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Seed Germination Studies. III. Properties of a Cell-free Amino Acid Incorporating System From Pea Cotyledons; Possible Origin of Cotyledonary α-Amylase[1]

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Abstract. **Pea cotyledonary α-amylase increases dramatically both in specific activity and total activity between days 7 to 10 when germination occurs in the dark. This enzymatic activity does not seem to appear as a consequence of release or formation of an activator, removal of an inhibitor, dissociation of an inactive amylase complex, or proteolytic decomposition of a zymogen precursor. The possibility remains that the α-amylase is newly synthesized during germination. The preparation and properties of a cell-free protein-synthesizing system from germinating pea cotyledons is described; polyuridylic acid must be added for L-phenylalanine incorporation. Active microsomal preparations can be obtained from cotyledons germinated 10 days.**

Seed germination is frequently accompanied by marked increases in the level of activity of certain enzymes (3, 4, 12, 17, 23, 32). It has been noted that these often dramatic increases in biocatalytic activity are not necessarily associated only with those parts of the seedling that are actively growing. Rather, the same phenomenon is also observed in the storage tissues of the endosperm or cotyledon (4, 17, 32).

We showed in previous reports (25, 26) that a number of enzymes required for the degradation of seed carbohydrate reserves are present in the cotyledon of the germinating pea, and at least 2 of these enzymes, an α-amylase and a phosphorylase, increase manyfold in total activity during the time period of germination. Since, in many instances, the cells of plant storage organs neither grow nor divide, the question arises as to what the origin of such new enzymatic activities may be. Other workers demonstrated that activation of latent enzyme occurs in the case of the β-amylase of wheat (21, 22), zymogen activation causes an increase in acid phosphatase and isocitritase activities of a variety of seeds (17), whereas isocitritase (6, 10), malate synthetase (10), tyramine methylpherase (12), and barley α-amylase (5, 28) increase in activity as a result of *de novo* enzyme synthesis.

We report here studies which show that cotyledonary α-amylase of peas most likely does not appear during germination as a consequence of the formation or release of a specific activator, the removal of an inhibitor, or the activation of a latent form (or zymogen precursor) of the enzyme. The possibility that the enzyme is newly synthesized is not eliminated. A cell-free protein-synthesizing system from the cotyledons of germinating peas has been prepared and its properties determined. A catalytically-active microsomal fraction can be prepared from cotyledons at a time during germination when the level of α-amylase activity is rapidly increasing.

Materials and Methods

Pea seeds (*Pisum sativum* L. var. Early Alaska) were germinated as previously described (25). Extracts of cotyledons were prepared and amylase activity was measured by procedures outlined earlier (25). One unit of amylase activity is defined as the quantity of protein that liberates 1 mg of reducing groups (calculated as maltose) in 10 min at 25°; specific activity is units of enzyme activity per mg of protein. Protein concentrations were deter-

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[3] Abbreviations: sRNA, soluble ribonucleic acid; mRNA, messenger ribonucleic acid.

mined by the method of Lowry *et al.* (11) with crystalline bovine serum albumin as standard. L-(^{14}C)leucine (170 μc/μmole) and L-(^{14}C)phenylalanine (252 μc/μmole), both uniformly labeled, were products of Nuclear Chicago Corporation. A mixture of L-amino acids and amides (13) was used to examine the requirements of the cell-free protein-synthesizing system. The protease present in pea cotyledon extracts (called arvensin) was purified to the status designated "Fraction G" by the procedure of Soedigdo and Gruber (24). Bromelain, pronase, and ficin were purchased from Boehringer Mannheim Corporation; papain from Merck and Company; trypsin and chymotrypsin from Worthington Biochemical Corporation and bovine pancreatic ribonuclease from Sigma Chemical Company.

Preparation of Cell Fractions. The methods of Marcus and Feeley (13) were used with the modifications noted. For the experiments in which the incorporation of labeled amino acids into aminoacyl sRNA alone was measured, the fraction designated "100,000*g* supernatant fluid" (called "dialyzed supernatant" by Marcus and Feeley) was prepared from 4 or 5-day-old seedlings. One ml of blending solution per cotyledon was used. In preparing this fraction, 0.05 M potassium phosphate buffer (pH 7.3) uniformly replaced the 0.01 M tris buffer (pH 7.9) prescribed (13).

