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Protein Synthesis in Cotyledons of *Pisum sativum* L.

I. CHANGES IN CELL-FREE AMINO ACID INCORPORATION CAPACITY DURING SEED DEVELOPMENT AND MATURATION[1]

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ABSTRACT

The changes in protein content of pea cotyledons have been followed during the period from 9 to 33 days after flowering. Initially protein content increased gradually with a rapid period of deposition occurring between days 21 and 27 after flowering. After the 28th day the rate of accumulation of protein declined as the seed dehydrated and matured. At maturity the pea cotyledon contained approximately 25% protein which was divided into albumins and globulins in the ratio of 1:1.4.

Analytical data and the incorporation of exogenously supplied ^{14}C-leucine indicated that albumins were synthesized early in cotyledon development whereas globulin synthesis predominated with increasing maturity.

Ribosomal preparations extracted from seeds during the period of rapid protein synthesis contained a high percentage of polysomes. Preparations from older cotyledons with a declining capacity for protein synthesis had few polysomes and an abundance of monosomes. The amino acid-incorporating capacity of ribosomal preparations from cotyledons of varying age was related to the polysomic content. The phenylalanine-incorporating capacity of ribosomal preparations from mature pea seed could be stimulated by the addition of polyuridylic acid. The distribution of polysomes and the *in vitro* incorporation data suggested that protein synthesis could be partially restricted by the availability of messenger RNA at maturity.

However, reciprocal mixing experiments of supernatant and ribosomal fractions from cotyledons of different developmental age indicated that the supernatant fractions have varying capacities to stimulate *in vitro* amino acid incorporation. Thus the possibility of the regulation of protein synthesis at the translational level was not precluded.

Approximately 25% of the dry weight of the mature pea seed is protein. This protein, which accumulates during development, may be divided on the basis of solubility characteristics into two major components, albumins and globulins (14). Preliminary developmental studies have shown that the rate of protein deposition and the type of protein synthesized vary with developmental age.

In view of these changes in the rate and pattern of protein synthesis during seed maturation, the developing pea cotyledon provides a convenient system in which to study the control of protein synthesis. In the present study variations in the capacity for exogenous amino acid incorporation *in vivo* have been examined, and attempts have been made to relate these changes to alterations in the capacity for cell-free amino acid incorporation.

MATERIALS AND METHODS

Seeds of an early dwarf variety of pea (*Pisum sativum* L., var. Burpeana) were planted in pots in a soil-peat-perlite (1:1:1) mixture and maintained in a greenhouse under a 16-hr photoperiod. Supplemental illumination was provided by fluorescent and incandescent lighting. Application of fertilizers N:P:K (200, 40, 160 μg/g) and 75 mM $Ca(NO_3)_2$ were made on alternate days.

Under this culture regime the plants flowered a month after the seeds had been sown. The date of full bloom was recorded for each individual flower and used as the zero date in developmental studies. At intervals following flower, the fruits were collected from the two lowest pod-bearing nodes. In general, 10 pods were collected for a specific developmental stage, and a random sample of the peas from these pods was then prepared. The testa and the embryonic axis were removed from the seed, and the cotyledons were used for analysis.

***In Vivo* Amino Acid Incorporation and Protein Fractionation.** Two microliters of ^{14}C-L-leucine (240 mc/mmole, 100 μc/ml) were injected through the pod into each cotyledon of intact pea seeds which were left *in situ* in the detached pods. After a 3-hr incubation the seeds were removed from the pod and the cotyledons were recovered.

Albumin and globulin fractions were prepared from the cotyledons by modifications of the method of Danielson (6). Twenty cotyledons from peas which had been injected 3 hr previously with ^{14}C-L-leucine were homogenized in a VirTis tissue homogenizer at medium speed for 15 min with 30 ml of 0.2 M NaCl in 20 mM phosphate buffer, pH 7.0. The homogenate was stirred at room temperature for 4 hr and then centrifuged at 10,000*g* for 30 min. The supernatant was brought to 70% saturation by the stepwise additions of $(NH_4)_2SO_4$ and allowed to stand in ice for 30 min. The precipitate was collected by centrifugation and dissolved in 20 ml of 0.2 M NaCl in 20 mM phosphate buffer, pH 7.0. This solution was then dialyzed for 12 hr at 4 C against 3 changes of 15 volumes of deionized H_2O to precipitate the globulin fraction which was collected by centrifugation. The protein content of the supernatant fraction was determined by the

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method of Lowry *et al.* (9) as an estimation of the albumin component, and the radioactivity of an aliquot of this fraction was determined using Bray's scintillant (5) and a Packard Tri-Carb spectrometer. The precipitated globulin was dissolved in 10 ml of 0.2 M NaCl in 20 mM phosphate buffer, pH 7.0, and the protein content and radioactivity were determined.

