

\ EFFECTS OF STRATIFICATION ON IN VITRO PROTEIN SYNTHESIS/
USING COMPONENTS FROM THE EMBRYOS OF PINUS LAMBERTIANA DOUGL.

by

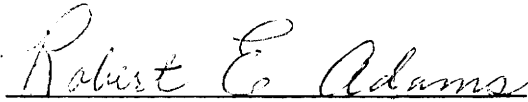
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MASTER OF SCIENCE


in

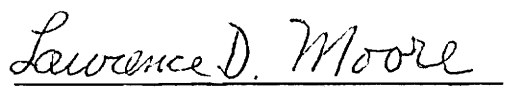
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INTRODUCTION

The seeds of many plant species are dormant when initially shed. Seeds showing "true" or "innate" dormancy will not germinate even under favorable environmental conditions until they have received various treatments to break this dormancy. True dormancy should not be confused with "imposed" dormancy or quiescence, where unfavorable environmental conditions have temporarily arrested active growth. True dormancy appears to be due to conditions within the dormant organism itself and endogenous changes must occur before germination can begin.

The genus Pinus includes many forest trees of economic importance. Seeds of many pine species show true dormancy when shed from the mother tree and a period of cold and moisture is necessary to overcome dormancy. In the laboratory, prompt germination (i.e. within 5-10 days) can be obtained by placing the seeds in moist sand and exposing to periods of cold temperature (4-10°C) in order to simulate the natural winter environment. This procedure is known as "stratification" and this term will be used henceforth to describe the moist, cold treatment necessary to break seed dormancy.

Much research has involved the physiology of dormancy in seeds of plants of the Subdivision Angiospermae but relatively little has been done with the seeds of plants of Gymnospermae. There are considerable differences in the seeds of these two plant sub-divisions (cf. Bell and Woodcock, 1971). Thus, generalizations cannot be made between the two plant types and a need exists for research with

gymnosperms, such as pine.

The seeds of sugar pine, Pinus lambertiana Dougl, were selected for investigation because 1) when shed, they possess true dormancy which is capable of being broken by stratification, 2) their relatively large size facilitates excision of sufficient homogenous tissue for study, and 3) the species is commercially important. Stone (1957a) reported that germination of unstratified sugar pine seeds was less than 50% after three months but 90-day stratified seeds showed 96% germination after seven days. He also noted that sugar pine is subject to periodic seed years and frequently it is necessary to rely on stored material for nursery and direct seeding operations. Since over two million acres of forest land in Oregon and California support sugar pine, Stone maintained that a better understanding of sugar pine seeds' dormancy and germination patterns could be justified on economic grounds. Understanding of the physiology of seed dormancy and germination could benefit the development of technologies involving pine seed storage, nursery operations, and direct seeding.

Protein synthesis is a major factor in plant growth and development and it is of interest to explore the changes that may occur at the translational stage of protein biosynthesis during seed stratification. This study characterizes an in vitro protein synthesizing system which includes components isolated from sugar pine embryos and uses Sephadex G-25 gel filtration to purify the 105,000 xg supernatant fraction. The study also explores the effects of sugar pine seed stratification on the ability of this polyuridylic acid-directed system to incorporate phenylalanine, and compares the results

with a similar system which uses isoelectric precipitation at pH 5.0 for 105,000 xg supernatant purification. An attempt is made to partially characterize changes occurring during stratification that may affect the translation process. Determinations are made of ribonuclease and protease activities in the separated fractions, the abilities of the extract systems to bind polyuridylic acid to the ribosomes, changes in the size of the ribosome polymers, and the abilities of these polymers to incorporate phenylalanine.

