PROTEIN SYNTHESIS IN AGING SOYBEAN COTYLEDONS
LOSS IN TRANSLATIONAL CAPACITY

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SUMMARY: Ribosomes and supernatant fractions from soybean cotyledons of different ages were prepared to study the Poly U (polyuridylic acid)-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 the older cotyledons. Substitution of wheat embryo supernatant fraction for soybean cotyledon supernatant fraction resulted in a several fold increase in amino acid incorporation activity, in ribosomes from all ages of soybean cotyledons. Such increase in activity was particularly significant in the older cotyledons. From these experiments it is concluded that in aging soybean cotyledons there is a loss in translational capacity.

It has been demonstrated that there is a rapid transition of the proportion of ribosomes to polysomes, with the washing (aging) of thin discs of storage tissues of plants (1,2,3). In addition to dramatic increases in metabolic activity in washed or aging discs of artichoke (4), carrot (5) and sugarbeet (6) their ability to incorporate labelled amino acids in ribosomal preparations from such storage tissues have been reported (7). The foregoing studies, though excellent in demonstrating the transition of an inactive-dormant system to a metabolically active system, does not necessarily reflect changes associated with aging. It may be erroneous to use the term "aging" in studies of storage organs where the emphasis is placed on the induction of cell dedifferentiation in resting, dormant mature inactive tissues.

This report deals with the rate of protein synthesizing ability of the cotyledons of the germinating soybean seedlings, as a function of cotyledon age and relate the changes in rate of protein synthesis to changes in *in vitro* amino acid incorporating capacity of ribosomal preparations. The cotyledon

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tissue provides a useful system to study protein synthesis, since there is extensive cytoplasmic differentiation dissociated with cell division.

MATERIALS AND METHODS

Plant material: Soybean seeds (Glycine max var. Harosoy 63) were surface sterilized in 10% chlorox, soaked in water for 18 hr and sown in moist vermiculite. Cotyledons were harvested after 1, 3, 5 and 10 days following germination in the dark at 27-29°C and used immediately.

Preparation of ribosomes: Isolation of ribosomes and the supernatant fraction was according to the procedure described by Verma et al. (8). 50 g tissue (soybean cotyledon frozen in liquid nitrogen) were extracted with 100 ml of extraction buffer containing 100 mM Tris-Acetate pH 8.0, 50 mM KCl, 20 mM MgAc, 5 mM β-mercaptoethanol and 0.5% sodium deoxycholate. The slurry was filtered through cheesecloth, centrifuged at 25,000 g for 15 min. The supernatant was filtered through miracloth and layered over a 3 ml sucrose cushion containing 50 mM Tris Ac pH 8.0, 25 mM KCl, 10 mM MgAc and 1.5 M sucrose and centrifuged at 105,000 g for 90 min. The supernatant was collected and diluted to 25% glycerol and stored at -15°C. The ribosomal pellet was resuspended in 50 mM Tris Ac pH 8.0, 25 mM KCl, 10 mM MgAc and 25% glycerol. The ribosomal concentration was determined at A_{254}, adjusted to 1.5 mg/ml and stored at -15°C until required.

Poly U-directed phenylalanine incorporation: The amino acid incorporation was carried out using a 0.5 ml reaction mixture incubated at 37°C. The mixture contained 0.3 mg ribosomes, 10 µl supernatant, 30 mM Tris Ac pH 8.0, 2 mM ATP, 40 µg Poly U, 60 mM KCl, 10 mM creatine phosphate, 30 µg creatine phosphokinase, 2 µl ^{3}H-phenylalanine, 0.02 mM GTP, 6 mM MgAc, 3.2 mM DTT, 1 µg each of the essential amino acids (except phenylalanine) and 80 µg wheat germ tRNA (9). At 10 min intervals 50 µl samples were dotted onto filter discs, dried and washed for 30 min each in 10% TCA containing 1 mM phenylalanine; 5% TCA; ethanol:ether (3:1) and finally 15 min in ether. Radioactivity of the dried discs was determined in a Nuclear Chicago Scintillation Counter.