Isolation of Ribosomes. Ten cotyledons were isolated from the developing peas and immediately dropped into a mortar containing liquid nitrogen and ground to a fine powder. Thirty milliliters of grinding medium (20 mM tris, pH 7.6; 0.25 M sucrose; 10 mM $MgCl_2$; 15 mM NaCl; 5 mM β-mercaptoethanol; 0.5% DOC [sodium deoxycholate]) and 2 mg of cycloheximide were added, and the partially frozen slurry was homogenized for 1 min. The homogenate was filtered through cheesecloth and Miracloth (Calbiochem), and the filtrate was centrifuged at 20,000*g* for 15 min. The resulting supernatant was layered over successive layers of 0.5 M (3 ml) and 1.8 M (10 ml) sucrose in solution A (20 mM tris, pH 7.6; 10 mM $MgCl_2$; 15 mM NaCl; and 5 mM β-mercaptoethanol) in 25-ml Spinco polycarbonate centrifuge tubes and centrifuged at 105,000*g* for 3 hr. The ribosomal pellet was gently rinsed in solution A and then suspended in solution A by stroking with a glass rod. This suspension was centrifuged at 1,000*g* for 2 min, and an aliquot of the supernatant was then layered onto a linear sucrose density gradient (10–34% sucrose in solution A) supported by a 1.5-ml cushion of 34% sucrose and centrifuged at 200,000*g* for 1 hr. The distribution of ribosomes within the gradient was measured at 254 nm using an ISCO density gradient fractionator with a 1-cm light path and flow rate of 0.6 ml/min.

Preparation of the Supernatant. Sixty cotyledons were frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar, 30 ml of 0.25 M sucrose in solution A were added, and the slurry was homogenized for 3 min. The homogenate was filtered through cheesecloth and Miracloth, and the filtrate was centrifuged at 20,000*g* for 15 min. The supernatant was recentrifuged at 140,000*g* for 90 min. The resulting supernatant could be stored for several days at −20 C without losing its capacity to stimulate *in vitro* amino acid incorporation.

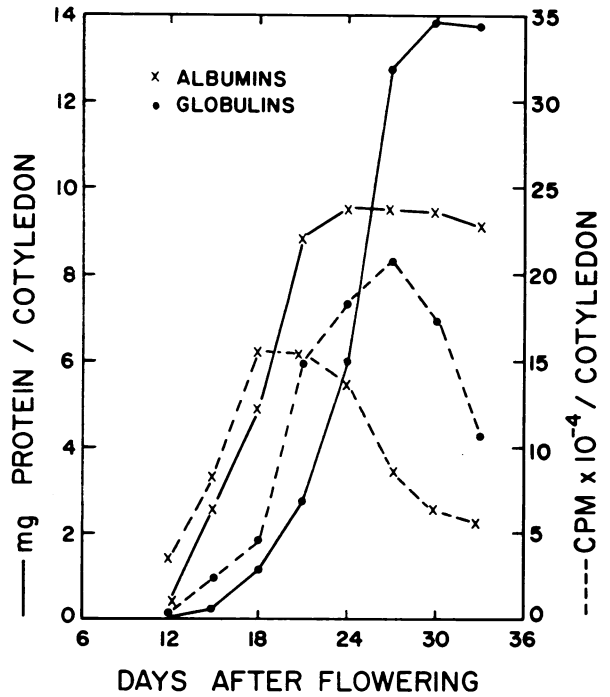

FIG. 1. Changes in the protein composition and incorporation of exogenously supplied ^{14}C-leucine into protein components in the pea cotyledon during development.