LITERATURE REVIEW

Dormancy and Protein Synthesis

The adaptation whereby certain plant seeds remain dormant during environmentally harsh periods (e.g. winter, drought) is clearly a means for increasing population fitness. The mechanisms controlling dormancy are not completely understood but true dormancy appears to be an endogenously controlled but environmentally imposed temporary suspension of growth, accompanied by reduced metabolic activity, and relatively independent of ambient conditions (Amen, 1968). There are several forms of seed dormancy, one of which involves the requirement for stratification. Seed dormancy has been intensively discussed in several reviews (cf. Amen, 1968; Wareing, 1969; Wareing and Saunders, 1971; Villiers, 1972).

Many researchers of seed germination have devoted their attention to the mechanisms of synthesis and activation of enzymes and proteins (cf. Mayer and Shain, 1974). General reviews of protein synthesis in higher plants were presented by Mans (1967a) and Zalik and Jones (1973). Study of the cytoplasmic sites of control, during the translation of RNA into protein, is of interest and seems justified. Clowes and Juniper (1968:388) stated, "It is ... possible that precise gene regulation occurs in the cytoplasm and not in the nucleus, i.e., in 'translating' the commands of the RNA base sequence into amino acid arrangements rather than in the transcription of the RNA."

Steinberg, et al. (1969) stated that the suppression of protein

synthesis during "cryptobiosis" ^{1/} is a well known phenomenon and the nature of the block is at the level of transcription in some organisms and at the level of translation in others. As the organism breaks dormancy, an increase in protein synthesis activity appears to occur and to play a role in the release from dormancy itself (Allende and Bravo, 1966; Steinberg, et al., 1969). There have been reports of increases in protein synthesis accompanying changes in the developmental states in many organisms, including spores (Horikoshi and Ikeda, 1968; Schmoyer and Lovett, 1969; Brambl and Van Etten, 1970; Kobayashi, 1972; Merlo, Roker, and Van Etten, 1972), sea urchin eggs (MacKintosh and Bell, 1969; Fellicetti, et al., 1971), and higher plant seeds (Sturani and Cocucci, 1965; Marcus and Feeley, 1965; Allende and Bravo, 1966; Sturani, 1968; Sasaki and Brown, 1971).

Research concerning the control of protein synthesis during germination of higher plant seeds has indicated that changes occur at the translational level of protein synthesis. The lack of an active messenger RNA-ribosome complex has been reported in the dry seeds of castor beans (Sturani, 1968), wheat (Marcus and Feeley, 1965; Allende and Bravo, 1966) and peanuts (Marcus and Feeley, 1965). Imbibition of peanut and wheat seeds seems to cause formation of an active complex (Marcus and Feeley, 1965) and an increase in poly-ribosome content (Marcus, Feeley and Volcani, 1966). Similar results

^{1/}Cryptobiosis is a term used by Keilin (1959) to define latent life or "The state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversably to a standstill." He also used a more general term, "hypobiosis", to define all states of an organism from simple low metabolism to cryptobiosis.

were obtained by Sasaki and Brown (1971) for red pine seeds and by Adams (1972) for sugar pine female gametophyte tissue. Tomé, Fellicetti and Cammarano (1972) reported that an inhibitory component was present in 6-day germinated seeds of Pisum sativum but not in 2-day germinated seeds. This inhibitor reduced the binding ability of messenger RNA and amino acyl - tRNA to the ribosomes. However, this reduction was not due to limiting amounts of transfer factors or to an increase in ribonuclease activity. Tao and Khan (1974) found that amino acyl-tRNA synthetases increased in activity during stratification of dormant pear embryos and this genetically regulated process led to the release from dormancy.

Black and Richardson (1967) found that protein synthesis occurred in dormant lettuce seeds and the addition of chloramphenicol promoted germination. They concluded that some protein(s) that inhibited germination of the embryo were being synthesized in the endosperm during dormancy. They admitted, however, that this possibility did not agree with all data and further research was needed.

Abscissic acid (ABA) has been closely linked with dormancy. Ihle and Dure (1972) found that ABA inhibited the translation of mRNA in cotton seeds and Bonnafous, Mousseron-Camet and Olive (1973) reported that ABA inhibited protein synthesis in barley coleoptiles by 30-40%. They were unable to determine the exact location of action but did suggest that it was at the level of elongation rather than initiation since the attachment of ribosomes to mRNA was unaffected.

