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Quantitative Changes in *In Vitro* and *In Vivo* Protein Synthesis in Aging and Rejuvenated Soybean Cotyledons[1]

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ABSTRACT

Cotyledons of light-grown soybean (*Glycine max* L. var Wayne) seedlings were used as a model system to study the possibility that aging requires qualitative changes in protein synthesis. Cotyledons reached a final stage of senescence and then abscised about 22 days after imbibition. Cotyledon senescence was reversed at 20 days after germination by epicotyl removal. Thereafter, the cotyledons regained much of the chlorophyll, RNA, protein, and polyribosomes lost during aging.

Total poly(A)mRNA was extracted from 4-, 12-, 20-day-old, and rejuvenated cotyledons and translated in a wheat germ system. Comparison of translation products on two-dimensional O'Farrell gels showed that many translation products increased in quantity during aging, while roughly half as many decreased. Rejuvenation returned the translation products to approximately 4-day-old levels in roughly half of those products which were diminished with age. Conversely, almost one-third of the products which had increased with age decreased with rejuvenation. None of the translation products were totally lost nor were newly synthesized products detected during aging. Therefore, aging in this system probably does not involve complete gene repression or depression. The observation that epicotyl removal causes a reversal in the levels of various proteins synthesized *in vitro* was corroborated by similar observations following *in vivo* labeling of cotyledon sections and analysis by SDS-polyacrylamide gel electrophoresis and fluorography. Densitometric scans of fluorograms revealed a gradual shift in profiles of both *in vitro* and *in vivo* translation products during aging. Rejuvenated cotyledon proteins had a profile resembling that of 4-day-old cotyledons. The overall level of [^{35}S]methionine incorporation into protein *in vivo* declined gradually during aging but was restored to 4-day-old levels within 2 days after epicotyl removal.

Many theories have been advanced to explain aging in plants and animals. A major concern is whether aging is controlled by genetically programmed mechanisms. Indirect evidence supports the concept that aging in plants is programmed (15, 24) while direct evidence is available for human cells (9, 16).

Changes in the ability to transcribe, process or protect RNA may result in declining protein synthesis, leaving cells unable to perform vital functions. Selective alterations in various transcription factors, tRNA turn-over rates, or aminoacyl tRNA synthetase activities may lead to qualitative changes in protein synthesis during aging (3, 22). Changes in translation at these levels, as well as in rates of mRNA transcription, could reduce the synthesis of vital proteins or even favor the production of destructive proteins. The initial agent which triggers the senescence process is unknown. Cytokinins appear to regulate the decline in various macromolecules (protein, nucleic acid, etc.) associated with aging in plants (6, 10, 13, 18), and the loss of membrane permeability (20, 23, 25).

We have examined changes in the spectrum of proteins synthesized during the aging and rejuvenation of soybean cotyledons. The aging of soybean cotyledons can be reversed simply by epicotyl removal, even after roughly 90% of the nucleic acids and proteins have been lost (11). Following epicotyl removal, Chl, RNA, protein, and polyribosome levels are increased (Skadsden and Cherry, unpublished results). If aging results from changes in genetic programs, a difference in detectable protein synthesis should be observed by two-dimensional polyacrylamide gels. If, for example, aging is regulated by specific proteins which increase with age, they should decline upon rejuvenation. Alternatively, if aging results from the loss of critical proteins, they should be replenished upon rejuvenation.

In the following study, the *in vitro* translation products from total poly(A)mRNA from aging and rejuvenated cotyledons were compared. Proteins were also labeled with [^{35}S]methionine *in vivo* via cotyledon sections to assess the validity of the *in vitro* experiments.

MATERIALS AND METHODS

Plant Material. Soybeans (*Glycine max* L. var. Wayne) were imbibed for 6 h in tap water and sown in 53- × 26- × 6-cm trays in a mixture of Vermiculite:Perlite (2:1). Each tray contained about 400 seedlings. Plants were grown in a greenhouse with day temperatures of 26 to 37°C and nights of 16 to 23°C. Supplemental overhead lighting was provided for 16 h during the day. Light intensities ranged from 200 to 2,000 $\mu E/m^2 \cdot s$. Rejuvenation was accomplished by excising the epicotyls of 20-d-old plants (20 d after imbibition) and leaving the plants an additional 20 d before harvesting the cotyledons. Axillary shoots were continuously pruned to prevent resumption of cotyledon aging. Cotyledons were excised, rinsed with deionized H_2O, and stored at −55°C.

mRNA Isolation. Total RNA was isolated by the method of Hall *et al.* (8), with the following changes. Cotyledons were ground in liquid N_2 to a fine powder. Proteinase K (EM Laboratories, Germany) was added to 0.5 mg/ml for 4-d-old cotyledons and 0.2 mg/ml for 12-, 20-d-old, and rejuvenated cotyledons. The suspension were incubated at 37 to 40°C with shaking for 1 h. A 10-ml solution of 0.2 M KCl was added, and the suspension was allowed to cool on ice prior to centrifugation at 12,000*g* for 10 min. The extracted RNA was redissolved in 10 mM Tris acetate buffer (pH 7.5), and the A_{260}/A_{280} was determined.

Poly(A)mRNA was prepared according to Aviv and Leder (1) with several modifications. Oligo(dT) cellulose type III (0.5 g, from Collaborative Research, Inc.) was equilibrated with binding

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buffer (10 mM Tris acetate, pH 7.5, 0.2% SDS, 0.5 M NaCl). The dissolved RNA was heated to 55°C for 1.5 min and allowed to cool to room temperature for 5 min. An equal volume of 2-fold concentrated binding buffer was added, and unconcentrated binding buffer was added to give a final concentration of 25 A_{260} units/ml. Less than 400 A_{260} units were used for each mRNA isolation. The solution was batch absorbed to the oligo(dT) in a 30-ml glass centrifuge tube with agitation at room temperature for 20 min. The mixture was washed several times by suspension and centrifugation in binding buffer until A_{260} units were not detected in the supernatant. The cellulose was poured into a 0.8- × 12-cm Teflon column connected to an ISCO optical analyzer. The cellulose was washed with binding buffer, and the wash was monitored at 254 nm to insure that no unbound RNA was present. Elution buffer (10 mM Tris acetate, pH 7.5, 0.04% [w/v] SDS) was heated to 55°C and applied to the column, and the poly(A)mRNA eluted at 3 ml/min. The elution peak was collected, and NaCl and SDS were added to 0.4 M and 0.16% (w/v), respectively. The solution was reheated, reapplied to the column, and eluted as above. The elution peak was cooled on ice for 15 to 30 min and centrifuged at 12,000*g* for 10 min to remove SDS. A solution of 4 M KCl was added to 0.3 M and the solution was again cooled and centrifuged as above. Ethanol was added to the supernatant to 2.5 volumes. The solution was allowed to stand overnight at −20°C. Total poly(A)mRNA was pelleted by centrifugation at 122,000*g* at 0°C for 2 h in a Beckman Ti 40 rotor. The pellets were lyophilized and dissolved in 0.1 ml dH_2O. Wavelength scans were performed on each sample using a Beckman DU-8 spectrophotometer. mRNA was frozen as pellets in liquid N_2 and stored at −55°C.