***In Vitro* Amino Acid Incorporation.** For studies of amino acid incorporation, ribosomal preparations similar to those used in the sucrose density gradient analysis were extracted. However, this extraction medium did not contain cycloheximide. The reaction medium used in the incorporation studies, unless otherwise indicated, was based upon that described by Mans and Novelli (11) and Williams and Novelli (17) and included the following, in micromoles: 50 tris-HCl, pH 7.6; 5 $MgCl_2$; 8 KCl; 2 β-mercaptoethanol; 0.5 ATP; 0.15 GTP; 6.4 P-enolpyruvate; 0.05 mg of crystalline pyruvate kinase; supernatant fraction (1.5 mg protein); ribosomes, 1 mg of protein; 0.5 μc of ^{14}C-L-leucine (240 mc/mmole) in a total volume of 0.5 ml. In the time course studies incorporation was determined by the filter paper disc method (10) using aliquots withdrawn from the reaction mixture. For the remainder of the studies incubation was at 37 C for 30 min when the reaction was terminated by the addition of 10% trichloroacetic acid containing 5 mM leucine. The trichloroacetic acid-insoluble material was collected and sequentially extracted with hot 5% trichloroacetic acid, absolute ethanol, ethanol-ether (2:1, v/v), and ether. The precipitate was dried at room temperature and dissolved in 0.2 ml of 0.1 N NaOH, and the radioactivity was determined in the Packard Tri-Carb spectrometer using 10 ml of Bray's scintillant.

RESULTS AND DISCUSSION

Protein Fractionation. In young cotyledons the bulk of the protein was water-soluble albumin (Fig. 1), while in the later stages of development globulins accumulated. Summation of the albumin and globulin fractions indicated that these components totaled 24 mg per cotyledon, which represents approximately 25% of the final dry weight of the mature cotyledon. In young cotyledons the exogenously supplied leucine was incorporated principally into the albumin fraction; however, by day 24 the amino acid was incorporated predominantly into the globulin fraction. Amino acid incorporation into the globulin fraction was sustained until day 30. In contrast, the capacity to incorporate exogenously supplied leucine into the albumin fraction declined from day 21.

Polysome Levels during Cotyledonary Development. Ribosome preparations isolated from pea cotyledons had an A_{260}/A_{280} ratio of about 1.8/1. This ratio decreased slightly in preparations from older cotyledons, but the RNA content of the ribosomes remained constant, and it thus appeared that there was increased contamination by nonribosomal protein rather than a change in ribosome composition *per se*.

The percentage of ribosomes present as polysomes was calculated from the area under the monosome and polysome tracing of the sucrose density gradient profiles. During early development approximately 85% of the ribosomes were present in polysomic configuration as typified by Figure 2,A and B, illustrating the distribution of polysomes and monosomes in ribosomal preparation from cotyledons 15 and 24 days after flowering. During the dehydration phase the monosome component increased with a concurrent decline in polysome content; consequently, at seed maturity the majority of the ribosomes existed as monosomes as indicated by the sucrose density gradient profile of ribosomes prepared from cotyledons 30 days after flowering (Fig. 2C).

The change in the relative distribution of polysomes to total ribosomes during the course of cotyledonary development is further depicted in Figure 3, and it is seen that the decline in polysome level, which commenced at dav 27, coincided with

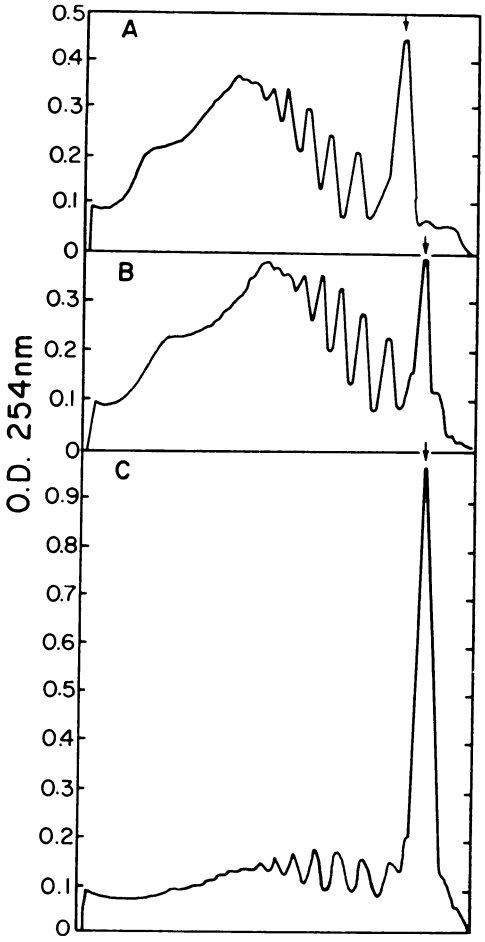

FIG. 2. Ribosome profiles prepared from pea cotyledons at varying ages after flowering. A: 15 days; B: 24 days; C: 30 days. Arrows denote monoribosome peak.

