Studies: on the nucleic acids of developing, dormant and germinating seeds of vicia faba (L)

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STUDIES ON THE NUCLEIC ACIDS OF DEVELOPING, DORMANT AND GERMINATING SEEDS OF VICIA FABA (L)

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ABSTRACT

Seeds of Vicia faba (L)take about 130 days from the fertilisation of the ovule to become fully mature. After an initial phase of cell division, storage protein is synthesised in the cotyledon cells and accumulates in organelles termed protein bodies. During the latter phase, the RNA contents of cotyledon cells increase sixfold. Methylated albumin-kieselguhr chromatography revealed that the newly synthesised RNA consists of 13% low molecular weight RNA and 87% ribosomal RNA, suggesting that storage protein synthesis may be mediated by the mRNA/tRNA/ribosome mechanism.

Isolated protein bodies contain only traces of RNA, indicating that storage protein is not synthesised in these organelles. Biochemical methods show that, during the phase of cell division, there are many free ribosomes and relatively few membrane bound ribosomes. Correlated with the onset of storage protein synthesis, there is a large increase in the numbers of membrane bound ribosomes, but the numbers of free ribosomes remain approximately constant. Experiments with (^{3}H) - uridine show that there is no interconversion of these two classes of ribosomes, and both were shown to incorporate amino-acids "in vivo". It is suggested, therefore, that each class of ribosomes may be synthesising different groups of proteins, the membrane

bound ribosomes being responsible for the synthesis of storage protein.

At the cessation of nutrient reserve accumulation, seeds dehydrate and there is a gradual loss of membrane bound ribosomes with a subsequent rise in the numbers of free ribosomes.

Upon germination, endoplasmic reticulum with associated ribosomes is formed once more. Experiments with (^{32}P) - ortho-phosphate indicate that these ribosomes are synthesised "de novo", rather than being formed by the attachment of pre-existing, free ribosomes to membranes.

The breakdown of various types of nucleic acids was studied during the senescence of testas in seed development, and cotyledons in seed germination.

CONTENTS

PAGE

PAGE

PAGE

PAGE

INTRODUCTION

The presence of nucleic acid in living organisms was discovered a little less than a century ago, by Miescher in 1871. Technical limitations over the next fifty years limited further research to simple chemical analyses. Miescher himself demonstrated that the compound was of high molecular weight and had a phosphorus content of 9.59%, a value which compares favourably with modern estimates. Miescher's students, and others, isolated and partially characterised the constituent purine and pyrimidine bases, though these components were not fully characterised until the advent of paper chromatography in 1947.

The heterogeneous nature of nucleic acid was discovered by Levene between 1910 and 1930, who identified the sugar in yeast nucleic acid as ribose and that in thymus nucleic acid as deoxyribose. This information initially led to the belief that nucleic acid containing ribose, ribonucleic acid *(RNA), occurred in plant tissues and deoxyribose containing nucleic acid, deoxyribonucleic acid (DNA), occurred in animal tissues. The development of two techniques, a basophilia histochemical test involving ribonuclease, Brachet (1940), for the detection of RNA, and the Feulgen

* The abbreviations and conventions used throughout this thesis are those recommended in "The Biochemical Journal". (Biochem.J., 66, 8 (1957).

nucleal reaction, Feulgen and Rossenberg (1924), for the detection of DNA, showed that DNA and RNA occurred in both animal and plant tissues. These authors also demonstrated that RNA occurred predominantly in the cytoplasm and DNA occurred in the nucleus of cells.

By about 1940, therefore, it was realised that nucleic acid consisted of three components, phosphate, a pentose sugar, and four organic bases. Three of the four bases are common to both DNA and RNA, namely adenine, guanine, and cytosine; the fourth base in DNA is thymine and in RNA is uracil. The early history of nucleic acids is reviewed by Chargaff & Davidson (1955).

The possible connection between DNA and genetic inheritance has been realised almost since the original discovery of nucleic acid. Miescher, between 1880 and 1890, discovered sperms to be an excellent source of nucleic acid, particularly salmon sperm heads which contained almost 50% nucleic acid. Positive proof that DNA is the genetic material developed from the classic transformation experiments of Avery, Macleod and McCarty (1944). They found that the transforming principle, which Griffith (1928) obtained from encapsulated Pneumococci and enabled non-encapsulated strains to make a capsule, was chemically identical to DNA. A chemical explanation of the ability of a molecule of DNA to store genetic information and to divide and so duplicate the

information was formulated by Watson and Crick (1953) on the basis of published chemical analyses and x-ray diffraction studies. The predictions made by the hypothesis have since been confirmed by experiment.

The first suggestions as to the function of RNA came from Caspersson (1941) using a quantitative ultra-violet spectrophotometry method for the estimation of RNA, and later by Davidson and Weymouth (1943) who estimated RNA by chemical methods. Both groups of workers showed that the cells richest in RNA were either growing rapidly, e.g. embryonic tissue, or synthesising protein, e.g. exocrine pancreas, and therefore concluded that RNA must be involved in protein synthesis.

The intracellular location of protein synthesis was not discovered until the advent of the electron microscope and the ultracentrifuge. Studies on the "in vivo" incorporation of labelled amino-acids into the livers of chicks, mice, and rats were made, followed by cell fractionation and differential centrifugation. The microsome fraction was more highly labelled than any other fraction (Keller, 1951). Rat liver fractions were incubated with (^{14}C) - alanine "in vitro" by Siekevitz (1952), and the highest specific activity was found in the microsomes. When the microsome fraction was treated with the detergent sodium deoxycholate to remove lipids, the radio-activity was found to be associated with small particles called ribosomes, which were characterised

by centrifugal analysis and electron microscopy.

Jacob and Monod (1961), in their hypothesis concerning induced enzyme systems of Escherichia coli, proposed that a messenger molecule is rapidly synthesised adjacent to, and complementary with, a strand of DNA, and is then transferred to the cytoplasmic ribosomes where it codes for the synthesis of a protein, and then breaks down. Volkin and Astrachan (1957) had previously detected an RNA molecule having such properties. They infected Escherichia coli with T2 phage and identified a particular type of RNA containing only a small percentage of the total RNA, as having a high rate of synthesis and breakdown, and a very similar nucleotide composition to the infecting phage DNA (after taking into account the substitution of uracil for thymine in RNA). The presence of this RNA, messenger RNA (mRNA), in uninfected bacterial systems, has since been confirmed by (i) "pulse" and "chase" labelling experiments designed to preferentially label RNA which is highly metabolic; (ii) molecular annealing experiments between RNA and homologous DNA, indicating the degree of complementary base sequences between the two molecules; (iii) cell free amino-acid incorporation systems which test the ability of RNA molecules to act as templates for protein synthesis, and (iv) sucrose gradient analysis and electron microscopy which distinguish ribosonal aggregates engaged in protein synthesis (polyribosomes)

and inactive ribosomes, e.g. Gross, Hiatt, Gilbert, Kurland, Risebrough and Watson (1961), Watson (1963), and Nakada (1963).

The final process in the synthesis of protein is the condensation of amino-acids into protein against the mRNA template. It is now certain that a sequence of three nucleotides of the mRNA codes for one amino-acid. Furthermore, all the possible sequences of three nucleotides (the codons) have been assigned to amino-acids for Escherichia coli with the exception of UAA, UAG, and UGA, which are chain terminator codes called ochre, amber, and opel respectively. It is apparent that as many as four codons can code for one amino-acid. Research relating to the genetic code is adequately reviewed by Woese (1967).

Crick (1957) thought it unlikely that an amino-acid would be capable of binding to a particular site on the mRNA template, and postulated adaptor molecules which would stereo-chemically recognise particular nucleotide triplets. Working with "in vitro" protein synthesising systems, Hoagland, Zamacnik, Sharon, Stulberger, Lipman and Boyer (1957) showed that their "pH 5 enzyme" fraction, obtained from rat liver, contained about 5% RNA. Experiments with (^{14}C) - leucine demonstrated an RNA labelled amino-acid complex, which in the presence of a microsomal suspension became dissociated, the radio-active amino-acid being incorporated into microsomal protein. This RNA fraction was called soluble RNA (sRNA) or more appropriately transfer (tRNA).

In the transfer process, an amino-acid is first activated with adenosine triphosphate (ATP) by an enzyme called an amino-acyl-tRNA synthetase which is specific for that particular amino-acid, so that an enzyme-AMP amino-acid complex is formed. The enzyme then binds the amino-acid to a specific tRNA molecule. The transfer of amino-acids to the ribosome-mRNA complex is reviewed by Simpson (1962) and Schweet and Heintz (1966).

tRNA molecules have a molecular weight of approximately 23,000 and a sedimentation coefficient of about 4s. There appears to be considerably more tRNA molecules than protein amino-acids and by 1962, Sueoka and Yamane showed by methylated albumin kieselguhr chromatography that some amino-acids become attached to two or more different tRNA molecules "in vitro". All tRNA molecules have a terminal cytidyl-cytidyl-adenosine sequence, and it is the final adenosine residue to which the amino-acid becomes attached by an ester linkage. Another characteristic of tRNA is the presence of unusual purine and pyrimidine bases, which in yeast-tyrosine-tRNA amounted to 20% of the total nucleotides, Madison, Everett and Kung (1966).

For a tRNA molecule to participate in protein biosynthesis, it must have at least two unique sites, one site which is recognisable solely by its specific amino-acyl-tRNA synthetase, and the other to recognise the appropriate codon on the mRNA template. Another site, common to all tRNA

molecules, may also occur which is able to recognise a particular site on the ribosome. These sites are no doubt intrinsic to the nucleotide sequence and tertiary structure of the tRNA.

Holley, Apgar, Everett, Madison, Marquisee, Merrill, Penswick and Zamir (1965) achieved a major advance into these problems when they purified and then determined the complete sequence of yeast alanine tRNA. The use of two enzymes, takadiastase ribonuclease T1 and pancreatic ribonuclease at 0° which cleave RNA at specific nucleotide bonds, and the presence of unusual nucleotides in small quantities which acted as markers in sequence determinations, were fundamental to elucidating the final sequence. Since then, several tRNAs have been sequenced, including yeast tyrosine tRNA (Madison et al, 1966), two yeast serine tRNAs (Zachau, Dutting, Feldman, Melchers and Kavan, 1966), and Escherichia coli N-formyl methionine tRNA (Dube, Marcker, Clark and Cory, 1968).

Holley et al (1965) suggested three possible secondary structures for alanine tRNA based on maximal base pairing, the unpaired nucleotides splaying out in the form of loops. Examination of other tRNAs whose sequences have been determined suggests the so-called clover leaf model is the most probable, in which three major loops occur at approximately right angles to one another. In all these tRNAs, a sequence of three nucleotides (an anticodon), capable of hydrogen bonding

with the relevant codon of the mRNA template, appears in the same loop of the clover leaf model. Lake and Beeman (1968) measured x-ray scattering at low angles by yeast tRNA in aqueous solution. Several published secondary structure conformations were compared with the results obtained, and the clover leaf model appeared most probable. A better fit with the results was obtained, however, if the loops were tightly accommodated against the central axis of the molecule.

It is probable that the tertiary structure of the tRNA will not be elucidated until it can be crystallised. Crick (1966) and Woese, Dugre, Dugre, Kondo and Saxinger (1966) are of the opinion that some of the unusual nucleotides present in tRNA are intimately involved in its tertiary structure.

When animal or plant cells are incubated with a labelled RNA nucleotide, tRNA molecules are first detected in the nucleus. Molecular annealing experiments between homologous DNA and denatured tRNA suggest that a small portion of the DNA codes for tRNA. Giancomoni and Spiegelman (1962), working with Escherichia coli demonstrated that 0.023% of the DNA is complementary to tRNA and more recently Morell, Smith, Dubnau, and Marmur (1967) obtained a similar result, 0.04% for Bacillus subtilis.

Pools of tRNA are found in isolated pea seedling nucleoli, Birnsteil, Borek & Fleissner (1963), and since 7-10% of the dry mass of the nucleolus is DNA (Birnsteil, 1967), it is possible that tRNA may be synthesised there, though this is a matter of dispute (see Birnsteil, 1967). The issue was complicated by the presence of a 5s

RNA which does appear to be synthesised in the nucleolus, and was thought to be a precursor of tRNA, but is now known to be a constituent of ribosomes. Two experiments strongly suggest synthesis of tRNA does not occur in the nucleolus. (i) Autoradiographs of L cell nuclei after incorporation of (^{3}H)-cytidine show that radio-activity is localised in the chromatin and not the nucleolus, Perry (1965). (ii) tRNA synthesis proceeds as efficiently in an anucleolate mutant of Xenopus as in the wild type, Brown and Gurdon (1964).

As much as 85% of the RNA in cells is ribosomal RNA (rRNA) and in nature occurs almost exclusively in ribonucleoprotein particles. There are three kinds of rRNA in the cytoplasm of plant cells. Two are large compared with tRNA and have molecular weights of about 1.7 million and 0.7 million, (Loening, in preparation) and sediment at 28s and 18s respectively, whereas the third is the same order of size as tRNA, has an approximate molecular weight of 41,000 (Lomb and Zehavi-Willner, 1967) and a sedimentation coefficient of 5s. Animal cells have similar rRNAs though the 28s and 16s molecules at least have slightly larger molecular weights, (Loening, in preparation). The three rRNAs of plant chloroplasts, plant and animal mitochondria, and prokaryotes are considerably smaller, Loening and Ingle (1967), Dyer and Leech (1968), and Loening (1968).

Neither the primary, secondary or tertiary structures of 28s and 18s rRNA are known, though base ratio determinations suggest little base pairing occurs. Brownlee, Sanger, and Barrell (1967), however, determined the nucleotide sequence of Escherichia coli 5s RNA uniformly labelled with (^{32}P). The molecule consists of 120 nucleotides and does not contain the minor nucleotides present in tRNA. A secondary structure was constructed on the basis of (i) maximal base pairing and (ii) the resistance of polynucleotide fragments to digestion by ribonucleases. Base pairing is less common than in tRNA, though, as with this molecule, it occurs between the two ends of the polynucleotide chain. Recently, Forget and Weissman (1967) determined the nucleotide sequence of a mammalian 5s RNA and suggested a similar secondary structure. They noticed a sequence of 5 nucleotides common to both bacterial and mammalian 5s RNA which was not involved in base pairing, but was complementary to a sequence of 5 nucleotides present in one of the unpaired loops of all the tRNAs so far sequenced, and suggested these sites may be responsible for the attachment of tRNA to the ribosome.

The ribosome has a sedimentation coefficient of 80s (chloroplasts, mitochondria, and prokaryotes have 70s ribosomes), and at low magnesium concentrations or at pH 8 or above, (Petermann, 1964) dissociates reversibly into two

sub-units, one sedimenting at 60s and the other at 40s. The rRNA of the larger sub-unit was originally thought to contain two molecules of 18s RNA, Midgley (1965), but recent work using more adequate ribonuclease inhibitors suggests one molecule of 28s (e.g. Click and Tint, 1967). In bacteria, at least, the 5s RNA component is also attached to the 60s sub-unit, Rosset, Monier and Julien, (1964). The 40s sub-unit on the other hand contains one molecule of 18s RNA.

Investigations into the structural proteins of ribosomes have been hampered by contamination of cytoplasmic proteins which become absorbed on to the ribosomes during extraction procedures. This problem has now been partially overcome by serially washing ribosomes, McQuillan and Bayley, (1966), or by subjecting ribosomes to DEAE cellulose chromatography (Stanley and Watiba (1967). Ribosomal proteins appear to be predominantly basic though a few are acidic, Osawa (1965). Moore, Traut, Noller, Pearson, and Delius (1968) have succeeded in purifying 13 of the 20 protein components of the smaller sub-unit of Escherichia coli ribosomes. The molecular weights of the proteins range from 4,500 to 27,600 and have an average molecular weight of 14,500. Quantitative methods suggest there is one molecule of each of the proteins to each sub-unit, making it possible that there are approximately 60 different proteins associated with an intact ribosome.

DNA - rRNA hybridisation studies by several workers, using a variety of organisms, suggest rRNA is synthesised against a DNA template. In pea seedlings (Chipchase and Birnsteil, 1963) about 0.3% of the total genome, that is about 500 cistons, is complementary to the rRNA. Birnsteil, Wallace, Sirlin and Fischberg (1966) isolated a minor component of DNA from the nucleolus of Xenopus by cesium chloride centrifugation in an angle rotor and it amounted to some 0.2% of the total DNA. It hybridised specifically to rRNA and was found to be absent in the anucleolate mutant. Huberman and Attardi (1967) demonstrated that the cistrons for rRNA occurred solely in the chromosomes involved in nucleolus formation. It seems likely therefore that rRNA is synthesised against a DNA template in the nucleolus.

Vaughan, Warner and Darnell (1967), working with HeLa cells, described pulse chase experiments with (^{3}H) - uridine in which a rapidly labelled RNA, having a sedimentation coefficient of 45s, could be detected in the nucleolus. The 45s RNA rapidly broke down to two molecules, 18s RNA and 32s RNA. The 18s RNA combined with its full complement of structural ribosomal proteins and passed into the cytoplasm as the 40s ribosome sub-unit after 10-15min. The 32s RNA broke down further to 28s RNA in the nucleolus, combined with ribosomal protein and passed into the cytoplasm as the 60s sub-unit, the whole procedure taking about 1 hr. Weinberg, Loening, Willems and Penman (1967) used polyacrylamide gel electrophoresis to detect ribosomal precursor RNAs instead of the more usual sucrose gradient

centrifugation

methods and detected several other short-lived RNA intermediates. Preliminary results by Loening (1967), using pea seedlings, suggest that a similar mechanism of rRNA synthesis occurs in plant tissues. Approximate molecular weight determinations by Weinberg et al (1967) suggest that just over one half of the 45s RNA becomes rRNA and the rest is lost during processing. This is in contrast to plant tissues, Loening (in preparation), where most of the 45s RNA appears to be ribosomal.

The mechanism of the coating of 28s and 18s RNA molecules with structural proteins is not clear. The process is a sequential one and requires some 15 to 20 min. "in vivo" with HeLa cells, Warner (1966). It presumably occurs in the nucleus since rRNA never occurs free of protein in the cytoplasm, Girard, Latham, Penman, and Darnell (1965) and Joklick and Becker (1965).

The presence and function of the nucleus in higher organisms complicates the definition and identification of mRNA proposed by Jacob and Monod (1961). After a history of conflicting ideas, it is now generally agreed that a large part of the messenger-like RNA of the nucleus is broken down in the nucleus and does not reach the cytoplasm, Birnboim, Pene and Darnell (1967) and Bramwell and Harris (1967). This RNA is not therefore a messenger in the strict sense since it does not direct protein synthesis in the cytoplasm. Experiments

attempting to study mRNA from extracted nuclei may therefore be difficult to interpret.

The identification of mRNA in the cytoplasm associated with ribosomes on the other hand is unequivocal. Since the expected length of mRNA appeared too long to be accommodated on one ribosome, the concept of the polyribosome, or polysome, arose as an aggregate of ribosomes joined by a strand of mRNA. Experimental evidence by electron microscopy and sucrose gradient analysis has confirmed such structures.

Electron micrographs of fixed tissues suggest that polysomes usually occur in coils or helixes attached to membranes, or free in the cytoplasm (e.g. for plant tissues, Bonnet and Newcombe, 1965). In many cases a fine thread is visible connecting the ribosomes together, and since it has the dimensions expected of mRNA and is very sensitive to ribonuclease, it has been concluded to be mRNA.

Single ribosomes and polysomes may be separated from one another by density gradient centrifugation. Brieff "in vivo" incorporation of labelled amino-acids into soya bean roots, (Lin, Key, and Bracker, 1966) and cotton seedlings (Waters and Duve, 1966) resulted in a preferential labelling of polysomes over the free ribosomes. Treatment of the microsome suspension with low concentrations of RNAase, prior to gradient centrifugation, resulted in a loss of the polysome fraction.

The polysome fraction appears to be heterogeneous in most tissues and electron microscopy shows that the number of ribosomes in a polysome is variable. This heterogeneity, it is believed, reflects the varying sizes of the mRNAs. In a reticulocyte cell, where effectively only one protein, namely haemoglobin, is formed, the polysomes predominantly consist of 5 ribosomes (Rich, 1963), as opposed to a range of 10-50 in plant cells. Since mRNA is unstable, very careful methods have to be employed to preserve the polysome structure, and it is possible that some of the heterogeneity obtained by sucrose gradients could be polysomal fragmentation. In spite of this, the polysome is a very good source for the isolation of mRNA, and indeed since polysomes of different sizes can be partially separated by density gradients, it makes possible the isolation of different mRNA molecules from the same tissue.

Polysomes, unfortunately, cannot be prepared in quantity, and since naked mRNA in particular is very susceptible to breakdown, mRNA has not yet been truly isolated. Drach and Lingrel (1966) were able to isolate an RNA fraction from reticulocyte polysomes which had a molecular weight expected for a mRNA acting as a template for haemoglobin synthesis. The fraction stimulated the "in vitro" incorporation of amino-acids into haemoglobin only when reticulocyte ribosomes were used. With Escherichia coli ribosome preparations, the RNA

stimulated incorporation but not into haemoglobin. The authors suggested therefore that RNA was acting as an activator rather than a messenger.

mRNA synthesis is catalysed by a specific enzyme, RNA polymerase; (for review see Singer and Loder, 1966). The mechanism by which mRNA is transferred from the nucleus to the cytoplasm is not understood. It is widely believed however that the mRNA travels in the form of a particle so that it is protected from ribonuclease activity. Two different particles have been claimed to contain mRNA. Work by Jocklick and Becker (1965), Latham and Darnell (1965) and McConkey and Hopkins (1965) on mammalian cells suggests that mRNA is transferred to the cytoplasm attached to the 40s ribosomal sub-unit. When the HeLa cell was infected with polio virus, Jocklick and Becker (1965) were able to detect a rapid pulse labelled RNA, presumably mRNA, in the nucleus, which after 30 sec. became attached to a particle sedimenting at a similar rate to the smaller ribosome sub-unit. This pulse could be very rapidly chased into the cytoplasm and finally into polysomes. They could not detect this mechanism in uninfected cells however. McConkey and Hopkins (1965) prepared naturally occurring free 40s ribosomal sub-units from the uninfected HeLa cell cytoplasm and found that the RNA hybridised with 8% of the homologous DNA suggesting that mRNA was present.

Spirin, Belitsina, and Ajtkozhin (1964) and Spirin and Nemer (1965) discovered a new particle in the cytoplasm of sea urchin embryos which had a similar sedimentation coefficient to the 40s ribosomal sub-unit and consisted of RNA and protein. Unlike the 40s sub-unit, the particle contained considerably more protein than RNA, and the RNA was conclusively shown not to be ribosomal but appeared to have similar properties to those expected of mRNA, since it stimulated amino-acid incorporation in an "in vitro" protein synthesising system, and annealed to 40% of the total DNA genome. Spirin et al (1964) appropriately called these particles "informosomes". Samarina, Krichevskaya, and Giorgiev (1966) obtained evidence that informosomes could be involved in transferring mRNA from the nucleus to the cytoplasm, when they identified such particles in the nuclei of rat liver. They experienced great difficulties in extracting and characterising the RNA because of its great instability, but suggested a sedimentation coefficient of approximately 30s. Recently, Infante and Nemer (1968) have further characterised these particles in the sea urchin embryo and by treatment with formaldehyde followed by cesium chloride centrifugation, demonstrated that they were heterogeneous, the heterogeneity being a function solely of the RNA, which varied from 10s to 40s.

It is difficult to assess the validity of these two mechanisms. Infante and Nemer (1968) suggested the 40s sub-unit-mRNA complex is an artefact of extraction since RNA extracted from informosomes of sea urchin embryos preferentially bound very tightly to 40s ribosomal sub-units. Although there is little doubt that informosomes exist, their function has not been fully substantiated. It is possible that they may solely serve as cytoplasmic storage organelles for mRNA and not be directly concerned with protein synthesis.

Although the mechanism of the transfer of mRNA from the nucleus to the cytoplasm is not understood, a clearer picture of the actual synthesis of protein on the ribosomes is now emerging. It is fully appreciated why the ribosome is so complex and is synthesised in a complicated though ordered and precise manner. The ribosome must have the ability to stereochemically recognise and bind with mRNA, tRNA and probably several enzymes and co-factors.

Takanami and Okomoto (1963) tested the ability of several synthetic polynucleotides to bind to isolated ribosomes. In all cases, they bound specifically to the smaller ribosome sub-unit. Takanami and Zubay (1964) discovered that when ribonuclease was added to a mixture of ribosomes and the polynucleotide, poly U "in vitro", a small amount of poly U, presumably that part which was chemically bound to the ribosome, was not degraded. Chromatographic methods revealed that the

undegraded portion of poly U consisted of 27 residues. Calculating the linear length of this chain, and knowing the dimensions of the 40s sub-unit, they were able to conclude that the polynucleotide could only bind along the long axis of the particle, i.e. at right angles to the long axis of the 60s sub-unit , assuming binding occurs in one plane only. The nature of the binding of mRNA to the ribosome was elucidated by Moore (1966) who chemically altered different chemical groups of the ribosomes and tested their ability to bind synthetic poly nucleotides. He was able to show that binding occurred by hydrogen bonding between the amino-groups of rRNA and the phosphate groups of the polynucleotide. Such specific bonding would not occur in the absence of ribosomal protein suggesting that the protein establishes a precise three dimensional structure for rRNA.

Cannon, Krug, and Gilbert (1963) established that amino-acyl tRNA "in vitro" attaches solely to the larger ribosome sub-unit. They went on to suggest that binding occurs through Mg^{++} bridges by phosphate groups of tRNA and 28s RNA. Recent work (see review by Schweet and Heintz, 1966) suggests that there are two sites on the ribosome for the attachment of tRNA. One has been called the "acceptor site" and here the amino-acyl tRNA, whose anticodon is complementary to the codon of the mRNA - 40s sub-unit complex, attaches itself to the ribosome. The amino-acyl tRNA - mRNA

complex simultaneously formed then becomes associated at a second ribosomal site, the "polymerisation site", where the amino-acyl residue forms a peptide bond with the adjacent amino-acyl residue of the growing peptide.

The mRNA appears in this mechanism to move in one direction relative to its attached ribosomes with the concomitant growth of the polypeptide chains at each ribosome. Wettstein, Stachlin, and Noll (1963) confirmed this movement of the mRNA relative to the ribosomes and further showed a stepwise movement, successive groups of three nucleotides becoming attached to the ribosome to be paired with the complementary nucleotides of the tRNA. It is uncertain as to which component of the polysome actually moves, but in the case of polysomes attached to membrane, it would seem probable that it is the mRNA strand.

Electron microscopic investigations suggest that some ribosomes occur attached to the endoplasmic reticulum. Blobel and Potter (1967), working with rat liver, treated membrane bound ribosomes both "in vivo" and "in vitro" to chemically remove mRNA and tRNA. The ribosome - membrane association was not affected, suggesting there is a binding site on the ribosome for the attachment of membrane. Sabatini, Tashiro, and Palade (1966) added increasing concentrations of EDTA to isolated guinea pig hepatic membrane bound ribosomes and showed a preferential detachment of the 40s sub-units.

Much higher concentrations of EDTA were required to detach 60s units from the membrane, and they concluded that the 60s sub-unit contained the membrane binding site. This was tentatively corroborated by electron microscopy.

Ribosomes, as well as being attached to endoplasmic reticulum, also exist free in the cytoplasm. Different cells or even the same cells at different stages in their life history have varying numbers of free and membrane bound ribosomes. Generally, cells which are actively dividing tend to have mainly free ribosomes, whereas undividing, differentiating cells have mainly membrane bound ribosomes. Secretory cells, e.g. from the liver, pancreas and mammary gland, have almost exclusively membrane bound ribosomes, and suggestions have been made that these are preferentially synthesising enzymes and proteins for "export", Campbell and Sargent (1967). Assuming interchange of the two ribosome species does not occur, a point which has as yet not been clarified, it might well be possible that there is a structural difference between the two ribosome species, one having a membrane binding site and the other lacking this site.

Although the cytoplasm can be regarded as the chief site of protein synthesis, organelles such as the chloroplast and mitochondrion are also capable of protein synthesis. Indeed, it has become established that these two organelles have their own nucleic acid complement which is distinct from

those of the cytoplasm. Recent evidence suggests that mitochondria and chloroplasts are only semi-autonomous, for although plastid DNA codes for some proteins, others are coded for by nuclear DNA (Kirk and Tilney-Basset, 1967).

Morton and Raison (1963) and Morton, Palk and Raison (1964) reported that the storage protein of wheat endosperm accumulates in plastids called protein bodies, from which could be isolated ribosomes, amino-acid activating enzymes, and tRNA. Preparations of protein bodies were found to incorporate radio-active amino-acids into several protein components separable by starch gel electrophoresis, Morton and Raison (1964). Unfortunately, no attempts were made to avoid bacterial contamination and subsequent work by Wheeler and Boulter (1967) using broad bean seed protein bodies and by Wilson (1966) using maize protein bodies, obtained high levels of amino-acid incorporation "in vitro" when bacteria were present, but when these were eliminated, only slight incorporation occurred. Electron microscopic investigations by Briarty (1967) suggested that broad bean protein bodies are not plastids since they appeared to arise "de novo" rather than arising from proplastids. He also could not detect ribosome-like particles in protein bodies, unlike Morton et al (1964).

These findings therefore cast doubts on the results of the Morton group, but the fact that the ribosomes removed

from wheat protein bodies by sonic disruption have a sedimentation coefficient less than that of cytoplasmic ribosomes (as has been reported for chloroplast and mitochondrial ribosomes) suggest that these are not contaminating cytoplasmic ribosomes. It is possible, therefore, that the protein bodies of wheat may be fundamentally different from those of maize and the broad bean.

Plants, in contrast to animals, often store energy reserves in the form of protein. This situation is probably most developed in the maturation stages of seeds of leguminous plants. In Vicia faba (L), the broad bean, for instance, massive protein accumulation occurs over a period of approximately 40 days, at the end of which 20% of the dry weight of the seed, i.e. 0.2g., is storage protein. Assuming, therefore, that nucleic acids are involved in storage protein synthesis, this tissue would be an ideal source for studying nucleic acid metabolism. Furthermore, since storage protein consists essentially of only two components, vicilin and legumin ((Danielson, 1952), the tissue may eventually be useful for attempting to isolate an mRNA coding for a known protein.

In the present study, the site of synthesis of storage protein has been investigated. This has involved RNA determinations of extracted protein bodies and an examination of the function and properties of free and membrane bound ribosomes throughout the development of the seed. A.

detailed analysis of the occurrence and change in levels of the major nucleic acids during development was also undertaken and related to other developmental processes occurring in the seed, reported by Grzesiuk, Mierzwinska, and Sojka (1962), Wheeler and Boulter (1966 & 1967), Davis (1967) and Yarwood (1968). The function and fate of the nucleic acids and free and membrane bound ribosomes during the germination of the seed, when the nutrient reserves of the cotyledon are mobilised for transport to the growing embryo, is also investigated.

In this thesis, heavy ribosomal RNA (the major RNA associated with the larger ribosomal sub-unit) is termed 28s RNA, and light ribosomal RNA (the major RNA associated with the smaller ribosomal sub-unit) is termed 18s RNA. These are descriptive terms and do not imply that these molecules sediment at exactly 28s and 18s respectively.

MATERIALS AND METHODS

1. Biological Material

A. The developing seed

Seeds of Vicia Faba (L) var. Triple White were obtained from Forizo & Co.Ltd., Liverpool. Plants were grown at The University of Liverpool Botanic Gardens, Ness, Cheshire, during 1966. The F1 generation were grown at the Science Laboratories, Durham City, during 1967. Seeds were sown in pots during February in heated greenhouses, and the young plants planted out of doors at the end of March. Other sowings after this date were made directly in the open. Crops were watered manually during drought.

Flowers were labelled on the first day on which they were observed to be fully open. At each age, pods of approximately the same size were selected for collection and the material further standardised by selecting seeds of approximately the same size from each age of pod. In all cases, large quantities of seed were picked and a random selection used for the different analyses. Seeds for all experiments were used within 18hr. of collection.

B. The dormant seed

130 day seeds of the 1966 crop were detached from the parent plants and dried in a ventilated warm room at 25°, to a constant weight. The dry seeds were stored in a dry cupboard at room temperature.

C. The germinating seed

Dormant seeds derived from the 1966 crop were imbibed in running tap-water for 24hrs., and surface sterilised by rapidly shaking with 5 vol. 10% /w/v) calcium hypochlorite solution for 20min. The seeds were directly placed in moist vermiculite at a depth of approx. 4 cm. below the surface, and were germinated at 20° with a 12hr. light, 12hr. dark cycle. The vermiculite, which contained a nutrient medium (Hewitt, 1952), was watered with sterile tap water periodically.

Large quantities of seeds were germinated, and cotyledons of average size and weight were selected. A strict examination for infection was maintained, and contaminated cotyledons were rejected.

2. Chemicals

Except for those listed below, chemicals were obtained from British Drug Houses Ltd., or May & Baker Ltd., and were of analytical grade when available.

Hyflo Super-cell	John-Manville Products Co., London
Whatman Chromedia CF 11	Baltson Ltd., London
Fibrous cellulose powder	
Bovine serum albumin, fraction V	Sigma Chemical Co.Ltd.
Sodium deoxycholate	London.
Nonidet, P.40	Shell Chemicals U.K. Ltd.
Oxoid nutrient broth No.2	Oxoid Ltd., London
Ringer solution tablets (quarter strength)	

2- mercaptoethanol) Kodak Ltd., London.

Chloramphenicol) Parke, Davis & Co., Hounslow, England.

2 - 5 diphenyloxazole (PPO).) Beckman Instruments Inc.,
)
1, 4 - bis (2 - (5 - phenyl) California, U.S.A.
)
oxazolyl) - benzene (POPOP).)

Dowex 1 x 4 (200 - 400 mesh)) Dowex Chemical Co.,
)
) Michigan, U.S.A.

Solid carbon dioxide) The Distillers Co.Ltd., Bootle, Lancs.,
)
("Cardice")) and Gateshead, Co. Durham.

Difco malt extract) Difco Laboratories, Detroit,
)
) Michigan, U.S.A.

Radio-active chemicals

Radio-active chemicals were purchased from The Radio-chemical Centre, Amersham, Bucks., and were :-

Uridine - 5 - (^{3}H). High specific activity (18 - 20 curies/mM) and sterilised.

D.L. - leucine - (^{3}H), uniformly labelled. High specific activity (20 - 25 curies/mM) and sterilised.

Orthophosphate - (^{32}P). High specific activity (43 - 95 curies/mg.phosphorus) and sterilised.

3. Preparation of reagents and solutions

Molybdivanadate solution for the determination of phosphorus

Solution A.

Ammonium metavanadate 1.25g.

Dilute nitric acid (1 part nitric acid conc. and 1 part water) ..... 100ml.

Solution B.

Ammonium molybdate 50g.

Distilled water 400ml.

Pour solution B into solution A, dilute to 1 l. with distilled water and mix.