Gibberellic acid (GA), which has been shown to aid in breaking dormancy in some seeds, was found to increase the ability of wheat

embryo ribosomes to promote protein synthesis in vivo but not in vitro, suggesting a co-factor is involved (Chen and Osborne, 1970). Chen and Osborne concluded that translation is the primary location of hormone effects and transcription is secondary. Evins (1971) reported that an increase in polyribosomes in barley aleurone grain was one of the earliest events following the application of gibberellic acid and the rate of protein synthesis thereafter doubled.

Dormancy in Sugar Pine Seeds

The seeds of sugar pine require 90 days of stratification at 2-10°C in moist sand or peat to give satisfactory germination (USDA, 1948; Villiers, 1972). Kozlowski (1971) stated that this dormancy appears to be due to an anatomically mature but physiologically immature embryo. The surrounding female gametophyte and two seed coats may also function in maintaining and controlling dormancy (Adams, 1972; Stone, 1957b).

Stone (1957b) found that unstratified sugar pine seeds germinated slowly and incompletely at both 25°C and 15°C, but after stratification, 90% of the seeds germinated within 30 days, even at 5°C. At 25°C, removal of the hard outer seed coat and inner papery seed coat proved to be almost as effective as stratification with 50% germination after 8 days. As the temperature was lowered, this method proved less effective. Stone concluded that at 25°C, there was no embryo dormancy since removal of the seed coat could substitute for stratification but at 5°C, the embryo was dormant as removal of the seed coats did not promote germination. He proposed a scheme

describing the process: Substrate (S) \rightarrow Intermediate (I) \rightarrow Growth (G), where S \rightarrow I is independent of the seed coats but I \rightarrow G is retarded by the seed coat's presence at 5°C. It is possible that a temperature-dependent diffusion process is involved in this phenomenon.

Baron (1966) reported that sugar pine seeds with coats removed germinated well on nutrient agar without stratification. However if the inner papery seed coat remained intact, germination was very low. He concluded that there was little evidence for inherent embryo dormancy, nor of chemical or hormonal stimulation or inhibition of germination and that embryo growth limitations were probably due to the unavailability of nutrients. Adams (1972) also found that the inner papery seed coat was a factor in the low germination of unstratified sugar pine seeds. The seed coat did not exert its effect by limiting gas exchange but appeared to limit the inward movement of some component such as moisture or nutrients or the outward movement of some inhibitor. He concluded that the latter proposal was the most likely and that stratification is probably accompanied by leaching of the inhibitory component. Removal of the seed coats allows a more rapid exit.

The evidence suggests that the sugar pine seed coats may act as a barrier to the leaching of some component that is inhibiting germination but gives no indication of the location of action of this component. It is possible that an inhibitor could reside in the embryo and be leached out during stratification or following seed coat removal. A possible site of action of a germination inhibitor is enzyme synthesis. Since this is a major controlling factor in

seedling development, it is of interest to study the effect of stratification on the ability of the embryo cells to synthesize protein.

An in vitro phenylalanine incorporating system from sugar pine seeds was isolated by Adams, Shih and Barnett (1970) which depended on polyuridylic acid as a messenger RNA. The 105,000 xg supernatant fraction was purified by isoelectric precipitation at pH 5.0 and suspension of the precipitate in Tris-HCl buffer. Barnett, Adams, and Ramsey (1974) further reported that using this system, phenylalanine incorporation in p-moles of phenylalanine incorporated per mg of ribosomes (Table 1) was approximately 13 times greater for the embryos of stratified seeds than for the embryos of unstratified seeds. The increase in activity with stratification was apparently due to changes in the components in the pH5 fraction as ribosomes from embryos from unstratified seeds were as active with the pH5 fraction of embryos from stratified seeds as ribosomes from the embryos of stratified seeds. They also found that stratification did not apparently increase the ability of the female gametophyte tissue to direct in vitro incorporation. However a drastic change occurred during the first 36 hours of stratification to the ribosomes of female gametophyte tissue. They were very active with the pH5 fraction from the embryos of stratified seeds at 0 days of stratification but lost 61% of their activity after 36 hours of stratification and lost 93% after 90 days. This loss of activity could not be explained but paralleled closely the polyribosome formation that occurred in this tissue upon seed imbibition (Adams, 1972). It was also reported

