

Ribonucleic Acid and Protein Metabolism in Pea Epicotyls¹

I. THE AGING PROCESS

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ABSTRACT

Aging of actively growing, etiolated pea *Pisum sativum* L. var Alaska plants was initiated by removing the plumules of plants in the third internode stage, and applying lanolin to the cut apices of otherwise intact plants. During the subsequent 4-day aging period, several degenerative events occurred in this apical 10-millimeter region. Ribosomal RNA and messenger RNA contents declined, polyribosomes disaggregated, and the protein synthesizing capacity of the polysomes decreased.

Two-dimensional, silver-stained protein patterns revealed that aging altered the relative amounts of specific cellular proteins accumulated *in vivo*. In addition, polypeptide patterns generated by cell-free translation of total and polysomal RNA, isolated from unaged and aged tissues, showed major modifications. More than 200 spots could be resolved by two-dimensional gel fluorography of translation products using RNA from fresh tissues. Of these 200 spots, about eight appeared or increased when total RNA from aged tissue was used, and about 58 disappeared or declined. When polysomal RNA from aged tissue was used as template, about 12 spots appeared or increased, whereas about 64 disappeared or decreased. In general, the products which increased after aging were lower molecular weight and those that decreased were higher molecular weight.

Aging of etiolated pea stem tissue is initiated by removal of the hook and plumule and application of lanolin to the cut end. During a 4-d period, this aging process changes actively growing tissue into nongrowing tissue presumably because of a depletion of endogenous auxin (7).

The terms aging and senescence are frequently used synonymously, but the system described here is not truly senescing. Here, aging is more aptly termed a maturation process, in contrast to senescing tissues which are undergoing a set of processes leading to death (13). The term 'aging' is also used when describing the aerobic activation of plant storage tissues (9). Since the latter is a rejuvenation event, aging in that context means nearly the opposite of what is defined here.

Because this aged pea system is relatively inactive metabolically, it has served as a control to study auxin effects (*e.g.* 3-5, 19) and wounding effects (6, 18), but little is known about the aging process itself. A few events which have been shown to

happen during aging are: a decline in the levels of soluble protein and nucleic acid and an increase in β -1,3-glucanase activity (5), a slight decrease in cellulase activity (2), and a disaggregation of free polyribosomes (4).

This study was initiated to provide a more detailed description of the decline in macromolecular processes which occur during aging as a prerequisite to understanding the reversal of these processes brought about by wounding (18) and by auxin treatment (19).

MATERIALS AND METHODS

Growth and Treatment of Plants. Pea seeds (*Pisum sativum* L. var Alaska) were soaked for 20 min in 10% (v/v) Clorox and then allowed to imbibe in tap water overnight. The seeds were sown in moist vermiculite and placed in a dark room for germination and seedling growth. The seedlings were watered twice, on day 3 and day 8. On the 8th d, the seedlings, which had third internodes longer than 10 mm, were either harvested (zero time samples) or treated for the aging process. Aging was initiated by excising the hooks and plumules and applying lanolin to the cut apex. The seedlings were aged for up to 4 d. At various times during the aging period, the plants were harvested and the apical 10 mm used for experimental analyses. All manipulations were carried out under dim green light.

Isolation of Polyribosomes. Polyribosome isolation was performed according to Larkins and Davies (11) with certain modifications. Apical 10-mm segments were ground in a mortar with 5 to 10 volumes of buffer A (0.2 M Tris-HCl, pH 8.5; 0.2 M sucrose; 50 mM KCl; 25 mM MgCl₂). The homogenate was strained through nylon cloth and the filtrate was centrifuged at 1000g for 5 min. The supernatant was made 2% (v/v) with Triton X-100, incubated for 10 min, and centrifuged at 27,000g for 5 min. The supernatant (4 ml) which contained the total population of polysomes (*i.e.* free and membrane-bound) was layered onto a 1-ml sucrose pad of 50% (w/v) sucrose in buffer B (50 mM Tris-HCl, pH 8.5; 20 mM KCl; 10 mM MgCl₂) and centrifuged for 3 h in a Beckman SW50.1 rotor at 300,000g. The pellet containing total ribosomes was rinsed with buffer B and frozen at -80°C until needed.

For polysome profile analyses, the pellets were resuspended in buffer B and 0.2 ml layered onto a 4.8-ml gradient of 15 to 60% (w/v) sucrose in buffer B. All manipulations were performed at 4°C. These gradients were centrifuged in a SW50.1 rotor for 50 min at 300,000g and scanned at 254 nm using an ISCO UA-5 monitor. The polysome profiles were used to calculate the relative amounts of subunits, monosomes, small polysomes, large polysomes, and mRNA by measuring areas under the peaks (4).

Extraction of RNA for Assaying rRNA Content. RNA was extracted from whole tissue segments using GPS⁴ as described

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⁴ Abbreviation: GPS, buffer composed of 0.1 M glycine, 0.3 M NaCl, 50 mM K₂HPO₄, pH 9.4.

by Larkins and Davies (11). The extract (0.2 ml) was layered onto 4.8-ml 7.5 to 30% (w/v) sucrose gradients in GPS and centrifuged at 300,000g for 4 h at 1°C in a SW50.1 rotor. Gradients were scanned at 254 nm and rRNA content measured from the area under the small and large rRNA peaks.

Extraction of Total and Polysomal RNA for *In Vitro* Protein Synthesis. RNA was extracted as described by Harris and Dure (8) with the following modifications. For total RNA isolation, pea stem segments were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. Five volumes of RNA extraction buffer (0.1 M Tris-HCl, pH 7.6; 1 mM Na₂ EDTA; 0.5% [w/v] SDS; 0.1 M NaCl) were quickly added and the tissue ground again at 4°C. An equal volume of phenol (buffer-saturated):chloroform:isopentyl alcohol (25:24:1) was added immediately, and the mixture was shaken for 20 min at room temperature. All further manipulations were carried out at room temperature unless otherwise indicated. After phase separation, the phenol phase was re-extracted with 0.5 volumes of re-extraction buffer (0.1 M Tris-HCl, pH 9.0; 1 mM Na₂ EDTA; 0.5% [w/v] SDS). The aqueous phases were combined and re-extracted with an equal volume of chloroform until no interphase remained. The aqueous phase was made 0.2 M with sodium acetate (pH 5.2) and the RNA precipitated with 2 volumes of absolute ethanol at -20°C overnight. The precipitate was washed three times with 3 M sodium acetate (pH 6.0) at 4°C to remove DNA and small mol wt RNA, resuspended in 0.1 M sodium acetate (pH 6.0), and precipitated with 2 volumes of ethanol overnight at -20°C. After washing with 70% (v/v) ethanol at 4°C, the RNA was resuspended in sterile, distilled H₂O. RNA was purified from polysomes in the same way, except that polysome pellets were resuspended directly in the RNA extraction buffer.