Solutions for Lowry's Folin method of protein determination

Stock solution A.

Sodium carbonate (anhydrous)	20.0g.
0.01N sodium hydroxide solution	to 1 l.

Stock solution B.

Copper sulphate ($5H_2O$)	1.0g.
Distilled water	to 100ml.

Stock solution C.

Sodium or potassium tartrate	2.0g.
Distilled water	to 100ml.

Working solution D. (Made up on the day of protein determinations).

Stock solution A.	50.0ml.
Stock solution B.	0.5ml.
Stock solution C.	0.5ml.

Solution B is mixed with solution C before solution A is added.

Working solution E.

1 N Folin and Ciocalteu's phenol reagent.

Extractant for protein body isolation

0.5M sucrose

0.002M magnesium chloride

0.1M potassium phosphate buffer, pH 7.0

Extractant for microsome isolation

0.45 M sucrose

0.005 M magnesium chloride

0.016 M potassium chloride

0.007 M 2 - mercaptoethanol

0.05 M tris buffer, pH 7.60 at 0°.

Solutions for sucrose density gradient analysis of microsomes and ribosomes

10% (w/v), 15% (w/v), 34% (w/v), and 68% (w/v) sucrose in :-

0.005 M magnesium chloride

0.016 M potassium chloride

0.05 M tris buffer, pH 7.60 at 0°.

Scintillation fluid

I.	PPO	5.0g.
	Naphthalene	100g.
	Dioxane	to 1 l.
II.	PPO	6.0g.
	Dimethyl POPOP	0.10g.
	Toluene	to 1 l.

Nutrient medium for germination of seeds

Calcium nitrate ($4H_2O$)	0.80g.
Potassium nitrate	0.34g.
Magnesium sulphate ($7H_2O$)	0.38g.
Sodium dihydrogen orthophosphate ($2H_2O$)	0.20g.
Ferric chloride	0.07g.
Distilled water	to 1 l.

Microbiological materials

I. Nutrient agar.

Oxoid No.2 nutrient broth powder	25g.
Davis New Zealand agar	6g.
Distilled water	to 1 l.

II. Malt agar.

"Difco" malt extract	20g.
Davis New Zealand agar	15g.
Distilled water	to 1 l.

III. Beef peptone agar.

"Bovril" beef extract	3g.
Peptone	10g.
Davis New Zealand agar	15g.
Distilled water	to 1 l.

4. Water content of seeds

50 or more seeds were selected from each of the various ages collected, separated into cotyledons and testas, and weighed before and after lyophilisation.

5. The extraction and estimation of RNA of seeds

A. Extraction

The extraction procedures of Smillie & Krotkov (1960) and Hutchinson & Munro (1961) were modified and combined for use with lyophilised bean meal in a similar manner to that of Wheeler (1965).

Approximately 0.5g. bean meal was stirred in a centrifuge tube with 10ml. methanol containing 1 drop of formic acid for 5min. at room temperature. The suspension was then centrifuged and the residue treated in a similar manner with two more aliquots of methanol and formic acid. The final residue was stirred with 10ml. 5% (w/v) trichloroacetic acid (TCA) at 2° for 1min., and the slurry centrifuged. The procedure was repeated twice on the residue.

The residue was next stirred with 10 ml. of each of the following solvents, centrifuging between each treatment :-

(i) Briefly with 10 ml. absolute ethanol.

(ii) 10 ml. ethanol for 10min.

(iii) 10 ml. ethanol/chloroform (3:1)(v/v) for 10min.

(iv) 10 ml. ethanol/ ,, ,, ,, ,, 15min.

(v) 10 ml. ethanol/ether (3:1)(v/v) for 10min.

(vi) ,, ,, ,, ,, ,, ,, 15min.

(vii) 10 ml. ether.

The final residue was dried by holding the centrifuge tube in a water-bath at 60° and gently stirring the residue. When dry, the contents of the tube were hydrolysed with 10 ml. 0.3N potassium hydroxide for 18hr. at 37°. The tube was then cooled at 0° and the contents acidified to pH 2 with perchloric acid solution (pH paper) and left to stand for 30min. in an ice-bath. The precipitate formed was removed by centrifugation and washed three times with perchloric acid solution, pH2 at 2°. The supernatant fluid plus washings were adjusted to pH 8 with a concentrated solution of potassium hydroxide and made up to a known volume with distilled water.

This solution, containing RNA nucleotides, was purified on a 5 x 1 cm. column of Dowex 1, chloride form. After applying a known volume of sample to the column, the column was washed with 20 ml. 0.01M sodium chloride solution and the RNA nucleotides eluted "en masse" by 50 ml. 0.37M sodium chloride, 0.77N hydrochloric acid, and made up to a known volume.

B. Estimation

RNA was estimated by its absorption at 260 mμ against the sodium chloride, hydrochloric acid eluting solution, assuming a molar extinction coefficient of 10.8×10^3.

C. The estimation of contaminating compounds during the extraction procedure

I. Free nucleotides by their phosphorus content.

Phosphorus was estimated by the method described in Method Sheet No.53 of Unicam Instruments Ltd. (1960), modified for use with RNA extracts from bean meal by Wheeler (1965).

A calibration graph was prepared (Fig.1) using solutions of "Analar" grade potassium dihydrogen orthophosphate, previously dried at 105° for 24hr., containing a range of phosphorus concentrations from 0 to 40 μg./ml.

II. Protein

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). A calibration graph was prepared (Fig.2) using solutions of bovine serum albumin, previously dried "in vacuo" over conc. sulphuric acid for 48hr., containing a range of protein concentrations from 0 to 120 μg./0.6ml.

6. Cell number determinations of developing cotyledons

Two methods were used; method I was used at all stages in cotyledon development, whereas method II was used only on cotyledsons aged between 27 and 35 days.

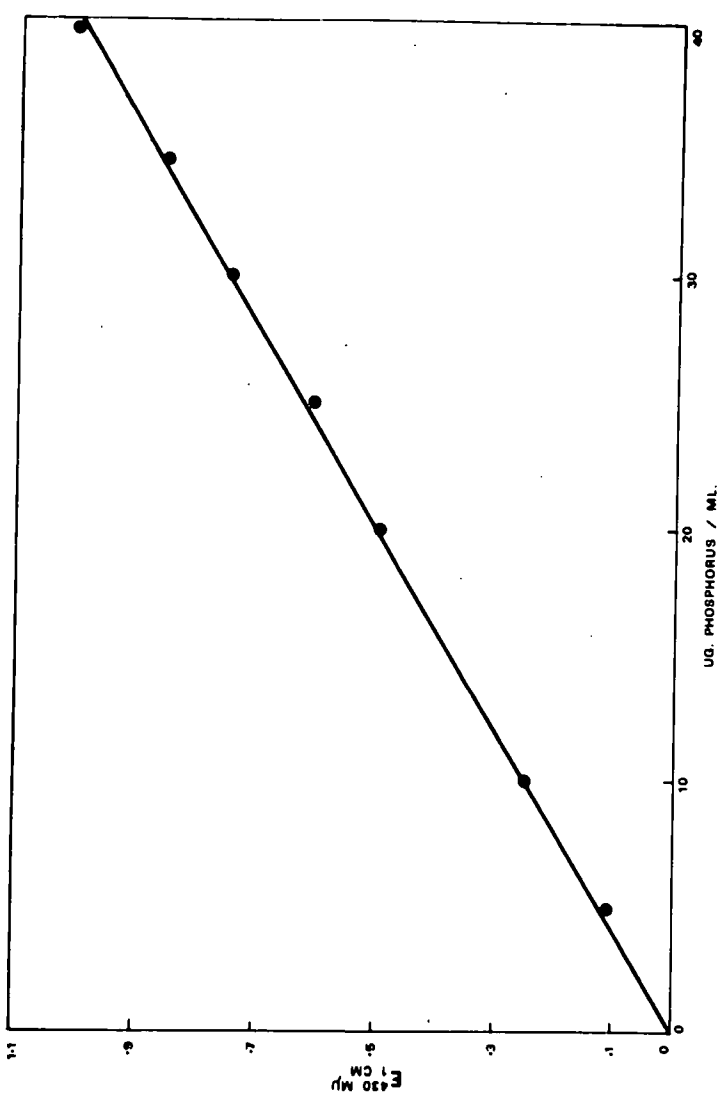
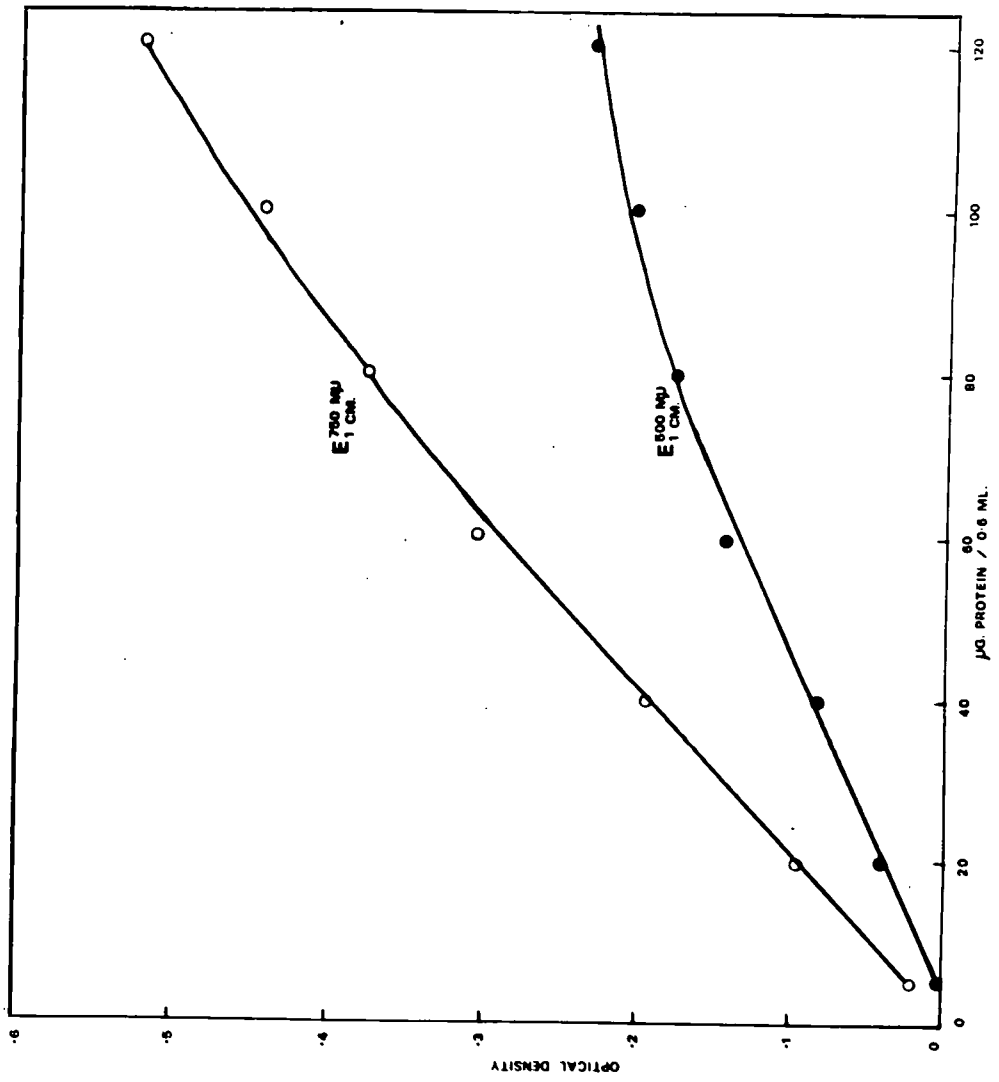


Fig.1. Calibration graph for the reaction of phosphorus with molybdivanadate

Fig.2. Calibration graphs for the determination of protein by the method of Lowry et al (1952)



Method I.

This method was modified from that of Loewenburg (1955).

60 to 80mg. of lyophilised cotyledon material were placed in a specially elongated Thunberg tube of internal diameter 1 cm., and 4ml. of 7.5% (w/v) chromic acid in 0.8N nitric acid was added. A drop of n-octanol was added to reduce frothing and the mixture was continuously infiltrated for 18hr. The tube was then violently shaken for 10min. and the solution reduced with excess sodium bisulphite. The sides of the tube were washed with distilled water and the volume reduced to less than 5ml. with an infra-red evaporator. Finally, a drop of 1% fast green was added, the volume was made up to 5ml., and the cells were counted on an haemocytometer slide.

Method II.

This method is similar to that of Brown and Rickless (1949) but has been modified to give a more vigorous maceration procedure.

60 to 80mg. of lyophilised cotyledon material were placed in a modified Thunberg tube described above, and continuously infiltrated with 4ml. of 5% (w/v) chromic acid and one drop of n-octanol for 12hr. The sample was then further treated as described in method I.

7. Methylated albumin-kieselguhr chromatography of seed nucleic acids

A. Esterification of serum albumin

Methylated serum albumin was prepared as described by Mandell and Hershey (1960).

B. Preparation of methylated albumin-kieselguhr columns

Methylated albumin-kieselguhr (MAK) columns were prepared as described by Mandell and Hershey (1960) with improvements suggested by Sueoka and Yamane (1962) and Yamane and Sueoka (1963). The columns were 1.8cm. in diameter and about 5cm. in height.

4g. kieselguhr (hyflo super-cell) were added to 20ml. of 0.1M sodium chloride in 0.05M sodium phosphate buffer, pH 6.7, in one beaker, and 0.5g. kieselguhr was added to 5ml. buffered 0.1M sodium chloride in a second beaker. The contents of both beakers were boiled to remove dissolved air and then cooled. To the first suspension, 1ml. 1% (w/v) methylated albumin in water was slowly added with stirring. Then, a further 7.5ml. of 0.1M sodium chloride was introduced.

The sinter of the glass column was covered with a 5mm. thick pad of moist cellulose powder. The methylated serum albumin kieselguhr suspension was then carefully poured into the column and packed under air pressure, /The column was and was followed by the introduction of the kieselguhr suspension.

washed with at least 100ml. 0.3M sodium chloride in 0.05M sodium phosphate buffer, pH 6.7, and stored in a cold room at 2 - 4° until required. A column was used for only one nucleic acid fractionation.

C. Extraction of nucleic acids

A modified phenol extraction method (Kirby, 1965) was used.

10 - 15g. of sliced cotyledons or testas were homogenised with 40ml. 6% (w/v) 4 amino-salicylic acid, sodium salt, and 60ml. 80% aqueous phenol (w/w) in an M.S.E. "Atomix" blendor at full speed for 30sec., at 4°. The homogenate was filtered through two layers of muslin and the filtrate centrifuged at 3,000 x g. for 10min. at 4°. The top, aqueous layer was carefully removed and the nucleic acids precipitated by the addition of two vols. of 95% (v/v) ethanol at -20° and by storing at -20° overnight. The precipitate was collected by centrifugation, re-suspended in 10ml. ethanol, water, 10% (w/v) NaCl, 2.5/1.0/0.1, (v/v/v) and pelleted again by centrifugation. The precipitate was washed two more times in the same solution.

D. Chromatography of the nucleic acids

The final nucleic acid precipitate was dissolved in 0.3M sodium chloride, 0.05M sodium phosphate, pH 6.7, and the solution adjusted to give a maximum concentration of 2mg.

nucleic acid in 40ml. of solution. 40ml. of the sample was passed through the column at a flow rate of about 2ml. per min. and the column was further washed with 0.3M buffered sodium chloride until ultra-violet light absorbing substances were no longer eluted. The nucleic acids were then eluted with 200ml. of a linear gradient of 0.3M to 1.15M sodium chloride, buffered with sodium phosphate at pH 6.7, at a maximum flow rate of 1ml. per min. using a peristaltic pump. Nucleic acids eluted from the column, were detected by their absorption at 254 mμ using an Isco model 222 ultra-violet continuous flow monitor, and collected into 1.5ml. fractions.

Chromatography was carried out at room temperature.

8. The determination of the nucleic acid content of protein bodies

A. Method I.

Protein bodies were prepared from cotyledons by the method of Morris (1968).

20g. cotyledons, cooled to 0 - 4°, were sliced into about 0.3mm. thick sections using a modified hand microtome, and immediately placed into 40ml. extractant at 0 - 4°. The slices were stirred gently for 10min. and then strained through gauze. All subsequent operations were performed at 0 - 4°.

The filtrate was centrifuged at 100 x g. AVE. for 4min. The supernatant was removed and centrifuged three more

times at 100 x g. AVE for 4min. The final supernatant was centrifuged at 2,500 x g.AVE for 6min., the sediment was removed and stored, and the supernatant again centrifuged at 2,500 x g.AVE for 6min. The two centrifuge pellets were re-suspended in 20ml. extractant and the suspension centrifuged at 3,900 x g.AVE for 10min. The sediment was re-suspended and centrifuged down again. Finally the sediment was suspended in 95% aqueous ethanol, centrifuged, and lyophilised.

The RNA content of the lyophilised powder was determined by the modified Smillie and Krotkov method (1960) described in "Materials and Methods", sections 5A and 5B.

B. Method II.

300g. cotyledons were homogenised at 0 - 4° in 1.4 l. of extractant using an M.S.E. Atomix blendor at full speed for 45sec. The homogenate was strained through 2 layers of muslin and the filtrate used to isolate protein bodies as described above, "Materials and Methods", section 8A.

Nucleic acids were extracted by the phenol method, and characterised by MAK chromatography as described in "Materials and Methods", section 7.

9. The isolation and RNA content of ribonucleoprotein particles of cotyledons

A. The isolation of microsomes

Microsomes were isolated from cotyledons by a similar manner to that described by Yarwood (1968).

5g. sliced cotyledons were rapidly ground in a crucible containing liquid nitrogen. The fine powder produced was suspended in 20ml. microsomal extractant at 0 - 4° and transferred to a rimless boiling tube. The suspension was homogenised by hand with 5 strokes of a teflon homogeniser with a tube clearance of ca.0.3mm. The homogenate was filtered through two layers of muslin and the filtrate centrifuged at ca. 1,000 x g. for 5min. The supernatant was centrifuged at 13,000 x g.AVE for 30min., and the supernatant obtained was centrifuged at 105,000 x g.AVE for 2hr. High speed centrifugation was carried out using an M.S.E. "superspeed 50" or "superspeed 65" centrifuge, and the temperature was controlled to 1 $\pm \frac{1}{2}$° using a calibrated temperature indicating system.

The sediment formed during the final centrifugation was defined as the microsomal pellet.

B. The isolation of free and membrane bound ribosomes

The microsomal pellet, prepared above, was carefully suspended in 0.5ml. 0.005M $MgCl_2$, 0.016M KCl, and 0.05M tris buffer, pH 7.60 at 0°. Approximately 0.2ml suspension was carefully layered on to the surface of a complex sucrose density gradient in a 23ml centrifuge tube. The gradient consisted of 4ml. 68% (w/v) sucrose, above which was 15ml. of a linear gradient of 10% (w/v) to 34% (w/v) sucrose in gradient solution

(see "Reagents and Solutions"). The gradient was centrifuged at 105,000 x g.AVE for 2hr. at 1 $\pm \frac{1}{2}^{o}$ using an M.S.E. 3 x 23 swing out rotor, catalogue no.59590.

After centrifugation, the gradient was fractionated by a modified method of Oumi and Osawa (1966). A thin steel rod was carefully inserted down the side of the tube until it rested at the bottom. The gradient was pumped out of the tube at a flow rate of 0.5ml per min. using a peristaltic pump, and the optical density at 254 mμ was monitored using an Isco Ultraviolet Analyser, model 222. The two main bands in the gradient, corresponding to free and membrane-bound ribosomes, were collected using a manually-operated fraction collector.

C. The RNA content of free and membrane bound ribosomes

Solid potassium hydroxide was added to the free or membrane bound ribosome fraction obtained above, to give a molarity of 0.3. The tube contents were incubated for 18hr. at 37° and the hydrolysed RNA purified and estimated as described in "Materials and Methods", sections 5A and 5B.

10. Measurement of Radioactivity

The radioactivity of all compounds was measured using a Beckman Liquid Scintillation Counter, model Ls-200B. The instrument is fully automated, operates at the ambient room temperature, is designed to count soft beta emission, and has a three channel counting capability.

A. (^{3}H) - compounds

0.5ml. of sample was added to 10ml. PPO/naphthalene/dioxane scintillation fluid, and then assayed for radio-activity, for 20min. at least three times. Samples containing intact ribosomes were solubilised with one drop of potassium hydroxide before the scintillation fluid was added. Each radio-active count was automatically calibrated with an external standard to give an external standard channels ratio. Corrections were made for background and also for quenching by means of an improvement of the channels ratio method (Baillie, 1960) as described in the Beckman manual to give absolute count rates. This required a calibration graph of the external standard channels ratio against the counting efficiency of the machine and was determined as described below.

(^{3}H) - uridine of known radio-activity (about 200,000 D.P.M.) was weighed out accurately on a five-place balance into each of five scintillation vials. 10ml of scintillation fluid was added to each sample followed by differing amounts of quenching agents, in this case sucrose gradient solutions. The samples were assayed for radio-activity and the results obtained were expressed graphically in the form of the counting efficiency of the machine against the external standard channels ratio (fig.3).

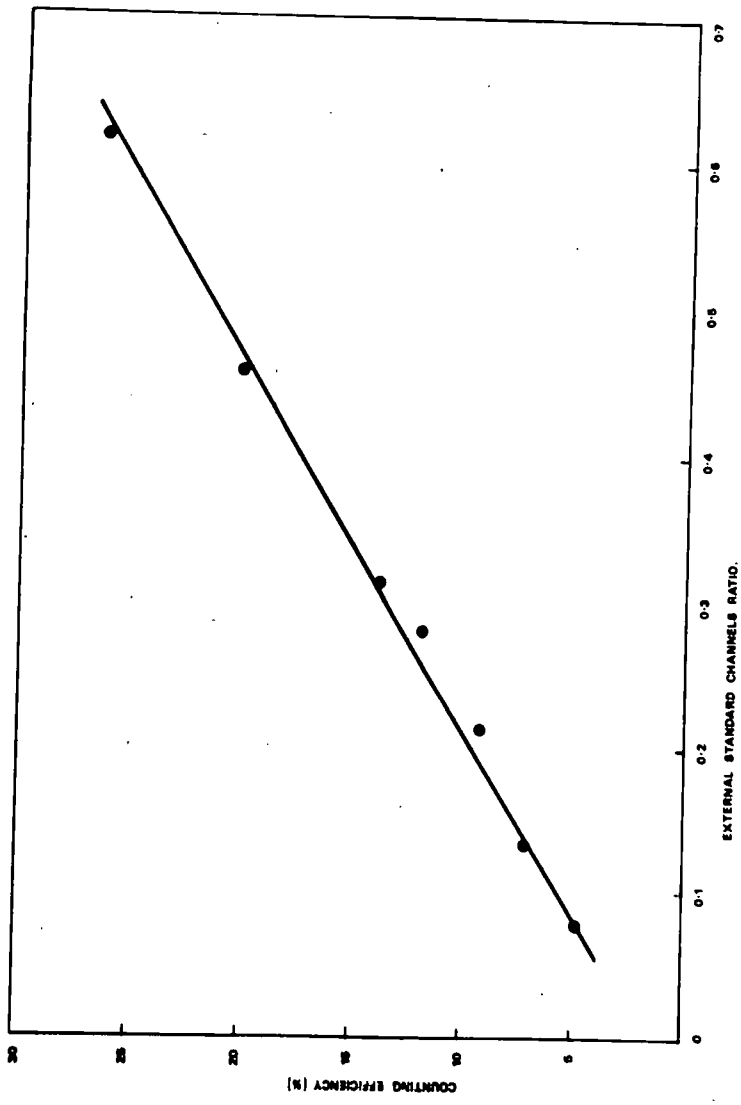


Fig.3. Calibration graph for the counting efficiency of tritium samples using a Beckmann Liquid Scintillation Counter, model Ls - 200 B

B. (^{32}P) compounds

0.5 to 1.0ml sample was added to a scintillation vial, evaporated to dryness, and treated with 10ml of PPO/POPOP/ toluene scintillation mixture. Samples were counted for 20min. at least twice using a fully open counting window. The efficiency of the machine in counting (^{32}P) emission was assumed to be 100%.

11. The counting of bacteria

Aliquots of incubations were checked for microbial contamination by a serial dilution method. Aliquots were diluted in quarter strength Ringer's solution and 0.1ml samples plated out on nutrient agar, and either malt or beef peptone agar. Triplicate samples of each dilution were made in each case. The plates were incubated at 24° for 3 days before the colonies were counted.

12. The short period incorporation of (^{32}P) - orthophosphate into developing cotyledon discs

A. The preparation and incubation of cotyledon discs

5g. of cotyledons were asceptically removed from developing seeds and sliced into 1 - 2mm. thick sections at 3° using a clean hand microtome, previously swabbed with absolute alcohol. The slices were washed with about 100ml sterile water and asceptically placed in a sterile flask containing 10ml 0.01M tris buffer, pH 7.1 at 30°. The

flask was incubated at 30° for 15min. and then 0.3mc (^{32}P) - orthophosphate was added asceptically. Incubation was continued for a further 3hr.

B. The determination of the radio-activity associated with the various nucleic acid fractions

At the completion of the incubation period, the cotyledon slices were removed and washed with at least 100ml of sterile water. Nucleic acids were extracted and fractionated as described in "Materials and Methods", sections 7C and D. 1ml aliquots of the fractions were assayed for radioactivity as described in "Materials and Methods", section 10B.

13. The incorporation of (^{3}H) - uridine into developing cotyledons "in vivo".

A. The injection of (^{3}H) - uridine into the pods of developing fruits

Pods were carefully swabbed with absolute ethanol and pierced by a sterile syringe needle in two places. One hole served as an air vent and the other used for the introduction of sterile (^{3}H) - uridine into the air spaces of the pod between the developing seeds. After removal of the syringe needle, the two holes were swabbed with absolute ethanol and smeared with lanolin. (^{3}H) - uridine was injected in the approximate proportions of 0.3mc (0.1mc/ml.) to 5g. fresh weight of seeds.

B. The determination of radio-activity associated with free and membrane bound ribosomes

After five to seven days of incubation, the cotyledons of the developing seeds were removed from the parent plants, and free and membrane bound ribosomes were extracted as described in "Materials and Methods", sections 9A and B. The radio-activity of fractions obtained by sucrose gradient centrifugation was assayed by the method described in "Materials and Methods", section 10A.

C. The extraction of RNA from free and membrane bound ribosomes and the measurement of its radio-activity

A suspension of ribosomes was made 3% (w/v) with respect to sodium 4 amino-salicylic acid by adding solid crystals and stirring. RNA was extracted by the phenol method described in "Materials and Methods", section 7C, with the exception that the nucleic acid fraction was treated with 2 volumes of absolute ethanol for 48hr. at -20°, instead of overnight treatment.

The final nucleic acid precipitate was dissolved in a small volume of liquid, estimated quantitatively by its absorption at 260 mμ, and assayed for radio-activity as described in "Materials and Methods", section 10A.

14. The short period incorporation of (^{3}H) - leucine into developing cotyledon discs

A. The preparation and incubation of cotyledon discs

60 day cotyledon discs were prepared and incubated in the presence of (^{3}H) - leucine for 4hr. as described in "Materials and Methods", section 12A.

B. Sucrose gradient centrifugation of the 13,000 x g.AVE supernatant

The cotyledon discs were washed with at least 100ml of cold sterile water and then treated as described in "Materials and Methods", section 9A, to obtain a 13,000 x g.AVE supernatant. 5ml of this supernatant was layered on to 17ml of a linear gradient of 15% (w/v) sucrose to 34% (w/v) sucrose in gradient solution (see "Reagents and Solutions"), and centrifuged at 105,000 x g.AVE for 2hr in a swing out rotor. The gradients were fractionated and monitored for ultraviolet light absorbing substances as described in "Materials and Methods", section 9B, taking care not to disturb any pellets formed at the bottom of the centrifuge tubes, and the fractions assayed for radio-activity as described in "Materials and Methods", section 10A.

C. Treatments of the 13,000 x g.AVE supernatant prior to sucrose gradient centrifugation

In some experiments, the mitochondrial supernatant was treated with detergent and/or ribonuclease prior to sucrose gradient centrifugation.

I. Detergent

A concentrated solution of detergent was added to the sample to give the required concentration and the mixture was stirred for a few minutes at 4^{o} prior to centrifugation. Two detergents were used, 0.4% nonidet P40 or sodium deoxycholate at a range of concentrations.

II. Ribonuclease

A concentrated solution of ribonuclease was added to the sample to give a concentration of 0.5 µg/ml. The sample was incubated for 1hr at 4^{o} before centrifuging.

15. The incorporation of (^{32}P) - orthophosphate into germinating seeds

A. The surface sterilisation and germination of seeds

Three dormant seeds were vigorously shaken with 4 volumes of absolute ethanol for 10min. and then with 4 volumes of 1% calcium hypochlorite for 20min. The seeds were placed directly into a sterile growth chamber without washing. The chamber, previously autoclaved at a pressure of 15lb/sq.in. for 15min., consisted of a 400ml rimless, lipless beaker enclosed by a glass Petri dish lid. The beaker contained 20ml sterile, distilled water and a small piece of absorbent cotton wool. 0.3mc of sterile (^{32}P) - orthophosphate was introduced using a sterile syringe, followed in some experiments by 800 µg of chloramphenicol.

The growth chamber was transfered to a growth room at 20° with a 12hr. light, 12hr. dark cycle.

B. The determination of radio-activity associated with free and membrane bound ribosomes

Free and membrane bound ribosomes were isolated as described in "Materials and Methods", sections 9A and B. Radio-activity of sucrose gradient fractions was assayed as described in "Materials and Methods", section 10B.

C. The determination of radio-activity associated with the RNA extracted from free and membrane bound ribosomes

Free and membrane bound rRNA was prepared as described in "Materials and Methods", section 13C and the radio-activity was assayed as in section 10B.

RESULTS

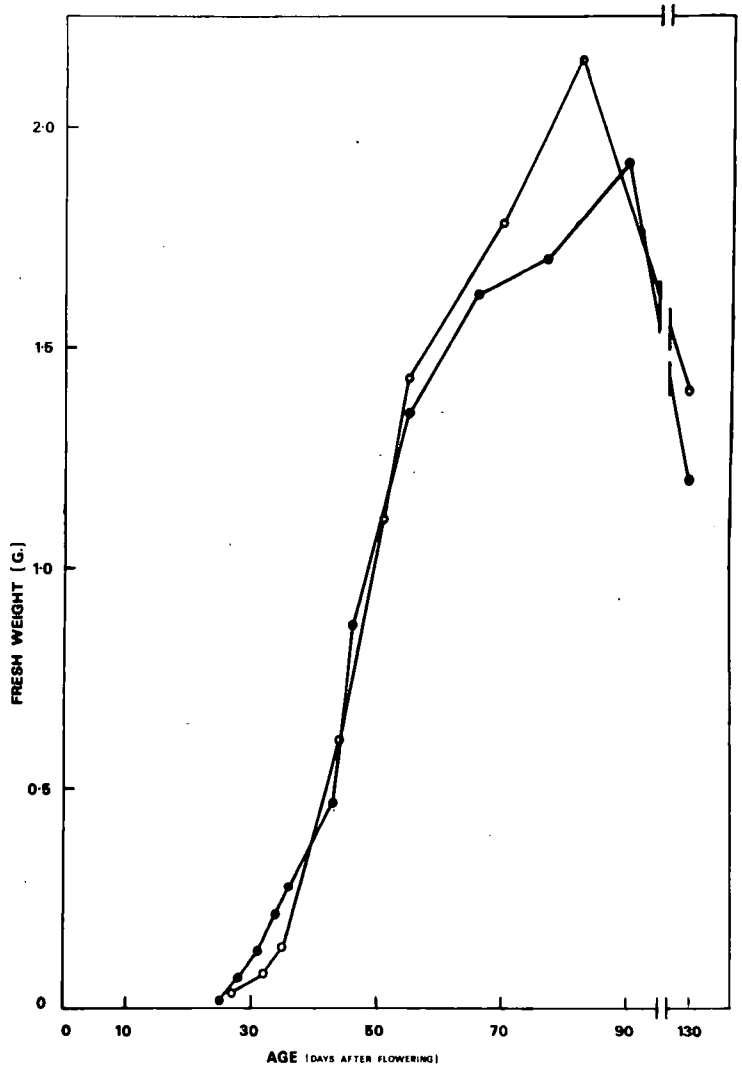
PART I. The developing seed

1. The fresh weight, dry weight, and water content of seeds

The average fresh and dry weights of cotyledons increased slowly to about 35 days after flowering, and then increased rapidly to about day 90 (figs. 4 and 5). After this age, the dry weight remained approximately constant but the fresh weight decreased to the final determination at day 130. Cotyledons collected during 1967 had larger fresh and dry weights than cotyledons of the 1966 crop during the early stages of development, but during later stages they became substantially smaller.

Changes in the fresh and dry weights of testas from seeds collected in 1967 are shown in fig.6. Initially the testa made the largest contribution to both the fresh and dry weight of the whole seed. At about day 40, however, the cotyledon became the heaviest component of the seed. The fresh weight of the testa reached a maximum at about day 55 and then started to decrease, while the dry weight of the testa reached a maximum between days 50 and 65 and then remained approximately constant. By day 90, the dry weight of the cotyledon was about 6 times greater than the dry weight of the testa.