RESULTS AND DISCUSSION

Data presented in Table 1 shows that the requirements for amino acid incorporation by ribosomes of aging cotyledons are similar to other plant systems (10,11). The activity of the system is strictly dependent on the presence of ATP, GTP, K^+, Mg^{2+}, an ATP generating system and upon the addition of the supernatant fraction. Ribonuclease, puromycin and cycloheximide inhibited the incorporation of ^{3}H-phenylalanine into trichloracetic acid-insoluble material. Ribosomal preparations from aging cotyledons have varying capacities for amino acid incorporation. Preparations from 3-, 5-, and 10-day-old cotyledons had a reduced capacity for amino acid incorporation compared to 1-day-old cotyledon preparations.

TABLE 1

Treatment	Amino Acid Incorporation/CPM/mg RNA Age of Cotyledons/Days 1	3	5	10
Complete System	8462	1987	926	245
- KCl	5835	1015	827	318
- $MgCl_2$	718	414	263	226
- Dithiothreitol	2651	821	472	345
- ATP	2516	948	769	264
- GTP	1962	413	348	270
- ATP, GTP, PEP, Pyruvate Kinase	250	233	259	280
- tRNA	521	302	254	275
- Supernatant	230	250	240	220
- Ribosomes	230	232	203	216
+ 0.5 mg RNase	197	-	-	-
+ 0.5 µg Cycloheximide	7028	-	-	-
+ 5.0 µg Cycloheximide	4418	-	-	-
+ 5.0 µg Puromycin	5855	-	-	-
+ 50.0 µg Chloramphenicol	5946	-	-	-

Requirements for cell-free amino acid incorporation by ribosomal preparations from aging soybean cotyledons. Incorporation of ^{3}H-phenylalanine in reactions involving ribosomes and 105,000 g supernatant fraction from soybean cotyledons of different ages. A 125 µl reaction mixture was used containing 50 µg ribosomes and 5 µl supernatant fraction. The reactions were incubated at 37°C for one hour.

In Figure 1 the kinetics of *in vitro* ^{3}H-phenylalanine incorporation of isolated ribosomes is presented. After an initial lag of 5 min, the rate of incorporation was almost linear over 60 min, when supernatant fractions and ribosomes from 1-day-old cotyledons were used. Within 3 days, the activity of the supernatant fraction and ribosomes was reduced by more than 50 per cent, possibly implying that most of the cotyledon protein synthetic activity occurred in the first 24-36 hr following imbibition. Between 3 and 10 days after germination, as the cotyledon aged, there was a gradual reduction with

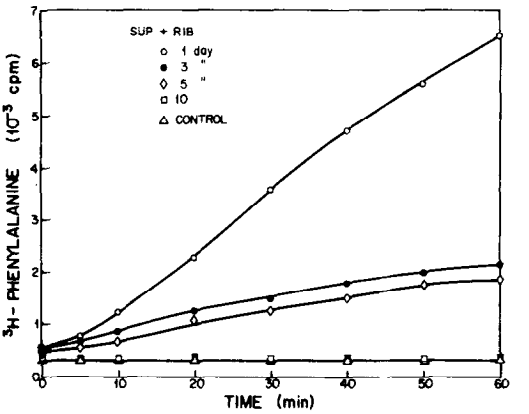
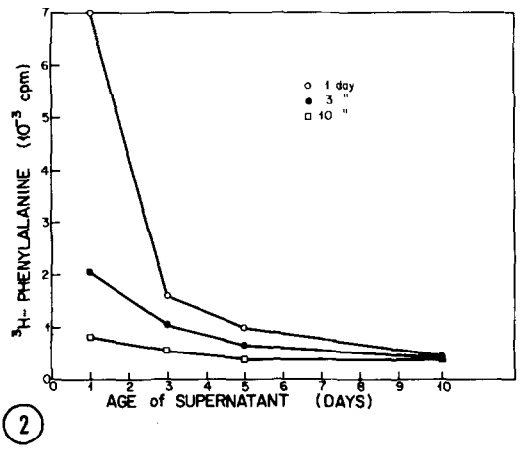


FIG. 1. Kinetics of *in vitro* ^{3}H-phenylalanine incorporation of ribosomes from 1- (○), 3- (●), 5- (◇) and 10- (□) day-old soybean cotyledons. Control (△) contained no ribosomes. Supernatant fractions were also prepared from cotyledons of different ages as described above. The reaction mixture of 0.5 ml contained 0.3 mg ribosomes, 10 µl supernatant, 40 µg poly U plus other ingredients as described under methods. At 10 min intervals 50 µl samples were dotted onto filter discs and assayed as described in Methods.

virtually very little activity left in 10-day-old cotyledons. Previous reports (12,13) of such declining capacities of ribosomal preparations from developing and maturing systems are in agreement with our results. In the presence of Poly U, a 15 fold increase in phenylalanine incorporation in 1-day-old ribosomal preparations and a reduced incorporating capacity in ribosomal preparations of aging cotyledons suggest limited availability of mRNA.