mRNA Translation. mRNA was translated with wheat germ extracts prepared according to Marcu and Dudock (14), except that 3 g of wheat germ was soaked in 4 ml of extraction buffer on ice for 15 min instead of being ground prior to centrifugation. The final extract was frozen as pellets in liquid N_2 and stored at −55°C. The translation system employed was also similar to Marcu and Dudock's (14). The final reaction mixtures in 25-μl volumes consisted of: 20 mM Hepes-KOH (pH 7.6), 2 mM DTT, 1 mM ATP, 20 μM GTP, 0.8 mM Mg acetate, 80 mM K acetate, 8 mM creatine phosphate, 6,520 units/ml creatine phosphokinase, 100 μg/ml spermidine, and 40 μM of each of the 19 unlabeled amino acids. [^{35}S]Methionine (1,090–1,315 Ci/mmol; Amersham Corporation) was used at 50 pmol per reaction, except where indicated. Incorporation increased linearly with increasing poly(A)mRNA until a concentration of 1.5 μg per reaction was reached; the reaction plateaued at 2.7 μg mRNA. In order to stay within the linear response range, 1.5 μg of mRNA was used in each reaction. A volume of 5.25 μl (105 μg protein) of wheat germ extract was used per reaction. Reactions were incubated at 25°C with shaking in 1.5-ml plastic Eppendorf centrifuge tubes. Aliquots of 2 μl were withdrawn at the times indicated, and at 90 min the remaining volumes were frozen at −55°C without further processing. The 2-μl aliquots were mixed with 30 μg BSA and precipitated with 1 ml of 10% (w/v) TCA containing 10 mM unlabeled methionine. Samples were heated to 94°C for 15 min, cooled on ice, and filtered by suction through glass fiber discs (Whatman GF/A). Discs were washed with 25 ml cold 5% TCA, followed by 10 ml ether, and dried. Incorporation was determined by liquid scintillation counting in 4 ml of a toluene scintillant. Counting efficiency was 90%.

***In Vivo* Protein Labeling.** [^{35}S]Methionine was incorporated into 4-, 12-, 20-d-old, and rejuvenated cotyledons as follows. A disc was cut from the center of each cotyledon with a 1-cm cork borer, and the epidermal layer was removed with sterile forceps. The discs were then placed with the mesophyll side downward in glass shell vials. Vials contained 140 μl of a sterile incubation medium containing 20 mM Na phosphate (pH 7.2), 50 mM mannitol, 100 μg/ml chloramphenicol, and 0, 1, or 10 μg/ml α-amanitin, as indicated (both from Sigma). After 15 min, the medium was removed and 135 ml of fresh medium was added, along with 5 μl of [^{35}S]methionine (1,090–1,315 Ci/mmol, 7.9–8.6 pmol/μl). The discs were incubated 5 cm above an incandescent Sylvania Gro-lux light at 27°C for 8.5 h.

At the termination of each reaction, the discs were rinsed with distilled H_2O for 5 min and the mesophyll layers were excised with a razor blade. The tissue samples were found in SDS sample buffer (11). Homogenates were clarified by centrifugation at 13,000*g* for 5 min. Two 2-μl aliquots from each sample were processed for scintillation counting as above. Total endogenous [^{35}S]methionine content was determined by placing 2-μl aliquots onto glass fiber discs (Whatman GF/A) and counting in a scintillation spectrometer.

Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (12). mRNA translation products or *in vivo* labeled proteins were loaded onto 12.5% polyacrylamide slab gels with a 5% stacking gel. Equal activities (cpm) of radioactive protein (hot TCA-insoluble) were loaded in each lane. Stacking was performed at a constant 50 v

Table I. *Total poly(A)mRNA Yield from Aging and Rejuvenated Cotyledons*

Age	mRNA Yields	A_{260}/A_{280}
d	μg/*cotyledon*	
4	1.85	1.97
12	0.56	1.93
20	0.13	1.80
Rejuv.	0.63	1.85

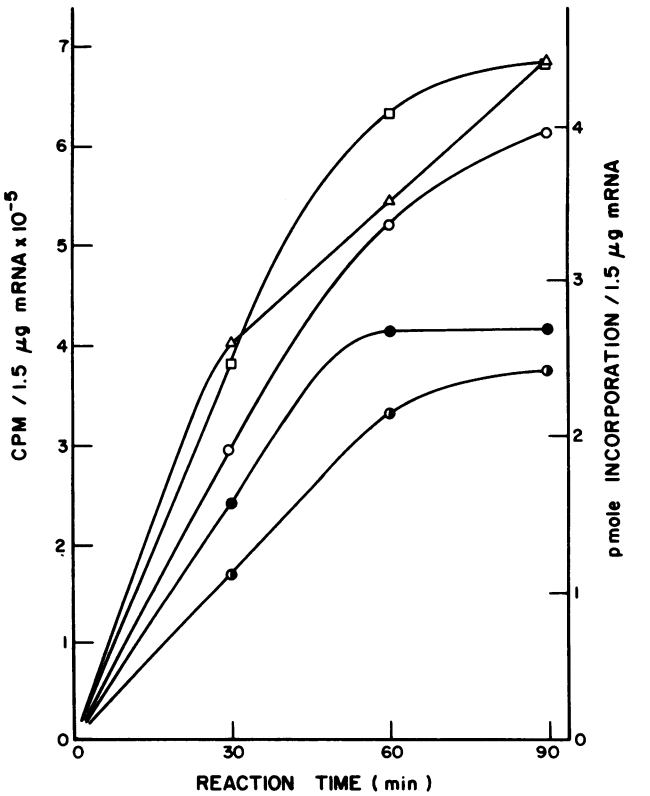

FIG. 1. Kinetics of mRNA translations. Each point represents [^{35}S]methionine incorporation into protein by 2-μl reaction samples. The following cotyledon mRNA were used at 1.5 μg/25 μl reaction: 4-d-old extracted with 0.2 mg/ml protease K (◑); 4-d-old extracted with 0.5 mg/ml protease K and precipitated two times in ethanol (●); 12-d-old (△); 20-d-old (○); and rejuvenated (□).

