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CHANGES IN POLYRIBOSOMES AND POLYADENYLATED
MESSENGER RNA IN AGEING SOYBEAN COTYLEDONS

by



Louise Elizabeth Barakett

A Thesis

submitted to the Faculty of Graduate Studies
through the Department of
Biology in Partial Fulfillment
of the requirements for the Degree
of Master of Science at
The University of Windsor

Windsor, Ontario, Canada

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To my uncle

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LIST OF ABBREVIATIONS

A	adenylic acid
ATP	adenosine-5'-triphosphate
BME	beta mercaptoethanol
CrP	creatine phosphokinase
DEAE-cellulose	(diethylaminoethyl)-cellulose
DMSO	dimethyl sulfoxide
(dT)	deoxythymidylic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(B-amino-ethyl ether) N,N'-tetraacetic acid
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
MgAc ₂	magnesium acetate
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein particle
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	hydroxymethylamino-methane
(U)	uridylic acid

ABSTRACT

Polysomes were isolated from the dark grown soybean cotyledons of different ages (1,3,5,7,10,15 and 20 days old and dry seeds) and their size distributions examined on sucrose density gradients. In younger cotyledons a high proportion of heavy polysomes was observed, while older cotyledons had a preponderance of monosomes and light polysomes. Further, two or possibly three bursts of polysomal activity was obtained between 0-20 days. The pattern of RNA distribution from polysome pellets of different ages, after treating the pellet with SDS and EDTA on sucrose gradients do not show a correspondence between polysomal activity and mRNA peak, which is questionable.

Extraction of the polysomal pellets with phenol and chromatography of the resultant polysomal RNA on oligo (dT) cellulose columns, demonstrated the presence of Poly A (+) or A (-) RNA, based on ^3H -Poly U hybridization techniques. The two oligo (dT) fractions (Poly A (+) and Poly A (-)) show template activity in a wheat germ cell-free assay system.

Ribosomes and supernatant fractions from soybean cotyledons of different ages were prepared to study the Poly U-directed phenylalanine incorporation. Ribosomes from younger cotyledons were more effective in phenylalanine incorporation compared to ribosomes from older cotyledons. Similarly, the supernatant fractions from younger cotyledons were more efficient, resulting in enhanced incorporation, than older cotyledons.

INTRODUCTION

Several reports, Kahl (1971), Leaver & Key(1967), Wasilewska & Cherry (1974) exist which indicate that excised tissues of storage organs, other plant parts, and leaf discs, when incubated in aerated water result in a rapid activation of mature, dormant tissue. Such activation includes increases in metabolic activity of washed or ageing discs of artichoke (Sparkuhl 1976), carrots (Erdman & Hall 1965), sugar beet (Wasilewska & Cherry 1974), including their ability to incorporate labelled amino acids in ribosomal preparation from such excised tissues. Further, a rapid transition of the proportion of ribosomes to polysome with ageing has been demonstrated. Proof of such polysomal activities was also obtained by cytological analysis (Fowke & Setterfield 1968) of ageing artichoke slices. Although the above studies clearly demonstrated the transition of the inactive-dormant system to a metabolically active system, this does not necessarily reflect changes associated with ageing. In a previous study (Pillay 1977), the germinating cotyledon was employed as a model for studying age related changes in ribosomal activities and protein synthesizing ability of the soybean cotyledons. It was shown that the decline in the capacity of the ribosomes and supernatant factors to incorporate labelled amino acid in vitro was due to a loss in translational capacity. Since it has been shown that polyadenylated mRNA and their associated polysomes have been widely studied in association with the synthesis of specific protein in plant cells (Payne 1977 , Verma & MacLachlan 1976) , and because of con-

tinued interest in the protein synthesizing ability of ageing soybean cotyledons, it was of interest to investigate the changes in poly-somes and their associated mRNA to determine the possible relationship of protein synthesis and the ageing process in the soybean cotyledons.

REVIEW OF LITERATURE

Ageing and Protein Synthesis

Several theories which attempt to explain the basic causes of ageing propose that one of the steps in the process of protein synthesis is impaired. One possible cause of senescence may be an accumulation of inactive or partially inactive ribosomes.

Wallach & Gershon (1979), studying ageing nematodes, found that the total amount of ribosomes remains constant with increase in age. However the percentage of ribosomes in polysomes per total ribosomes decreased from 63% in 5 day old animals to 35% in 53 day old animals. Furthermore, through mixing experiments, it was determined that the decrease in amount of polysomes and the accumulation of 60 S particles are not caused by increased ribonuclease activity in the older nematodes.

Perhaps a certain process results in an age dependent accumulation of altered monomers while the amount of active normal monomers decreases. These altered monomers may be modified in their proteins, RNA or both and such modifications may disturb the functioning of a specific active site on the ribosome.

Biswas (1969) studied the ribosomes in the cotyledons of ageing mung beans. As the cotyledons age, their ribosomes were more readily dissociated. It is believed that protein components are associated with the 80 S ribosome to prevent its dissociation in younger tissues. Two protein bands were detected electrophoretically which were not

found in root ribosomes. In cotyledons there was a decrease in the quantity of these proteins as the tissue aged.

While studying rabbit globin mRNA Nokin and co-workers (1975) found that old mRNA molecules differ from relatively young messages in their ability to direct cell-free globin synthesis. Modifications reducing template activity in vitro seem to take place during mRNA ageing. The decreasing ability of a message to direct protein synthesis occurs at the same time as the poly (A) tract becomes shorter but these two events appear unrelated. In peanut cotyledons Cherry (1967) found that as peanut seeds germinate and deplete their storage materials, there is an increase in enzyme and mitochondrial activity to about 8 days followed by a reduction in activity. The activity of the enzyme isocitritase is one which increases following germination. This increase in isocitritase activity in oil seeds can be related to the disappearance of lipid and the production of carbohydrate. RNA content increased threefold by the 8th day then declined rapidly. After 9 days of germination ribonuclease activity increased several fold.

Mariotti and Ruscitto (1977) employed double labelling (^3H -leucine- ^{14}C -phenylalanine) techniques to determine alterations in translation in a cell free poly-U directed protein synthesis system. As the age of the rat liver cells increased the amount of ^3H -leucine incorporated by the system increased. While many of the changes in accuracy were attributed to changes in the tRNA portion of the system, an increase in the ^3H -leucine binding ability is also shown when a standard system is added to the ribosomes from differently aged animals. Though this increase is less than that described for tRNA, it may be explained

on the basis of the ribosomes function in the complex formation. Aged ribosomes contribute as well to the observed increase in errors.

Polysomes

The pathway of mRNA transport to the cytoplasm and control of the formation of polysomes on specific messengers is not well understood. It is believed that the messenger may be transported in association with specific proteins as a ribonucleoprotein particle which may enter the polysomes directly (Schochetman & Perry (1972), Shore & Tata (1977), Barrieux et al., (1976)). It is suggested that proteins associated with heterogeneous nuclear RNA are exchanged before or during release of the mRNA from the nucleus; remaining mRNA-associated proteins are added in the cytoplasm, followed by removal of most of these proteins during polysome formation (Roy et al., (1979)). Involvement of the 3'-terminal poly-A is also possible.

Jain & Sarkar (1979) isolated poly A containing cytoplasmic messenger ribonucleoprotein particles from embryonic chick muscles. A protein of about 78,000 M.W. is associated with the Poly A portion of mRNP of both polysomal and non-polysomal derivatives suggesting that the association of this protein to the poly (A) tracts is not influenced by translation. However, specific polypeptides associated with the non Poly (A) portions of the free (non-polysomal) mRNP may act as a stabilization mechanism against cleavage by specific nucleases when the mRNA is not protected by binding to ribosomes.

Membrane Associated Polysomes

It has been demonstrated that specific classes of proteins synthesized on either free or membrane bound polysomes may be distinct from one another (Bulova & Burka 1970, Brennessel & Goldstein 1975, Ganoza & William 1969, Sparkuhl et al., 1976). It is proposed that proteins destined for export from the cell may be translated preferentially on membrane bound messengers. (Uenoyama & Ono 1972). Larkins et al., (1975, 1976) have demonstrated the preferential synthesis of zein, a storage protein of maize on membrane bound polysomes. Using the same procedure, Beachy, Thompson and Madison (1978), found that polysomes isolated from the developing cotyledons of soybean were capable of synthesizing soybean storage proteins.

Payne, et al., (1969, 1971) found that in the development of the seeds of Vicia faba, a broad peak of membrane bound material is present only in the 50 to 60 day old preparations. This corresponds to a period of intense synthetic activity in the developing seed, when the storage proteins and starch accumulate (approximately 40-70 days). The increased ribosome activity at 60 days was possibly attributed to the presence of protein factors which are associated with the ribosomes at this stage in seed development making them more active. During seed dehydration there was a loss in membrane bound ribosomes with a concomitant increase in free ribosomes.

Loeb & Yeung (1978) found that in regenerating rat liver, the substantial increase in the concentration of free ribosomes is con-

sistent with an enhanced need for the synthesis of protein destined for intracellular use.

Poly Adenylated mRNA

A majority of mRNA molecules and some heteronuclear RNA (hnRNA) species contain a discrete segment of adenylic acid residues (Darnell et al., 1973, Lee et al., 1971) attached at their 3'-OH end. The poly-A tail from 50 to 250 nucleotides in length has been found to be associated with mRNA's of animals, (Lizardi et al., 1975, Galau et al., 1974, Collier 1975), plants (Tobin & Klein 1975, Filiminov et al., 1977) and yeast (McLaughlin et al., 1973, Reed 1973, Hereford and Rosbach 1977).

The particular exception of histone mRNA of higher organisms apparently lacks poly A as do other select classes of mRNA (Sonenshein et al., 1976).

Nemer and co-workers (1974) demonstrated that non-histone mRNAs lacking and containing poly (A) have similar base compositions and sizes but they differ widely in their nucleotide sequences. However, Gray and Cashmore (1976) when studying the mRNA of plant leaf tissue, confirmed that both poly A (+) and poly A (-) messenger RNA code for the same major polypeptides. The possibility that the poly A (-) mRNA in this case, arose from digestion of the poly A tail was considered. While de-adenylated messenger has been found to retain its translational capacity (Bard et al., 1974) and the poly A does not contribute to secondary structure required for protein synthesis (Munoz & Darnell

1974) the question of functional stability of the message arose. Nudel and co-workers (1976) experimentally demonstrated that globin mRNA retained functional stability when the poly (A) segment contained 32 or more adenylate residues. If the mRNA contained 21 adenylate residues the functional stability decreased to 30% of the native mRNA. Huez and Marbaix et al., (1974, 1975, 1977) found that the 3' poly (A) segment of globin mRNA ensures the stability of the message. When injected into *Xenopus* oocytes, deadenylated globin mRNA was translated only for a short period of time and then rapidly degraded. Native poly (A) containing mRNA is considerably more stable and is translated for extended periods of time. Moreover, readdition of the poly (A) segment to the previously deadenylated message restores its functional stability. Poly (A) itself or the proteins which are known to bind to the poly A segment of mRNA as part of the messenger ribonucleoprotein (Mendecki et al., 1972, Bergmann and Brawerman, 1977) may have a role in protecting mRNA against degradation. The results suggest that the Poly (A) sequence is normally protected from nucleases by virtue of association with protein. The slow reduction in Poly (A) size can be accounted for by a factor capable of interfering with the Poly (A) protein interaction. This is also dependent on the structural integrity of the polysomes or messenger ribonucleoproteins. It is suggested that a polynucleotide segment adjacent to the Poly (A) can modulate the affinity of the protein for the Poly A sequence controlling Poly (A) stability.

Key and Silflow (1975) have shown that in soybean hypocotyl

tissue, at least a part of the mRNA (greater than 50% in polysomes) contain a Poly A sequence. This is in keeping with the finding in sea urchin embryos (Nemer, 1975) where 50% of the mRNA was found to be polyadenylated.

Mori et al., (1968) found that non messenger RNA fractions isolated from soybean cotyledons had template activities. Both ribosomal RNA and a tRNA-like RNA fraction demonstrate a high template activity when introduced into an amino acid incorporating system. The roles of this template-like RNA are still not certain.

MATERIALS AND METHODS

Plant Material

Seeds of Glycine max. (var. Harosoy 63) were soaked in distilled water for three hours. The seeds were then planted in vermiculite and grown in darkness at room temperature. Cotyledons were removed from the seedlings at various ages (1,3,5,7,10,15 and 20 days old). Harvested cotyledons were placed directly into liquid nitrogen.

Isolation of Total Polysomes

Polysomes were isolated using combined methods of Verma and MacLachlan (1976) and Jackson and Larkins (1976). Ten grams of cotyledons were frozen in liquid nitrogen and then ground for 10 seconds in an electric grinder to a fine powder. This powder was homogenized gently in 25 ml of polysome extraction buffer (150 mM Tris Ac, pH 8.5, 20 mM KCl, 5 mM MgAc₂, 200 mM sucrose, 7 mM B-mercaptoethanol, 0.5% sodium desoxycholate, 25 mM EGTA, 1 mM DTT) in a mortar and pestle which was kept on ice. The slurry was centrifuged for 10 minutes at 3,000 xg. The supernatant was filtered through Miracloth and centrifuged at 20,000 xg for 10 minutes. The supernatant was decanted and again filtered through Miracloth to remove the lipid layer, carefully layered over 3 ml of sucrose cushion (50 mM Tris Ac, pH 8.5, 1.5 M sucrose, 20 mM KCl, 5 mM MgAc₂, 7 mM B-mercaptoethanol, 1 mM DTT) in a polycarbonate centrifuge tube and then centrifuged for 90 minutes at 105,000 xg using a Beckman 60 Ti rotor. The supernatant was discarded and the tube was cleaned carefully by rinsing with resuspension buffer and

drying by wiping with a tissue. The remaining glassy pellet was resuspended in 0.4 ml of resuspension buffer (50 mM Tris-Ac, pH 8.5, 25 mM KCl, 10 mM MgAc₂, 7 mM B-mercaptoethanol) using a 1 ml disposable syringe and a fine needle, making sure that all the pellet was removed from the side of the tube. An aliquot of 10 O.D.₂₅₄ units was layered onto a 10-45% sucrose gradient and centrifuged for 90 minutes at 150,000 xg in an SW 41 swinging bucket rotor. The gradient was fractionated using an ISCO density gradient fractionator and ultraviolet analyzer with a 55% sucrose chaser. The RNA concentration was determined by measuring the absorbance at 254 nm.

Extraction of Membrane Bound Polysomes

The method of extraction of membrane bound polysomes was a modification of the procedure used for the total polysome isolation. The ground cotyledons were homogenized in extraction buffer which contained no EGTA or sodium desoxycholate and the slurry was centrifuged for 10 minutes at 3,000 xg. The supernatant was filtered through Miracloth and centrifuged at 20,000 xg for an additional 10 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of extraction buffer containing 1% Triton X-100. The sample was incubated for 15 minutes, stirring constantly, then centrifuged at 20,000 xg for 5 minutes to remove the debris. The supernatant was then carefully removed and layered upon 3 ml of sucrose cushion. Subsequent steps in the procedure were those used in the isolation of total polysomes.

Fractionation of RNA from the Polysomal Pellet

RNA was fractionated according to the procedure of Wasilewska and

Cherry (1974). To the polysomal pellet, 0.3 ml of resuspension buffer (50 mM Tris-Ac, pH 8.5, 25 mM KCl, 10 mM MgAc₂, 7 mM B-mercaptoethanol) was added and the pellet resuspended using a 1 ml disposable syringe and a fine needle. An equal volume of RNA release buffer (25 mM Tris-Ac, pH 8.0, 200 mM NaCl, 5 mM EDTA, 1% SDS) was added and the suspension was vigorously shaken for 10 minutes. An aliquot of 10 O.D.₂₅₄ units was layered onto a 5-25% sucrose gradient and then centrifuged at 80,000 xg for 16 hours using a Beckman SW 41 rotor. The gradients were fractionated as previously described.

Extraction of RNA from Polysomal Pellet

RNA was extracted for further separation using oligo (dT) chromatography using the procedure described by Brawerman (1974). The polysome pellet was diluted to less than 100 O.D. per ml using resuspension buffer. To the sample, 0.1 volume of 1 M Tris-HCl, pH 9.0 and 5% SDS was added. An equal volume of 80% (v/v) aqueous phenol was also added and then incubated at 0-5°C for 5 minutes stirring constantly. The sample was centrifuged for 10 minutes at 12,000 xg. The aqueous layer was carefully removed and stored on ice. The phenol phase plus interphase was re-extracted with an equal volume of 0.5% SDS-0.1 M Tris-HCl, pH 9.0, stirring vigorously for 5 minutes and then centrifuged at 12,000 xg for 10 minutes. The aqueous phase was carefully removed and pooled with the previous sample. The total aqueous sample was re-extracted using an equal volume of 80% phenol, incubation and centrifugation as before. The aqueous layer was removed and re-extracted twice with equal volumes of fresh aqueous phenol.

To the final aqueous phase, 0.1 volume of 1 M NaCl and 2.5 volumes of ethanol were added, then stored overnight at 4°C. The sample was centrifuged at 12,000 xg for 10 minutes and then washed with cold 66% ethanol in 0.1 M NaCl. The pellet was dissolved in distilled water, the O.D. determined and then stored in the freezer at -20°C.

Oligo (dT) Cellulose Chromatography

Oligo (dT) cellulose chromatography was carried out according to the method described by Gordon and Payne (1976). Approximately 0.25 g of oligo (dT) cellulose (Collaborative Research, Mass. U.S.A.) was prepared according to instructions provided by the supplier. The oligo (dT) cellulose column (1 x 5 cm) was first equilibrated using the high salt buffer (0.01 M Tris HCl, pH 7.5, 0.5 M KCl). One hundred and sixty (160) A₂₆₀ units of RNA extracted from the polysomes were dissolved in 5 ml of 0.5 M KCl - Tris buffer and applied to the column. Non-bound material was eluted by continued washing with the same buffer, collecting 1 ml fractions. The RNA concentration for each fraction was determined by measuring the absorbance at 254 nm. Those tubes showing high RNA concentration were pooled and referred to as peak 1. Washing continued with the same buffer until a constant baseline was achieved. Material retained on the column was eluted using buffers of decreasing ionic strength. Peak 2 fractions were eluted using 0.1 M KCl - Tris buffer (0.01 M Tris-HCl, pH 7.5, 0.1 M KCl) and the poly (A) rich RNA of peak 3 was eluted with Tris buffer (0.01 M Tris-HCl, pH 7.5). Again the absorbance of each fraction was determined and washing continued

until a constant baseline was re-established. The three fractions were stored in two volumes of ethanol at -20°C . Following each run, the column was regenerated by elution with 0.1 M KOH and then equilibrated with the starting buffer.

Hybridization with ^3H - Poly (U)

From each fraction, aliquots of 0.1 A_{254} units were added to 50 μl of a poly U salts mixture (0.1 M Tris-Ac, pH 7.6, 2 M NaCl, 0.05 M MgCl_2 , 1 $\mu\text{Ci/ml}$ ^3H -poly (U)). The volume of the reaction mixture was brought to 0.5 ml with water and then incubated at room temperature for 15 minutes. Following incubation, 20 μl of RNase (0.3 mg/ml pancreatic RNase A (Sigma), 20 $\mu\text{g/ml}$ RNase T_1 (Boehringer) was added and incubation was continued for a further 30 minutes. The reaction was terminated by adding 1 ml of cold 15% TCA with 100 μg of yeast RNA as carrier. The precipitate was collected on Whatman GF/A filters, washed with cold 5% TCA and dried. The radioactivity was measured in a Beckman scintillation counter.

Wheat Germ Cell-Free Protein Synthesis (Micro Scale)

Preparation of Sephadex Column

At least 24 hours before packing, 19 g of dry Sephadex (G-25 fine) were soaked in a beaker with 200 ml of column buffer (20 mM HEPES, 100 mM KCl, 3 mM MgAc_2 , 6 mM BME) and left covered in the coldroom. The column used was 34 cm high with a 75 ml capacity. The Sephadex was packed to a height of 20 cm with 34 cm remaining as headroom. Two column volumes of buffer were passed, at an unrestricted flow rate and the buffer was

allowed to empty to the level of the Sephadex.