The concentration of RNA was determined using a Beckman DB spectrophotometer, assuming 20 A₂₆₀ units were equivalent to 1 mg/ml RNA. The 260/280 nm ratios were calculated to determine the purity of the RNA and were always close to 2.0.

The integrity of the RNA was determined by analysis of rRNA on GPS-sucrose gradients. RNA was heated to 65°C for 5 min, cooled rapidly, and layered on the gradients. All glassware and solutions were autoclaved or heat sterilized prior to use.

Extraction and Measurement of Unlabeled Protein. Extraction of unlabeled protein accumulated *in vivo* was performed according to Van Etten *et al.* (21) with the following modifications. One g pea stem segments was ground in a mortar with 20 ml extraction buffer (0.7 M sucrose; 0.5 M Tris; 30 mM HCl; 50 mM EDTA; 0.1 M KCl; 2% [v/v] mercaptoethanol). After a 10-min incubation at 4°C, the homogenate was strained through nylon cloth and an equal volume of water-saturated phenol added. This was shaken for 10 min at room temperature, followed by centrifugation at 6000g for 10 min at room temperature to separate the phases. The phenol phase was re-extracted with an equal volume of extraction buffer and the re-extracted phenol phase was precipitated with 5 volumes of 0.1 M ammonium acetate in methanol at -20°C overnight. The precipitate was washed three times with 0.1 M ammonium acetate in methanol and once with 100% acetone. The pellets were air dried and resuspended in lysis buffer (16).

Protein was measured by the Coomassie blue protein quantification method described by Kochert (10) and compared to values given by BSA standards, which were also dissolved in lysis buffer.

[³H]Poly(U)-Poly(A) Hybridization. [³H]poly(U) (Amersham, 500 mCi/mmol of nucleoside residue) was hybridized to poly(A) in polysomes according to Wilt (23) with the following modifications. The reactions were performed in a final volume of 1 ml of hybridization buffer (10 mM Tris-HCl, pH 7.6; 0.3 M NaCl) containing 0.2 A₂₆₀ units of polysomes and 0.0625 μCi (39 ng) [³H]poly(U). The mixture was incubated at 45°C for 15 min,

cooled to room temperature, and RNase A added to a final concentration of 20 μg/ml. This mixture was incubated for an additional 20 min to cleave any single stranded RNA. The hybrids were precipitated on ice for 30 min with an equal volume of 20% (w/v) TCA containing 100 μg unlabeled, carrier RNA. The mixture was filtered through a Whatman GF/C filter and the hybrids which were retained on the filter were washed with cold 5% TCA.

Preparation of Wheat Germ Extract. The preparation of the wheat germ extract was done according to the methods of Marcu and Dudock (14) with the following modifications. Wheat germ, obtained from General Mills (Vallejo, CA) was floated on carbon tetrachloride:cyclohexane (2.5:1), filtered through a Büchner funnel until dried, weighed, and transferred to a Corex tube. Each gram of wheat germ was extracted in 2.3 ml of extraction buffer (0.1 M Hepes, pH 6.0; 50 mM K-acetate; 2 mM Mg-acetate; 5 mM DTT), stirred on ice for 5 min, centrifuged at 27,000g, and the supernatant applied to a G-25 Sephadex column (1.5 × 40 cm) equilibrated with column buffer (20 mM Hepes, pH 7.4; 5 mM Mg-acetate; 0.12 M K-acetate; 5 mM DTT). The extract was washed through with column buffer and the eluate collected. Fractions containing greater than 80 A₂₆₀ units/ml were pooled and centrifuged as before. The supernatant was divided into small aliquots and stored at -80°C.

***In Vitro* Protein Synthesis.** The conditions for *in vitro* protein synthesis were similar to those described by Marcu and Dudock (14). Reactions were typically performed in a final volume of 100 μl containing: 20 mM Hepes, pH 8.1; 80 mM K-acetate; 1

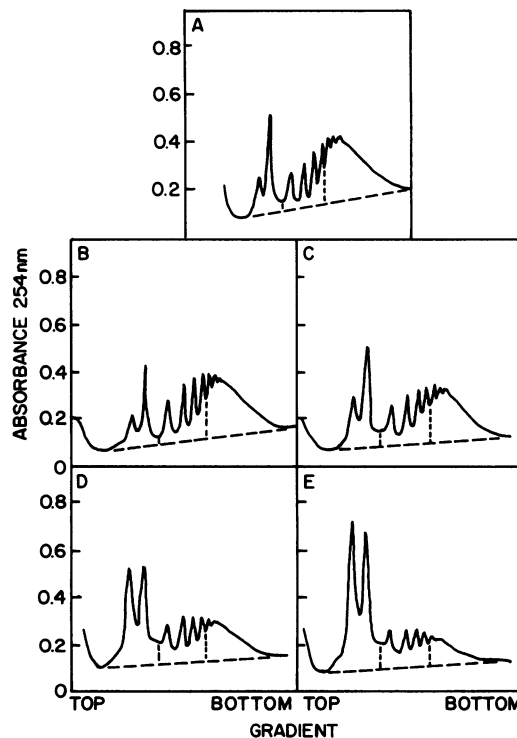


FIG. 1. Polyribosome content declines during aging. Eight-d-old etiolated pea seedlings were decapitated and the apical 10 mm either harvested immediately (zero time) or lanolin applied to the cut ends and the peas allowed to age for various lengths of time. Polysomes were isolated from 10-mm segments and 0.5 A₂₆₀ units applied to each gradient. The time of treatment (h) and number of segments used for each gradient was: A, 0, 2.2; B, 12, 3.2; C, 24, 3.5; D, 48, 8.4; E, 96, 12.0. The vertical dashed lines divide the profiles into three categories, which from the top of the gradient are: subunits plus monosomes (S + M); small polysomes (SP); and large polysomes (LP).