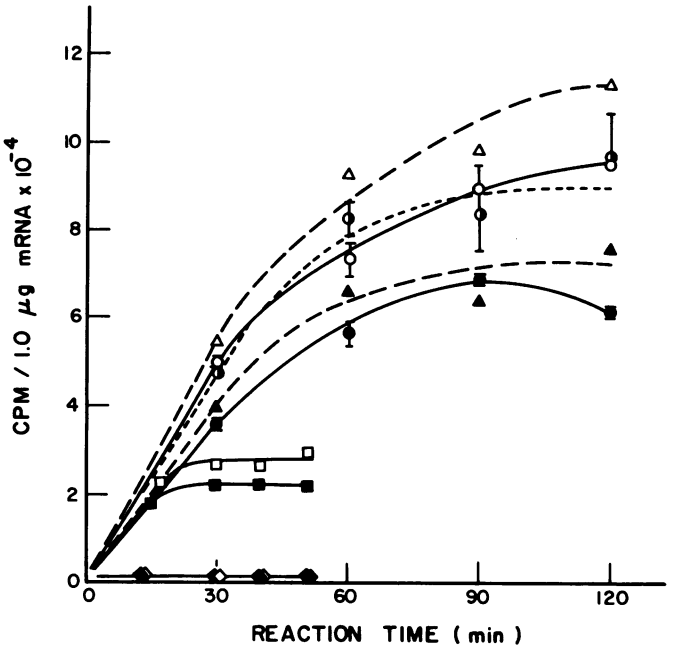

FIG. 2. Comparison of 4- and 12-d-old mRNA translation capacities following various treatments. Reactions were conducted as described in "Materials and Methods," except that 70 pmol of [^{3}H]leucine per reaction was used (72 Ci/mmol; Amersham), 1.0 µg total mRNA was used in each reaction, and 4-µl aliquots were withdrawn. Cotyledon ages and treatments: 4-d-old, alone (●——●); 12-d-old, alone (○——○); 0.5 µg of 4-d-old plus 0.5 µg of 12-d-old (◑- - -◑); 4-d-old, heated to 55°C for 90 min and cooled in ice water before translation (▲- - -▲); 12-d-old, heated and cooled (△- - -△); 4-d-old, plus 100 µM ATA added prior to mRNA (◇——◇); 12-d-old, plus ATA added prior to mRNA (◆——◆); 4-d-old, plus ATA added 20 min after mRNA (■- - -■); 12-d-old, plus ATA added 20 min after mRNA (□- - -□). Each point is the average of two repetitions. Bars represent 1 SD.

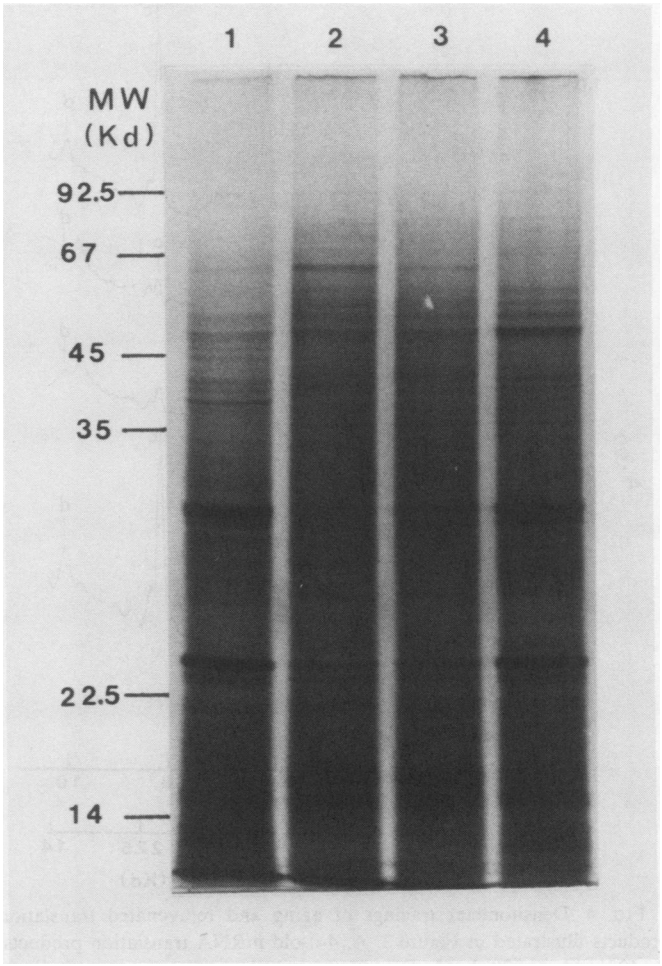

FIG. 3. Aging and rejuvenated cotyledon mRNA translation products. Lanes 1 through 4 were loaded with 500,000 cpm of [^{35}S]methionine labeled protein from ages 4, 12, 20 d, and rejuvenated, respectively. Electrophoresis was as described in "Materials and Methods." Fluorograms were developed for 6 h.

and subsequent electrophoresis was at a constant 150 v.