Preparation of the S₃₀ Fraction

Approximately 3 g of washed baked long form pasteur pipette tips were broken into chips using mortar and pestle. The wheat germ (3 g, Old Stone Mill, Niblack Foods Inc., Rochester, N.Y.) was added and then ground vigorously until powdered. About 14 ml of extraction buffer (20 mM HEPES, 100 mM KCl, 1 mM MgAc₂, 2 mM CaCl₂ and 6 mM BME) was added in two instalments, the first of about 10 ml was added and mixed with the pestle until an even consistency was obtained, then rapidly stirring in the remaining 4 ml. The homogenate was centrifuged at 30,000 xg for 12 minutes. The tubes were transferred carefully to an ice bucket to avoid disturbing the pellet or floating fatty layer.

Later the tubes were transferred to the cold room and 6 ml of supernatant was removed using a pasteur pipette, avoiding both the floating scum and the pellet. The 'S₃₀' fraction was pre-incubated by the addition of 60 µl of 250 mM MgAc₂, 60 µl of "DGAC x 100 stock" (200 mM DTT, 2 mM GTP, 100 mM ATP, 800 mM CrP) and 60 µl of creatine phosphokinase (4 mg/ml). The S₃₀ fraction was stirred thoroughly with a glass rod as each item was added and then placed in a 30°C water bath for 15 minutes, stirring occasionally for the first few minutes. Following pre-incubation the sample was placed on ice and returned to the coldroom. When the S₃₀ fraction had been chilled, (2-3 minutes) it was added dropwise to the prepared column slightly above the Sephadex

surface using a long pasteur pipette. The bottom of the column was released at this stage. The sample was loaded and the meniscus allowed to reach the level of the Sephadex. With another pipette, 5ml of column buffer was added to the column and allowed to enter the Sephadex and then the head of the column was filled up. The S_{30} fraction was collected 30 minutes later in 15-20 test tubes each with about 15 drops per tube. The fast moving beige or straw coloured region was separated from the slow moving yellow fraction and saved. The tubes with the most concentrated fractions were pooled over ice until a volume of 4.5 ml was obtained. The sample was dispensed dropwise into tubes holding liquid nitrogen and then stored in liquid nitrogen until ready for use.

Cell-Free Protein Synthesis Assay

Cell-free protein synthesis assay was carried out in a 10 μ l reaction mixture, which included 2 μ l RNA, 5 μ l wheat germ S_{30} fraction and 3 μ l of a solution mixture containing essential ions and organic compounds including the labelled amino acids (0.9 M KCl, 24 mM $MgAc_2$, 640 μ M spermine, 40 mM DTT, 400 μ M GTP, 20 mM ATP, 160 mM CrP, 1 mM each amino acid except leucine and 3H -leucine. Polystyrene tubes cut to a length of 20 mm were placed in a test tube rack on ice. With Oxford automatic ultramicropipettes each assay component was added (S_{30} fraction was added last. The droplets in each tube were mixed using micropipette tips as disposable stirring rods. Each tube was covered with a small

piece of parafilm and suspended in a water bath at 30°C for one hour. After incubation the tubes were cooled on ice for at least five minutes. Aliquots of 1 to 5 μ l were pipetted onto small pieces of Whatman filter paper. The filters were washed in a beaker of ice cold TCA (10%) containing 15 g/l of unlabelled amino acid (200 ml solution / 40 filters) for 30 minutes. They were then rinsed in 5% TCA (+ cold amino acids) at room temperature for 15 minutes, decanted and then added to boiling 5% TCA and boiled for 10 minutes. The filters were decanted and rinsed twice with cold 5% TCA, dried and then placed in scintillation vials for counting.

Extraction of Monoribosomes for Amino Acid Incorporation

Approximately 20 g of cotyledons were frozen in liquid nitrogen and then ground to a fine powder. This was homogenized in 50 ml of monosome extraction buffer (100 mM Tris-Ac, pH 8.0, 500 mM KCl, 25 mM MgAc₂, 5 mM BME, 200 mM sucrose, 0.5% sodium desoxycholate) filtered through 4 layers of cheesecloth and then centrifuged at 20,000 xg for 20 minutes. The supernatant was filtered through Miracloth, layered onto 3 mls of sucrose cushion (100 mM Tris-Ac, pH 8.0, 50 mM KCl, 25 mM MgAc, 1.5 M sucrose, 5 mM BME) and centrifuged at 105,000 xg for 90 minutes. The supernatant was carefully removed and dialyzed overnight. The pellet was resuspended in 0.4 ml of 0.5 M KCl, 20 mM MgAc₂, 200 mM sucrose, 5 mM BME), using a disposable syringe. The volume of the resuspended pellet was made up to 5 ml with the same buffer and layered onto 3 ml of sucrose cushion. Following centrifugation as before, the pellet was resuspended in 0.4 ml of buffer (50 mM Tris-Ac, pH 8.0,

25 mM KCl, 10 mM MgAc₂, 25% glycerol, 0.06 M DTT), and then centrifuged at 23,000 xg for 10 minutes to clarify the suspension. The clear ribosomal suspension was then measured for absorbance at 254 nm, concentration adjusted to 1.5 mg/ml and then stored in small aliquots at -10°C.

Purification of Protein Synthesizing Factors

This procedure is a modification of the method of Marcus, Seal and Weeks (1974). Following dialysis, 7 ml of the supernatant obtained from the monosome isolation were passed through a 0.7 x 5 cm column equilibrated with Buffer A (1 mM Tris-Ac, pH 7.0, 2 mM MgAc₂, 4 mM BME). The column was eluted with the same buffer, 2 ml void volume was collected and discarded. An additional 7 ml was collected (crude fraction C) and stored on ice. The column was washed with 5 ml of buffer, and then 1 ml of Buffer A + 0.3 M KCl was discarded. The next 3.8 ml were collected (crude fraction D) and also stored on ice. Crude fraction C was dialyzed against Buffer B (1 mM Tris-Ac, pH 7.6, 1 mM MgAc₂, 4 mM BME, 0.1 mM EDTA) for 45 minutes; crude fraction D was dialyzed against buffer C (1 mM Tris-Ac, pH 7.6, 150 mM KCl, 4 mM BME, 1 mM MgAc₂), for 60 minutes. Sample C was passed through a 9 x 0.9 cm DE-23 column equilibrated with Buffer B. The column was eluted with Buffer B + 0.3 M KCl. The first 10 ml wash volume was collected and retained for elongation factor T₁. The buffer was changed to Buffer B + 0.15 M KCl, and the 6 ml following a 2 ml void volume was collected as Factor C, which was divided into aliquots and frozen. Crude sample D was passed through a 5 x 0.7 cm DE-23 column equilibrated with Buffer C and then 15 ml

collected and retained for elongation factor T_2 . An additional 4 ml were collected and discarded. Factor D was eluted in 3 ml from the column with Buffer A + 0.3 M KCl, divided into aliquots and frozen. The samples collected as elongation factors T_1 and T_2 were combined and dialyzed overnight against dialysis buffer (1 mM Tris-Ac, pH 7.3, 50 mM KCl, 4 mM BME) then frozen in small aliquots.

Poly U-Directed Phenylalanine Incorporation

The amino acid incorporation was carried out using a 125 μ l reaction mixture incubated at 37°C. The mixture contained 25 μ l ribosomes, 50 μ l reaction cocktail (75 mM Tris-HCl, pH 7.6, 2.5 mM ATP, 75 mM KCl, 125 mg/ml creatine phosphate, 150 mg/ml creatine phosphokinase, 25 mM $MgAc_2$, 0.05 M GTP, 8 mM DTT, 5 μ g/ml each of 19 amino acids, 250 μ g/ml tRNA, 10 μ g/ml 3H -phe, 200 μ g/ml Poly U, and 50 μ l recombined factors (20 μ l Factor C, 20 μ l Factor D, 10 μ l Factors T_1 and T_2). Following incubation for one hour the reaction was stopped by the addition of 1-2 ml 10% TCA and the tubes were placed on ice. The samples were filtered on glass filters and the radioactivity was determined using a Beckman Scintillation counter.

RESULTS

Polyribosome Isolation

Isolation of polyribosomes from germinating soybean cotyledons was carried out using various buffers at a pH of 8.5 as previously established (Jackson & Larkins, (1976)). The combination of high ionic strength and high pH have been found to reduce polysome degradation in some germinating seeds. From the results presented in Figure 1A, initial recovery of polysomes from the cotyledons of Glycine max. was poor in the absence of the chelator EGTA and the detergent (sodium desoxycholate). The independent additions of EGTA and sodium desoxycholate were tested (Figures 1B & C), as well as the addition of both substances to the extraction buffer (Figure 1D). Results indicate that the presence of EGTA in the extraction buffer was critical to the recovery of polysomes from the soybean cotyledons, this recovery was further enhanced in the presence of the detergent. This addition was not essential for the extraction of the polyribosomes from other tissues of the germinating seedling. Polysomes were successfully isolated from 10 g of soybean hypocotyl in the absence of both EGTA and sodium desoxycholate (Figure 1E). Attempts were made to isolate membrane bound ribosomes from the soybean cotyledons at various ages of germination. Extraction was carried out using extraction buffer that was free of 1) desoxycholate or 2) desoxycholate and EGTA. In both cases membrane bound ribosomes could not be extracted from the cotyledons of any age tested.

The presence of detergent has been found to be inhibitory to the

isolation of membrane bound ribosomes in the cotyledons of broad beans (Payne & Boulter, 1969). However, in soybean cotyledons our results indicate the requirement for sodium desoxycholate in the extraction buffer would eliminate any membrane bound material.

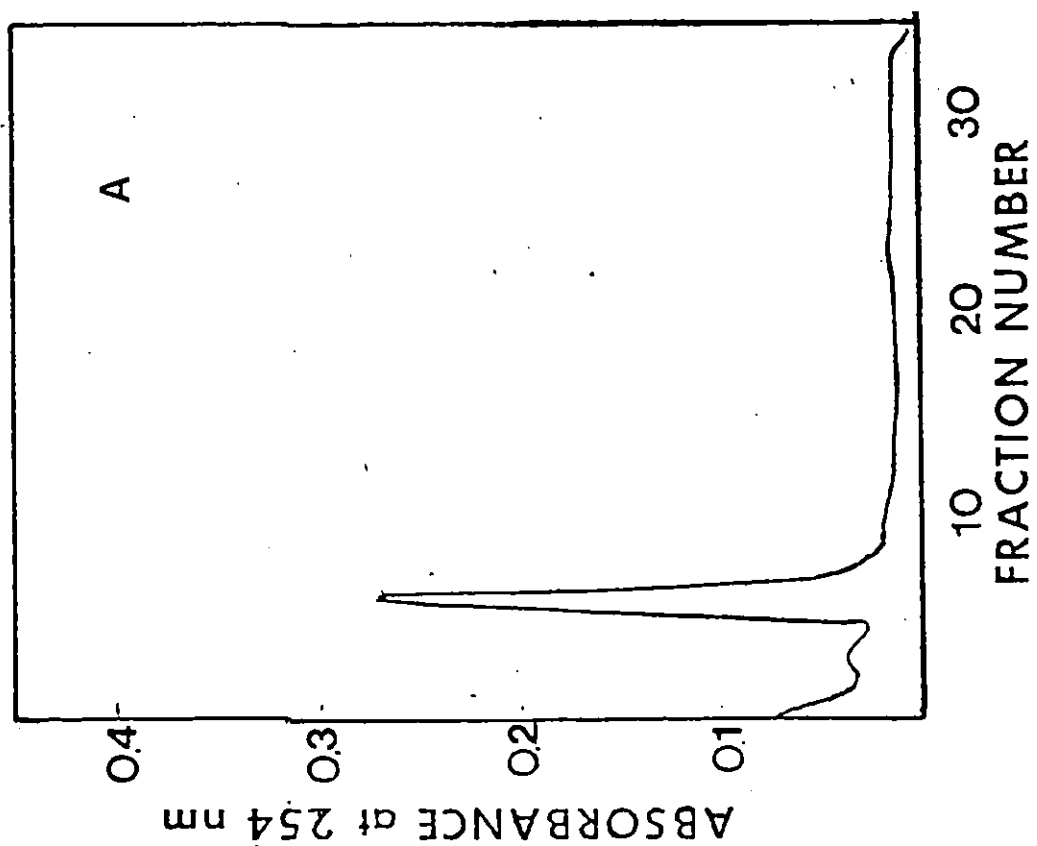
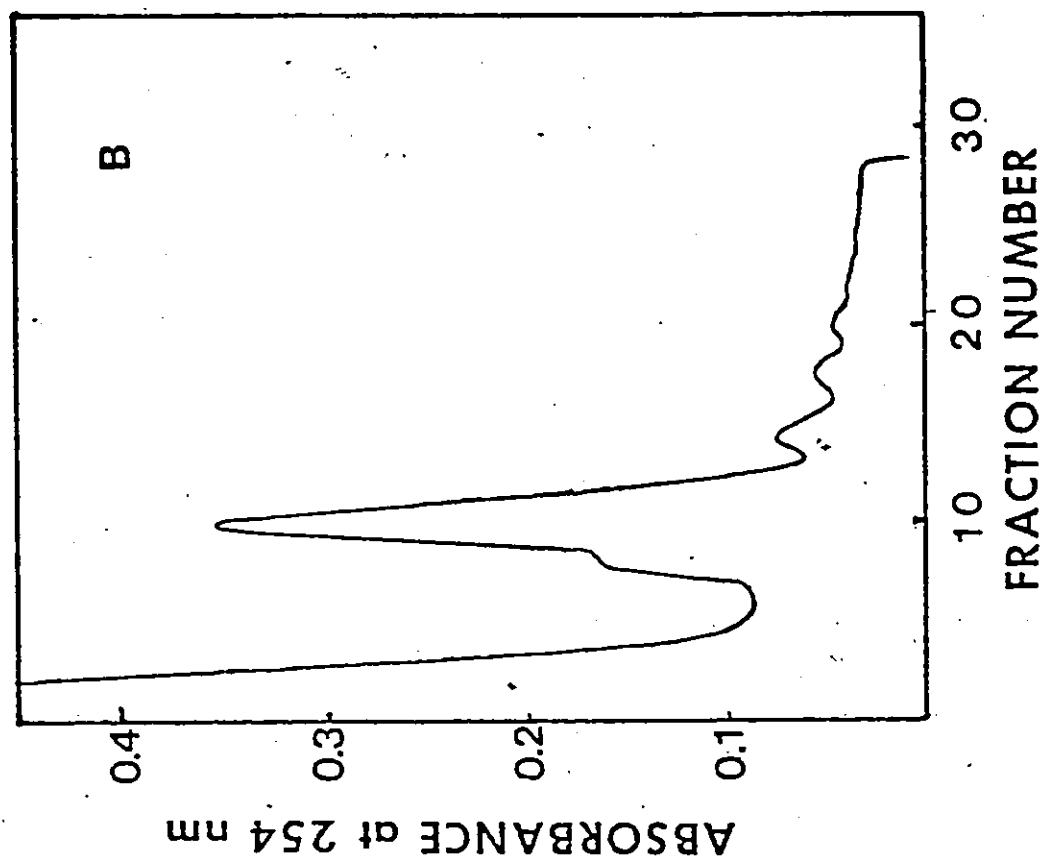
Polysomes of high molecular weight were believed to be lost during centrifugation because of the formation of aggregates. Protease K which had previously been shown by Larkins and Tsai (1977) to dissociate aggregated polysomes active in zein synthesis without resulting in polysome disassembly, was added to the resuspension buffer before gradient centrifugation. Sucrose density gradient profiles of total polysomes obtained in the presence and absence of protease K are shown in Figure 2. The protease K treatment had no effect on the recovery of heavy polysomes and was therefore not added to subsequent extractions.

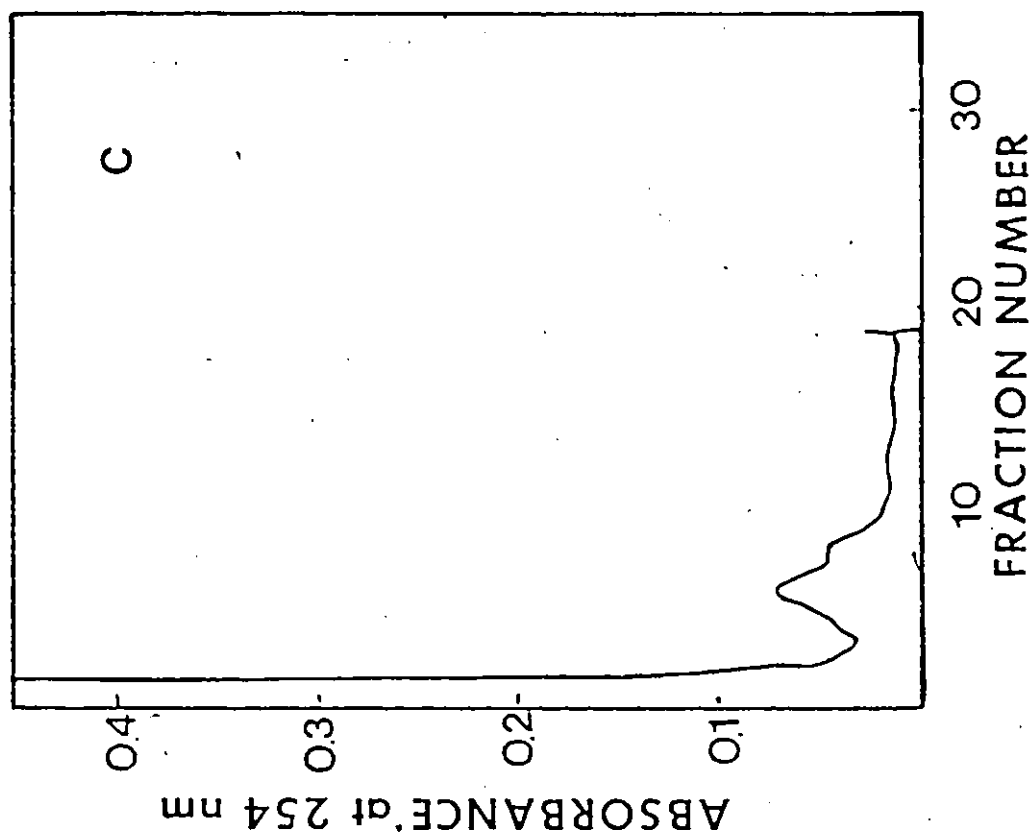
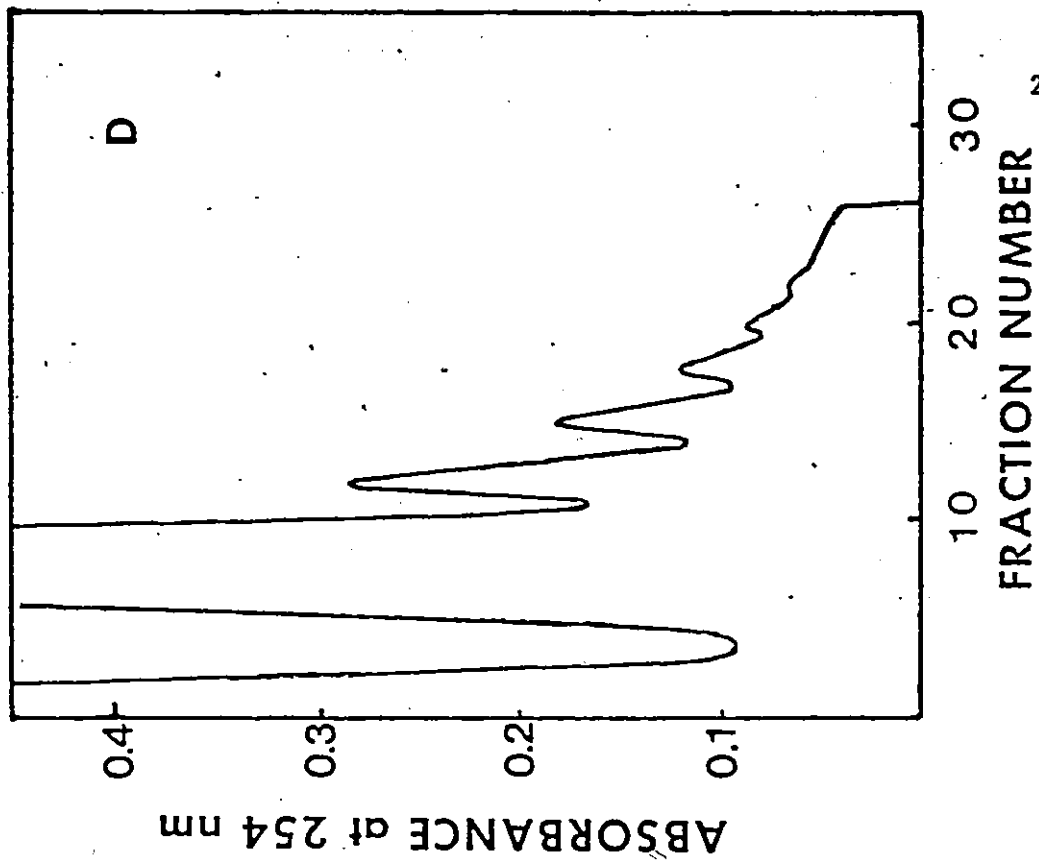
Ageing in the cotyledons of Glycine max appeared to have definite biochemical manifestations. Polysome metabolism and mRNA synthesis may be expected to be altered in seeds undergoing changes in metabolism during germination. Sucrose density gradient profiles of total polysomes isolated and analyzed as described previously from 0,1,3,5,7,10,15 and 20 day old cotyledons are shown in Figures 3-10. (Also Summary Figure, page 31)

Figure 1.

