin; tracks 3, 4 and 5, translation products in the presence of 10^{-3}M, 5 x 10^{-4}M and 10^{-5}M ATA; track 6, control, (no ATA present). There is an apparent difference in mol.wt. of the 60,000 mol.wt. polypeptide between tracks 1 and 2, and tracks 3 and 4. The translation products were labelled with [^{35}S] methionine.

2-2-2 The Reticulocyte Lysate System

The inhibitory effect on polysomal protein synthesis of increasing ATA concentrations is illustrated in Fig. 15. No inhibition was demonstrated when the ATA concentration was 10^{-4}M or less, but there was a sharp increase in inhibitory effect at concentrations above this.

Preincubation of the system at 30°C for 10 min eliminated the effect of ATA, whereas preincubation at 0°C had little effect on the level of inhibition, (Fig. 16).

The fluorograph of the products of the inhibited systems, (Fig. 17), showed a loss of several small mol.wt. bands (ie 30,000 mol.wt.), and the loss of one of the bands (probably the upper) of the 47,000 mol.wt. doublet, when compared to the controls. The 60,000 mol.wt. polypeptide appeared to be a doublet in both inhibited and control samples, with the upper band more strongly labelled in the uninhibited samples.

2-2-3 The Pea Axes and the Pea Cotyledon Cell-free System

When ATA was present in the axes system and the cotyledon system at 10^{-4}M, polysomal protein synthesis was inhibited by 34% and 44% respectively. Complete inhibition was achieved at 10^{-3}M ATA, (Fig. 18).

As in the other systems, preincubation of the axes and cotyledon systems at 30°C resulted in a decrease in inhibition, of about 50% after 15 min preincubation in these examples (Fig. 19). The decrease in inhibition was mainly during the first 5 min of preincubation of the axes system, though it continued at a steady rate for the remainder of the time course, whereas there was a more uniform decline in inhibition with increased preincubation time in the cotyledon system. Preincubation at 0°C only decreased the inhibitory effect by 1%.

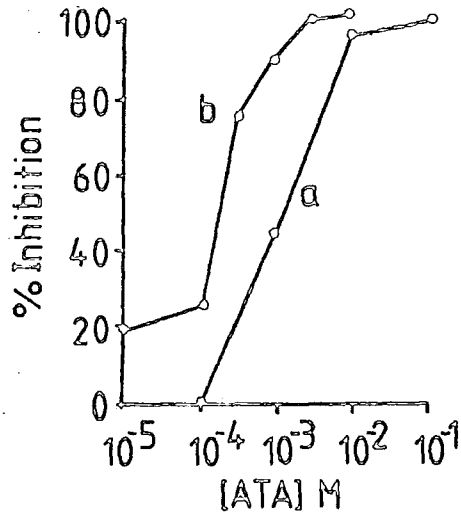


Fig. 15. The Effect of ATA Concentration on Polysome Driven Protein Synthesis in the Reticulocyte Lysate System.

Values are the % inhibition of the incorporation into polypeptides of [^{3}H] leucine, using the Reticulocyte system (a) compared with that of [^{35}S] methionine using the Wheat-germ system (b), in the presence of ATA.

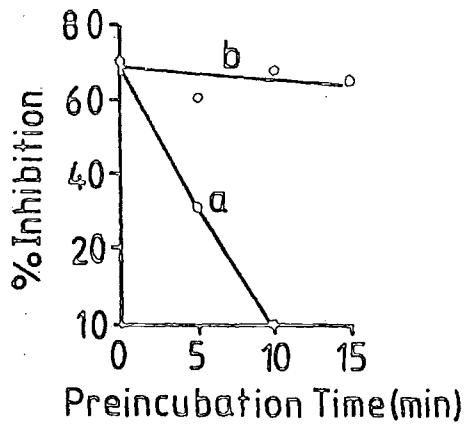


Fig. 16. The Effect of Preincubation of Samples before ATA Addition to the Polysome Driven Reticulocyte Lysate System.

Values are the inhibition by 10^{-3}M ATA of [^{3}H] leucine incorporation into polypeptides during a 45 min incubation. Preincubation was at (a) 30°C, and (b) 0°C.

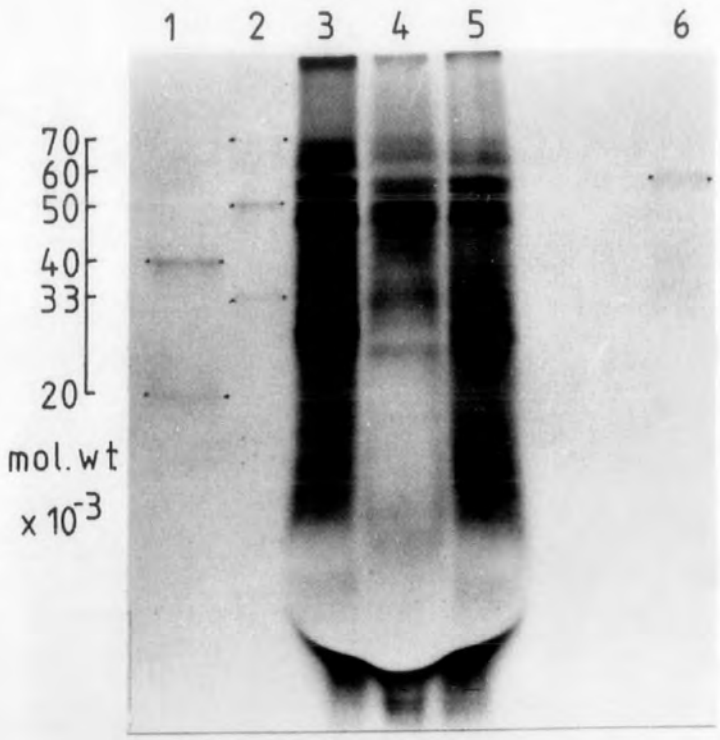


Fig. 17. Translation Products of 14 day Polysomes in the Reticulocyte Lysate System in the Presence of ATA.

Track 1, reduced legumin standard; track 2, vicilin standard; track 3, control translation products (ie no ATA added); track 4, 10^{-3}M ATA present throughout incubation of the translation system; track 5, ATA (final concentration 10^{-3}M) added after 7 min incubation of the translation system; track 6, unreduced legumin standard. There is a loss of low mol.wt. polypeptides in the presence of ATA if no preincubation is allowed. The translation products are labelled with [^{3}H] leucine.

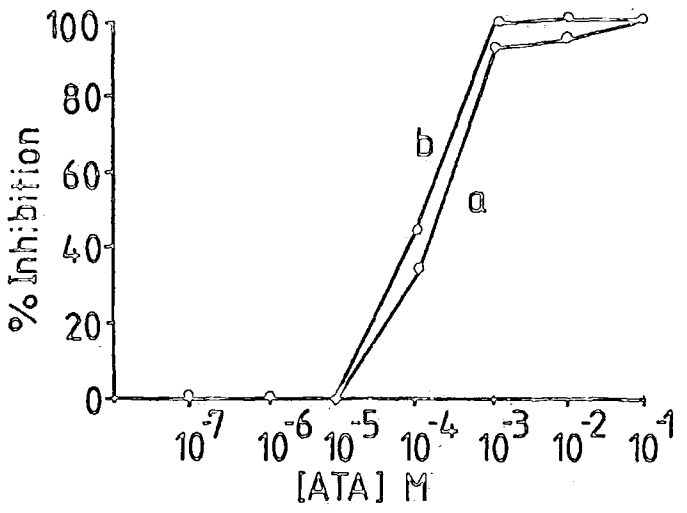


Fig. 18. The Effect of ATA Concentration on Polysome Driven Protein Synthesis in the Pea Axes and Pea Cotyledon Systems.

Values show the inhibition by ATA of incorporation of [^{3}H] leucine into TCA precipitated counts, after 45 min incubation of (a) the Pea Axes System and (b) the Pea Cotyledon System.

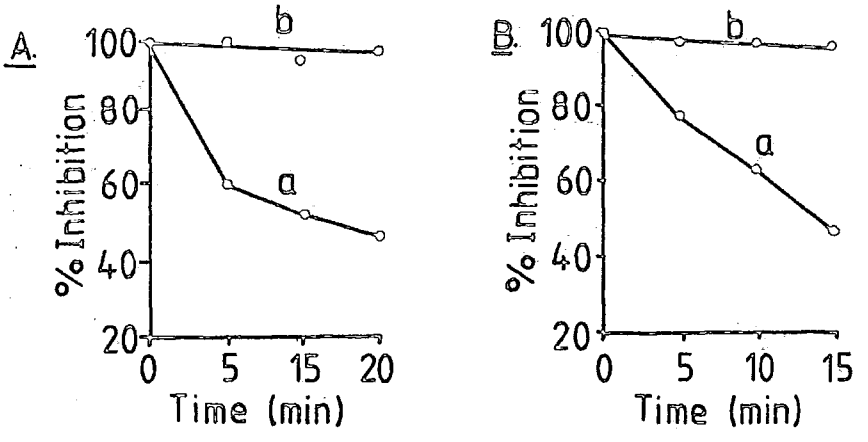


Fig. 19. The Effect of Preincubation of Samples before ATA Addition to the Polysome Driven Pea Axes and Pea Cotyledon Systems.

Values show the inhibition of [^{3}H] leucine incorporation into TCA precipitable polypeptides, by 10^{-4}M ATA added at different times during a 45 min incubation of (A) The Axes System and (B) The Cotyledon System. Preincubation at (a) 30°C or (b) 0° C. Time 0 was considered to be 100% inhibition here, this corresponds to approximately 40% inhibition in Fig. 18.

2-3 Post-translational Modification of Polypeptides In Vitro

2-3-1 Characterisation of 'Templates'

The results presented in Fig. 20 show the sedimentation profiles of various polysome preparations on sucrose gradients. Each sample contained monosomes and different sized polysomes, up to 10 mer.

The percentage of ribosomes present as polysomes, calculated by the relevant peak areas, was 79% for 'total' polysomes, and 95% for 'released' polysomes, but only 30% for 'free' polysomes.

The relative spectral characteristics of the preparations measured at 260nm and 280nm (Table 1) showed that the membrane-containing samples had a lower relative absorbance than membrane-free preparations.

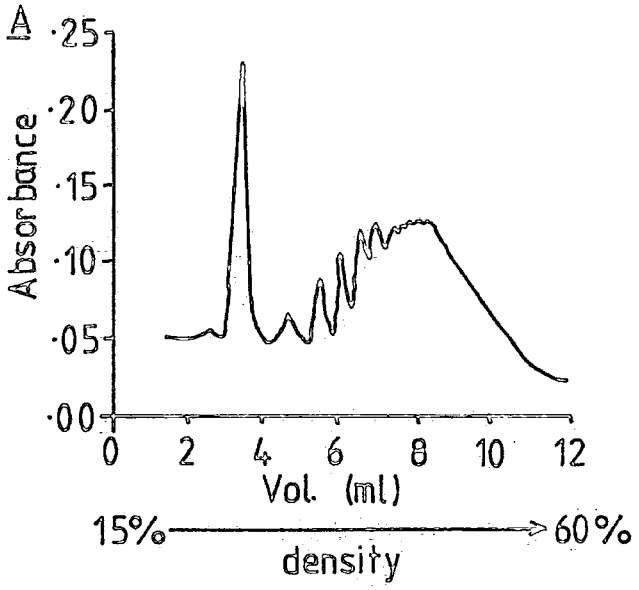
The profiles of 19 day and 9 day poly(A)$^+$-RNA are shown in Fig. 21, and it can be seen that some material, which absorbs at 254nm, was lost between purification steps. Approximately 0.5% of polysomes (by weight) was recovered as 2x purified poly(A)$^+$-RNA.

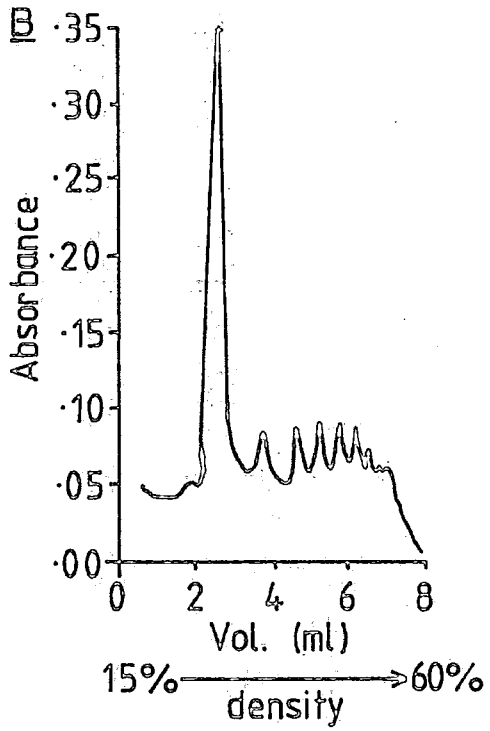
An electron micrograph of a microsome preparation showed that most polysomes were associated with vesicular-like membrane structures (Fig. 22). The preparation was slightly contaminated with free polysomes and electron dense protein granules.

Fig. 20. Sedimentation Profiles of Polysome Preparations.

Analysis of 400µg of polysome preparations separated on a 15-60% sucrose density gradient, measured at an absorbance of 254nm. A, 'total' polysomes; B, 'free' polysomes; C, 'released' polysomes (these are also equivalent to microsomes - see extraction procedure).