For those experiments in which the incorporation of labeled amino acids into protein was measured, microsomal and supernatant fractions were prepared as outlined (13) except that tris-HCl buffer (pH 7.6) of the same concentration replaced the phosphate buffer in every case. Our "microsomal suspension" corresponds to the "microsome" preparation of Marcus and Feeley.

Isolation of Aminoacyl sRNA and Protein; Estimation of Radioactivity. When the incorporation of labeled amino acids into aminoacyl sRNA only was measured, the reaction was terminated by the addition of a mixture containing 0.2 ml of 2 % (w/v) casein solution, 0.5 ml of 0.1 M DL-leucine (or DL-phenylalanine, depending on the radioactive amino acid used), and 0.4 ml of 30 % (w/v) trichloroacetic acid solution. The precipitate obtained by centrifugation was washed 3 times with 4 ml portions of 5 % (w/v) trichloroacetic acid solution, 1 time with 4 ml of 95 % (v/v) ethanol, and finally once with 4 ml of ether. The washed precipitate was then dissolved in 1 ml of 91 % (v/v) formic acid and 0.5 ml aliquots were plated and counted with the use of a Nuclear-Chicago gas-flow detector (Model D-47). The incorporation of labeled amino acids into both protein and aminoacyl sRNA was measured as described (13). All data were corrected by control incubation mixtures which were precipitated at zero time.

Preparation and Determination of RNA. sRNA from extracts of pea cotyledons was prepared by the method of Holley *et al.* (7). A yield of 142 mg was obtained from 454 g (wet wt) of pea seeds. This material apparently was not pure since the absorbancy (at 260 mμ) of a 0.01 % (w/v) solution was only 0.4; the value for pure sRNA from yeast is reported to be 1.9 (7). This preparation, however, was effective in studies dealing with amino acid incorporation into protein when used in place of yeast sRNA (table IV); it was completely ineffective in this regard after being digested by ribonuclease. RNA was estimated by assuming that the absorbancy of a 0.01 % (w/v) solution is equal to 2.0 at 260 mμ.

Results

A quantitative measure of the increase in cotyledonary α-amylase activity, both in etiolated seedlings and in seedlings grown in a normal day-night cycle, is shown in Fig. 1.

Test for an Activator or Inhibitor. Table I indicates that extracts prepared from cotyledons at various stages of germination have no potentiating or inhibitory effect on the activity of the partially purified α-amylase. Identical results were obtained when extracts prepared from axis and cotyledonary tissues were tested individually and in combination; no diffusible amylase activator or inhibitor, therefore, seems to have its origin in axis tissue. In other tests, soluble extracts were prepared from cotyledons germinated for 2 and 9 days. The total activity for the individual extracts before they were combined was 3.8 amylase units; that observed immediately after mixing was 3.5 units. One half of the mixed

Table I. *Effect of Extracts Prepared From Cotyledons of Different Ages on α-Amylase Activity*

Partially purified α-amylase, specific activity = 126(25), was added to extracts prepared from cotyledons germinated 1, 3, and 8 days (A, B, and C, respectively). Cotyledon extracts were prepared as described before (25). After 0.03 ml of the partially purified enzyme was added to the cotyledon extract, the mixture was incubated for 30 min at 25° before the amylase assay was performed.