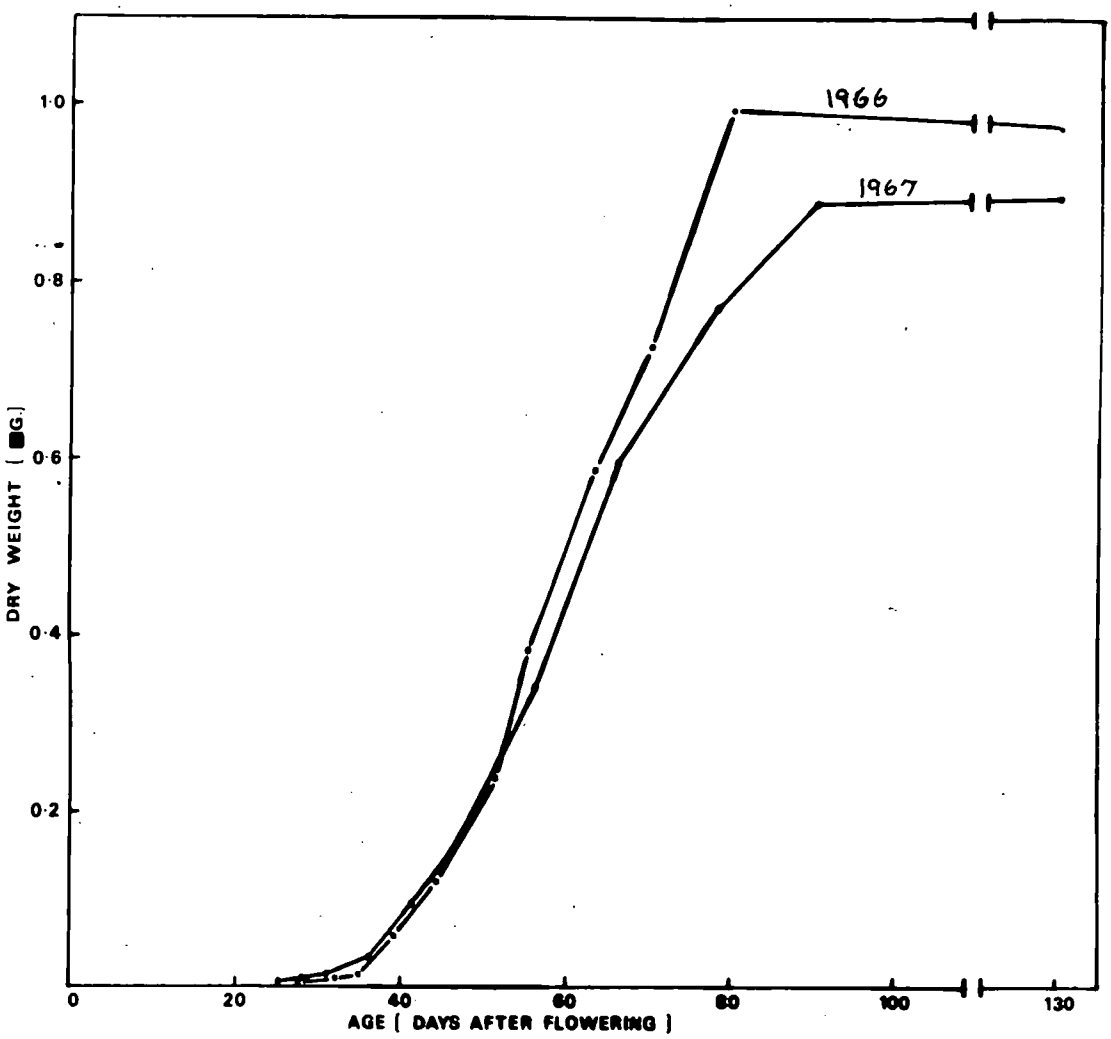
Fig.4. The variation in the fresh weight of developing cotyledons collected during 1966 and 1967



o ——— o 1966 season

● ——— ● 1967 season

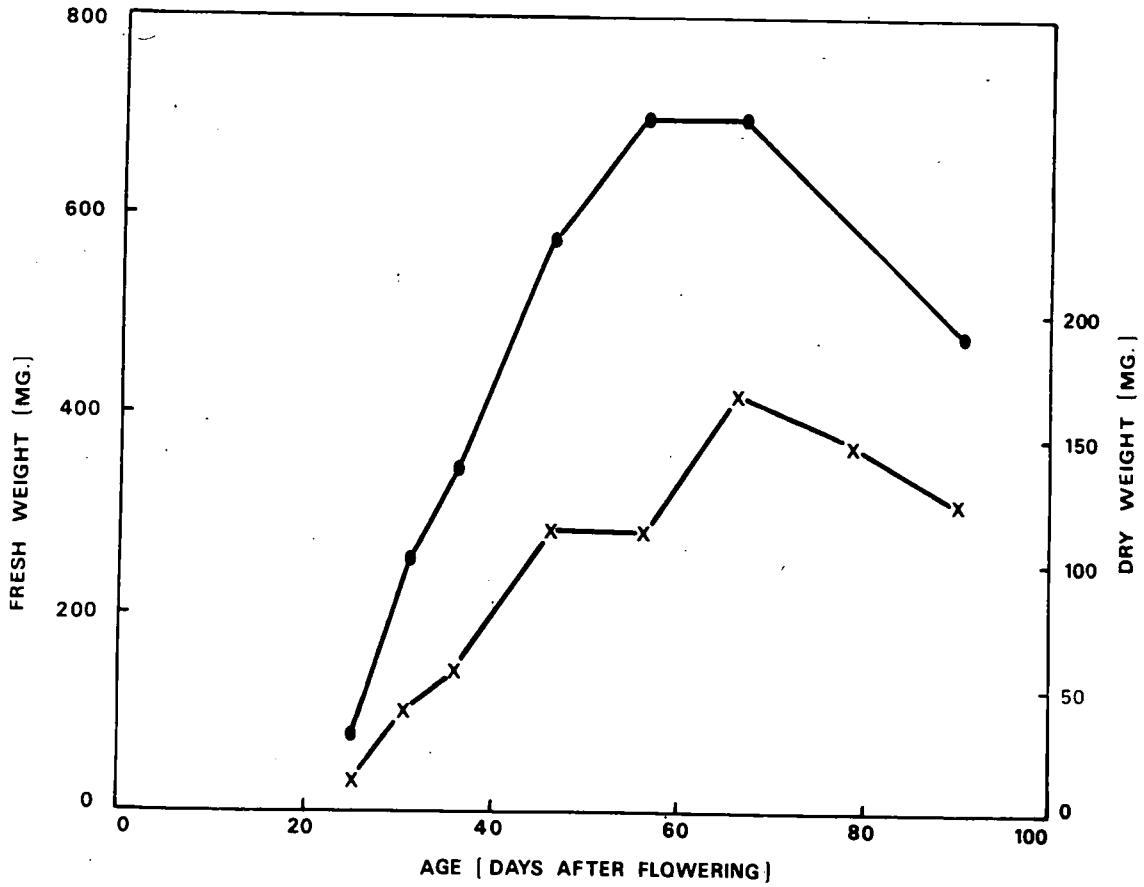
Fig.5. The variation in dry weight of cotyledons separated from developing seeds collected in 1966 and 1967



■------■ 1966 season

0-------0 1967 season

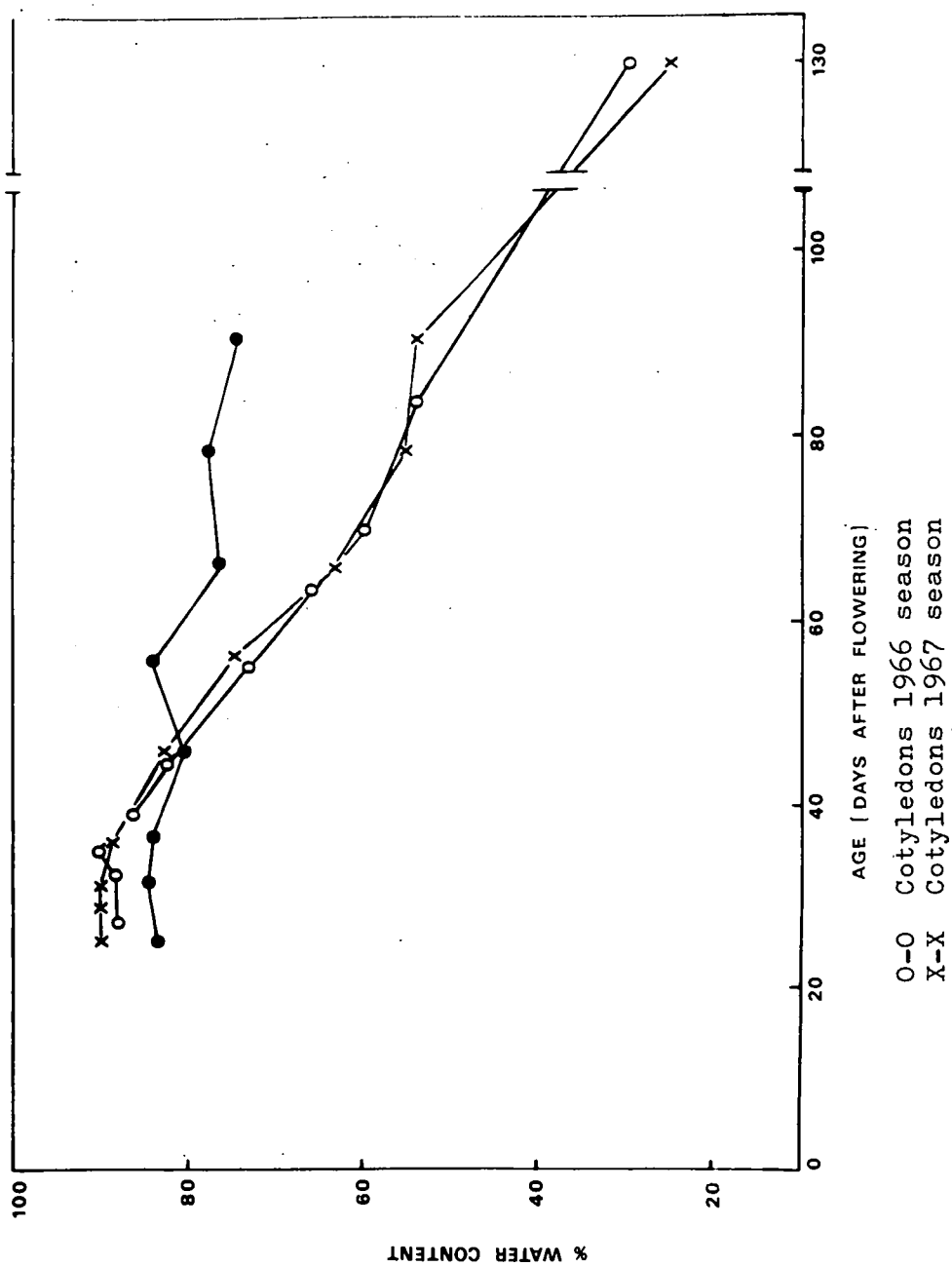
Fig.6. The variation in the fresh and dry weight of testas, collected from developing seeds in the 1967 season



● - ● Fresh weight

X - X Dry weight

Fig.7 The variation in the % water content of testas and cotyledons separated from developing seeds collected in 1966 and 1967



The water content of the cotyledons, expressed as a percentage of the fresh weight, was very similar in both growing seasons, fig.7. At early stages of development the water content was high, about 88%, and at about day 40 began to fall constantly to about day 130, when it was 30% of the fresh weight. Further loss of water did not occur in the field. The water content of the testas on the other hand varied only slightly during those stages of seed development sampled, fig.7, though a slight decrease with age did occur.

2. Cell number determinations of cotyledons

A. The reproducibility of method I

Using mature seeds, soaked for 24hr, it was found that eight separate counts for each of three macerations, giving a total of 24 values for each determination, gave reproducible results. Table 1 shows four such determinations, the values of which correspond within $\pm 3\frac{1}{2}\%$.

TABLE 1

Cell number determinations of lyophilised cotyledons, from mature seeds imbibed for 24hr

Determination	Cells per mg.dry weight of cotyledon			Average cells per mg.dry weight
	Maceration 1	Maceration 2	Maceration 3	
1	5,470	5,977	6,020	5,822
2	4,718	5,471	6,484	5,556
3	6,237	6,078	5,326	5,800
4	6,383	5,543	5,902	5,943
			Mean	5,780
			% difference between maximum and minimum average values	6.7%

Cell numbers were determined by method I as described in the methods.

The eight haemocytometer counts for each maceration involved counting cells in 40 large squares, and the distribution of cells within these squares was shown to approximate to a calculated Poisson probability distribution, table 2. The first three examples represent macerations of the same tissue, and the fourth represents the combined experimental results of the first three, and a re-calculated Poisson distribution. The experimental and theoretical

values of example 4 were compared by the χ^2 test, table 2, and shown to have a high probability of correlation.

This suggests sufficient squares were counted to give a reliable cell count determination.

B. The comparison of methods I and II

Method I, involving a relatively rigorous maceration procedure, was compared with the mild maceration procedure of method II for early stages of the developing cotyledon. Both methods gave approximately similar cell number values, and there was no evidence of cell disruption, though cells, treated by method I especially, had contracted protoplasts, (Table 3).

C. The change in the numbers of cells per cotyledon pair during the development of the seed.

The cell numbers of cotyledons, collected in 1966, increased steadily from the first determination at 27 days after flowering and reached a constant value, approximately 5.6×10^6 cells, by about day 55, though the exact age is difficult to assess, fig.8.

TABLE 2

The comparison of the theoretical and observed distribution of cells in suspension, on a haemocytometer slide

Sample No.		The number of cells per large square						
		0	1	2	3	4	5	6
1.	Probability distribution	0.056	0.161	0.233	0.233	0.161	0.093	0.044
	Frequency expected in 40 samples	2	6	9	9	6	4	2
	Actual frequency	1	11	6	10	3	4	5
2.	Probability distribution	0.119	0.252	0.269	0.190	0.101	0.043	0.015
	Frequency expected in 40 samples	5	10	11	8	4	2	1
	Actual frequency	5	11	11	5	5	1	2
3.	Probability distribution	0.146	0.281	0.271	0.174	0.084	0.032	0.010
	Frequency expected in 40 samples	6	11	11	7	3	1	0
	Actual frequency	8	11	7	7	5	1	1
4.	Probability distribution	0.100	0.230	0.265	0.204	0.118	0.055	0.021
	Frequency expected in 120 samples	12	28	32	24	14	7	3
	Actual frequency	14	33	24	22	13	6	8
4.	Probability of correlation between frequency of actual and expected by χ^2 test	0.99-1.00	0.99 1.00	0.99 0.95	0.99 1.00	0.99 1.00	0.99 1.00	ca. 0.80

Samples were derived from 48hr soaked mature seeds.

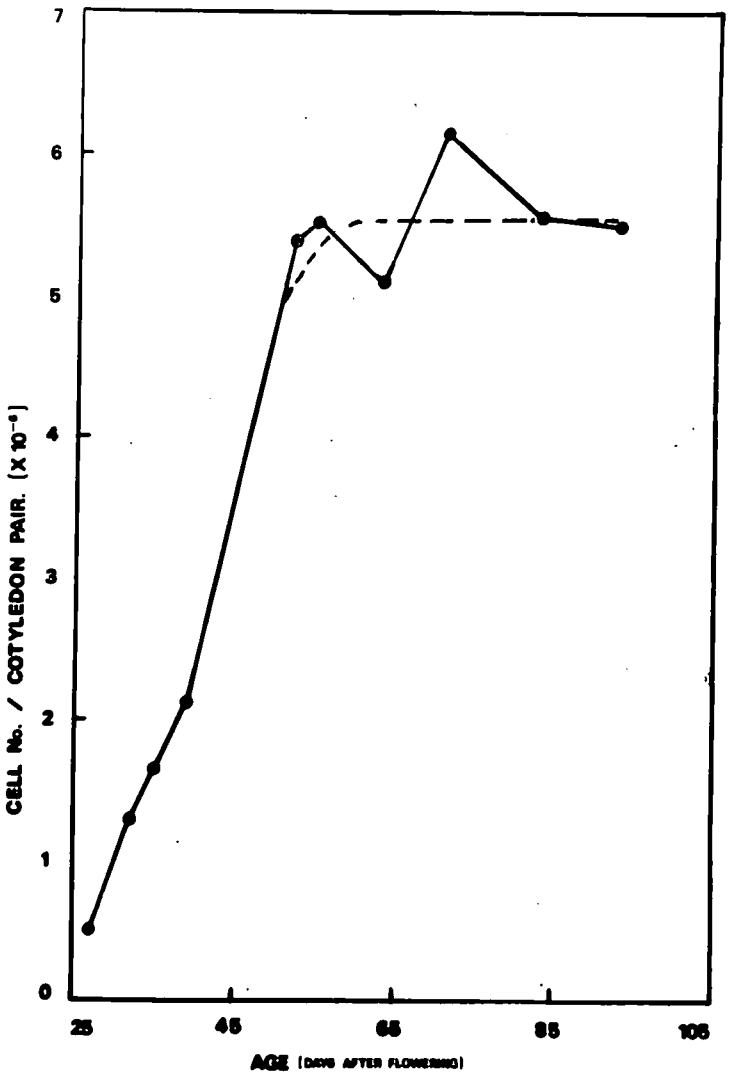
The Poisson probability distribution was calculated from the formula $1 = e^{-z}\left(1 + z + \frac{z^2}{2!} + \frac{z^3}{3!} + \frac{z^4}{4!} + \ldots\ldots\right)$, where z = the mean no. of cells per large square, for a determination. χ^2 was determined for each value by the formula $\chi^2 = \frac{(A-E)^2}{E}$ where A is the observed frequency and E is the expected frequency. The probability of correlation was determined by χ^2 tables.

TABLE 3

Cell number determinations of developing cotyledons by methods I and II

Age of developing seed	Method I (cells/Cotyledon pair)	Method II (cells/cotyledon pair)	Mean Value	Variation between Methods I and II
27	5.15×10^5	5.41×10^5	5.28×10^5	4.9%
32	1.23×10^6	1.28×10^6	1.26×10^6	4.0%
35	1.64×10^6	1.60×10^6	1.62×10^6	2.5%
39	2.12×10^6	Maceration incomplete	-	-
44	2.88×10^6	Maceration incomplete	-	-

Fig.8. The variation in the cell numbers of developing cotyledons, collected in 1966



----------estimated mean.

Assuming at the fertilisation of the flower, i.e. at day 1, the embryo consists of one cell only, the change in the number of cell generations of cotyledons during seed development can be calculated, by the formula

$$\frac{\log_{10} C}{0.301 (\log_{10} 2)} = g - 1$$

where C = the number of cells per cotyledon and g = the number of cell generations. Fig.9 is a plot of these results, and reveals that the rate of cell division was relatively low for all ages determined and was virtually zero by day 45.

3. The RNA content of seeds

A. The demonstration of the complete removal of contaminating free nucleotides from RNA in the extraction process

Since RNA is ultimately purified and estimated as free nucleotides, naturally occurring nucleotides must be completely removed from the sample, prior to the hydrolysis of RNA. In this method, 5% (w/v) TCA is used to extract nucleotides and related compounds. The results in Table 4 show that effectively all soluble phosphorus containing substances were extracted after four TCA treatments.

B. The purity of the final RNA nucleotide fraction

Fig.10 compares the absorption spectrum of a purified sample of RNA nucleotides derived from developing cotyledons, with the absorption spectrum of a mixture of pure nucleotides

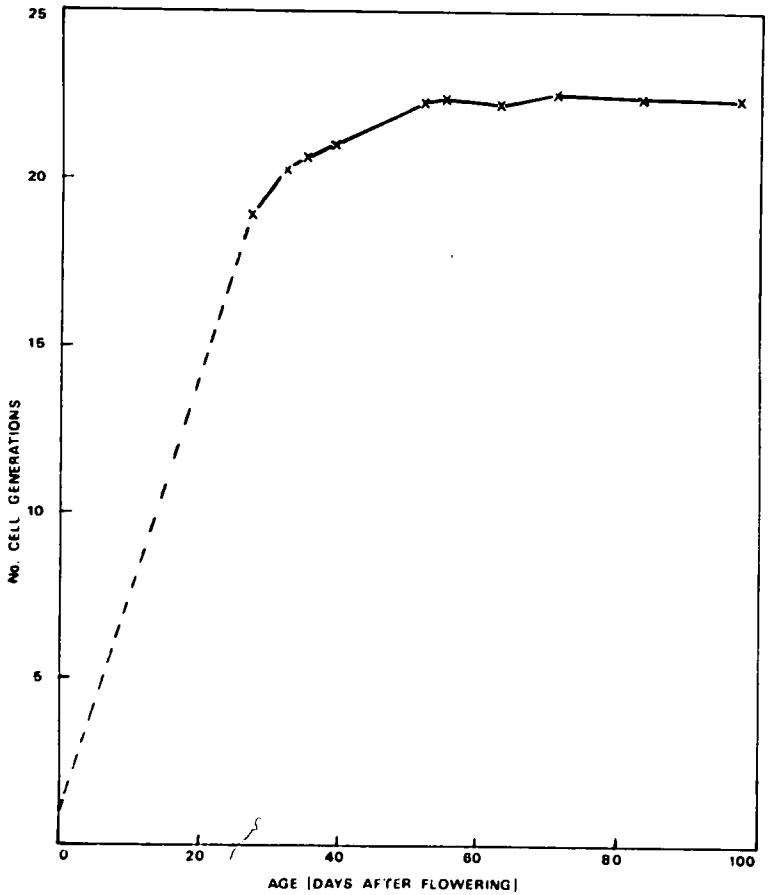
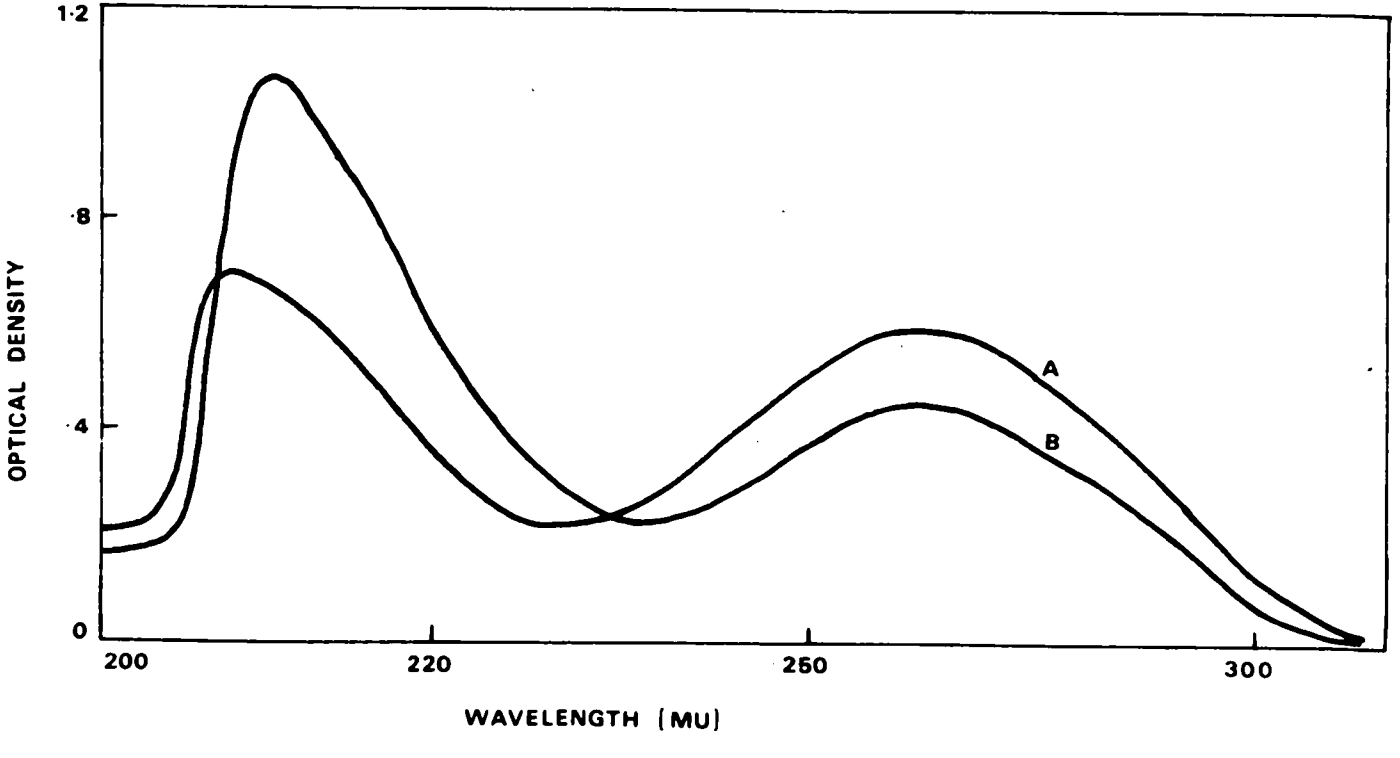


Fig.9. The variation in the number of cell generations of cotyledons, collected from developing seeds in 1966.

The change in the number of cell generations of cotyledons during seed development was calculated as described in the results section.

-·-·- x-x Experimental determinations. ------- Extrapolated values.

Fig.10. Absorption spectra of RNA nucleotides



A. A pure mixture of the four RNA nucleotides (B.D.H.Ltd.) present in equal molar concentrations.

B. The final RNA nucleotide fraction for RNA estimation (from 56 day developing cotyledons). Absorption spectra were determined in 0.37 M Sodium chloride, 0.77 N hydrochloric acid.

TABLE 4

The Phosphorus content of compounds extracted from bean-meal by serially washing with 5% (w/v) TCA

TCA treatment number	Experiment 1		Experiment 2	
	µg phosphorus	% total	µg phosphorus	% total
1	362.5	56.6	410.0	52.2
2	192.5	30.1	232.5	29.7
3	80.0	12.5	137.5	17.5
4	5.0	0.8	5.0	0.6
5	0.5	0	0.5	0

In both experiments, 0.5g samples of bean-meal, derived from mature seeds, were used. Samples were pre-treated with methanol and formic acid as described in the methods. The supernatants of the TCA washes were evaporated to dryness in an infra-red evaporator prior to phosphorus determinations.

present in equal molar concentrations. The absorption spectra are quite similar and each has an absorption minimum at ca.232mµ and a maximum at ca.260mµ. However, there is evidence of contaminating materials absorbing at wavelengths around 220mµ, but the absorption at 260mµ, where RNA is estimated, is unlikely to be affected.

The RNA nucleotide fraction was routinely tested for the presence of protein by the Lowry Folin method, and was never at a concentration liable to affect the absorption of the sample at 260 mμ.

C. The reproducibility of the extraction procedure

Five samples of seed meal, derived from dormant, mature seeds, were quantitatively analysed for RNA, Table 5. The percentage difference between the greatest value and the smallest value, expressed per g.dry weight of bean-meal, was 4.0%.

D. The change in total RNA per cotyledon pair and per testa during development

The change in the RNA content of cotyledons during development, fig.11, follows quite closely the change in dry weight (fig.5). There was little increase in RNA between days 25 and 35, but after day 35 there was a rapid increase to days 65-70. By day 80, the increase in RNA had ceased, and had attained a value of 5.6mg/ cotyledon pair, and between then and day 130, there was a loss of some 25% of this RNA. There was a marked similarity of RNA levels in cotyledons for the two growing seasons, as has been shown for other analyses. The RNA content of the cotyledons in the 1967 season was slightly higher than the 1966 cotyledons at early stages of development, but by day 55 the RNA level was lower, and remained so for the rest of the developmental period.

TABLE 5

The RNA content of dormant seed bean-meal

Determination	Mass of bean-meal(g)	Mass of RNA nucleotides(mg)	Mass of RNA nucleotides (mg) per g. of bean-meal
1	0.566	0.968	1.731
2	0.555	1.001	1.803
3	0.715	1.273	1.780
4	0.438	.767	1.752
5	0.542	.974	1.797
		Mean	1.77
		% difference between maximum and minimum value	4.0%

RNA was extracted and estimated as described in the methods section.

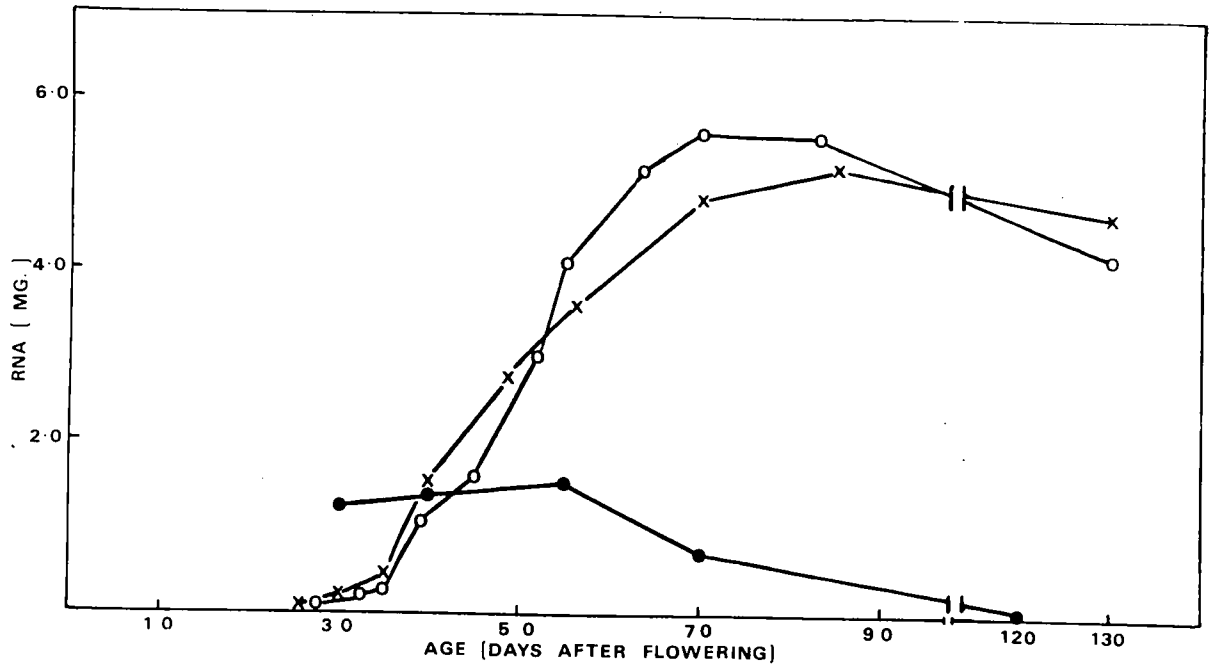
At early stages of development, around 30 days after flowering, the testas contain considerably more RNA than the cotyledon, fig.11. This level does not increase with age, however, and by day 60 began to decrease, until by day 120 it was approaching zero.

E. The change in total RNA per cotyledon cell during development

A combination of the cell number and RNA content of cotyledons from the 1966 season enables a graph to be constructed of the RNA content of the average cotyledon cell against the age of the developing seed, fig.12. This curve is similar to the plot of RNA per cotyledon pair against age (fig.11), except that there is a more abrupt increase in the RNA at day 35 and an earlier tailing off of the RNA synthesis rate.

Also illustrated in fig.12 is the change in dry weight per cotyledon cell with age. The dry weight increases rapidly from day 35 and unlike the respective RNA curve continues to rise steadily to day 85 and then remains constant.

Fig.11. The variation in the RNA content of cotyledons and testas collected from developing seeds in 1966 and 1967



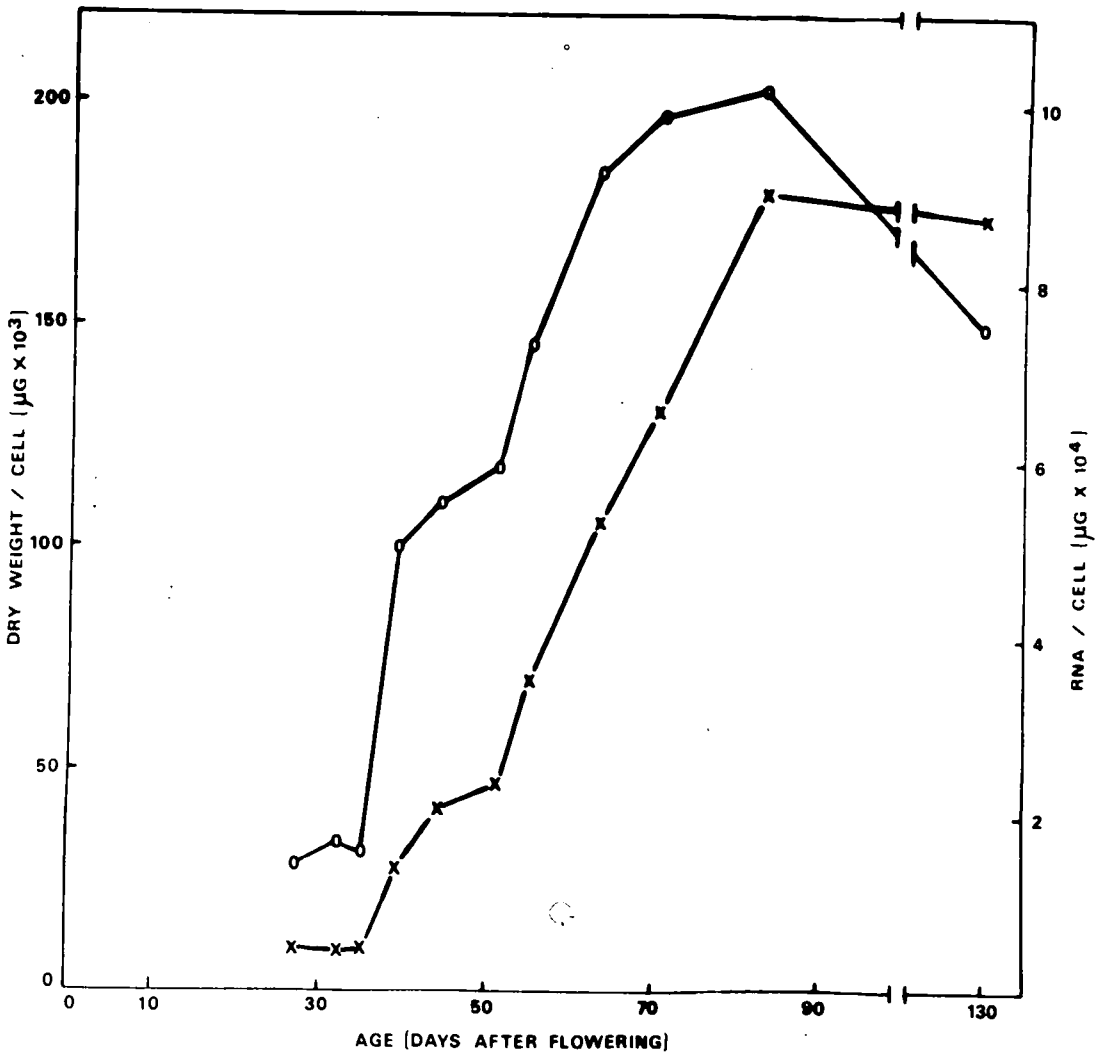
RNA was quantitatively extracted from seeds and estimated as described in the methods section.

O - O RNA/cotyledon pair, 1966 season

X - X RNA/cotyledon pair, 1967 season

● - ● RNA/testa, 1967 season

Fig.12. The variation in the RNA content per average cotyledon cell, and the dry weight per average cotyledon cell, of developing seeds collected in 1966



RNA was quantitatively extracted from cotyledons and estimated as described in the methods section. The cell number content of cotyledons was determined essentially by the method of Loewenberg (1955), (method I in the methods section).