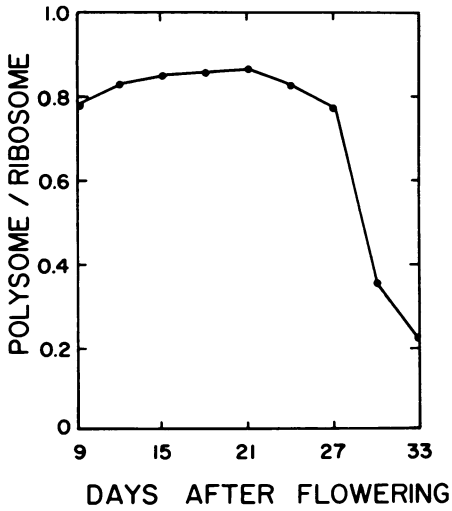

FIG. 3. The distribution of polysomes in the ribosomal preparations from pea cotyledons. Results are expressed as the proportion of the total ribosome profile occurring in polysomic configuration after sucrose density centrifugation.

the observed decreased capacity of the intact mature cotyledon to incorporate exogenously supplied leucine (Fig. 1).

Marré (13) has reported a similar decrease in polysome level during the maturation of the castor bean endosperm. However, in contrast to the situation in pea cotyledons, Marré (13) reported that the decline in polysome content was associated with a reduction in total ribosome content of the castor bean endosperm at maturity. A similar situation appears to exist in the developing maize seed. Ingle *et al.* (8) recorded an extensive depletion of endosperm RNA during seed maturation, indicating ribosome degradation.

The observed accumulation of monosomes (Fig. 2C) coupled with our unpublished findings that RNA content remained high at maturity indicated that ribosomal RNA was not extensively degraded during dehydration and maturation of the pea cotyledon. The extraction of ribosomes from the cotyledons of mature dry seeds of *Pisum arvense* (3) and peanuts (12) and the ultrastructural studies of Bain and Mercer (2) demonstrating the occurrence of free ribosomes in mature pea cotyledons provide further confirmation that ribosome destruction is not an integral part of the maturation of the cotyledons in these species.

***In Vitro* Amino Acid Incorporation.** In general it was found that the requirements for amino acid incorporation by ribosomes from developing pea cotyledons were similar to those reported in other plant systems (4). The reaction was dependent upon the presence of ATP, GTP, K^+, Mg^{2+} and P-enolpyruvate (Table I) with a broad pH optimum of 7.6. Studies using varying amounts of different monovalent ions indicated that K^+ was the most effective in stimulating amino acid incorporation with 8 μmoles of KCl per 0.5 ml being optimal. Na^+ and NH_4^+ were slightly less effective in stimulating amino acid incorporation at their optimal concentrations of 6 and 5 μmoles per 0.5 ml, respectively. RNase, puromycin and cycloheximide inhibited the incorporation of ^{14}C-L-leucine into trichloroacetic acid-insoluble material; however, chloramphenicol did not affect amino acid incorporation, thus indicating that the polypeptide synthesis was achieved by 80S ribosomes (1). This suggestion is confirmed by our unpublished observation that the ribosomal RNAs in the ribosomal preparation have molecular weights of 1.3×10^5 and 0.7×10^6 daltons as determined by polyacrylamide gel electrophoresis.

The incorporation of ^{14}C-leucine by the ribosomal prepara-

Table I. *Requirements for Cell-free Amino Acid Incorporation by Ribosomal Preparations from Developing Pea Cotyledons*

The complete system is that described in "Materials and Methods." Both the ribosomal and supernatant fractions were prepared from cotyledons 21 days after flowering.