Effect of EGTA and sodium desoxycholate on the extraction of polysomes from soybean cotyledons or hypocotyls (13 day). Polysomes were extracted in 150 mM Tris Ac, pH 8.5, 20 mM KCl, 5 mM MgAc₂, 200 mM sucrose, 7 mM BME.

- A. no EGTA or sodium desoxycholate
- B. 25 mM EGTA
- C. 0.5% sodium desoxycholate
- D. 25 mM EGTA & 0.5% sodium desoxycholate
- E. Polysomes extracted from 5 grams soybean hypocotyl; buffer contains no EGTA or sodium desoxycholate.





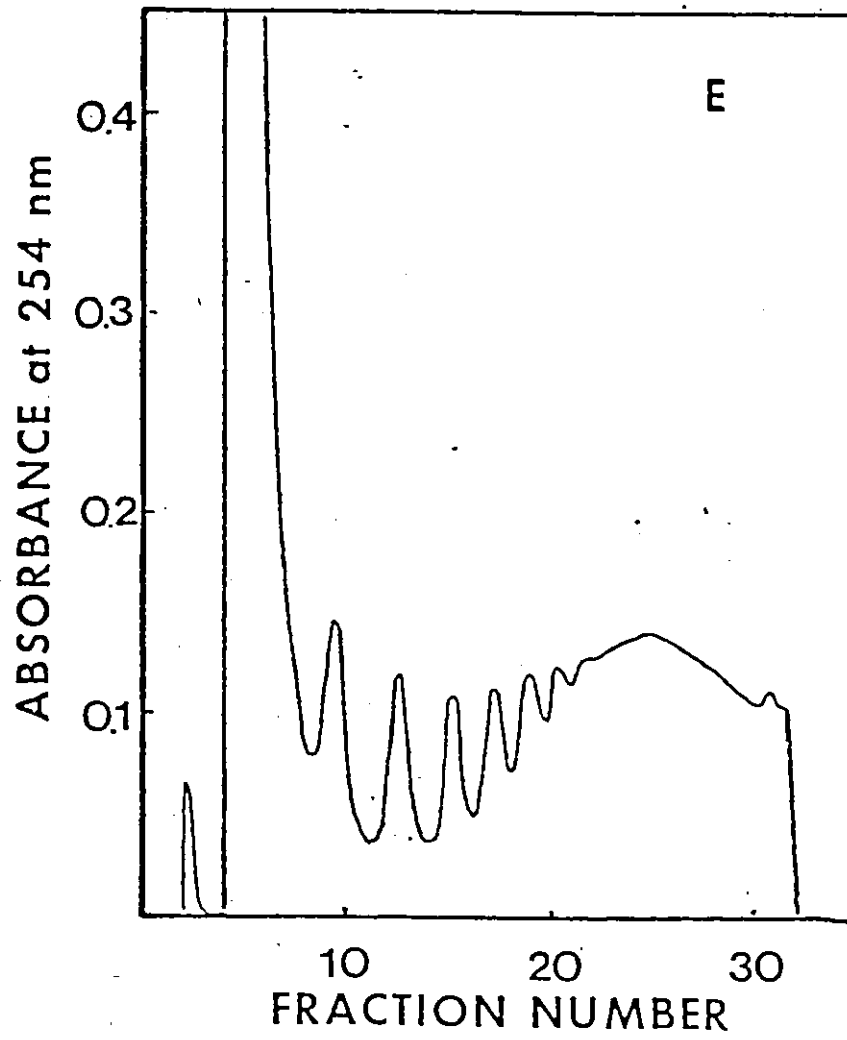


Figure 2.

Effect of Protease K treatment on the extraction of polysomes from 7 day old soybean cotyledons.

- A. Polysomes were extracted in 150 mM Tris Ac, pH 8.5, 20 mM KCl, 5 mM MgAc₂, 200 mM sucrose, 7 mM BME, 25 mM EGTA and 0.5% sodium desoxycholate.
- B. Seven day old polysomes (10 A₂₅₄ units) were treated with 10 ug Protease K (Beckman) for 10 minutes incubation at room temperature prior to density gradient centrifugation and subsequent analysis.

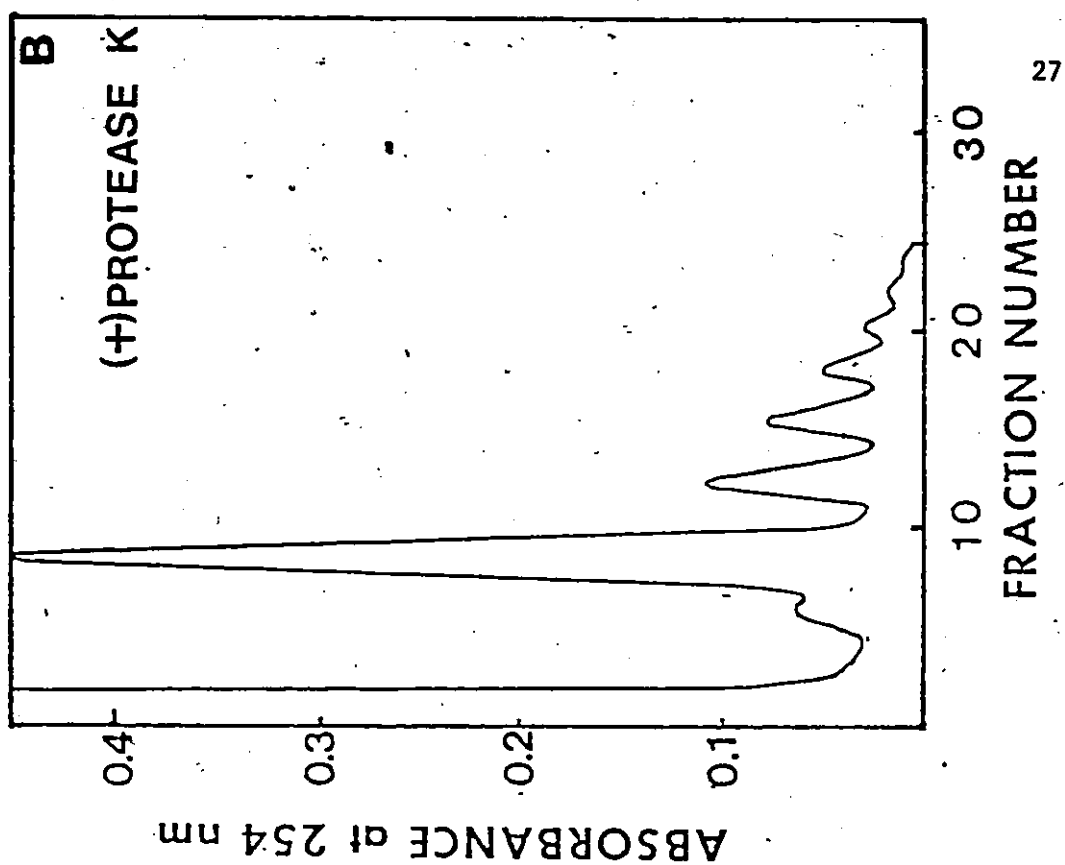
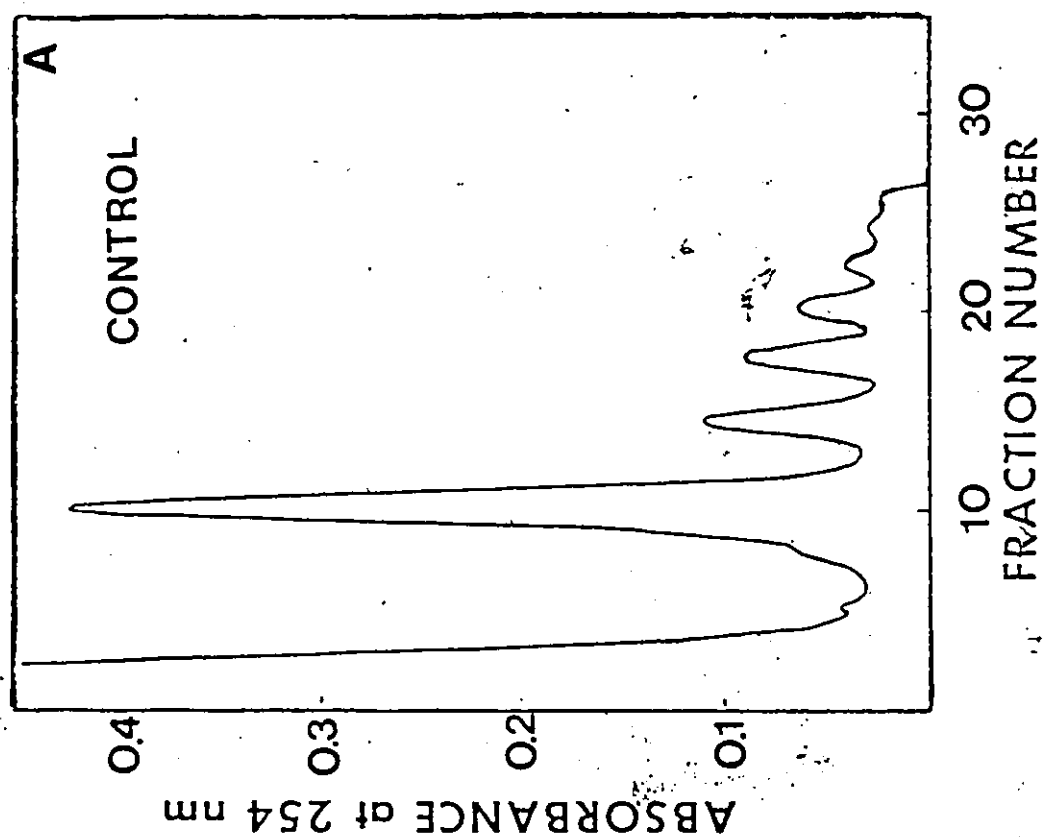
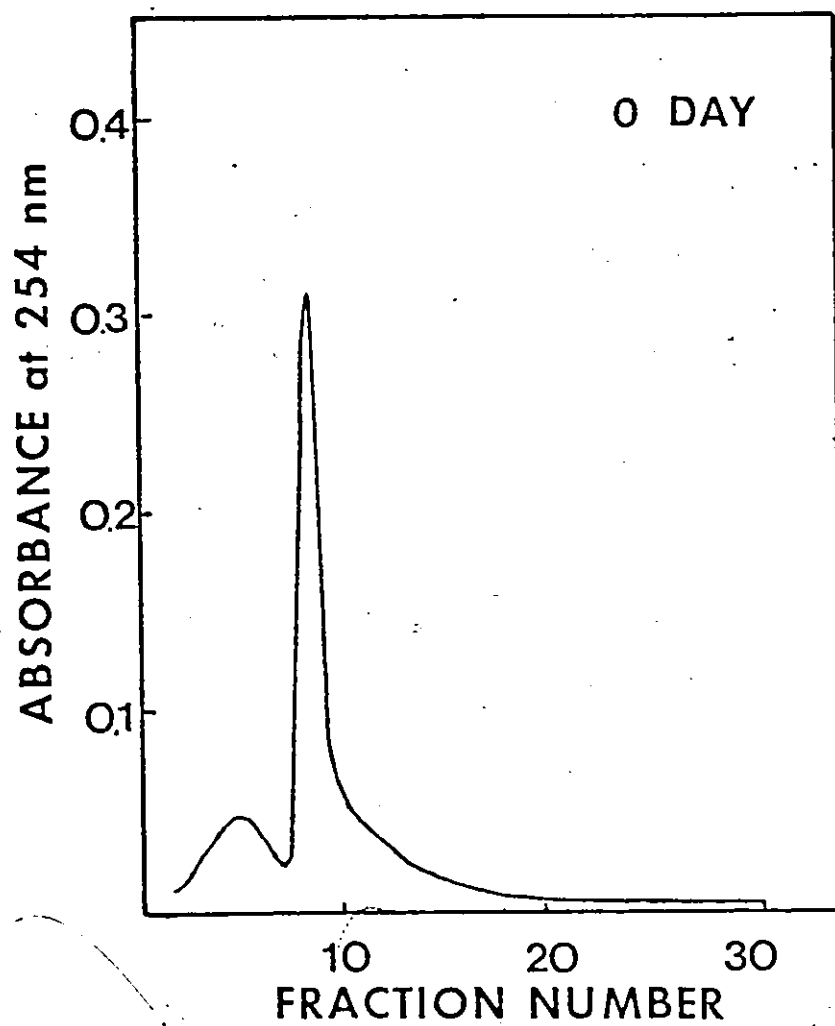


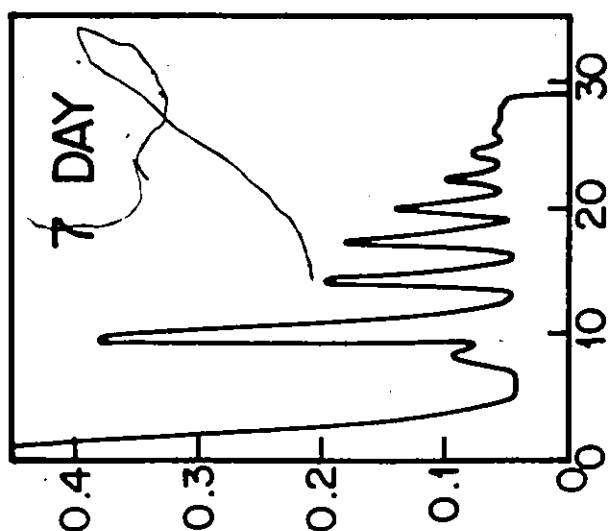
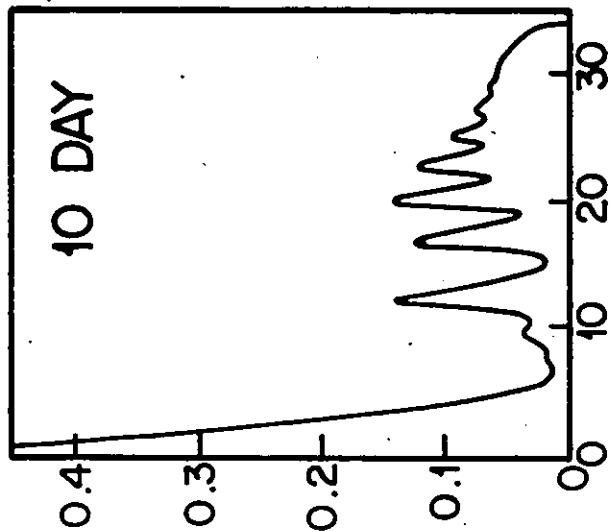
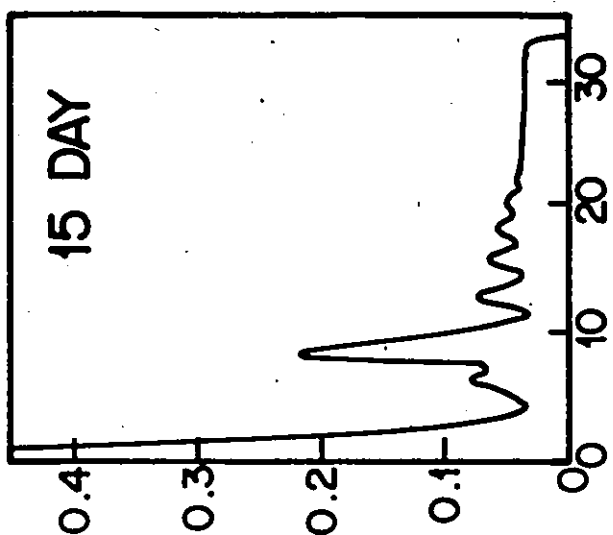
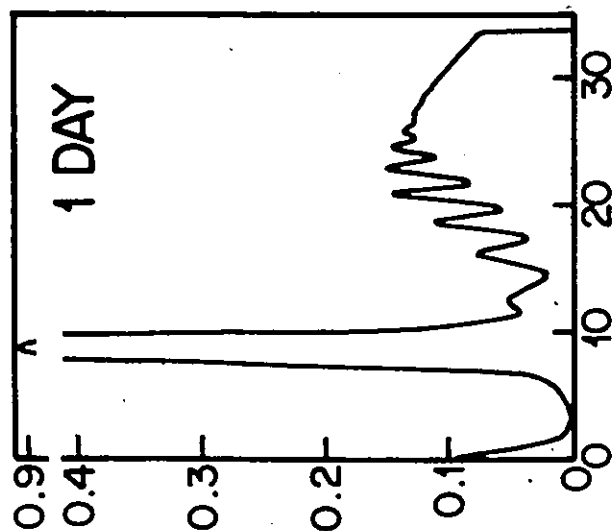
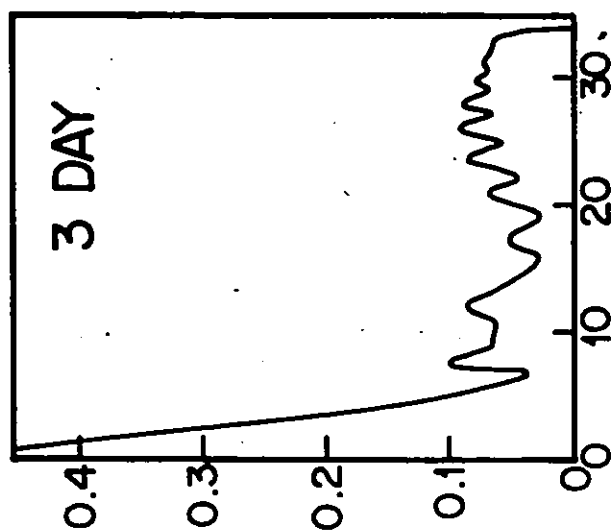
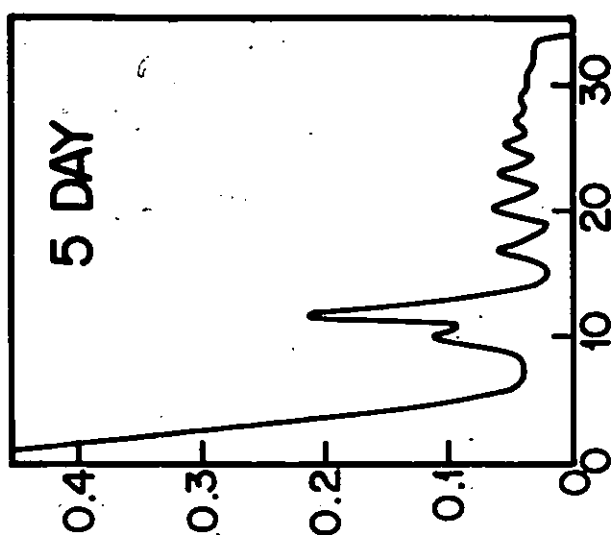
Figure 3.

Sucrose density gradient analysis of total polysomes from dry soybean cotyledons. Total polysomes ($10 A_{254}$ units) isolated from cotyledons of dry soybean cotyledons as described in Methods, were layered on a 10-45% linear sucrose gradient containing 50 mM Tris Ac, pH 8.5, 25 mM KCl, 10 mM MgAc. Gradients were centrifuged at 150,000 $\times g$ for 90 minutes and then analyzed by continuous monitoring of absorption at 254 nm with an ISCO automatic density gradient fractionator.