Extract	Units α-amylase added (a)	Endogenous activity (b)	Sum (a+b)	Total activity observed
ml			*mg maltose/10 min*	
A (1.0)	1.0	0.3	1.3	1.2
B (1.0)	1.0	0.6	1.6	1.5
C (0.2)	1.1	0.6	1.7	1.6

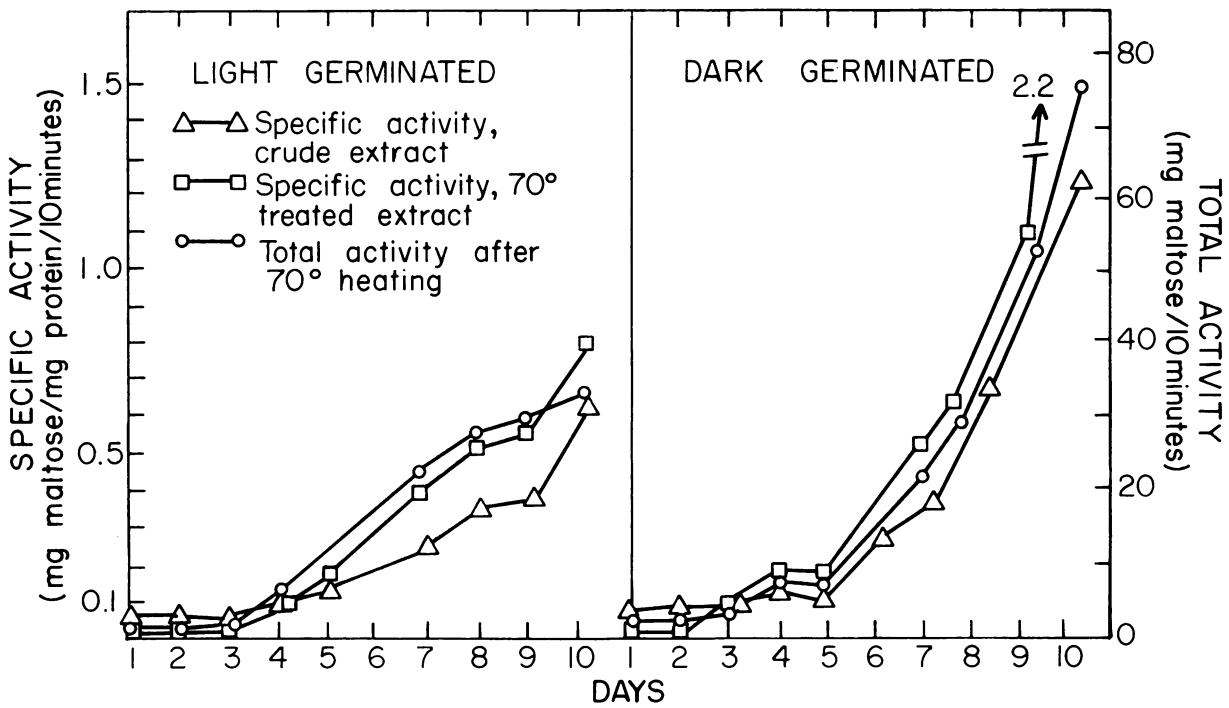

FIG. 1. Increase in α-amylase activity in the cotyledons of germinating peas. Each day, 20 cotyledons were excised and homogenized with 20 ml of 0.1 M acetate buffer (pH 5.8). The homogenate was centrifuged at 18,000g for 25 min. Calcium chloride (0.2 ml, 0.5 M) solution was added to the supernatant fluid and the mixture heated with constant stirring at 70° for 15 min. After centrifugation, this supernatant fluid and that of the untreated extract were assayed.

extracts was stored at 4° and the other half incubated for 12 hr at 25°. Both solutions were then assayed again and found to contain 3.5 units of α-amylase activity. Such results suggest that pea seeds do not contain a tightly bound amylase-inhibitor complex which is dissociated or degraded during germination.

Tests for Activation by Physical or Chemical Means. Experiments were performed to determine whether α-amylase activity might be tightly bound to sub-cellular components or to starch granules and, as a consequence, would not be able to manifest itself during the early stages of germination. Extracts,

Table II. *Effect of Protease Treatment on Cotyledonary α-Amylase Activity*

Extracts used for protease digestion were made from cotyledons germinated for 2 or 3 days (25). Similar extracts were prepared from cotyledons germinated for 8, 9, and 11 days, which extracts served as controls for measuring the stability of the amylase to the protease under the conditions employed. Incubation mixtures contained 2 ml of extract and these amounts of protease preparations: bromelain, 1 mg; ficin, 1 mg; pronase, 1.2 mg; papain, 1.2 mg; trypsin, 2 mg; α-chymotrypsin, 2 mg; pea cotyledon protease, 13.6 mg of protein. All mixtures were incubated for 12 hr at 32°; zero-time controls were frozen immediately after adding protease. At the end of 12 hr, 1 ml of each incubation mixture was added to 1 ml of 15 % (w/v) trichloroacetic acid solution. After centrifugation, the absorbancy and the protein content of the supernatant fluids were determined.