Fig. 20


A
·25
·20
·15
·10
·05
·00
Absorbance
0
2
4
6
8
10
12
Vol. (ml)
15%
60%
density


B
·35
·30
·25
·20
·15
·10
·05
·00
Absorbance
0
2
4
6
8
Vol. (ml)
15%
60%
density

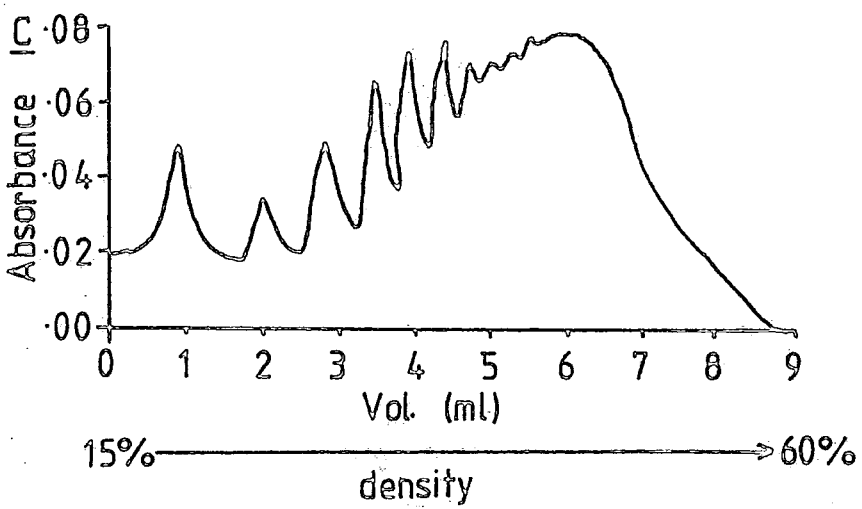

C
·08
·06
·04
·02
·00
Absorbance
0
1
2
3
4
5
6
7
8
9
Vol. (ml)
15%
60%
density

Table 1 The Absorbance Properties of 'Polysome Preparations'

	'Total' Polysomes			Microsomes		'Free'	'Released'
	9day	14day	19day	I	II	Polysomes	Polysomes
Absorbance at 262nm	.23	.98	.68	.62	.52	.91	.22
Absorbance at 280nm	.13	.55	.40	.50	.43	.55	.14
Ratio $\frac{262}{280}$	1.77	1.78	1.70	1.24	1.21	1.65	1.63

The absorbance of samples of 'polysome preparations' were measured, as described in methods.

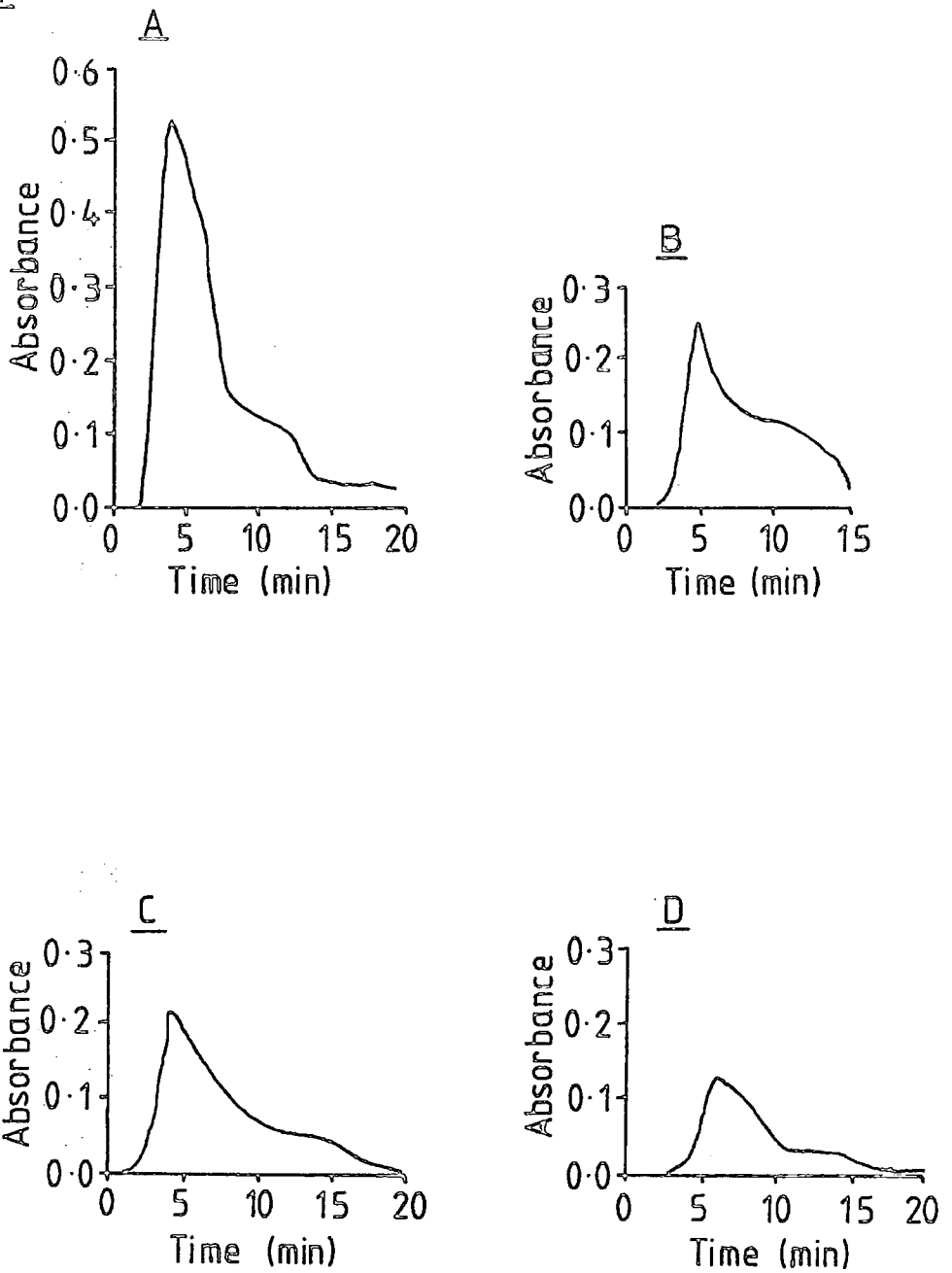
The relative absorbance of 262:280 of 'pure' polysomes is 1.8 according to Beevers and Poulson (1972) and 1.7 according to Noll (1966).

The low relative absorbance values obtained for microsome samples is due to the presence of membrane proteins. There was low yield of 'released' polysomes and 9 day polysomes.

Fig. 21. Profiles of Poly(A)$^+$-RNA from an oligo-(dT)-cellulose column.

9 day poly(A)$^+$-RNA (A, B) and 19 day poly(A)$^+$-RNA (C, D) were eluted from the first oligo-(dT)-cellulose column (A, C) and then a second oligo-(dT)-cellulose column (B, D). The elution profile was measured as absorbance at 254nm.

Fig. 21


A
Absorbance
0·6
0·5
0·4
0·3
0·2
0·1
0·0
0
5
10
15
20
Time (min)
B
Absorbance
0·3
0·2
0·1
0·0
0
5
10
15
Time (min)
C
Absorbance
0·3
0·2
0·1
0·0
0
5
10
15
20
Time (min)
D
Absorbance
0·3
0·2
0·1
0·0
0
5
10
15
20
Time (min)

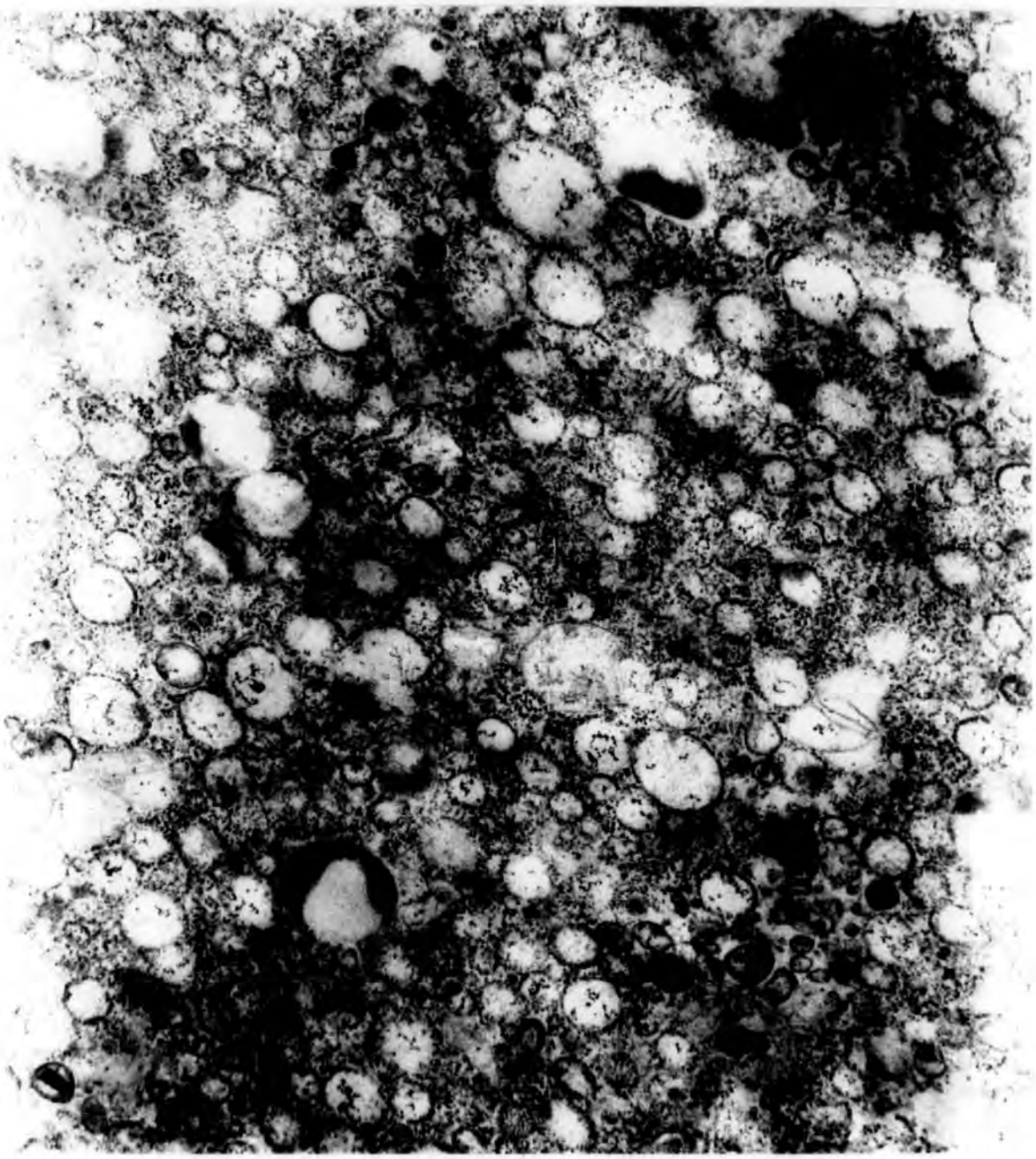


Fig. 22. An Electron Micrograph of a Microsome Sample.

The electron micrograph shows ribosome and polysome studded vesicles contaminated with protein bodies and free ribosomes. Magnification x 27,500.

2-3-2 Translation Products

Band II microsomes (see methods) gave a higher percentage incorporation of total counts added to the assay than did band I microsomes, though the translation products appeared to be identical when visualised on a fluorograph. These two samples were subsequently mixed and used as the microsome fraction.

The optimal conditions for translation of the microsomes in different cell-free systems are shown in table 2.

Microsomes and 'released' polysomes incorporated 6-10% of the total counts, provided by either [^{35}S] methionine or [^{3}H] leucine, into polypeptides when translated in either the wheat-germ or reticulocyte lysate systems, these values compare with about 14% incorporation with 'free' polysomes and 12-40% with 'total' polysomes. Endogenous incorporation was always less than 1% during incubation times of 45 to 90 min. Membranes stripped of ribosomes were inactive in protein synthesis.

Figure 23A shows a fluorgraph of the reticulocyte translation products of different 'polysome preparations', labelled with [^{3}H] leucine. The major translation products in each sample were the 60,000, 50,000 and 47,000 mol.wt. polypeptides of storage proteins. The 50,000 and 47,000 mol.wt. polypeptide bands were doublets, (ie each band actually consisted of 2 bands of approximately 1,000 mol.wt. difference) which varied in their relative intensity depending on the preparation (ie microsome, polysome etc.) used as a 'template'. Microsomal products showed the lower band of each doublet the more intense, whilst 'released' polysomes produced polypeptides in which the upper band was the more intense; bands in the doublets of 'free' polysome products appeared to be of equal intensity.

Table 2 The Optimisation of Components Added to Microsome Driven Cell-free Translation Systems

	Wheat-germ	Axes
KCl mM	100	50
MgOAc mM	5	1
Spermine μM	32	16
CPKμg/ml	20	6
Time min.	60	60
% incorporation	6-10	5-30

The concentrations shown were the optimal levels for translation using microsomes in the cell-free systems. The optimal concentrations of components used in the microsome driven Reticulocyte System were similar to those used in the polysome driven system.

Fig. 23. Reticulocyte Lysate Translation Products of Different 'Templates'

A. Different preparations were translated using [^{3}H] leucine as the labelled amino acid.

Tracks 1 and 12, unreduced legumin standards; tracks 2 and 10, reduced legumin standard; tracks 3 and 11, vicilin standards; track 4, microsome products; track 5, 'free' polysome products; track 6, 'released' polysome products; tracks 7, 8 and 9, as 4, 5 and 6 except 'stripped membranes' were added to the translation systems. The presence of membrane alters the relative intensity of the bands in the doublets of the 50,000 and 47,000 mol.wt. polypeptides.

B. Translation Products labelled with [^{35}S]methionine

Tracks 1 and 7, vicilin standards; tracks 2 and 3 'free' polysome products - and + 'stripped membranes'; track 4, microsome products; tracks 5 and 6, 'released' polysome products - and + 'stripped membranes'; track 8, reduced legumin standard. No doublets are visible. The 'free' polysome products do not appear to contain 47,000 mol.wt. polypeptides, but they do contain a large amount of material in the 25,000 - 29,000 mol.wt. range.