Two-dimensional gel electrophoresis was performed according to O'Farrell (17). Aliquots of 3 µl (about 15 µg of protein) of mRNA translation products were processed according to O'Farrell (17), but the DNase digestion step was omitted. Concave exponential gradient gels (12.5–16%) were used in the second dimension. A final processed volume of 20 µl was loaded onto each IEF[4] rod. Proteins were focused at 300 v for 3,800 v-h. The gels were extruded into SDS sample buffer in screw cap tubes, immediately frozen in a bath of ethanol and liquid N_2, and stored at −55°C. Prior to electrophoresis in the second dimension, the IEF gels were equilibrated in sample buffer for 1 h. The pH along the IEF gels was determined on unequilibrated rods sliced into 7.5-mm sections. These had been focused without protein. The sections were immersed in 2 ml of degassed 10 mM NaCl (pH 7.0) and, after agitating for 2 h, the pH was determined. Mol wt standards for all gels were: phosphorylase a, 92.5 kD; BSA, 67 kD; ovalbumin, 45 kD; glyceraldehyde-3-P dehydrogenase, 35 kD; soybean trypsin inhibitor, 22.5 kD; and lysozyme, 14 kD.

Gel Analysis. Gels were fixed for 1 h in a solution of 50% methanol, 10% glacial acetic acid, and 0.1% (w/v) Coomassie Brilliant Blue. They were destained in 30% methanol and 7% acetic acid overnight. Gels were prepared and fluorographed as described by Bonner and Laskey (2). Two-dimensional gels were not stained. Water was removed by three 30-min baths in DMSO, followed by PPO impregnation in a 90-min bath of 20% (w/v) PPO in DMSO. After rinsing in several changes of deionized H_2O for 3 h, the gels were vacuum-dried and fluorographed. The x-ray film was not preflashed. Fluorograms from two-dimensional gels of translation products from 4-d-old cotyledons were exposed for 9 d, while all others were exposed for 4 d. Stained proteins retained most of their stain throughout the processing steps.

Densitometric scans of fluorograms were done with a Beckman DU-8 spectrophotometer at a wavelength of 490 nm.

RESULTS

mRNA Yield and Activity. The amount of extractable poly(A)mRNA declined more than 10-fold from 1.85 µg per cotyledon at 4 d to 0.13 µg at 20 d (Table I). Rejuvenation caused a 4-fold increase in mRNA to 0.63 µg per cotyledon. To obtain functional mRNA, 0.5 mg/ml proteinase K was essential during the extraction of 4-d-old cotyledons. If the concentration of proteinase K was lowered to 0.2 mg/ml, the yield of mRNA was reduced by 92%. Even though 4-d-old mRNA had the highest A_{260}/A_{280} ratio, it had the lowest translational activity per µg (Fig. 1). The activity of the 4-d-old mRNA was not improved after reprecipitation in ethanol (data not shown), and it remained only 60% as active as mRNA from other ages. The activities for 12-d-old and rejuvenated mRNA were roughly equal, while 20-d-old mRNA was only 10% less active, based on total incorporation

[4] Abbreviations: IEF, isoelectric focusing; DMSO, dimethyl sulfoxide; ATA, aurintricarboxylic acid.

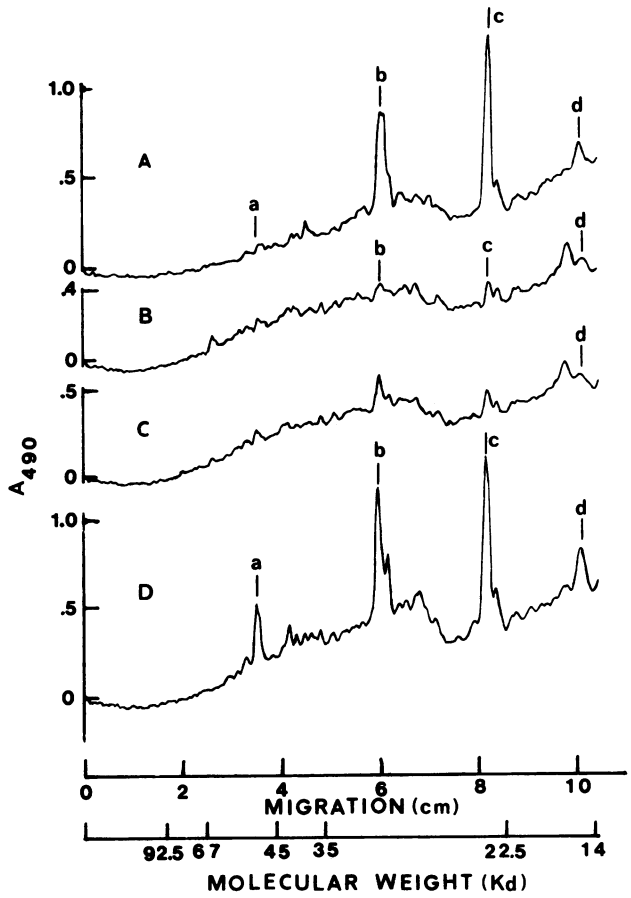

FIG. 4. Densitometer tracings of aging and rejuvenated translation products illustrated in Figure 3. A, 4-d-old mRNA translation products; B, 12-d-old; C, 20-d-old; D, rejuvenated. Lower case letters indicate identical protein peaks between samples.

after 90 min. Control reactions, containing no mRNA, had 1.5 to 3.0% as much activity as the mRNA-containing reactions.

To insure that the low activity of mRNA from 4-d-old cotyledons was not due to inhibitors, reaction mixtures were prepared with equal amounts of mRNA from 4- and 12-d-old cotyledons (Fig. 2). No evidence of inhibition was found. The 4-d-old mRNA did not reduce the amount of translation expected from the 12-d-old moiety. In fact, the mixture translated as well as 1 μg of the 12-d-old mRNA alone. In another experiment, 4- and 12-d-old mRNA were heated to 55°C for 90 s and rapidly cooled in ice water prior to translation to relieve possible conformational hindrance. This treatment improved the activity of 12-d-old mRNA by 19% while that of 4-d-old mRNA was improved by 11%. To determine whether the mRNA was contaminated with bound polyribosomes, ATA was added either at the beginning of translation or after 20 min of incubation. When ATA was added at the beginning of incubation, translation was completely inhibited. However, the addition of ATA at 20 min stopped translation within an additional 5 to 10 min. In these experiments, mRNA from 4- and 12-d-old cotyledons gave similar results. Therefore, ribosomal contamination was not present.