Enzyme	ΔA[1]	Δ Trichloroacetic acid[2] soluble protein	Δ Amylase activity	Percent initial[3] amylase activity
		mg/incubation tube	*units/ml*	
Bromelain	0.475	5.0	0	100
Ficin	0.455	5.0	0	100
Pronase	0.515	8.0	0	85
Papain	0.398	6.5	0	100
Trypsin	0.347	6.0	0	100
Chymotrypsin	0.430	6.5	0	100
Cotyledon protease	0.981	5.0	0	88

[1] Increase in absorbancy(A) at 280 mμ relative to zero-time control.
[2] Increase in trichloroacetic acid-soluble protein relative to zero-time control; measured by Lowry method (11).
[3] Control tube containing extract prepared from cotyledons germinated 8, 9, or 10 days.

prepared from 5 to 8 day germinated cotyledons, were separately subjected to (A) freezing and thawing, (B) sonic oscillation, (C) mixing for 1 hr with sodium deoxycholate (0.05 % (w/v), final concn) and then dialysis, (D) mixing for 1 hr with calcium acetate (0.2 M, final concn) and dialysis, and (E) mixing with urea (4.0 M, final concn) for 4 hr followed by dialysis. Regardless of the treatment, no bound amylase activity was released or activated; starch degradation occurred to the very same extent in the various test solutions and in controls.

Tests for Proteolytic Activation. Table II (column 4) shows that treatment of pea cotyledon extracts with a wide variety of proteases also does not result in any increase in α-amylase activity. Enzymatic digestion of a zymogen, therefore, does not appear to take place.

Effects of Metabolic Inhibitors and Amino Acid Analogs. It was found that a number of metabolic inhibitors and amino acid antagonists inhibit the growth of the seedling stem and the development of amylase activity in the cotyledon (table III). Actinomycin D is a very powerful inhibitor of both processes; puromycin showed no effect.

Properties of a Cotyledonary Cell-free Protein-Synthesizing System. Formation of Aminoacyl sRNA. Table IV shows that cell-free extracts prepared from germinating pea cotyledons are able to catalyze the formation of aminoacyl sRNA with either L-(^{14}C)leucine or L-(^{14}C)phenylalanine. The complete system exhibits a strict requirement for added ATP, sRNA, and native 100,000g supernatant fluid. No counts whatever are incorporated when either ribonuclease (600 μg), NH_2OH (0.76 M), PP_i (0.01 M), or EDTA (0.01 M) is added to complete incubation mixtures. Other experiments (Swain, R. R. and E. E. Dekker, unpublished results) clearly demonstrate that the observed incorporation of L-(^{14}C)phenylalanine and L-(^{14}C)leucine into aminoacyl sRNA is additive, and that the presence of unlabeled L-phenylalanine in the reaction mixture has no effect on the formation of (^{14}C)leucyl sRNA.

Fig. 2A demonstrates proportionality of the amino acid activating system with amount of sRNA added before the system becomes saturated with this component; 2B, the effect of added protein in the form of 100,000g supernatant fluid; 2C, the time course of aminoacyl sRNA formation.

Table IV. *Requirements for the Formation of Aminoacyl sRNA*

Complete incubation mixtures contained (in μmoles): potassium phosphate buffer (pH 7.5), 20; KCl, 40; 2-mercaptoethanol, 20; ATP, 0.5; creatine phosphate, 10; L-(^{14}C)leucine, 73,500 cpm (or, in one instance, the same number of cpm as L-(^{14}C)phenylalanine), creatine phosphokinase, 50 μg; yeast sRNA, 400 μg; and dialyzed pea cotyledon 100,000g supernatant fluid (0.8 mg protein). Final incubation volume: 1.0 ml. Incubation time was 60 min at 30°.