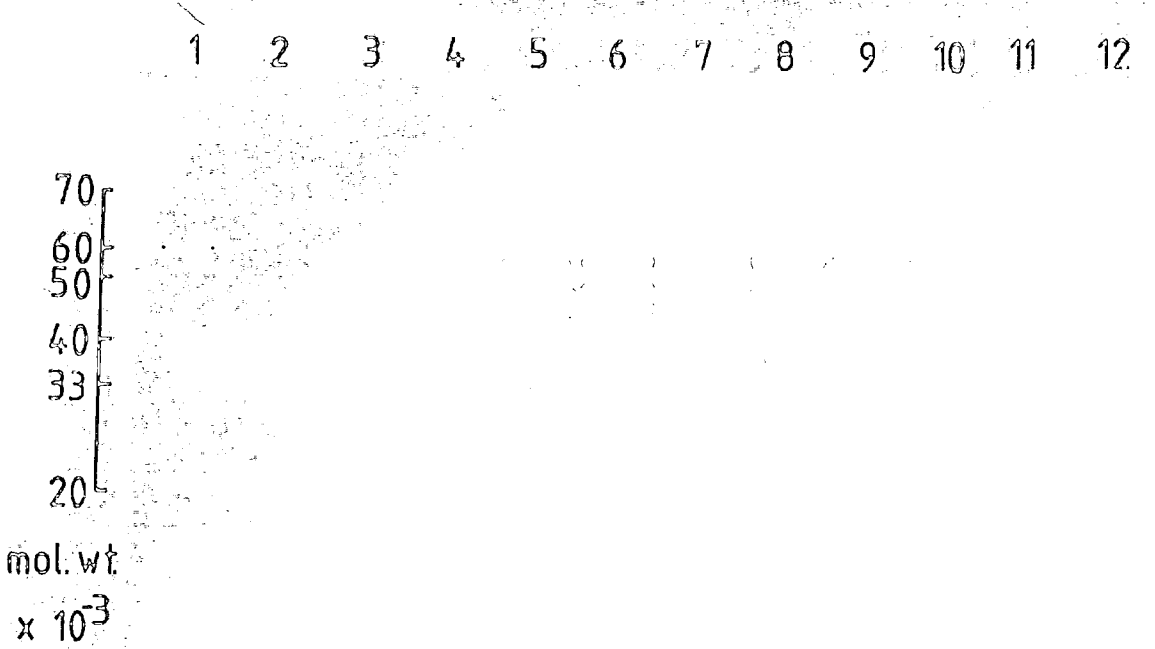


Fig. 23A

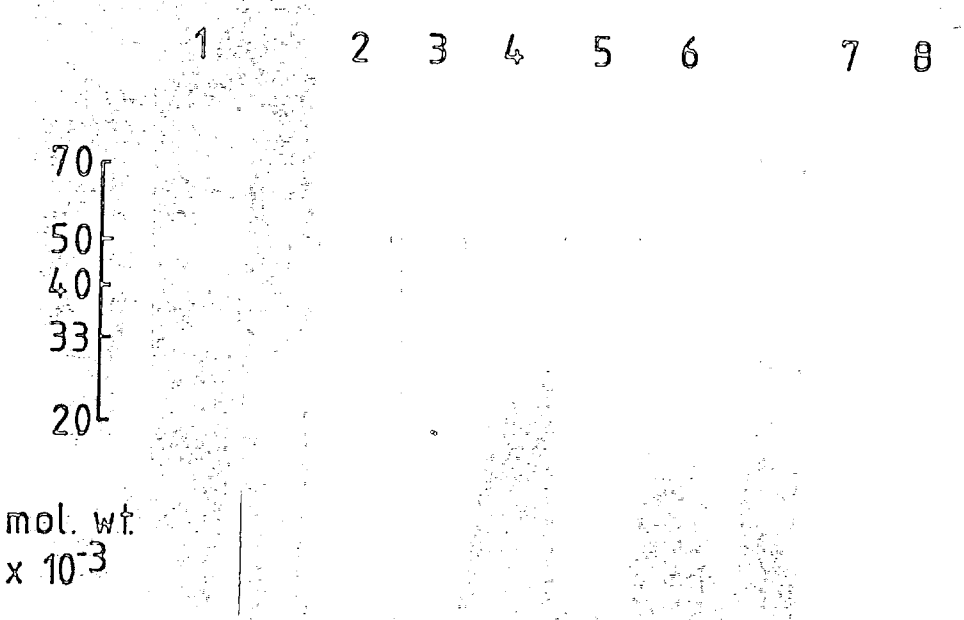


Fig. 23B

The addition of membranes to the translation system had no effect on the microsome products, but appeared to affect the intensity of the bands in the doublet of the 'released' polysome products, so that both bands were of equal intensity (Fig. 23A, tracks 7-9).

A fluorograph of the translation products of different preparations labelled with [^{35}S] methionine showed a similar basic pattern to those of [^{3}H] leucine labelled products, except no doublets were apparent in the 50,000 and 47,000 mol.wt. polypeptide bands (Fig. 23B).

A comparison of [^{3}H] leucine labelled translation products from the wheat-germ system and the reticulocyte system, when driven by 'total' polysomes, showed that the 50,000 and 47,000 mol.wt. polypeptide bands synthesised in the wheat-germ system were single and coincided with the lower of the two bands in each doublet synthesised in the reticulocyte system. The upper band of each doublet synthesised in the reticulocyte system was more intense than the lower, (Fig. 24). Reticulocyte translation products of poly(A)$^{+}$-RNA showed only single polypeptide bands of 50,000 and 47,000 mol.wt., which coincided with the upper bands of the doublets synthesised using 'total' polysomes in the reticulocyte system, (Fig. 9).

2-3-3 The Addition of Protein Bodies, and of An Albumin Extract from Cotyledons to the Translation Systems

The protein body fraction, isolated from cotyledons, contained the major storage protein subunits of legumin and vicilin, (Fig. 25). The 60,000 mol.wt. legumin subunit was reduced in the presence of 2ME to give a 40,000 mol.wt. subunit and a 20,000 mol.wt. subunit, as is characteristic for native legumin from mature cotyledons.

When the globulin and albumin preparations, which were extracted from cotyledons, were fractionated on a PAG, the globulin fraction was seen to contain the storage proteins, which were almost entirely absent from the albumin fraction, (Fig. 26).

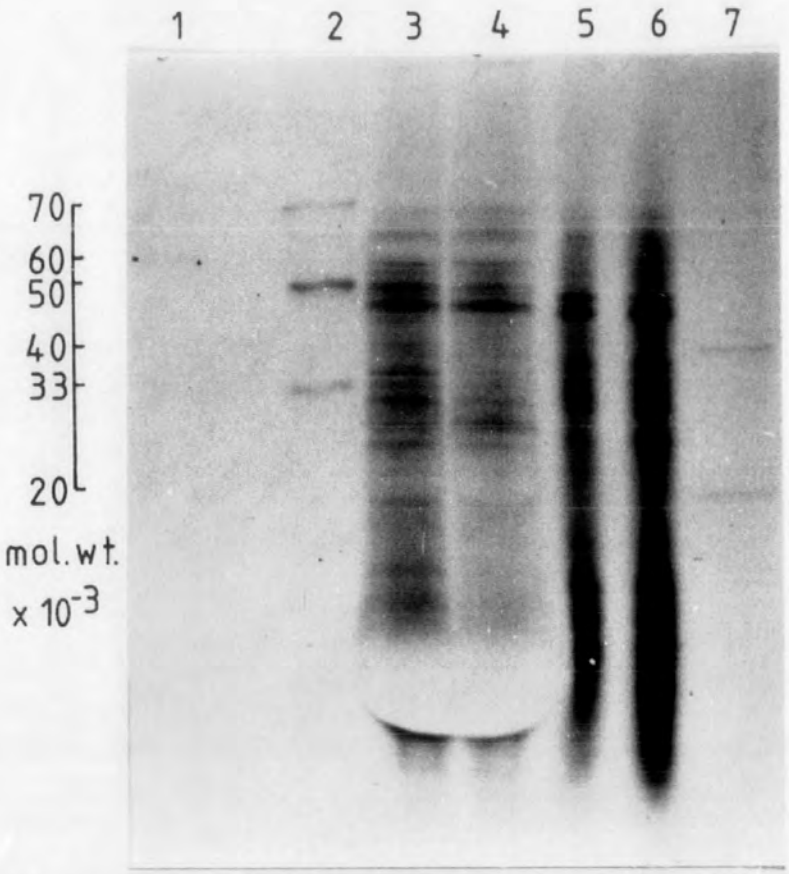


Fig. 24. A Comparison of Wheat-germ Translation Products and Reticulocyte Lysate Translation Products.

Total polysomes were translated in the cell-free systems using [^{3}H] leucine as the labelled amino acid.

Track 1, legumin standard (unreduced); track 2, standard vicilin; tracks 3 and 4, reticulocyte lysate products; tracks 5 and 6, wheat-germ products; track 7, reduced legumin standard. Tracks 3 and 5 are products of Kelvedon Wonder variety polysomes, tracks 4 and 6 are products of Mangetout polysomes.

Fig. 25. A Polyacrylamide Gel of Protein Bodies.

Samples of protein bodies were electrophoresed on a 17% P.A.G. and stained with Coomassie Blue.

Tracks 1 and 6, vicilin standards (25µg/track); tracks 2 and 3, reduced protein body samples (30µg and 15µg loaded); tracks 4 and 5, unreduced protein body samples (30µg and 15µg loaded).

Fig. 26. A Polyacrylamide Gel of Globulin and Albumin Extracts.

Samples of globulins and albumins extracted from 14 day pea cotyledons were electrophoresed on a 17% P.A.G., under reducing conditions, and stained with Coomassie Blue.

Tracks 1 and 7, vicilin standard (25µg/track); tracks 2 and 8, legumin standard (25µg/track); tracks 3 and 4, globulins extracted with and without Triton X-100 (37.5µg/track); tracks 5 and 6, albumins extracted with and without Triton X-100 (37.5µg/track). The presence of Triton X-100 did not affect the globulins extracted, but it appeared to destroy the albumin fraction.

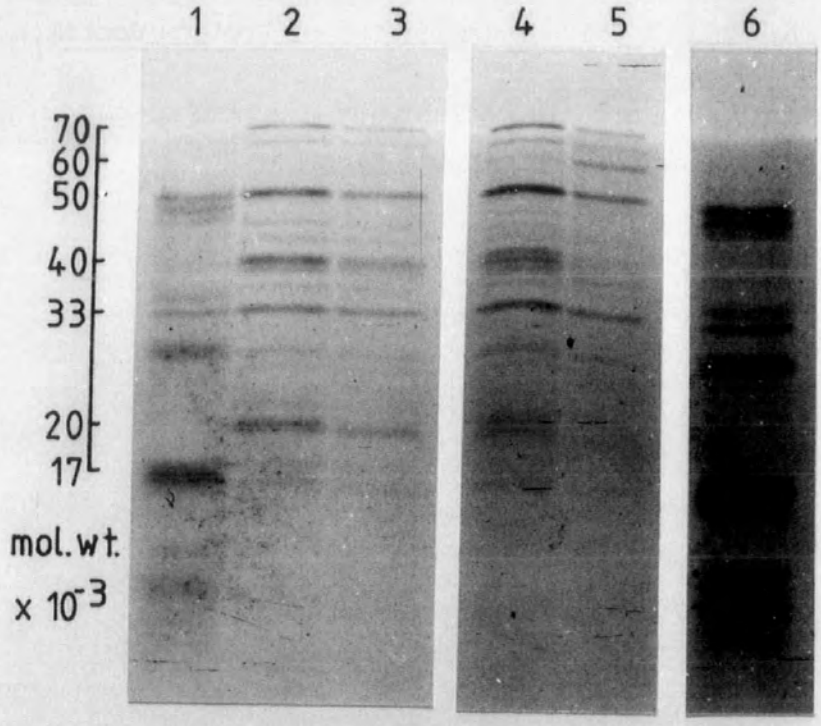

1
2
3
4
5
6
70
60
50
40
33
20
17
mol.wt.
x 10⁻³

Fig. 25.

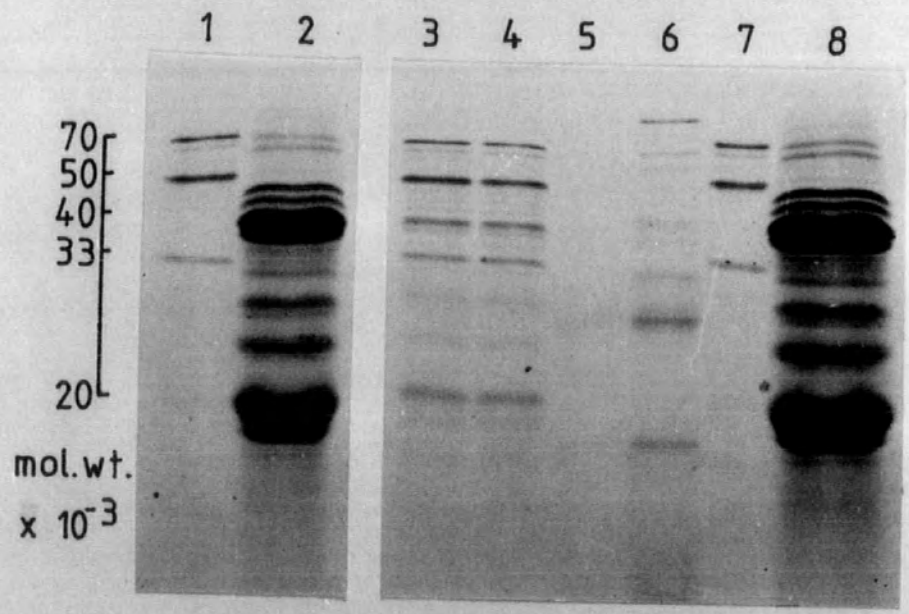

1
2
3
4
5
6
7
8
70
50
40
33
20
mol.wt.
x 10⁻³

Fig. 26.

The addition of protein bodies, or of the albumin fraction, to the different translation systems at different stages of incubation had no visible affect on the storage protein subunits produced *in vitro*, when either microsomes or polysomes were used as 'templates'.

2-3-4 Extended Incubation Times of the Translation Systems

Extending the incubation period of the different translation systems, with or without the addition of albumin, led to the production of many new polypeptide bands, including some which coincided with the 40,000 and 20,000 mol.wt. markers. However, the addition of D-threo-chloramphenicol to the systems resulted in only those polypeptides which are produced in 90 min incubations being produced, even after 22h.

2-4 Characterisation of Poly(A)$^+$-RNA

2-4-1 [^{3}H]cDNA Synthesis

When globin mRNA was used as a template for AMV reverse transcriptase 25% of the mRNA was transcribed into cDNA. This compared with 8%, 4% and 12% of 9 day, 14 day and 19 day poly(A)$^+$-RNA when these preparations were transcribed by the same enzyme. Each [^{3}H]cDNA sample had an activity of approximately 1.4×10^7 c.p.m. per μg cDNA.

2-4-2 S1-nuclease Activity

There was no significant difference in the efficiency of digestion of single stranded [^{3}H]cDNA by S1 nuclease when incubation times of 90 min or 30 min were used, or at an incubation temperature of 37^{o}C compared to 30^{o}C. However, the presence of carrier, single stranded, DNA did increase the efficiency of digestion of non-duplexed [^{3}H]cDNA; although there was no difference in effect when concentrations above 20μg/ml were used.