O - O RNA content per average cotyledon cell

X - X Dry weight per average cotyledon cell

4. Methylated albumin kieselguhr chromatography of seed nucleic acids

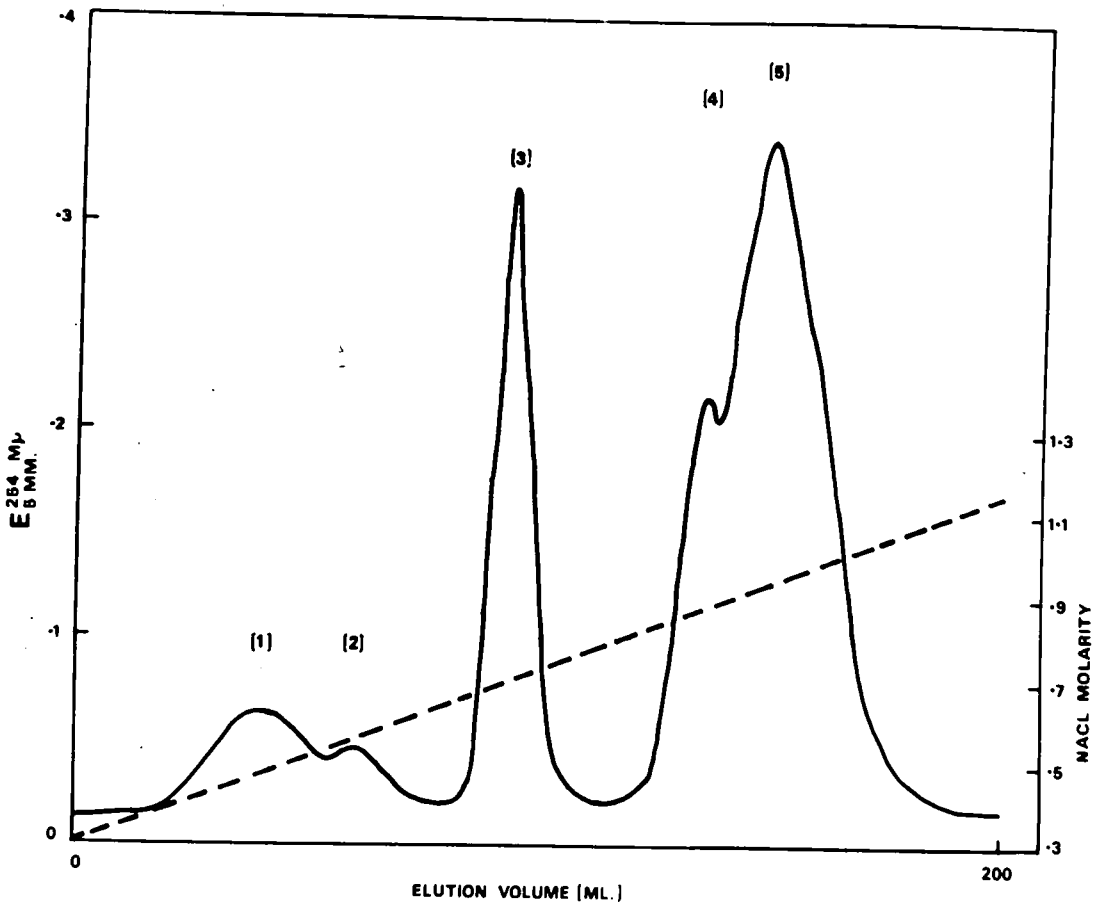
A. Identification of the major fractions

Fig.13 represents a typical MAK fractionation of nucleic acid extracted from mature seed cotyledons, soaked for 48hr in running tap-water. Three main fractions occur. The first fraction to elute from the column is relatively small and broad, and is partially separated into two regions, (1) and (2). The next fraction, (3), appears to be homogeneous, whereas the final, large fraction (5) has a distinct shoulder (4). All fractions had absorption spectra typical of nucleic acids in aqueous solution, fig.14.

The nucleic acids from a 105,000xg pellet and a 105,000xg supernatant of 48hr soaked seed cotyledons were also chromatographed on MAK columns. The results of such investigations are summarised in Table 6.

Fractions (4) and (5) are microsomal RNA (rRNA); fraction (3) is DNA since it gives a positive diphenylamine test; fraction (1) is "supernatant RNA", and fraction (2) appears to be associated with both the microsomal pellet and the high speed supernatant. Since MAK columns elute RNAs with increasing molecular weights, the following identifications were assumed and are universally accepted by other workers, e.g. Cherry (1964), and Wood and Bradbeer (1967); fraction (1) is 4s RNA (tRNA), (2) is a mixture of 5s rRNA and tRNA, (4) is 18s RNA and (5) is 28s RNA.

Fig.13. MAK chromatography of 48hr soaked mature cotyledons



Nucleic acids were extracted from cotyledons by a modified Kirby method (1965). The optical density of the effluent was automatically recorded at 254 mμ using a U.V. monitor and a chart recorder, and the flow rate was adjusted and maintained at 1ml/min by a peristaltic pump. The salt concentration of the effluent was periodically tested using a 0% to 50% sugar refractometer (Bellington and Stanley Ltd.).

Identifications of the fractions are as described in the results section.

TABLE 6

The characterisation of the major peaks obtained by MAK chromatography

Material	The % area of fractions to total peak area			
	1	2	3	4 + 5
Cotyledons	7.3	3.1	20.9	68.7
105,000 x g supernatant	79.3	5.6	0.0	15.1
105,000 x g pellet	0.0	4.0	0.0	96.0
Chemical test	The test reaction			
	1	2	3	4 + 5
Diphenylamine reaction (Burton, 1956)	Negative	Negative	Positive	Negative

The 105,000 x g pellet and supernatant were prepared as described in the methods. Areas under the peaks were calculated by tracing the peaks of the recorder chart, cutting them out, and weighing them. Results were expressed as a percentage of the weight of a fraction to the total weight of all the fractions.

B. The purity of the extracted nucleic acid sample for MAK chromatography

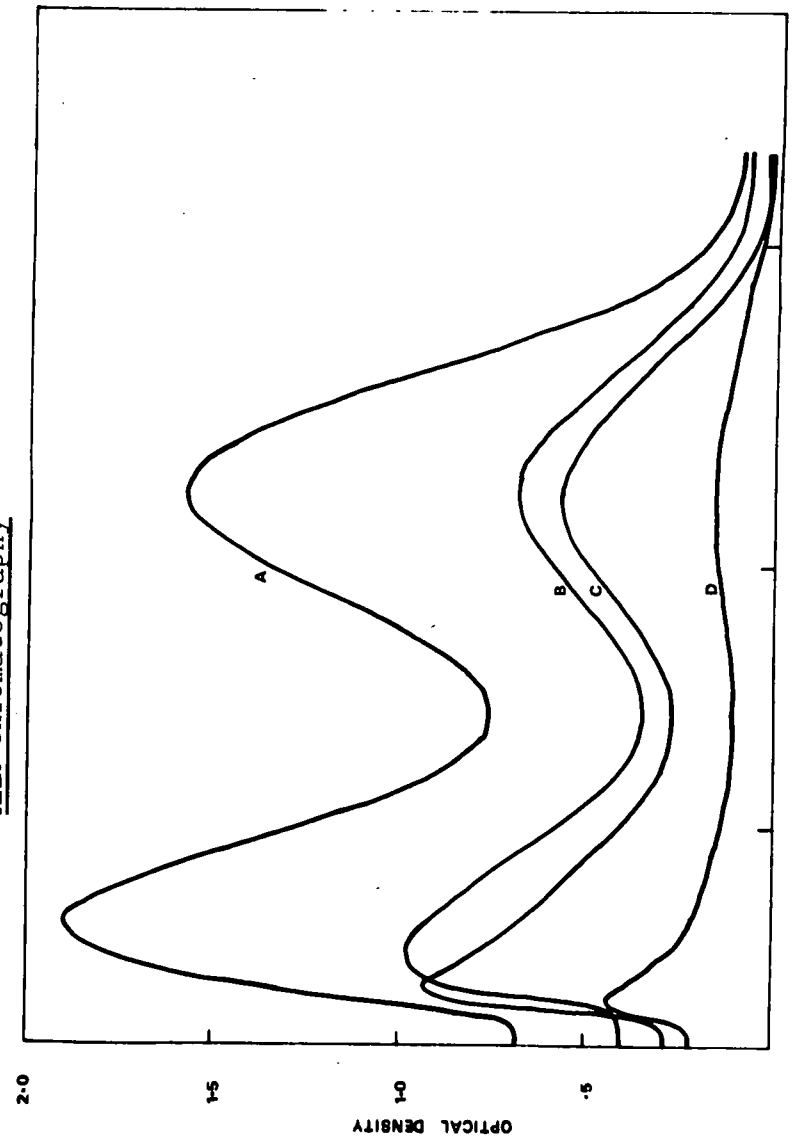
Absorption spectra of extracted nucleic acid fractions from all stages in the development of cotyledons and from the

early stages in the development of testas gave typical curves of pure nucleic acids, fig.14. The absorption spectra of nucleic acids from senescing testas, however, contained an additional shoulder at about 300mμ, fig.15. When such a sample was applied to a MAK column, the 300mμ absorbing material was not retained by the methylated albumin and was immediately eluted from the column. Its absorption spectrum is shown in fig.15. It was not further studied.

C. The fractionation of nucleic acids at different stages in the development of cotyledons and testas

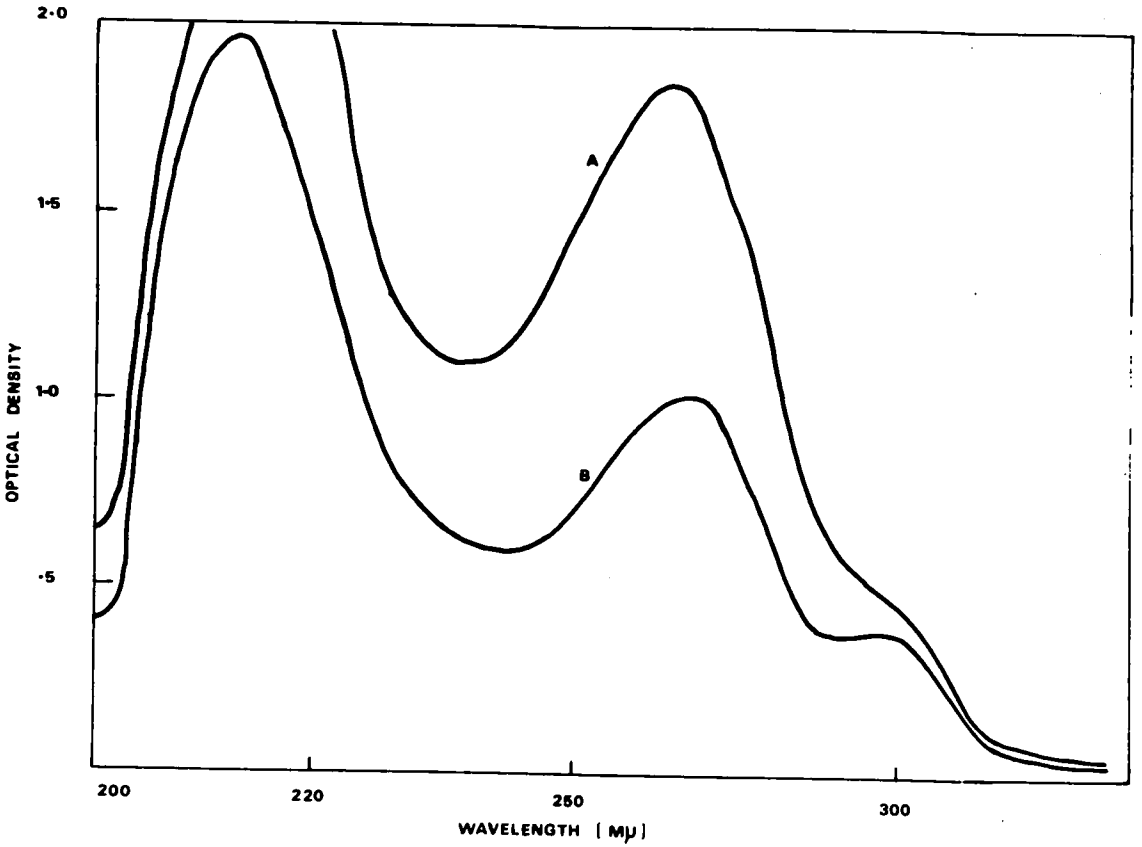
MAK chromatography of developing cotyledon and testa nucleic acids produced up to two additional fractions compared with mature seed nucleic acids. Firstly, a heterogeneous DNA fraction, occasionally discernible into two distinct peaks, was present in tissues which were actively metabolising, e.g. 28 day, 60 day, and 91 day developing cotyledons (figs.16, 17 and 18 respectively) and 28 day testas (fig.19), but was absent in dormant or senescing tissues, e.g. 130 day cotyledons and 43 day testas (fig.20). The second fraction constituted a third large molecular weight RNA, fraction (6), eluting at slightly higher salt concentrations than 28s RNA, and was first detectable in 28 day cotyledons (fig.16). This fraction becomes increasingly apparent and was most pronounced around day 60 (fig.17), and then began to decline in quantity and was absent by day 91, (fig.18). The fraction was not detected at any of the stages examined in the development of the testa.

Fig.14. The absorption spectra of fractions separated by MAK chromatography



A. The original nucleic acid fraction. B. rRNA fraction.
C. DNA fraction. D. LMW RNA fraction.
Absorption spectra were determined in 0.3M NaCl, 0.05M phosphate buffer, pH 6.7.

Fig.15. The absorption spectra of a nucleic acid fraction prepared from 70 day developing testas

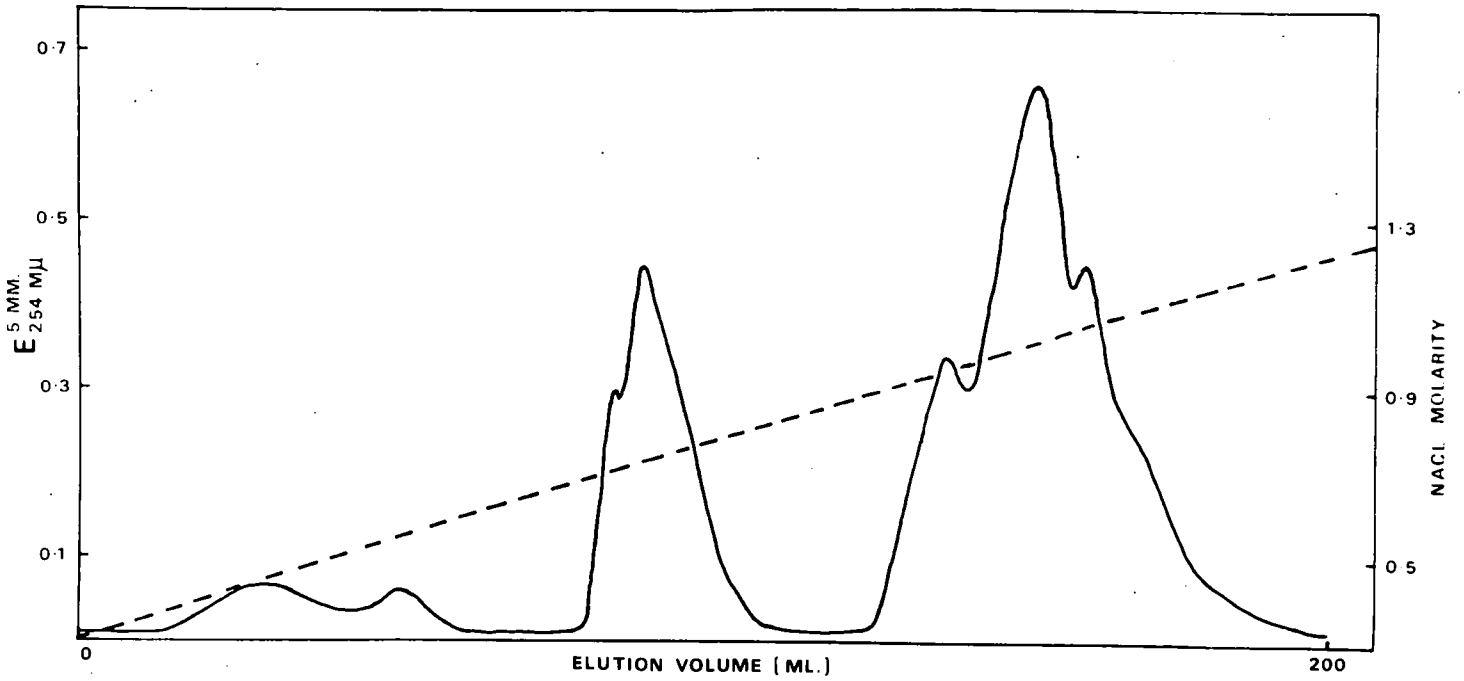


A. The absorption spectrum of the nucleic acid fraction for MAK chromatography.

B. The absorption spectrum of the fraction which immediately elutes from the column.

Absorption spectra determined in 0.3M NaCl, 0.05 phosphate buffer, pH 6.7.

Fig.16. MAK chromatography of 28 day developing cotyledon nucleic acids

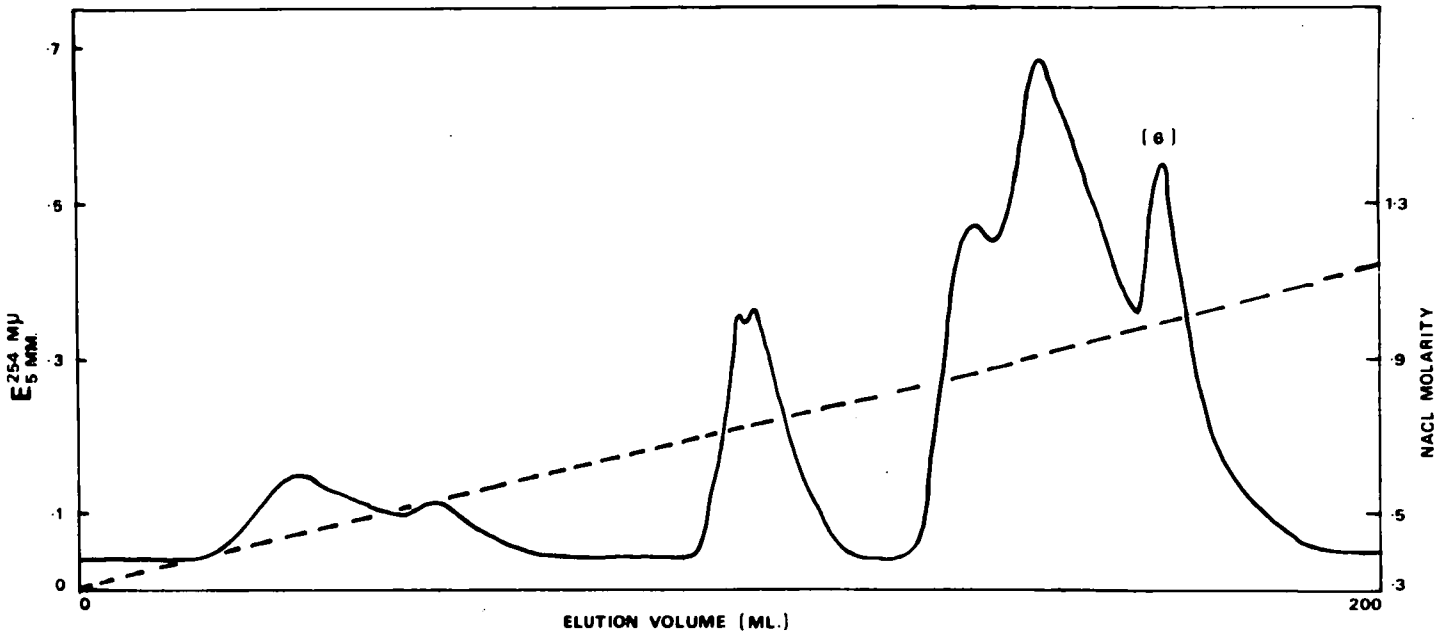


Nucleic acids were extracted from cotyledons and chromatographed as described in the legend of Fig.13, and the methods section.

———— E^{254}_{5mm}

---------- Sodium chloride molarity

Fig.17. MAK chromatography of 60 day developing cotyledon nucleic acids

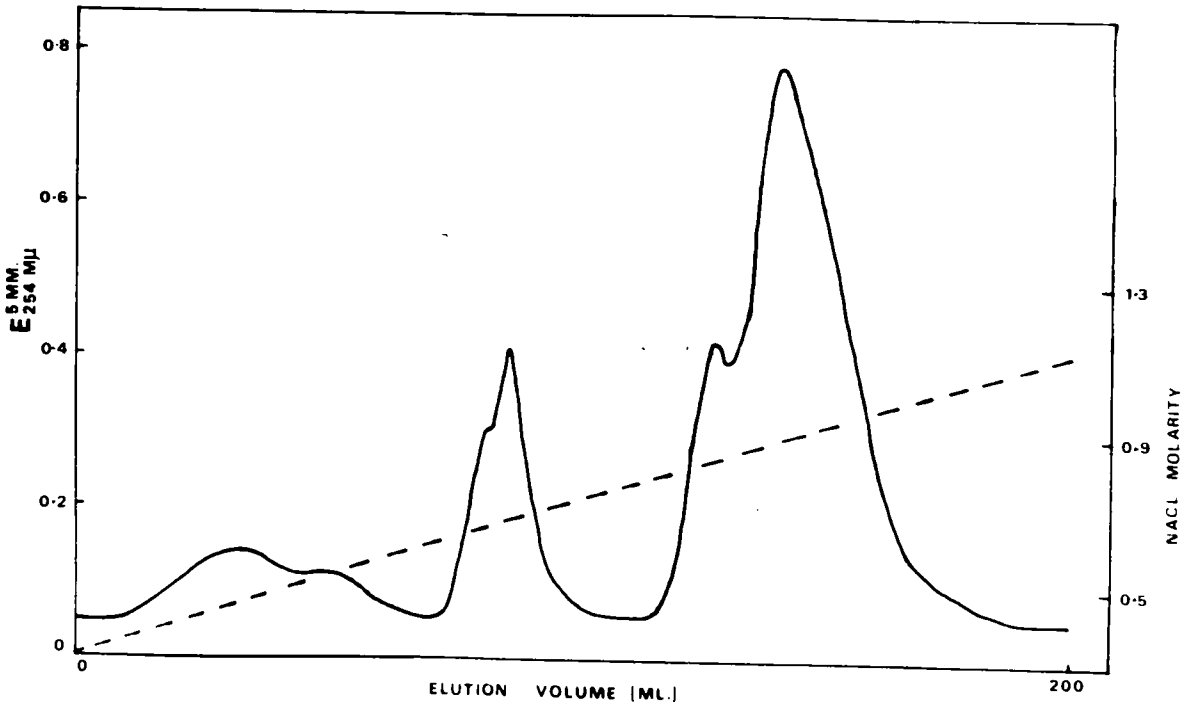


Nucleic acids were extracted from cotyledons and chromatographed as described in the legend of fig.13, and the methods section.

$\frac{254}{5mm.}$ E

------ sodium chloride molarity

Fig.18. MAK chromatography of 91 day developing cotyledon nucleic acids

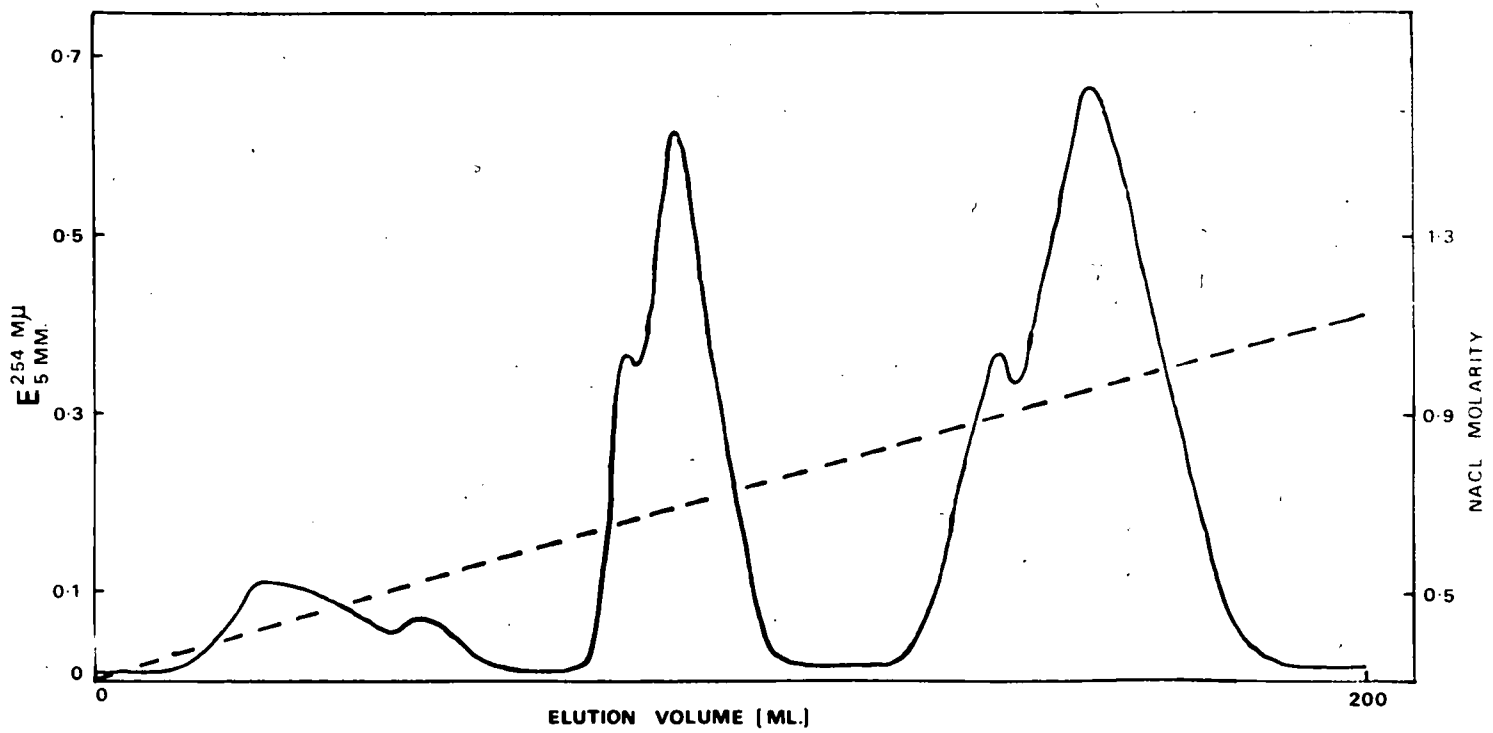


Nucleic acids were extracted from cotyledons and chromatographed as described in the legend of fig.13, and the methods section.

———— E^{254}_{5mm}

---------- sodium chloride molarity

Fig.19. MAK chromatography of 28 day testa nucleic acids

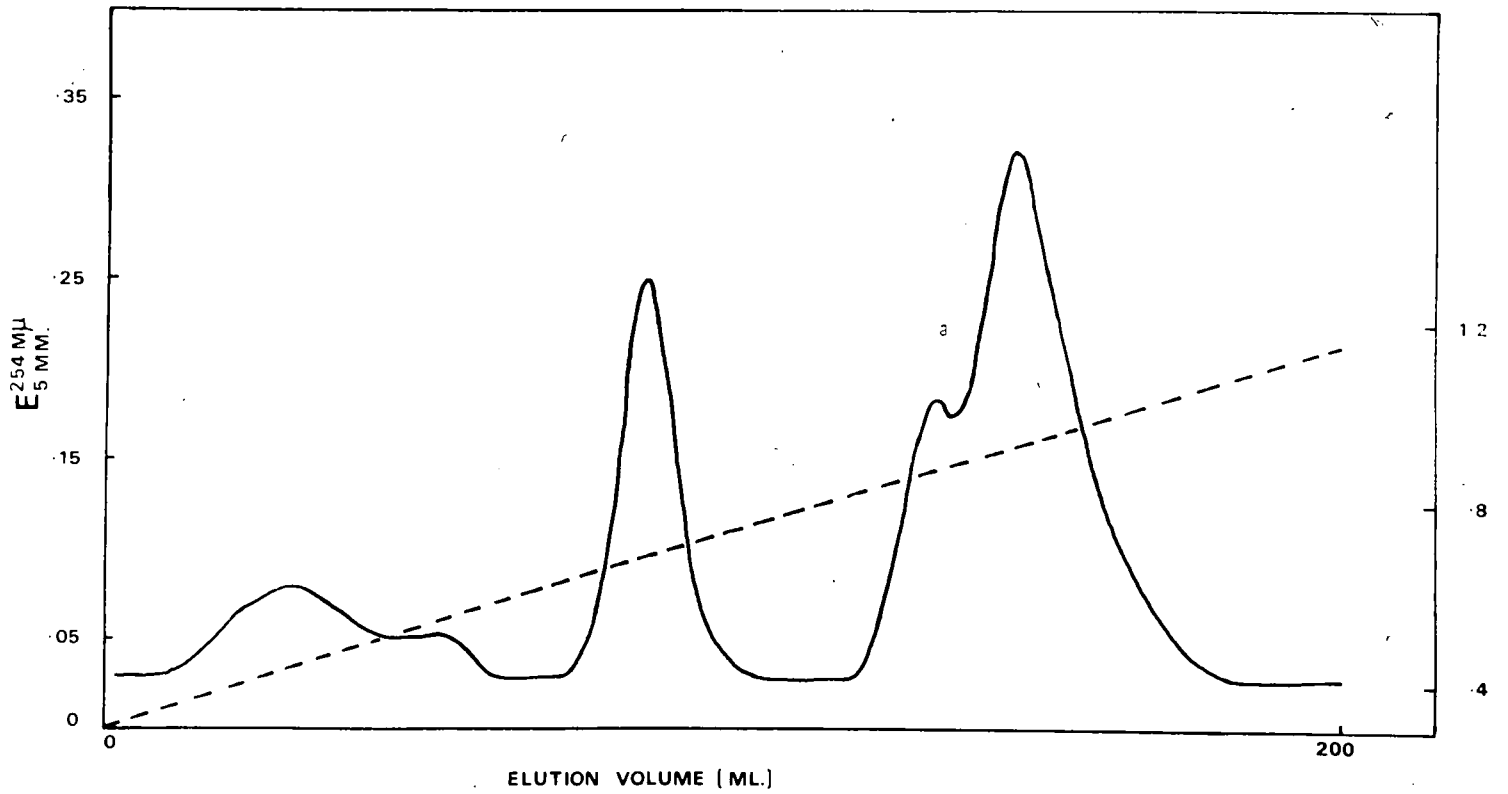


Nucleic acids were extracted from testas and chromatographed as described in the legend of Fig.13, and the methods section.

———— E^{254}_{5mm}

--------- Sodium chloride molarity

Fig.20. MAK chromatography of 43 day testa nucleic acids



Nucleic acids were extracted from testas and chromatographed as described in the legend of Fig.13, and the methods section.

———— E^{254}_{5mm}

--------- Sodium chloride molarity

D. The short period incorporation of (^{32}P) - orthophosphate into developing cotyledon nucleic acids

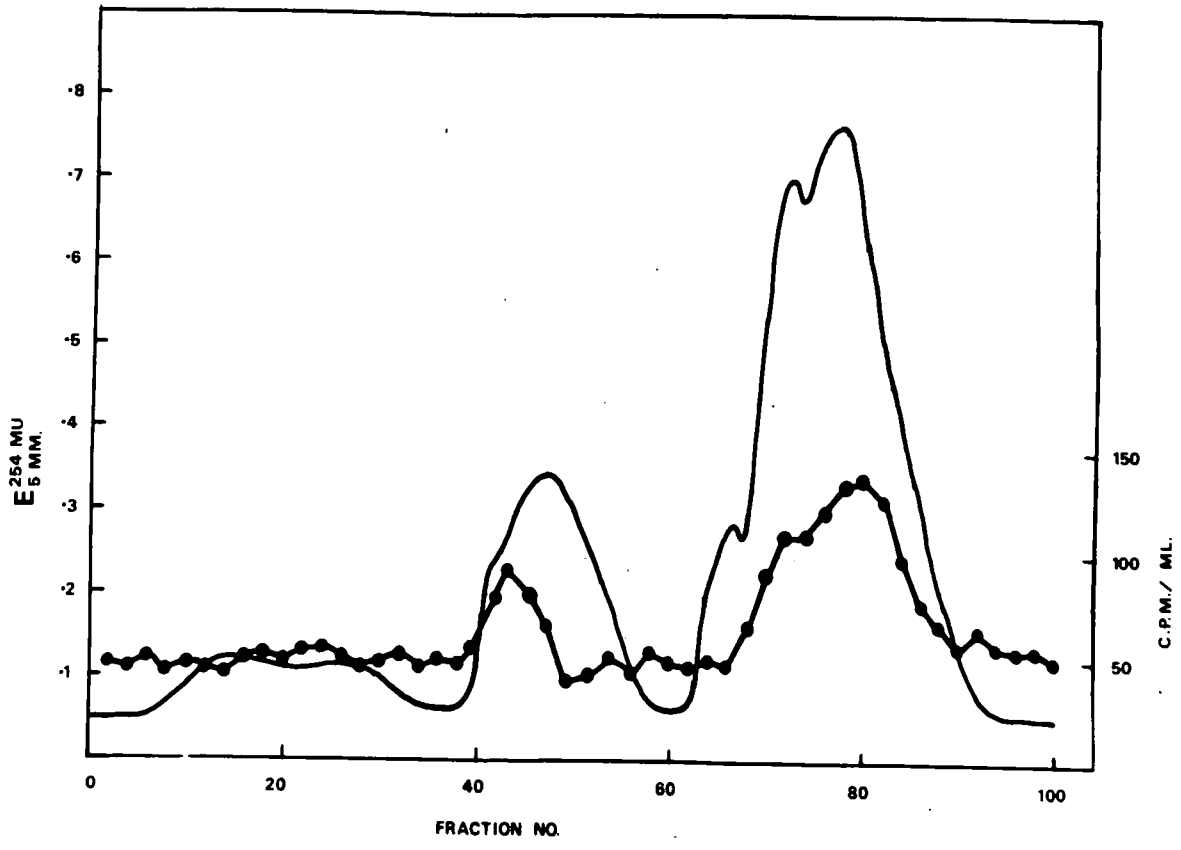
Cutting cotyledons into slices and incubating them at 30° in an incubation medium containing (^{32}P) - orthophosphate produced a unique elution profile upon MAK chromatography of the extracted nucleic acids. 60 day cotyledon material treated in this way produced a much broader DNA fraction, a less distinct 18s RNA fraction, and a very large fraction (6) compared with similar material not incubated with (^{32}P) - orthophosphate, fig.21.

Little (^{32}P) - phosphorus became incorporated into nucleic acid. Radioactivity was associated with part of the DNA Fraction (that which elutes first from the column), Fraction (6) and possibly, the 28s RNA fraction, fig. 21

E. The reproducibility of percentage composition determinations of the nucleic acids

The percentage composition of the nucleic acids of imbibed seed cotyledons was determined three times, Table 8, and all gave similar values.

Fig.21. The incorporation of (^{32}P) - orthophosphate into the nucleic acids of 60 day developing cotyledons



Nucleic acids were fractionated by MAK chromatography as described in the legend of fig.13, and the methods section.

——————— optical density

● ——— ● radioactivity

Bacterial content of incubation medium = 75/ml.