Quantitative Changes in mRNA Products. The translation products from mRNA isolated from 4-d-old cotyledons were heterogenous. Three proteins (b, c, and d) were predominant when products were analyzed on one-dimensional 12.5% SDS-polyacrylamide gels (Figs. 3 and 4). However, translation of mRNA from 12- and 20-d-old cotyledons revealed that synthesis of proteins b, c, and d was greatly reduced while a higher mol wt protein adjacent to d was enhanced. Translation of mRNA from rejuvenated cotyledons gave a profile very similar to that of mRNA from 4-d-old cotyledons except that an additional protein, a, was greatly enhanced.

The same general trend in changes in mRNA translation products was observed when products were analyzed on two-dimensional gels (Fig. 5). Approximately 470 proteins were identified on each gel. Of these proteins, 133 increased in relative abundance from 4 to 20 d, whereas 74 decreased. Of those which decreased, 36 again increased over 20-d-old levels after rejuvenation. Of those which had increased during aging, 40 again declined after rejuvenation. No translation product was found to completely disappear at any age, although many appeared very faint even after prolonged x-ray film exposure. Synthesis of new protein species was not noted during aging or rejuvenation. The prominence of at least two translation products was transitory between 4 and 20 d. These could be seen to either increase or decrease from 4 to 12 d and then revert to 4-d-old levels at 20 d (Fig. 5, proteins 1 and 14). Rejuvenation caused five major products to vastly increase in prominence beyond the levels found at any age (Fig. 5, proteins 1–5).

Quantitative Changes in *In Vivo* Translation. A comparison of *in vivo* labeled proteins was made to determine whether the *in vitro* system reflected the same general changes occurring *in vivo*. Chloramphenicol was added to the incubation medium to inhibit bacterial and chloroplastic protein synthesis. The latter are labeled to a much greater extent in young mesophyll cells than in older cells. Their reduction provided a better comparison of strictly cytoplasmic protein synthesis between tissues of various ages. The major chloroplastic protein, ribulose bisphosphate carboxylase, is translated from mRNA which may not be bound by oligo(dT)-cellulose (5). Thus, we expect comparisons between *in vivo* and *in vitro* protein synthesis with age to be valid, since one of the main products in young and rejuvenated tissue (but not old tissue) would be removed in both systems.

Maximal inhibition of [^{35}S]methionine incorporation into plastid proteins occurred at approximately 100 μg/ml chloramphenicol with no effect on net synthesis of cytoplasmic proteins (data not shown). In the first series of *in vivo* labelings, α-amanitin was used at 10 μg/ml to inhibit RNA polymerase II activity and thereby prevent possible new gene transcription resulting from wounding. No qualitative differences in protein synthesis between α-amanitin-treated and nontreated tissue were detected on SDS-polyacrylamide gels (Fig. 6, lanes 1 and 2). Also, when the outer ring of the mesophyll discs, which contained wounded tissue, were excised after incubation and compared to the unwounded tissue in the inner discs, banding patterns of both were virtually the same (Fig. 6, lanes 5 and 6, respectively).

Since α-amanitin at 10 μg/ml greatly inhibited [^{35}S]methionine uptake (data not shown), these experiments were repeated with 1 μg/ml while using only mesophyll layers for protein analysis (Table II). Without the bulky palisade layer, the specific activity of the labeled proteins was increased which allowed for a better comparison between the age groups (Fig. 7). Four-d-old samples previously had very high amounts of relatively unlabeled palisade protein. This caused band distortion and quenching in the fluorograms. The main difference between labeled proteins from 4-, 12-, and 20-d-old cotyledons is that the majority of radioactivity was confined to relatively few bands in 4-d-old tissue while the activity was more evenly distributed among many bands in the 12- and 20-d-old tissue. Rejuvenation resulted in a partial reversal of the aging effect as the majority of radioactivity was again confined to fewer bands (Fig. 7). The changes in *in vivo* labeling of proteins observed with aging and rejuvenation are more readily seen in densitometer tracings of the fluorogram (Fig. 8). These results are consistent with the results of *in vitro* mRNA translations (Figs. 3–5). Figure 8 shows that the major protein bands (d and e; f, g, and i; l and m; and p) are greatly reduced during aging but