Summary of Figures 4 - 9

Sucrose density gradient analysis of total polysomes from cotyledons of 1,3,5,7,10 and 15 day old soybeans (20 day cotyledons excluded). The graphs represent fractionation of sucrose gradients to which 10 O.D. units of resuspended polysome pellet had been applied. Polysomes were initially extracted in a buffer containing 150 mM Tris-Ac, pH 8.5, 200 mM sucrose, 20 mM KCl, 5 mM MgAc_2 , 7 mM BME, 0.5% sodium desoxycholate, 25 mM EGTA, 1 mM DTT. Following centrifugation at 150,000 xg for 90 minutes and then analyzed by continuous monitoring of absorption at A_{254} with an ISCO automatic density gradient fractionator.



ABSORBANCE at 254 nm

FRACTION NUMBER

Figure 4.

Sucrose density gradient analysis of total polysomes from cotyledons of 1 day old soybeans. Total polysomes ($10 A_{254}$ units) isolated from cotyledons of 1 day soybean seedlings as described in Methods, were layered on a 10-45% linear sucrose gradient containing 50 mM Tris Ac, pH 8.5, 25 mM KCl, 10 mM MgAc. Gradients were centrifuged at 150,000 xg for 90 minutes and then analyzed by continuous monitoring of absorption at 254 nm with an ISCO automatic density gradient fractionator.

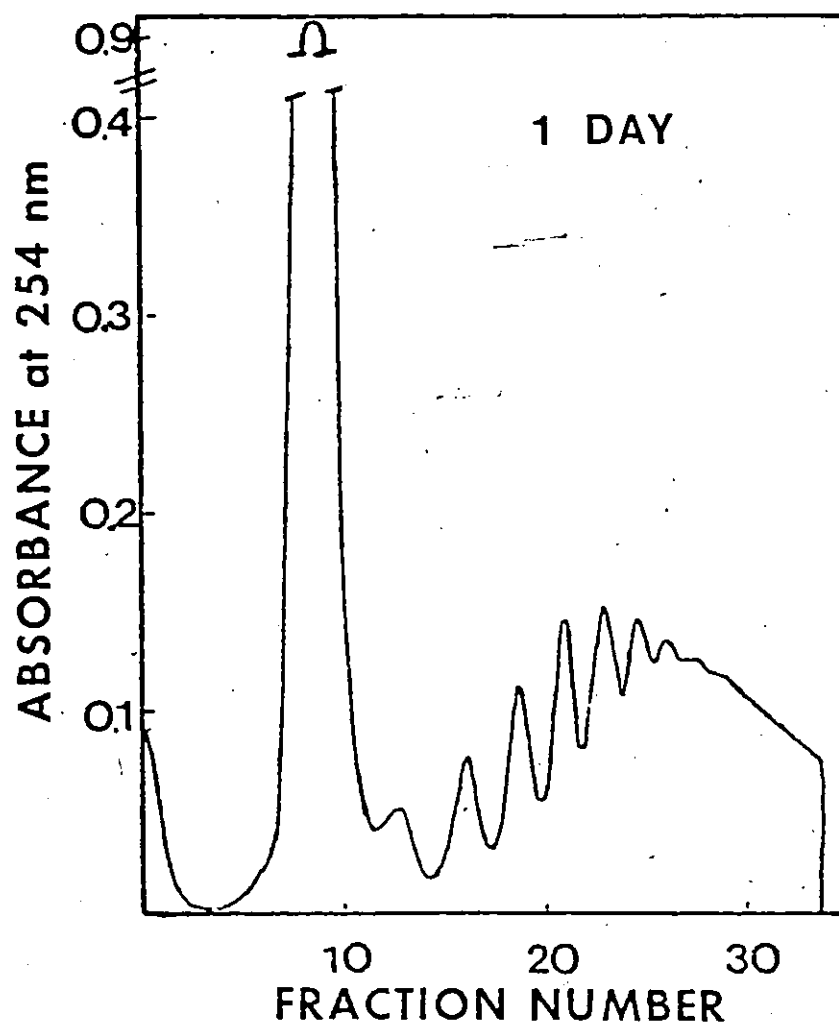


Figure 5.

Sucrose density gradient analysis of total polysomes from cotyledons of 3 day old soybeans.

Total polysomes (10 A₂₅₄ units) isolated from cotyledons of 3 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.

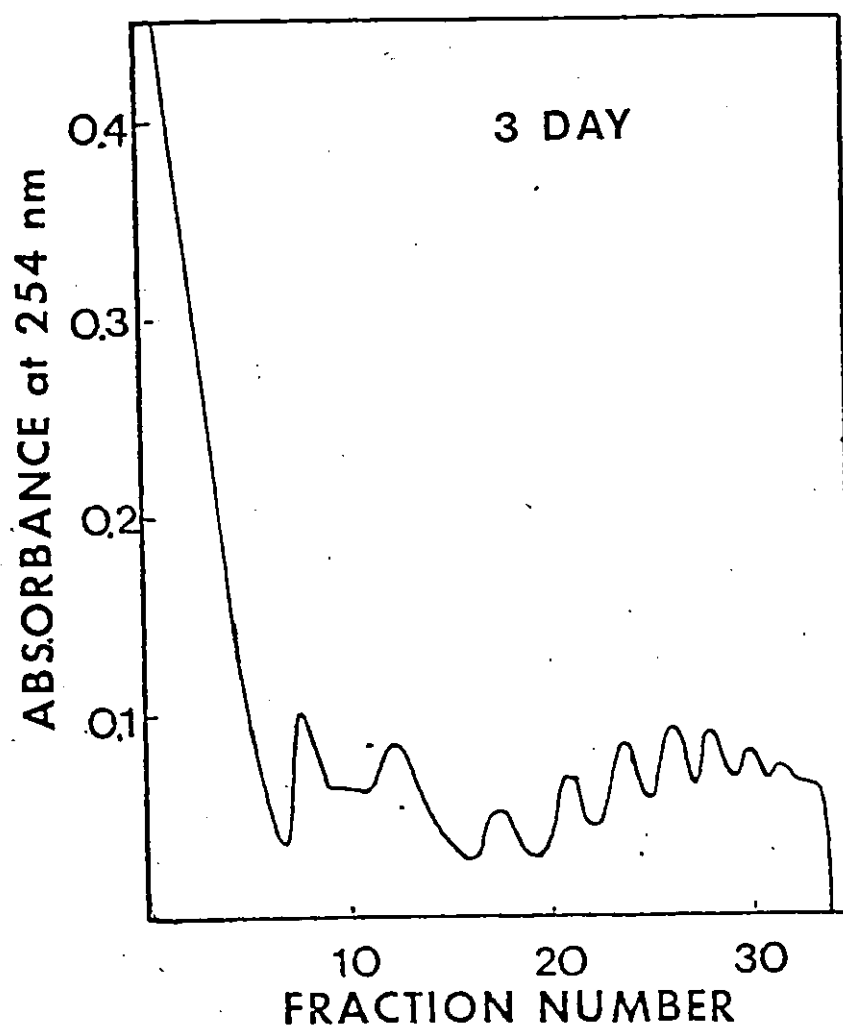



Figure 6.

Sucrose density gradient analysis of total polysomes from cotyledons of 5 day old soybeans. Total polysomes (10 A_{254} units) isolated from cotyledons of 5 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.



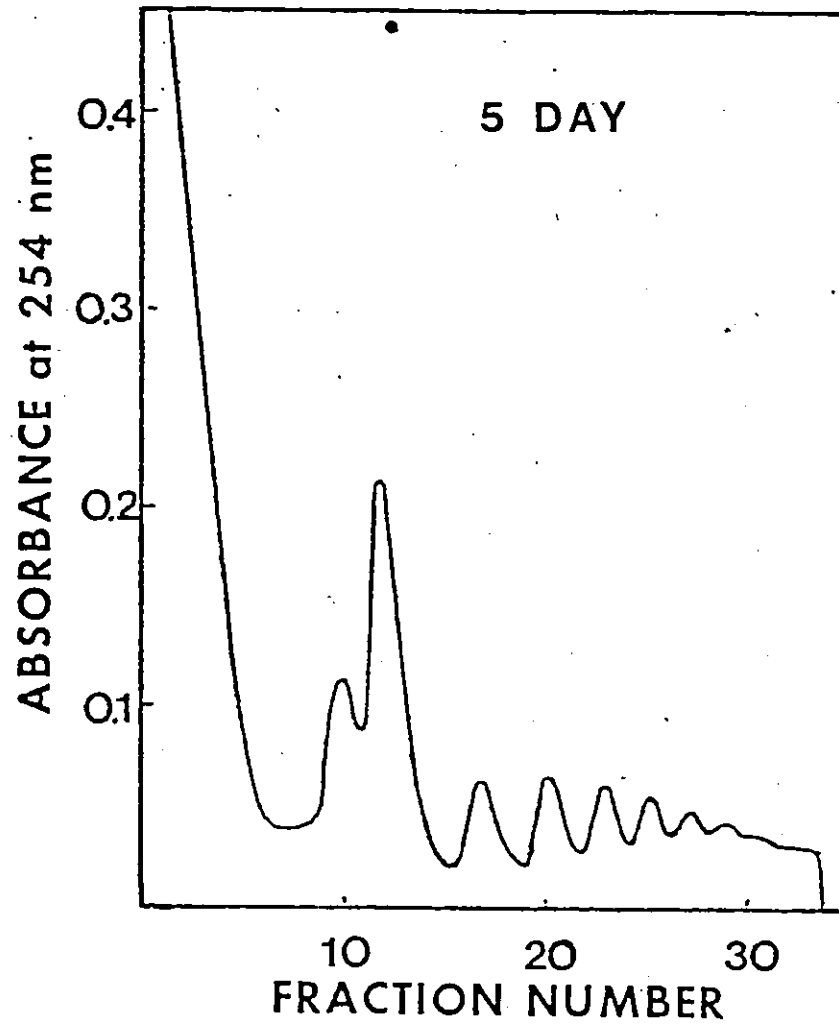


Figure 7.

Sucrose density gradient analysis of total polysomes from cotyledons of 7 day old soybeans. Total polysome (10 A_{254} units) isolated from cotyledons of 7 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.

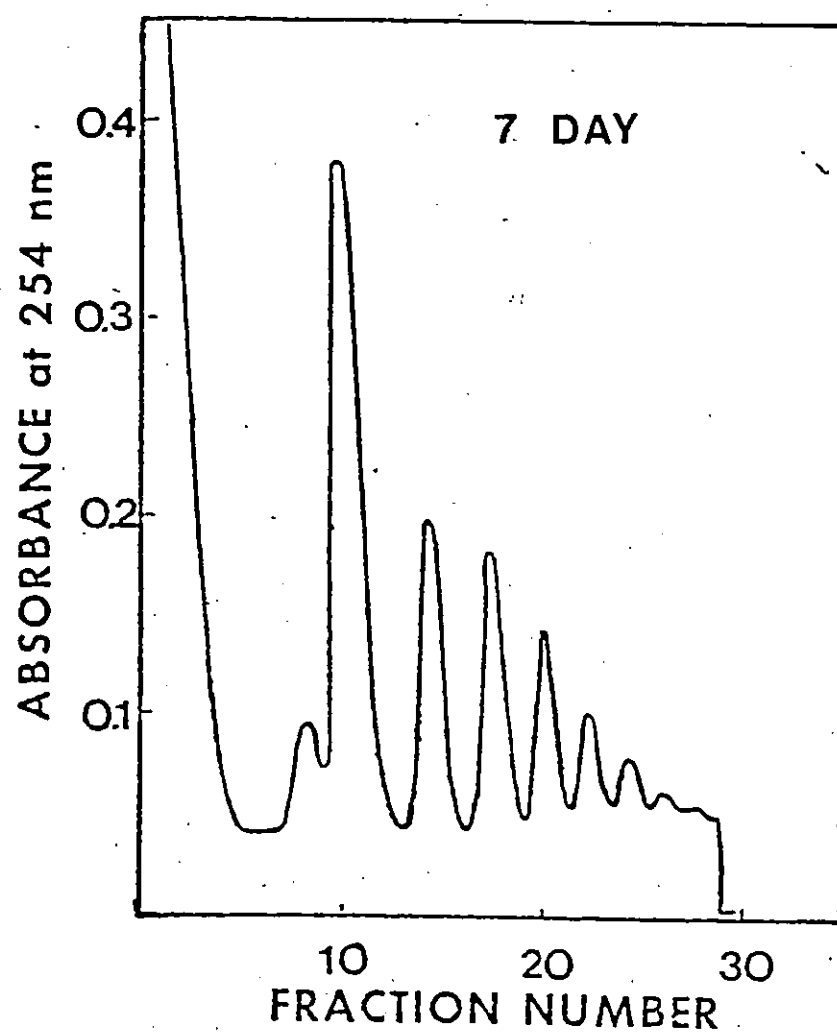


Figure 8.

Sucrose density gradient analysis of total polysomes from cotyledons of 10 day old soybeans. Total polysomes (10 A₂₅₄ units) isolated from cotyledons of 10 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.

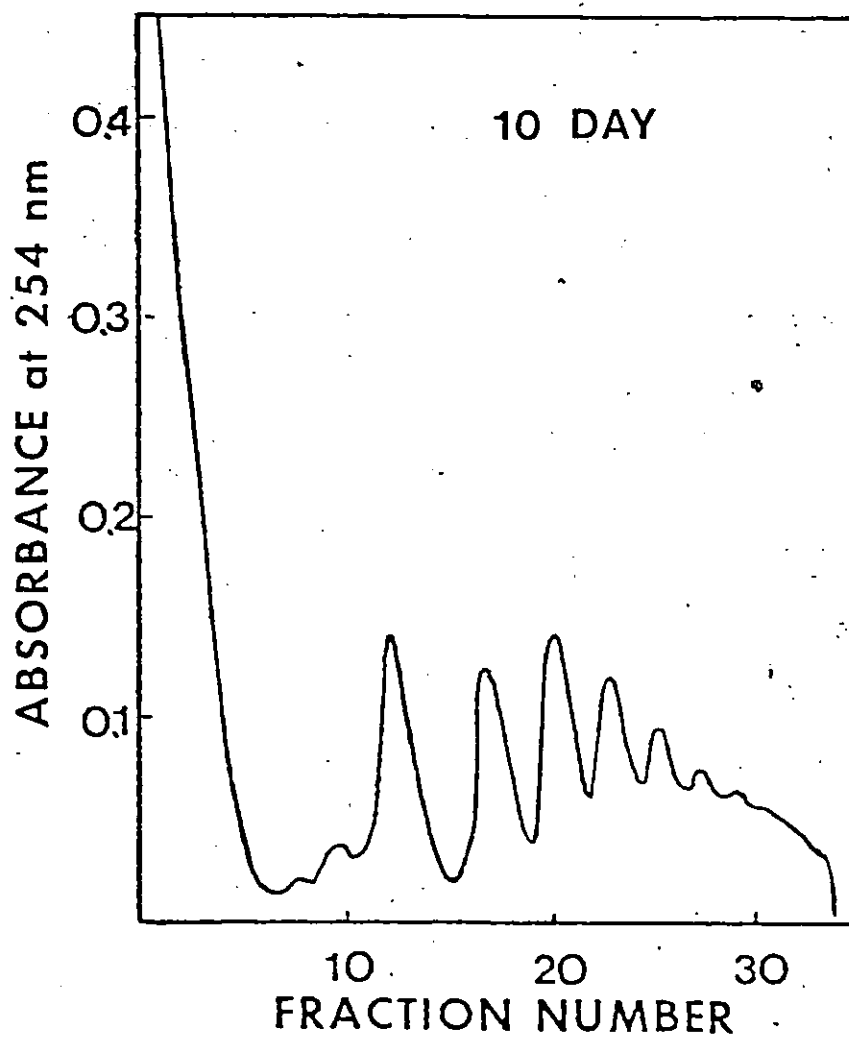


Figure 9.

Sucrose density gradient analysis of total polysomes from cotyledons of 15 day old soybeans. Total polysomes (10 A₂₅₄ units) isolated from cotyledons of 15 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.

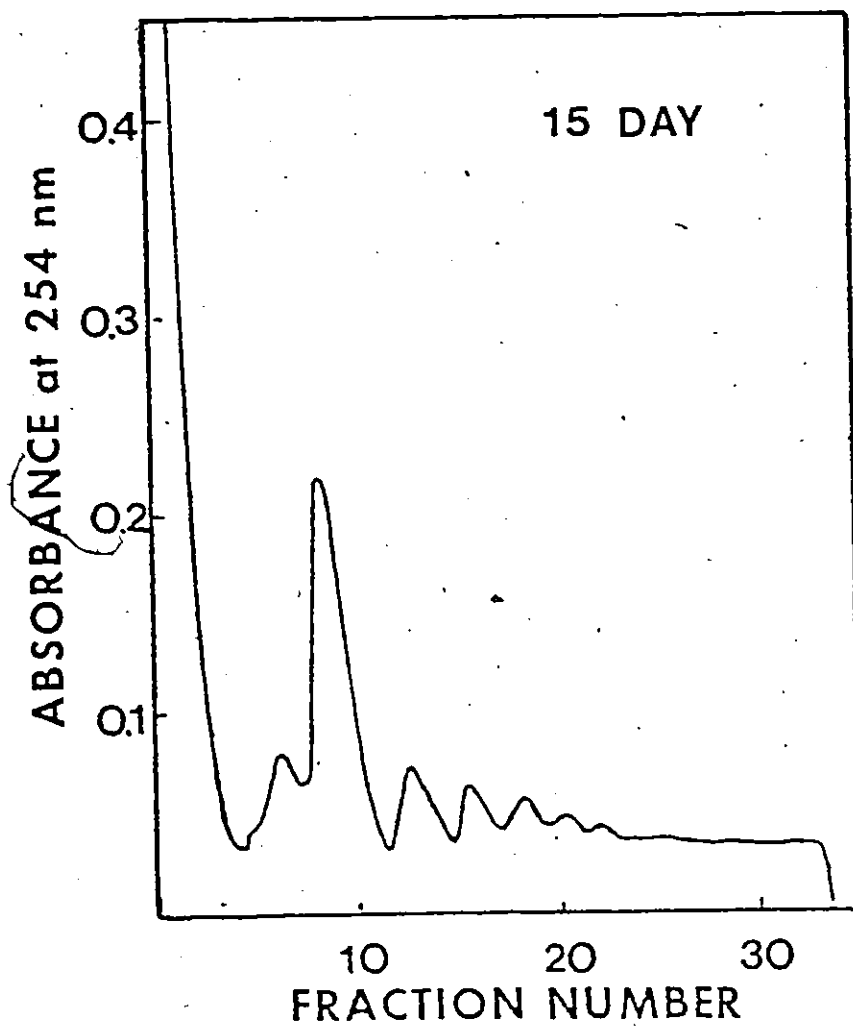


Figure 10.

Sucrose density gradient analysis of total polysomes from cotyledons of 20 day old soybeans. Total polysomes (10 A₂₅₄ units) isolated from cotyledons of 20 day old soybeans were separated by density gradient centrifugation and analyzed as described in Figure 3.