To determine the influence of the supernatant fraction, on the capacity of ribosomal preparations from different ages of cotyledons, to incorporate ^{3}H-phenylalanine, results obtained in Fig. 2 clearly show a differential capacity to support amino acid incorporation. Supernatant fraction from older cotyledons 3-, 5- and 10-days was less effective than supernatant from 1-day old cotyledon. It is clear that the enhanced incorporation associated with supernatant from younger cotyledons was independent of the age of the ribosomal preparation. One could speculate that with age, the supernatant fraction loses some essential factor or some other inhibiting product accumulates during the aging process.



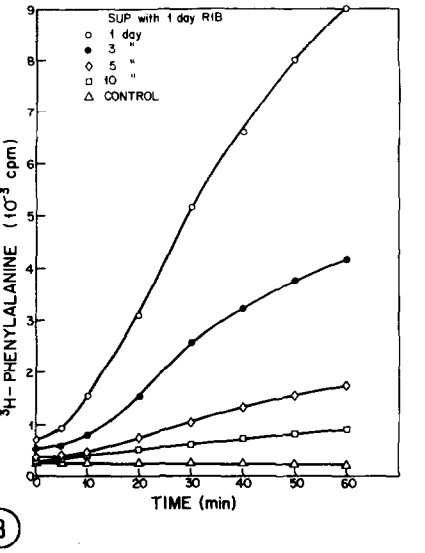


FIG. 2. Influence of supernatant fraction on amino acid incorporation by ribosomes from soybean of different ages; 105,000 g supernatant was isolated from 1-, 3-, 5- and 10-day soybean cotyledons, ribosomes were isolated from 1- (○), 3- (●) and 10- (□) day soybean cotyledons. A 125 µl reaction mixture was used with 75 ug ribosomes and 5 µl of supernatant fraction. The reactions were incubated at 37°C for one hour. The reaction was stopped by the addition of 0.5 ml of 10% TCA and filtered onto GF/A filter discs and counted in a Nuclear Chicago Scintillation Counter.

FIG. 3. Kinetics of *in vitro* ^{3}H-phenylalanine incorporation of 1-day-old ribosomes and 105,000 g supernatant from 1- (○), 3- (●), 5- (◇) and 10- (□) day-old soybean cotyledons. The incorporation medium was similar to that described under Fig. 2. Control (△) reaction contained no ribosomes.

To further test the relative efficiency of supernatant fractions from young and old cotyledons the following experiment was performed. In addition to the standard reaction ingredients, Poly U, and ribosomal preparations from 1-day-old cotyledons, supernatant from different ages was added to the reaction mixture. The kinetics of the rate of incorporation is presented in Fig. 3 which illustrates that between 1 and 3 day supernatant there is a 50 per cent decrease in ^{3}H-phenylalanine incorporation. Activity of supernatant from 10-day cotyledon is less than 10 per cent of the activity, observed with 1-day supernatant. These results further demonstrate that the decreased incorporation associated with the age of the supernatant is independent of the age of the

TABLE 2

Source of Supernatant	Source of Ribosomes	Age of Ribosomes/Days/CPM			
		1	3	5	10
Soybean Cotyledon (1 day old)	Soybean Cotyledon	5,058	2,897	864	409
Soybean Cotyledon	Wheat Embryo	867	400	373	286
Wheat Embryo	Soybean Cotyledon	42,874	25,877	17,251	9,186

The effect of combining the wheat embryo system with soybean cotyledons on amino acid incorporation. Freeze-dried wheat embryos were extracted using the same methods as those used for extraction of soybean cotyledons. A 125 µl reaction mixture was employed using 40 µg ribosomes and 5 µl of 105,000 x g supernatant fraction. The reaction was incubated at 37°C for 60 minutes.

ribosome, since a 1-day ribosome was used in all cases. However, results from a storage tissue such as the carrot discs sliced and washed (aged) show enhanced incorporation as a feature of the ribosome quite independent of the supernatant source (14). The difference here is that the soybean cotyledon is an aging organ in situ, while the mature quiescent carrot tissue has been triggered into metabolic activity following slicing and washing.