Treatment	Amino Acid Incorporation	
	cpm/mg rRNA	*% of control*
Complete system	21,903	100
−KCl	10,420	47
$-MgCl_2$	7,345	33
−β-mercaptoethanol	7,155	32
−ATP	8,085	36
−GTP	4,890	22
−ATP, GTP, P-enolpyruvate, pyruvate-kinase	880	4
−supernatant	815	3
−ribosomes	795	2
+19 L-amino acids	19,890	90
+0.5 mg RNase	890	4
+0.5 μg cycloheximide	12,485	57
+5.0 μg cycloheximide	1,800	8
+5.0 μg puromycin	2,630	12
+50.0 μg chloramphenicol	19,955	95

tions was linear for the first 10 min, and the reaction was essentially complete after 15 min (Fig. 4). The causes of the limited reaction time are not known, but such reaction times are characteristic of cell-free amino acid-incorporating systems from plants free of bacterial contamination (4).

Amino acid incorporation was dependent upon the addition of the supernatant fraction (Table I and Fig. 5). Ribosomal preparations obtained from cotyledons of different ages which showed differences in amino acid-incorporating capacity (Table II) had similar requirements and saturation kinetics for the supernatant component (Fig. 5). These observations indicated that the variation in activity of the ribosomal preparations was not due to variable contamination by the supernatant fraction.

Ribosomal preparations isolated from cotyledons of various developmental ages possessed varying capacities for amino acid incorporation (Table II and Fig. 6). Preparations from cotyledons of days 27 to 33 had a reduced capacity for amino acid incorporation. A similar decline in the capacity of ribosomal preparations from maturing seeds to support amino acid

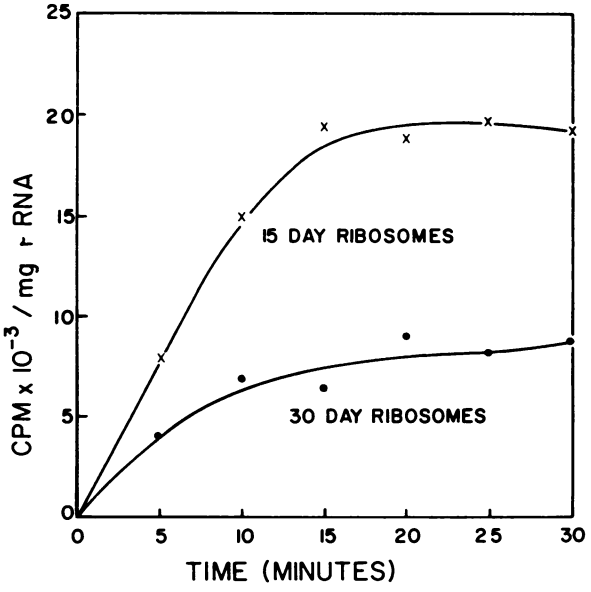

FIG. 4. The reaction time course for the incorporation of ^{14}C-leucine by ribosomal preparations from pea cotyledons 15 days and 30 days after flowering.

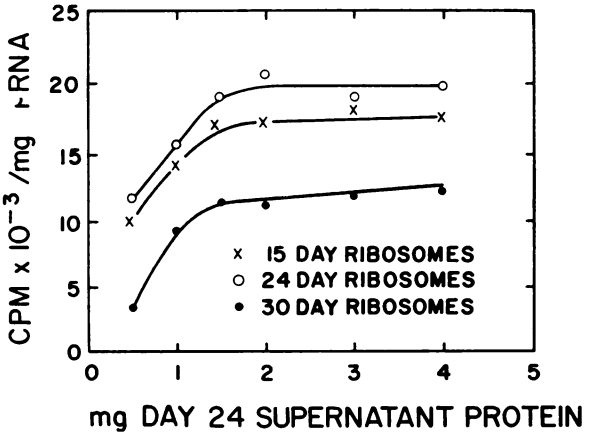

FIG. 5. The influence of the supernatant fraction on the capacity of ribosomal preparations from 15-, 24-, and 30-day cotyledons to incorporate ^{14}C-leucine into trichloroacetic acid-insoluble product.

Table II. *Amino Acid-Incorporating Capacity of Ribosomal Preparations Prepared from Cotyledons of Differing Developmental Age*

The standard incorporating system was used as described in "Materials and Methods" along with the supernatant fraction prepared from cotyledons 21 days after flowering.