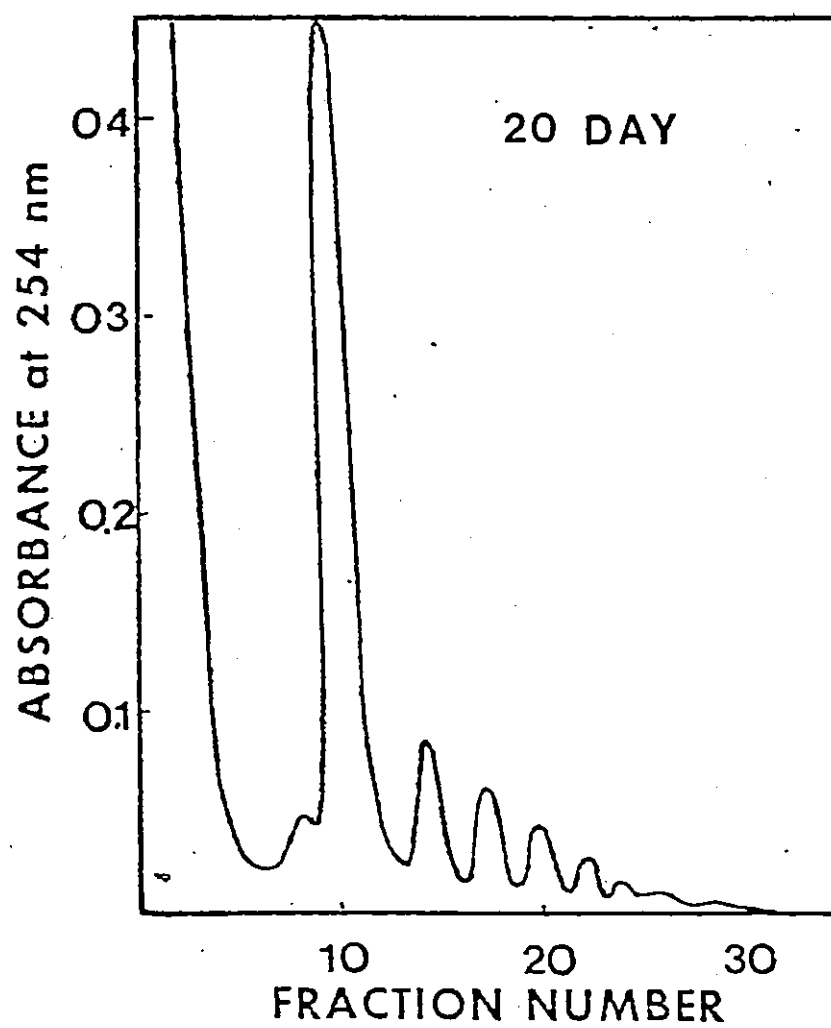


TABLE 1: . PERCENTAGE DISTRIBUTION OF TOTAL RIBOSOMES AS
MONOSOMES, LIGHT, MEDIUM AND HEAVY POLYRIBOSOMES

AGE OF COTYLEDONS (DAYS)	MONOSOMES	POLYSOMES		
		LIGHT	MEDIUM	HEAVY
1	48.5	3.3	9.4	28.3
3	29.0	17.8	24.2	16.7
5	64.6	16.4	12.2	4.6
7	50.4	32.6	12.0	2.5
10	25.1	39.2	29.5	11.7
15	53.0	34.5	12.7	-
20	74.8	17.4	11.4	-

Results presented in Figures 4-10 are computed and presented here. (Ten O.D. units (500 ug) of total polysomes were layered on a 10-45% linear sucrose gradient and centrifuged at 105,000 xg for 90 minutes and A_{260} monitored).

Percentage distribution was calculated based on the area under the curves, (Monosomes plus polysomes equal to 100%). Polysomes possessing 2 or 3 ribosomes per mRNA were designated as 'light, those possessing 4 or 5 ribosomes per mRNA were designated as 'medium' and those possessing greater than 5 ribosomes per mRNA were designated as 'heavy' polysomes.

The alteration of polysome profiles with increased seed age is immediately apparent. In dry seeds (Figure 3) there is very little polysomes, since all the material is present as monosomes. From 0 to 1 day of germination, following the imbibition of water, there is a rapid transition of monosomes to polysomes (Fig. 4). Data presented in Table 1 for 1 day cotyledons represents the highest percentage (28%) of heavy polysomes for any of the ages tested. In 3 day old cotyledons we observed the smaller percentage (29%) of monosomes. As the cotyledons aged from 3 to 7 days a decrease in the percentage of heavy polysomes was obtained compared to the light and medium polysomes and monosome populations. On a quantitative basis these species tend to increase during this period. In the 5 and 7 day old systems (Fig. 6,7) a separate peak attributed to the breaking down of monosomes into ribosomal subunits appeared during this period. Results in Figure 8 further show that at 10 days there is a second burst of polysomal activity with a four fold increase in the heavy polysome population compared to the 7 day heavy polysome and an accompanying sharp decrease in the quantity of monosomes (from 50% to 25%) during the same period. After 10 days there is a rapid increase in the percentage of monosomes from 53% to 74% in 15 and 20 day old cotyledons respectively. With the elimination of heavy polysomes, the formation once again of a significant proportion of ribosomal subunits (Fig. 9,10) is apparent.

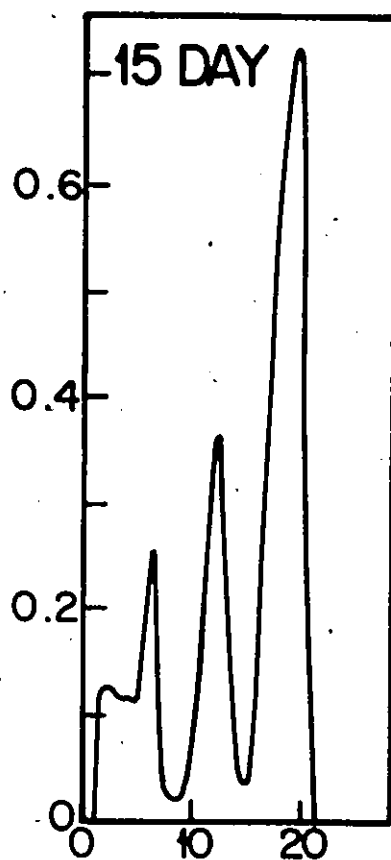
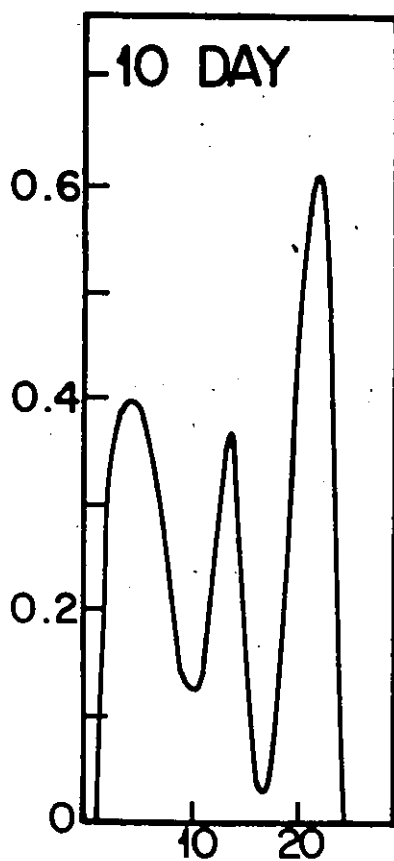
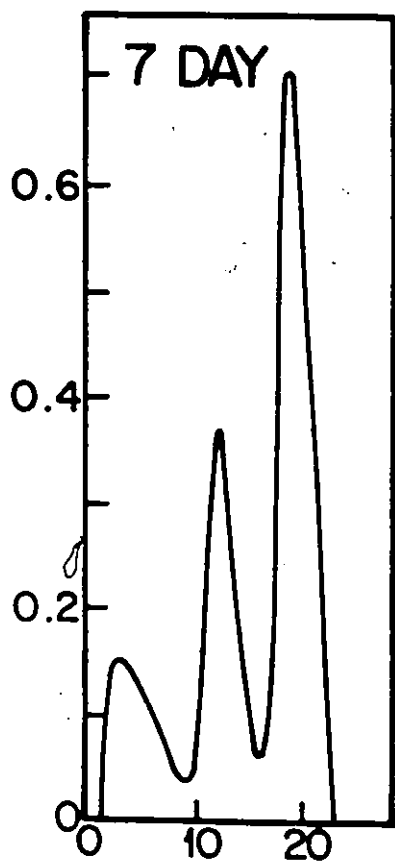
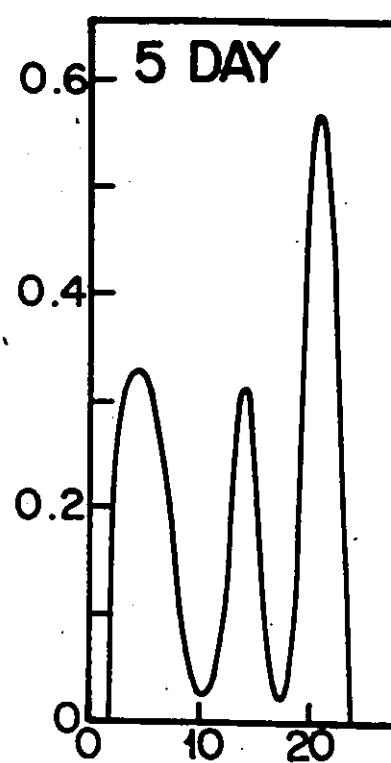
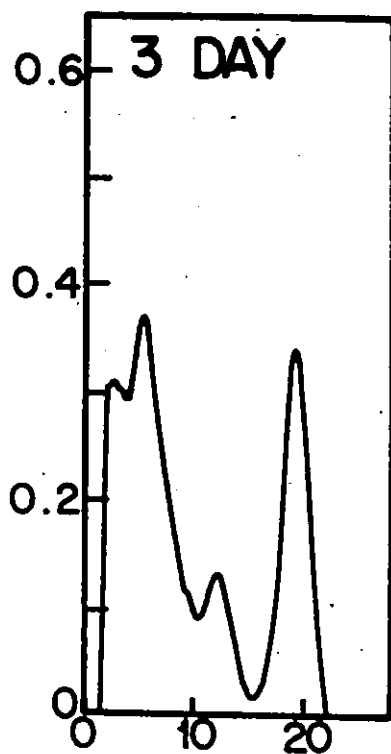
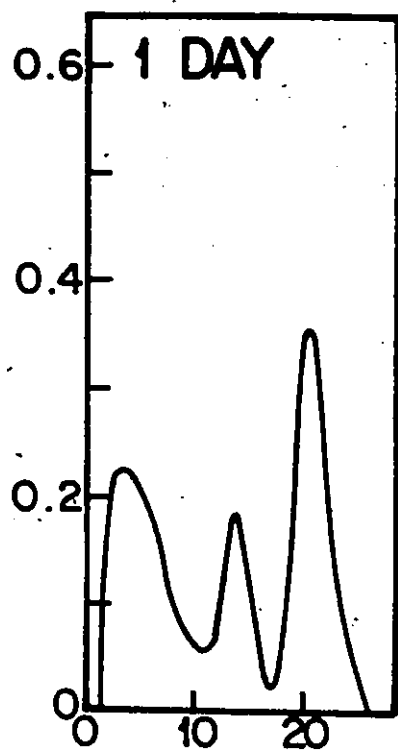
RNA Sucrose Gradient Analysis (Also Summary Figure, page 49)

In order to demonstrate the distribution of the various classes

Summary of Figures 11 - 16

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 1,3,5,7,10 and 15 day old soybean cotyledons (20 day soybeans excluded). Total RNA was extracted from polysome pellets using a buffer containing 25 mM Tris Ac, pH 8.0, 200 mM NaCl, 5 mM EDTA, and 1% SDS. An aliquot of 10 A_{254} units (500 ug) was layered on a 5-25% sucrose gradient and then centrifuged at 22,000 rpm for 16 hours and then analyzed by continuous monitoring absorption at A_{254} with an ISCO automatic density gradient fractionator.

ABSORBANCE at 254 nm



FRACTION NUMBER

Figure 11.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 1 day old soybean cotyledons. Total RNA was isolated from the polysome pellets using an EDTA-SDS containing buffer. The sample (10 A_{254} units) was layered onto a 5-25% sucrose gradient, centrifuged at 80,000 xg for 16 hours and the O.D. monitored.

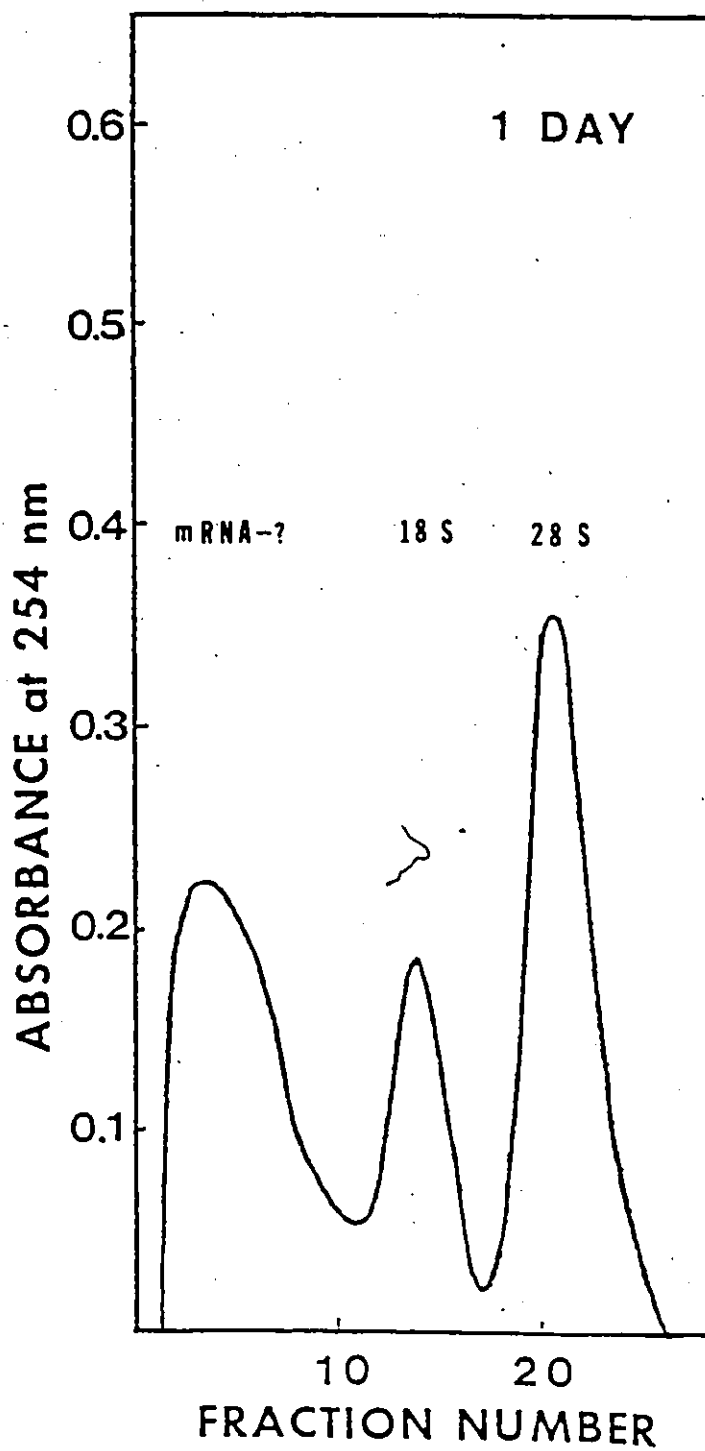


Figure 12.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 3 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.

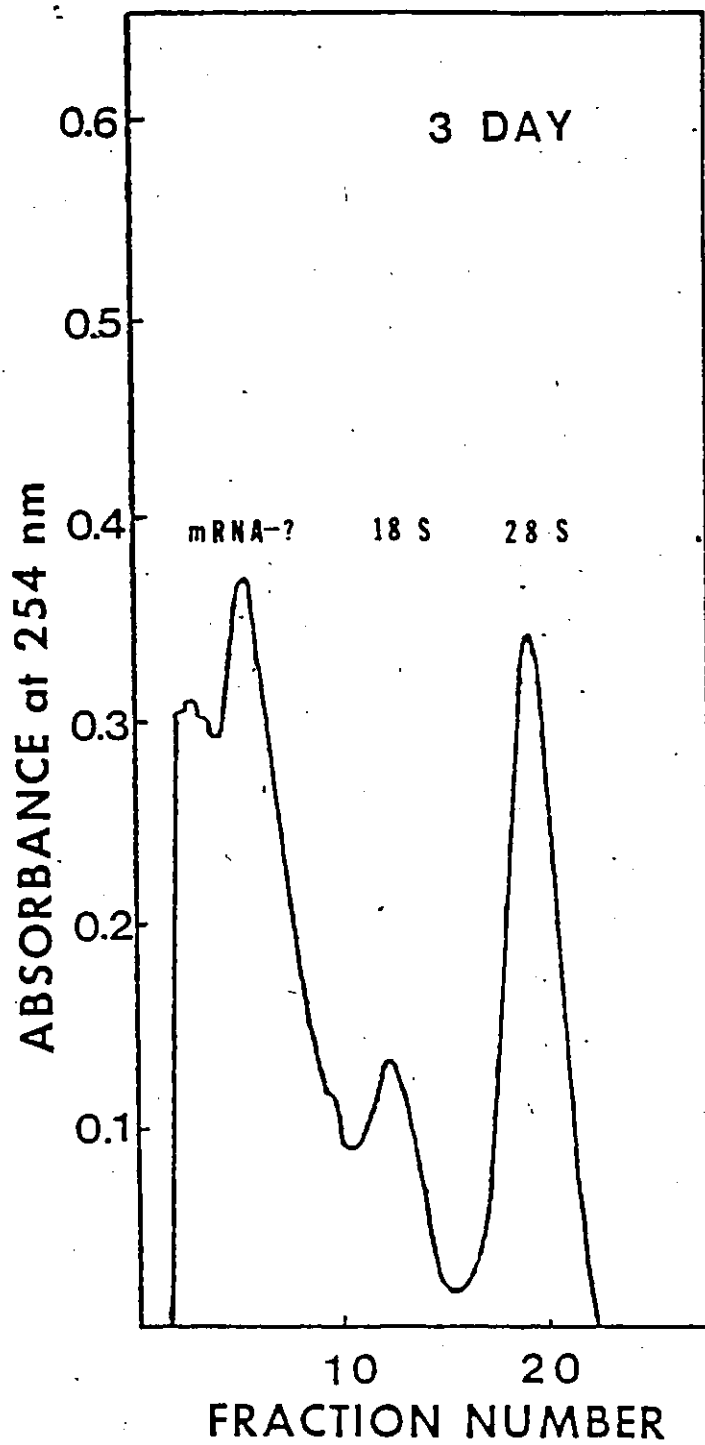


Figure 13.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 5 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.

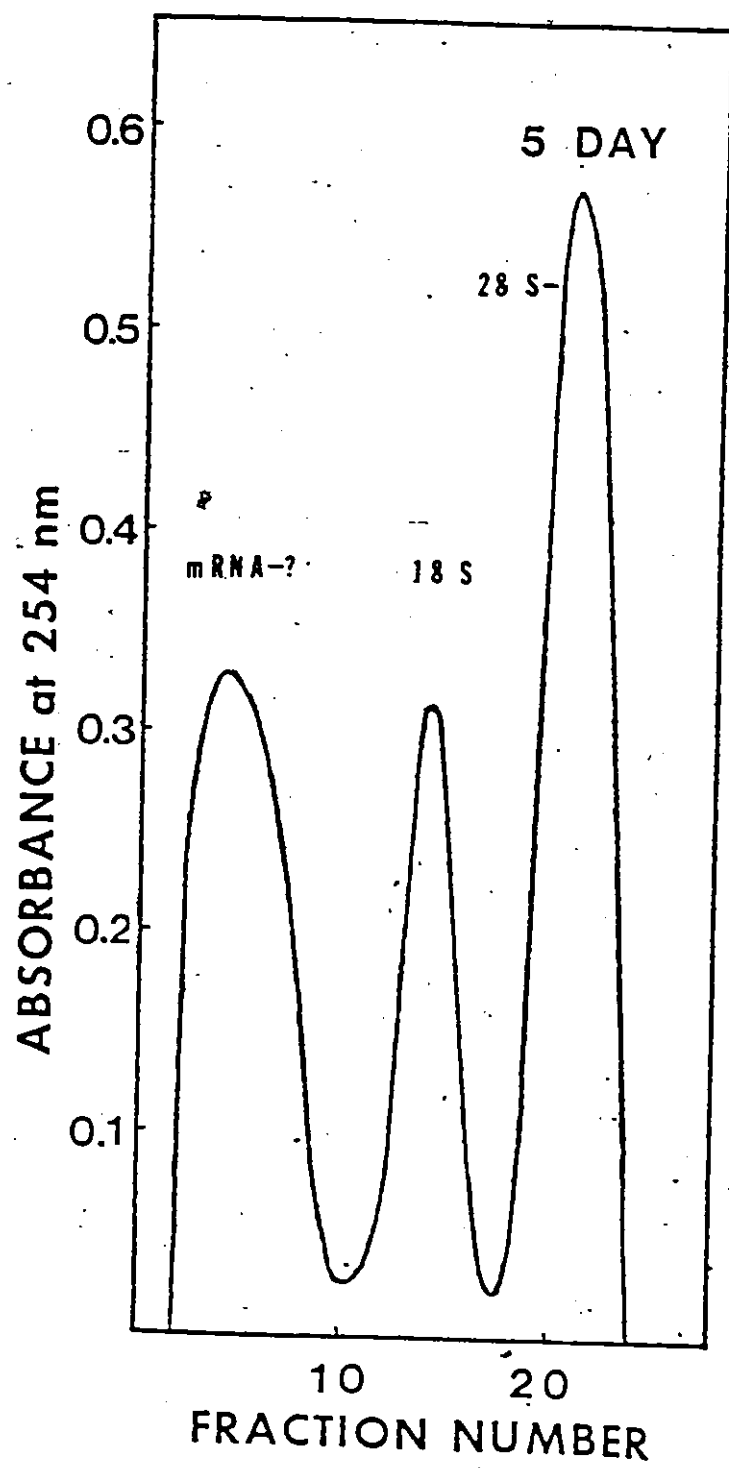


Figure 14.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 7 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.