Table 1. Protein synthetic activity* of ribosomal and pH5 fractions from embryo and female gametophyte tissues of dry and 90-day stratified seeds.**

Source of pH5 fractions	Source of ribosomal fractions			
	Embryo (Dormant)	Embryo (Stratified)	Female Gametophyte (Dormant)	Female Gametophyte (Stratified)
Embryo (Dormant)	24 ± 12	56 ± 26	48 ± 30	4 ± 3
Embryo (Stratified)	341 ± 54	326 ± 11	302 ± 82	13 ± 5
Female Gametophyte (Dormant)	2 ± 1	10 ± 4	2 ± 1	4 ± 1
Female Gametophyte (Stratified)	1 ± 1	4 ± 1	2 ± 2	8 ± 1

Data expressed in value ± standard error of mean.

* Activity expressed in picomoles ¹⁴C-phenylalanine incorporated per mg of ribosomes.

**Data from Barnett, Adams and Ramsey (1974).

(Adams, 1972) that this increase in polysome content was independent of messenger RNA synthesis and thus probably due to ribosome association with pre-existing messenger RNA.

TECHNIQUES AND PROCEDURES

Sugar pine seeds were obtained from a single seed source in Plumas County, California (Latitude 40°15'N, Longitude 121°15'W) at an elevation of approximately 5000 feet. They were stored at -20°C in sealed containers until used. Moisture content was 7.8% (ODW).