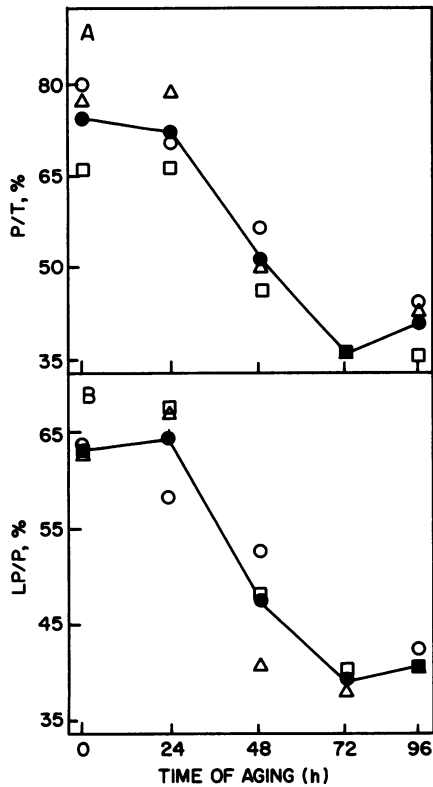


FIG. 2. Time course for the decline in polyribosomes during aging. The protocol was the same as for Figure 1. Polysome profiles were analyzed and the areas corresponding to subunits plus monosomes (S + M), small polysomes (SP), and large polysomes (LP) were measured. Values were recalculated to yield total polysomes (P), *i.e.* SP + LP, and total ribosomes (T), *i.e.* S + M + P. Data from three experiments were plotted as: A, per cent polysomes ($100 \times P/T$); and B, per cent large polysomes ($100 \times LP/P$). Symbols represent experiment 1 (Δ), experiment 2 (\circ), experiment 3 (\square) and the means for the three experiments (\bullet).

mm ATP; 20 μ M GTP; 40 μ g/ml creatine phosphokinase; 2 mM DTT; 8 mM phosphocreatine; 25 μ M of 19 unlabeled amino acids, and either 10 μ Ci [35 S]methionine (Amersham, greater than 600 Ci/mmol) or 2.5 μ Ci [3 H]leucine (Schwarz/Mann, 62 Ci/mmol). The optimal concentration for Mg-acetate was 1.5 mM with [3 H]leucine or 2.5 mM with [35 S]methionine. The optimal amount of wheat germ extract was routinely 10 μ l. The amount of RNA added varied depending on the form of the RNA. Polysomes were resuspended in water and 0.4 A_{260} units were typically added, whereas for total or polysomal RNA, 10 μ g RNA were typically added. After the reaction had proceeded for 60 min at 25°C, an aliquot was spotted onto a Whatman 3 MM filter and processed as described by Roberts and Paterson (17) to determine the amount of radioactivity incorporated. Using RNA or polysomes from fresh tissue, incorporation was typically stimulated 10- to 15-fold above background.

With the samples to be prepared for electrophoresis, the reaction was stopped on ice and diluted with an equal volume of 20 mM Hepes (pH 8.1) and centrifuged for 60 min at 100,000g at 4°C in a SW50.1 rotor. Ten volumes of 80% (v/v) acetone were added to the supernatant and the polypeptides precipitated overnight at -20°C. The precipitate was washed with 100% acetone, air dried, and resuspended in lysis buffer (16). A sample of this was spotted onto a filter and processed as above to determine the amount of radioactivity present. These samples were stored at -20°C until electrophoresis.

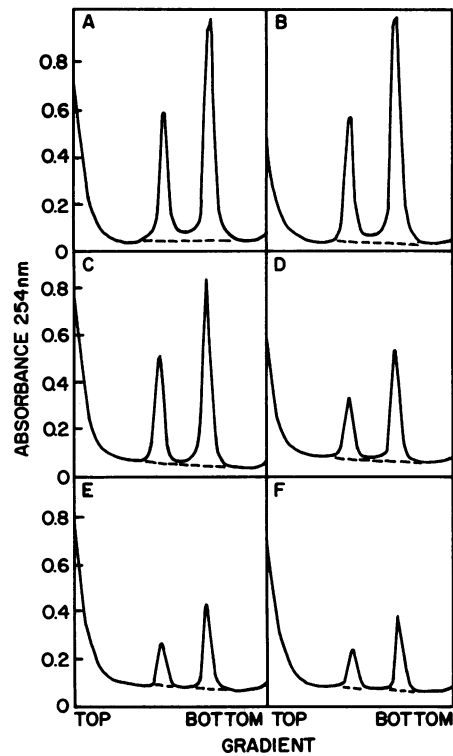


FIG. 3. Ribosomal RNA content declines during aging. RNA was extracted in GPS buffer from 10-mm apical segments at various times during aging. An amount equivalent to 0.8 segments was applied to each GPS-sucrose gradient from tissue aged for (h): A, 0; B, 12; C, 24; D, 48; E, 72; F, 96.

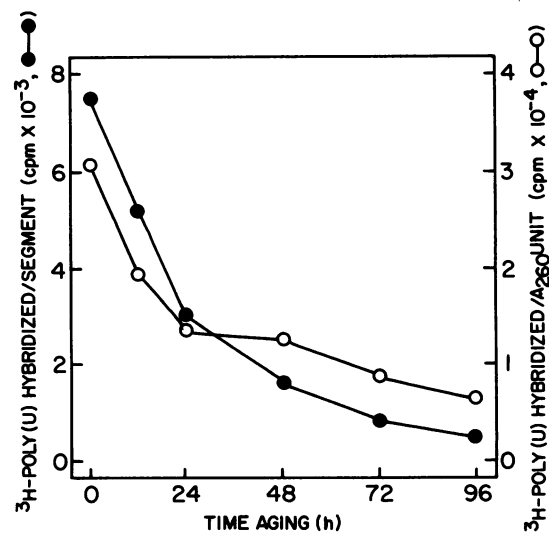


FIG. 4. Polysomal poly(A) content declines during aging. Polysomes were isolated from apical 10-mm pea stem segments at various times during aging. [3 H]poly(U) was hybridized to poly(A)RNA from 0.2 A_{260} units of total ribosomes. Results represent one typical experiment.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Two-dimensional gel electrophoresis was done according to O'Farrell (16) with slight modifications. The isoelectric focusing gels were run at 400 v for 16 h, equilibrated for 2 h in equilibration buffer, and stored at -80°C until used for the second dimension. The second dimension gels were 7.5 to 15% (w/v) polyacrylamide gradient gels. The running buffer was 25 mM Tris; 1.9 M glycine;