2-4-3 Hybridisations of [^{3}H]cDNA and Poly(A)$^+$-RNA

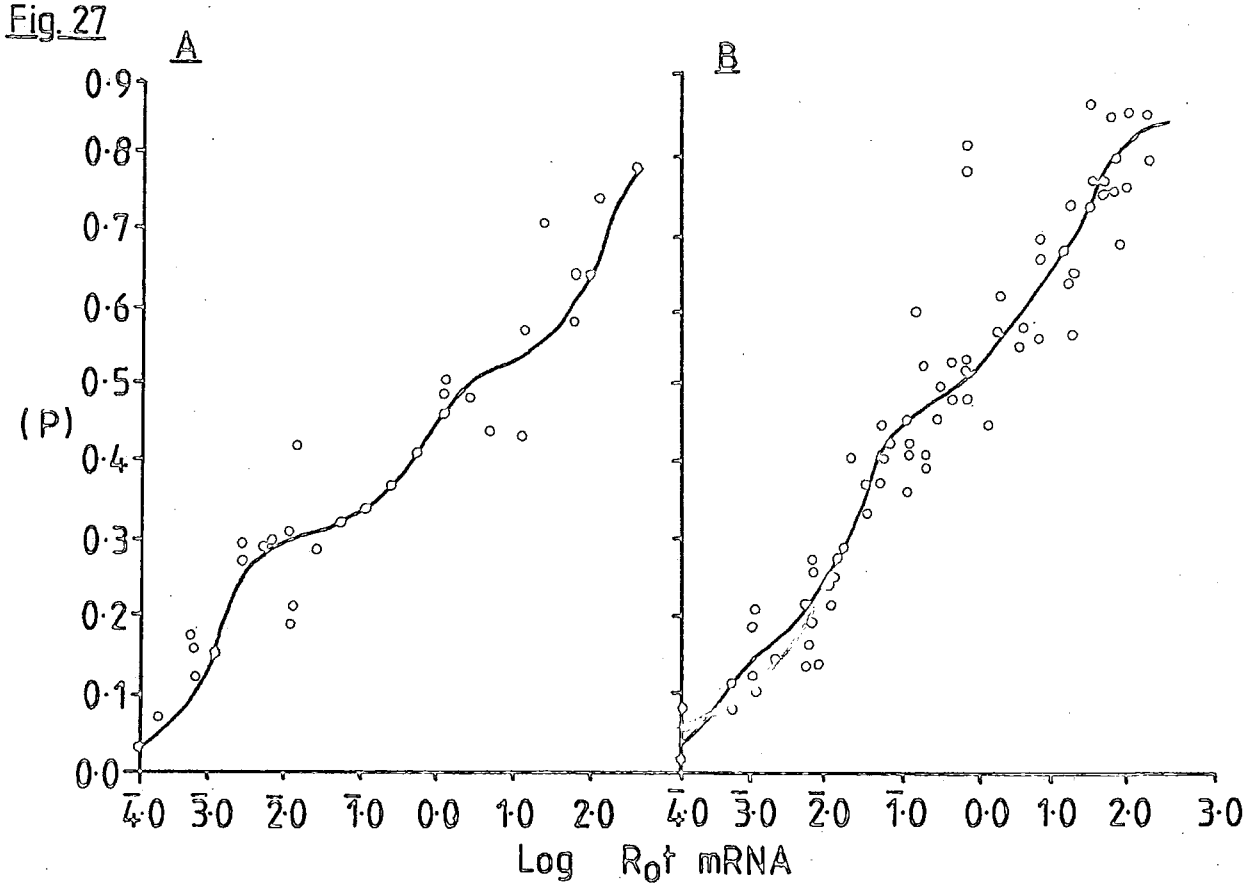
The results presented in Fig. 27 show the hybridisation curves of [^{3}H]cDNA with homologous poly(A)$^+$-RNA using 9 day, 14 day and 19 day materials; the standard curve from the hybridisation of globin mRNA and cDNA is also shown. The hybridisation values are corrected for self-annealing of the [^{3}H]cDNA, which was approximately 0.5-2.0%.

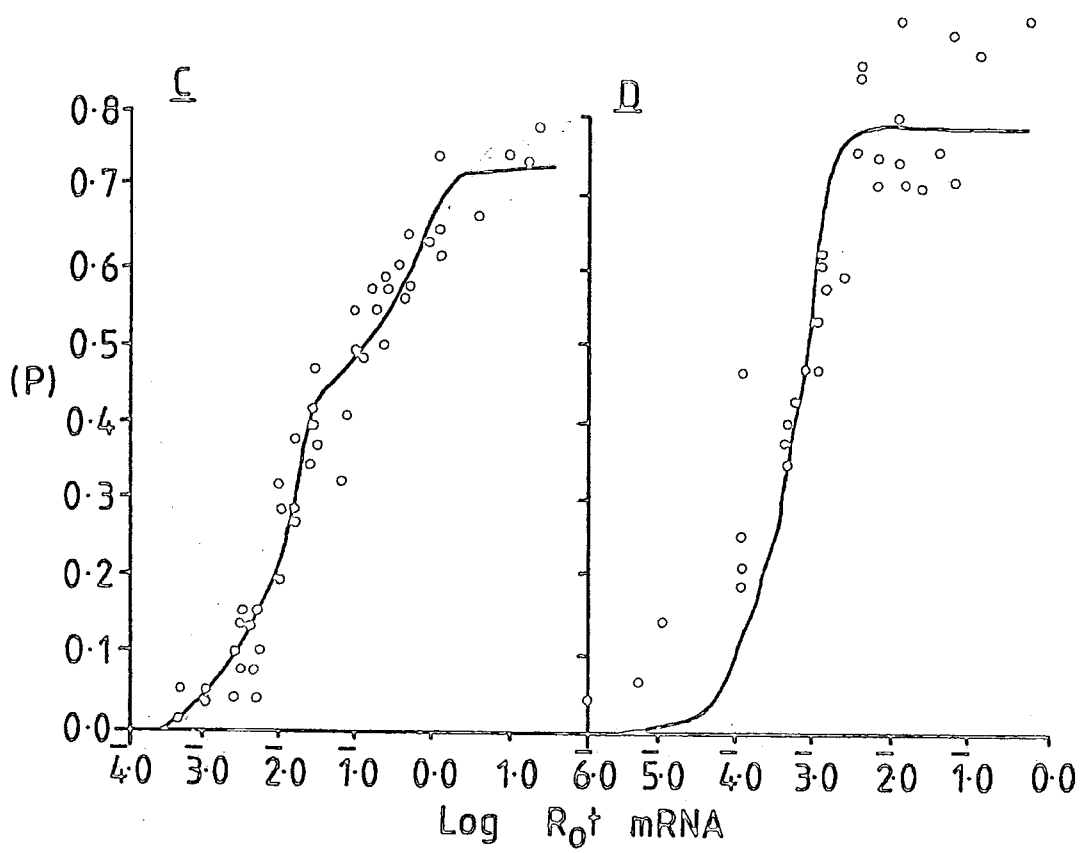
The standard curve spreads over 2.0 log units, and shows the hybridisation kinetics of a single mRNA species, which has a complexity of 1178 nucleotides (Efstratiadis et al. 1977). The $Rot_{\frac{1}{2}}$ value obtained for this mRNA was 4×10^4, a value which is similar to that obtained by Hastie and Bishop (1976).

Fig. 27. The Hybridisation of [^{3}H]cDNA to Poly(A)$^+$-RNA.

[^{3}H]cDNA synthesised from poly(A)$^+$-RNA, which was extracted from cotyledons of different ages, was hybridised to its homologous poly(A)$^+$-RNA. Nucleic acids were from A, 9 day old cotyledons; B, 14 day old cotyledons; and C, 19 day old cotyledons. D shows the hybridisation curve for Globin mRNA and Globin [^{3}H]cDNA (standard). The curves drawn are best fit curves computed from a least squares analysis of the data. (P) is the proportion of [^{3}H]cDNA hybridised.

Fig. 27


A
B
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
(P)
4·0 3·0 2·0 1·0 0·0 1·0 2·0
4·0 3·0 2·0 1·0 0·0 1·0 2·0 3·0
Log R0t mRNA


C
D
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
(P)
4·0 3·0 2·0 1·0 0·0 1·0
6·0 5·0 4·0 3·0 2·0 1·0 0·0
Log R0t mRNA

The hybridisation curves of the other samples cover between 4 and 6 log units, and therefore contain a mixtureof mRNA species in various concentrations. The number of abundance classes assumed to be correct was chosen by comparing the SSD (sums of squares of deviations) for increasing class numbers; if no improvement in SSD was gained by increasing the number of classes from x, that number of classes, (ie x), was assumed to be representative of the population.

The data obtained from the hybridisation curves ($Rot_{1/2}$ values and proportions of mRNA in each abundance class) was used in the calculations shown in Table 3, which were based on the approach of Goldberg et al.,(1981).

The hybridisation kinetics of polysomal poly(A)$^+$-RNA and homologous cDNA show that each developmental stage has a distinct distribution of poly(A)$^+$-RNA. The data for the 9 day old cotyledon poly(A)$^+$-RNA can be best divided into 3 abundance classes. The first contains 'less than 1' diverse RNA species in a 'very abundant' concentration and probably represents highly homologous sequences and degraded mRNA. The 'abundant' class represents 26% of the poly(A)$^+$-RNA mass and contains a calculated 78 different species of mRNA, while the 'rare' sequence class is large, representing 37% of the poly(A)$^+$-RNA mass and containing over 19,000 different mRNA species. The poly(A)$^+$-RNA from the 14 day old cotyledon is divided into 4 abundance classes, the first of which is a 'superabundant' class, probably analagous to the 'very abundant' class of the 9 day cotyledon poly(A)$^+$-RNA. The 'very abundant' class consists of only 6 diverse mRNA's, which account for 37% of the poly(A)$^+$-RNA mass, while the 'abundant' and 'rare' classes contain 124 and about 4,800 different species, respectively, which represent 16% and 30% of the poly(A)$^+$-RNA mass. The analysis of the data from 19 day old cotyledon poly(A)$^+$-RNA showed only 2 abundance classes to be present. The 'very abundant' class was similar to that of the 14 day poly(A)$^+$-RNA as it contained 6 diverse mRNA species, but it accounted for over 50% of the poly(A)$^+$-RNA mass, while the remainder of the poly(A)$^+$-RNA belonged to an 'abundant class' and contained 173 mRNA species.

Table 3 Abundance Classes in Poly(A)$^+$ -RNA from Different Aged Cotyledons

1		2	3	4	5	6	7	8	9
poly(A)$^+$ -RNA source	Class	Fraction labelled cDNA (P)	Fraction of RNA Mass	Rot 1/2 (A) M.sec	$K_{obs.}$ $M^{-1}sec$	K_{pure} $M^{-1}sec$	Complexity nucleotides	No. of diverse RNAs	No. of molecules $cell^{-1}seq$
9 day	1 (v.ab.)	0.291	0.372	0.0007	985.7	264.9	6.68×10^2	~1	2.6×10^6
	2 (ab.)	0.204	0.261	0.2323	2.97	11.38	1.56×10^5	78	7.7×10^3
	3 (rare)	0.287	0.367	41.48	0.017	0.046	3.85×10^7	19,300	4.4×10^1
14 day	1 (s.ab.)	0.138	0.167	0.0002	3450	20671	85	<1	4.5×10^7
	2 (v.ab.)	0.308	0.372	0.013	53.1	142.9	1.24×10^4	6	6.9×10^5
	3 (ab.)	0.133	0.161	0.600	1.15	7.16	2.47×10^5	124	1.5×10^4
	4 (rare)	0.249	0.301	12.622	0.055	0.183	9.65×10^6	4825	7.1×10^2
19 day	1 (v.ab.)	0.428	0.589	0.0075	92.0	156.3	1.13×10^4	6	6.4×10^5
	2 (ab.)	0.299	0.411	0.3273	2.108	5.124	3.45×10^5	173	1.5×10^4

Calculations are based on data from Fig. 27 using the methods of Goldberg et al. (1981).
s.ab. - superabundant; v.ab.- very abundant; ab. - abundant

(1) Age of the cotyledon from which the poly(A)$^+$-RNA was isolated.

(2) The fraction of [^{3}H]cDNA hybridised in each class. This is the value P from the computed curve.

(3) The fraction of poly(A)$^+$-RNA mass hybridised - ie (2) normalised to 100% cDNA reactivity.

(4) $Rot_{1/2}$ for each class - a value computed from the kinetic curve.

(5) K observed. The pseudo first-order rate constant, where $K = \frac{0.69}{Rot_{\frac{1}{2}}} M^{-1}sec^{-1}$.

(6) K pure. The pseudo first-order rate constant, if the abundance class was 100% pure. K pure = $\frac{Kobs}{(3)}$

(7) Complexity = $\frac{Kglobin \times Cglobin}{Kpure} \times \left(\frac{L}{600}\right)^{1/2} \times \left(\frac{L}{M}\right)^{1/2}$

The standard curve obtained for rabbit globin mRNA had a $Rot_{1/2}$ of 0.0004 M.sec. The pseudo first-order rate constant, K, was $1725 M^{-1}sec^{-1}$. The complexity of rabbit globin mRNA is 1178 nucleotides (Efstratiadis et al., 1977). The last 2 terms of the equation correct for differences in lengths of cDNA and poly(A)$^+$-RNA, which affects reaction rates. (Globin cDNA was assumed to havea complexity of 600 nucleotides, Goldberg et al.,(1978). L, the modal cDNA size was 3.1×10^5 mol.wt. approximately 954 nucleotides; M, the number-average poly(A)$^+$-RNA size at the beginning of hybridisation, was 6.5×10^5 mol.wt., approximately 2,000 nucleotides (Evans et al. 1980).

Legend to Table 3 continued

(8) Number of diverse poly(A)$^+$-RNAs = $\frac{\text{Complexity of the abundance class}}{N}$

where N is the number-average polysomal poly(A)$^+$-RNA size, approximately 2,000 nucleotides (Evans et al., 1980).