Stratification Procedures

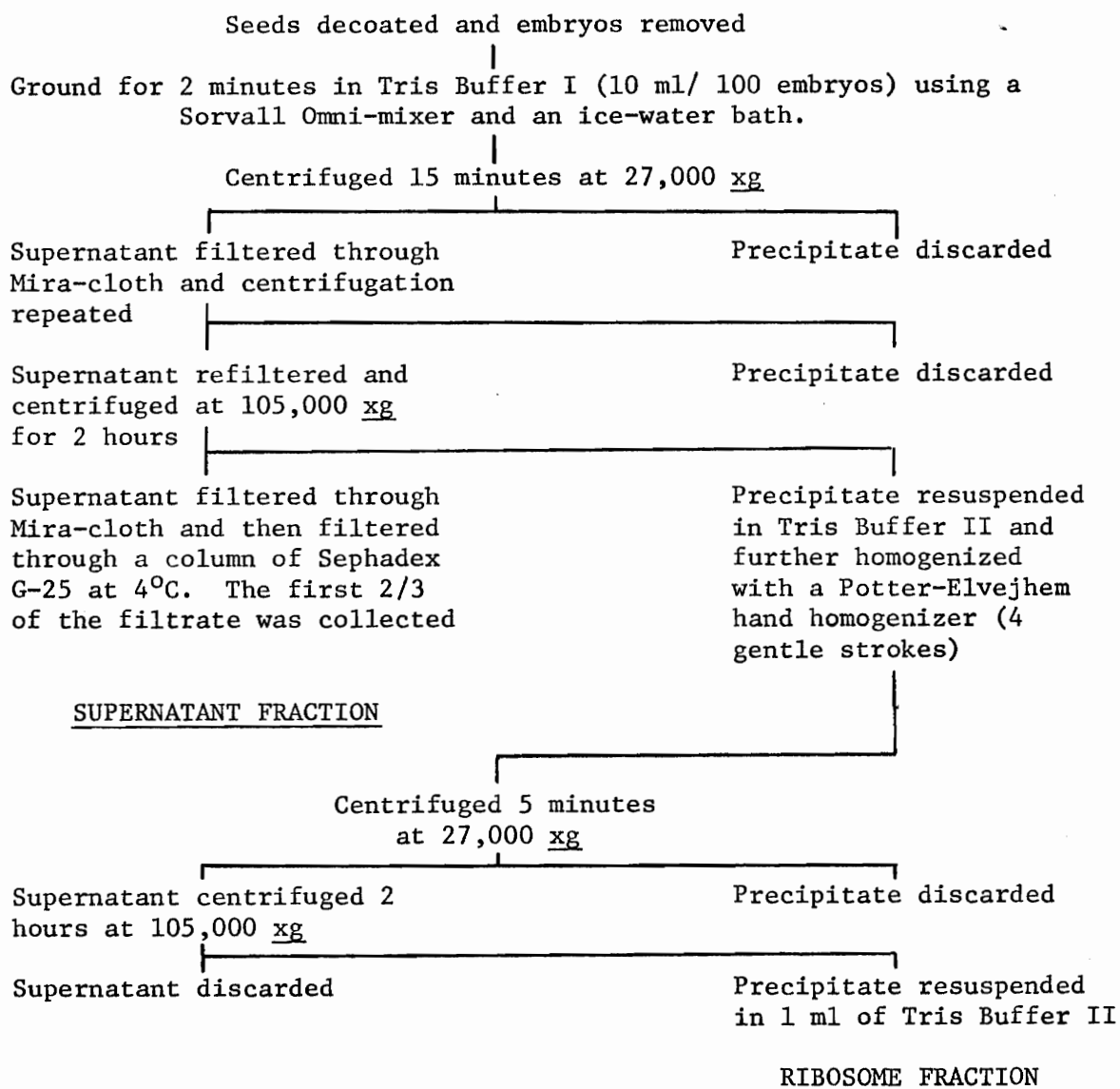
The sugar pine seeds were stratified in moist, sterile sand at 4°C in the dark.

Isolation of System Components from Sugar Pine Embryos

Components for an amino acid incorporating system were isolated from sugar pine embryos using a modification of the procedure of Adams, Shih and Barnett (1970). Figure 1 is a flow sheet of the isolation procedure. The major modification involved purification of the 105,000 μ g supernatant by filtering it through Sephadex G-25 rather than precipitation of protein at pH 5.0. Plastic gloves were worn during excision of the seeds to prevent contamination by hydrolytic enzymes on the hands. All steps were performed at 4°C.

Two Tris-HCl buffers were used in the isolation procedure. Buffer I, the homogenizing buffer, contained 0.0125M Tris-HCl, pH 8.5, 0.015M KCl, 0.0075M MgCl₂, 0.4M sucrose, 0.6mM β -mercaptoethanol. Buffer II, the general-use buffer, consisted of 0.02M Tris-HCl, pH 8.0, 0.015M KCl, 0.0075M MgCl₂, 0.6mM β -mercaptoethanol.

Figure 1. Method of isolation of the components for an amino acid incorporating system from sugar pine seeds.