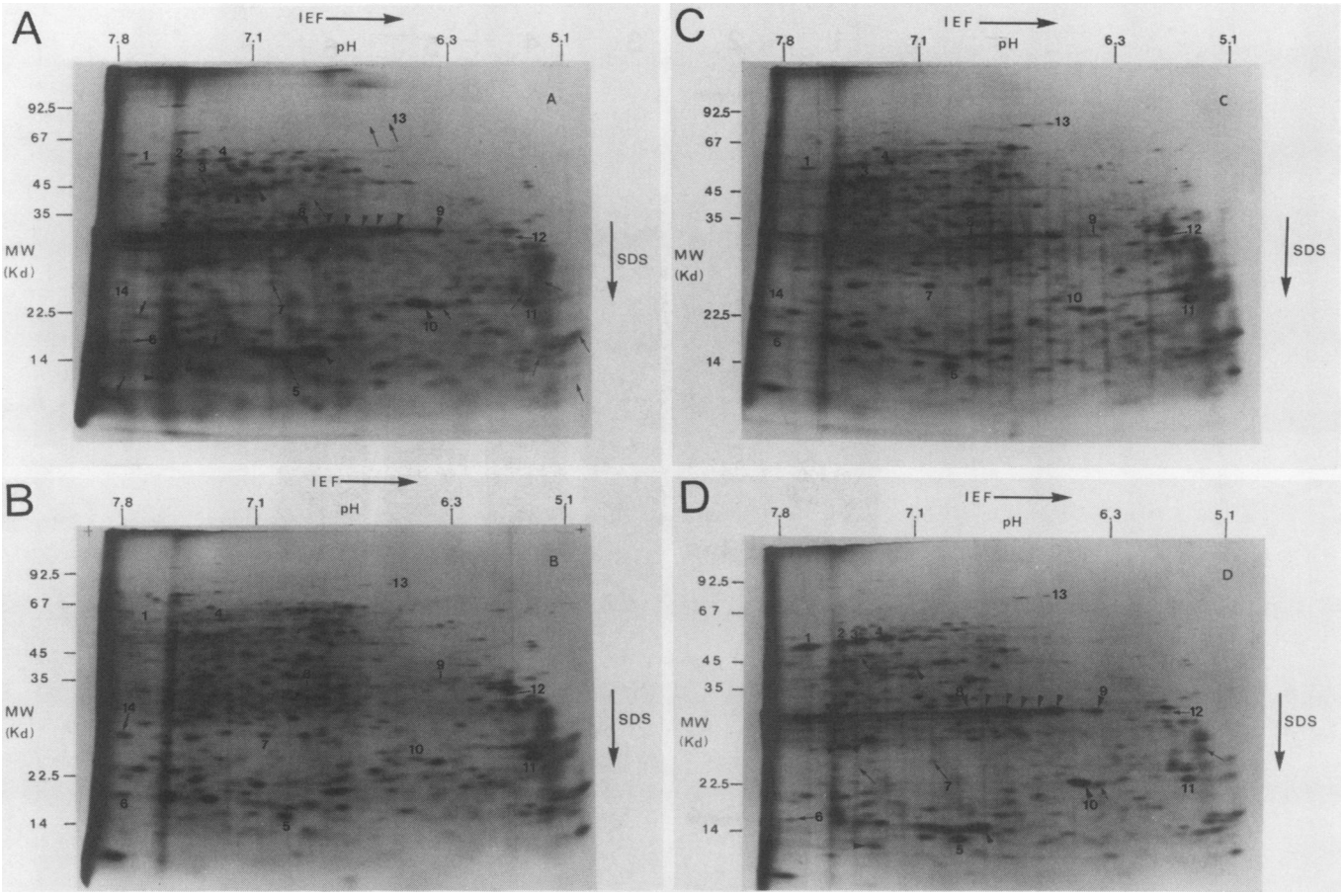

FIG. 5. Two-dimensional gel electrophoresis of aging and rejuvenated cotyledon mRNA translation products. Translation and electrophoresis were as described in "Materials and Methods." A, 4 d old; arrows denote proteins which increase in intensity from 4 to 20 d; wedges denote proteins which decrease in intensity. B, 12 d old; arrow denotes a protein which increases in intensity from 4 to 12 d but decreases again by 20 d. C, 20 d old. D, rejuvenated; arrows denote proteins which had increased in intensity during aging but decreased upon rejuvenation; wedges indicate proteins which decreased during aging but increased again after rejuvenation; numbers 1 through 5 indicate major rejuvenation proteins which were found in lower amounts at other ages. Numbers 1 through 14 indicate identical proteins in each fluorogram. Mol wt standards were electrophoresed on one-dimensional gradient gels.

are greatly restored during rejuvenation. Protein band n decreased with age but did not recover with rejuvenation, while band o increased greatly with age but reverted almost to 4-d-old levels during rejuvenation. Furthermore, the prominence of peaks a and b is a unique feature of the rejuvenated cotyledons. This is similar to the appearance of a unique protein band, a, in rejuvenated *in vitro* products (Fig. 4). Figure 8 also shows a shift from high to low mol wt proteins occurring with age, which is reversed by rejuvenation. The differences between the *in vitro* and *in vivo* translation products is to be expected due to protein processing, turnover, and differences in the selection of mRNA for translation.

Cotyledons normally abscise with aging. To determine whether such cotyledons synthesize proteins, abscised 20-d-old cotyledons were labeled with [^{35}S]methionine (Fig. 7, lane 9). Although these abscised cotyledons are just as active in protein synthesis as 20-d-old cotyledons which had been intact, it can be seen that they produce a distinctly different banding pattern. In the excised cotyledons, there is a great shift toward smaller protein products as compared with 20-d-old intact cotyledons (Fig. 7, lanes 5 and 9, respectively).

Quantitative aspects of *in vivo* labeling of cotyledons is of interest even though pool and turnover considerations make it impossible to rigorously compare activities between ages (Table II). Methionine incorporation into protein declines during aging but is greatly increased after rejuvenation. This increase in protein synthesis by epicotyl removal occurs within 2 d. We are particularly interested in the regulation of cotyledon aging and rejuvenation as mediated by the presence or absence of factors in the epicotyl of the soybean seedling.

DISCUSSION

Many of the translatable poly(A)mRNA vary in relative concentration during aging and rejuvenation of soybean cotyledons (Fig. 5). We suggest that aging in this system does not involve total repression or derepression of gene activities. Instead, quantitative shifts in individual species of the mRNA population and/or their relative rates of *in vitro* translation occur after 4 d and are maintained during early and late senescence (12–20 d). While 56% of the 470 translation products detected by two-dimensional gels maintained their original rates of synthesis at all ages, based on their relative degrees of x-ray film exposure, 28% increased, and 16% decreased with age. Of the proteins (44% of total) which changed, 37% returned after rejuvenation to roughly 4-d-old levels (Fig. 5). Therefore, about 28% of the total proteins irreversibly change with aging. During the process of rejuvenation, there must be a mechanism capable of shifting many gene activities to favor survival of the organ. This reversible shift in protein synthesis was also found by *in vivo* labeling experiments (Figs. 6–8). The electrophoretic protein band profiles were greatly altered with cotyledon age when compared to 4-d-old tissue, while rejuvenation