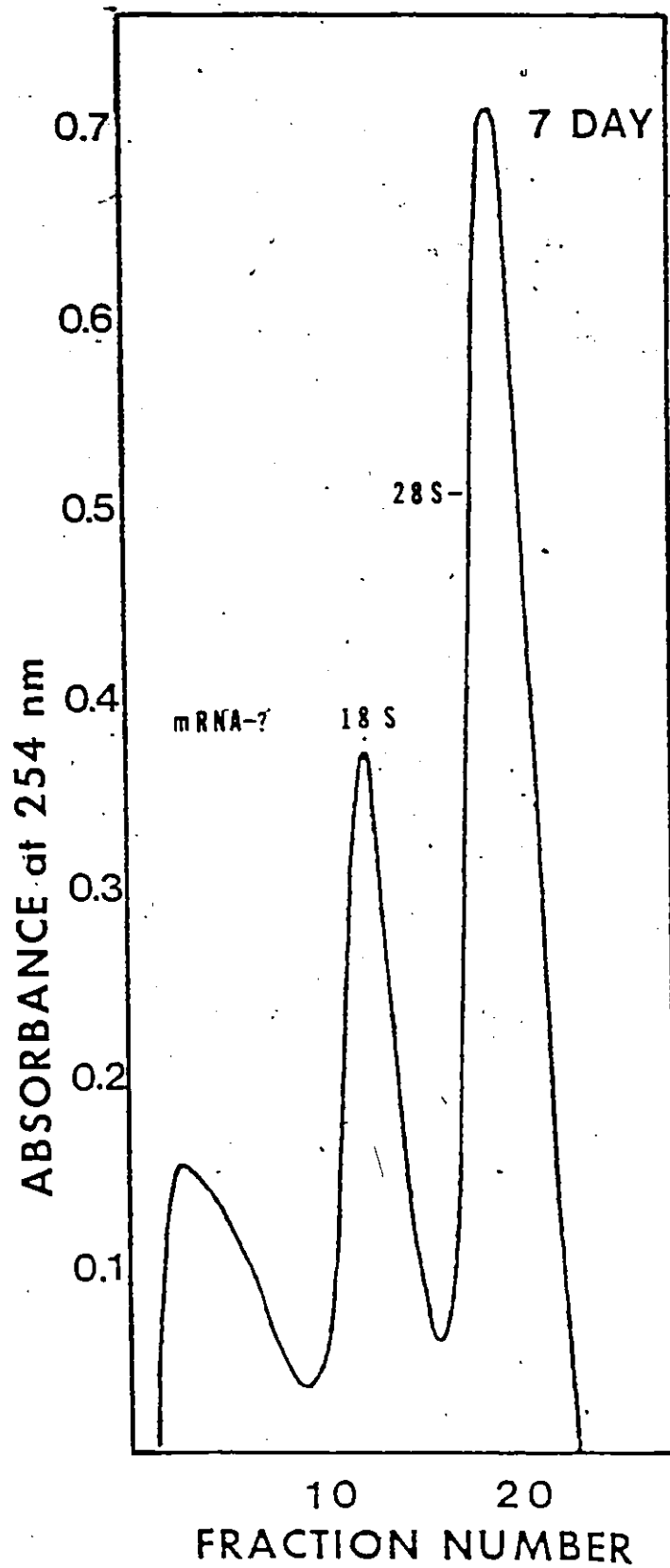


Figure 15.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 10 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.

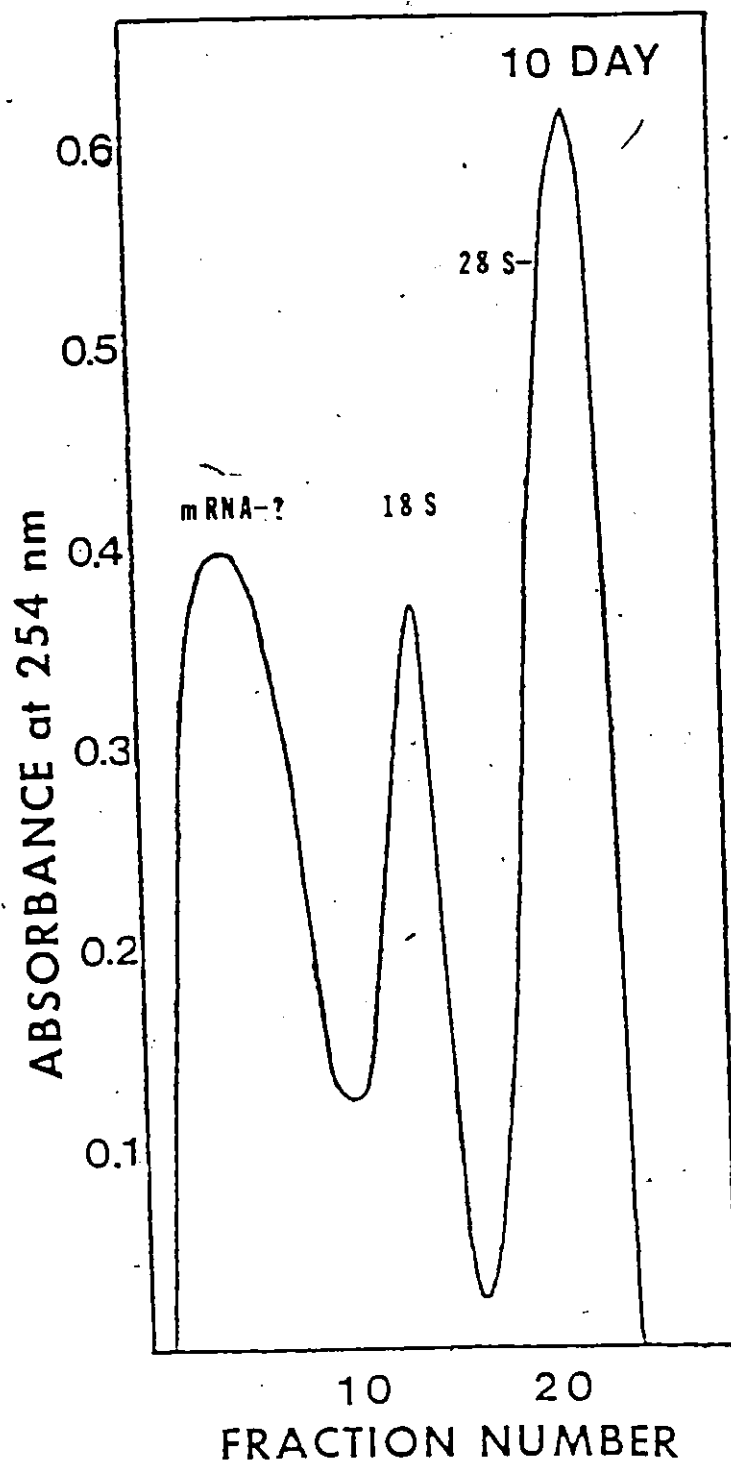


Figure 16.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 15 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.

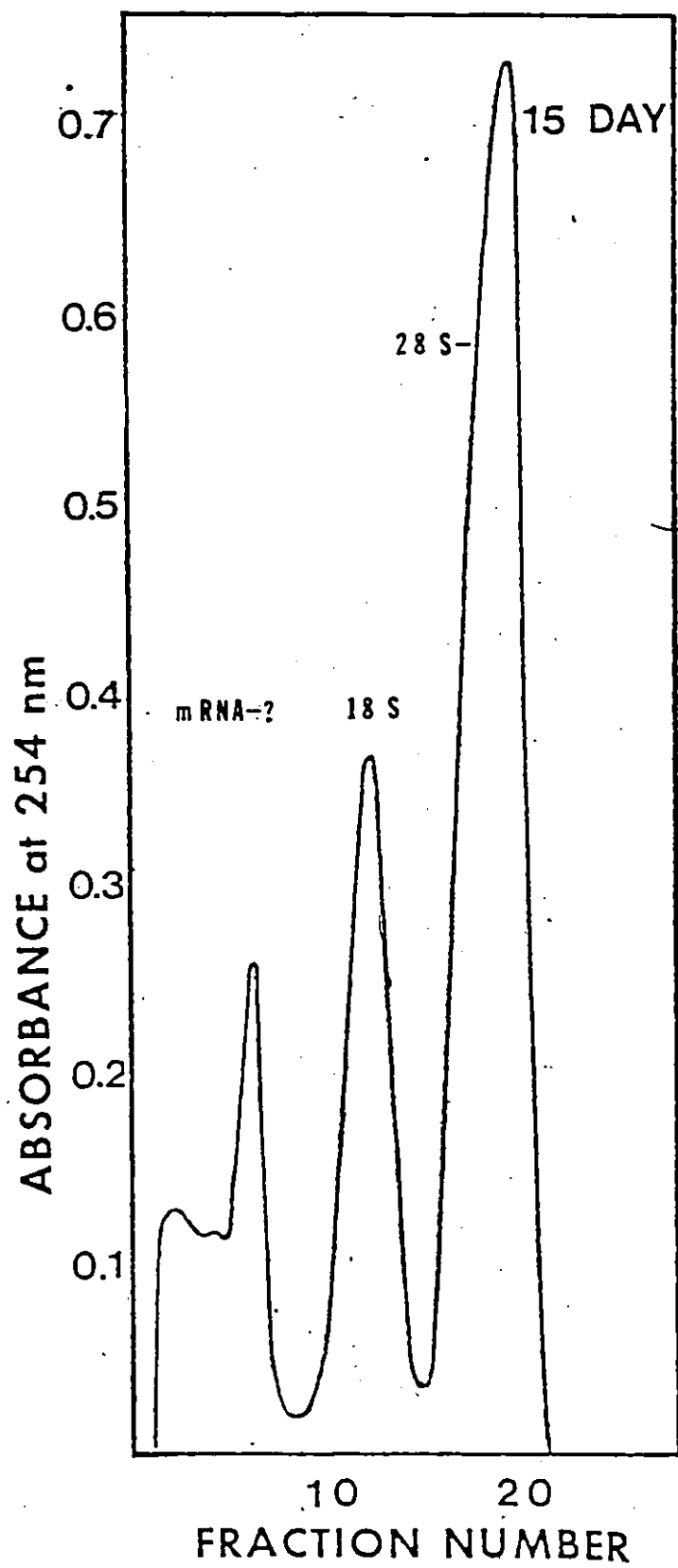
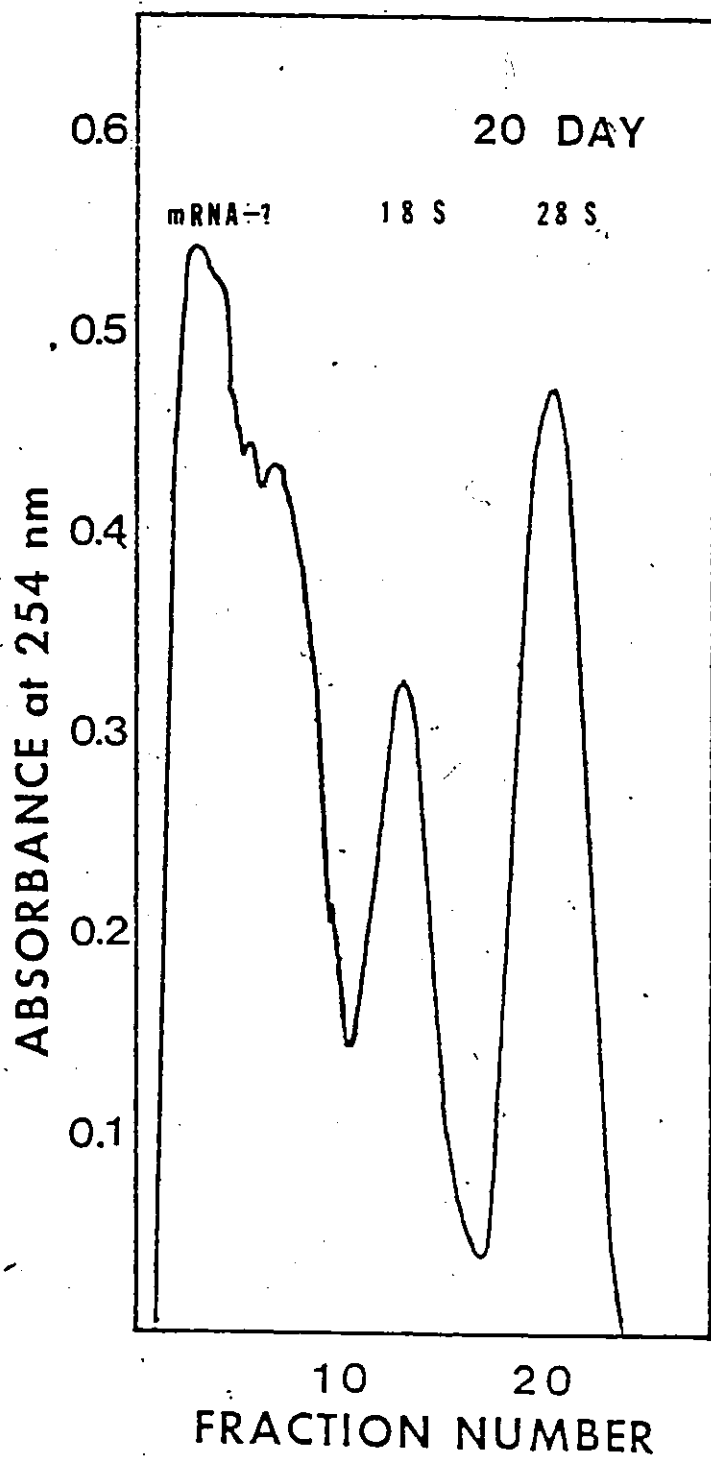


Figure 17.

Sucrose gradient sedimentation of total RNA isolated from polysome pellets obtained from 20 day old soybean cotyledons. Total RNA was isolated and analyzed as described in Figure 11.



of RNA associated with the polysomes, RNA was released from the pellet using EDTA-SDS treatment and then subjected to sucrose gradient analysis. It should be pointed out here that we are unable to show the correspondence between polysome profiles and the RNA species in Figures 11-13, particularly the mRNA peaks, which are present in high proportions in 5 and 10 day old cotyledons (Fig. 13,15). In 7 day old cotyledons (Fig. 14) the amount of mRNA decreased considerably, relative to 18 S and 28 S RNA species. Again in 10 day old cotyledons we note an increase in mRNA which decreased in 15 day old cotyledons. Lack of correspondence in results between the polysome profiles and mRNA peaks suggests that higher mRNA peaks may be due to degradation.

Oligo (dT) Cellulose Chromatography

Figures 18-24 show the profiles of Poly A (+) and Poly A (-) RNA for ageing cotyledons. It can be seen that from 1 to 5 days of germination there is a four fold increase in the amount of Poly A (-) mRNA recovered from the oligo (dT) cellulose column while the amount of Poly A (+) mRNA decreases only slightly. At 7 days (Fig. 21) the amount of Poly A (-) mRNA also decreases. In 10 day old cotyledons (Fig. 22) there is a doubling in the amount of Poly A (+) mRNA recovered from the oligo (dT) column. This indicates the possibility that the mRNA is highly polyadenylated with the second burst of polysomal activity. After 10 days the quantity of both Poly A (+) and Poly A (-) mRNA is considerably reduced. (also Summary Figure, page 66)

³H-Poly (U) Hybridization Assay

In order to determine the presence of Poly A (+) mRNA in peak 3 from the oligo (dT) cellulose column, ³H-Poly (U) was quantitatively

Summary of Figures 18 - 23

Fractionation of RNA from polysome pellets of 1,3,5,7,10 and 15 day old soybean cotyledons (20 day soybeans excluded). by oligo (dT) cellulose chromatography. RNA was phenol extracted from the polysome pellets and 160 A_{254} units (8 mg) were loaded onto an oligo (dT) cellulose column. The buffers were changed as indicated by the arrows to elute the different fractions. a. 0.5 M KCl-Tris buffer b. 0.01 M KCl-Tris buffer c. 0.1 M Tris HCl, pH 7.5. The RNA which passed through the column was monitored for absorption with A_{254} using an optical unit.

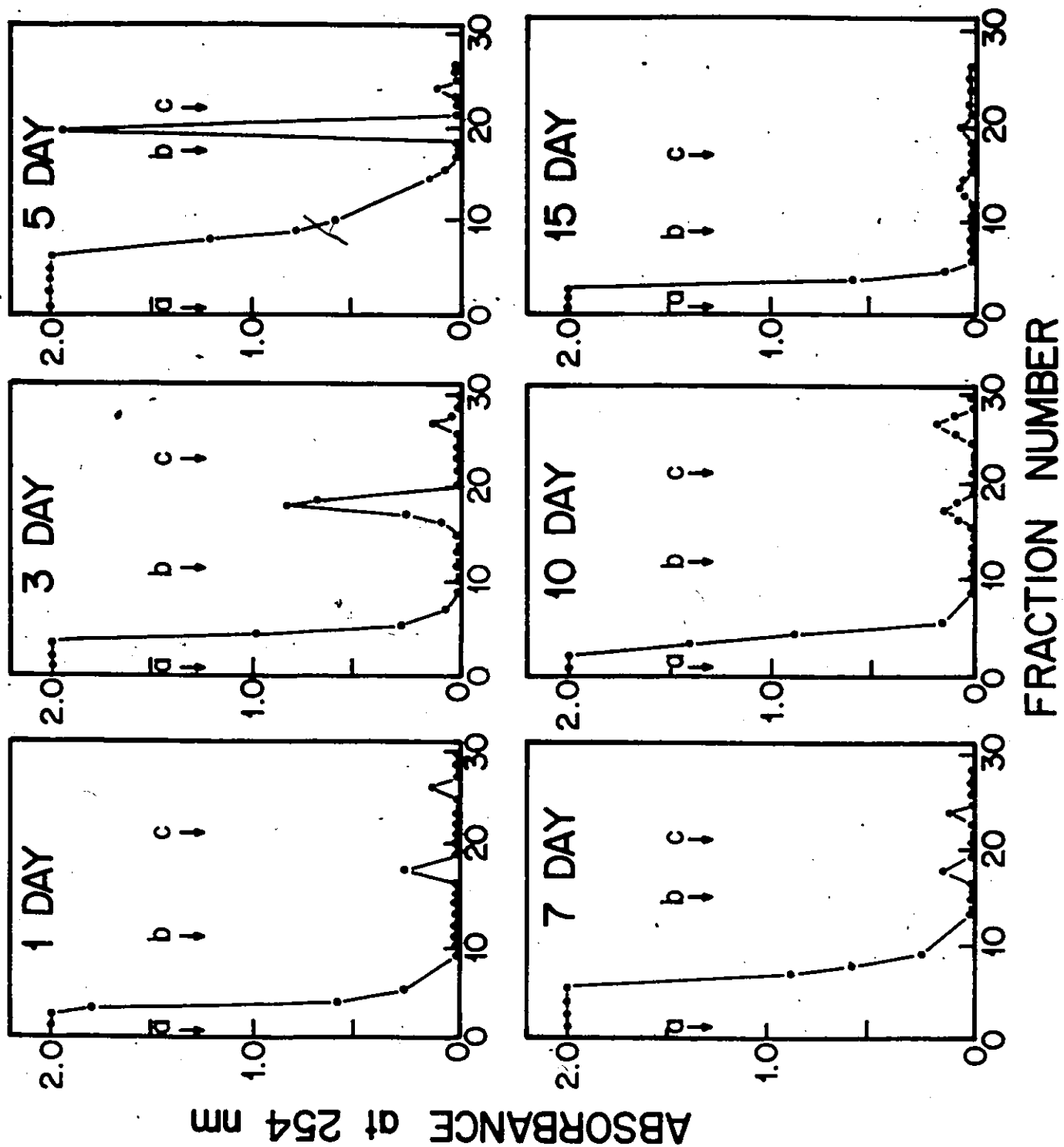


Figure 18

Fractionation of RNA from polysome pellets of 1 day soybean cotyledons by oligo. (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A_{254} units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