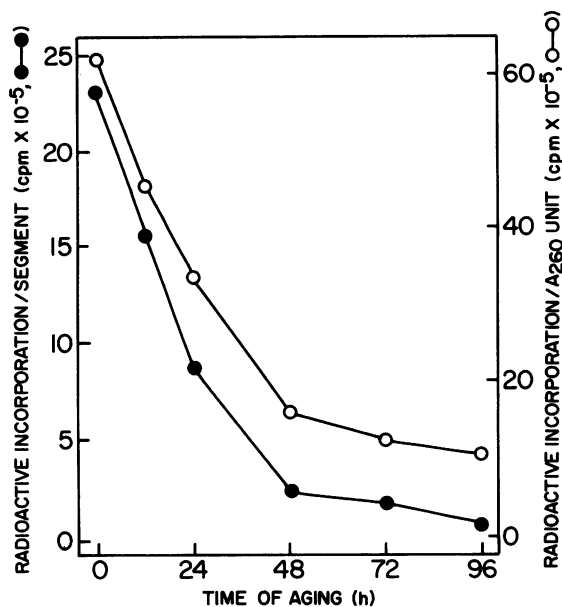


FIG. 5. Ribosome-primed *in vitro* protein synthesizing capacity declines during aging. Total ribosomes (*i.e.* subunits + monosomes + polysomes) were isolated from 10-mm apical pea stem segments aged for various times and used for *in vitro* protein synthesis. Reactions were performed in a final volume of 100 μ l containing 0.4 A_{260} units of ribosomal material and 20 μ Ci [35 S]methionine. The data were displayed as cpm/ A_{260} unit of total ribosomes (○) or as cpm/segment (●). Results from one typical experiment.

0.1% SDS; 0.08% mercaptopropionic acid. Gels were run at constant voltage, between 60 and 80 v, until the bromophenol blue reached the bottom of the gel.

Mol wt markers (Biorad) ranging from 14,400 to 92,500 D were used to assess the mol wt of the polypeptide spots (22). The pH range of the isoelectric focusing gels was determined by cutting the gels into 5-mm slices, which were placed in vials containing 1 ml degassed, distilled H₂O and soaked for 2 h prior to measuring the pH.

Silver Staining of Unlabeled Proteins. After completion of the second dimension, the gels were fixed and silver stained according to the method of Oakley *et al.* (15). Typically, the best results were obtained when 50 to 80 μ g of protein were loaded onto the isoelectric focusing dimension. Protein extractions and electrophoresis were repeated at least three separate times for each tissue type.

Fluorography of Labeled Polypeptides. Aliquots containing 750,000 cpm of *in vitro* translation products labeled with [35 S] methionine were loaded onto the isoelectric focusing dimension. Following electrophoresis of the second dimension, gels were stained in a solution of 0.1% (w/v) Coomassie brilliant blue R, 45.5% (v/v) methanol, 9% (v/v) acetic acid for 2 h. The gels were destained with two or three changes of 7% (v/v) acetic acid. If the stained patterns of unlabeled wheat germ proteins exhibited good separation, the gels were processed for fluorography using the method of Bonner and Laskey (1), except that three changes of dimethyl sulfoxide were used rather than two. The gels were exposed to pre-flashed Kodak X-Omat AR film (12) and stored at -80° C for 144 h. Experiments, using separate batches of RNA isolated at different times, were repeated on at least three occasions.

RESULTS AND DISCUSSION

Decline in Components Involved in Protein Synthesis during Aging. Figure 1 shows representative profiles of total ribosomes

Table 1. Magnitude of the Decline in Protein Synthesizing Capacity *In Vitro* During Aging Depends upon the Source of Primer

Results are the averages of three experiments \pm SD.

| Source of Primer | Protein Synthesis at Following Times of Aging | | |
|---|---|-----------------|-----------------|
| | Zero time | 96 h | 96/0 h |
| | <i>cpm</i> $\times 10^4$ /segment | | % |
| Total ribosomes ^a | 345 \pm 106 | 6.6 \pm 1.7 | 1.9 \pm 0.9 |
| Whole tissue RNA ^b | 529 \pm 84 | 51.3 \pm 12.4 | 9.7 \pm 0.5 |
| RNA from ribosomal pellets ^c | 523 \pm 43 | 35.1 \pm 5.2 | 6.6 \pm 2.1 |
| | / A_{260} unit ^f | | |
| Total ribosomes ^a | 674 \pm 123 | 85.6 \pm 14.2 | 12.7 \pm 4.0 |
| Whole tissue RNA ^b | 164 \pm 26 | 89.7 \pm 12.7 | 54.7 \pm 3.2 |
| RNA from ribosomal pellets ^c | 164 \pm 13 | 91.8 \pm 21.1 | 56.0 \pm 11.3 |
| | / A_{254} unit ^g | | |
| Total ribosomes ^d | 679 \pm 162 | 66.5 \pm 19.6 | 9.8 \pm 3.6 |
| Polysomes ^e | 910 \pm 120 | 161 \pm 38.5 | 17.5 \pm 5.8 |

^a Ribosomal pellets containing unfractionated subunits, monosomes, and polysomes.

^b RNA extracted by phenol/chloroform from whole tissue.

^c RNA extracted by phenol/chloroform from ribosomal pellets.

^d Calculated from subunits + monosomes + polysomes displayed on gradient profile.

^e Calculated from area under polysome region displayed on gradient profile.

^f Measured at 260 nm on a Beckman DB spectrophotometer.

^g Measured at 254 nm on an Isco UA-5 absorbance monitor.

extracted from apical pea stem tissue during the course of aging. The areas of the various components of the profiles (*i.e.* subunits, monosomes, small polysomes, large polysomes) were measured (Fig. 2). The results in Figures 1 and 2A show that the proportion of polysomes, which comprise approximately 75% of the total ribosomal material at zero time, was maintained during the first 24 h of aging, but then diminished to about 40% after 96 h. Similar kinetics were also seen for the proportion of large polysomes, which declined from about 65% at zero time to 40% by 96 h (Fig. 2B). By 96 h, the total ribosome content declined to about 20% ($19.2 \pm 7.4\%$) of the zero time value (data not shown). This decline in total ribosomal material in conjunction with the decline in the proportion of polysomes (Fig. 2A) caused the amount of polysomes to decline about 10% ($10.6 \pm 3.8\%$) over the 96-h aging period. The amount of polysomal mRNA, measured according to Davies and Larkins (4), where the area of the dimer was divided by 2, the trimer by 3, etc., also declined to about 10% ($11.8 \pm 4.6\%$) during the aging period.

Ribosomal RNA from whole tissue extracts was analyzed by sucrose density centrifugation and the rRNA profiles depicted in Figure 3. These profiles show that the amount of rRNA per segment was maintained during the first 12 to 24 h of aging, but dropped sharply between 24 and 48 h and declined steadily until 96 h after aging, at which time it was about 20% ($21.1 \pm 5.1\%$) of the zero time value.