Phenylalanine Incorporating System:
Preparation and Incubation Procedures

The incorporating system utilized ^{14}C -labelled phenylalanine as the amino acid and polyuridylic acid as a synthetic messenger RNA. The system contained the following components in varying amounts: an ATP generating system (10.6mM ATP, 24 mM phosphoenolpyruvate, and 0.176 mg/ml pyruvate kinase), 1.2mM GTP (added with the ATP mixture) polyuridylic acid, 0.0075M MgCl_2 , 0.015M KCl, 0.6mM β -mercaptoethanol, 0.02M Tris-HCl, pH 8.0, ^{14}C -phenylalanine, supernatant fraction and ribosome fraction. Concentrations of several of the components were varied in order to determine optima for phenylalanine incorporation. The preparations were incubated in a water bath and 0.1 ml samples placed on paper discs (Whatman No. 3 filter paper - 2.3 cm diameter). These were dried for 15 seconds under a warm air stream and placed in a solution of 10% TCA and 0.1M ^{12}C -DL-phenylalanine to stop protein synthesis and to dilute the unincorporated labeled phenylalanine. The discs were treated by the method of Mans and Novelli (1961a), placed in vials with 10 ml of PPO-POPOP-Toulene solution and counted in a Beckman Scintillation Counter. The amount of ^{14}C -labelled hot TCA insoluble material was used as an indication of protein synthesis.

In each experiment, comparisons were made between the phenylalanine incorporating abilities of components from embryos of dormant and stratified sugar pine seeds. Dormant seeds were taken from the -20°C storage immediately before use and neither stratified nor imbibed. They will be referred to henceforth as dry seeds. Seeds which received 90 days of stratification will be referred to henceforth as stratified

seeds. Incubation mixtures were prepared using the four possible combinations of supernatant and ribosome fractions separated from the embryos of dry and stratified seeds. Several experiments were also made using 40-hour stratified seeds, referred to as imbibed seeds. Replications of each test were made to insure repeatability.

Calculation of the Activity of the Phenylalanine Incorporation System

The amount of ^{14}C -phenylalanine incorporated into hot TCA insoluble material was quantitated by determining the counts per minute detected by the scintillation counter and adjusting for specific activity of the phenylalanine and the efficiency of the counter. The quantity of ribosomes and ribosomal protein in each tube was calculated from the absorbance at 260 nm, using an absorbtivity ($a_{1\%}^{1\text{cm}}$) of 99 and a protein content of 58% (Shih, Adams, and Barnett, 1973). Activity was expressed in pico-moles of ^{14}C -phenylalanine incorporated per milligram of ribosomal protein.

Protein Analysis

Supernatant protein was analyzed using the method of Lowry, et al. (1951). Standard reference protein solutions were prepared using bovine serum albumin.

Ribonuclease Activity Assay

The activity of ribonuclease in the supernatant fractions was measured using the method of Ambellan and Hollander (1966). Standard RNase was prepared using Ribonuclease A crystallized from bovine pancreas (Sigma Chemical Co.). Quantities were expressed in milligrams

RNase detected per mg protein. The substrate used was yeast ribonucleic acid (Mann Research Laboratories).

Protease Activity Assay

The activity of protease enzymes in the supernatant and ribosome fractions was measured using the method of Wiley and Ashton (1967). Quantities were expressed in units of absorbance at 280nm per mg protein. The substrate used in the assay reaction was A.N.R.C. Reference Protein (Nutritional Biochemicals Co.).

Effect of Protease on the Amount of Hot TCA Insoluble Material

In order to verify that polypeptides were being produced by the phenylalanine incorporating system, protease (Sigma Chemical Co., Type VI, Bacterial Protease purified from Streotomyces griseus) was added to the samples following 60-minute incubation at 37°C, and the samples "post-incubated" for 15 minutes at 37°C. Protease should cause hydrolysis of any polyphenylalanine produced, when added after incubation, and thus result in a lower activity since the free amino acids would be soluble in TCA.

Sucrose Density Gradient Profiles

Five absorbance units (260nm) of ribosomes from the embryos of dry, imbibed and stratified seeds were layered on 10-40% sucrose density gradients and spun for 2 hours at 95,000 $\times g$ ($R_{av} = 11.8$ cm) using a Beckman SW-27 swinging bucket rotor and a Beckman LS-50 ultracentrifuge. The gradients were analyzed using an ISCO Gradient

Analyzer. Ribosome distribution, determined by absorbance at 254nm, was plotted on an ISCO chart recorder.