System	Total counts incorporated into sRNA
	cpm
Complete	688
Complete, less yeast sRNA, plus pea cotyledon sRNA (0.4 mg)	1786
Complete, less L-(^{14}C)leucine, plus L-(^{14}C)phenylalanine	188
Complete, less ATP	14
Complete, less yeast sRNA	12
Complete (with boiled 100,000g supernatant fluid)	0

Table III. *Effect of Metabolic Inhibitors and Amino Acid Antagonists on Development of α-Amylase Activity*

Experiment 1) each analog was tested by placing 10 seedlings (age, 6 days) in a petri dish containing 50 ml of a solution of the amino acid analog. The plants were then allowed to germinate until the age of 10 days. Additional solution (25 ml) containing the amino acid analog was added at day 8. Control seedlings received only H_2O. Experiment 2) The amount of amino acid analog or inhibitor indicated was dissolved in water and allowed to imbibe completely into dry seeds over a period of 18 hr with subsequent addition of more water as necessary to keep the seeds moist. These seeds were then germinated for 7days. Cotyledon extracts were prepared in the usual manner (25).

Experiment	Inhibitor or analog	Concn	Stem elongation	Amylase activity
		M	*% control*	*units/extract*
1	DL-Ethionine	5×10^{-3}	50	15
	DL-*p*-Fluorophenylalanine	5×10^{-3}	55	15
	DL-Allylglycine	5×10^{-3}	78	35
	None	...	100	47
2	L-Azetidine-2-carboxylic acid	8[1]	74	16
	Actinomycin D	0.5[1]	28[2]	2
	Actinomycin D	1.0[1]	13[2]	0
	Puromycin	2.8[1]	100	28
	None	...	100	27

[1] mg per 100 seeds.
[2] Root elongation measured in this instance.

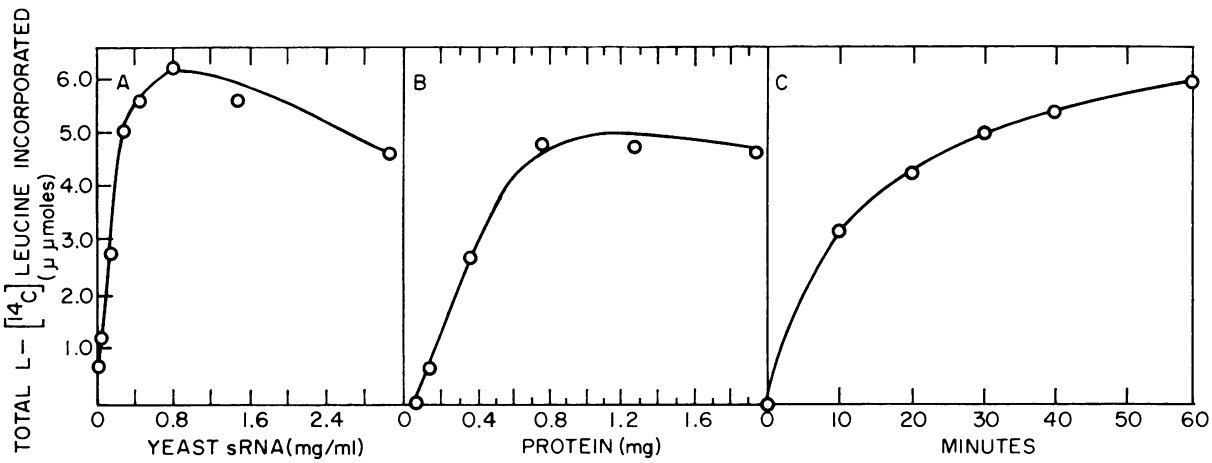

FIG. 2. A) Requirement of the amino acid activating system for yeast sRNA; B) for dialyzed 100,000*g* supernatant fluid; C) effect of time of incubation. The incubation mixtures contained all the components listed in the legend of table IV, except for the one varied as indicated. Incubation time was 60 min, or the times shown, at 30°.