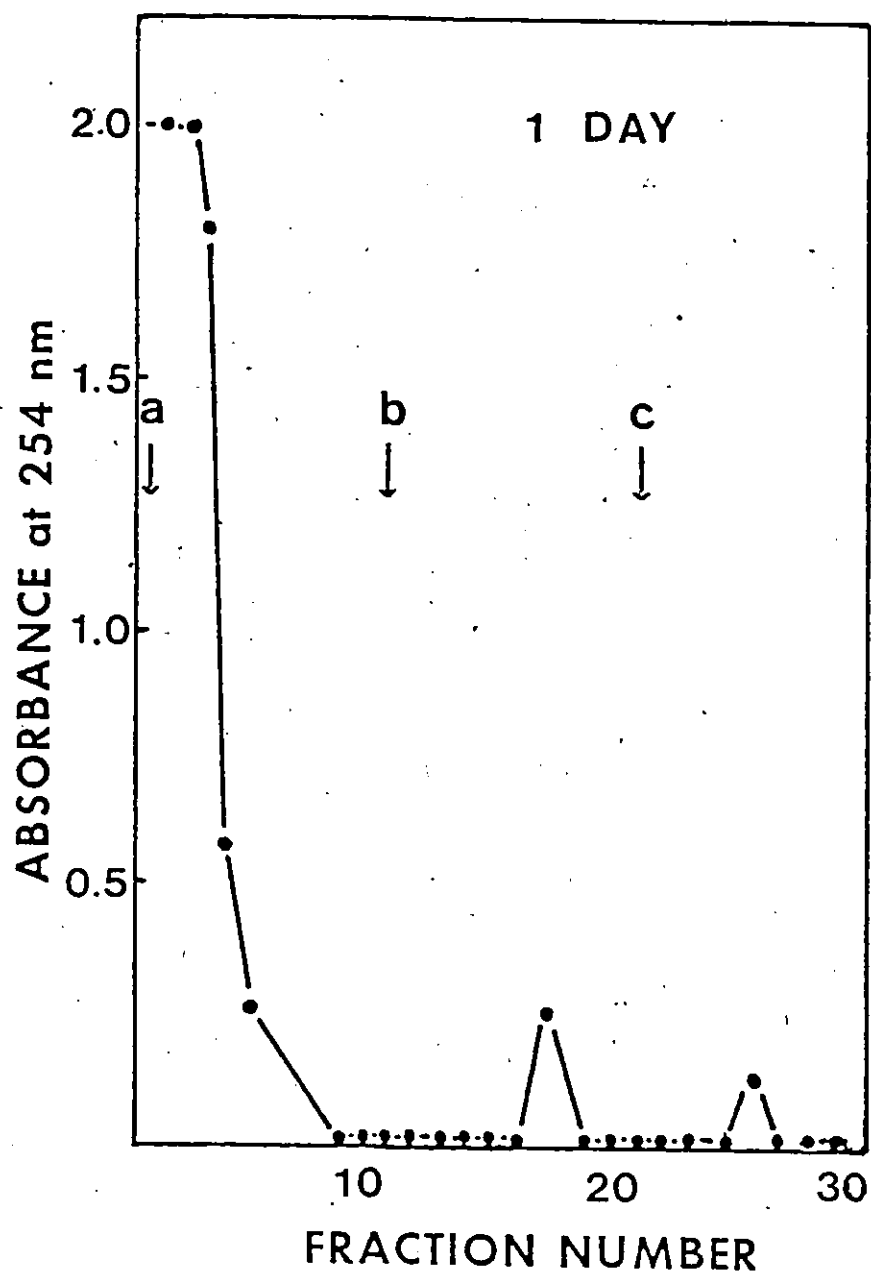


Figure 19

Fractionation of RNA from polysome pellets of 3 day soybean cotyledons by oligo (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A_{254} units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

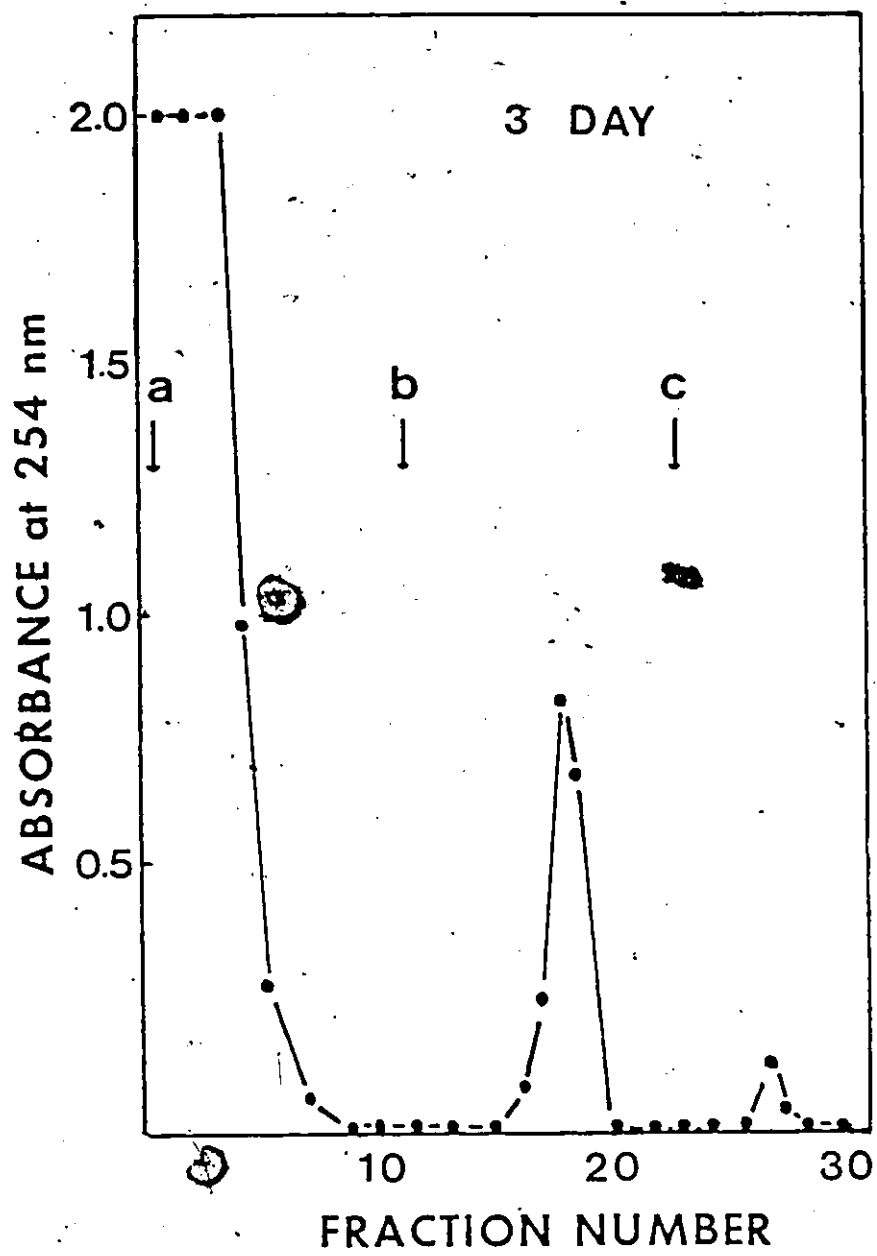


Figure 20

Fractionation of RNA from polysome pellets of 5 day soybean cotyledons by oligo(dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A₂₅₄ units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

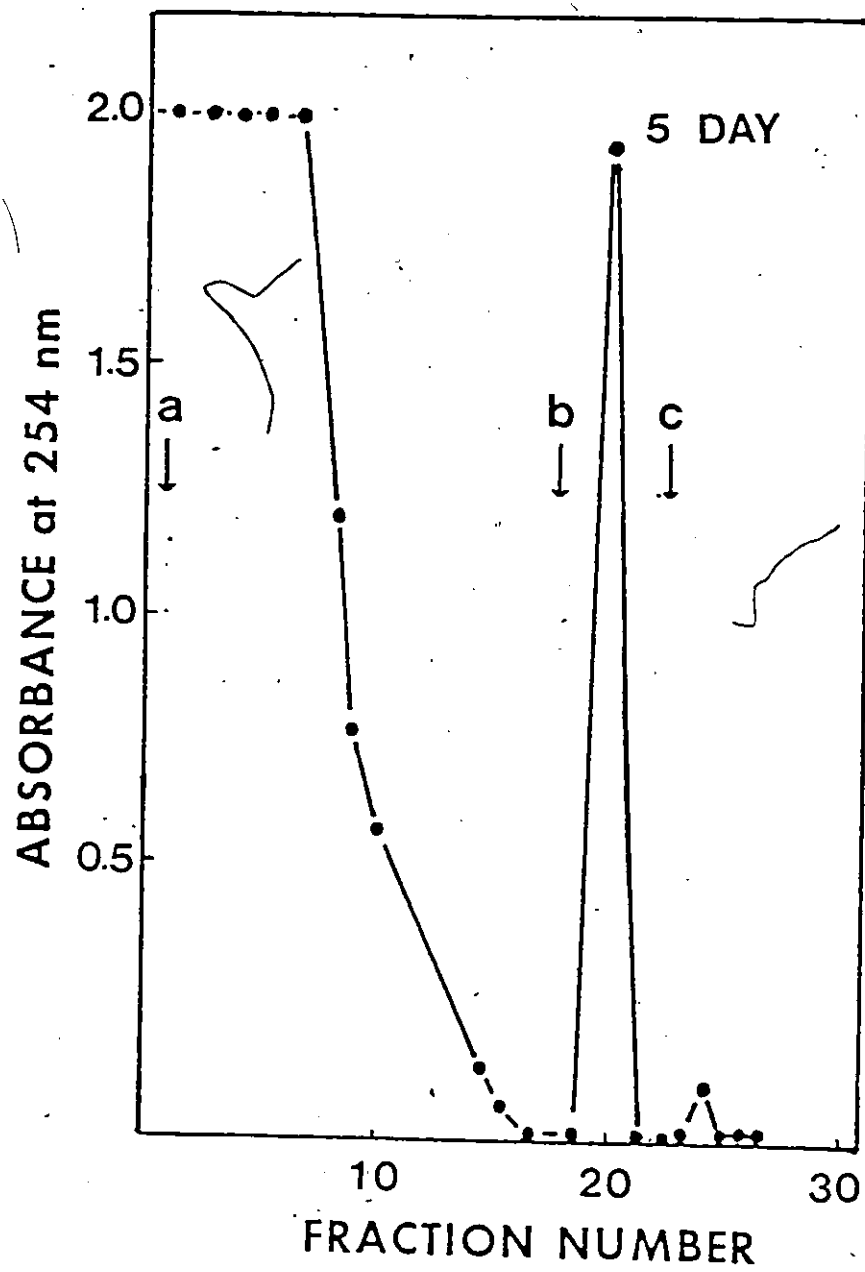


Figure 21

Fractionation of RNA from polysome pellets of 7 day soybean cotyledons by oligo (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A₂₅₄ units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

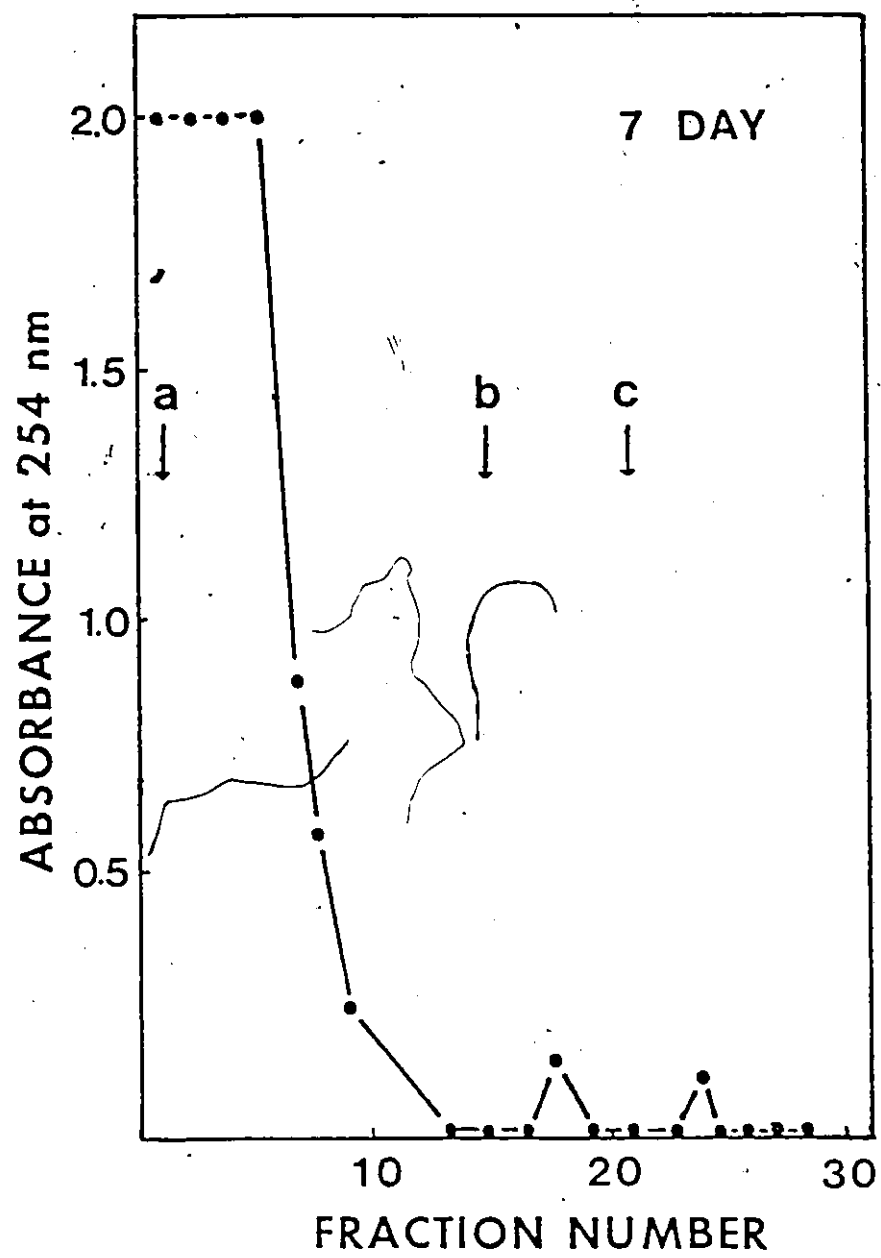


Figure 22

Fractionation of RNA from polysome pellets of 10 day soybean cotyledons by oligo (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A_{254} units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

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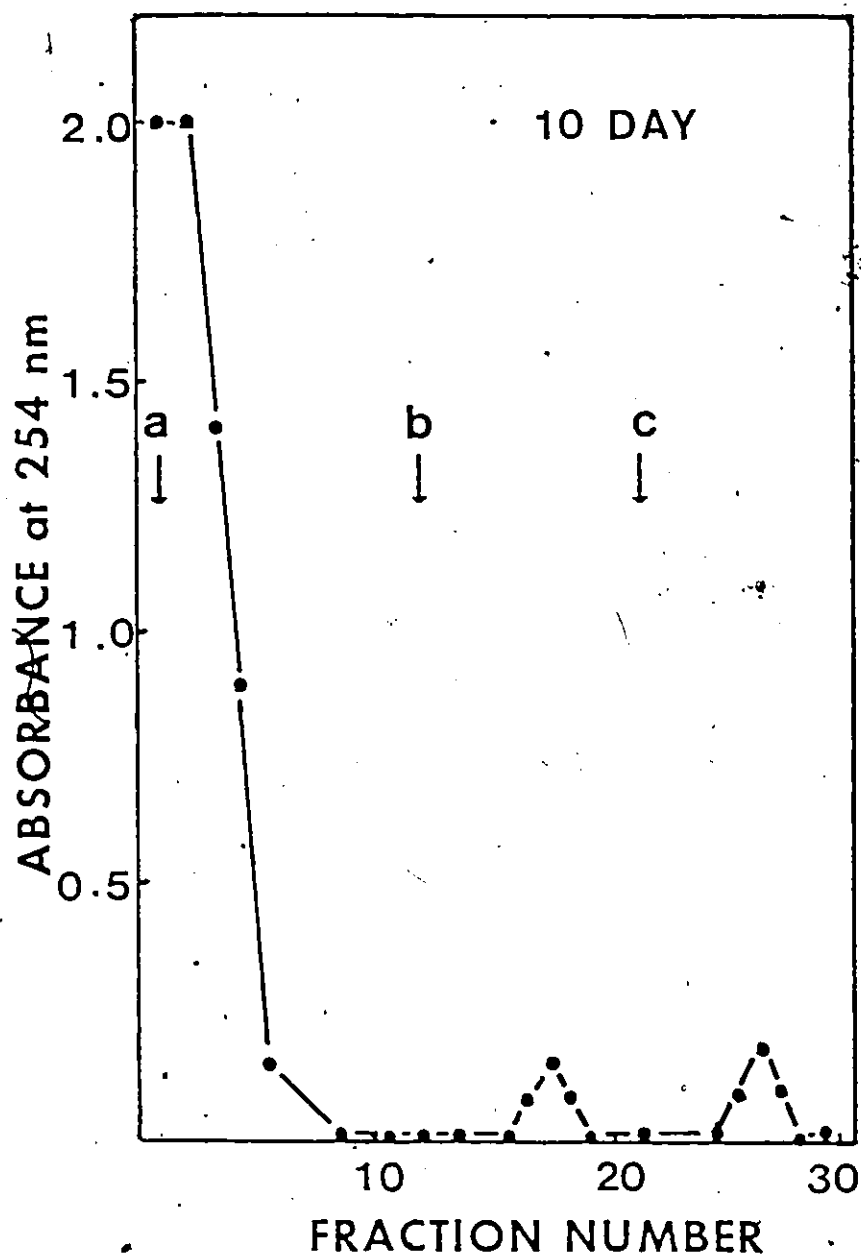


Figure 23

Fractionation of RNA from polysome pellets of 15 day soybean cotyledons by oligo (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A_{254} units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

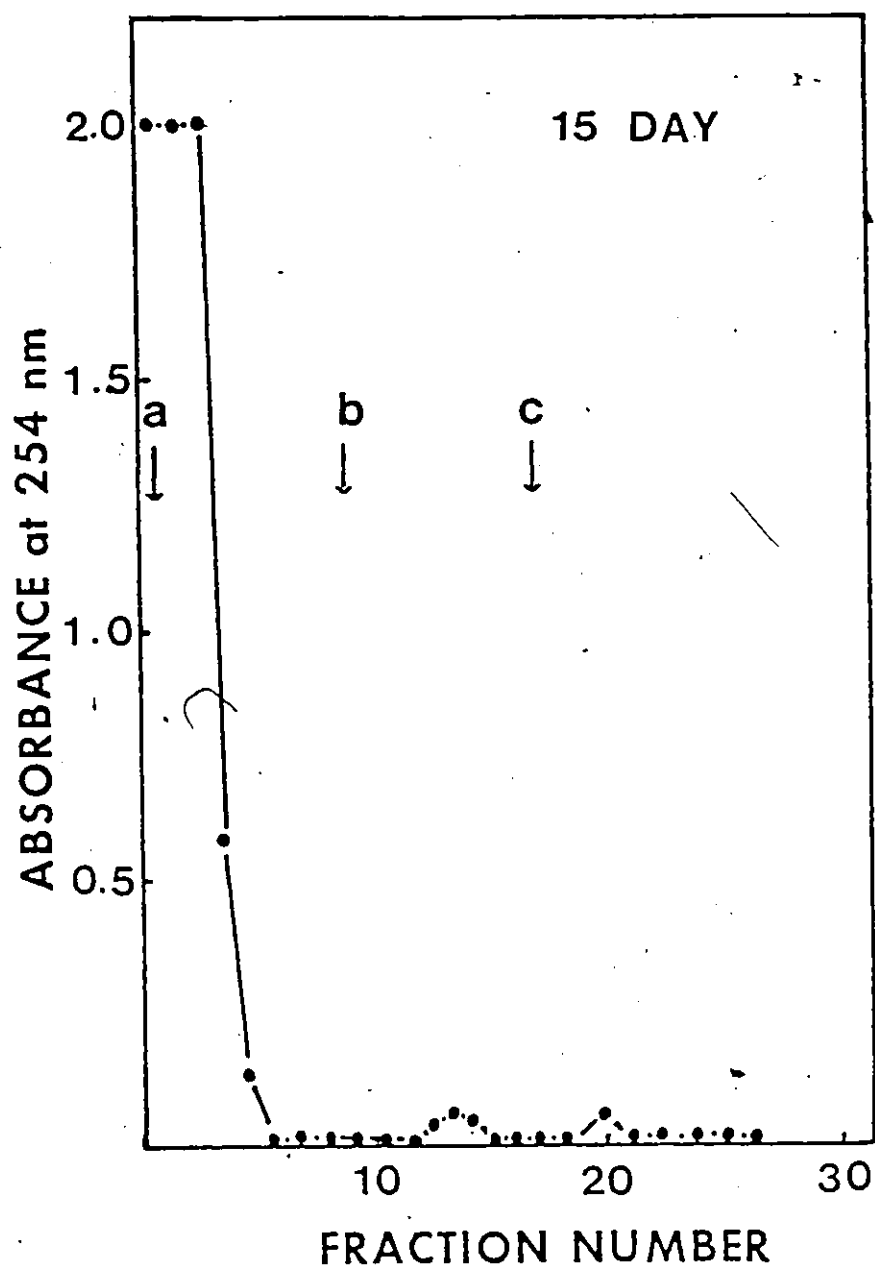


Figure 24

Fractionation of RNA from polysome pellets of 20 day soybean cotyledons by oligo (dT) cellulose chromatography.