Individual incubation mixtures, containing 0.5 μ Ci of ^{14}C -phenylalanine and 5 absorbance units (260 nm) of ribosomes in 0.5 ml were also analyzed in a similar manner. One ml fractions were collected. One-half ml of each sample was mixed with 0.5 ml of distilled water and 10 ml of Aquasol (New England Nuclear Corp.) and the radioactivity determined in a scintillation counter. The amount of radioactivity in each sample was compared with the ribosome distribution pattern on the gradients. In some tests, polyvinyl sulfate, a known nuclease inhibitor (Davidson, 1972:189), was added to the samples prior to application to the gradients.

Ribosome - RNA Binding Assay

The amount of messenger RNA bound to the ribosomes was determined by a modification of the method of Roberts and Coleman (1971). Incubation mixtures were prepared as previously described but with ^3H -polyuridylic acid, plus the usual ^{14}C -phenylalanine. The tubes were incubated for 10 minutes at 37°C and filtered through Millipore filters (EGWP-0500) using a vacuum pump to insure a slow, constant suction. The filters were washed with 2, 4-ml portions of Tris Buffer II, air dried overnight and placed in vials with 10 ml of PPO-POPOP-toluene solution. The vials were counted in a Beckman Scintillation Counter. The discriminator-ratio method of Okita, et al. (1957) was used to separate ^3H and ^{14}C counts. Efficiency factors were determined using known concentrations of the isotopes on the same millipore filters.

The amount of polyuridylic acid bound to the ribosomes was calculated from known isotope concentrations and the detected counts. Also, using the ^{14}C -phenylalanine that was bound to the filters, the amount of amino acyl-tRNA bound to the ribosomes was calculated.

RESULTS

Characteristics of the In Vitro Phenylalanine Incorporating System

Adams, et al. (1970) used 0.5 mg of pH5 purified supernatant protein per ml of sample (0.25 mg/0.5 ml sample) to yield maximum incorporation of phenylalanine. In order to check the optima with the gel-filtered supernatant, a protein concentration study was performed, using the four possible combinations of gel-filtered supernatant and ribosomes from dry and stratified sugar pine embryos. Figure 2 shows the results of this study. Not only was the amount of supernatant protein necessary for optimum phenylalanine incorporation different from that reported by Adams, et al. (1970), but the amount varied with the source of the supernatant and the ribosomes. Ribosomes from the embryos of stratified seeds required less supernatant protein than did the ribosomes from the embryos of dry seeds, and with a single ribosome type, slightly more of the supernatant from the embryos of dry seeds was required for maximum incorporation than from the embryos of stratified seeds.

Table 2 shows phenylalanine incorporation at supernatant protein concentrations yielding maximum incorporation. The activity of the embryo system from dry seeds was significantly higher, as determined by analysis of variance, than that from the stratified seeds. However, using the ribosomes from embryos of dry seeds, the incorporation was significantly higher using either supernatant than corresponding systems using ribosomes from the embryos of stratified seeds. Also the supernatant from the embryos of stratified seeds had significantly