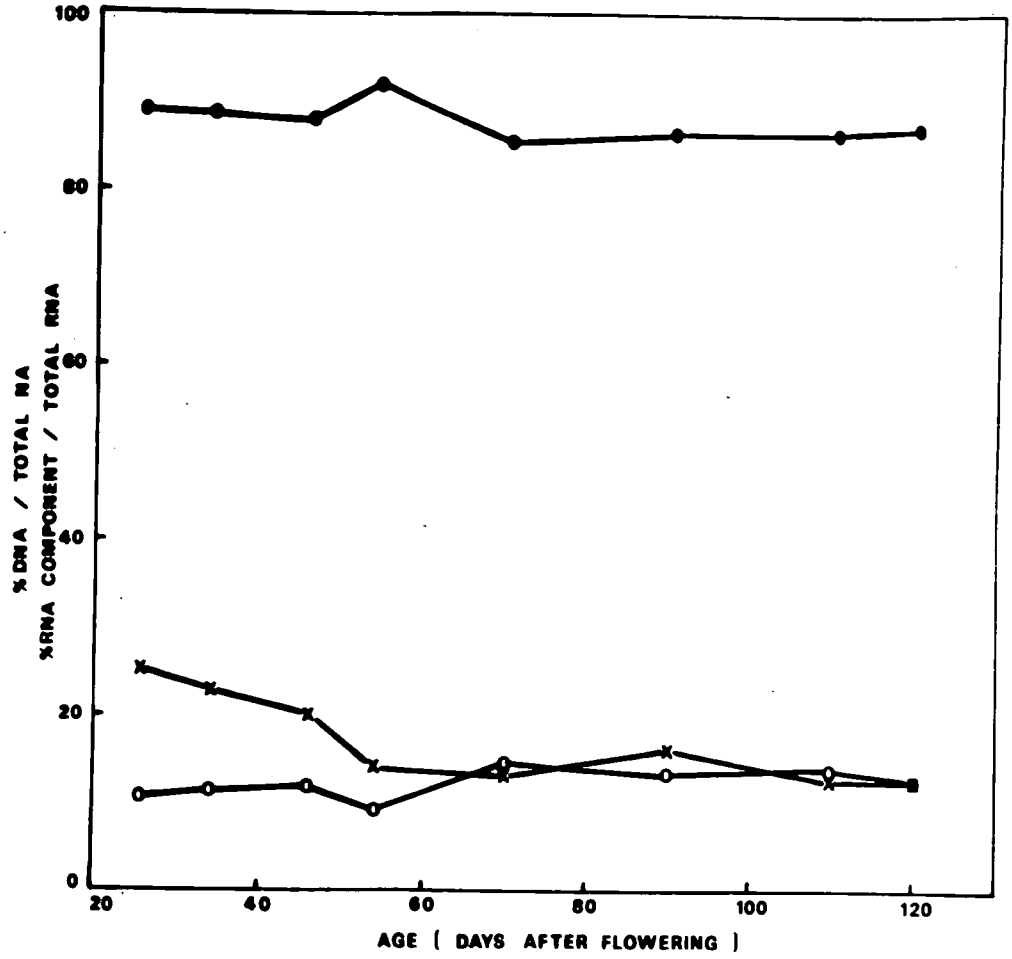
F. Changes in the composition of nucleic acids during the development of cotyledons and testas

There was little change in the composition of the cotyledon RNAs during development, fig.22, though there was a slight increase in low molecular weight (LMW) RNA between days 50 and 70, and a corresponding decrease in rRNA, relative to the total RNA. The percentage of DNA to total nucleic acid (NA), on the other hand, decreased from the first determination at day 28, to day 70, and then remained approximately constant.

These were greater changes in the nucleic acid composition of developing testas, fig.23. At early stages of development, the composition of RNA was very similar to that of cotyledons throughout their developmental period, namely 13% LMW RNA and 87% rRNA. As development progressed in the testa, there was a relative increase in LMW RNA compared with rRNA, up to the final determination at 90 days, when the LMW RNA/total RNA was 25%. The variation of DNA to total NA was considerable. At 28 days, the DNA composition was 30%; it then fell slowly to 43 days and then began to rise again, slowly at first, but then rapidly at day 70. By day 90, the DNA to total NA was 64%.

Since the change in total RNA during testa development is known, the change in weight of the component nucleic acids per testa can be calculated, fig.24. During later stages of seed development, there was a prefferential breakdown of rRNA over LMW RNA. DNA on the other hand was much more stable, and indeed there was little breakdown for the whole of the developmental period studied.

Fig.22. The nucleic acid composition of cotyledons, collected from developing seeds in 1967.



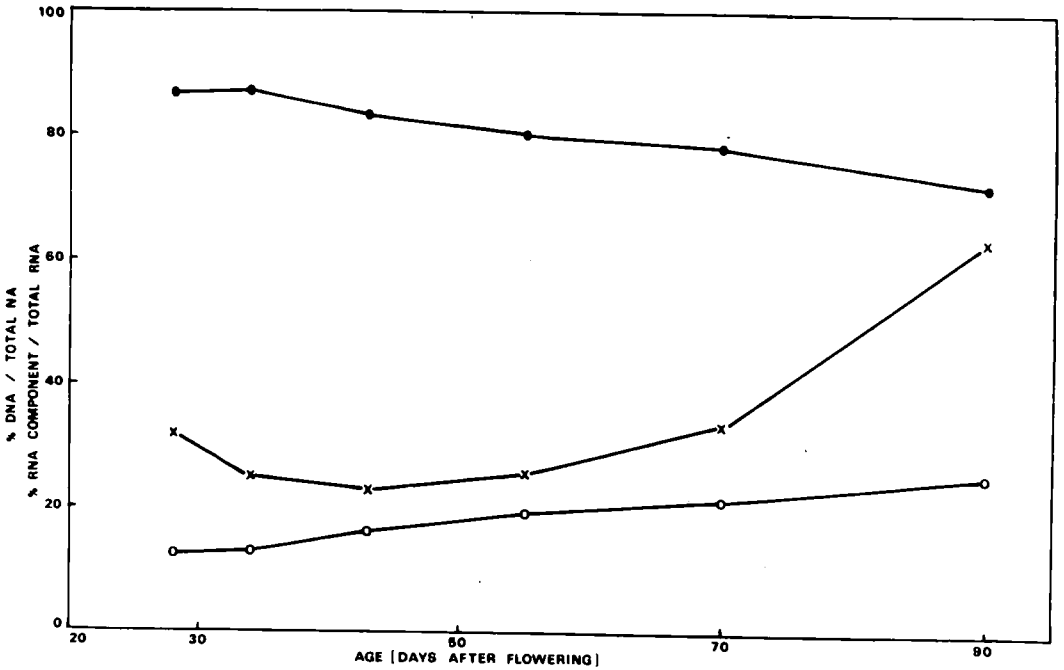
The nucleic acid composition of cotyledons was calculated from MAK column elution profiles as described in the legend of table 6.

● - ● % rRNA/total RNA

o - o % LMW RNA/total RNA

x - x % DNA/total NA

Fig.23. The nucleic acid composition of testas, collected from developing seeds in 1967



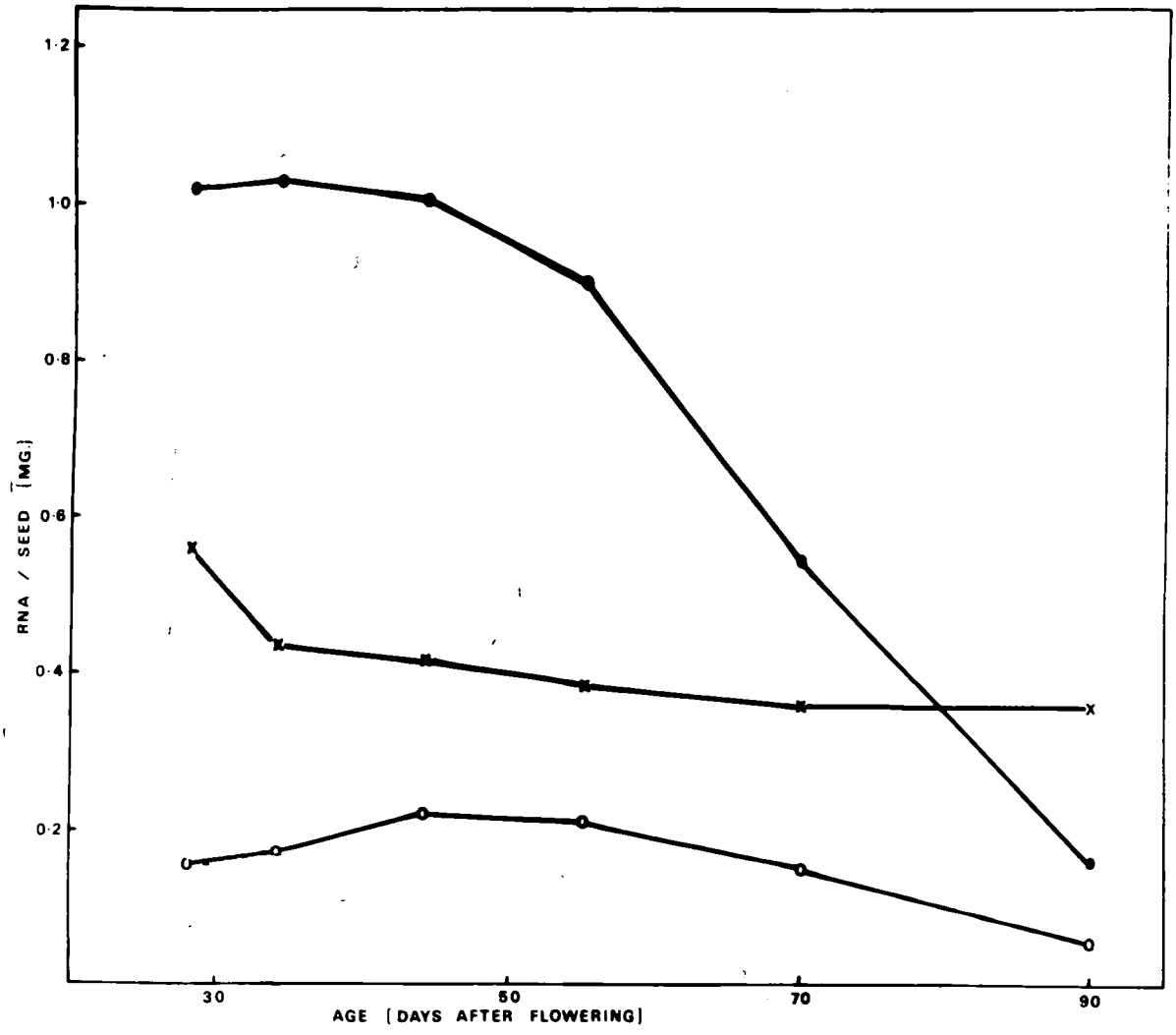
The nucleic acid composition of testas was calculated from MAK column elution profiles as described in the legend of table 6.

● - ● % rRNA/total RNA

o - o % LMW RNA/total RNA

x - x % DNA/total NA

Fig.24. The variation in the nucleic acid contents of testas, collected from developing seeds in 1967.



The nucleic acid contents/testa were calculated from the total RNA content and the nucleic acid composition of testas.

● - ● rRNA

o - o LMW RNA

x - x DNA

TABLE 8

The percentage composition of imbibed seed nucleic acids

Sample	% LMW RNA total RNA	% rRNA to total RNA	% DNA to total NA
1	13.2	86.8	18.5
2	13.2	86.8	19.4
3	14.5	85.5	18.6
Mean	13.6	86.4	18.8
% difference between maximum and minimum values	9.0	1.5	4.7

Nucleic acids were extracted and fractionated as described in the methods section. The % composition of each fraction was calculated as described in the legend of Table 6.

5. The nucleic acid content of protein body preparations

A. By Method I

RNA determinations by method I on several protein body preparations, isolated from 50, 60, and 70 day developing cotyledons, gave negative results. The absorption spectrum of the final RNA nucleotide fraction showed some absorption at 220mμ, but very little at 260mμ, and was not characteristic of the absorption spectra of nucleotides.

B. By Method II

MAK chromatography of the nucleic acid fraction of protein bodies, extracted from developing seeds between 50 and 80 days old, produced three fractions. Two eluted at the salt concentrations expected of 18s and 28s RNA and were just detectable. The third peak was appreciable and eluted at a similar salt concentration to DNA; its identity was confirmed to be DNA by the diphenylamine test (Burton, 1956).

Assuming the phenol extraction method extracts 100% of the nucleic acids in the protein body fraction, the percentage of nucleic acid components to the dry weight of protein bodies was calculated, Table 9. Values for the rRNAs are very small, but that for DNA, namely 0.05%, is significant.

6. The ribonucleoprotein particles of seeds

A. Characterisation of the microsome fraction

I. Absorption spectra

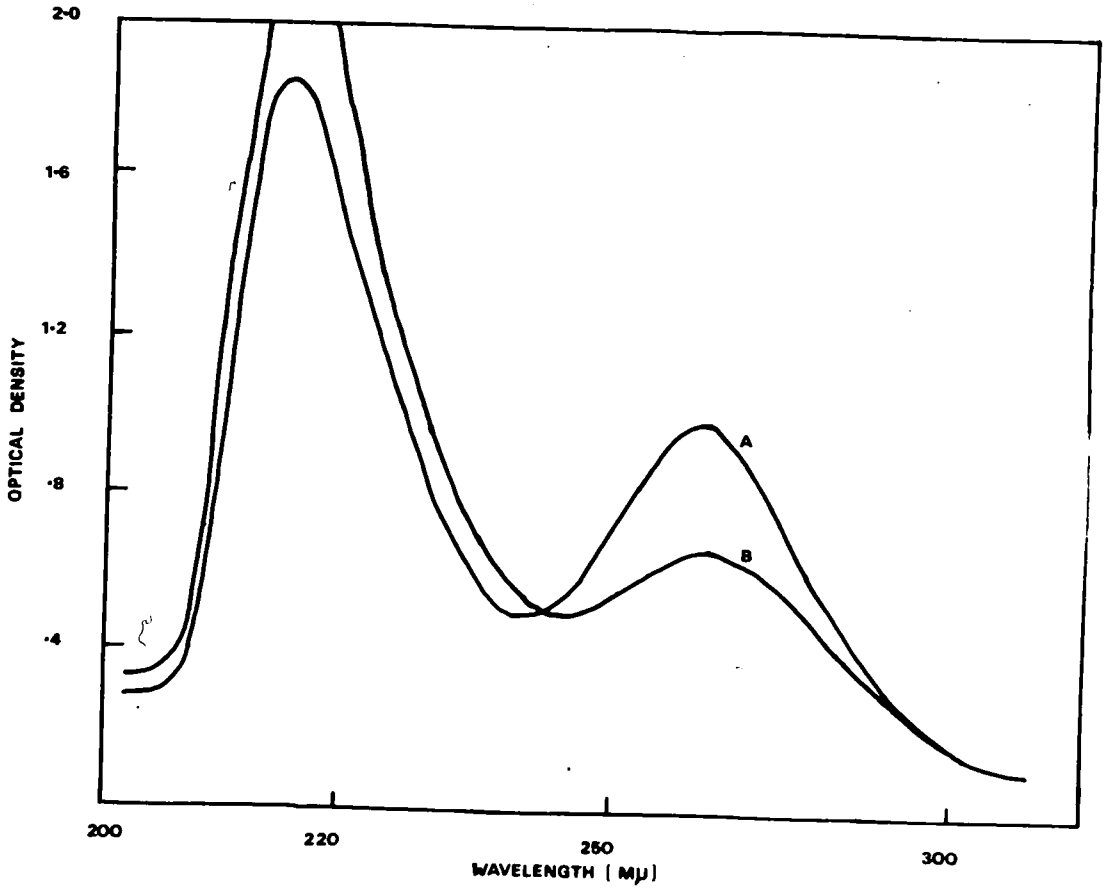
The absorption spectrum of the microsomal pellet,

dissolved in sodium hydroxide, pH 8, varied considerably with different tissues. The absorption spectra of microsomal suspensions, prepared for 60 day and 95 day developing cotyledons, are shown in fig.25. That from 60 day cotyledons had a maximum absorption at 261mμ, a minimum absorption at 245 mμ, and a ratio of absorption at 260 to 235 mμ of 1.18; 95 day cotyledon preparations on the other hand had an absorption maximum at 261 mμ, an absorption minimum at 238 mμ, and a ratio of absorption at 260 to 235 mμ of 1.60.

II. Sedimentation coefficient determinations

The sedimentation coefficient of microsomes, isolated from 3 day germinating plumules of Vicia faba, was determined in conjunction with A. Yarwood (see Yarwood, 1968) by an identical method used here, to isolate cotyledon microsomes. A mean value of 82.1 s was obtained from 4 determinations with a standard deviation of 1.97 and a coefficient of variation of 2.4%.

Fig.25. The absorption spectra of microsomal preparations from developing cotyledons



A microsomal preparation was dissolved in water, adjusted to pH 7 with dilute NaOH, and the absorption spectrum of a suitable dilution determined.
A. From 95 day cotyledons. B. From 60 day developing cotyledons.

TABLE 9

The nucleic acid content of protein bodies

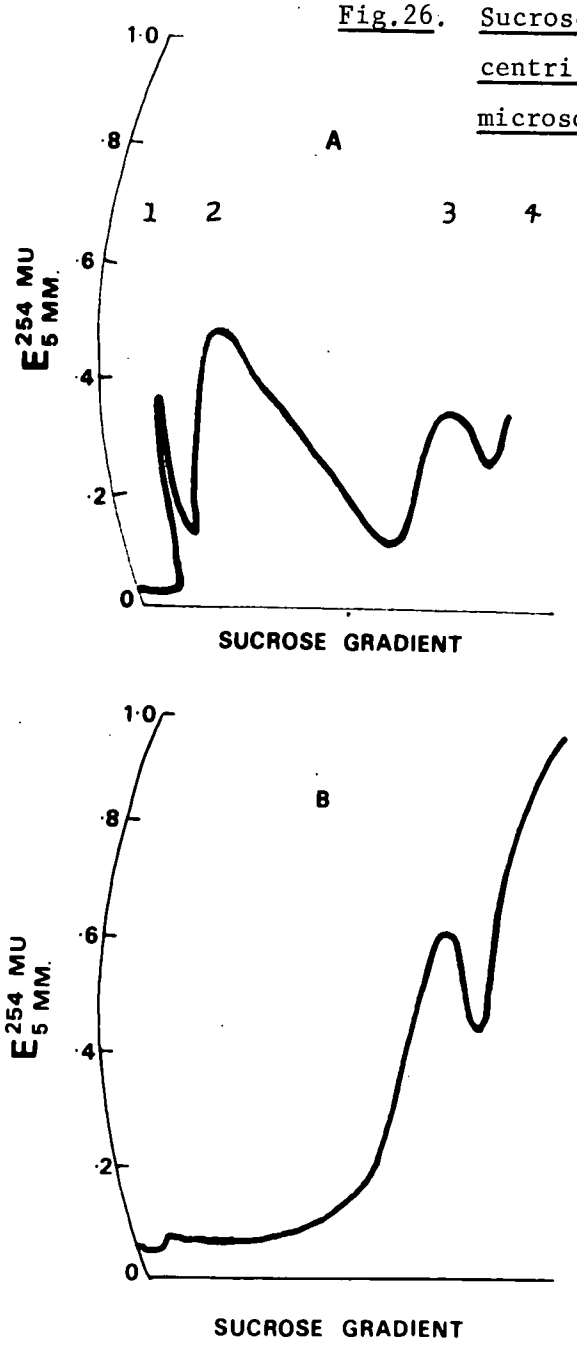
Experiment No.	Dry weight of protein bodies (g.)	Wt. LMW RNA. (µg)	Wt. rRNA (µg)	Wt. DNA (µg)	% NA to dry weight of protein bodies		
					LMWRNA	rRNA	DNA
1	2.32	0	11.5	1048	0	4.95×10^{-4}	0.045
2	2.82	0	19.6	1532	0	6.95×10^{-4}	0.054

Protein bodies were isolated, and nucleic acids extracted and estimated, by method II as described in the methods section. The concentrations of the NA fractions were determined from their $E_{1\ cm}^{260\ m\mu}$, assuming an optical density of 20.0 for a 1mg/ml solution.

B. Characterisation of the major fractions of microsomes, separated by sucrose gradient centrifugation

A typical sucrose gradient centrifugation of microsomes is illustrated in fig.26A. Four fractions are present, numbered (1) to (4), fraction (1) being nearest to the bottom of the gradient. These fractions were characterised by three methods.

Fig.26. Sucrose gradient centrifugation of microsomes.



Microsomes of 75 day developing cotyledons were centrifuged in sucrose gradients as described in the methods section.

A. Untreated microsome sample.

B. An equivalent microsome sample treated with 0.4% (w/v) nonidet. P.40.

I. Electron microscopy

Fig.27A, B, and C are electron micrographs of fractions (1), (2), and (3) respectively. Fraction (3) consists exclusively of small particles having the dimensions expected of single ribosomes. Fraction (2) contains similar particles to those of fraction (3), but are attached to membranes. Fraction (1) on the other hand appears to be heterogeneous, and consists mainly of membranes, either smooth or with ribosomes bound, though a few mitochondria are also present.

II. Absorption spectra

The absorption spectra of fractions (1), (2), and (3), are shown in fig. 28. Fraction (3) has an absorption maximum at 261 mμ and an absorption minimum at 239 mμ; the ratio of absorption at 260 to 235 mμ is 1.72. The absorption spectrum of fraction (2) is similar, but the absorption trough is less well defined and the absorption minimum is displaced slightly to greater wavelengths. The 260 mμ, 235 mμ ratio of absorption is also glower. Fraction (1) has only a slight absorption peak at 255 mμ, and the 240 mμ absorption trough is absent.

III. The effect of detergent

Fig.26 shows two sucrose gradient profiles of microsomes, the microsomes of 26(B) being identical to those of 26(A), except that they were treated with the detergent nonidet P.40 prior to gradient centrifugation. The effect of nonidet P.40, which solubilises membrane, is to cause a loss of fractions (1) and (2)

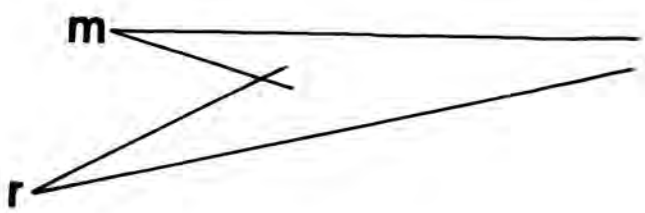
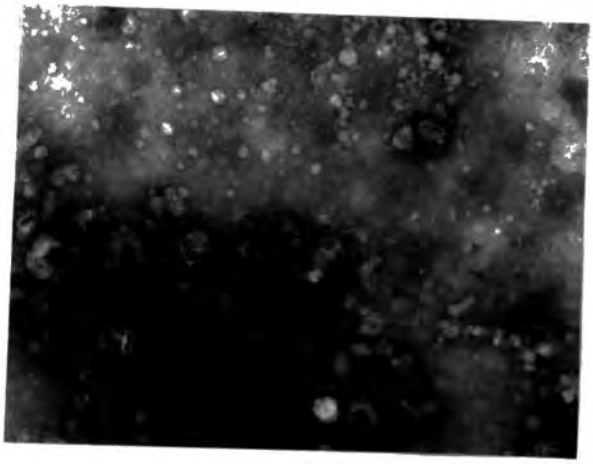
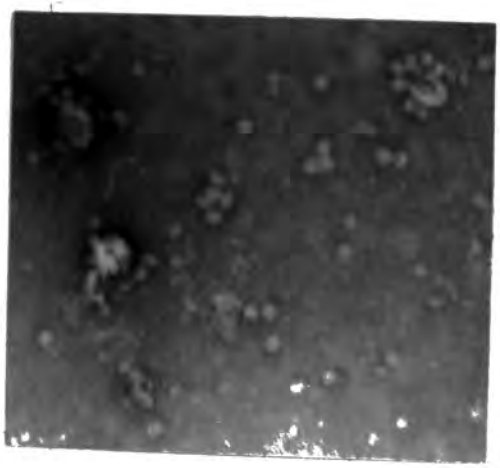

m
r

Fig.27. Electron microscopy of sucrose gradient microsome fractions

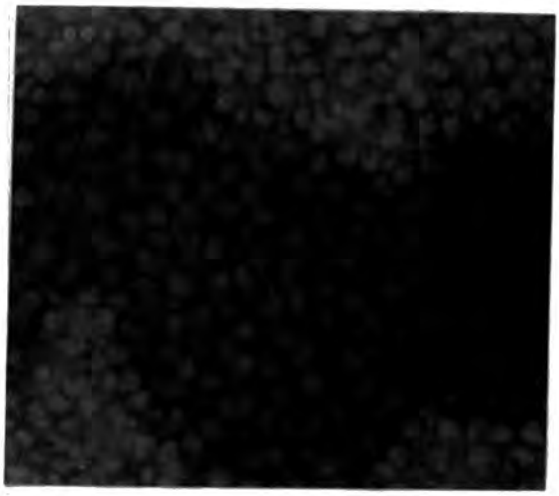
Fraction 1



Fraction 2



Fraction 3

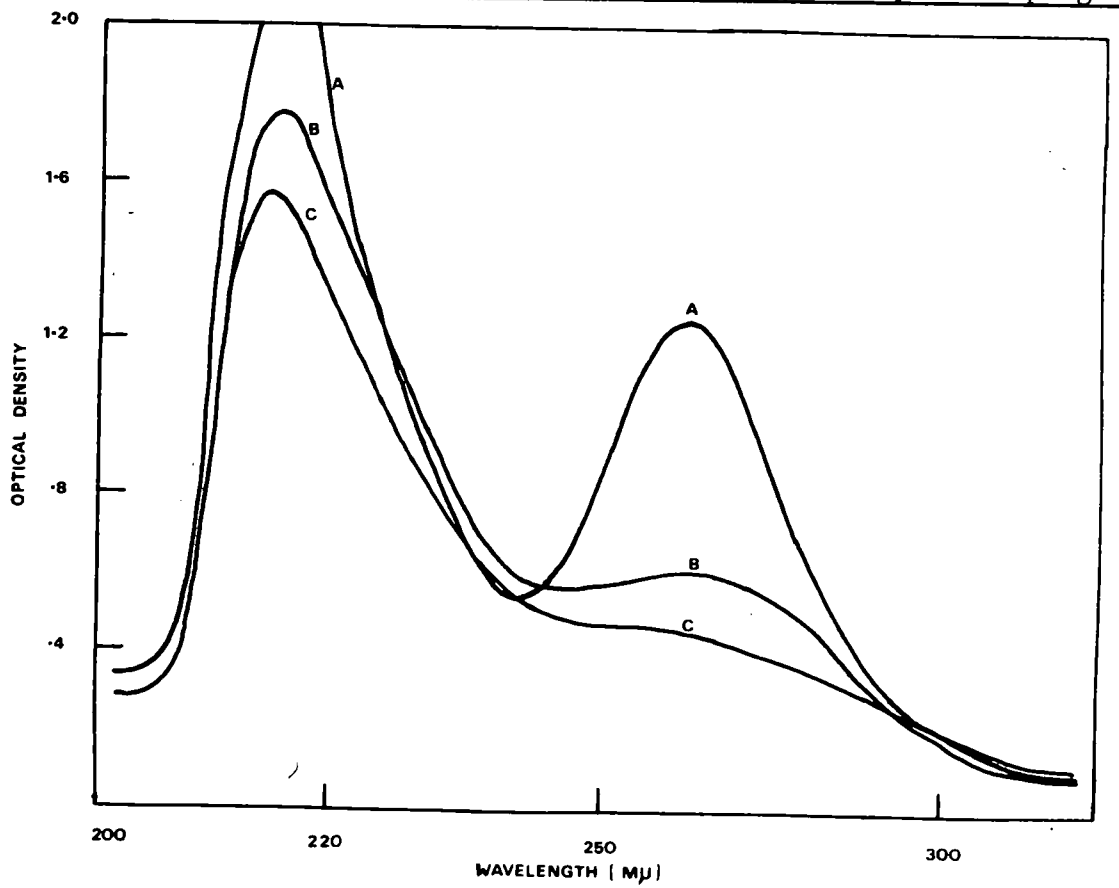


The sucrose gradient fractions were fixed and stained on grids as described by Marcus et al (1967).

M = membrane
R = ribonucleoprotein particle.

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

Fig.28. The absorption spectra of the bands obtained after sucrose gradient centrifugation of microsomes, prepared from 60 day developing cotyledons



A. Fraction (3). B. Fraction (2). C. Fraction (1).
Absorption spectra determined at pH 8.

with a relative increase in fractions (3) and (4).

From all these experiments, it was concluded that fraction (3) consisted of free ribosomes and fraction (2) consisted of membrane bound ribosomes. As fraction (1) appeared to contain some membrane bound ribosomes, it was included with fraction (2) in free to membrane bound rRNA ratio determinations.

C. The reproducibility of free to membrane bound rRNA ratio determinations

Three separate determinations, from 48hr soaked cotyledons, gave reproducible results within $\pm$ 4%, table 10.

D. Changes in the ratio of free to membrane bound rRNA during the development of the cotyledon

It was assumed that ribosomes contained the same weight of RNA, i.e. one molecule of each of 5s, 18s, and 28s RNA, throughout the developmental period of the cotyledon. The experimentally determined ratio of free to membrane bound rRNA is therefore equivalent to the ratio of free to membrane bound ribosomes.

At 30 days after flowering, the ratio was at a value of 5.5 to 1, fig.29, but it then fell to a steady value by 60 days, when the number of membrane bound ribosomes exceeded free ribosomes by 1.4 to 1. At day 75, however, the ratio began to increase, rapidly so by day 90, so that by day 130, the free ribosomes outnumbered the membrane bound ribosomes by 14 to 1.

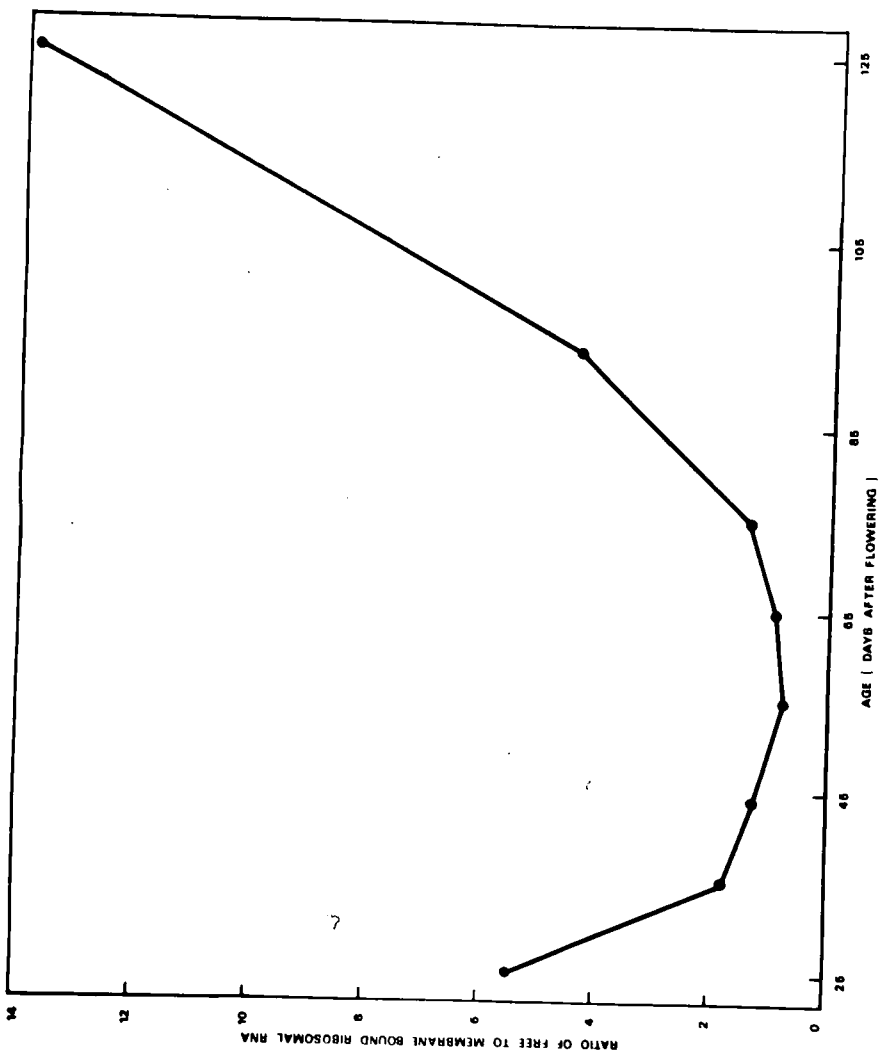


Fig.29. The variation in the ratio of free to membrane bound rRNA of cotyledons, collected from developing seeds in 1967

TABLE 10

The reproducibility of free to membrane bound rRNA ratio determinations

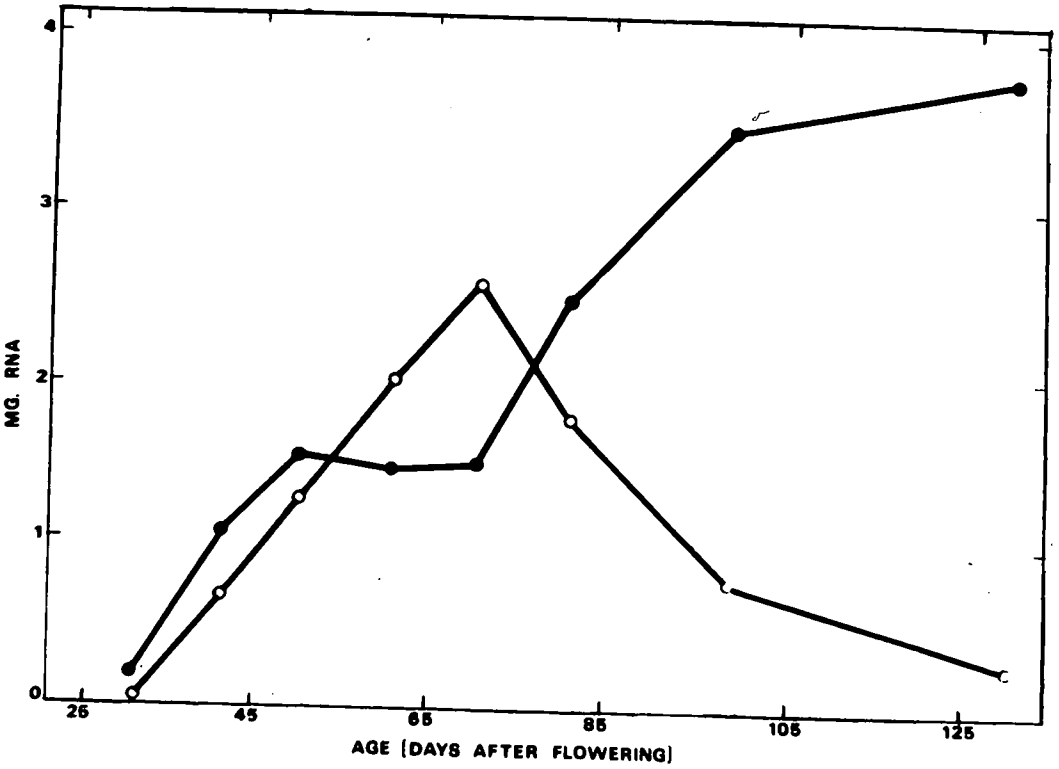
Determination number	Ratio of free to membrane bound rRNA
1	7.17 : 1
2	6.91 : 1
3	7.46 : 1
Mean ratio	7.18 : 1
% difference between maximum and minimum values	7.5%

The ratio of free to membrane bound rRNA was determined for 48hr soaked seed cotyledons as described in the methods section.