The amount of polysomal poly(A)RNA was estimated by hybridization to [3 H]poly(U) and the results are displayed in Figure 4 as [3 H]poly(U) hybridized per A_{260} unit of ribosomal material and per segment at different times during aging. Both sets of data show that there was a decline in the amount of poly(A), with the most dramatic changes occurring between zero and 24 h. By 96 h of aging, the amount of poly(A) per A_{260} unit

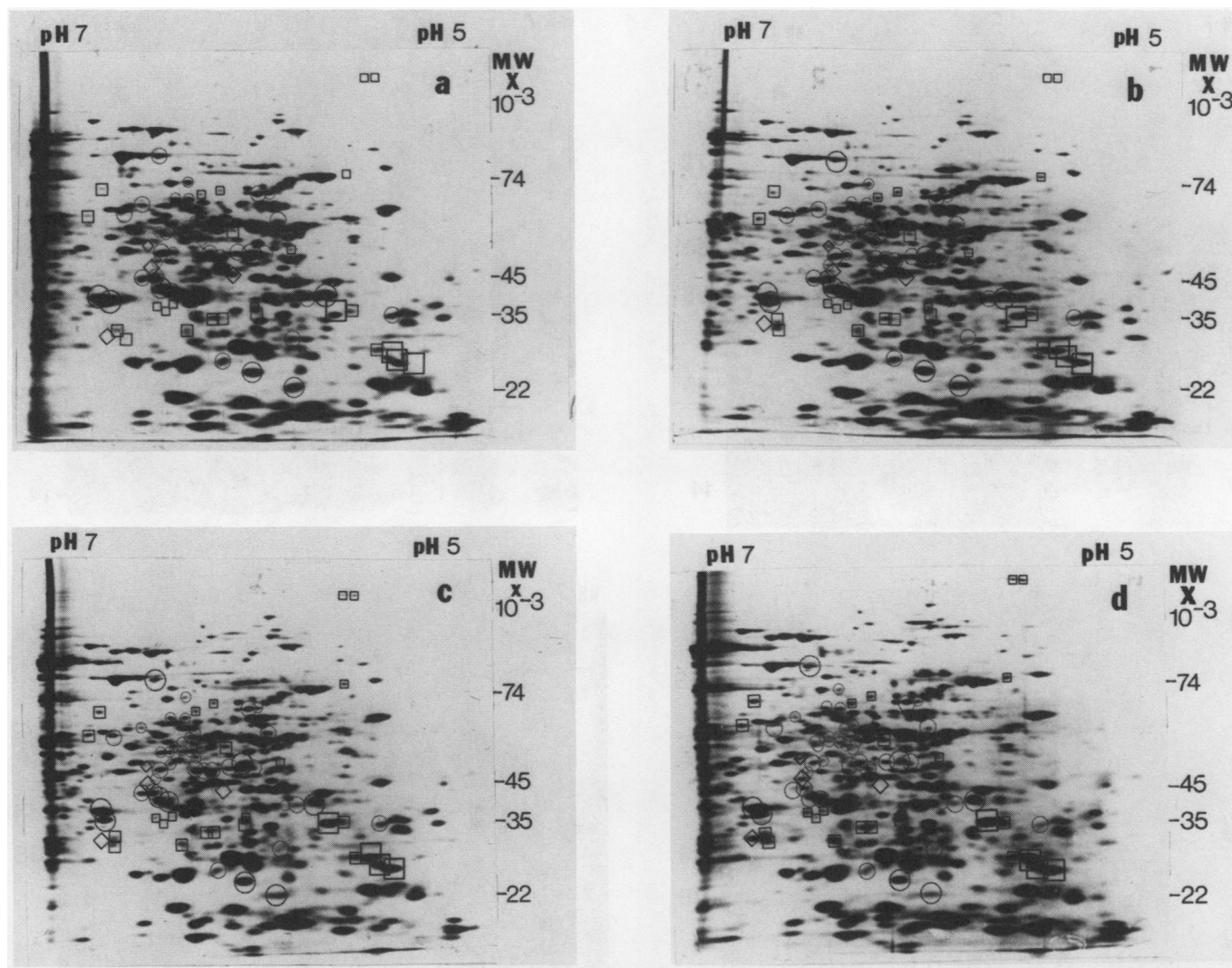


FIG. 6. Relative abundancies of various endogenous proteins change during aging. Apical 10-mm segments from peas aged for various lengths of time were homogenized and total, unlabeled protein was extracted. Eighty μg of protein were loaded onto the isoelectric focusing dimension, electrophoresed, and silver stained after the second dimension. (\square), Protein spots which increase in abundance during aging; (\circ), spots which decrease in abundance during aging; (\diamond), locations of spots which appear or disappear during aging. The gels correspond to samples isolated from tissue aged for (h): a, 0; b, 24; c, 48; d, 96. The photographs are representative of three experiments, and only those changes which were reproducible are indicated here.

of ribosomal material had declined approximately 5-fold when compared to the zero time value. On a segment basis, the decrease was more evident, since the poly(A) content declined about 14-fold compared to the zero time value (Fig. 4).

Decline in the *In Vitro* Protein Synthesizing Capacity during Aging. Of all the parameters measured, the ability of the isolated ribosomes (*i.e.* unfractionated polysomes, monosomes, and subunits) to support protein synthesis *in vitro* declined to the greatest extent during aging (Fig. 5). Equal A_{260} units of total ribosomes isolated from tissue at different times during aging were used to prime the cell-free wheat germ system. The capacity of the ribosomes to incorporate radioactive amino acids into polypeptides quickly diminished during aging, especially between zero and 24 h, whether expressed on an A_{260} or on a segment basis (Fig. 5). This contrasts with the percentage of polysomes which remained constant during the first 24 h (Fig. 2). By 96 h, the protein synthesizing capacity per segment of isolated ribosomes declined to about 2% ($1.9 \pm 0.9\%$) of the zero time value.