Incorporation of Amino Acids Into Protein. When tris-HCl buffer replaced potassium phosphate in the preparation of extracts and reaction mixtures, the incorporation of labeled amino acid into trichloroacetic acid-insoluble material was routinely observed (table V). Puromycin and ribonuclease are potent inhibitors. The time course of L-phenylalanine incorporation into protein is shown in figure 3A; approximately 0.5 mg of microsomal protein is required to saturate this system (Fig. 3B).

The amino acid incorporating system from pea cotyledons described here does not show a requirement for added 100,000*g* supernatant fluid when tested under the conditions outlined in table V. Presumably, formation of aminoacyl sRNA is accomplished by enzymes in the supernatant fluid that adhere to the microsomal pellet. When, however, the microsomal pellet obtained from the initial 100,000*g* centrifugation is washed, the system shows a definite requirement for the supernatant fraction (table VI). The complete protein-synthesizing system is capable, therefore, of being resolved into 2

Table VI. *Requirement for Supernatant (100,000g) and Microsomal Fractions for the Incorporation of L-(14C)phenylalanine Into Protein*

Incubation mixtures contained the same basic components listed in the legend of table V. The microsomal fraction was a washed preparation from 4-day pea cotyledons (0.5 mg protein, 0.3 mg RNA); the supernatant fraction was dialyzed 4-day pea cotyledon 100,000*g* supernatant fluid (0.4 mg protein). Final incubation volume: 1.03 ml. Incubation time was 40 min at 30°.

System	Total counts incorporated sRNA	Protein
	cpm	*cpm*
Complete	1200	880
Complete, less microsomal suspension	1100	0
Complete, less dialyzed supernatant fluid	200	38
Complete (boiled supernatant fluid)	115	17
Complete (washed microsomes from 10-day cotyledons; 0.4 mg protein, 0.3 mg RNA)	890	582

Table V. *Requirements for Incorporation of L-(14C)phenylalanine Into sRNA and Into Protein*

Complete reaction mixtures contained (in μmoles): tris-HCl buffer (pH 7.6), 40; KCl, 40; 2-mercaptoethanol, 20; $MgCl_2$, 5; ATP, 0.5; GTP, 0.5; creatine phosphate, 10; L-(14C)phenylalanine, 73,500 cpm; creatine phosphokinase, 50 μg; yeast sRNA, 0.4 mg; polyuridylic acid, 100 μg; pea cotyledon microsomal suspension (1.3 mg protein, 0.7 mg RNA). Final incubation volume: 1.03 ml. Incubation time was 40 min at 30°.

System	Total counts incorporated sRNA	Protein
	cpm	*cpm*
Complete	280	213
Complete, plus 19 amino acids (125 μmoles of each)	230	205
Complete, less yeast sRNA	0	40
Complete, less creatine phosphate	80	10
Complete, less GTP	328	13
Complete, less polyuridylic acid	200	22
Complete, plus puromycin (10^{-3} M)	300	22
Complete, plus ribonuclease (200 μg)	0	0

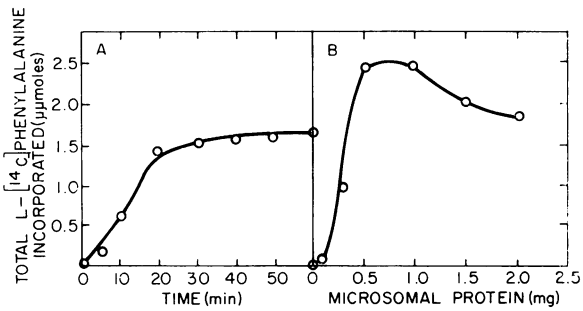

FIG. 3. A) Time course of L-(^{14}C)phenylalanine incorporation into protein; B) dependency on microsomal protein. The reaction mixtures contained all the components listed in the legend of table V. Incubation time was 40 min, or the times shown, at 30°.

components, 1 microsomal and 1 supernatant. Both of these fractions are necessary for the incorporation of L-(^{14}C)phenylalanine into a form precipitated by trichloroacetic acid.