(9) Number of molecules per cell per sequence is the number of nucleotides in an abundance class per cell, divided by the complexity of that abundance class.

$$\text{ie No. molecules cell}^{-1}\text{sequence}^{-1} = \frac{(Fn) \times (g \text{ poly(A)}^+\text{-RNA cell}^{-1}) \times (6\times10^{23} \text{ nucleotides mole}^{-1})}{(Cn) \times (325g \text{ mol}^{-1} \text{ nucleotides})}$$

The prefix n refers to the abundance class, F is the fraction of poly(A)$^+$-RNA mass (3), C is the complexity of the class expressed in nucleotides (7), g poly(A)$^+$-RNA cell^{-1} was 2.5×10^{-12}g for 9 day old cotyledons, 12.4×10^{-12}g for 14 day old cotyledons and 6.7×10^{-12}g for 19 day old cotyledons, (Gatehouse et al., 1982). Avogadro's number divided by the approximate mol.wt. of nucleotides gives the number of nucleotides per gramme.

The overall picture of the change in poly(A)$^+$-RNA distribution during development was of an increase in the number of mRNAs present in the 'very abundant' class from zero to about 6, an increase in numbers of copies per cell of these molecules, and a concomitant decrease in 'rare' species of poly(A)$^+$-RNA.

The results of heterologous hybridisations between 9 day and 14 day poly(A)$^+$-RNA and [^{3}H]cDNA are shown in Fig. 28, and the calculations from them in Table 4. Eighty-two percent of 9 day [^{3}H]cDNA was hybridised to 14 day poly(A)$^+$-RNA, relative to the homologous reaction of 14 day material, whereas 92% of 14 day [^{3}H]cDNA hybridised to 9 day poly(A)$^+$-RNA, relative to the 9 day homologous hybridisation. The 9 day cDNA: 14d poly(A)$^+$-RNA reaction was initially about 10 times slower than the reciprocal reaction which reflects a difference in the 'very abundant' and 'abundant' poly(A)$^+$-RNA classes, ie the 9 day material contained fewer diverse species of poly(A)$^+$-RNA, in fewer numbers, than did the 14 day material.

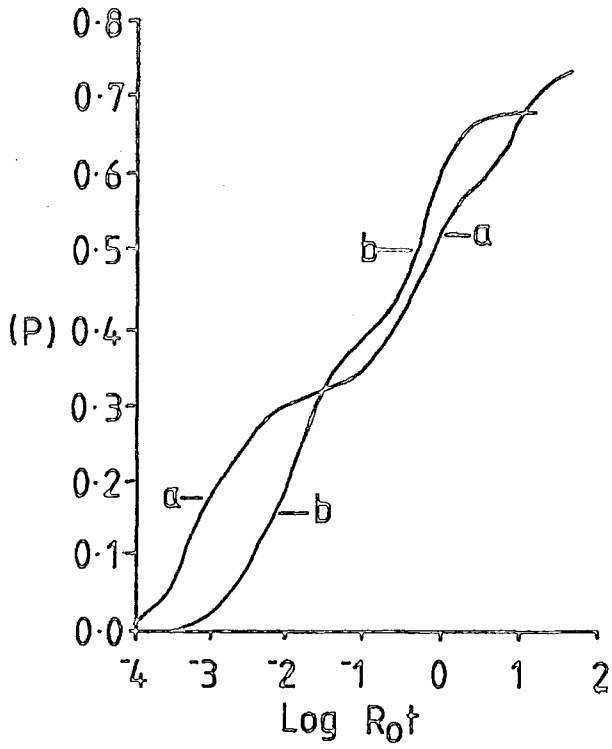


Fig. 28. Heterologous Hybridisations of [^{3}H]cDNA to Poly(A)$^+$-RNA.

(P) is the proportion of [^{3}H]cDNA hybridised. The lines were drawn from a best fit curve for the data analysed by a least squares fit programme. (Original data points were omitted for clarity, but were of a similar distribution to those of Fig. 27.)
(a) [^{3}H]cDNA synthesised from 14 day poly(A)$^+$-RNA hybridised to 9 day poly(A)$^+$-RNA.
(b) the reciprocal reaction (ie 14 day poly(A)$^+$-RNA and 9 day [^{3}H]cDNA).

Table 4 The Analysis of Heterologous Hybridisations between Poly(A)$^+$-RNA and [^{3}H]cDNA from 9 day and 14 day cotyledons

poly(A)$^+$-RNA and [^{3}H]cDNA	Class	Fraction of labelled cDNA (P)	Fraction of poly(A)$^+$-RNA mass	$Rot_{1/2}$ mol.sec. (A)
9 day poly(A)$^+$-RNA and 14 day cDNA (a)	1	0.295	0.321	0.0009
	2	0.239	0.260	0.2562
	3	0.199	0.216	5.1797
14 day poly(A)$^+$-RNA and 9 day cDNA (b)	1	0.326	0.40	0.0063
	2	0.351	0.43	0.3875

Calculations are based on data from Fig. 28. The fraction of poly(A)$^+$-RNA mass was calculated relative to the hybridisation of the homologous poly(A)$^+$-RNA and [^{3}H]cDNA.

2-5 Comparison of Nuclear Nucleic Acids to Cytoplasmic Poly(A)$^+$-RNA

2-5-1 Single-copy DNA

When [^{32}P] end labelled sc DNA was hybridised to a high Rot value, with a vast excess of 14 day poly(A)$^+$-RNA, 5% of the [^{32}P] scDNA formed duplexes, Fig. 29.

2-5-2 Nuclear RNA

Approximately 0.07% of nuclear poly(A)$^+$-RNA template was transcribed into [^{3}H]cDNA by AMV reverse transcriptase. When [^{3}H]cDNA was hybridised to 9 day polysomal poly(A)$^+$-RNA at a high Rot value, approximately 50% of the [^{3}H]cDNA formed duplexes with the poly(A)$^+$-RNA, Fig. 30. This value was corrected for self annealing of the cDNA, which was approximately 13%.

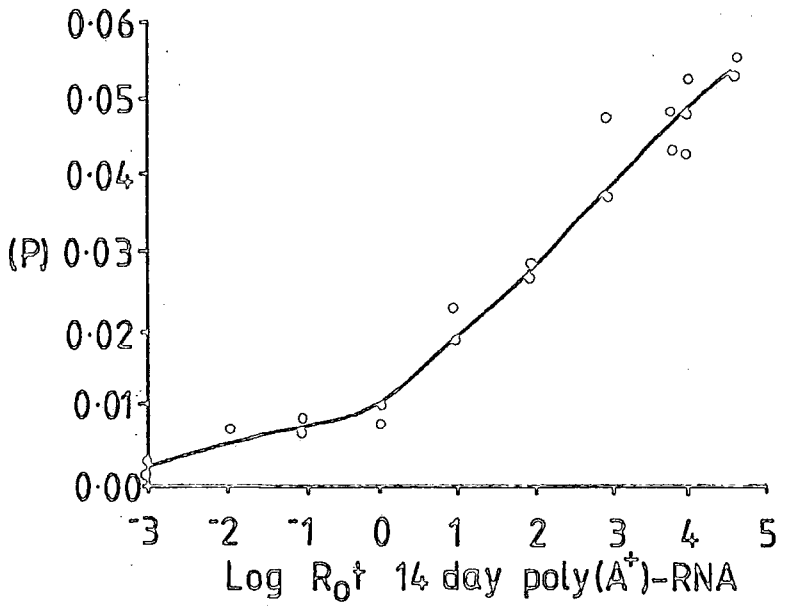


Fig. 29. The Hybridisation of scDNA to Poly(A)$^+$-RNA.

(P) is the proportion of [^{32}P]scDNA which has been hybridised to 14 day poly(A)$^+$-RNA.

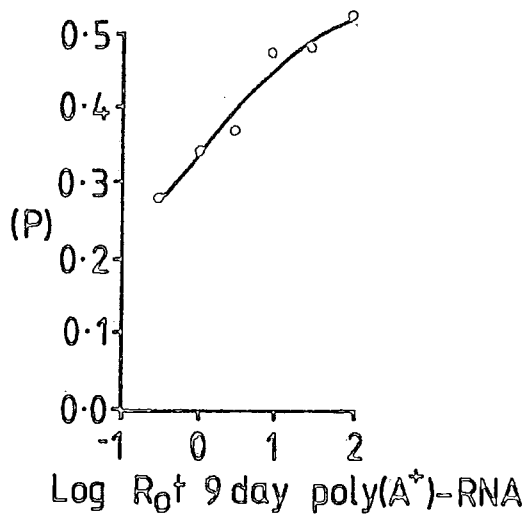


Fig. 30. The Hybridisation of Nuclear[^{3}H]cDNA to 9 day Poly(A)$^+$-RNA.

[^{3}H]cDNA synthesised from nuclear poly(A)$^+$-RNA was hybridised to polysomal poly(A)$^+$-RNA extracted from 9 day cotyledons. (P) is the proportion of cDNA duplexed.

3. DISCUSSION

DISCUSSION

The use of cell-free protein synthesising systems to examine the products of mRNA translation is a technique frequently employed in both plant and animal research, (Mans, 1967, Allende, 1969, Boulter, 1970, Taylor, 1979, and Shields et al., 1981). Cell-free systems enable components of protein synthesis to be manipulated more readily than do whole cell systems, where rate of uptake of an added component has to be accounted for and so have advantages in elucidating mechanisms. However, the possible removal of cell regulatory mechanisms, (eg. involved in initiation, elongation, and termination of the polypeptide), could alter the polypeptide products and therefore careful comparison with the *in vivo* situation must be made before conclusions can be drawn from *in vitro* results. The use of polysomes rather than purified mRNA to drive cell-free systems ensured that the translation products reflected polypeptides being actively synthesised in the cell at the time of their extraction, assuming that the extraction procedures did not select any particular population of polysomes; although it is possible that centrifugation of polysomes through a sucrose pad, an essential step in the preparation, reduces the number of smaller polysomes being pelleted, (Larkins and Davies, 1975). This would result in the *in vitro* translation products containing relatively more high mol.wt. polypeptides than *in vivo*. As the major storage protein subunits of pea are of relatively high mol.wt., this problem probably did not directly affect the results of this study of their synthesis.

The four translation systems used had a very low endogenous protein synthesising ability, indicating that the large number of polypeptides synthesised, on addition of the various templates were the products of the exogenous message. It has been reported, (Senger and Gröss, 1976), however, that the addition of exogenous templates stimulated the translation of endogenous message, so, clearly a comparison of products of four systems was valuable, (a point also mentioned by Hall, 1979), and demonstrated that the major translation products of each template were common to all systems and therefore were due to the translation of the exogenous template. The protein synthesis measured in the systems over the optimal incubation period was not due to bacterial contamination, as

the addition of D-threo-chloramphenicol, (a gift from J. Tuffnel, which was known to inhibit bacterial protein synthesis at the concentrations used, Ellis, (1969), Rendi and Ochoa, (1962) and Pestka (1971b).), had no effect on the incorporation of radioactivity into polypeptides or on the composition of polypeptides produced. However, some protein synthesis during long incubations (>10h), especially in the presence of membrane fractions, (ie. microsomes and protein bodies), was due to prokaryote contamination, and so it was necessary to include D-threo-chloramphenicol in these incubation "mixes". Wilson, (1966), and Burr and Burr, (1976), also reported that bacterial contamination and ribonuclease (RNase), activity in cell-free systems was associated with protein bodies of maize. Wheeler and Boulter, (1966), noted that bacterial contamination was associated with Vicia faba protein bodies; this was probably because bacteria and protein bodies were of similar density and therefore are sedimented together, (Mans, 1967).

The question that arose next was whether in vitro synthesised polysome translation products represented only 'run-off' polypeptides (ie. completion of polypeptides in vitro which were initiated in vivo before the polysomes were extracted), as suggested by Higgins and Spencer, (1980), or whether initiation of protein synthesis occurred on the mRNA of the polysomes in the cell-free system, so that translation products contained both 'run-off' and 'initiated' polypeptides. Re-initiation in the cell-free systems, would mean that the in vitro translation products did not solely reflect polypeptides being synthesised in the cell, but also reflected the 'in vitro' environment with respect to the initiation rates of the cell-free system, (which may well be different from in the cell, ie. may be selective towards a specific mRNA).

Poly(A)$^+$-RNA (isolated by oligo(dT) chromatography, Aviv and Leder, 1972, Nakazato and Edmonds, 1972), was translated in the wheat-germ, reticulocyte lysate, and pea axes system, indicating that initiation occurs in these systems, and that the necessary initiation factors (shown by Yarwood et al., 1971, Zalik and Jones, 1973, and Wells and Beevers, 1973 and 1974, to include methionyl-tRNA, proteinaceous factors which associate with ribosomes, and GTP), must be available in

the systems in order to enable the process to continue. Re-initiation of protein synthesis on mRNA of polysomes should therefore also be possible unless the initiation factors, (eIF-2 was found to be important in the regulation of initiation, Jagus et al., 1981), or mRNA were degraded, or the energy available used, during elongation of the 'run-off' products.

Aurintricarboxylic acid (ATA) is a triphenylmethane dye which prevents initiation of protein synthesis in eukaryotic systems when present in low concentrations (10^{-3}M or less), (Pestka 1971a and b). The mechanism of the inhibition is unknown, but it is thought that the attachment of mRNA to the 40s ribosomal subunit is prevented (Huang and Grollman, 1972), possibly by the allosteric action of the ATA, or by neutralisation of negatively charged phosphates on the nucleic acids (Gonzalez et al., 1980, Grollman and Stewart, 1968). At higher concentrations, (>10^{-3}M), ATA also inhibits the elongation of the nascent polypeptide chains, (Huang and Grollman, 1972, Siegelman and Apirion, 1971). The level of inhibition is also dependent on the number of ribosomes present, (the addition of ribosomes can overcome the inhibitory effects), and on the Mg^{2+} concentration (Grollman and Stewart, 1968). The fact that commercial ATA contains inactive, isomeric impurities has been well documented (Stewart et al. 1971, Gonzalez et al., 1980), and this results in variation in the absolute active concentration of ATA from batch to batch; unfortunately the purified form of ATA is unstable, (Huang and Grollman, 1972).