The decline in protein synthesizing capacity might be attrib-

uted to several factors including a net loss of RNA, ribosomes, or polysomes per segment, as well as a loss of activity per unit RNA, per ribosome, or per polysome. Table I contains zero time (control) and 96 h data as well as values at 96 h expressed as percentages of zero time values for protein synthesis per segment, per unit RNA, per ribosome, and per polysome. Aging caused a decline in protein synthesis regardless of the basis for its calculation, but it declined to the greatest extent when calculated on a segment basis. The protein synthetic activities of total ribosomes (*i.e.* crude polysomes), total RNA (phenol-extracted RNA from whole tissue), and polysomal RNA (phenol-extracted RNA from ribosomal pellets), isolated from tissue aged for 96 h, were about 2, 10, and 7%, respectively, of the zero time values. Protein synthetic activities declined less dramatically when calculated on the amount of RNA or ribosomal material that was present. Total ribosomal activity decreased approximately 13%, while RNA from whole tissue and from ribosomal pellets both retained greater than 50% of their ability to direct protein synthesis after 96 h of aging. An alternative way of measuring the protein

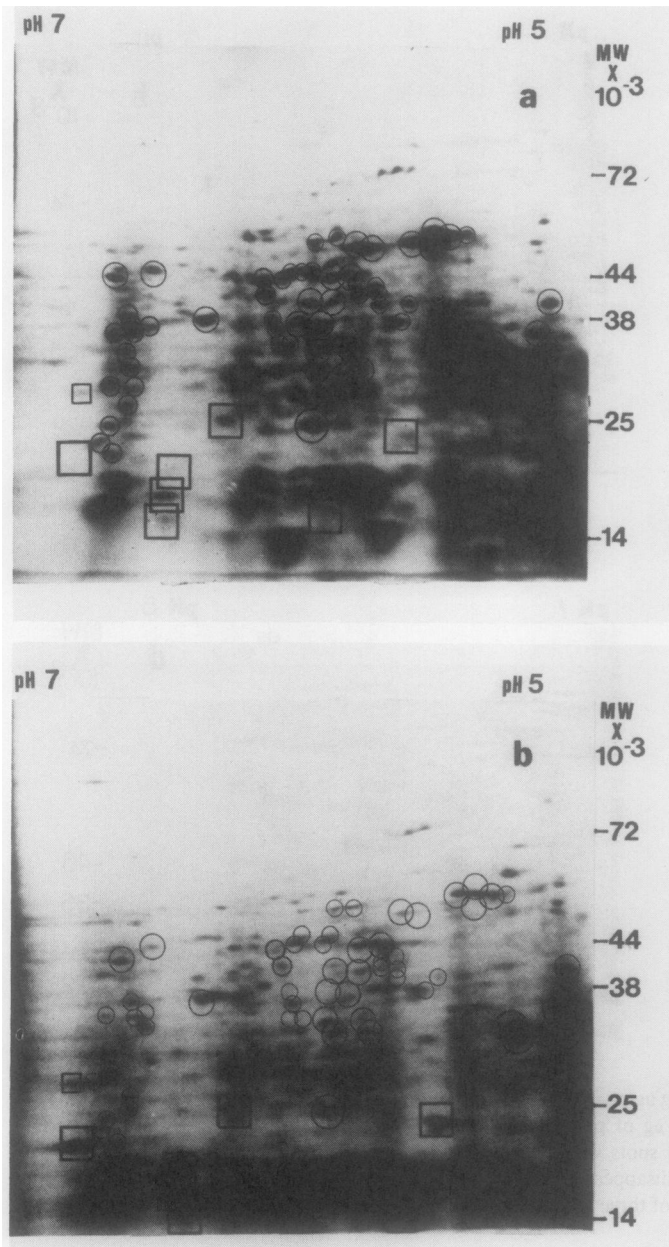


FIG. 7. Total RNA extracted from aged tissue evokes the synthesis of polypeptides different from those synthesized by RNA from unaged tissue. Total RNA was extracted and $10 \mu\text{g}/100 \mu\text{l}$ reaction used for *in vitro* translation with the resulting polypeptides subjected to electrophoresis. Radioactivity equivalent to 750,000 cpm was applied to each isoelectric focusing gel. The second dimension gels were stained with Coomassie brilliant blue R to provide internal markers, processed for fluorography and exposed to x-ray film for 144 h. (O), Polypeptides which are more intense in the unaged sample; (□), polypeptides which are more intense in the aged sample. Fluorographs correspond to patterns generated by RNA extracted from the equivalent of: a, 0.15 segments of unaged (zero time) tissue; b, 1.5 segments of 96-h aged tissue. The photographs are representative of three experiments, and only those changes which were reproducible are indicated here.

synthesizing abilities based on the amount of RNA present is shown as a measure of activity per A_{254} unit (*i.e.* from areas of polysome profiles). When calculated in this manner, the protein synthetic activity for total ribosomes from tissue aged for 96 h was about 10% of the zero time value. When calculated on a