Attention is called to the finding that cotyledons germinated for 10 days yield a microsomal preparation that is capable of catalyzing the incorporation of L-phenylalanine into protein (table VI, line 5). This result is significant in view of the fact that the level of α-amylase is still rapidly increasing in cotyledons of this age (see Fig. 1).

Discussion

Seed germination is associated with a number of interesting biochemical phenomena, *i.e.* degradation of seed reserves, synthesis of new plant materials, a changing array of enzymes and of new and novel metabolites, *etc.* Previous work (25) showed that, during germination, pea cotyledons develop potent amylase activity of the α-type which enzyme can be obtained in pure form. The results reported here deal with 2 phases of pea seed germination. Firstly, what factors may be responsible for the dramatic rise in cotyledonary α-amylase activity? Secondly, the non-growing cells of the cotyledon have indirectly been implicated in *de novo* enzyme synthesis (32) and have been suggested as a useful biological system for studying cellular senescence processes (27). Although cell-free protein synthesis has been observed in pea seedling extracts by other workers (18, 29, 30), the systems previously described used cell fractions from those parts of the plant that remained *after* excision of the cotyledons; unfortunately, results obtained with these systems have not been reproducible (8, 9, 31). It seemed desirable, therefore, to establish in detail the parameters of a well-defined protein-synthesizing system from pea cotyledons.

It is very clear that cell fractions prepared from the cotyledons of germinating peas are capable of catalyzing the formation of aminoacyl sRNA and, subsequently, the incorporation of the amino acid into protein. Aminoacyl sRNA formation requires ATP, sRNA, and native enzymes present in the 100,000*g* supernatant fluid. Pea cotyledon microsomal preparations, in turn, catalyze the incorporation of an amino acid into a form precipitated by trichloroacetic acid. This amino acid incorporating system requires GTP in addition to the microsomal fraction and the substrates necessary for aminoacyl sRNA formation. Puromycin, a highly-specific inhibitor of protein synthesis, and ribonuclease both inhibit amino acid incorporation into protein. Collectively, these results show that the cotyledon of the pea, in spite of being a senescent organ, is capable of catalyzing the synthesis of protein by the usual sequence of reactions (16). The following points rule out the possibility that microbial contaminants significantly influenced the results reported here: A) the peas used in these studies were germinated under essentially sterile conditions; B) the requirement for GTP and for polyuridylic acid (table V) is strict; and C) the incorporation of L-(^{14}C)phenylalanine into protein, as a function of length of time of incubation (fig 3A), does not increase indefinitely but reaches a finite level after a period of time.

The cell-free amino-acid incorporating system from pea cotyledons always requires the addition of polyuridylic acid; peanut cotyledon preparations show this requirement only before imbibition of water by the seed (13, 14). This difference is most likely due to significant ribonuclease activity in pea cotyledon extracts; we showed in separate experiments that our extracts readily degraded sodium ribonucleate to an acid-soluble ultraviolet-absorbing form [assay of McDonald (15)]. The absolute requirement of the system from peas for added sRNA (table IV and V) can be explained on the same basis. Furthermore, we could not detect by sucrose density gradient centrifugation (14) any polyribosomes in washed microsomes prepared from germinated pea cotyledons. Secondly, the presence of nucleoside triphosphatases in pea cotyledon preparations (20) makes the requirement for added GTP more demonstrable in this instance (table V) than with the analogous amino acid incorporating system from peanuts.

In conclusion, it should be noted that some enzymatic activities present in germinating seeds do, indeed, appear to be derived by modification of an inactive or zymogen-like precursor (17, 21). The demonstration, however, that protein synthesis occurs in the cotyledon at a time when the levels of certain enzymes are increasing dramatically in activity is consistent with the possibility that some enzymatically-active proteins are also synthesized *de novo* during seed germination. Varner and Schidlovsky, using 6 to 18-day germinated pea cotyledons, observed a low level of incorporation of labeled amino acids into partially purified α-amylase (personal communication from J. E. Varner). Further studies along similar lines and directed toward the question of

what initiates the increase in α-amylase in pea cotyledons must be done.

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