The concentration of ATA necessary to prevent initiation of poly(A)$^+$-RNA in the wheat-germ system (10^{-4}M), was comparable to the concentration Huang and Grollman, (1972), found necessary to inhibit the initiation of globin synthesis on mRNA in the reticulocyte lysate system, although it was higher than the concentration that Marcus et al., (1970), found necessary to inhibit poly(A)$^+$-RNA in the wheat-germ system (2.5×10^{-5}M). At this concentration of ATA, (10^{-4}M), re-initiation of protein synthesis on polysomal mRNA should also be inhibited, but elongation of the already initiated polypeptides should continue, so only 'run-off' products would be synthesised. These will

reflect the polypeptides which were actually being synthesised in the cotyledon cells, and are not affected by the inability of the cell-free system to initiate protein synthesis. The difference in numbers of ribosomes, and in the Mg^{2+} concentrations, between the polysome and poly(A)$^{+}$-RNA driven systems were unavoidable, as the different Mg^{2+} concentrations were important in preventing premature termination of the polypeptides during synthesis, (Tse and Taylor, 1977).

Using this approach it was estimated that 25% of protein synthesis conducted by polysomes in the wheat-germ system was a consequence of re-initiation of the mRNA. This was comparable with results obtained by Sun et al., (1975), who estimated that 29% of in vitro protein synthesis in wheat-germ, driven by polysomes of Phaseolus vulgaris, was due to re-initiation and also with the results of Lebleu et al., (1970), who found that 20% of polysome globin synthesis in the reticulocyte lysate system was due to re-initiation. However, Bollini and Chrispeels, (1979), found that initiation was not involved with the synthesis of P. vulgaris storage proteins when using polysomes in their wheat-germ system, and ATA at 10^{-4}M. Beachy et al, (1978), stated that 10^{-4}M ATA had no effect on the translation of soyabean polysomes in wheat-germ system, and Higgins and Spencer, (1977), working with peas, found the wheat-germ system insensitive to ATA, but they used a concentration of 2×10^{-5}M. Jagus et al., (1981), stated that little initiation occurred in nucleated cell-free extracts. These differences were probably due to the impurity of ATA, and the number of ribosomes present in the systems as well as to possible differences in the wheat-germ preparations used. They could also be explained by the fact that optimal conditions for elongation of polypeptides in vitro were different to those required for initiation, as reported by Algranati, (1980), in relation to Mg^{2+} concentrations in the wheat-germ system, and it is possible therefore that the conditions used by some authors may have actually inhibited initiation.

The large decrease in protein synthesis at ATA concentrations above 10^{-4}M, in the wheat-germ system, was due to inhibition of polypeptide elongation, leading to premature termination of polypeptides; this was

confirmed by the presence of additional low mol.wt. polypeptides seen in the translation products of cell-free systems containing 10^{-3}M ATA, (Fig. 14).

Re-initiation also occurred in the pea axes and cotyledon systems, (representing about 34% and 44% of the polypeptides translated), and as these were homologous systems the correct initiation factors were likely to be present. Peumans et al., (1980), reported the inhibition of initiation of protein synthesis on mRNA in the pea axes system at 10^{-4}M ATA.

Only a limited amount of initiation can occur in a cell-free system because certain essential factors are depleted during incubation; however, elongation of the polypeptide continues after initiation has stopped. The fact that initiation in cell-free systems decreased with incubation time was also noted by Jagus et al., (1981). This enabled the length of time during which initiation occurred <u>in vitro</u> to be estimated. Each of the cell-free systems were incubated for varying time periods either at 30°C, (normal incubation temperature), or at 0°C, then ATA was added and incubation of the sample continued at 30°C. When the uninhibited incubation step was at 30°C initiation proceeded, and the exhaustable factors were depleted, consequently the longer this step the lower the remaining capacity for initiation in the system and therefore the less effect the addition of ATA had on the subsequent incubation step. When initiation was completed, (ie. the necessary factors were depleted), during this first incubation step the addition of ATA had no effect on the final level of protein synthesis, when compared to control experiments (ie. no ATA added). By comparison, because initiation was retarded when the first incubation was at 0°C, most of the initiating capacity of the system was present at the beginning of the second incubation step, and therefore the addition of ATA inhibited the level of protein synthesis, regardless of the length of time of this first incubation step.

Initiation of protein synthesis by mRNA in the wheat-germ system occurred mainly during the first 25 min of incubation, while re-initiation of polysome driven protein synthesis in the axes and cotyledon systems mainly occurred during the first 15 min of incubation, (at 30°C).

Re-initiation of polypeptide synthesis (accounting for about 20% to 45% of total protein synthesis), occurred in the reticulocyte lysate system during the first 10 min of incubation at 30°C; however this system required a higher concentration of ATA, (10^{-3}M), to cause inhibition of protein synthesis than did the other systems. (Initiation of poly(A)$^{+}$-RNA protein synthesis was completely inhibited by concentrations of ATA between 2x 10^{-4}M and 10^{-3}M, Evans personal communication). This may be a consequence of the Mg^{2+} concentration or of the animal origin of the system. It was unlikely that elongation of the polypeptides was affected at this concentration of ATA, (as was suspected initially), because the inhibitor had no effect on the final level of protein synthesis in this system if added after 10 min of incubation, (at 30°C), when elongation was proceeding, (which continued for a further 50 min).

Re-initiation of protein synthesis on mRNA from pea cotyledons occurred in the 4 systems used; comparisons of 'run-off' and total products, however suggested little difference in the basic patterns of storage protein sub-units produced, but a difference in the overall quantities synthesised. As more storage protein polypeptides were synthesised when re-initiation occurred it was beneficial to allow this process to continue. The ability of the cell-free system to re-initiate protein synthesis also emphasised the extent to which the in vivo situation can be simulated.

The translation products of the cell-free systems were shown to contain authentic polypeptides of pea storage proteins, namely the legumin 60,000 mol.wt. subunit, the vicilin 50,000 and 47,000 mol.wt. subunits and convicilin 70,000 mol.wt. subunit, (Croy et al., 1980c). This was demonstrated by immunoprecipitation of the polypeptides by

antibodies raised against 'native' storage proteins, (a method of identification which is commonly used, eg Graham and Gunning, 1970, Blagrove and Gillespie, 1975, and Greene, 1981), and by the similar mobilities of immunoprecipitates and native subunits during polyacrylamide electrophoresis.

A degree of cross reaction of antibody:polypeptide, (ie. antivicilin precipitating legumin and vice versa), was detected in some instances, (Graham and Gunning, 1970, also reported cross contamination of bean storage proteins), this was probably due to contamination of the original proteins used to raise antibodies. This problem was noted by Millerd, (1975), who stated that some vicilin subunits of pea remained associated with legumin even after several purification steps. Jackson et al., (1969), reported that considerable portions of amino acid sequences of vicilin and legumin were similar, this could lead to cross reactivity of antibodies. However this was using less sophisticated purification methods than those now available and the generally held view is that the vicilin and legumin have no antigenic determinants in common. It has been reported that of a variety of immunoprecipitation techniques used none were 100% satisfactory, (Evans et al., 1979, Spencer et al., 1980); Taylor, (1979), claimed that nonspecific immunoprecipitation was proportional to the bulk of the immunoprecipitate, while Higgins and Spencer, (1977) explained non-specificity as being a result of using mature, fully modified, proteins to manufacture the antibodies, while *in vitro* synthesised polypeptides were likely to be in a precursor form.

However, the immunoprecipitation method employed here involved a refinement of that used by Evans et al., (1979), (ie. the attachment of the antibody:polypeptide complex to sepharose beads to enhance precipitation, a method also used by Shapiro et al., 1974, and 1975.), and provided satisfactory results for both *Pisum* and *Vicia faba* storage protein subunits. *In vitro* synthesised polypeptides do, therefore, have similar antigenic properties to those of their mature storage protein subunits synthesised *in vivo*. Schechter, (1974), also found that, in mouse myeloma cells, polypeptide precursors were recognised by antibodies raised against 'mature' proteins, if the polypeptide sequence was long enough to allow some degree of conformation to be obtained.

The labelling of native storage proteins by acetylation (to provide labelled standard proteins), did not appear to affect the mobility of the polypeptides during polyacrylamide gel electrophoresis, however occasionally there was a lack of coincidence between *in vitro* and standard polypeptides, especially if electrophoresis was over a long time period, this could be explained by unavoidable differences within gels. Slight differences in the secondary structure of *in vitro* polypeptides compared to native polypeptides, perhaps caused by lack of glycosylation or phosphorylation *in vitro*, could also cause a difference in mobility which would be amplified during longer electrophoresis times. Matthews et al., (1981), reported that phaseolin (the storage protein of *Phaseolus vulgaris*) was not glycosylated when synthesised in the wheat-germ cell-free system. Hall et al., (1980), also noted the inability of the wheat-germ or reticulocyte lysate systems to glycosylate polypeptides. An excess of protein in a sample caused distortion of polypeptide bands on polyacrylamide gels, this was clearly seen in microsome translation products where the 50,000 mol.wt. polypeptide was slightly displaced, probably by unlabelled membrane associated proteins. Although precise coincidence of native and *in vitro* synthesised polypeptides could be achieved, (Fig. 1), it was the immunoprecipitation of the polypeptides by specific antibodies which actually identified the polypeptides as being related to the storage proteins.

The apparent similarity of the *in vitro* translated polypeptides of the reticulocyte lysate system, to those of native storage proteins was confirmed by experiments in which polypeptides were specifically cleaved by cyanogen bromide (CNBr), a method also used by Peeters et al., (1979). Under the acidic conditions employed, CNBr cleaved polypeptides at methionine sites, (Gross, 1967). The cleavage products of tritiated *in vitro* translation products were of similar mol.wt. to some of the cleavage products of native storage proteins, which strongly implies that the original arrangement of methionine in each was the same. The native 33,000 mol.wt. subunit was uncleaved, as was the 50,000 and the 47,000 mol.wt. polypeptide of the *in vitro* products, (mature 50,000 mol.wt. vicilin does not contain methionine, Croy et al., 1980c), this weakly supports the idea from the *in vivo* labelling studies of Croy et al., (1980c), and Gatehouse et al., (1981), that the native 33,000 mol.wt.

polypeptide and the *in vitro* synthesised 47,000 mol.wt. polypeptide are related and that a post-translational modification step, (about 4h after synthesis), cleaves the 47,000 mol.wt. polypeptide producing a 33,000 mol.wt. subunit. Clearly there must not be any internal methionine sites available for cleavage in these polypeptides. The loss of radioactive label from *in vitro* synthesised polypeptides containing [^{35}S] methionine, as a consequence of CNBr treatment, also indicated that a majority of the methionine sites were near the ends of the polypeptides and were consequently released as very small fragments, which were not detected by PAGE. Smith and Ellis, (1979), suggested that the loss of labelling with [^{35}S] methionine, due to CNBr cleavage, in a low methionine polypeptide indicates the methionine is located in the 'signal sequence', but it could be just the terminal amino acid as, so far as is known, all eukaryotes synthesise proteins with N-terminal methionine, which is often subsequently removed, (Lodish et al., 1971).

Trypsin digestion of *in vitro* synthesised and native proteins produced polypeptides of similar mol.wt. in both cases, which reinforced the proposal that the *in vitro* synthesised and native storage protein polypeptides were of a similar composition. In both trypsin digestion and CNBr cleavage experiments the native products contained more polypeptides than did the *in vitro* products, possibly because native products had been subjected to post-translational modifications and so some polypeptides were subtly different in minor aspects of composition. The heterogeneity of native legumin and vicilin of pea has been reported by Croy et al., (1980a,b,c), Gatehouse et al., (1980), and Thomson et al., (1978). Soyabean storage protein also appeared to be heterogenous, (Hill and Breidenbach, 1974), as did the storage proteins of *Vicia faba*, (Wright and Boulter, 1972, Bailey and Boulter, 1972, Krishna et al., 1979, and Casey, 1979).

The 60,000 mol.wt. subunit of legumin, which was produced in cell-free systems, did, however, differ from the native subunit in one important aspect, which was that under reducing conditions it did not break down to give a 40,000 and a 20,000 mol.wt. polypeptide. The native subunit was composed of these two polypeptides linked by a disulphide

bond, which was readily reduced in the presence of 2ME, (Croy et al., 1980a). Similar results were obtained with *in vitro* synthesised *Vicia faba* legumin, (Croy et al., 1979). Tumer et al., (1981), also described this phenomenon in soyabean translation products and concluded that the 57,000 mol.wt. polypeptide was a long-lived precursor to the 40,000 and 20,000 mol.wt. polypeptides, which was cleaved post-translationally, following disulphide linkage; however, Beachy et al., (1978), claimed to demonstrate the cleavage process *in vitro*, but only used coincidence of polypeptides during PAGE and not immunoprecipitation to substantiate their results, as did Higgins and Spencer, (1977), when working with legumin of peas. There are other examples of post-translational modifications occurring slowly, *in situ*, e.g. Dure and Galau, (1981), showed that the cottonseed 60 kilodalton subunit produced a 52 kilodalton subunit in this manner, while Roberts and Lord, (1981a and b), demonstrated that the cleavage of the 66-69,000 mol.wt. subunit of castor bean agglutin produced subunits of 31-37,000 mol.wt., slowly, as a post-translational event.

Post-translational cleavage of the *Pisum* legumin 60,000 mol.wt. subunit, however, was undetected in the cell-free systems driven either by polysomes or microsomes even when supplemented with varying concentrations of protein bodies isolated from 19 day old cotyledons. The protein bodies contained legumin in the reducible form so the modification process must have occurred before or during deposition of the polypeptide in the structure, and Croy et al., (1980a), using *in vivo* labelling techniques, showed the presence of a 60,000 mol.wt. legumin 'precursor' in mature pea cotyledons as well as processed 40,000 and 20,000 mol.wt. subunits. It can be concluded that either the necessary enzyme or cofactor was not present in protein bodies, or their membranes, or that conditions in the cell-free systems were unsuitable for the modification to occur. Possibly '19 day old' protein bodies have a lower ability to process legumin, than perhaps '14 day old' protein bodies, because they are approaching maximum size; it would be interesting to investigate the use of 'younger' protein bodies in these systems, however, extraction is difficult because of their lower density.

It was also possible that the mechanism which modifies the legumin polypeptide was located in the cytosol, rather than in the membrane fraction. Smith and Ellis, (1979) and Dobberstein et al., (1977) found the

small subunit precursor of ribulose bisphosphate carboxylase of pea and Chlamydomonas were post-translationally modified by a cytosol enzyme, and Langridge, (1981), found that the large subunit of spinach chloroplast, (which is coded for and synthesised in the chloroplast), was converted to its native form in the presence of a water soluble chloroplast extract and not the thylakoid membrane. However, as the addition of albumin fractions to the reticulocyte lysate translation system had no effect, and translation using polysomes or microsomes in either a 'total' extract of pea cotyledons, or a 'purified' pea cotyledon system, did not alter the legumin subunit synthesised, it would seem that the processing mechanism was not active in the in vitro conditions.