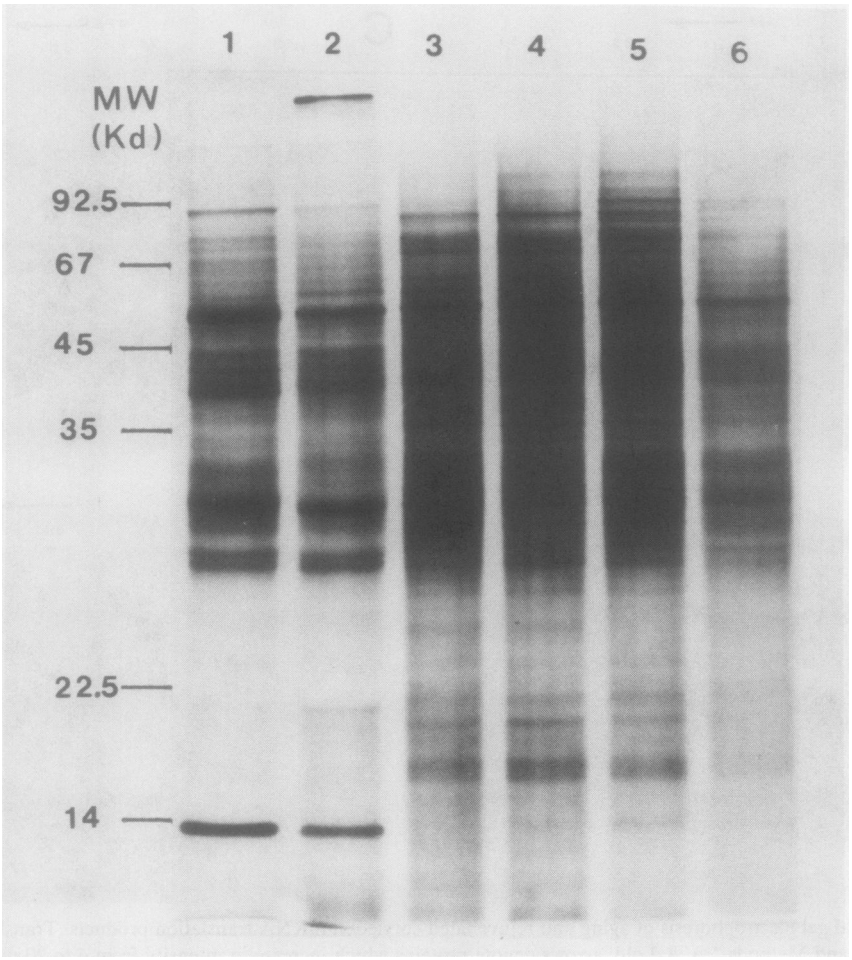

FIG. 6. *In vivo* labeling of aging and rejuvenated cotyledon proteins. Procedures were as described in "Materials and Methods," but epidermal layers were not removed, and mesophyll and palisade layers were extracted together. α-Amanitin was used only in the experiment presented in lane 1. Lane 1 represents 4-d-old products from medium supplemented with 10 μg/ml α-amanitin. Lanes 2 through 5 represent 4-, 12-, 20-d-old, and rejuvenation products (respectively) from the outer 1-mm ring of cotyledon discs. Lane 6 (rejuvenated) represents products from inner 8-mm disc. The disc was cut with an 8-mm cork borer from within the initial 10-mm disc following incubation. Note that the same bands are found in lanes 1 and 2 and in 5 and 6. Each lane contained 200,000 cpm of labeled protein.

Table II. *In Vivo Labelings of Aging and Rejuvenated Cotyledon Mesophyll Tissue*

Samples were labeled for 8.5 h. Pluses followed by a number indicate number of days following epicotyl removal from 20-d-old seedlings.

Age	[^{35}S]Methionine Incorporation	[^{35}S]Methionine Accumulation	[^{35}S]Methionine Incorporation into Protein
d	*cpm* × 10^{-5} *into protein*	*cpm* × 10^{-6}	*% of accumulation*
4	25.37	45.94	55.2
12	15.10	36.87	41.0
20	5.07	36.48	13.9
20 (abscised)	3.55	22.68	15.6
20 + 2	30.85	51.27	60.2
20 + 22	22.48	48.89	46.0
20 + 45	41.73	69.48	60.1

partially reversed the effect of age.

Much work has been done on protein synthesis during germination in castor bean cotyledons. From the fluorograms of Roberts and Lord (21), it appears that the mRNA population, as judged by *in vitro* translation products, undergoes little qualitative change throughout germination. This is similar to our *in vitro* results (Fig. 3). We know of only one other study of qualitative changes in protein synthesis during plant aging (7). Unlike soybean cotyledons, the senescence of somatic cells in *Volvox* is accompanied by the cessation of synthesis of most protein species (7). In the *Volvox* system, only one senescing cell type is involved, and the aging and death of the cell population is closely synchronized. It is difficult to compare the two systems since the soybean cotyledon involves the functional death of an organ of heterogenous nonsynchronized cells. In human fibroblast cultures, the only qualitative change to accompany cell senescence was the appearance of two new proteins (4).

Aging of any organism may result from the loss of required proteins (structural proteins, enzymes, or cofactors) or from an increase in deleterious proteins (*e.g.* hydrolases). However, not all proteins which either increase or decrease in quantity may influence the aging process. An advantage of the rejuvenated cotyledon system is that it helps to screen possible proteins required for survival. Those which decline with age but fail to increase upon rejuvenation would seem not to influence survival. The above approach narrows the range of possible causal proteins to more restrictive subsets of either the increasing or decreasing sets of proteins.

Rejuvenation likely involves more than the simple restoration of the translational activities of a few proteins. In this study, five

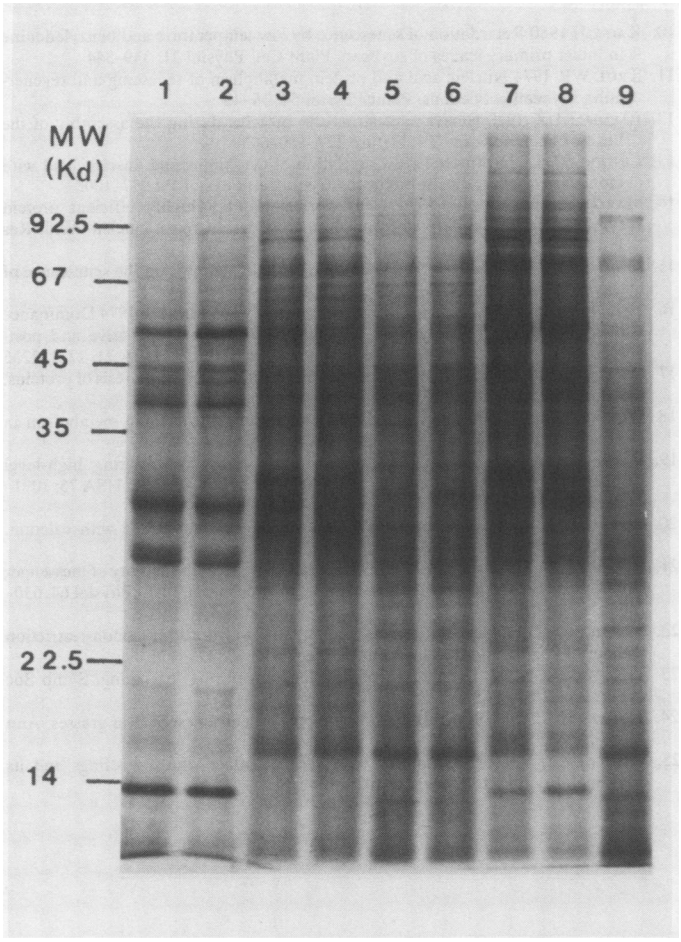

FIG. 7. *In vivo* labeling of aging and rejuvenated cotyledon proteins in the presence of α-amanitin. Incubations were conducted according to "Materials and Methods." α-Amanitin was used as a concentration of 1 μg/ml. Lanes 1, 3, 5, and 7 were loaded with 4-, 12-, 20-d-old, and rejuvenated cotyledon proteins (respectively) following 8.5 h of incubation. Lanes 2, 4, 6, and 8 were loaded with 4-, 12-, 20-d-old, and rejuvenated cotyledon proteins (respectively) following 7.5 h of incubation and subsequent 2 h cold methionine chases. Lane 9 contained protein from a 20-d-old abscised cotyledon incubated for 8.5 h. Each lane contained 200,000 cpm of labeled protein.

