The Influence of Axis Removal on Protein Metabolism in Cotyledons of *Pisum sativum* L.1

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**ABSTRACT**

The protein metabolism of cotyledons attached to the embryonic axis has been compared with that in cotyledons removed from the axis at the initiation of a 6-day imbibition. Total protein declined in the attached but not in the detached cotyledons. Concurrent with the decline in protein level in the intact cotyledons there was an increased capacity to incorporate exogenously supplied leucine into protein. In contrast, detached cotyledons showed a restricted capacity for protein synthesis. It was demonstrated that ribosomal preparations from cotyledons of intact seedlings contained an increasing proportion of polyribosomes as germination progressed and such ribosomes were active in *in vitro* amino acid incorporation. Ribosomal preparations from detached cotyledons contained few polyribosomes and had a restricted capacity to incorporate amino acids *in vitro.* The *in vitro* incorporation of phenylalanine was restricted in intact cotyledons. The results suggest that an axis component may regulate the availability of messenger RNA in the cotyledons during germination.

Characteristically during seed germination the principal reserve materials, starch, lipid and protein, in the cotyledons or endosperm are degraded and the products arising from the reserve tissue are translocated to the developing axis (4, 19, 23). However, it has been demonstrated that the utilization of the reserve components and normal development of the reserve tissues are dependent upon the presence of an attached embryonic axis (7, 10, 12, 20, 21, 24). Endosperm tissues or cotyledons from which the embryos have been removed fail to show the characteristic loss of reserve compounds. The situation has been most extensively studied in cereal grains (12, 20, 25, 26), where it has been demonstrated that the normal onset of reserve hydrolysis is prevented by removing the half of the seed containing the embryo. Associated with the restricted reserve hydrolysis, it has been demonstrated that there is a failure to produce various hydrolytic enzymes.

An analogous situation was demonstrated to occur in pea seeds in which the reserve components are deposited predominantly in the cotyledon. Removal of the cotyledons from the axis resulted in a decreased level of hydrolytic enzymes (24) and a restricted ultrastructural modification (1) of the cotyledon.

These observations on the influence of axis removal on enzyme level of both endosperm and cotyledonary tissue suggest that protein metabolism in the reserve tissue is regulated, in some manner, by the axis. As part of our investigation into the protein metabolism in pea cotyledons we have studied this possibility and attempted to determine at what points protein synthesis may be impaired following axis removal.

**MATERIALS AND METHODS**

**Culture of Seeds and Cotyledons.** Pea seeds (*Pisum sativum* L., cv. Burpeeana) were surface-sterilized with 1% sodium hypochlorite solution for 5 min and rinsed several times with sterile distilled water before being allowed to soak in sterile distilled water for 3 hr. After this initial imbibition phase the seeds were divided into two groups. In one group, the seeds were left intact whereas in the other group the embryonic axis and testa were removed from the seed to produce the detached cotyledons. Sets of 5 intact seeds or 10 detached cotyledons were placed in culture dishes (10 cm × 5 cm with a cover) containing two sheets of Whatman No. 2 filter paper moistened with 10 ml of sterile distilled water. The seeds or detached cotyledons were incubated in the dark at 20 C for varying periods of time up to 144 hr. In order to produce cotyledons which were free from visible bacterial or fungal contamination at the end of the 144-hr incubation period, it was essential that all the manipulations of axis excision, etc., were performed in a sterile hood.

**In Vitro Protein and RNA Synthesis.** Protein synthesis was measured by determining the capacity of the cotyledons to incorporate exogenously supplied radioactive leucine. Two microliters of 3H-leucine (240 μCi/μmole; 100 μCi/ml) were injected by means of a microsyringe into individual cotyledons. The injected cotyledons were then incubated in the dark at room temperature in sterile culture dishes on moistened filter papers. After the incubation the cotyledons were rinsed with deionized water and extracted with boiling ethanol (80%, v/v) for 3 min. The material and ethanol were then transferred to a VirTis tissue homogenizer and homogenized for 2 min at medium speed. The homogenate was centrifuged at 12,000g for 10 min, and the supernatant fraction was collected. The resulting residue was resuspended and washed twice in 80% (v/v) ethanol. The original supernatant fractions and washings were combined and diluted to volume and used...
for the determination of radioactivity using a Nuclear-Chicago gas flow counter. The alcohol-insoluble residue was re-extracted in sequence with cold 10% trichloroacetic acid, absolute ethanol, ether-chloroform-ethanol (2:2:1, v/v/v), and ether. The final precipitate of the ether extraction was dried in air and suspended in 1 N NaOH at 37°C for 16 hr. After this time the insoluble residue was removed by centrifugation at 3,000g for 5 min, and the resulting supernatant was used for the determination of protein (16) and radioactivity using a Nuclear-Chicago gas flow counter.