Microsomes did not provide an efficient template for protein synthesis, compared to polysomes, this was possibly due to the presence of RNases and proteases. Ryan, (1973), described the presence of endopeptidases and peptide hydrolases in association with protein bodies of a variety of seeds; protein body fragments were present in the pea cotyledon system, however protein synthesis in this system was more probably limited by RNases, rather than proteases, as the time course of incorporation of amino acids into polypeptides plateaued when protein synthesis ceased, indicating no significant degradation of polypeptides. Kamine and Buchanan, (1978), and Larkins and Hurkman, (1978), also mentioned an inhibitory effect of membranes on in vitro protein synthesis. It would be worthwhile modifying this system, so that microsomes could provide an efficient template, as it seems probable that the processing legumin follows a temporal sequence of events which would be sensitive to the presence and concentration of various 'factors' (eg. enzymes, cofactors, membrane sites), and which would be difficult to maintain in a heterologous system. The conformation of the legumin polypeptide could also be an important factor in its subsequent modification, Kreil, (1981), mentioned the interaction of a completed polypeptide with a membrane during post-translational modification could involve any part of the molecule, not just the terminal region, so the 3 dimensional structure of the polypeptide could be critical; Lingappa et al., (1980), demonstrated that the ovalbumin signal sequence was in the middle region of the molecule. Hershko and Fry, (1975), working on viral polypeptides, noted that specific scissions of the polypeptides depended on the conformation of the polypeptide as did the correct sequential assembly of the viral components.

The translation of pea storage protein mRNA in Xenopus oocytes (a translation system developed by Gurdon et al., (1971), would be interesting as the system can continue protein synthesis for long time periods, (days), without bacterial contamination, and would therefore possibly allow post-translational as well as co-translational modification to proceed. Larkins et al., (1979), showed that zein, (the storage protein of maize), synthesised in this system was transported into vesicles where it remained stable; Matthews et al., (1981), demonstrated that phaseolin, (the storage protein of Phaseolus vulgaris), was synthesised, processed, (glycosylated), and localised in membrane fractions when mRNA was translated in oocytes. If transport and modification of legumin could be demonstrated in vitro using the oocyte system, the factors involved in the process would be non-specific, if specific enzymes were necessary, the modification would not occur in a heterologous system.

When cell-free systems were incubated for long periods (>10h) without D-threo-chloramphenicol, polypeptides were produced which coincided with the 40,000 and 20,000 mol.wt. subunits of legumin. It is feasible that the bacteria contaminating the system produced enzymes capable of modifying the legumin, so, on reduction of the disulphide bond the two components separated.

A comparison of polysomal translation products of the wheat-germ and the reticulocyte lysate systems revealed differences between the vicilin 50,000 and 47,000 mol.wt. polypeptides of the two systems when labelled with [^{3}H] leucine, (ie. doublet bands in the reticulocyte lysate and single bands in the wheat-germ). This provided evidence that the vicilin sub-units were produced as precursors, and were processed, by the removal of about 10 amino acids, (as judged from the spacing of the doublets), during translation in the wheat-germ system, but not in the reticulocyte system, which would appear to lack a processing component, (probably a membrane fraction); Burka, (1974) mentioned that reticulocytes do not contain endoplasmic reticulum.

Mumford et al., (1981), examined proteases present in the wheat-germ and reticulocyte lysate systems and found that, although there were high levels of proteases in both systems there were higher levels of amino-

peptidases and elastase-like enzymes in the wheat-germ system than in the reticulocyte lysate. Wheat-germ lysates also showed trypsin-like activity and post-proline cleavage activity which was absent in the reticulocyte lysate. They concluded that the presence of these proteases while probably accounting for the 'relative inefficiency' of translation in the wheat-germ system could also account for the removal of the initiator methionyl residue. Some of the protease activity could be associated with the membrane fraction of the wheat-germ system, (ie could be the 'modification enzyme'). This phenomenon has been demonstrated in the number of experiments, Walk and Hock, (1978), reported that water melon proteins were processed in the wheat-germ system but not the reticulocyte lysate system, while Milstein et al., (1972), noted processing of light chain mRNA translation products in an ascites S-30 system but not in the reticulocyte lysate system. Peeters et al., (1979), showed processing of human pre-lactogen in the wheat-germ system, which they concluded was due to the presence of membranes, and Ellis, (1981), demonstrated post-translational processing, which required ATP, of ribulose bisphosphate carboxylase and chlorophyll a/b binding protein, in a wheat-germ system. Mertens and Verhoeven, (1981), also showed partial processing of pre-αsu-globulin in a wheat-germ system, but no processing in the reticulocyte system, however, they reported complete processing and secretion of the polypeptide in Xenopus oocytes, injected with mRNA. Although Larkins et al., (1979), could not demonstrate processing of zein polypeptides in either wheat-germ or reticulocyte systems, in the absence of added membranes, they also showed processing of mRNA products in Xenopus oocytes.

The doublets, (of vicilin polypeptides), produced in the reticulocyte lysate system were due to the presence of 'run-off' products, which had been processed before polysome extraction, and unprocessed polypeptides, which were products of re-initiation, or unprocessed 'run-off' polypeptides. The fluorograph of polysome products in the reticulocyte system where initiation was inhibited, (by ATA), showed the 50,000 and 47,000 mol.wt. subunits probably coincided with the lower (processed) bands of the doublets in the control samples, which would indicate that most 'run-off' polypeptides were already processed. The products of mRNA

translation were unprocessed, as expected, because no 'run-off' products would be present. The pea axes and cotyledon systems also showed some processing ability, which implies that the S-30 fraction, from which these systems and the wheat-germ system, originated, contains some material, (possibly membranous), capable of processing polypeptides.

The method of processing, which is most likely to account for these results is that ascribed to the 'Signal Hypothesis' described originally by Redman and Sabatini, (1966), in relation to animal secretory proteins, and which was expanded by Blobel and Dobberstein, (1975), Campbell and Blobel, (1976), and Jackson and Blobel, (1980). They postulated that the NH_2 terminal 10-20 amino acids of a nascent polypeptide of a secretory protein form a signal sequence, which recognises a receptor site on the endoplasmic reticulum, attaches to it and passes through into the lumen, followed by the remainder of the polypeptide, as it is synthesised; the signal sequence is enzymatically removed from the polypeptide during its translocation, (a canine pancreatic signal peptidase has been isolated by Jackson and Blobel, 1980). Where analysed the signal sequence has been found to have a hydrophobic sequence, (Burstein et al., 1976, Shore and Tata, 1977, von Heijne and Blomberg, 1979, Steiner et al., 1980), and this would assist entrance of the polypeptide into a membrane. Kreil et al., (1981), noted that a decrease in hydrophobicity of a signal sequence lowered the transport efficiency of the polypeptide across membranes. There have been refinements suggested with regard to the kinetics, membrane behaviour, and recognition mechanisms of the hypothesis, (von Heijne and Blomberg, 1979, Shore and Tata, 1977, Prehn et al., 1981), but the basic hypothesis has been supported by evidence from many animal secretory proteins, (Shore and Harris, 1977, Lingappa et al., 1978, Prehn et al., 1981, Kamine and Buchanan, 1978, Craig et al., 1979, Steiner et al., 1980, Shields et al., 1981), which are processed, packaged and eliminated from the cell. Recently evidence has accumulated which shows the 'Signal Hypothesis' to apply to plant proteins, which are isolated from the general cell environment, especially storage proteins, (Cameron-Mills et al., 1978, and Matthews and Miflin, 1980, working on

hordeins in barley, Burr et al., 1978, and Larkins and Hurkman, 1978, zein in maize, Roberts and Lord, 1981a, b, castor bean, Gatehouse et al., 1981, and Higgins and Spencer, 1981, vicilin in peas, Greene, 1981, gliadin in wheat.), although Bollini and Chrispeels, (1979), working with Phaseolus vulgaris, and Beachy et al., (1980), working with soyabean have found no such evidence.

The addition of a membrane fraction (ie. microsomes or stripped microsomes), to the reticulocyte lysate system increased the production of processed polypeptides confirming that the 50,000 and 47,000 mol.wt. polypeptides were initially synthesised as precursors and were post- or co-translationally modified in the presence of membranes to produce 'native' storage protein subunits in a method analagous to the 'Signal Hypothesis'. Some reconsititution of microsomes occurred in vitro, as also shown by Cameron-Mills and Ingversen, (1978), clearly the polysome complex is capable of recognising membrane receptor sites, (the structure of which has been a subject of much discussion, Blobel and Potter II, 1967, Redman and Sabatini, 1966, Kreibich et al., 1980, and Kreil, 1981), even in the in vitro conditions provided.

Translation products labelled with [^{35}S] methionine did not show processing of the polypeptides which suggests that the processed forms contained insignificant amounts of radioactivity. As pea storage proteins are known to be deficient in the sulphur amino acids, methionine and cysteine, (Derbyshire et al., 1976, Jackson et al., 1969), especially the vicilin subunits, (Casey and Short, 1981, Croy et al., 1980c), it would seem probable that most of the label was present in the signal sequence of the precursor polypeptides (associated with the initiator sequence), and therefore was removed from the polypeptide during processing, as suggested by Matthews and Miflin, (1980), consequently only the unprocessed polypeptides would be visualised on fluorographs. This is supported by the results of the CNBr cleavage of the storage proteins discussed earlier.

Higgins and Spencer, (1981), detected processing of a 75,000 mol.wt. vicilin subunit assumed to be equivalent to our 70,000 mol.wt. subunit, as well as the 50,000 and 47,000 mol.wt. subunit in translation products of cell-free systems. No processing of a 75,000 mol.wt. polypeptide, or of the 70,000 mol.wt. convicilin subunit (Croy et al., 1980c), was demonstrated here.

No conclusive evidence was found to show in vitro processing of the legumin 60,000 mol.wt. subunit, in the presence of microsomal membranes, by removal of a signal sequence and, Higgins and Spencer, (1981), could not detect processing of this polypeptide either. However, occasionally the polypeptide appeared as a doublet in translation products, as seen in the reticulocyte lysate translation products in the ATA inhibition experiments, (Fig. 17); the doublet could represent processed and unprocessed polypeptides. In the samples where re-initiation had occurred the upper, (unprocessed), polypeptide band was produced in preference to the lower, (processed), band, indicating that processing, which was occurring in vivo was not occurring in this translation system.

It is unclear why there was an apparent difference in mol.wt. of the legumin 60,000 mol.wt. polypeptide synthesised in the wheat-germ system in the presence of ATA, (10^{-3}M or $5x10^{-4}$M), compared to the polypeptide synthesised in the absence of ATA, (this difference was not seen in reticulocyte products). It could have been due perhaps to processing of the polypeptide in the control wheat-germ system, but not in the inhibited system since ATA is known to prevent polysome attachment to membranes, (Blobel and Dobberstein, 1975), which is a necessary step in processing. However, as 'run-off' products would be expected to contain some polypeptides which had been processed before the polysomes were extracted, (Matthews and Miflin, 1980), and these were not apparent in the inhibited samples, it is possible that the difference noted was an artefact.

If processing of the legumin does occur by removal of a short amino acid sequence, it seems that either the microsome fraction was not directly involved in these modifications or the techniques used were not sensitive enough to detect the process efficiently, (the processing of vicilin subunits, Gatehouse et al., 1981, Higgins and Spencer, 1981, was overlooked for some time).

No protection of the processed polypeptides against tryptic digestion could be demonstrated, even when additional microsome vesicles were present in the cell-free systems. This was in contrast to results obtained by Higgins and Spencer, (1981), and most other results concerned with the Signal Hypothesis, (Cameron-Mills et al., 1978, Shore and Harris, 1977, Katz et al., 1977, Lingappa et al., 1978, Roberts and Lord, 1981a).

It is possible that the modification process is not linked with transport of the polypeptide across the membrane, (i.e. is a different mechanism to that of the 'Signal Hypothesis'), however the results obtained do show that the process involves cleavage of a specific length of amino acid (as a sharp band of 'processed' polypeptides is formed), and that the mechanism is membrane associated. It therefore seems more probable that pea microsomes are less robust than dog pancreas microsomes, which are used by most researchers, and perhaps the extraction procedure used to isolate the cotyledon microsomes damaged the membranes, resulting in them being 'leaky' with regard to trypsin, yet intact enough to process polypeptides. Kamine and Buchanan, (1978), had difficulties in demonstrating protection of processed polypeptides of Rous sarcoma virus in the reticulocyte lysate system, and Higgins and Spencer, (1980), concluded that the addition of membranes to a system was inefficient unless large quantities of dog pancreas microsomes were added.

Attempts to demonstrate glycosylation of any of the storage protein polypeptides in vitro were unsuccessful, as were Hall et al., (1978, 1980), Sun et al., (1975), Bollini and Chrispeel, (1978), and Matthews et al., (1981), when working with Phaseolus vulgaris. Glycosylation in the wheat-germ system has been reported by Katz et al., (1977), Shore and

Tata, (1977a) and Lingappa et al., (1978a,b), while Spencer and Higgins, (1979), suggested the rough endoplasmic reticulum was the site of glycosyl transferase which could be used in glycoprotein formation, and Roberts and Lord, (1981b), found a core glycosylating enzyme in the ER membrane, and demonstrated incorporation of [^{3}H]N-acetylglucosamine into polypeptides. Glycosylation of vicilin does occur *in vivo*, though the protein only contains approximately 0.3% mannose and 0.2% glucosamine, (Basha and Beevers, 1976), and there may be a time lapse between translation and glycosylation; glycosylation was not essential for synthesis, processing or assembly of the vicilin oligomer *in vivo*, according to Badenoch-Jones et al., (1981). Conflicting data exists about the level of glycosylation of legumin *in vivo*, which Hurkman and Beevers, (1980), suggest is due to the difference in extraction procedures employed, but it is agreed that it is very low, Basha and Beevers, (1976), suggested 1% mannose and glucose, and 0.1% glucosamine. The low levels of glycosylation *in vivo* probably account for the lack of results using *in vitro* systems. If glycosylation was necessary as a co-translational or an immediate post-translational event the inability of the system to glycosylate proteins could cause a block in translation, as seen in castor bean, (Roberts and Lord, 1981b), and the synthesis of the immunoglobulin heavy chain, (Bergman et al., 1981), or prevent a further post-translational modification which could be dependent on a specific configuration of the polypeptide, (Kreil, 1981). Matthews et al., (1981), demonstrated synthesis and glycosylation of phaseolin when mRNA was injected into Xenopus oocytes, this system would perhaps enable *in vitro* glycosylation of pea storage protein subunits to occur.