E. Changes in the free and membrane bound rRNA content of cotyledons during development

Fig.30 shows the change in quantities of free and membrane bound rRNA during development. The free ribosomes increased in number from 30 to 48 days and then remained constant

Fig.30. The variation in the levels of free and membrane bound rRNA of cotyledons from developing seeds, collected in 1967.



Values were calculated by equating the following experimental determinations : (i) RNA/cotyledon (Fig.11); (ii) percentage rRNA/total RNA (Fig.22); and (iii) the ratio of free to membrane bound rRNA (Fig.29).

●———● free rRNA o———o membrane bound rRNA.

until about day 70, when they increased rapidly to day 130. Membrane bound ribosomes on the other hand increased rapidly from day 30, reached a maximum at day 70, and then declined rapidly to the final determination at day 130.

7. The incorporation of (^{3}H)-uridine into developing cotyledons "in vivo"

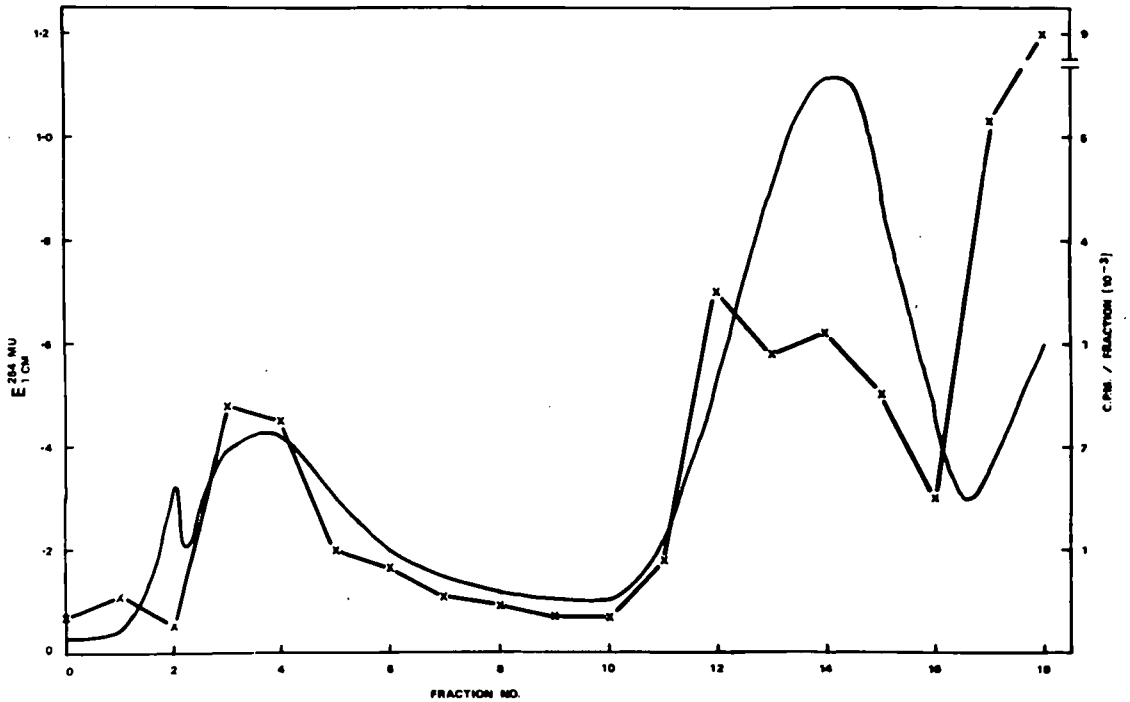
A. The incorporation of radioactivity into free and membrane bound ribosomes

Cotyledons, injected with (^{3}H)-uridine at 27 days, and harvested at 32 days, contained radioactivity in both ribosome fractions, fig.31. The specific activity of the membrane bound ribosomes was about twice that of free ribosomes, table 11. Cotyledons, injected at 60 days and harvested at 65 days however, only contained radioactivity in the membrane bound ribosome fraction, table 11.

B. The incorporation of radioactivity into membrane bound rRNA

RNA, extracted from membrane bound ribosomes of cotyledons, previously incubated with (^{3}H)-uridine between 60 and 65 days after flowering, contained radioactivity. Its specific activity was about 38% lower than the specific activity of the original membrane bound ribosomes, table 11.

Fig.31. Sucrose gradient profile of microsomes, prepared from developing cotyledons, injected with (^{3}H) uridine between days 27 and 32



——————— Optical density, determined using a U.V. monitor.

x ——————— x radioactivity, determined as described in the methods section.

TABLE 11

The incorporation of (^{3}H)-uridine into the ribosomes of developing seed cotyledons

Experiment no.	Age of material during incubation period (days)	Fraction	Specific activity	
			C.P.M/mg RNA	µµM/mg RNA
1.	27 - 32	Free ribosomes	114,062	2.78
	27 - 32	Membrane bound ribosomes	249,568	6.08
2.	27 - 32	Free ribosomes	56,532	1.38
	27 - 32	Membrane bound ribosomes	138,938	3.39
3.	60 - 65	Free ribosomes	Undetectable	-
	60 - 65	Membrane bound ribosomes	103,147	2.51
4.	60 - 65	Free ribosomes	Undetectable	-
	60 - 65	Membrane bound ribosomes	160,328	3.90
5.	60 - 65	Membrane bound ribosomes	127,622	3.11
	60 - 65	Membrane bound rRNA	79,125	1.93

Free and membrane bound ribosomes were isolated as described in the methods section. Each ribosome fraction was divided into two equal aliquots; one was assayed for radioactivity and the other was assayed for RNA by a method modified from that of Smillie and Krotkov (1960).

Membrane bound rRNA was extracted from membrane bound ribosomes by a modified phenol method, as described in the methods section. The fraction was dissolved in a small volume of water,

assayed spectrophometrically to determine its concentration, assuming an $E_{1\ cm}^{260\ m\mu}$ of 20.0 for a 1mg/ml solution of RNA, and finally assayed for radioactivity.

C. The bacteria content of developing cotyledons

Cotyledons of ages 25, 32, 50, and 62 all gave negative tests for bacteria, whether or not the homogenates were directly plated, or serially diluted. Only two bacterial colonies appeared on any of the test plates, and as these were not in the vicinity of a sample streak, they were considered to have arisen by atmospheric contamination. After the incubation period, all plates were purposely infected and shown to be capable of supporting the growth of microbes.

8. The short period incorporation of (^{3}H)-leucine into developing cotyledon discs

A. The determination of the minimum concentration of sodium deoxycholate required to dissolve membranes

The ability of various concentrations of sodium deoxycholate (DOC) to dissolve the membranes of microsomes is listed in table 12. The minimum concentration of DOC required was 0.5%.

B. The distribution of radioactivity in sucrose gradients after centrifuging with a post-mitochondrial supernatant

The post-mitochondrial supernatant was separated into four fractions by sucrose gradient centrifugation, fig.32.

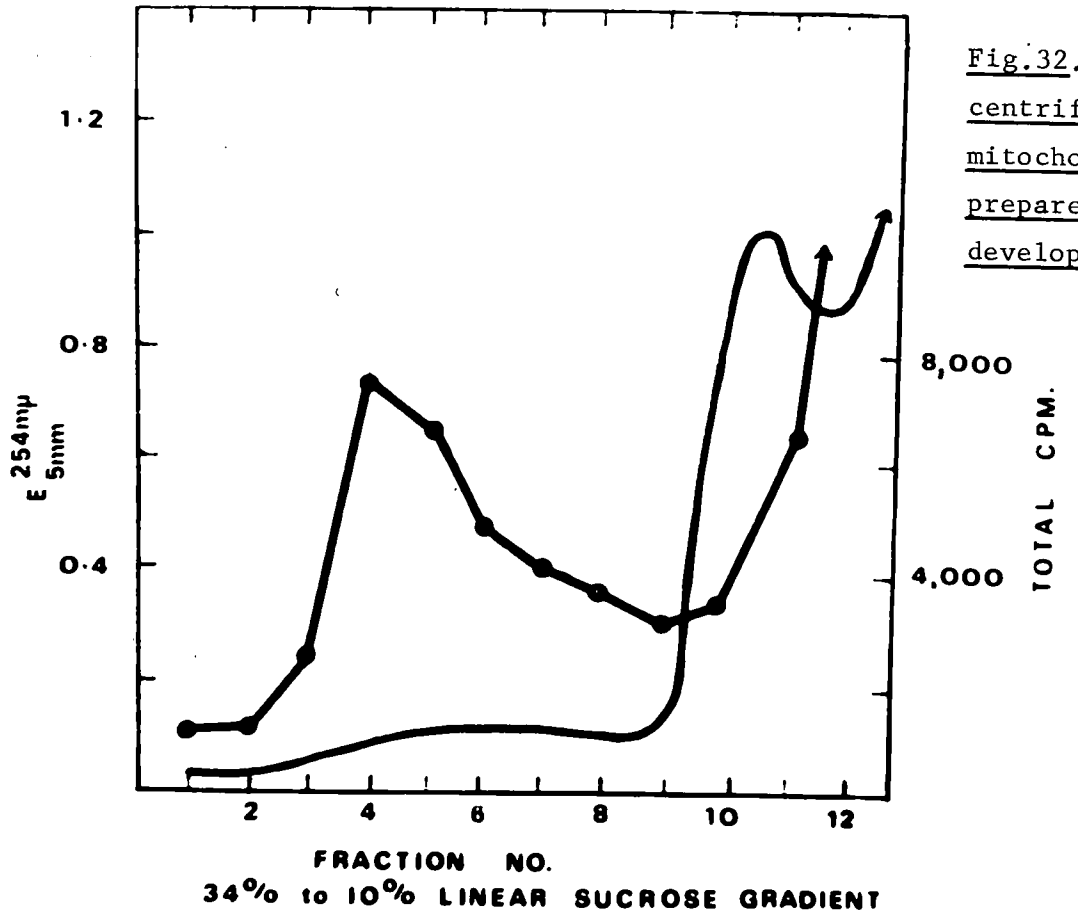


Fig.32. Sucrose gradient centrifugation of a post-mitochondrial supernatant, prepared from 60 day developing cotyledons.

Fraction (1) was a pellet at the bottom of the gradient and consisted of membrane bound ribosomes. Fraction (2) had a very low absorption at 254 mμ, but has an appreciable quantity of radioactivity associated with it. Fraction (3) sedimented at the rate expected of single, free ribosomes but did not appear to be radioactive, whereas fraction (4), the supernatant, was highly radioactive.

TABLE 12

The ability of various concentrations of sodium deoxycholate to dissolve microsomal membranes

Experiment No.	Concentration of DOC	Solubilisation of membrane
1.	0%	-
2.	0.3%	Incomplete
3.	0.4%	Incomplete
4.	0.45%	Incomplete
5.	0.5%	Virtually complete
6.	0.6%	Virtually complete

60 day developing cotyledon material was used in all experiments. The 13,000 x g ave. supernatant was prepared as described in the methods section. 5 ml of supernatant was layered on to 15 ml of a 10% to 34% (w/v) linear sucrose gradient. A complete solubilisation of membrane resulted in no pellet being formed upon centrifuging at 105,000 x g for 1½hr.

When an equivalent post-mitochondrial supernatant was pre-treated with 0.5% DOC, a similar sucrose gradient profile was produced, except that fraction (1) was absent, and the heterogeneous fraction (2) was noticably larger, table 13. When 0.4% nonidet P40 was used instead of 0.5% DOC, fraction (1) was again absent, but fraction (2) was much reduced and was only just detectable, table 13.

C. The distribution of radioactivity in sucrose gradients after centrifuging with a post-mitochondrial supernatant treated with ribonuclease

When the post-mitochondrial supernatant was treated with 0.5 µg/ml ribonuclease and then centrifuged in a sucrose gradient, fraction (2) lost at least 80% of its radioactivity, table 13.

It was concluded that fraction (2) constituted polysomes. It consisted solely of polysomes derived from the cytoplasm, or, if the post-mitochondrial supernatant was treated with DOC, a mixture of free and membrane bound polysomes.

TABLE 13

(^{3}H)-leucine experiments with developing cotyledons

Experiment No.	Treatments of post-mitochondrial supernatant prior to sucrose gradient analysis	Radioactivity in fraction (2) of sucrose gradients (TOTAL CPM)	Bacterial concentration in incubation solutions (colonies/ml)
1(a)	Untreated	110,575	
(b)	0.5% (w/v) DOC	204,564	25
(c)	0.4% (w/v) nonidet P40	16,475	
2(a)	Untreated	64,055	
(b)	0.5% (w/v) DOC	126,188	230
(c)	0.4% (w/v) nonidet P40	17,324	
3(a)	Untreated	66,230	
(b)	0.5 µg/ml ribonuclease	15,500	83
4(a)	Untreated	45,327	
(b)	0.5 µg/ml ribonuclease	10,292	105

In all experiments, 60 day developing cotyledons were used. The post-mitochondrial supernatant was prepared as described in the methods. All apparatus, where possible, was autoclaved before use. Polythene gloves were worn at all stages in the isolation procedure.

PART 2. The dormant seed

9. The effect of artificially drying mature seeds

(a) Dry weight

In the 1966 season, 130 day developing seeds had a water content of 24.5%. This did not decrease in the field. When the seeds were removed from the withered pods and transferred to a ventilated warm room they lost a further 15% of water over a period of weeks, table 14.

(b) The RNA content

Developing seed cotyledons lost a considerable quantity of RNA between days 90 and 130, fig.11. No further RNA was broken down when seeds were dried indoors, however, table 14.

The RNA contents of dried cotyledons remained constant for the 18 month period studied.

10. Methylated albumin-kieselguhr chromatography of the dormant seed nucleic acids

(a) The MAK column elution profile

The MAK columns profile of dormant seed nucleic acids was noticably different from profiles derived from developing cotyledons, fig.33. The LMW fraction was very poorly separated into its two sub-fractions and the 18s RNA formed a barely distinguishable shoulder to the 28s RNA fraction. The DNA fraction was homogenous and eluted as a sharp peak.

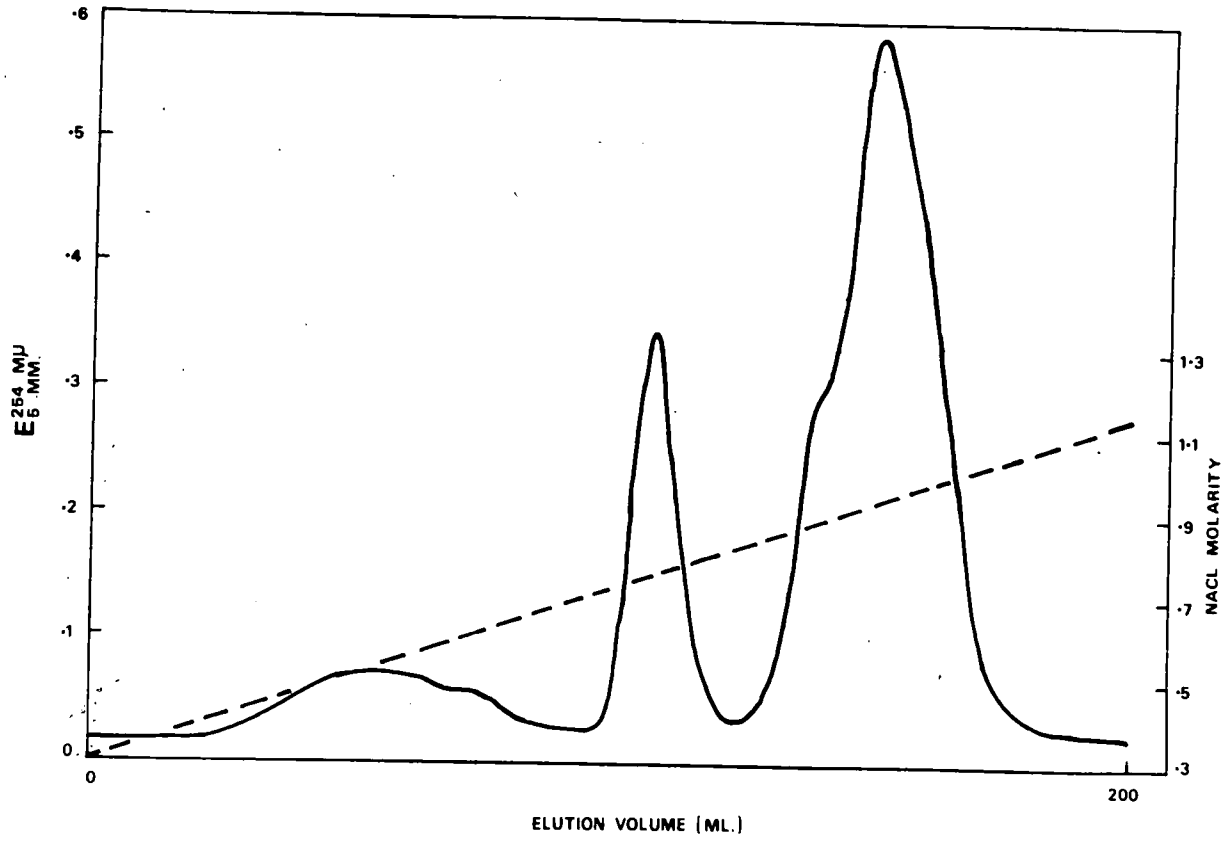


Fig.33. MAK chromatography of dormant cotyledon nucleic acids

Nucleic acids were extracted and estimated as described in the legend of fig.13 and in the methods section.

——————————— optical density

---------- sodium chloride molarity

(b) The percentage composition of the nucleic acids

The percentage composition of the nucleic acids, as determined by MAK chromatography, was :-

% LMW RNA/total RNA = 14.7%

% rRNA/total RNA = 85.3%

% DNA/total NA = 18.6%

The LMW RNA and rRNA percentages were very similar to those of 90 day developing cotyledons. The DNA percentage was higher, however, and was no doubt a reflection of the breakdown of some RNA during the dehydration of the seed.

TABLE 14

The RNA content of mature seed, subjected to drying

Drying period (weeks)	Fresh wt/ cotyledon pair (g)	Dry wt/ cotyledon pair (g)	Moisture content (%)	RNA content/ cotyledon pair (mg)
0	1.515	1.144	24.5	4.13
1	1.357	1.170	13.8	4.15
2	1.344	1.189	11.5	4.26
3	1.259	1.132	10.0	4.17
5	1.286	1.164	9.5	4.23

Dry weights and RNA contents were determined as described in the methods section

PART III. The germinating seed

11. The dry weights of seed cotyledons

There was a constant decrease in the dry weight of cotyledons with age of germinating seedlings, up to about 25 days (fig.34). The rate of weight loss then declined and was approaching completion by about 31 days after the onset of germination.

12. The RNA content of cotyledons

A. The purity of the final RNA nucleotide fraction

Fig.35 compares the absorption spectrum of the RNA nucleotide fraction from 21 day germinating seeds, with the absorption spectrum of a pure mixture of the four RNA nucleotides present in equal molar concentrations. Considerable quantities of substances other than nucleotides were present in the extracted fraction, which absorbed strongly in the far ultraviolet, but only slightly, if at all, at 260 mμ. As germination progressed, the final nucleotide fraction for RNA estimation became increasingly contaminated with such compounds.

B. The change in the total RNA per cotyledon pair during germination

The RNA of cotyledons did not increase in quantity at any of the stages of germination studied, fig.34. Between days 0 and about 8, there was a slight decrease in the RNA content. RNA loss became rapid by about day 12 and this continued to the final determination at day 31.

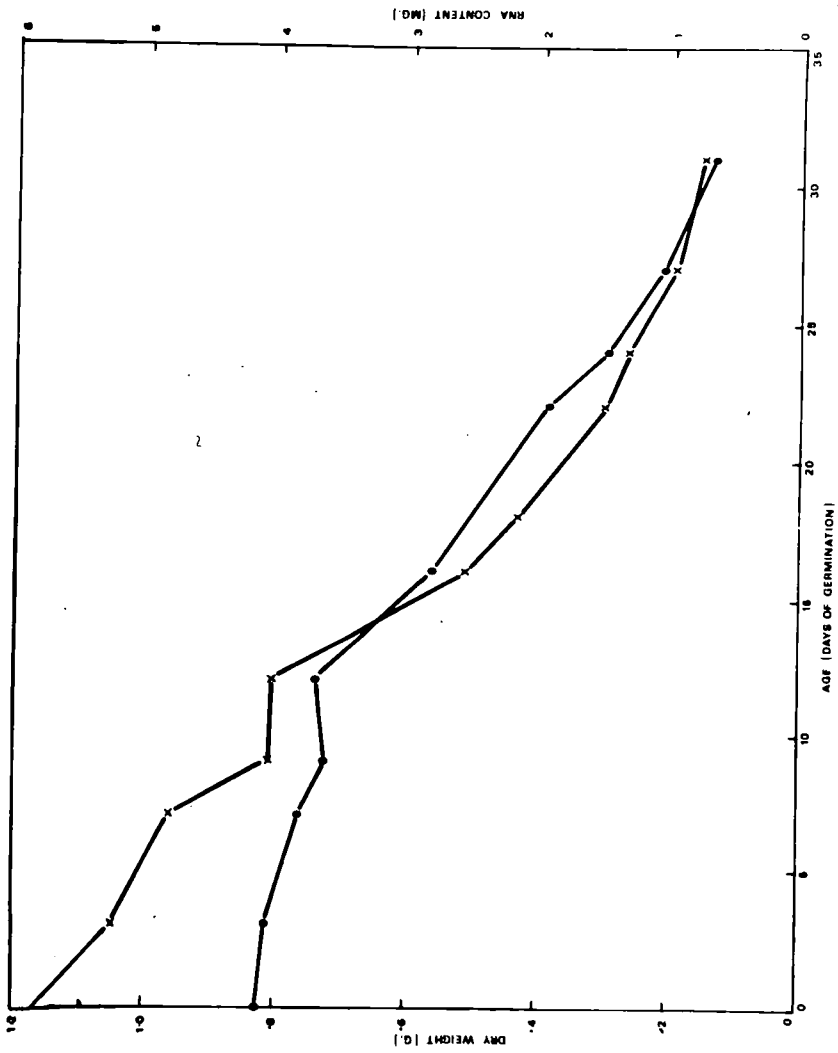
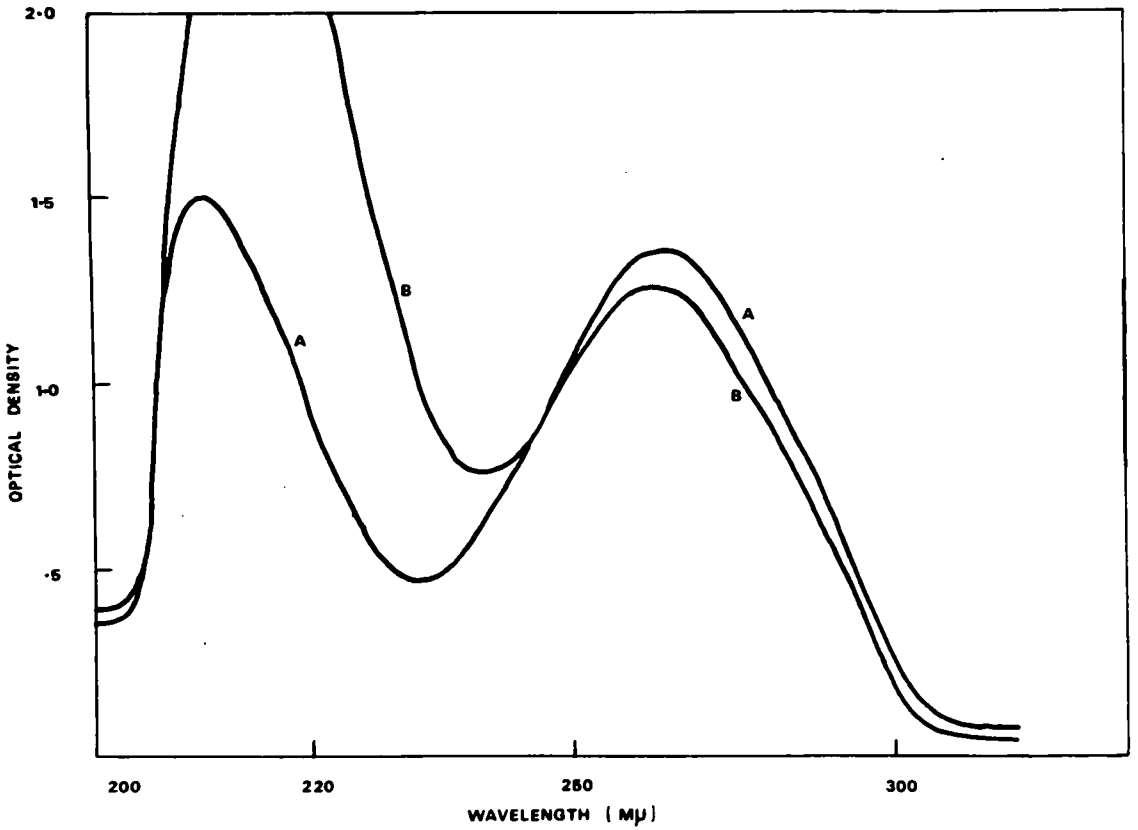


Fig.34. The dry weight and RNA content of cotyledons during seed germination

x - x dry weight per cotyledon pair

● - ● RNA content per cotyledon pair

Fig.35. Absorption spectra of RNA nucleotides



A. A pure mixture of the four RNA nucleotides (B.D.H.Ltd.) presentin equal molar concentrations.

B. The final RNA nucleotide fraction for RNA estimation (from cotyledons of 21 day germinated seeds.

Absorption spectra were determined in 0.37 M sodium chloride, 0.77 N hydrochloric acid.

(c) The change in the total RNA per g.dry weight of cotyledon, during germination

When expressed as a per g.dry weight basis, the RNA increased in quantity during the early stages of germination, fig.36, despite the fact that there is an overall loss of RNA in each cotyledon (fig.34). The RNA content reached a maximum at day 22, but then decreased to the final determination at day 31.

13. Methylated albumin kieselguhr chromatography of the nucleic acids of cotyledons

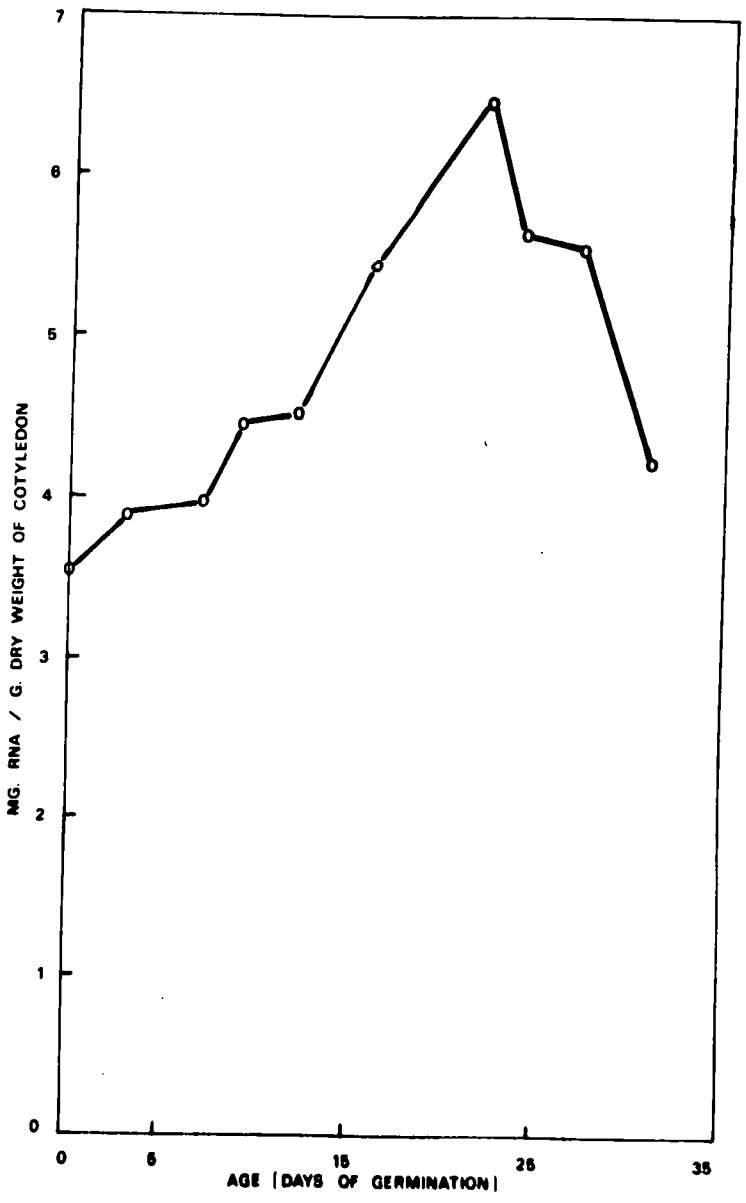
(a) The purity of the phenol extracted nucleic acid fraction

The nucleic acid fraction extracted from cotyledons at early stages in germination possessed an absorption spectrum typical of pure nucleic acid with an absorption maximum at 260 mμ and an absorption minimum at 233 mμ. Nucleic acids extracted at later stages in germination, from day 17 onwards, contained an additional absorption shoulder at ca.295 mμ (fig.37) as was noted for nucleic acids extracted from senescing testas (fig.15). This material was not retained by the methylated albumin when the sample was applied to a MAK column, and was immediately eluted (fig.37).

(b) The fractionation of nucleic acids at different stages in the germination of seeds

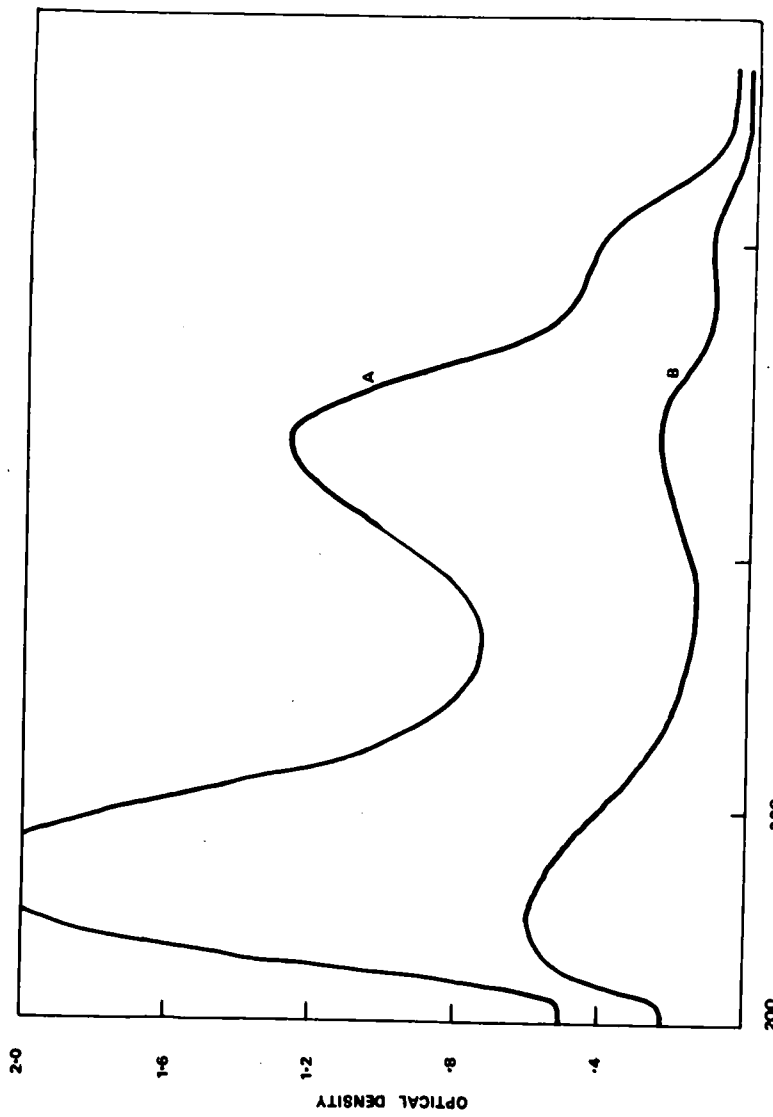
None of the eight MAK column chromatographs of nucleic acids, extracted from cotyledons aged between 2 days and 37 days, contained the double DNA fraction or the high molecular weight

Fig.36. The RNA content of cotyledons, expressed per g.dry weight of cotyledon material, during seed germination



The dry weight and RNA content of cotyledons were determined as described in the methods section.

Fig.37. Absorption spectra of nucleic acid fractions prepared from cotyledons of 26 day germinated seeds



A. The absorption spectrum of the nucleic acid fractions for MAK chromatography.

B. The absorption spectrum of the fraction which immediately elutes from the column.

Absorption spectra determined in 0.3M NaCl, 0.05 phosphate buffer, pH 6.7.

RNA fraction of developing cotyledons. 48hr soaked cotyledons (fig.13) contained a distinct 18s RNA fraction upon chromatography of their nucleic acids, unlike dormant seeds (fig.33). Apart from the relative increase in the DNA peak of the MAK elution profile as germination progressed, no other significant changes took place (fig.38).

(c) Changes in the composition of nucleic acid during the germination of seeds

At early stages in the germination of the seed, the RNA composition of the cotyledons was quite similar to that of cotyledons during seed development, and of actively metabolising testas. By day 15, however, there was a progressive increase in the percentage of LMW RNA and a corresponding decrease in the percentage of rRNA throughout the stages of germination studied (fig.39), as was seen for senescing testas (fig.23). By day 37, the percentage of LMW RNA/total RNA was 19%, an increase of 5% from the dormant seed stage.

The percentage of DNA to total NA remained constant for the first 17 days of germination, at a value of 21% (fig.39). It then began to increase very rapidly, in a similar manner recorded for senescing testas (fig.22), so that by the final determination at 37 days it had reached a value of 79%.

Fig.40 illustrates graphically the change in quantities of the different nucleic acids during the germination of bean seeds.