For an assessment of total RNA synthesis 2 μl of "H-adenine (1 mc/ml) were injected into the cotyledons. Alcohol-soluble fractions were prepared as indicated above, and the alcohol-insoluble residue was extracted with trichloroacetic acid, ether-chloroform-ethanol, and ether. The washed residue was incubated for 16 hr with 0.3 N KOH at 37°C. After incubation the solution was adjusted to pH 1.5 with perchloric acid, and the insoluble residue was removed by centrifugation. Radioactivity of aliquots of the ethanol-soluble extracts and alkaline hydrolysates were determined in a Packard TriCarb spectrometer using Bray's scintillator (5).

Fractionation and Characterization of "P-Labeled Ribonucleic Acid. In studies aimed at characterizing RNA synthesis, 5 μl of carrier-free "P-orthophosphate (0.1 mc/ml, neutralized by NaOH) were injected into each cotyledon. The injected cotyledons were incubated for 5 hr and rinsed with deionized water. The total RNA was extracted from the cotyledons using the sodium deoxycholate-phenol method described by Click and Hackett (9), except that the extraction medium was adjusted to pH 8.0 and the concentration of bentonite was increased to 1.0%. The extracted RNA fraction was dissolved in electrophoresis buffer (40 mm tris, 20 mm sodium acetate, 2 mm sodium EDTA, adjusted to pH 7.8) containing 10% sucrose to give a concentration of approximately 1 mg/ml.

The radioactive RNA was fractionated by gel electrophoresis on 2.5% polyacrylamide following the detailed procedure described by Loening and Ingle (15), except that 0.2% sodium lauryl sulfate was included in the buffer system. The RNA sample (25 μg/25 μl) was applied onto the gel in Plexiglas tubes (9 cm length, 6 mm diameter) and electrophoresis was carried out at a constant current of 5 ma per gel for 2 hr with a Shandon electrophoresis apparatus. The distribution of RNA in the gel was then determined at 260 nm using a Gilford gel scanner attached to a Beckman DU spectrophotometer.

After optical scanning, the gel was transferred to an aluminum foil trough and frozen with powdered solid CO₂. The frozen gel was transversely sectioned into slices (0.5 mm thick) by means of a Mickel gel slicer. The slices were placed on planchets and dried. Subsequently the radioactivity in the slices was determined on a Nuclear-Chicago gas flow counter.

Preparation of Ribosomes. The isolation and characterization of ribosomes from the two groups of cotyledons was conducted by a modification of the method of Jacoby and Cherry (13). Thirty cotyledons were rinsed with deionized water and were frozen immediately with liquid nitrogen and ground into a fine powder with a precooled mortar and pestle. The powder was then homogenized with 30 ml of grinding medium (0.25 M sucrose, 10 mm MgCl₂; 20 mm tris-HCl, pH 7.6; 15 mm KCl; 5 mm β-mercaptoethanol; 0.5% deoxycholate; and 1 mg of cycloheximide) to produce a partially frozen slurry. The ice-cold homogenate was strained through cheesecloth and Miracloth (Calbiochem), and the filtrate was centrifuged at 15 min at 20,000g. The resulting supernatant was layered over an upper layer of 0.5 M sucrose (3 ml) which in turn was layered over 10 ml of 1.8 M sucrose in a 25-mi Spinco polycarbonate ultracentrifuge tube. Both the sucrose solutions contained solution A (20 mm tris-HCl, pH 7.6; 10 mm MgCl₂, 15 mm KCl; and 5 mm β-mercaptoethanol). The tube and contents were centrifuged at 105,000g in a Spinco 30 rotor for 3 hr. The resulting supernatant was discarded. The ribosomal pellet was gently suspended in 0.5 ml of solution A. This suspension was centrifuged at 1,000g for 1 min in order to remove unsuspended material, and an aliquot of the supernatant fraction containing approximately 100 μg of RNA was layered onto a linear sucrose gradient (11 ml of 10–34% [w/v] sucrose in solution A) suspended on a cushion made of 1.5 ml of 34% (w/v) sucrose solution in a cellulose nitrate centrifuge tube. The mixture was centrifuged at 200,000g in an SW 41 rotor of the Beckman model L2-50 ultracentrifuge for 70 min. The distribution of ribosomes in the sucrose gradient was determined at 254 nm by means of the ISCO model 180 density gradient fractionator and a recording spectrophotometer (model UA-2) with a 1-cm light path at a flow rate of 0.6 ml/min.