In a comparison of the supernatant fraction from the soybean system with that of the wheat embryo system, data presented in Table 2 shows that the efficiency of wheat embryo ribosome to incorporate ^{3}H-phenylalanine to be 20 per cent of a 1-day ribosome and supernatant of soybean and 50 percent of a 10-day-old ribosome/1-day supernatant. However, when a wheat embryo supernatant was used, with soybean ribosome, several fold increase in incorporation was obtained for all ages of soybean ribosomes. It should be pointed out here that the activity of the older ribosomes from 5- and 10-day cotyledons was proportionally greater than 1- and 3-day-old cotyledons. It is suggested here that the activity of the ribosomes from aging soybean cotyledons is greatly improved by supplying an active supernatant fraction from the wheat embryo.

The decrease in ribosomal capacity of aging cotyledons seems to be under

TABLE 3

Age of Purified Factors Days	Age of Ribosomes / Days/ CPM					
	1	3	5	7	10	15
1	11,353	10,382	4,126	3,101	2,903	2,775
3	8,426	5,735	3,419	2,964	2,302	2,342
5	5,295	3,819	3,041	2,597	2,385	2,347
7	5,075	3,500	3,007	2,046	2,219	1,650
10	4,736	3,232	2,914	2,150	2,100	1,655
15	3,723	3,070	2,880	2,431	2,325	2,069

The effect of age of purified supernatant factors on amino acid incorporation in ribosomes of different ages from soybean cotyledons. Dialyzed supernatant (105,000 x g) fractions were separated on DEAE columns and fractions collected as described by Marcus et.al. (9). A 125 µl reaction mixture was employed using 40 µg ribosomes and 5 µl of purified factors. The reaction was incubated at 37° C for 60 minutes.

the influence of supernatant factors. This could be explained by data presented in Table 3 which provides additional proof that purified (initiation and elongation) factors are more active than crude supernatant factors. Activity of purified factors from 15-day cotyledons is 30 per cent of that observed for purified factors from a 1-day ribosome preparation. Whereas with crude supernatant factors the activity in a 10-day ribosome preparation is only 10 per cent. In the case of ribosome preparations from 5- and 7-day cotyledons the activity with purified 15-day factors is about 50 per cent compared to 1-day factors, which again demonstrates that older ribosomes remain active, provided purified supernatant factors are used. However, with 10- and 15-day ribosomes, the age of supernatant had no significant effect, since the activity between 1- and 15-

day factors was roughly equal. Therefore, substitution of crude factors with purified factors and the resulting increase in ribosomal efficiency in aging cotyledons possibly demonstrates the presence of a translation inhibitory factor in the crude supernatant that greatly inactivates the protein synthesizing system.

Preliminary results on the isolation of poly A segments on oligo (dT) cellulose columns and the determination of their size by ^{3}H-Poly U hybridization indicates that the length of the poly A message shortens with age in soybean cotyledons (Data not presented here). In the absence of a well defined function for poly A it is possible that with age there is an accumulation of hydrolytic enzymes or inhibiting substances. These could result in the digestion of poly A segments and a decrease in the stability of the message with possible hindrance of the efficiency of the translation machinary. Further work is necessary to determine the exact role of poly A in aging systems.

Results presented here demonstrate that with age there is a decline in the capacity of ribosomes and supernatant factors to incorporate amino acids in vitro and thus a loss in translational capacity is observed. The events accompanying aging in soybean cotyledons could be due to any one or all of these possibilities 1) limited availability of mRNA 2) decrease in polyadenylated mRNA 3) decrease in polysome content 4) inactivation of ribosome and translation factors due to loss of an essential factor or possibly accumulation of an inhibiting factor and finally resulting in a decrease in net protein synthesis. Further work is required to examine in detail each of these possibilities.

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