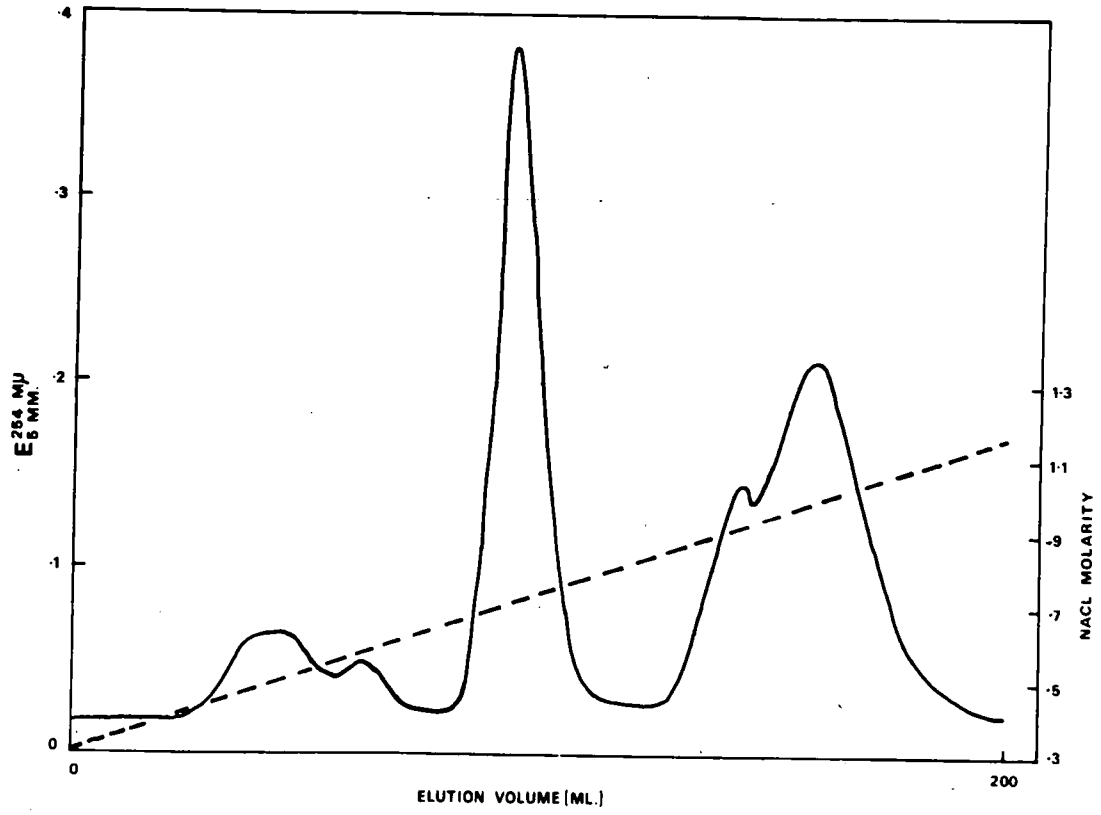


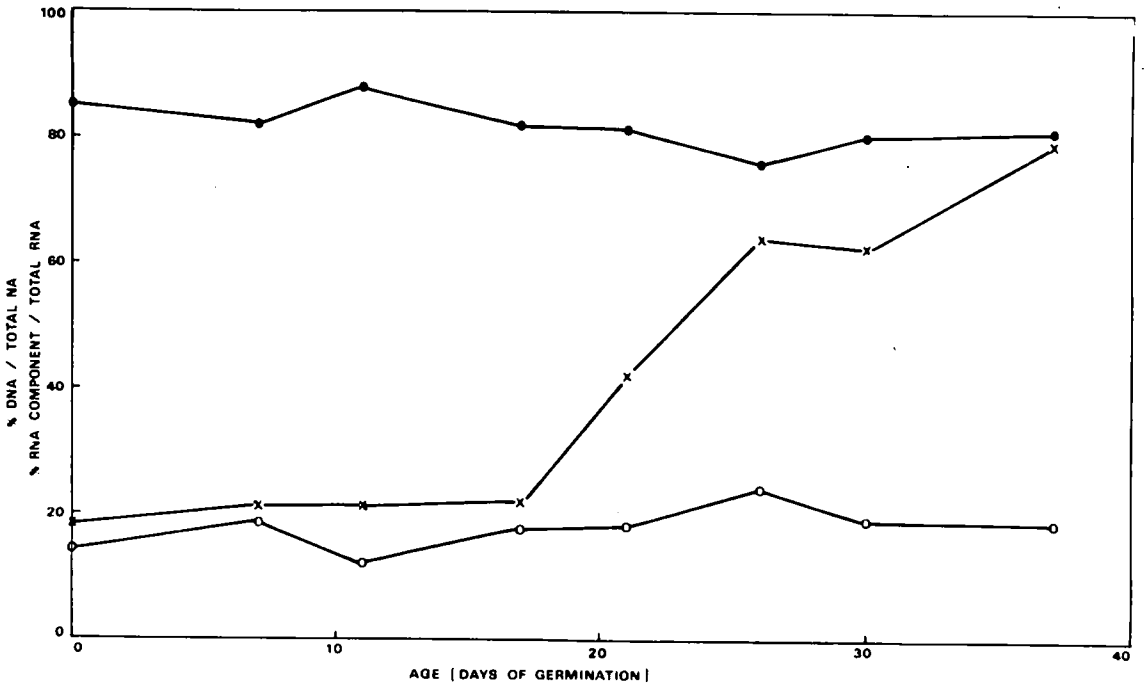
Fig.38. MAK chromatography of the nucleic acid fraction extracted from cotyledons of 21 day germinating seeds

Nucleic acids were extracted and estimated as described in the legend of fig.13, and in the methods section.

——————— optical density

---------- sodium chloride molarity

Fig.39. The nucleic acid composition of cotyledons during the germination of seeds

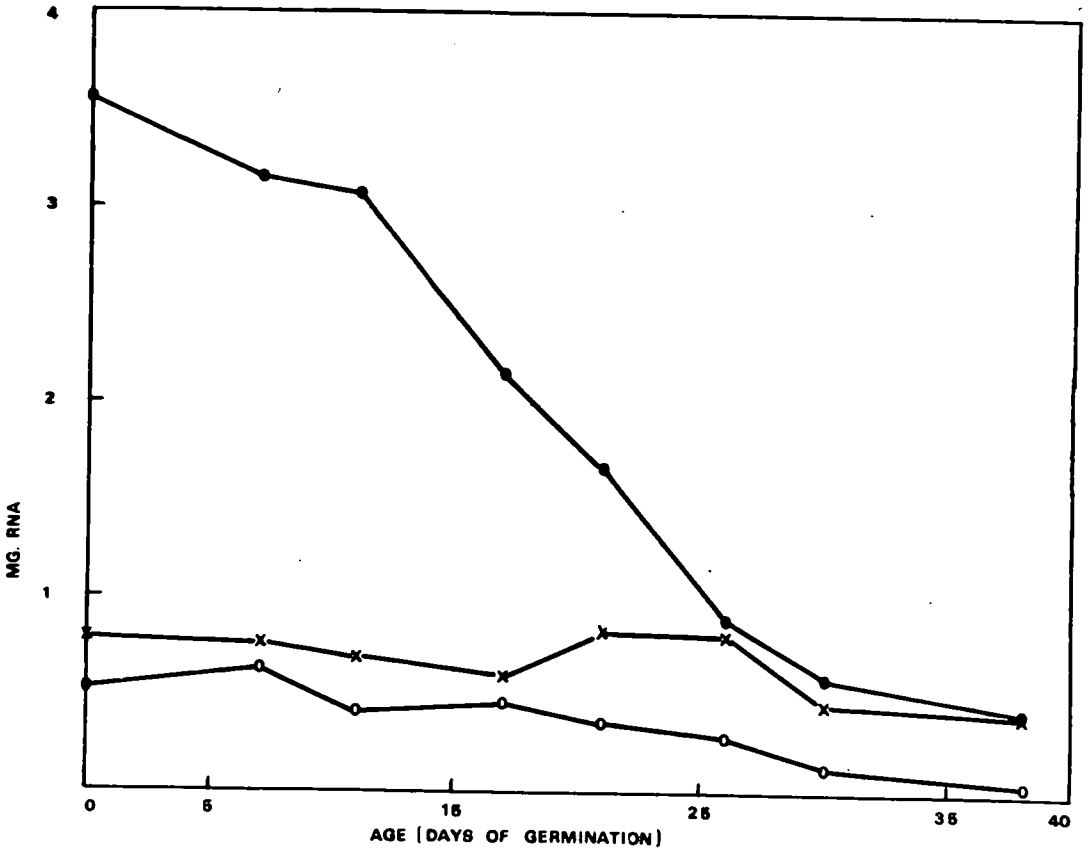


The nucleic acid compositions of cotyledons were calculated from MAK column elution profiles as described in the legend of table 6.

● - ● % rRNA/total RNA. o - o % LMW RNA/total RNA.

x - x % DNA/total NA.

Fig.40. The variation in the nucleic acid contents of cotyledons, from germinating seeds



The nucleic acid contents/cotyledon were calculated from the total RNA content and nucleic acid composition of cotyledons.

● - ● rRNA

o - o LMW RNA

x - x DNA

The rRNA content decreased rapidly at all stages in cotyledon senescence. LMW RNA also decreased in quantity, but at a much slower rate. The DNA content in contrast remained approximately constant until later stages of senescence, when it slowly decreased.

14. Free and membrane bound ribosomes of cotyledons

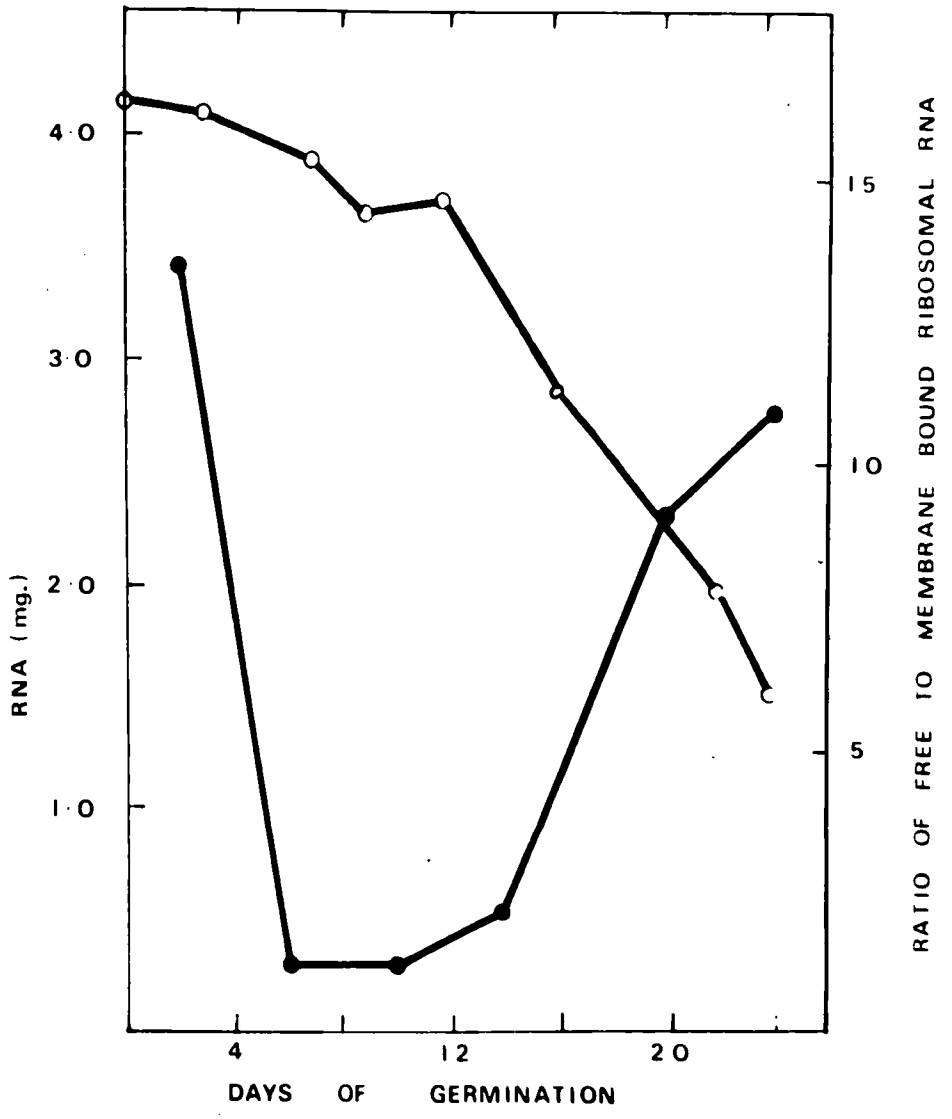
(a) Changes in the ratio of free to membrane bound rRNA during germination

The ratio of free to membrane bound rRNA decreased during early stages of germination, fig.41. Between days 6 and 12, the ratio remained approximately constant at a value of 1.2 : 1, but later began to increase to the final determination at day 24. In contrast to developing cotyledons, the numbers of membrane bound ribosomes never exceeded the numbers of free ribosomes.

(b) Changes in the contents of free and membrane bound rRNA

Between days 2 and 6, there was an increase in membrane bound rRNA despite a decrease in the total RNA content of cotyledons, fig.42. The level of membrane bound RNA then remained approximately constant for the next 4 days, but later was rapidly degraded. Free rRNA decreased rapidly at first, and then more slowly at the period when membrane bound rRNA was being broken down. At later stages in seed germination the remaining free rRNA was rapidly degraded.

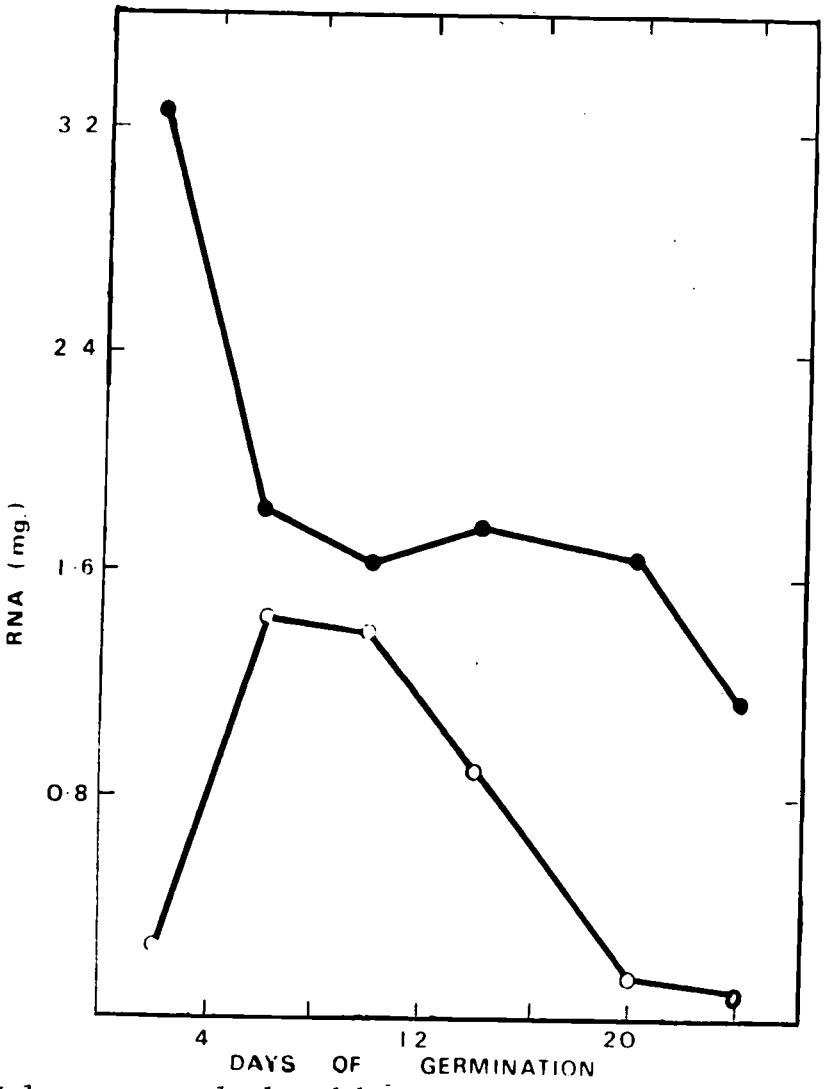
Fig.41. The total RNA content and the ratio of free to membrane bound rRNA of cotyledons during seed germination



o - o Total RNA

● - ● The ratio of free to membrane bound rRNA.

Fig.42. The levels of free and membrane bound rRNA in cotyledons during seed germination



Values were calculated by equating the following experimental determinations : (i) RNA/cotyledon (fig.34); (ii) percentage of rRNA/total RNA (fig.39); and (iii) the ratio of free to membrane bound rRNA (fig.41).

⊙ - ● free rRNA

o - o membrane bound rRNA

15. The incorporation of (^{32}P) - orthophosphate into germinating seeds

(a) The bacteria content of the growth chamber after the incubation period

In no experiments were seeds successfully germinated for 4 days under sterile conditions. In the first two experiments, table 15, considerable quantities of bacteria were present in the solution surrounding the seedlings (about 1 x 10^7/ml) but in the second two experiments, in which chloramphenicol was used, less bacteria were present (about 5 x 10^3/ml).

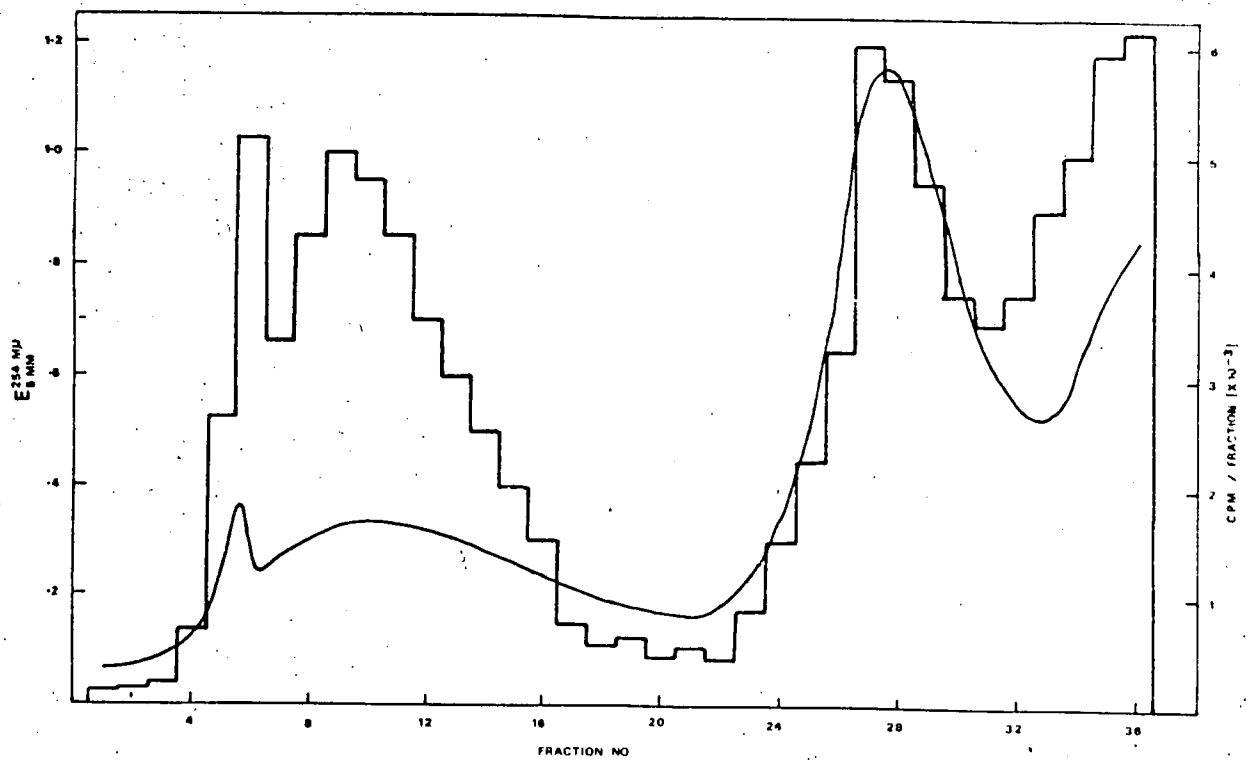
(b) The incorporation of radioactivity into free and membrane bound ribosomes of the cotyledons

Radioactivity could be detected both in the free and membrane bound ribosome fractions, fig.43. The amount of radioactivity in each fraction varied considerably, especially the fraction containing the free ribosomes which was relatively large when prepared from heavily contaminated seedlings.

(c) The incorporation of radioactivity in free and membrane bound rRNA

Considerable quantities of (^{32}P) - orthophosphate were incorporated into the RNA of free and membrane bound ribosomes isolated from cotyledons heavily contaminated with bacteria. The specific activity of free rRNA in this material was 2 - 14 times greater than the specific activity of membrane bound rRNA.

Fig.43. The incorporation of (^{32}P) - orthophosphate into the microsome fractions of cotyledons, during seed germination



The microsome fraction of cotyledons was analysed by sucrose gradient centrifugation as described in the methods section.

——— Optical density

Histogram. Radio-activity.

Bacterial content of growth chamber = 7.6×10^6/ml. growth medium.

In experiments where the levels of bacteria were reduced to about 5×10^3/Ml, there was over a ten fold decrease in the specific activity of the free rRNA. The specific activity of membrane bound rRNA, on the other hand, remained approximately constant and was 6-10 times greater than that of the free rRNA.

TABLE 15

The incorporation of (^{32}P) - orthophosphate into the RNA of free and membrane bound ribosomes

Experiment No.	Specific activity of membrane bound rRNA		Specific activity of free rRNA		Bacteria content of growth chamber
	C.P.M./ mg.RNA	µµM/ mg.RNA	C.P.M./ mg.RNA	µµM/ mg.RNA	(colonies/ml.)
1	436,952	0.15	655,428	2.21	3.5×10^7
2	230,672	0.07	576,680	0.17	7.6×10^6
* 3	348,629	0.05	47,757	7.3×10^{-3}	2×10^3
* 4	541,800	0.14	50,508	1.3×10^{-2}	8×10^3

* The growth chamber contained 40 µg/ml of chloramphenicol.

Specific activities of (^{32}P) - orthophosphate :- experiment 1 = 43 curies/mg.phosphorus, experiment 2 = 48 curies/mg.phosphorus, experiment 3 = 95 curies/mg.phosphorus, experiment 4 = 56 curies/mg.phosphorus.

Bacteria were estimated by a serial dilution method. The rRNA pellets, prepared as described in the methods section, were dissolved in a small volume of water. An aliquot was diluted and assayed spectrophotometrically to determine its concentration, assuming an $E^{260\ m\mu}_{1cm}$ of 20.0 for a 1mg/ml RNA, and the rest assayed for radioactivity.

DISCUSSION

The seeds of many members of the Papilionaceae are characteristic in containing protein as a major component of their nutrient reserves. In Vicia faba two storage proteins, vicilin and legumin, have been reported (Daniellson, 1952) and they amount to some 20% of the dry weight of the mature seed (Grzesiuk, Mierzwinska, and Sojka, 1962). The developing seed should serve therefore as a convenient tissue for studying both RNA metabolism and protein biosynthesis.

On the basis of anatomical and biochemical studies of developing seeds of Vicia faba, Grzesiuk et al (1962) divided the developmental period into three phases :-

(i) Endosperm and proembryo formation (1 to 24 days after pollination),

(ii) Embryo formation and the initial accumulation of food reserves in the cotyledon (24 - 42 days after pollination), and

(iii) The stage of intensive accumulation of nutrient reserves (42 days to maturity at 60-70 days after pollination).

Davis (1967), Briarty (1967), and Davis and Boulter (1968), by light and electron microscopy and by chemical analyses, confirmed the validity of dividing the developmental period into these three phases. In all cases, material was grown in the open, since greenhouse conditions do not favour fruit set, and was therefore subject to the inevitable fluctuation in environmental conditions. Davis (1967), for instance, found that storage protein synthesis

commenced intensively at 38 days after flowering in the 1964 season, but in the 1963 season it commenced 8 days earlier.

A similar variation between crops is reported here, (see fresh weight, dry weight, and RNA content measurements, figs. 4, 5, and 11). It is perhaps surprising that more variation did not occur, since plants in 1966 were grown in Cheshire, which enjoys a mild, moist climate, and plants in 1967 were grown in the drier, colder climate of Durham. In an attempt to reduce variation as much as possible, a random sample of mature seeds, harvested in 1966, were dried and sown in Durham to yield the 1967 crop. It is apparent from these preliminary findings that precise, analytical results obtained here only apply to a particular harvest, although the pattern of changes evidenced are applicable to all growing seasons.

As a basis for timing development, flowers were labelled as day 0 when they were first noticed to be fully open. The actual time of the fertilisation of the ovule was suggested by Davis not to occur in the field until 2 or 3 days after flowering, since 4-celled embryos were generally obtained on the fourth day. A similar conclusion was reached by Grzesiuk et al (1962). After this age, cell division proceeds and it is not until about day 25 that the developing seed is of sufficient size to be subjected to chemical analysis.

Briarty (1967) suggested, on the basis of the presence of mitotic chromosomes, that the bulk of cell division was complete in the developing seed by day 25 after flowering. Wheeler and Boulter (1967) determined the DNA content of seeds during development and showed that DNA synthesis was complete in the cotyledons by about day 53 and concluded that cell division had ceased by this age.

This study employed a more direct method of characterising the stage of cell division. Cell numbers of cotyledons were determined during development by the basic procedure of Brown and Rickless (1949). This method involved quantitatively macerating tissue into single cells, suspending them in a known volume of water, and counting the number of cells on a haemocytometer slide. It is possible in this technique that, during the process of maceration, some cells are broken down. However, no evidence of this was detected by light microscopy, and using 48hr soaked cotyledon material three separate experiments gave reproducible results (table 1).

The actual technique adopted was developed using cotyledons from 48hr soaked dormant seeds, and their cells, like those of cotyledons in later stages of development, contain secondary thickening in their walls. Cotyledons in early stages of development only contain primary thickening in their cell walls, and the possibility arose that some of these cells might be broken down by the vigorous maceration procedure adopted. To check this, the cell number content of young cotyledons was determined using a milder maceration procedure, slightly modified from that of Brown

and Rickless (1949), designed to macerate young pea root tips. The two methods gave similar results (table 3) suggesting no breakdown had occurred.

The graph of the number of cells per cotyledon pair against age of seed (fig.8) shows that there is a rapid increase in cell numbers between days 27 and about 48. After this period, the cell division rate slows down rapidly and ceases by about day 57. The variation of cell number determinations at later stages in development is considerable, and is too great to be attributed to the experimental error inherent in the technique adopted, (see table 1). The cotyledon during later stages in maturation is apparently much more variable than 48hr soaked mature cotyledons. This variability was effectively standardised in other analyses by gathering large quantities of material and analysing a representative sample. In these determinations, however, a maximum of only 80mg freeze dried cotyledon tissue could be used, making effective standardisation impossible. Nevertheless, it may be concluded that cell division continues in the cotyledon to about day 57, and this is in general agreement with the conclusions of Wheeler and Boulter (1967) but is in disagreement with those of Briarty (1967).

When the data from cell number determinations is recalculated to give the number of cell generations of cotyledons at different stages in their development (fig.9), it is evident that cell division proceeded at a relatively low rate during those

stages measured. Between days 25 and 57, approximately five generations of cells are formed and mitotic chromosomes should be detectable by electron microscopy. Unfortunately, electron microscopic studies are even more prone to sampling errors than cell number determinations as an even smaller sample is examined. It is suggested that differences in sampling may be a contributing factor to the discrepancies in the conclusions of Briarty (1967) and the present study.

The first nucleic acid experiment reported here involved the determination of the RNA contents of cotyledons and testas. Since the methods of estimation of RNA are subject to gross interference by other cell constituents, RNA must be quantitatively extracted and purified before it can be estimated. Holdgate and Goodwin (1965) critically reviewed the various extraction methods available and concluded that the method of Smillie and Krotkov (1960), when combined with the lipid extraction procedure of Hutchinson and Munro (1962), was the most satisfactory. In this procedure, the ground plant tissue is first given an initial lipid extraction, and is then treated with cold 5% (w/v) TCA to remove acid soluble compounds. Lipids are next removed by a series of organic solvents and the residue is digested with alkali. Upon acidification, the RNA, hydrolysed to nucleotidyl material, remains in solution and is purified by passing through a small Dowex 1 resin column.

Holdgate and Goodwin (1965), working with various organs of rye and wheat seedlings, suggested further modifications to

this method. They discovered that the hot ethanol treatment in the lipid extraction process degraded a small percentage of the RNA and suggested replacing hot ethanol with cold ethanol. The final nucleotide solution from most extracts was found to contain considerable impurities when analysed spectrophotometrically. These impurities were effectively removed by subjecting the sample to activated charcoal column chromatography.

In the present study, RNA was extracted basically by the method suggested by Holdgate and Goodwin (1965). In all cases, however, the RNA nucleotide fraction, derived from both developing and germinating seeds, was free of non-nucleotidyl material absorbing in the vicinity of 260 mμ after purification on Dowex (figs. 10 and 35). The activated charcoal purification stage was consequently not used.

Three methods are currently used to estimate RNA, (1) ultra-violet light measurements, (2) the orcinol reaction for the determination of ribose, and (3) the molybdivanidate reaction for the determination of phosphorus. Wheeler and Boulter (1966) extracted RNA from developing seeds as described in the present study except that the hot ethanol stage was included and the Dowex purification stage was omitted. RNA was estimated by two methods :- (A) the phosphorus content and (B) separating mononucleotides by ion-exchange chromatography, and by the summation of their absorbances at 260 mμ. Method (A) gave values about twice as great as method (B), and the authors suggested the

presence of phosphorus-containing substances, other than nucleotides, in the RNA extract. Cherry (1962) found that the RNA content of mature and germinating pea-nut cotyledons, determined by analysis of phosphorus or ribose, was 3 to 5 times greater than when ultra-violet light measurements were used for the determinations. Since in the work recorded here, the absorption spectra of the RNA extracts indicate the absence of non-nucleotidyl material absorbing at 260 mμ, RNA was simply estimated by the absorbance at this wavelength.

RNA determinations revealed that during the early stages of development the RNA content remained approximately constant, fig.11. At day 35 after flowering, there was a rapid increase in the dry weight of the cotyledons (fig.5) and a concomitant rapid synthesis of RNA which continued to about 70 days after flowering.

Bisson and Jones (1932), working with Pisum sativum, showed by chemical methods that the developing seed started to accumulate starch in the cotyledons several days before it started to accumulate storage protein. More recently, Opik (1968), by histochemical staining techniques, came to the same conclusions with developing seeds of Phaseolus vulgaris. Davis (1967) also inferred a similar situation in Vicia faba, since storage protein accumulation commenced rapidly 8 days after the initiation of a rapid increase in the dry weight of the seed. If the information of Davis is incorporated into the present results, it is clear that the commencement of the rapid increase in the RNA content of cotyledons at 35 days after flowering, precedes the initiation of

storage protein synthesis by about 8 days. Since figs. 5 and 11 show that RNA synthesis is also complete about 10 days before the completion of nutrient reserve accumulation, it is concluded that RNA mediates the synthesis of storage protein. A similar conclusion was reached by Wheeler (1965) and Wheeler and Boulter (1967).

Since some cells are still undergoing cell division at the commencement of food reserve accumulation, a clearer understanding of the relationship between RNA and storage protein is obtained by expressing the RNA content results on a per average cotyledon cell basis, rather than a per cotyledon pair basis (fig.12). During nutrient reserve accumulation, the RNA content/cell increased approximately six-fold, from 1.5 $\mu g \times 10^{-4}$ to 10 $\mu g \times 10^{-4}$. Heyes (1963) determined the RNA content/average cell of developing pea roots, and obtained values up to 2.7 $\mu g \times 10^{-4}$, which compare favourably with the results presented here for cotyledons in early stages of development. The atypical quantities of RNA in cotyledon cells at later stages in development are no doubt due to the equally unusual rate of synthesis of protein.

During the later stages of rapid RNA synthesis in the cotyledons, there was a concomitant hydrolysis of the RNA in the surrounding testa (fig.11). As suggested by Wheeler and Boulter (1967), the nucleotidyl material produced is no doubt translocated to the cotyledon for use, either in the production of nucleotides to be utilised in the general metabolism of the cell, or in the incorporation into RNA.

To obtain a fuller understanding of the involvement of RNA in storage protein synthesis the various components of nucleic acids were studied in detail at all stages in the development of the cotyledon. At the commencement of these studies, methylated albumin kieselguhr (MAK) chromatography appeared to be the most suitable method of characterising nucleic acids. According to Ellem (1966), "only column chromatography on MAK offers a single step separation of all the major recognised nucleic acid species".

MAK chromatography was initially developed by Mandell and Hershey (1960) to separate species of DNA. Sueoka and Yamane (1962) and Yamane and Sueoka (1963) modified the method for the separation of all the major nucleic acid components.

The active part of the column is the methylated albumin, and this is absorbed on to the surfaces of the inert kieselguhr particles. The negatively charged nucleic acids became bound to the positively charged serum albumin and are selectively eluted from the column by increasing concentrations of sodium chloride. The ribonucleic acids elute mainly as a function of their molecular weight, but base composition is a secondary factor.

A typical MAK fractionation of a nucleic acid extract from plant tissues is illustrated in fig.13. Three main fractions occur. The first fraction is LMW RNA and is partially separated into two regions containing tRNA and 5s RNA respectively. The second fraction is homogeneous and consists of DNA, but the third is composite

and is subdivided into two overlapping peaks of 18s RNA and 28s RNA.

The profile of the LMW RNA fraction was similar at all stages in seed development. The DNA fraction on the other hand became noticably heterogeneous when isolated from cotyledons or testas which were actively metabolising.

A similar heterogeneity in the DNA fraction has been reported in the literature. Sampson, Katoh, Hotta, and Stern (1963) detected two DNA fractions from wheat seed extracts. One had a relatively low molecular weight of $2 - 3 \times 10^5$, was rapidly labelled, and eluted at slightly lower salt concentrations than the major DNA fraction, which had a molecular weight of $4 - 6 \times 10^6$, and was not rapidly labelled. The authors termed this DNA "metabolically labile DNA", and although they showed it was probably not a breakdown product, and was not an intermediate in the synthesis of the major DNA, they were not able to assign a function for it. Cherry (1964) also detected this fraction from peanut cotyledons.

Another DNA component in the DNA fraction was described by Holoubek (1967). Rapidly dividing Ehrlich ascite cells were incubated with (^{3}H) - thymidine, nuclei were isolated and DNA extracted. A highly labelled DNA was detected by MAK chromatography, eluting at higher salt concentrations than the main, unlabelled fraction, and concluded that it consisted of a newly synthesised DNA.

Several workers have also shown that the DNA fraction contains RNA, both by chemical analysis and by the incubation of

tissues with (^{3}H) - 5 - uridine. Cherry (1964) determined that 25% of the nucleic acid in the DNA fraction obtained from peanut cotyledons was RNA. Ingle, Key, and Holm (1965) on the other hand obtained a value of 5% RNA for soya bean hypocotyles.

Cherry (1964), and Galling and Richter (1966) demonstrated that this RNA eluted immediately before the main DNA fraction, and showed that it was complexed to DNA. Cherry (1964), in addition, gave evidence that the DNA - RNA complex was in the form of a precise hybrid, and postulated that it consisted of natural mRNA - DNA duplexes. The validity of this hypothesis has not yet been confirmed.