Preparation of Supernatant Fraction. Sixty cotyledons were frozen with liquid nitrogen and ground to a fine powder with a precooled mortar and pestle. The powder was homogenized with 30 ml of 0.25 M sucrose in solution A for 3 min. The slurry was filtered through cheesecloth and Miracloth and the filtrate was centrifuged at 140,000g for 90 min. The supernatant was used for the studies of amino acid incorporation in vitro.

In Vitro Amino Acid Incorporation. The ribosomal preparations used for this study were extracted from the cotyledons as described for preparation of ribosomes, except that cycloheximide was not added to the grinding medium. The incubation medium for amino acid incorporation was essentially the same as described by Mans and Novelli (17) and included the following in micromoles: 50 tris-HCl, pH 7.6; 5 MgCl₂; 8 KCl; 2 β-mercaptoethanol; 0.5 ATP; 0.15 GTP; 6.4 PEP; 0.05 mg crystalline pyruvic kinase; supernatant fraction (1.5 mg protein); ribosomes 1 mg protein; 0.05 μC-[3-C]-leucine (240 mc/mmole, 100 mc/ml) in a total volume of 0.5 ml. The amino acid-incorporating system was incubated at 37°C for 30 min. The reaction was then terminated by the addition of 10% trichloroacetic acid containing 5 mm leucine. The trichloroacetic acid-precipitated material was collected by centrifugation and extracted sequentially with hot 5% trichloroacetic acid, absolute ethanol, ethanol:ether (2:1 v/v), and absolute ether. The resulting residue was dried and dissolved in 0.2 ml of 0.1 N NaOH. The determination of the radioactivity of the residue was undertaken by means of a Packard TriCarb spectrometer with Bray's scintillator (5).

RESULTS AND DISCUSSION

Protein. During the course of the experiment it was observed (Fig. 1) that, in the cotyledons of the intact seedling, protein content declined in a manner similar to that described previously for total nitrogen (4). In contrast, the protein content of the detached cotyledons remained almost constant during the period of study. These chemical analyses confirm the ultrastructural observations of Bain and Mercer (1), which indicated that protein body disruption did not occur in detached cotyledons. Coincident with the decline in protein in the cotyledons from the intact seedlings there was an increase in alcohol-soluble α-amino nitrogen. There was only a small increase in this component in the detached cotyledons (Fig. 1).

These findings were consistent with the concept that protein degradation in the cotyledons from intact seedlings proceeds by way of hydrolysis to amino acids. The lack of an accumulation of α-amino nitrogen in the detached cotyledons
However, in attached radioactive leucine by deons interfered indicated whereas there was little change in this 4.
corporate the time the showed cotyledons necessarily exogenously supplied during germination, cotyledons supplied the protein this following incubation reached counts axis.
The variable accumulation of soluble amino acids could foreseeably interfere with the determination of protein synthesis by using exogenously applied labeled amino acids. However, in spite of this complication exogenously supplied radioactive leucine was incorporated into protein by cotyledons from intact seedlings. The capacity to incorporate the supplied amino acid expressed as the percentage of the available counts incorporated into protein increased rapidly and then reached a plateau (Fig. 2). However, this did not necessarily indicate that the rate of protein synthesis became constant in the detached cotyledons, since with increasing germination time the exogenously supplied amino acid was diluted with increasing quantities of endogenous amino acids in the cotyledons from the intact seedlings. The detached cotyledons showed an initial increase in the capacity to incorporate the supplied leucine at 24 hr, similar to that observed in the cotyledons from the intact seedlings; however, following this time the incorporation of the radioactivity into protein declined progressively during the experimental period.

RNA. The RNA content of the attached cotyledons declined by about 20% during the 6-day germination period, whereas there was little change in this component in the detached cotyledons (Fig. 3). Although the RNA content declined during germination, there was also an increase in the capacity for RNA synthesis in the attached cotyledons (Fig. 4). This was evident from the increasing ability to incorporate exogenously supplied adenine into RNA. This finding was consistent with the previous observation by Beevers and

FIG. 1. Changes in protein and α-amino nitrogen content of attached (□—□) and detached (○—○) cotyledons during a 6-day incubation period.

FIG. 2. Changes in the capacity of cotyledons to incorporate exogenously supplied 14C-leucine into protein. The attached cotyledons (□—□) were removed from seedlings at 12, 24, 48, 96, and 144 hr after the beginning of imbibition; injected with 14C-leucine; and incubated for 3 hr. The detached cotyledons (○—○) were detached from the embryonic axis at the initiation of the experiment and incubated on moistened filter paper for the assigned period prior to being exposed to 14C-leucine for 3 hr. The results are expressed as counts incorporated into protein as a percentage of the total ethanol-soluble and protein counts.