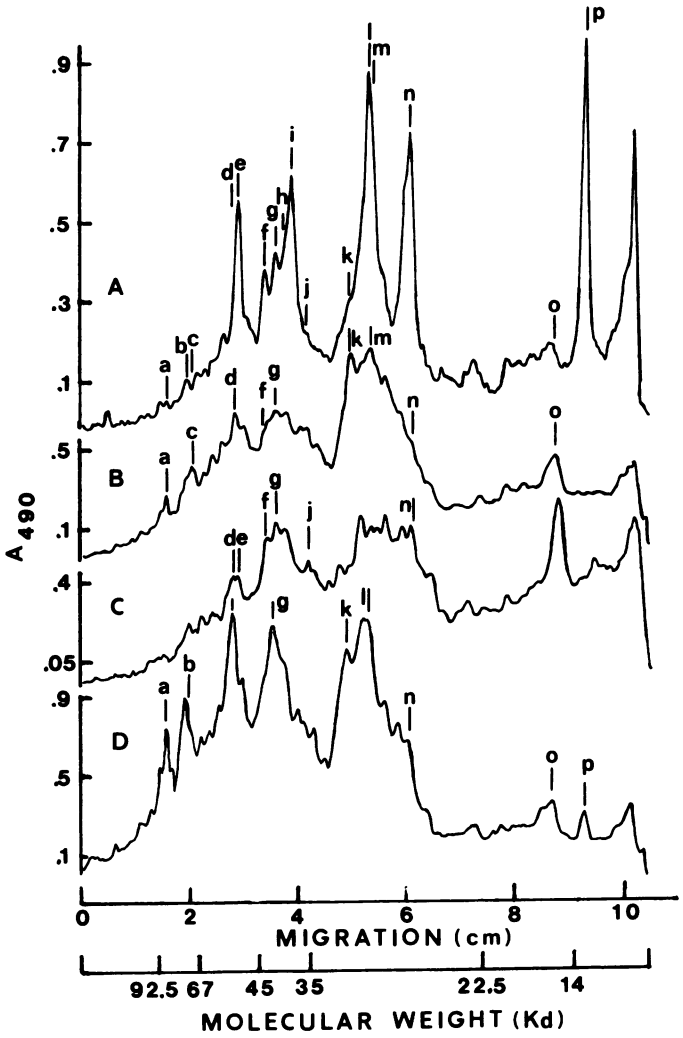

FIG. 8. Densitometer tracings of aging and rejuvenated proteins from Figure 7. Each tracing is from the 8.5 h incubation proteins. A, 4-d-old mesophyll disc protein; B, 12-d-old; C, 20-d-old; D, rejuvenated. Signal amplification (vertical axis span) was adjusted to provide similar tracing areas for each age. Lower case letters indicate identical protein peaks between samples.

proteins which were not prominent at any age became very prominent among rejuvenated mRNA translation products (Fig. 5D, proteins 1–5). It is not known whether any of these proteins are directly involved in the rejuvenation process, but they deserve further study. The possibility should be acknowledged that these proteins are involved in the differentiation of the rejuvenated cotyledon to a state unrelated to the young cotyledon. Thus, the mechanism by which young tissue fails may not be the converse of that by which old tissue is rejuvenated.

A quantitative decline in protein synthesis is correlated with aging, and it is likely that protein synthesis is controlled by availability of mRNA. mRNA levels fell by 70% from 4 to 12-d-old cotyledons. Twelve-d-old cotyledons showed a major decline in Chl, protein, RNA, and polyribosome content and size (Skadsden and Cherry, unpublished results). However, mRNA content in rejuvenated cotyledons was similar to 12-d-old cotyledons—66% below that of 4-d-old cotyledons. Thus, the total mRNA content alone cannot explain the declining metabolic activity accompanying aging.

Maximum poly(A)mRNA *in vitro* translation product size increased after day 4, and there was no tendency for the average product size to decrease with age (Fig. 4). This contrasted with the *in vivo* results in which the average size of translation products declined with aging in intact cotyledons and continued to decline in abscised cotyledons (Fig. 7). Furthermore, a diffuse background of the labeling pattern occurred in gels of *in vivo* products from aged cotyledons, perhaps due to an increase in proteolysis (Figs. 6 and 7). However, sharper protein bands were evident after rejuvenation. Rejuvenation also increased the relative amount of high mol wt protein synthesis *in vivo* and reduced the low mol wt background (Figs. 7 and 8). In a previous study (Skadsen and Cherry, unpublished results), polyribosomal poly(A)mRNA in young, aging, and rejuvenated tissue was found to be of the same size. This and the above *in vitro* translation data provide compelling evidence that mRNA size does not decline during aging. The decline in *in vivo* product size may therefore be the result of increased proteolysis or a decline in net synthesis.

No evidence for increased errors in the production of translatable mRNA was found with age. No 'stuttering' of proteins was found to emanate for any of the proteins seen on two-dimensional gels. It is still quite possible that errors are encountered during translation in the *in vivo* system. The loss of amino acids accom-

panying aging is certainly a factor which could cause errors (19).

Rejuvenated cotyledons were usually assayed 20 d after epicotyl removal. However, the increase in apparent *in vivo* translational activity reached a maximum within 48 h of epicotyl removal. Additional work is required to determine which factor is essential for the restoration of protein synthesis during the early stage of rejuvenation. We suspect that this may also be related to the cause of aging in this system.

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