Following phenol extraction of the RNA from the polysome pellets, 160 A_{254} units of RNA were applied to an oligo (dT) cellulose column. The buffers were changed to elute the different fractions.

- a. 0.5 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- b. 0.1 M KCl-Tris buffer (0.01 M Tris HCl, pH 7.5)
- c. 0.01 M Tris HCl, pH 7.5

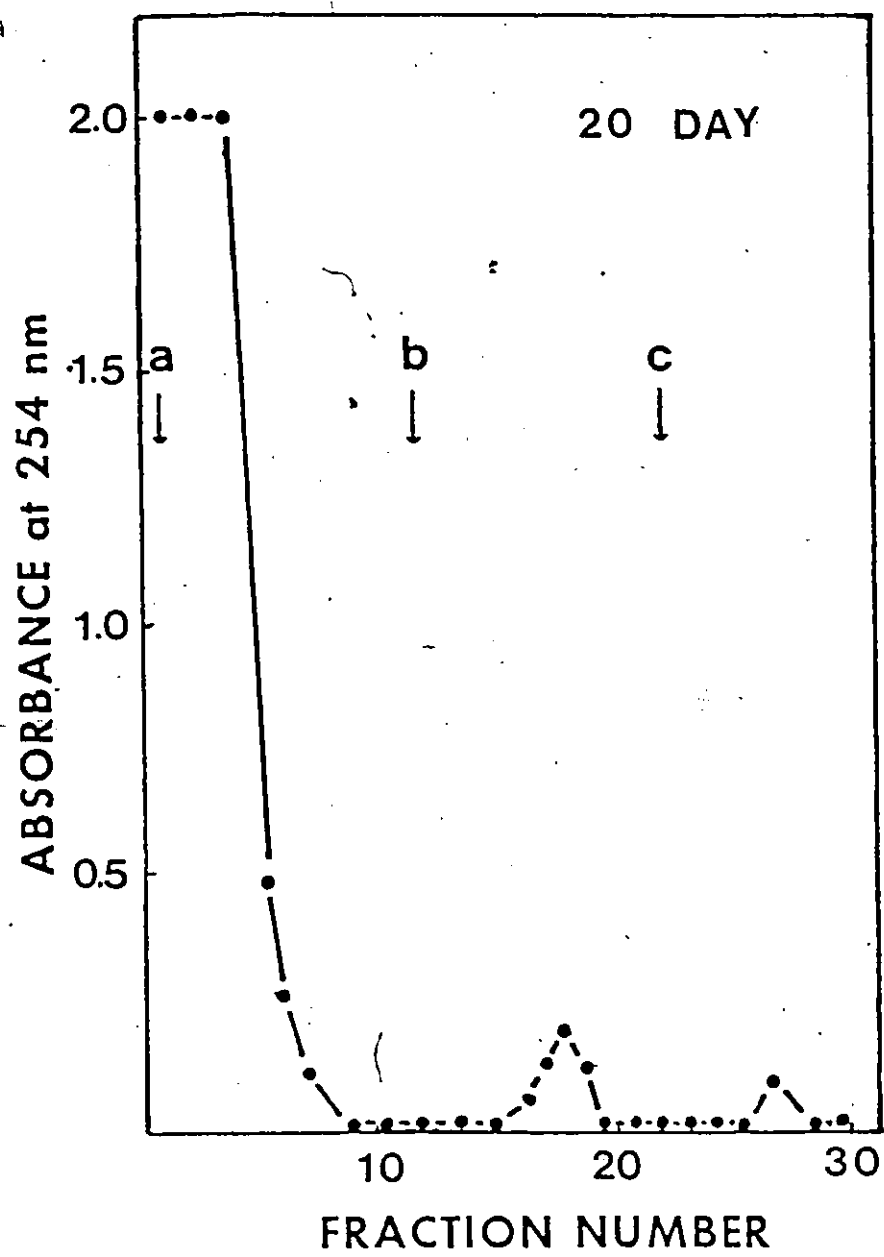


TABLE 2: ^3H -Poly U HYBRIDIZATION OF RNA FRACTIONS
OBTAINED BY OLIGO (dT) CELLULOSE CHROMATOGRAPHY

AGE OF COTYLEDONS (DAYS)	^3H -Poly (U) Bound (cpm)		
	Peak 1	Peak 2	Peak 3
1	293	563	1656
3	228	554	3095
5	517	211	1549
7	303	244	1272
10	220	158	3026
15	189	230	82
20	237	97	107

RNA was phenol extracted from the polysome pellets and 160 A_{254} units (8 mg) were loaded onto an oligo (dT) cellulose column. The buffers were changed to elute the three fractions. From each fraction aliquots of 0.1 A_{254} units were added to 50 μl of a Poly U salts mixture (as described under 'Methods'). The volume of the reaction mixture was brought to 0.5 ml with water and incubated for 15 minutes at room temperature. Following incubation 20 μl of RNase (0.3 mg/ml pancreatic RNase A, 20 μl /ml RNase T_1) was added and incubation continued for a further 30 minutes. The reaction was terminated by the addition of 1 ml of cold 15% TCA with 100 μg of yeast RNA as carrier. The precipitate was collected on filters, washed with cold 5% TCA, dried, and placed in scintillation vials for counting.

hybridized in vitro to unlabelled mRNA from the three peaks.

Results presented in Table 2 indicate that the amount of ^3H -Poly (U) hybridized to RNA with the highest incorporation of radioactivity was in the Poly A (+) mRNA fraction of Peak 3. Low incorporation in peaks 1 and 2 indicates a peak 3 binding specificity with Poly (U). When ^3H -Poly (U) hybridization assay was performed using mRNA preparation from soybean cotyledons of different ages, some interesting observations were made. Based on the incorporation of radioactivity maximum hybridization occurred in mRNA samples from 3 and 10 day old cotyledons. This is a very interesting observation since this result corresponds with the occurrence of heavy polysomal activity for the same ages (Figures 5,8). Hybridization studies of mRNA assays for 15 and 20 day cotyledons show very little incorporation (Table 2) which allows us to conclude that with age there is a loss in Poly A (+) RNA as well as a decrease in polysomal activity.

Template Activity of Peaks Obtained by Oligo (dT) Cellulose Chromatography

While the results obtained by ^3H -Poly (U) hybridization indicate the presence of Poly A (+) mRNA obtained in peak 3 and its absence in Peaks 1 and 2, it was necessary to demonstrate the template activities of the three RNA fractions as designated by Gordon and Payne (1976). It was observed as shown in Table 3 that both Poly A (-) and Poly A (+) mRNA demonstrate template activity with peaks at 3 and 10 days following germination. This corresponds to peak activity in hybridization of ^3H -Poly (U) and distribution of heavy polysomes observed at these ages. The RNA from Peak 1 also shows some template activity, however

such activity at all ages is lower than the activities of Peaks 2 and 3. It is possible that this high activity is in part due to the high concentration of Peak 1 RNA (500 ug/ml) supplied to the system, relative to the much lower amounts (5 ug/ml) from Peaks 2 and 3. Since we were interested in only peaks 2 and 3, no effort was made to adjust the RNA concentration in the Peak 1 fraction. However, if one simply dilutes the Peak 1 value by ten, the average CPM ranges between 200-300 which is almost background. This in no way alters our conclusions with respect to Peaks 2 and 3. Similar results were also reported by Gordon & Payne (4) where they observed template activity in the Peak 1 fraction which was attributed to Poly A (-) mRNA that did not bind to the oligo (dT) column, or mRNA lacking Poly A or with Poly A tracts too short to bind to the oligo (dT) or finally, to the template activity of RNA fragments which would elute in Peak 1.

Amino Acid Incorporation in Ribosomes and Supernatant Factors of Different Ages

The data presented in Table 4 shows the effect of age of purified supernatant factors on amino acid incorporation in ribosomes of different ages from soybean cotyledons. The 1 day system was most efficient in its ability to incorporate ^3H -phenylalanine. This efficiency decreased with both the increasing age of the supernatant factors supplied and the increasing age of the ribosomes, indicating a contribution by both components of the in vitro system. Again, as was the case with the template activity of the message, incorporation increases or remains the same when 10 day ribosomes are supplied to the system, as compared to the level when 7 day ribosomes are used.

TABLE 3: TEMPLATE ACTIVITIES OF RNA FRACTIONS OBTAINED
BY OLIGO (dT) CELLULOSE CHROMATOGRAPHY

AGE OF COTYLEDONS (DAYS)	INCORPORATION OF ^3H LEUCINE (CPM)		
	RNA 500 ug/ml	RNA 5 ug/ml	
	Peak 1	Peak 2	Peak 3
1	2424	2957	3795
3	3295	3362	4418
5	915	1111	1935
7	2276	4990	3644
10	3355	4717	5952
15	2823	4407	4733
20	2473	2781	3441

RNA was phenol extracted from the polysome pellets and 160 A₂₅₄ units (8 mg) were loaded onto an oligo (dT) cellulose column. The buffers were changed to elute the three fractions. The concentration of RNA eluted in Peak 1 was adjusted to 500 ug/ml. The concentration of RNA obtained from peaks 2 and 3 were adjusted to 5 ug/ml. The cell free protein synthesis assay was carried out in a 10 ul reaction mixture (5 ul of wheat germ S₃₀ fraction, 2 ul RNA and 3 ul of a mixture containing essential organic and inorganic compounds including ^3H -leucine). Following 30 minute incubation, aliquots were pipetted onto filter discs, washed in TCA, dried and radioactivity determined.

TABLE 4: THE EFFECT OF AGE OF PURIFIED SUPERNATANT
FACTORS ON AMINO ACID INCORPORATION IN
RIBOSOMES OF DIFFERENT AGES FROM SOYBEAN
COTYLEDONS

AGE OF PURIFIED FACTORS (DAYS)	<u>AGE OF RIBOSOMES/DAYS/CPM</u>					
	1	3	5	7	10	15
1	5302	5185	4882	4800	3945	3770
3	5140	4570	4155	3635	3725	2562
5	3541	3325	2936	2562	2795	2410
7	2520	2480	2440	2052	2165	2010
10	2090	2030	1890	1810	1710	1530
15	1990	1940	1800	1790	1700	1460

The effect of age of purified supernatant factors on amino acid incorporation (^3H -phenylalanine) in ribosomes of different ages from soybean cotyledons. Dialyzed supernatant (105,000 xg) fractions were separated on DEAE columns and fractions were collected as described under Methods. A 125 μl reaction mixture contained: 40 μg ribosomes, 50 μl purified factors (20 μl Factor C, (30 $\mu\text{g}/\text{ml}$) 20 μl Factor D (30 $\mu\text{g}/\text{ml}$), 10 μl $\text{T}_1 + \text{T}_2$, (20 $\mu\text{g}/\text{ml}$) and 50 μl reaction cocktail including 10 $\mu\text{g}/\text{ml}$ ^3H -phe and 200 $\mu\text{g}/\text{ml}$ Poly U. The reaction was incubated at 37°C for 60 minutes and then stopped by the addition of TCA. Following filtration on glass filters and the radioactivity determined using a Beckman Scintillation counter.

DISCUSSION

The soybean cotyledon is the primary storage organ of the seed, which contains protein and fat at levels of 31 and 22% by weight, respectively. Extraction of polyribosomes from the cotyledons of Glycine max required the combination of high ionic strength and high pH as well as the presence of a chelator and detergent (RNase inhibitor) in the extraction buffer. Jackson and Larkins (1976) found that difficulties in recovering polysomes from mature leaf tissue may have been due to causes other than RNase activity such as polysome precipitation due to the accumulation of divalent cations. High levels of Ca^{2+} in extraction media have been found to cause the aggregation of polysomes in expanding leaves. This Ca^{2+} accumulation also stimulates the degradation of pea polysomes possibly by activating a heat labile nuclease. The addition of EGTA (ethylene glycol-bis-(B-aminoethylether)-tetraacetic acid), a cation chelator with a high affinity for Ca^{2+} enhances the polysome yield from both expanded and unexpanded tobacco leaves. Its addition to the extraction buffer used in this report allowed for the recovery of polysomes, this recovery being further enhanced by the addition of sodium desoxycholate. The inability to recover large polysomes at various cotyledon ages does not appear to be due to the formation of aggregates and hence the subsequent loss of the heavy polysomes, but rather to their total absence in these tissues. The addition of protease K, which would dissociate

such aggregates, to the resuspended pellet had no effect on the resultant profile. It is therefore concluded that heavy polysomes are present only at selected times during the ageing of the cotyledon.

Conditions necessary for the extraction of membrane bound ribosomes (i.e. the absence of detergent such as sodium desoxycholate) led to no recovery of membrane bound polysomes and a very poor yield of free polysomes. This detergent requirement when working with the cotyledons of Glycine max may be based on the high concentration of fat which is present in the tissue and needs to be removed. It is also possible that no membrane bound polysomes exist (at least not in recoverable quantities) in the dark grown tissue. Payne and Boulter (1969) studying the dispersion of free and membrane bound ribosomes in the cotyledons of broad beans conclude that the membrane bound ribosomes of the germinating cotyledon arose de novo in response to the requirement for the synthesis of new protein. They also concluded that free and membrane bound ribosomes were responsible for the synthesis of different groups of proteins. Our inability to isolate membrane bound ribosomes from the soybean cotyledons, led us to speculate that perhaps the proteins associated with membrane bound ribosomes arose largely in response to the photosynthetic function of the cotyledon or for storage protein synthesis. Treffry et al., (1967) studied the structural changes in the cotyledons of Glycine max, during early germination (grown in the dark). They could not detect membrane bound ribosomes in the germinating cotyledon before 3 days. This time span corresponds well with the time of onset of greening in the coty-

ledons, with a maximum in both greening and the quantity of membrane bound ribosomes reached at 7 days that may partially account for our inability to recover membrane bound polysomes from the cotyledons grown only in the dark. The ability of oligo (dT) cellulose to accurately separate mRNA fractions has been criticized by Bantle et al., (1976) where non-specific types of binding occur due to the formation of aggregates between rRNA and Poly (A) RNA. These aggregates can be disrupted if the RNA sample is first treated with heat and DMSO. Treated and untreated RNA samples were fractionated by oligo (dT) cellulose chromatography. There was no difference in the elution patterns or amounts of the various RNA fractions. Bantle et al., did not attempt to translate the treated RNA so its efficiency as template is questionable. As the heat-DMSO treatment appeared to have no effect on the separation of cotyledon RNA, the RNA samples were not subjected to treatment.

Polysomes isolated by sucrose density gradient centrifugation show that a higher percentage of heavy polysomes are present during the early stages of germination which decrease with age, and a corresponding increase in medium and light polyribosomes and monosome populations occur. The high percentage of messenger RNA recovered at 3 days decreases with age with respect to the amounts of light and heavy ribosomal RNA recovered by gradient centrifugation. Initially the amount of Poly A (-) mRNA increases until 5 days after germination, after which both Poly A (+) and Poly A (-) mRNA tend to decrease in amount. There is one exception to this decreasing pattern in polysomes and poly A (+) RNA which occurs in 10 day old cotyledons. A

four fold increase in the percentage of heavy polysomes occurs, accompanied by an increase in the amount of mRNA relative to amounts of light and heavy ribosomal RNA recovered by gradient centrifugation. This increase in mRNA quantity is further reflected in the increase of Poly A (+) mRNA fraction isolated by oligo (dT) cellulose chromatography and by hybridization of ^3H -Poly (U) to the Peak 3 Poly A (+) mRNA.

Both the Poly A (+) and Poly A (-) mRNA fractions served as template when introduced into the wheat germ cell free system. The Poly A (+) mRNA was more efficient however with enhanced activities at 3 and 10 days. Ribosomes and supernatant factors showed an age dependence. Younger ribosomes and purified supernatant factors were more efficient in the incorporation of radioactive amino acids than those from older tissues. There was some recovery demonstrated by 10 day ribosomes and factors whose activities were higher or equal to those found in 7 day tissues.

Results obtained here lend support to a previous observation by Pillay (1977) dealing with the protein synthesizing ability in ageing soybean cotyledons, where it was shown that a 1 day system was most efficient in its ability to incorporate ^3H -phenylalanine which then decreased with the increasing age of the tissue. However, this decline in efficiency was reversed at 10 days, where 10 day old ribosomes with 10 day factors was as efficient as the 7 day homogeneous system. A combination of 10 day ribosomes with 7 day factors was more efficient than the 7 day homologous system. Based on these results, changes in

incorporating ability have been attributed to the changes in both ribosome and supernatant factors. Somewhat similar results were obtained by Treffry et al., (1967) for the incorporation of ^{14}C -glycine into protein, in light grown soybean cotyledons. While the reliability of comparison of light and dark grown cotyledons is questionable, it allows us to examine the sequence of events in ageing cotyledons grown either in light or dark. Treffry et al., (1967) also found higher protein synthetic activity in 1 day cotyledons and then another increase in 10 day cotyledons. They report peak activity at 10 days for several biochemical and metabolic events with concurrent peaks in fresh weight, respiration rate, protein synthesis and chlorophyll a & b content. In addition, at 10 days the vacuoles are empty and lipid droplets are lost from the cytoplasm.

After 10 days degenerative changes were seen in more cells of the cotyledon; disruption and breakdown of the tonoplast, plastid membrane, mitochondria and nucleolus. The nuclear membrane and stroma degenerated and the cytoplasmic content was lost. During this period protein synthesis became minimal, the chlorophyll and protein content of the cotyledon was greatly reduced and there was a sharp fall in fresh weight.

Not all of these metabolic changes may contribute to the results demonstrated here, from the cotyledons of dark grown seeds. Further investigation is required to determine the metabolic activity centered around 10 days after germination which would account for the cotyledon's increased capacity to synthesize protein.

It should be pointed out here that the amount of Poly A (-) mRNA, recovered from oligo (dT) chromatography increases in parallel to higher amounts of light polysome populations in the ageing cotyledons. Similarly, the amounts of Poly A (+) mRNA increases correspond to higher levels of heavy polysome population. These results tempt us to tentatively conclude that two or possibly three classes of mRNA, each associated with a specific polysome population appear during different periods of ageing of the cotyledons. It is also possible that these two polysome populations and the mRNA species control the synthesis of different types of proteins. Further work to elucidate the existence of different polysome populations, mRNA species and in vitro translation products is necessary.

SUMMARY

Polysomes and their associated ribonucleic acids were extracted from ageing soybean cotyledons. Density gradient analysis showed that the ribosomes of older cotyledons were present largely as monosomes and light polysomes while younger cotyledons possessed a high proportion of heavy polysomes. Increased polysomal and mRNA activities were observed in 3-5 day and again 10 day old cotyledons. The presence of polyadenylated mRNA was determined by oligo (dT) cellulose chromatography and confirmed by hybridization with ^3H polyuridylic acid.

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