FIG. 3. Changes in total RNA content of attached (□—□) and detached (○—○) cotyledons during a 6-day incubation period.
comparable to cotyledons from intact seedlings; however, with progressive incubation time this ability rapidly declined and by 48 hr the detached cotyledons showed only a limited incorporation of the supplied nucleic acid precursor.

RNA Fractionation. In view of the heterogeneous nature of RNA it was necessary to determine if the synthesis of all RNA components was restricted or whether axis removal limited the synthesis of only certain RNA species. This possibility was checked by extracting RNA from cotyledons incubated in the presence of $^{32}P$-labeled orthophosphate and fractionating the labeled RNA by polyacrylamide gel electrophoresis.

Ultraviolet scanning of the gels indicated that the RNA from both attached and detached cotyledons contained three principal components comprising the heavy (25S) and light (18S) ribosomal RNA and soluble RNA. These RNA species were present in preparations from both types of cotyledons at all of the incubation stages studied.

Radioactivity arising from the incorporation of $^{32}P$ principally associated with the three regions corresponding to heavy ribosomal (25S), light ribosomal (18S), and soluble RNA (Fig. 5). This finding agreed with the recent observations of Hewson et al. (11). The incorporation of $^{32}P$ into all of the detectable RNA components of both attached and detached cotyledons was similar after 24 hr. However, with progressive incubation times the incorporation of $^{32}P$ into all of the detectable RNA species was reduced in the detached cotyledons in comparison to cotyledons excised from intact seedlings, which showed an increasing capacity to incorporate $^{32}P$ up to 96 hr. In addition to the three major peaks of radioactivity there was additional radioactivity heterogeneously dispersed along the gels (Fig. 5), and this constituent was much more evident in RNA prepared from attached cotyledons 48 hr and 96 hr following imbibition.

Polyribosome Level. In view of the demonstrated differences in capacity for in vivo amino acid incorporation, attempts were made to determine the causes of differences in the protein synthesis in detached and attached cotyledons. Since Marcus and Feeley (18) have shown that the enhanced amino acid incorporation following imbibition is associated with an increased polyribosome level, it was of interest to determine if the differences in protein synthesis in the cotyledons could be related to polyribosome formation.

Sucrose density fractionation of the ribosomal preparations from detached cotyledons and cotyledons excised from intact seedlings indicated that the monoribosome to polyribosome ratio changed during the experimental period (Fig. 6). In cotyledons from the intact seedlings there was a progressive increase in the ribosomes occurring in polyribosomal configuration, with the result that by the 4th day of germination 60% of the 254 nm absorbing material was associated with the polyribosome region of the sucrose density gradient. In contrast in the detached cotyledons, although the percentage of ribosomes occurring as polyribosomes was initially the same as that in the cotyledons from intact seedlings, there was a progressive decrease in polyribosomes.

Amino Acid Incorporation $V$. Using the ribosomal preparations from the cotyledons of peas it was possible to demonstrate amino acid incorporation. The capacity to incorporate radioactive leucine into hot trichloroacetic acid-precipitable material by the ribosomal preparations from detached cotyledons and from cotyledons excised from intact seedlings is shown in Figure 7. In these studies the supernatant components, which we have previously shown (3) to be necessary for amino acid incorporation, were extracted from cotyledons excised from intact seedlings 72 hr after the commencement of imbibition.

Ribosomal preparations from both types of cotyledons 24 hr after imbibition showed similar capacities for leucine incorporation. Subsequently with increasing imbibition time it was observed that ribosomal preparations from the cotyledons from intact seedlings showed an increased capacity for leucine incorporation. In contrast, the amino acid-incorporating capacity of the ribosomal preparations from detached cotyledons declined with time of imbibition.

Influence of Supernatant Fraction on Amino Acid-Incorporating Capacity. Although the results of the in vitro amino acid incorporation studies were consistent with the suggestion that the capacity for protein synthesis both in vivo and in vitro is related to polyribosome level, it was considered that control of protein synthesis in the two types of cotyledon could be exerted by components in the necessary supernatant fraction. Supernatant fractions were prepared from the two types of cotyledon at various times following imbibition. Subsequently the capacity of these fractions to support amino acid incorporation by ribosomal preparations from cotyledons from intact seedlings 96 hr after imbibition was investigated. The various supernatant fractions all showed similar capacities to support leucine incorporation (Fig. 8). Thus it appeared that amino acid incorporation and hence protein synthesis were not restricted by components of the supernatant fraction but were more closely related to the percentage of polyribosomes.