Age of Cotyledons	Amino Acid Incorporation
days	*cpm/mg rRNA*
12	20,310
15	19,180
18	20,550
21	20,250
24	20,200
27	15,320
30	9,735
33	3,495

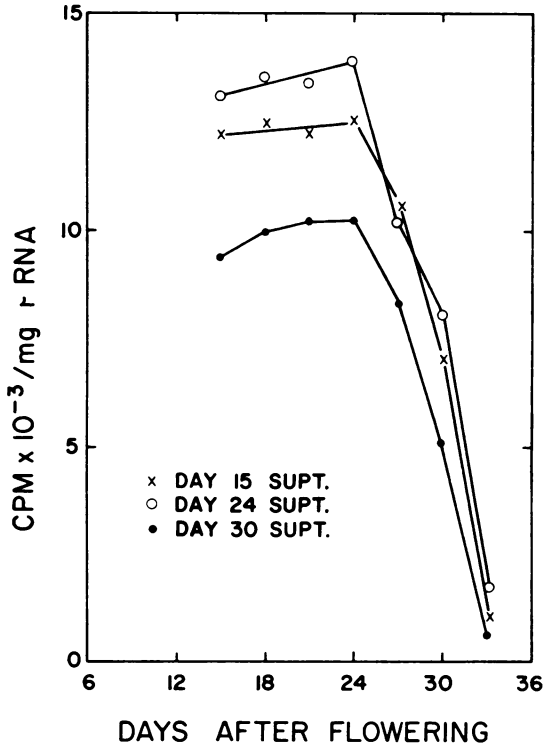

FIG. 6. The influence of the supernatant fraction from 15-, 24-, and 30-day cotyledons on the capacity of ribosomal preparations prepared from cotyledons of varying age to incorporate ^{14}C-leucine.

incorporation has been reported by Rabson *et al.* (16) in their studies of developing maize kernels. However, the observed high incorporating capacity of the ribosomal preparations from the young developing pea seed cotyledons differs from that in maize (16), where it was observed that particulate preparations from the young kernel had a restricted capacity for amino acid incorporation.

Addition of Synthetic Messenger. The close relationship between polysome level and the incorporating capacity of ribosomal preparations indicated that ribosomal activity may be a function of the availability of mRNA in these ribosomes. Alternatively, the low amino acid-incorporating capacity of ribosomal preparations from mature cotyledons may be a function of enzyme deficiency. In the presence of Polv UJ[4] the

[4] Abbreviation: Poly U: polyuridylic acid.

Table III. *Effect of Polyuridylic Acid on Phenylalanine Incorporation*

Ribosomal preparations from pea cotyledons of differing developmental ages were fortified with varying supernatant components in the reaction system outlined in "Materials and Methods" in which 0.5 μc of ^{14}C-phenylalanine (375 mc per mmole) replaced the ^{14}C-leucine. In addition a parallel set of reactions contained 50 μg of polyuridylic acid per assay.

Age of Cotyledons		Phenylalanine Incorporation		Increase
Ribosome	Supernatant	−Poly U	+Poly U	
days				%
15	15	16,200	18,800	16
15	24	15,000	17,800	18
15	30	11,800	14,000	18
24	15	16,800	18,200	8
24	24	15,200	16,400	7
24	30	12,400	13,400	8
30	15	6,400	14,000	118
30	24	7,400	15,500	109
30	30	6,000	12,800	113

Table IV. *The Influence of Cotyledonary Development Age on the Capacity of Ribosomes and Supernatant Fractions to Stimulate the Incorporation of Various Amino Acids*

Standard assay conditions were used in the presence of 0.5 μc of the following amino acids: ^{14}C-leucine, 344 mc per mmole; ^{14}C-phenylalanine, 375 mc per mmole; ^{14}C-arginine, 250 mc per mmole; glutamic acid, 200 mc per mmole.

Age of Cotyledons		Amino Acid Incorporated			
Ribosome	Supernatant	Leucine	Phenylalanine	Glutamic acid	Arginine
days		*pmoles/mg rRNA*			
15	24	127.6	81.6	132.2	104.1
24	24	155.3	104.3	142.1	117.4
30	24	66.5	36.2	128.7	80.6
24	15	124.8	82.2	140.7	103.3
24	24	147.8	105.1	141.7	111.5
24	30	80.1	56.9	137.5	82.6

incorporation of phenylalanine was increased by only 15% in ribosomal preparations from 15- and 24-day-old cotyledons (Table III). These ribosomal preparations were apparently saturated with mRNA. In contrast, the incorporation of phenylalanine by ribosomal preparations from 30-day-old cotyledons was stimulated 115% by the addition of Poly U. The amount of phenylalanine incorporation by ribosomes from 30-day-old cotyledons in the presence of Poly U approached the rate observed in ribosome preparations from 15- and 24-day-old cotyledons. These data suggest that ribosomes from 30-day-old cotyledons were not enzymatically deficient but their capacity for amino acid incorporation was limited by the available mRNA.