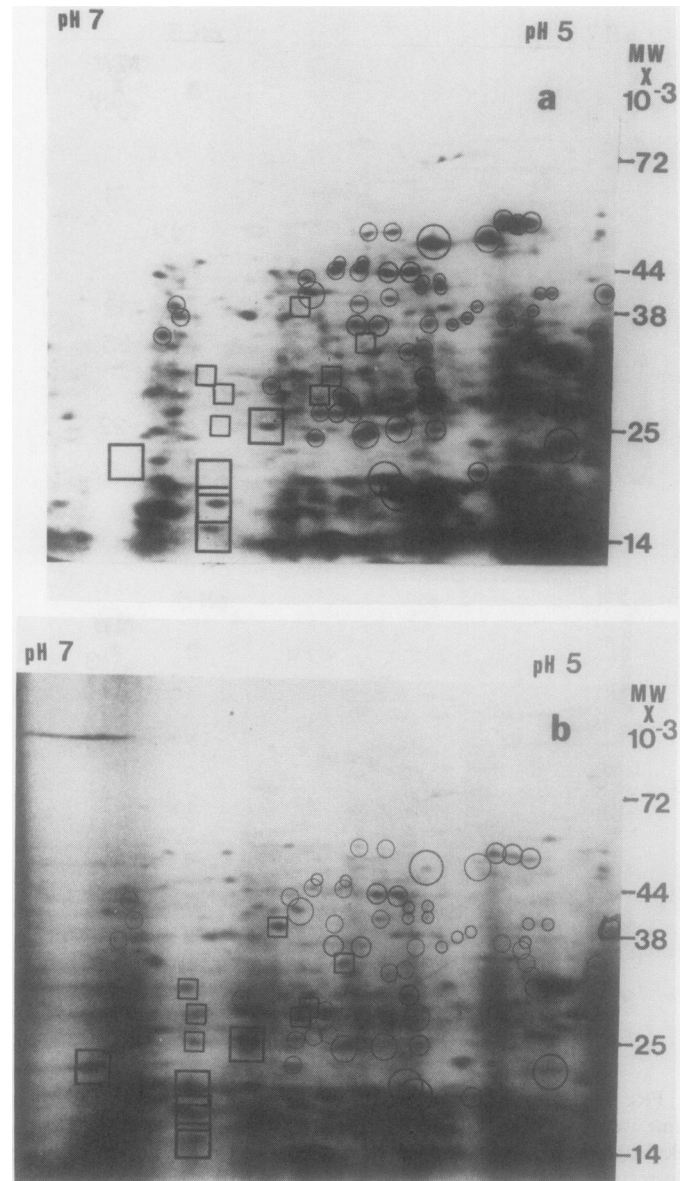


FIG. 8. Polysomal RNA extracted from aged tissue evokes the synthesis of polypeptides different from those synthesized by RNA from unaged tissue. The protocol was identical to that described in Figure 7, except that polysomal RNA ($10 \mu\text{g}/100 \mu\text{l}$ reaction) was used as a template for protein synthesis. (O), Polypeptides which are more intense in the unaged sample; (□), polypeptides which are more intense in the aged sample. Fluorographs correspond to patterns generated by RNA extracted from the equivalent of: a, 0.14-segments of unaged (zero time) tissue; b, 2.3 segments of 96-hour aged tissue.

polysome basis (*i.e.* from polysome profiles but excluding subunits and monosomes), the 96-h aged tissue retained approximately 18% of the zero time activity. The decline in priming capacity of this RNA from aged tissue is almost certainly the result of a decline in the proportion of mRNA in the total or polysomal RNA extract, since the polysomes and polysomal mRNA declined twice as much as did the total ribosomes and rRNA. This conclusion is reinforced when the protein synthesizing activity per A_{254} unit of total ribosomes is compared with the protein synthesizing activity per polysome (Table I). Again, a 2-fold difference is evident. Alternative explanations for this 2-fold reduction in activity appear less likely. For example, it seems

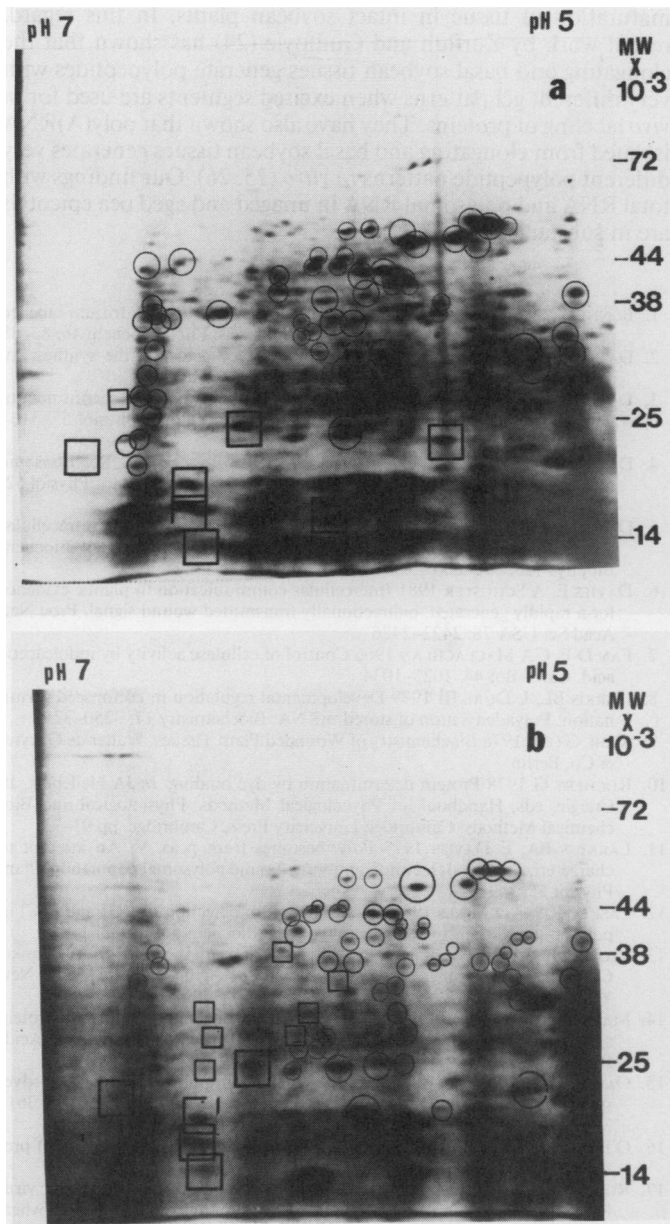


FIG. 9. A mixture of RNA extracted from zero time and aged tissues evokes the synthesis of a polypeptide pattern intermediate between the two individual patterns. The protocol was identical to that described for Figures 7 and 8, except that 5 $\mu\text{g}/100 \mu\text{l}$ reaction of RNA from both zero time and 96-h aged tissues were combined and used for *in vitro* protein synthesis. Fluorographs represent patterns generated by: a, total RNA; b, polysomal RNA. (\square , \circ) in 'a' correspond to similar spots in Fig. 7, a and b. (\square , \circ) in 'b' correspond to similar spots in Fig. 8, a and b.

unlikely that ribonucleases or proteases encoded by RNA from aged tissue and active *in vitro* reduced the radioactive incorporation rates by half (Fig. 9).

In summary, the factors accounting for the 98% reduction in the protein synthesizing activity of total ribosomal material per segment by 96 h of aging seem to be: (a) the 5-fold reduction in amount of ribosomal material per segment (Fig. 1); (b) the halving in the proportion of mRNA within the RNA population (Table I); and (c) the 4- to 5-fold reduction in efficiency or integrity of the translation complexes (Table I).

Patterns of Unlabeled Proteins Accumulated and Degraded *In Vivo* during Aging. Protein content declines only 30% by 96 h

of aging, while the results described here indicate that the protein synthesizing activity of the aged tissue is reduced to a much greater extent. This suggests that the bulk of the protein contained within the tissue during aging was relatively stable, but the components required for the ongoing synthesis of new proteins were not stable. To confirm (or deny) this supposition, two-dimensional gel patterns of unlabeled, silver-stained proteins isolated from tissue at different times during aging were examined. Unlabeled proteins were analyzed, since attempts using intact plants to radiolabel the proteins *in vivo* (by applying labeled amino acids in lanolin to the apex) yielded samples with specific activities too low for two-dimensional gel fluorography. Somewhat higher specific activities were attained by incubating excised tissue segments in solutions containing radioactive amino acids, but this treatment generated a wound response in which poly-some formation and enhanced protein synthesis were observed (6, 18).