In the present investigation, the heterogeneity in the DNA fraction appeared to be due to a minor component eluting at a slightly lower salt concentration than the major fraction. When 60 day developing cotyledon discs were pulsed with (^{32}P) - ortho-phosphate under near sterile conditions, a small though distinct peak of radioactivity became preferentially associated with the minor DNA component. This suggests that this component is either the metabolically labile DNA of Sampson et al (1963) or a DNA - RNA complex.

The MAK column elution profiles of nucleic acid extracts from metabolically active developing cotyledons were atypical in containing a third, high molecular weight (HMW) RNA peak. When tissues were pulse-labelled with (^{32}P) - orthophosphate, this region of the profile became preferentially labelled. The fraction

was most pronounced in cotyledons undergoing storage protein synthesis (fig.17), but was completely absent in testas.

A similar fraction, though usually only detectable by radioactivity, has been demonstrated from various plant tissues which were actively metabolising, e.g. Cherry and van Huystee (1965), Galling and Hemleben-Vielaben (1967), Gressel and Galun (1967), and Loomis and Sussman (1966). It has usually been referred to as mRNA, or as D-RNA (in order to avoid the functional implications of a messenger). Some supporting evidence comes from the fact that it often has a base composition intermediate between DNA and rRNA.

Cherry and Lessman (1967) reported that MAK chromatography of several monocotyledon seed nucleic acids separated a third HMW - RNA fraction detected by its optical density at 260 mμ, and this may therefore be comparable with the fraction isolated in this study. The authors suggested that this fraction, which consisted of 34% of the total HMW RNA, constituted a store of long-lived mRNA which would direct protein synthesis at the onset of germination.

It has been realised for some time that the high salt concentrations of sodium chloride required to elute the HMW RNAs could cause aggregation between molecules, and this is evidenced most clearly in the separation of 28s from 18s RNA. Since these RNAs occur in equal amounts in cells, and as the molecular weight of one is approximately twice that of the other, a ratio of

absorbance of 2 : 1 in favour of 28s RNA would be expected upon fractionation. In actual fact, the ratio is often as much as 4 or 5 : 1 (see fig.13) suggesting most of the 18s RNA has aggregated with the 28s RNA.

Polyacrylamide gel electrophoresis has recently been developed for the fractionation of both high and low molecular weight RNA by Loening (1967), and has a much greater resolution than MAK chromatography without having the drawbacks of salt concentration and aggregation. Ingle and Key (1968) applied this technique to analyse the high molecular weight fractions separated by MAK chromatography from soya-bean hypocotyl extracts. RNA eluted from the 18s region of the MAK column contained 18s and 16s RNA components by subsequent gel electrophoresis. The whole of the 28s region of the MAK elution profile contained not only 28s RNA, but also a considerable amount of the 18s component together with a trace of 23s RNA. From these experiments it was not possible to decide whether aggregation of the 18s RNA was caused by a 28s - 18s or an 18s - 18s association. 16s and 23s RNA were assumed to be of plastid or mitochondrial origin. Gel fractionation of the "mRNA region" eluted from MAK columns revealed mainly 28s and 18s RNA but in addition there were two other fractions corresponding to 32s and 30s RNA. A broad radioactive peak, ascribed as D-RNA, occurred in this region when the tissues were pulsed with (^{32}P) - orthophosphate. The authors considered that these two large molecular weight RNAs were not aggregates, but did

not make positive suggestions as to their identities.

Loening and Ingle (1967) fractionated the RNAs of several seeds of the Monocotyledons by polyacrylamide gel electrophoresis and did not detect any fractions of higher molecular weight than that of 28s RNA, or any unusual fractions. The third HMW RNA fraction of Cherry and Lessman (1967), detected by MAK chromatography, has therefore probably arisen by an aggregation of RNAs of lower molecular weight and is most unlikely to contain mRNA.

It is probable then that at least part of the third HMW fraction detected in this investigation is due to aggregation. This is suggested experimentally as cotyledons, when incubated with (^{32}P) - orthophosphate for 3hr in the form of discs, produced a much larger third HMW fraction upon chromatography, than equivalent cotyledons not treated in this way. Since, however, the fraction only occurs at certain stages in the development of cotyledons and as it becomes preferentially labelled when tissues are pulsed with (^{32}P) - orthophosphate, it is possible that other RNAs are contributing to the formation of this fraction.

This fraction was kindly analysed by Dr. U.E. Loening (University of Edinburgh), by polyacrylamide gel electrophoresis. The third HMW RNA fraction, isolated by MAK chromatography, was shown to be quite heterogeneous (fig.44). The largest components were 28s RNA and 18s RNA, and these were, no doubt, in the form of duplexes, either 18s - 18s or 28s - 18s RNA during MAK chromatography (Ingle and Key, 1968). Fractions (D) and (F) are either breakdown

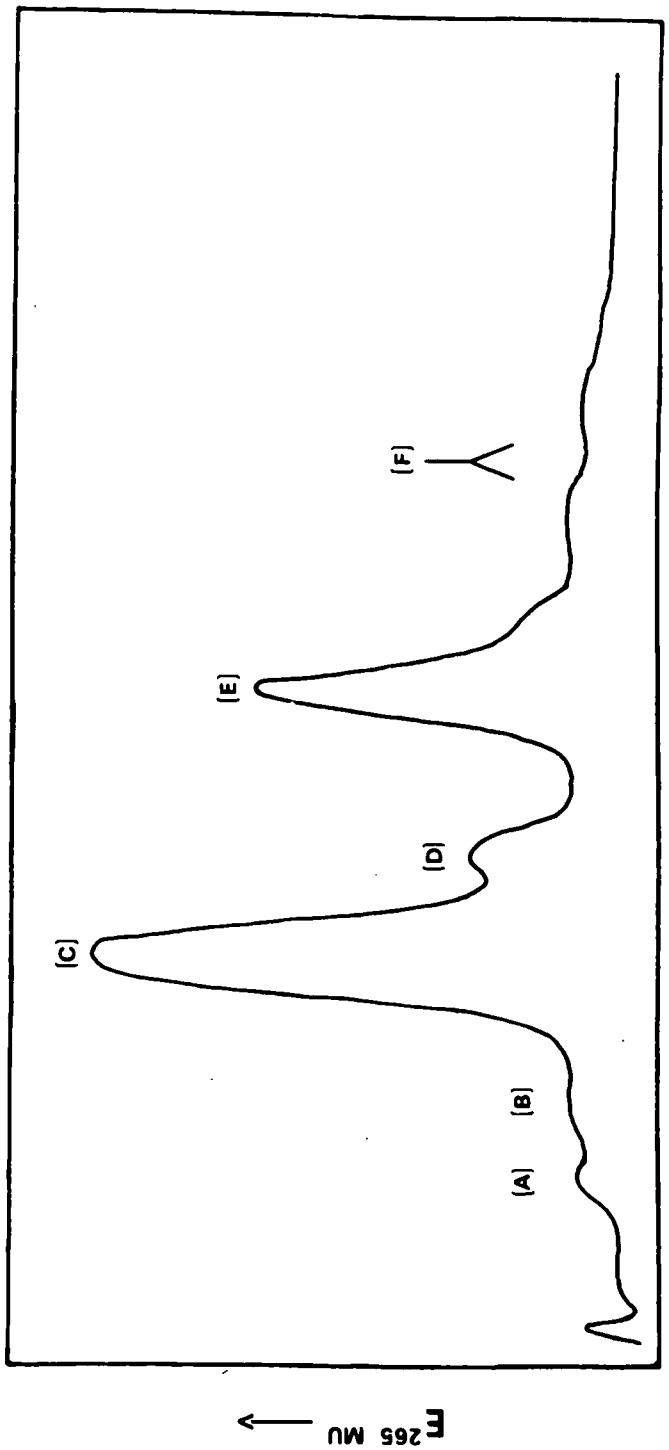


Fig.44. Polyacrylamide gel electrophoresis of the third high molecular weight RNA fraction, separated by MAK chromatography

Legend for fig.44

60 day developing seeds were collected from the 1968 harvest (grown at Durham) and their nucleic acids were extracted by a method modified from that of Kirby (1965). The nucleic acid extract was chromatographed on MAK as described in the methods section and the third high molecular weight RNA fraction obtained was centrifuged at 138,000 x g.max. for 16hr using a swing out rotor.

The RNA pellet was dissolved in 0.15 M sodium acetate containing 0.5% sodium dodecyl sulphate, and precipitated by adding 2 volumes of ethanol at 0^{o}. This RNA fraction was subjected to polyacrylamide gel electrophoresis by Dr. U.E. Loening (University of Edinburgh) by the method of Loening (1967).

Fraction A Molecular weight = 2.20 million
" B " " = c.a. 1.93 million
" C " " = 1.28 million (28s RNA).
" E " " = 0.70 " (18s RNA).

Identifications for fractions D and F are suggested in the text.

products of 28s and 18s RNA respectively or are of plastid origin. Two small RNA fractions, (A) and (B), occur which have a greater molecular weight than 28s RNA. Fraction (B) has a molecular weight very close to that of a 28s - 18s RNA duplex (actual = 1.93 million, theoretical = 1.98 million). Fraction (A) on the other hand has a molecular weight which cannot be accounted for by duplex formation of rRNA molecules.

Fraction (A) may consist, therefore, of one or more of the following alternatives, (1) an rRNA molecule combined with mRNA, (2) polycistronic mRNA (D-RNA), and (3) a ribosomal precursor RNA. All these RNAs would be expected to rapidly incorporate (^{32}P) - orthophosphate "in vivo". The presence of such RNAs in the third high molecular weight RNA fraction of MAK elution profiles would hence explain the tendency of this fraction to become rapidly labelled.

The possibility that mRNA would elute from MAK columns at high salt concentrations arose from the work of Asano (1965). He isolated T 4 phage mRNA and demonstrated that it became strongly bound to rRNA in sodium chloride solutions above a molarity of 0.5. Upon MAK column chromatography, phage mRNA eluted both with, and after, 28s RNA.

Yoshikawa-Fukada, Fukada, and Kawade (1965) pulse labelled cultures of human amnion (FL) cells with (^{32}P) - orthophosphate. They obtained two distinct radioactive fractions (q_1 and q_2) by MAK chromatography, which eluted after the bulk of the 28s RNA. Both fractions broke down rapidly (especially q_2) and were shown, by

sucrose gradient centrifugation in the presence of EDTA, to consist of RNA having a greater molecular weight than that of 28s RNA. From these results, and from base composition determinations, the authors suggested that q_1 contained precursor rRNA and q_2 contained polycistronic mRNA. q_1 and q_2 RNA have also been isolated by MAK chromatography of HeLa cell nucleic acids (Ellem, 1966). Subsequent characterisations of these two RNA molecules led to the same conclusions as those reached by Yoshikawa-Fukada et al (1965).

Scherrer, Marcaud, Zajdela, London and Gros (1966) and Loening (1968), suggested that the presence of a polycistronic mRNA of very short half life would explain the observed RNA turnover at the nuclear level. Polycistronic mRNA may, therefore, be a precursor of cytoplasmic mRNA; specific mRNA molecules required by the cell are detached from the polycistronic mRNA in the nucleus and are transported to the cytoplasm. The remaining fragments of the polycistronic mRNA would break down in the nucleus.

In the particular phenol extraction procedure employed in this study, RNAs of the nucleus are extracted in addition to those of the cytoplasm (Kirby, 1965). The nucleic acid preparations for MAK chromatography may, therefore, contain polycistronic RNA and also precursor rRNA.

The presence of this small fraction (A) cannot completely account for the large third high molecular weight fraction detected at particular stages in the life history of the cotyledons. Ingle

and Key (1968) showed that after soya bean hypocotyls were labelled with (^{32}P) - orthophosphate, the specific activity of the non-aggregated 18s RNA was much higher than that of the 18s RNA located in the rapidly labelled region of the 28s RNA fraction. It is possible, therefore, that in the present study the third high molecular weight RNA fraction is formed by an aggregation of 18s RNA synthesised at earlier stages in cotyledon development. This does not explain the virtual absence of this MAK fraction in cotyledons during the dehydration or germination of the seed however.

Clearly, the fractionation of RNAs by MAK chromatography at high salt concentrations is a very complex process, and some of the results reported here are very difficult to interpret.

Despite these disadvantages, the percentage composition of the nucleic acids can be reliably estimated by measuring the peak areas of the individual fractions. The method assumes that the phenol extraction procedure quantitatively extracts nucleic acids from cells which are ruptured during homogenisation. Similar nucleic acid composition determinations have been made by Chroboczek and Cherry (1966) using cotyledons of germinating peanuts, and by Wood and Bradbeer (1967) using cotyledons of dormant and germinating hazel seeds.

In the present study, the third HMW RNA fraction for these purposes was regarded as consisting solely of rRNA. The large increase in total RNA which occurred immediately before, and during, storage protein synthesis was shown to consist of 13%

LMW RNA and 87% rRNA (fig.22). The percentage composition of a range of metabolically active plant cells is closely similar to that recorded above, e.g. peanut cotyledons (Chroboczek and Cherry, 1966), hazel cotyledons (Wood and Bradbeer, 1967), and broad bean leaves (Dyer and Leech, 1968).

It is concluded therefore that storage protein is synthesised by the mRNA/tRNA/ribosome mechanism universally accepted for the synthesis of enzymes.

The storage protein which accumulates in the developing seeds of Vicia faba is deposited in organelles termed protein bodies. Protein bodies have a bounding lipoprotein membrane and appear to originate by the sub-division of vacuoles, Briarty (1967). Similar findings have been reported, by Opik (1968) for seeds of Phaseolus vulgaris, Englemann (1966) for cotton, and Bain and Mercer (1966) for Pisum sativum. Protein appears to accumulate in these bodies at two or three regions at the periphery. These areas increase in size until eventually the whole organelle is densely filled with protein.

Wheeler and Boulter (1967) detected an appreciable incorporation of (^{14}C) - leucine into peptidyl material by protein body extracts contaminated with 10^8 bacteria per ml of incubation. Incorporation was reduced by 98% when protein bodies were isolated using asceptic techniques so that bacteria were present in the order of 10^4/ml. These findings have been confirmed by Yarwood (1968), who also succeeded in reducing the bacteria present in the protein body fraction to 2 - 4 x 10^3 per ml. Under these conditions, it was

not possible to demonstrate any incorporation of (^{14}C) - leucine into peptidyl material. Both authors concluded that protein body preparations of Vicia faba are probably incapable of autonomous protein synthesis. This is in contrast with the findings of Morton and his colleagues (Morton and Raison, 1963, Morton, Palk and Raison, 1964, and Morton and Raison, 1964) for protein body preparations of developing wheat seeds. They obtained high levels of amino-acid incorporation into peptidyl material by protein bodies "in vitro" (though they did not assay for bacterial contamination) and further showed that the isolated protein body fraction contained the major components required for protein synthesis.

In the present investigation, protein bodies were extracted by a method known to preserve their integrity (Morris, G., 1968), and several RNA extractions and estimations by the modified method of Smillie and Krotkov (1960) gave negative results. It is concluded, therefore, that protein bodies contain little or no RNA.

Unfortunately, this method of protein body isolation employed gives very low yields, and sufficient material could not be obtained for a reliable analysis by MAK chromatography. Protein bodies were isolated in quantity, in the second procedure, by initially disrupting the tissues with a Waring blender. MAK column chromatography of the phenol extracted nucleic acid fraction indicated the presence of very small quantities of 28s and 18s RNA, but the complete absence of LMW RNA. These results confirm the

findings of the first method. DNA, on the other hand, was present in appreciable quantities, and amounted to some 0.04% of the dry weight of the protein body isolate.

Barker and Reiber (1967) prepared a crude chromatin fraction from blended pea seedlings, by centrifuging at a slightly greater centrifugal force to that used in the present study to pellet protein bodies. It is possible, therefore, that the DNA in the protein body fraction is due to contaminating chromatin rather than the DNA being a constituent part of protein bodies.

The accumulated evidence suggests, therefore, that it is most unlikely that storage protein of broad-bean seeds is synthesised within protein bodies. Briarty (1967) reported that there was an intensive synthesis of endoplasmic reticulum immediately prior to the onset of storage protein synthesis. A similar finding was detected by Opik (1968) in developing seeds of Phaseolus vulgaris. Since the ribosome is accepted as the major site of protein biosynthesis, the occurrence of ribosomes, both free in the cytoplasm and attached to membrane, was studied biochemically during cotyledon development.

Free and membrane bound ribosomes were isolated by the method of Tatal and Exum (1966). This initially involved the preparation of a microsomal fraction. This was partially characterised, and was shown to contain ribonucleoprotein particles. The amount of protein present varied at different stages of development, but in all determinations exceeded the RNA content as determined by the ratio of absorbance at 260 mμ to 235 mμ (Petermann, 1964).

When a microsome preparation was centrifuged on sucrose gradients, two major fractions separated and sedimented at the rates expected of free and membrane bound ribosomes. Their identities were confirmed by subsequent studies.

The results from the free and membrane bound ribosome determinations of developing cotyledons are similar to the findings of Briarty (1967) in that there is a large increase in membrane bound ribosomes immediately before, and during, storage protein synthesis (fig.30). What is more apparent, however, is that the free ribosomes persist throughout the period of nutrient reserve accumulation, at early stages increasing in number, but later remaining constant. From this finding, it would appear that free and membrane bound ribosomes may constitute two individual classes of ribonucleoprotein particles.

This hypothesis could be directly tested, since, between days 55 and 70, there is a rapid increase in the level of membrane bound ribosomes, but little or no increase in the free ribosomes. (^{3}H) - uridine, injected into developing seeds at 60 days after flowering, and incubated for five days, contained radioactivity solely in the membrane bound ribosome fraction. In the control experiment, most of the radioactivity was shown to be incorporated in the RNA moiety of the ribosomes. Seeds injected at 27 days, and harvested at day 32, contained radioactivity in both ribosome fractions since both ribosome species were being synthesised.

To check that the results of these radioactive experiments were not invalidated by the presence of bacteria, the bacterial content of developing seeds was determined. Seeds at several stages of development were shown not to contain bacteria, or at least none that could be demonstrated by the usual microbiological procedures, provided the fruit pod was perfectly healthy and not damaged.

From these experiments with (^{3}H) - uridine, therefore, it was concluded that free and membrane bound ribosomes form separate classes, and do not interchange, at least during the stage of storage protein synthesis. If interchange had taken place between days 60 and 65 after flowering, the free ribosome fraction, in addition to the membrane bound ribosome fraction, would have been radioactive.

Experiments were designed to demonstrate whether these two classes of ribosomes are both capable of synthesising protein "in vivo". These experiments were based on those designed to demonstrate protein synthesis by polysomes of (i) rabbit reticulocytes (Warner, Rich and Hall, 1962), (ii) soya bean roots (Lin, Key and Bracker, 1966), (iii) cotton seeds (Waters and Dure, 1966), and (iv) slime molds (Phillips, Rich and Sussman, 1964).

Ribosomes actively undergoing protein synthesis occur as aggregates, termed polysomes (Rich, 1963), which are connected by a molecular strand of mRNA. When tissues which are actively synthesising protein are given a pulse of a labelled protein amino-

acid, the polysome fraction, isolated by sucrose gradient centrifugation, becomes preferentially labelled. Treatment of the post-mitochondrial supernatant with 0.5 µg/ml ribonuclease will result in a loss of the polysome fraction with a subsequent increase in the numbers of single ribosomes, due to the hydrolysis of mRNA.

In the experiments reported in this investigation, a very similar method was employed to demonstrate the presence of free polysomes from 60 day developing cotyledons. To show the possible presence of membrane bound polysomes, ribonucleoprotein particles must first be detached from membranes. 0.4% sodium deoxycholate (Doc) is principally used to effect this (Blobel and Potter, 1967), but preliminary experiments demonstrated that cotyledon membranes were incompletely dissolved at this concentration; 0.5% DOC was required. Other tissues are also known to require minimal concentrations other than 0.4% DOC to dissolve their membranes (O'Neal Nicolson and Flamm, 1965).

Experiments in which the post-mitochondrial supernatant was treated with 0.5% DOC, clearly demonstrated an increase in the size of the polysome fraction, when compared with an untreated sample, indicating the presence of membrane bound polysomes in addition to free polysomes. In some experiments, 0.4% (w/v) of the detergent nonidet P40 was used in place of 0.5% DOC to dissolve membranes, but a nearly complete loss of both free and membrane bound polysomes resulted.

In all these experiments, whether detergent was used or not, the proportion of polysomes to single ribosomes was very small. Similar findings have been reported for many plant tissues, and is due, not only to the presence of endogenous nucleases released upon homogenisation, but also to minor treatments in the handling of the material. Clark, Matthews and Ralph (1964) showed that a slight wilting of Chinese cabbage leaves caused a complete and rapid loss of polysomes. Lin and Key (1967) merely removed soya bean seedlings from the growing medium and incubated them in aerated water with shaking for an hour, and this resulted in an appreciable, though temporary, loss of polysomes. In the present study, developing cotyledons were sliced into discs and these incubated with (^{3}H) - leucine; this treatment may have affected the polysome concentration. The rigorous method used, through necessity, to disrupt cotyledon cells, may also have disrupted some polysomes. Rich (1963) was one of the first to isolate polysomes using reticulocyte cells, and this was primarily due to disrupting the cells by a very gentle homogenisation technique.

In the present experiments with (^{3}H) - leucine, the incubation medium was routinely tested for the presence of bacteria at the end of the incubation period. The levels of bacteria present ($2.5 \times 10^1 - 10^2$/Ml. incubation medium) made it extremely unlikely that they could contribute significantly to the experimental results.

It is concluded, therefore, that both the free and membrane bound ribosome fractions contain polysomes and that both are capable of protein synthesis "in vivo". Now, as it has already been

proposed that free and membrane bound ribosomes constitute two discrete classes in the cell, it is further suggested that each class synthesises different groups of proteins. Since membrane bound ribosomes increase rapidly in number immediately before and during nutrient reserve accumulation, it is suggested that all the storage protein is synthesised on membrane bound ribosomes. Free ribosomes are predominant at early stages in cotyledon development (Briarty, 1967) and as they persist with slightly greater numbers during the stage of nutrient reserve accumulation, it is suggested that they are synthesising enzymes and protein for general metabolism.

Similar studies investigating the significance of free and membrane bound ribosomes in animal secretory cells, particularly those of the rat liver, are in progress in several laboratories. Work was initiated by electron microscopists who demonstrated that rapidly dividing cells contain abundant quantities of free ribosomes, but very few membrane bound ribosomes. Upon the completion of cell division, and during cell differentation, cells commence to secrete proteins and there is a dramatic increase in the numbers of membrane bound ribosomes until they eventually amount to some 90% of the total ribosome population. With the realisation that the ribosomes are the site of protein synthesis, they suggested that ribosomes bound to membranes were responsible for the synthesis of protein for secretion, whereas the ribosomes unattached to membranes (free ribosomes) were involved in the synthesis of protein for intracellular purposes (Porter, 1954, Palade, 1955, Howatson and Ham, 1955,

Slautterback and Fawcett, 1959, and Birbeck and Mercer, 1961).

Early biochemical work refuted this idea, since although membrane bound ribosomes were shown to synthesise protein both "in vivo" and "in vitro", free ribosomes were incapable of such synthesis (Henshaw, Bojarski and Hiatt, 1963, and Campbell, Cooper and Hicks, 1964). In "in vitro" cell-free synthesising systems these free ribosomes were stimulated to incorporate amino-acids upon addition of a synthetic mRNA such as poly U. Hallinan and Munro (1965) further suggested that new ribosomes first enter the free ribosome pool and are then continuously exchanged for ribosomes attached to polysomes in the membranes of the endoplasmic reticulum. This hypothesis is in contradiction to the experimental results presented in this thesis.

However, more recent comparisons between free and membrane bound ribosomes of rat liver have revealed that both classes undergo protein biosynthesis with approximately equal efficiency (Marganiello and Phillips, 1965, and Bloemendal, Bont and Benedetti, 1967). Campbell's laboratory (Campbell, Serck-Hansson and Lowe, 1965) obtained similar results, and accounted for their previous finding that free ribosomes do not synthesise protein, by having too low a concentration of Mg^{++} in their extraction medium, which caused a selective degradation of the unstable, free polysomes (Campbell et al, 1964).

Sargent and Campbell (1965) isolated the microsome fraction from rat liver and demonstrated by radioactive tracer methods,

followed by tryptic digests and autoradiography, that it synthesised serum albumin in a cell free system. A constructive sequel to this experiment would be to first isolate free and membrane bound ribosomes and then to determine which species of ribosome synthesises serum albumin "in vitro".

Hallinan, Murty and Grant (1968) claimed to have achieved "the first, clearcut demonstration of qualitative differences in the pattern of protein biosynthesis by free and membrane bound ribosomes isolated from the same tissue". (^{14}C) - D glucosamine was injected intraperitoneally into rats, and at various time intervals free and membrane bound ribosomes were isolated and highly purified. Radioactivity was associated almost completely with the membrane bound ribosome fraction, indicating that they alone are involved in the synthesis of glycoprotein apopolypeptides. The authors, unfortunately, did not test the ability of the free ribosome fraction to incorporate amino-acids "in vivo".

Upon the completion of nutrient reserve accumulation, seeds dehydrate and begin to lose their metabolic activity. Between the commencement of this process and complete dormancy, some 25% of the total RNA of cotyledons is hydrolysed. Commoner (1964) suggested that during cell division and DNA synthesis, nucleotides, which could otherwise be used as an energy source for synthetic processes, are sequestered into DNA molecules. In a similar manner the intensive rate of RNA synthesis which occurs during cotyledon development will significantly deplete the free nucleotide pools of cells. An

hydrolysis of some RNA immediately after the completion of nutrient reserve accumulation would result in larger nucleotide pools to be utilised at the onset of germination.

During the dehydration process chemical methods showed that there was a loss of membrane bound ribosomes and a concomitant increase in free ribosomes. A similar observation was noted by Briarty (1967) in his electron microscopic studies of the developing broad bean. Since metabolic activity was progressively reduced during this period, it was considered that membrane bound ribosome loss occurred by the detachment of ribosomes from the endoplasmic reticulum, rather than a degradation of membrane bound ribosomes and a "de novo" synthesis of free ribosomes. O'Neal, Nicolson and Flamm (1965), working with cultured tobacco cells, produced convincing evidence that in ageing cells there was a progressive detachment of ribosomes from membranes and a resultant increase in the free ribosome pool. In contrast to developing cotyledons, therefore, dehydrating cotyledons show a uni-directional interchange of free and membrane bound ribosomes.

The MAK column elution profile of dormant seed nucleic acids (fig.33) is unique in that it contains a very poorly defined 18s RNA fraction. Wood and Bradbeer (1967) noticed a similar situation with dormant hazel seeds and suggested the rRNA may be wholly or partly non-functioning. This is very unlikely to be the case in the Papilionaceae as Marcus and Feeley (1964) for peanut, and Barker and Rieber (1967) for pea, showed that ribosomes of dormant

seeds were apparently functional, since incorporation of phenylalanine was stimulated by poly U in a cell-free system.

The sole requirement of mature seeds of Vicia faba to germinate is water. Seeds swell during the first two days and the radicle emerges from the seed on the third day. Almost immediately, the nutrient reserves in the cotyledon begin to break down and their sap-soluble degradation products are translocated to the growing embryo.

The cytology of cotyledon senescence in the Papilionaceae has been studied in several laboratories. Briarty (1967), working with Vicia faba, showed that breakdown of protein bodies first occurs in cells close to the endodermis and the vascular bundles. At later stages storage protein hydrolysis is initiated in cells which are progressively distant from these tissues, so that eventually a complete spectrum of cells at different stages of protein mobilisation is reached. Similar findings were reported by Smith and Flinn (1966) for Pisum sativum. Biochemical studies dealing with whole cotyledons will, therefore, reflect the average state of all the individual cells.

During the first few days of germination, the RNA content of cotyledons of Vicia faba decreased slightly, but at later stages it decreased rapidly. Similar findings have been reported for other seeds whose germination is hypogeal, e.g. Pisum sativum (Barker and Hollinshead, 1964, and Beevers and Gernsey, 1966), Vigna sesquipedalis (Oota, Fujii and Osawa, 1953), corn (Cherry and Hageman, 1961) and the tomato (Hall and Cocking, 1966).

When the RNA content is expressed per g. dry weight of cotyledon material, there is initially an increase in the RNA to the twenty-second day of germination, followed by a rapid decrease. Evidently, between days 1 and 22, food reserves are being broken down and transported to the embryo at a faster rate than the hydrolysis of RNA, possibly indicating that RNA still plays an active role in cellular activity.

When nucleic extracts from senescing cotyledons were analysed by MAK column chromatography, no additional fractions were detected to those found in mature cotyledons, and those fractions present occurred as sharp peaks on the elution profiles. That fraction which immediately elutes from the column upon the introduction of the sample increased significantly as senescence progressed. Altmann, Dole and Fetter (1967) considered that this fraction contained low molecular weight nucleic acid precursors so that, by analogy, the corresponding fraction in senescing cotyledons probably contains small polynucleotide fragments.

There is every indication, therefore, that intermediates between molecules of the major RNA species and small polynucleotide chains are only present in low concentrations, suggesting that RNA molecules, when once exposed to digestion by ribonuclease, are rapidly broken down. Similar conclusions may be reached with senescing testas during seed development.

It is interesting that as senescence progressed in the cotyledon and the testa, the composition of the RNAs remained

approximately constant. There was, however, a slight, though significant, increase in the proportion of LMW RNA, implying that rRNA was being broken down at a slightly faster rate than LMW RNA. Similar observations were recorded by Wood and Bradbeer (1967) for senescing hazel cotyledons, and Cherry (1967) for senescing pea-nut cotyledons.

As might be expected by its genetic properties, DNA was very resistant to break down in either of the two senescing tissues. By the thirty-seventh day of germination, when the last MAK analysis was performed, only a fraction of the DNA had hydrolysed. Cotyledons at this age became very prone to attack by micro-organisms, despite the fact that seeds were surface sterilised and germinated in sterile vermiculite. There is no doubt that if grown in the field the DNA of the cotyledons would be used as a supply of nucleotides for invading organisms rather than for the growing axis of the plant.

During the first few days of germination the levels of several enzymes in the cotyledon of seeds increase considerably, e.g. ribonuclease (Barker and Douglas, 1960), and phosphatase and α-amylase (Varner, Balce and Huang, 1963). Using widely different techniques, Cherry (1967), and Filner and Varner (1966) showed that the respective enzymes isocitritase from pea-nut, and α-amylase from barley, are synthesised "de novo" in the cotyledons during germination and not during the later stages of seed development. Morris, J. (1968) on the other hand provided evidence that in Pisum sativum there is no gross synthesis of protease on germination, and

suggested that hydration caused the activation of a latent enzyme which was laid down during the maturation of the cells.

In the present study, the increase in the numbers of membrane bound ribosomes at the commencement of germination suggests that protein synthesis is occurring in cotyledons. Similar increases were found for the broad bean by Briarty (1967), for the pea by Chapman and Rieber (1967), and for the soya bean by Teffrey, Klein and Abrahamsen (1967). These membrane bound ribosomes must have arisen either by a "de novo" synthesis or by some pre-existing free ribosomes becoming attached to the newly formed endoplasmic reticulum. For the former suggestion to be correct, rRNA synthesis must be occurring in the cotyledons of germinating seeds with a simultaneous degradation of RNA, as the level of total RNA is constantly decreasing.

In an attempt to assess the validity of the two hypotheses, seeds were germinated in the presence of (^{32}P) - orthophosphate. A high specific activity of the membrane bound rRNA compared to that of the free rRNA would indicate a "de novo" synthesis of membrane bound ribosomes, but a low specific activity of both components would imply an attachment of pre-existing free ribosomes to the endoplasmic reticulum. Initial experiments yielded a remarkable result; the specific activity of the RNA of free ribosomes was 2 - 14 times greater than the specific activity of the membrane bound RNA.

The effect of contaminating bacteria in experiments involving the incorporation of (^{32}P) - orthophosphate into the

nucleic acids of seedlings has been extensively studied by Lonberg-Holm (1967). He concluded that bacterial nucleic acids are labelled in preference to those of plant cells and that as little as one part in 10^7 by weight of contaminating bacteria can alter the labelling characteristics. Similar findings have also been reported by Barber (1966).

As the bacteria content of the growth containers in which seeds in the above experiments were germinated was approximately 1×10^7/ml growth medium, the unexpected results obtained may have arisen by the presence of bacteria. This high concentration of bacteria was present despite stringent attempts to surface sterilise the seeds prior to germination. Unfortunately, any bacteria not killed at this stage would be surrounded by a medium fully able to support their growth and division (^{32}P-orthophosphate and exudates from germinating seedlings) for a period of four days.

In the second series of experiments, chloramphenicol was added to the growth chamber at a concentration known to hinder bacterial replication but not to affect higher plants (Weisberger and Wolfe, 1964). In these experiments, the bacteria content was reduced to ca. 5×10^3/ml growth medium and resulted in a much lower specific activity of free rRNA but virtually unaffected the specific activity of the membrane bound rRNA, table 15. It is concluded that contaminating bacteria cause a preferential labelling of their ribosomes which, during extraction processes, become segregated into the free ribosome fraction. Membrane bound ribosomes

of the germinating cotyledon have, therefore, probably arisen by a "de novo" synthesis, indicating again the individuality of the two classes of ribosomes.

Further experiments to determine whether either or both species of ribosomes synthesise protein during seedling germination were not undertaken because of possible difficulties in the interpretation of results due to contaminating bacteria.

In contrast to germinating seeds, seeds undergoing development were shown not to contain bacteria provided they were enclosed by a healthy, undamaged pod. Personal observation indicates that microbes become associated with seeds during the drying out process in the field. Pods at this stage dry and blacken, and their inner surfaces often become infested with bacteria and fungi. A possible method to reduce the bacterial flora of dormant seeds is to detach maturing seeds from pods which are still green and healthy, and to transfer them to a warm, ventilated room.

Evidence has been presented in this thesis that free and membrane bound ribosomes constitute two separate classes of particles which are involved in the synthesis of different groups of proteins. Evidence has also accumulated in the literature suggesting that a similar division of labour occurs amongst ribosomes in the cells of rat liver.

If these theories are accepted, then mechanisms hitherto undiscovered must be in operation to transfer a newly synthesised mRNA from the nucleus to the eytoplasm and to form polysomes located

either free in the cytoplasm or attached to the membranes of the endoplasmic reticulum, according to the protein for which it codes.

It is hoped that the experiments described in this thesis may serve as a basis for the eventual proof that membrane bound ribosomes alone synthesise storage protein in the cotyledons of the developing seeds of Vicia faba. The membrane bound ribosome fraction extracted from such tissues is capable of synthesising protein in a cell free protein synthesising system (Yarwood, 1968). Partial hydrolysates of the newly synthesised proteins should produce peptide maps (prepared by two dimensional paper chromatography/high voltage electrophoresis) which are similar to those of authentic storage protein preparations, whereas peptide maps of partial digests of proteins, synthesised by the free ribosome fraction in a cell free system, should not.

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