Supernatant Fraction. Supernatant fractions obtained from cotyledons 15, 24, and 30 days after flowering have varying capacities to support amino acid incorporation. Supernatant from 30-day-old cotyledons was consistently less effective than the supernatant prepared from 15- or 24-day-old tissue (Fig. 6). Since only crude unpurified supernatant preparations were used, it is not possible, at this stage, to account for the reduced efficiency of this component from 30-day-old cotyledons to support amino acid incorporation. Clearly, further intensive investigations are required on this aspect.

Variations in Amino Acids. In view of the different amino acid composition of the albumins and globulins (7), it was surprising that greater differences were not observed in the *in vitro* incorporation of various amino acids. Ribosomal preparations from the same developmental stage showed similar capacities to incorporate leucine and phenylalanine with preparations from older cotyledons showing a reduced capacity for incorporation of both of these amino acids (Table IV). Ribosomal preparations from both young and mature cotyledons had a similar capacity to incorporate glutamic acid. The capacity of the ribosomal preparations to incorporate arginine declined with increasing developmental age, but the decrease was less rapid than that observed for leucine and phenylalanine. Similar changes were observed in the capacity of the supernatant components, prepared from 15-, 24-, and 30-day-old cotyledons, to support incorporation of the various amino acids (Table IV).

It is significant that the capacity to incorporate glutamic acid and arginine was retained at relatively higher levels than leucine and phenylalanine incorporation in ribosomal preparations from mature cotyledons. The former amino acids are present in greater amounts in the reserve globulins synthesized at maturity. The potential of the ribosomes from different developmental stages to incorporate the various supplied amino acids at varying rates may be partially masked by the free amino acids associated with the ribosomal or supernatant fractions. In addition, the isolated ribosomes may not be those functioning in reserve protein synthesis. Payne and Boulter (15) have suggested that reserve proteins are synthesized on membrane-bound ribosomes. If this class of ribosomes was not isolated, then the incorporation of amino acids characteristic of the reserve protein would not be observed. However, the utilization of sodium deoxycholate during the isolation procedures in the present study should have resulted in the release of the membrane-bound ribosomes. The *in vitro* amino acid incorporation should therefore have reflected the synthetic capabilities of both free and bound ribosomes.

CONCLUSIONS

Developmental studies have indicated that the rate of protein deposition and the type of protein synthesized vary with the developmental age of the pea cotyledon (Fig. 1). From the experimental results some conclusions can be drawn concerning the loci at which the rate of protein synthesis is regulated during the developmental process.

During the initial phase of development the cotyledons had only a limited capacity to incorporate exogenously supplied amino acids (Fig. 1). However, the ribosomal preparations from young cotyledons had a high proportion of polysomes and were active in amino acid incorporation (Fig. 3 and Table II). The supernatant components prepared from young cotyledons were also capable of stimulating amino acid incorporation in the *in vitro* system (Fig. 6). Thus, the low capacity for incorporation of exogenously supplied amino acids by young cotyledons was apparently not due to any major deficiency in any specific component required for protein synthesis but appeared to be related to the over-all low level of protein-synthesizing components. This contention was supported by the observations that the young cotyledons are low in protein (Fig. 1) and RNA (unpublished results).

During later stages of development of the pea cotyledon the capacity for protein synthesis and incorporation of exogenous amino acids (Fig. 1) may be restricted by deficiencies

in the protein-synthesizing mechanism *per se*. The reciprocal mixing experiments of supernatant and ribosomal fractions of different developmental age (Fig. 6) indicated the possibility that protein synthesis at later stages of seed development could be restricted by deficiencies in components of the supernatant fraction. In addition, the ribosomal preparations from the dehydrating seeds show a decreasing abundance of polysomes, suggesting that mRNA availability may be restricted. The observation that ribosomal preparations from maturing seeds were progressively less efficient in supporting cell-free amino acid incorporation but could be restored to activity by addition of exogenous polynucleotide was consistent with a declining mRNA component.

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