The literature offers many conflicting views on specialisaton of polysomes, i.e. whether membrane-bound polysomes constitute a different population to free polysomes, (Bollini and Chrispeels, 1979, Boulter, 1970, and Payne and Boulter, 1969), but the controversy may arise from the techniques used to separate the 'different' populations and the inevitable cross-contamination of samples, (Bollini and Chrispeels, 1979, Taylor, 1979, Galau and Dure, 1981). Evans et al., (1979), determined that, at 14daf. 67% of pea cotyledon polysomes were membrane-bound. The method used here to extract microsomes, (that of Cameron-Mills et al., 1978), would minimise contamination by free polysomes as the 37,000g

centrifugation should only pellet membrane-bound polysomes, if polysomes subsequently detached the flotation step at 96,000g should cause them to pellet. However, in spite of these methods the EM did show the presence of some apparently free polysomes among the microsomes, these were probably trapped among the membranes, (Mechler and Vassalli, 1975). The free polysomes could very easily be contaminated with polysomes detached from membranes during extraction procedures. Blobel and Potter, I, (1967), noted a loss of membrane-bound polysomes which did not sediment under high speed centrifugation, some membrane-bound polysomes may also be lost if they sediment with mitochondria or nuclei, (Ramsay and Steele, 1976, Shore and Tata, 1977b). The translation products of the various 'templates' contained storage protein polypeptides, though microsomes appeared to preferentially synthesise the 60,000, 50,000 and 47,000 mol.wt. subunits, as non-storage protein polypeptides were not apparently synthesised in significant amounts; released polysomes also contained a higher proportion of large polysomes (up to 10 mer), than did the free polysome samples, (the maximum size polysomes detected were 7 mer). This supports evidence of Shore and Tata, (1977a,b), which suggests that there is not a discreet population of polysomes which translate storage proteins but initiation and polypeptide elongation begins in the cytoplasm producing 'free' polysomes, which subsequently attach to membranes (ER), if the nascent polypeptide has a signal sequence, (Craig et al., 1979, Kreil, 1981). Consequently in a system actively synthesising storage proteins the majority, but not all, of the mRNA's coding for these proteins will be in a polysome complex and will be attached to a membrane; mislocalisation of a polypeptide does occur, (Kreil, 1981), probably due to lack of membrane receptor sites. This situation has been described by Larkins and Dalby, (1975), in maize, Beachy et al., (1978), in soyabean and Püchel et al., (1979), in Vicia faba.

During the course of experiments comparing poly(A)$^+$-RNA with polysome driven cell-free systems it became clear that not only were there differences in the quality of the products, (+ and - leader sequences), but also quantitative differences with respect to amounts of vicilin and legumin synthesised. A particularly striking example was the

differences in the two systems using '19 day' material. In the poly(A)$^+$-RNA driven system legumin and vicilin were both strongly represented in the products whereas in the polysome driven system legumin predominated. The most likely explanation of that result is that the legumin message is less stable than the vicilin one and during the course of purification of poly(A)$^+$-RNA from the polysomes the proportion of vicilin mRNA relative to legumin mRNA was increased. Sun et al., (1978), reported similar results regarding translation products of polysomes and mRNA of Phaseolus vulgaris cotyledons in a wheat-germ system; a 53,000 mol.wt. subunit which was poorly synthesised in the polysome driven system was absent from products of the mRNA driven system.

In order to investigate the change in mRNA population during development of the pea cotyledon a preliminary analysis of the abundance and complexity of poly(A)$^+$-RNA extracted from 9, 14 and 19 day old cotyledons was undertaken, using the techniques of RNA: complementary DNA(cDNA), hybridisation, (Bishop and Rosbash, 1974). There are several points concerning the methods employed which need to be mentioned and which emphasise the preliminary nature of the investigation.

cDNA synthesised from a mRNA population using AMV reverse transcriptase requires a starter sequence, (Verma et al., 1972, Ross et al., 1972). It is fortunate that plant mRNA is polyadenylated so oligo(dT) can be used as a primer and therefore this method can be employed. The synthesis of storage protein polypeptides in poly(A)$^+$-RNA driven cell-free systems and the production of vicilin and legumin clones using poly(A)$^+$-RNA from polysomes, (Croy et al., 1982), showed that the storage protein mRNA's have poly(A) tails. However, this method would not lead to synthesis of cDNA from messenger which did not have a poly(A) tail, although there is no evidence that such messengers exist in the seed cytosol. It is perfectly true that people have separated a poly(A)$^-$ fraction, (Moffet and Doyle, 1981, Evans et al., 1979), but the general consensus of opinion is that this messenger is poly(A)$^+$-RNA degraded. Evans results, (Evans et al., 1979), showed that some storage protein mRNA was in a poly(A)$^-$ fraction and was therefore possibily unstable, so less would be copied into cDNA. As mentioned before, legumin mRNA was probably less stable than vicilin mRNA

and consequently would be underrepresented in the cDNA population.

The use of polysomal poly(A)$^+$-RNA in the kinetic experiments also excluded non-polysomal mRNA; this did ensure that cytoplasmic mRNA, which was probably being translated in the cell at that stage, was used in the experiments and not stored mRNA, (Baglioni, 1974), or nuclear poly(A)$^+$-RNA, an assumption which Bishop et al., (1974), had to make when using total poly(A)$^+$-RNA.

It was assumed that AMV reverse transcriptase transcribed all the poly(A)$^+$-RNA with equal efficiency independent of the abundance of the template, so the cDNA population was an accurate reflection of the RNA population; Hastie and Bishop, (1976), showed this was approximately so using a mixture of liver and globin mRNA.

The average size of poly(A)$^+$-RNA and cDNA were presumed to be similar to those obtained by Evans et al., (1980), because the materials and techniques used were identical. The assumption was also made that the average size of nucleic acids would be constant throughout development; Galau and Dure, (1981), assumed this was so during cotton cotyledon development but Goldberg et al., (1981), noted a small difference in average size of cDNA and mRNA during soyabean development.

The size of cDNA manufactured is important as the smaller the cDNA the greater will be the proportion of poly(T) sequence present, (because reverse transcriptase begins copying at the 3' hydroxyl end of the mRNA), and therefore cross hybridisation is more likely to occur, also Weiss et al., (1976), claimed that less stable cDNA:RNA hybrids would be produced. The cDNA manufactured (954 nucleotides long), was approximately half the length of the mRNA, probably because of RNase activity. In support of this Buell et al., (1978), concluded that the length of cDNA obtained depended on the RNase activity in the reverse transcriptase used and found that this varied with different lots. The presence of RNasin, an RNase inhibitor has since been found to enable full length cDNA to be synthesised. A correction factor was used in calculations to compensate for the difference in cDNA and mRNA lengths.

The use of S_1 nuclease, which selectively degraded single stranded polynucleotides, (Wiegand et al., 1975, Vogt, 1973), provided a rapid, reproducible, method of estimating the proportion of hybrids in a mixture of polynucleotides.

The fact that the rate of hybridisation (of cDNA:mRNA), is inversely proportional to the base sequence complexity of the mRNA, (Bishop, 1969, Bishop et al., 1974, Monahan et al., 1976), was used to calculate the complexity of pea cotyledon poly(A)$^+$-RNA.

The hybridisation curves derived from the least squares solutions of the data are not unique solutions but represent the lowest SSD, (sum of squares deviation), with the fewest abundance classes, (Bishop et al., 1974). This assumes that the poly(A)$^+$-RNA is arranged in classes of different complexity and abundance, (ie 'very abundant', 'moderately abundant', 'abundant' and 'rare'), rather than in a continuous gradation of abundances, a concept discussed by Quinlan et al., (1978).

The abundance curves obtained for poly(A)$^+$-RNA at each stage of development studied were interpreted, taking these approximations into consideration, using the methods of Goldberg et al., (1981), and the following conclusions were drawn.

The distribution of classes of poly(A)$^+$-RNA was shown to change during development. Both the '9 day' and '14 day' poly(A)$^+$-RNA contained a class which hybridised very rapidly, especially the 'superabundant' class of the '14 day' samples, this was probably because the sequences were either highly homologous, or perhaps degraded. Goldberg et al., (1981), observed a class of mRNA which behaved in a similar manner and which they concluded contained highly homologous sequences and subsequently included in a 'very abundant' class. The examination of poly(A)$^+$-RNA on glyoxyl gels (by Dr. J.A. Gatehouse), revealed a polydispersed pattern which indicated the presence of some degraded material, which could account for this class.

The presence of a large 'rare' class of complex poly(A)$^+$-RNA, as found in the '9 day' material, was expected in a situation where cells are still dividing and growing, (Millerd and Spencer, 1974), as these

processes require a large number of diverse enzymes and structural proteins. The storage protein poly(A)$^+$-RNAs are probably present in the 'abundant' class at the '9 day' stage, because their translation products are detectable both *in vivo* and *in vitro*, but not in large quantities. At the '14 day' stage the 'very abundant class' contains 6 mRNAs which represent 37% of the poly(A)$^+$-RNA mass. As the major *in vitro* translation products of '14 day' poly(A)$^+$-RNA are 4-6 storage protein sub-units, they are probably the products of the 'very abundant' mRNAs.

There was a marked decrease in 'rare' sequences during development, (none at 19 daf), which reflects the processes of a system changing from a growing and developing tissue to one mainly concerned with the accumulation of storage proteins. If a more advanced developmental stage had been studied, (eg. 22 daf), a decrease in 'very abundant' poly(A)$^+$-RNA, as seen by Dure and Galau, (1981), in cottonseed, may have been observed, corresponding to the cessation of storage protein synthesis. Both Beevers and Poulson, (1972), and Bollini and Chrispeels, (1979), reported that a decrease in protein synthesis at maturity of pea and bean, (*Phaseolus vulgaris*), seeds was due to the limited availability of mRNA. The total number of poly(A)$^+$-RNA sequences expressed at the '9 day' stage of cotyledon development was calculated, from hybridisation data, to be about 19,000. This was of the same order as the number of sequences calculated to be present in cottonseed, (Galau and Dure, 1981, 25,000), and in mid-embryo soyabeans, (Goldberg et al., 1981, 32,000).

A further refinement of techniques would have been to fractionate the poly(A)$^+$-RNA and cDNA kinetically and study the hybridisation kinetics of each fraction separately, which was a method employed by Hastie and Bishop, (1976), Clissold et al., (1981), Goldberg et al., (1981), and Bathurst et al., (1980a and b). This would yield more substantial data on the composition of poly(A)$^+$-RNA in the different abundance classes, and would enable the abundance class of storage protein mRNAs to be located. Another improvement not carried out due to lack of time would have been the isolation of purer storage protein mRNA by immuno-precipitation of storage protein polypeptides on polysomes, (Legler and Cohen, 1976, Jost and Pehling, 1976 and Schechter, 1974). The

hybridisation kinetics of such purified mRNAs would provide more precise information on their abundance during development.

Heterologous hybridisation reactions which were also carried out, although difficult to interpret, (as mentioned by Galau and Dure, in press), did show that the majority of mRNA sequences were present both at '9 day' and '14 day' stages, supporting the polysomal poly(A)$^+$-RNA translation results. 18% of the '9 day' poly(A)$^+$-RNA was unique to the '9 day' stage while only 8% of the '14 day' poly(A)$^+$-RNA was unique to the '14 day' stage; these were similar results to those of Galau and Dure, (1981), who found that in cottonseed at least 90% of cDNA reacted with mRNA from all developmental stages.

About 5% of single-copy DNA of the pea genome was estimated to be present as transcripts, which were being translated, in the pea cotyledon at 14 daf; this compares with 2% for soyabean, (Goldberg et al., 1981), 1.4% for sea urchin, (Galau et al., 1974), 4.7-5.2% for tobacco cells, (Kamalay and Goldberg, 1980), and 1.05% for HeLa cells, (Bishop et al., 1974). Only small amounts of material were available for experiments, (owing to difficulties in preparation), and therefore not enough data was available to construct a curve. However, the hybridisation reaction was taken to a value of Rot 10^5. At a Rot value of 10^2 the homologous cDNA:poly(A)$^+$-RNA reaction for '14 day' material had reached completion, so all the 'rare' poly(A)$^+$-RNA sequences should have hybridised to homologous single-copy DNA molecules before Rot 10^5. The hybridisation reactions occurred over Rot values for 'rare' sequences of poly(A)$^+$-RNA (as determined by homologous cDNA:poly(A)$^+$-RNA hybridisations). It would therefore seem that the majority of single-copy DNA transcripts are unique mRNAs, rather than abundant mRNAs. However, Croy et al., (1982), have shown, using cloning techniques, that legumin and vicilin genes are not highly reiterated in the pea genome. The sensitivity of the hybridisation of single-copy DNA to poly(A)$^+$-RNA was probably too low to detect the formation of hybrids between storage protein mRNAs in the 'abundant class', (perhaps only 10 hybrids), to single-copy DNA.

Approximately 50% of nuclear poly(A)$^+$-RNA appeared in polysomal poly(A)$^+$-RNA, at 9 daf, although, again due to low yields of material, insufficient data was available to obtain a computed curve. The reaction was taken to Rot 10^2, which, (as mentioned above), should allow the hybridisation of most 'rare' species of poly(A)$^+$-RNA to proceed. Molloy and Puckett, (1976), reported that at least 40% of nuclear poly(A)$^+$-RNA sequences were transported to the cytoplasm, while Bathurst et al., (1980), noted that about 40% of nuclear poly(A)$^+$-RNA was represented in the cytoplasm of Guinea Pig mammary glands. Moffett and Webb, (1981), found significant regulation of genetic expression occurred at the 'post-transcriptional nuclear level', and estimated that 10-25% of nuclear mRNA in the liver was represented in the cytoplasm. The poly(A)$^+$-RNA sequences present in the nucleus but absent from the cytoplasm could either be stored in the nucleus, or could represent sequences of introns which are removed from nuclear mRNA before transport into the cytoplasm.

In spite of the constraints and limitations of the reassociation experiments it was shown that as protein synthesis increased so did 'abundant' and 'very abundant' mRNA classes, although strict quantitative results were not available regarding mRNA and protein levels.

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