About 500 protein spots could be visualized at each time point (Fig. 6). The most obvious change that occurred during the first 24 h was an increase in some of the large proteins (squares in upper middle part of Fig. 6, a and b). The most obvious changes occurring at later stages include an increase in some of the small proteins (squares towards bottom of Fig. 6, b-d) and a decline in a wide range of proteins (Fig. 6, a, c, and d, \circ). A number of distinct differences were seen in the protein composition of the 96-h aged tissue (Fig. 6d) compared with the zero time tissue. Overall, after 96 h of aging there were at least 44 proteins which declined in relative amount, one protein which disappeared, at least 25 proteins which increased and 3 proteins which appeared. Those proteins which seem to be altered in their relative abundancies during aging were heterogeneous in terms of their mol wt and isoelectric points. These gel patterns also show that the majority of the proteins remain comparatively unchanged in their relative concentrations during aging.

Patterns of Labeled Polypeptides Synthesized *In Vitro* by RNA from Aged and Unaged Tissues. The protein synthesizing activity of isolated polysomes was reduced dramatically during aging (Fig. 5, Table I). Because these polysomes were so inefficient at supporting protein synthesis, attempts at analyzing the polypeptides generated *in vitro* by isolated polysomes using two-dimensional gel electrophoresis were unsuccessful. Additional efforts were made to isolate poly(A) RNA from isolated polysomes as an alternative source of message but, because the poly(A) content declines so extensively during aging (Fig. 4), the yields of poly(A)RNA from aged tissues were too low to generate sufficient incorporation. Therefore, total phenol-extracted RNA from whole tissue as well as from ribosomal pellets was used to prime the wheat germ cell-free system. The resulting polypeptides were subjected to two-dimensional gel electrophoresis and visualized by fluorography. Figure 7 demonstrates the differences in the polypeptide patterns when total RNA isolated from whole tissue at zero time (Fig. 7a) and after 96 h of aging (Fig. 7b) were used for *in vitro* translation. Of the more than 200 spots, there were at least eight polypeptides which increased in intensity or appeared during the aging process (Fig. 7, \square). These were all smaller mol wt polypeptides, ranging from about 15,000 to 30,000 D. There were at least 58 spots which decreased in intensity or disappeared during aging (Fig. 7, \circ). Most of these were in the mol wt range of 25,000 to 60,000 D.

Figure 8 shows the polypeptide patterns when RNA extracted from total ribosomes was used for *in vitro* translation. The polypeptide patterns representing the zero time (Fig. 8a) and the 96-h aged (Fig. 8b) samples showed that at least 12 polypeptides, ranging in mol wt from about 15,000 to 40,000 D, increased in intensity or appeared during aging (Fig. 8, \square). At least 64 polypeptides decreased in intensity or disappeared during aging and were typically larger mol wt products (Fig. 8, \circ).

Figure 9 shows the polypeptide patterns resulting when total RNA (Fig. 9a) or polysomal RNA (Fig. 9b) from zero time and 96-h aged tissues were mixed in equal proportions (A_{260} units) and used for *in vitro* translation. The combined patterns appear to be intermediate between the individual patterns (cf. Fig. 7, a and b, with Fig. 9a, and Fig. 8, a and b, with Fig. 9b), representing the zero time and 96-h aged samples, respectively. This suggests that the decrease in large polypeptides and increase in small polypeptides in the aged tissue sample is not an artifact resulting from RNase or protease degradation occurring during translation *in vitro*.

The polypeptide patterns generated by polysomal RNA and by total RNA were amazingly similar when either zero time tissue (cf. Fig. 7a and Fig. 8a) or aged tissue (cf. Fig. 7b and Fig. 8b) were used. This implies that most of the messages present in the whole tissue extracts were present within the polysomes and hence being translated at the time of tissue extraction. This was confirmed for the zero time tissue, since little RNA could be found in the postribosomal supernatant and it supported virtually no protein synthesis *in vitro* (data not shown). More RNA was present in the postribosomal supernatant fraction from aged tissue, but this RNA did not support protein synthesis *in vitro*; in fact it suppressed protein synthesis primed by polysomal RNA (data not shown) and might have contained mRNA degraded during the aging process. We believe that the results using polysomal RNA (and to a lesser extent, total RNA) reflect the situation occurring *in vivo* more accurately than would have been the case had whole tissue poly(A)RNA been used. Polysomal RNA contains only that fraction of the mRNA being translated at a given time, regardless of whether it is polyadenylated or not, whereas poly(A) RNA from whole tissue could include mRNA not being translated and exclude polysomal mRNA that is non- (or under) polyadenylated.

Relation to 'Aging' in Other Plant Systems. The aging phenomenon described here is fundamentally different from that described by other workers using different systems. Aging is defined here as a reduction in certain physiological activities (e.g. growth) accompanied by a reduction in protein synthetic activity. However, the tissue so aged is easily reactivated upon addition of auxin (3, 4, 19) or by wounding (6, 18). Other researchers use the term aging when referring to: (a) a reactivation of metabolic activities, typically in plant storage tissues; (b) senescence; or (c) the maturation of certain tissues.

The first case refers to a process opposite to that described here. Aging of plant storage tissues (e.g. Jerusalem artichoke, potato, carrot) is typically initiated by excising the tissue and incubating it in solution. This stimulates several processes (which may or may not be related), for example, the formation of callus, an increase in polysome levels, an increase in respiration rates, and this is, in the opinion of some, primarily a wound response (9).

The second case, senescence, refers to an irreversible process culminating in death (13). This is markedly different from the aging process investigated here in which reactivation takes place in response to wounding (18) or auxin treatment (19).

The third case, maturation of tissue in intact plants, seems most similar to the process investigated here. Like the zero time (unaged) pea tissue described here, apical soybean hypocotyl tissue is actively growing and contains high levels of polysomes (20). Furthermore, like the aged pea tissue, basal 'mature' hypocotyl tissue is non-growing and contains low levels of polysomes and a high proportion of monosomes (20). In addition, like the aged pea tissue, the mature hypocotyl tissue forms polysomes in response to excision and incubation, *i.e.* wounding (6, 18) as well as to auxin treatment (4, 19). Hence, aging in the decapitated pea stem tissue is analogous in many respects to the

maturation of tissue in intact soybean plants. In this regard, recent work by Zurfluh and Guilfoyle (24) has shown that the elongating and basal soybean tissues generate polypeptides with very different gel patterns when excised segments are used for *in vivo* labeling of proteins. They have also shown that poly(A)RNA isolated from elongating and basal soybean tissues generates very different polypeptide patterns *in vitro* (25, 26). Our findings with total RNA and polysomal RNA in unaged and aged pea epicotyls are in substantial agreement.

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