Amino Acid Incorporation in the Presence of Synthetic Polyribonucleotides. The accumulation of polyribosomes in cotyledons from intact seedlings but not in detached cotyledons suggested that the ribosomal preparations from detached cotyledons may be deficient in messenger RNA. If this were the case, it should be possible to stimulate amino acid incorporation by the addition of synthetic polyribonucleotides to serve as a template for polypeptide synthesis. In experiments designed to test this it was found (Table 1) that polyuridylic acid stimulated the incorporation of $^{14}C$-phenylalanine by ribosomal preparations from detached cotyledons and also by ribosomal preparations from cotyledons excised from intact seedlings for 24 hr following imbibition.
Fig. 5. The distribution of radioactivity in polyacrylamide gels following electrophoresis of $^{32}$P-labeled RNA prepared from detached and attached cotyledons. The attached cotyledons were removed from the germinating seedlings at the times indicated and incubated for 5 hr with $^{32}$P-orthophosphate. The detached cotyledons were removed from the embryonic axis at the beginning of the experiment and incubated on moist filter paper for the designated periods of time prior to exposure to $^{32}$P.

Seedlings. The stimulation of phenylalanine incorporation was similar in preparations from both types of cotyledon after 24 hr of imbibition. However, the stimulation of incorporation of ribosomal preparations from detached seedlings decreased at later stages of imbibition. In contrast, there was a greater stimulation of phenylalanine incorporation in ribosomal preparations from detached cotyledons. The resulting effect of the differential stimulation was that all of the
riboosomal preparations show similar capacities for phenylalanine incorporation in the presence of polyuridylic acid.

These findings suggested that the ribosomal preparations from detached cotyledons have the same enzymatic potential for polypeptide synthesis as preparations from cotyledons from intact seedlings. However, their capacity for amino acid incorporation was restricted because of a deficiency of associated messenger RNA. Also, the stimulation of phenylalanine incorporation by ribosomal preparations from cotyledons of intact seedlings indicated that these ribosomes were not fully saturated with messenger RNA. In other studies (3) we have indicated that in ribosomal preparations containing 35% polyribosomes phenylalanine incorporation was stimulated 120% whereas in preparations containing 85% polyribosome phenylalanine incorporation was only slightly stimulated by polyuridylic acid.

Fig. 6. The distribution of 254 nm absorbing material in sucrose density gradients following centrifugation of ribosomal preparations from attached and detached cotyledons at 24, 48, and 96 hr after imbibition.
Plant preparations from prepared cotyledons. Ribosomal preparations were fortified with supernatant component from attached cotyledons (72 hr after imbibition). The reaction system is outlined in "Materials and Methods," but 0.5 μc of 14C-phenylalanine (375 mc/mmole) replaced 14C-leucine. In assays with polyuridylic acid (poly U) the reaction mix contained 50 μg of polyuridylic acid.

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**CONCLUSIONS**

Pea cotyledons detached from the embryonic axis at the commencement of imbibition fail to hydrolyze the protein and RNA reserve components of the cotyledon. This reduced hydrolytic activity was associated with a restricted capacity to incorporate exogenously supplied leucine into protein. Similar earlier studies of Varner et al. (24) indicated a limited accumulation of hydrolytic enzymes which was associated with a restricted respiratory activity of detached cotyledons. The observations of Cherry (8), Breidenbach et al. (6), and Koloffel and Stuys (14) indicated that the increased respiratory activity in cotyledons during germination was associated with an enhanced level of various mitochondrial enzymes, and it was thus possible that the restriction in hydrolytic enzyme production and reduced respiratory activity in detached cotyledons could have resulted initially from an over-all failure of protein synthesis.

In comparing the in vitro amino acid incorporation of ribosomal preparations from detached cotyledons and from cotyledons excised from intact seedlings, it was found that ribosomes from detached cotyledons show a restricted protein synthesis. This reduced amino acid incorporation was associated with a reduced polyribosome level in ribosomal preparations from detached cotyledons, suggesting that such preparations had a reduced messenger RNA content. The observation that the amino acid incorporation in ribosomal preparations from detached cotyledons showed greater stimulation by synthetic polynucleotide than similar preparations from cotyledons excised from intact seedlings was consistent with this proposition. At this stage of the investigation, however, it cannot be determined whether axis removal reduced messenger RNA production in detached cotyledons or in some way limited the capability of the ribosomes to bind to and utilize existing messenger RNA components.

**LITERATURE CITED**