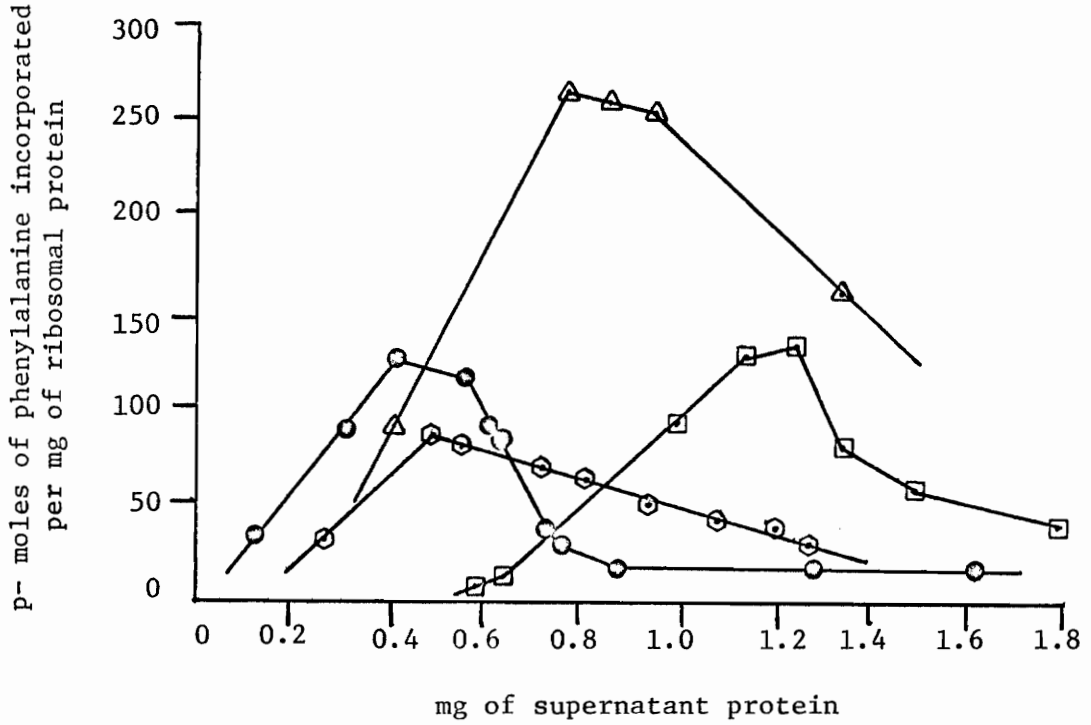


Figure 2. The effect of variation of supernatant protein concentration on the amount of phenylalanine incorporated in the sugar pine embryo in vitro system.

	<u>Source of Supernatant</u>	<u>Source of Ribosomes</u>
○ — ○	Stratified	Stratified
△ — △	Stratified	Dry
⊙ — ⊙	Dry	Stratified
□ — □	Dry	Dry

Table 2. Maximum phenylalanine incorporation activities* of the sugar pine embryo in vitro system from dry seeds and from 90-day stratified seeds.**

Source of Ribosomes	Source of supernatant	
	Stratified Seeds	Dry Seeds
Stratified seeds	112 ± 26***	79 ± 15
Dry seeds	260 ± 19	144 ± 22

* Expressed in p-moles of phenylalanine incorporated per mg of ribosomal protein.

** Supernatant protein concentrations were used that yielded maximum phenylalanine incorporation for that mixture.

***Values expressed as the average ± standard deviation.

higher incorporation activity than the supernatant from the embryos of dry seeds when systems utilizing ribosomes from either dry seeds or stratified seeds were compared. Thus it appears that the supernatant from the embryos of stratified seeds was more active in promoting phenylalanine incorporation than that from dry seeds, while the ribosomes from the embryos of dry seeds were more active than those from the embryos of stratified seeds.

These results contrast sharply with those reported by Barnett, et al. (1974) using the same seeds but with a pH5 supernatant preparation. Even though a supernatant protein concentration study was run using only dry seeds with the pH5 system, the stratified embryo system still showed 13 times greater incorporation activity over that from dry seeds at optimum supernatant protein concentration for dry seeds. It is obvious from Figure 2 that this would not be possible with the gel-filtered supernatant system at optimum supernatant protein concentration for dry embryos.

Because of the differences between the pH5 and the gel-filtered supernatant systems and in order to better understand the present system, further tests of optimum conditions and component concentrations were performed. Optimum incorporation of phenylalanine occurred with 0.3-0.6 mg of ribosomal protein in the 0.5 ml samples (see Figure 3). The amount of phenylalanine incorporated declined at higher concentrations of ribosomes. The system had an optimum polyuridylic acid concentration of 0.0375 - 0.050 mg per 0.5 ml sample (see Figure 4). Additional polyuridylic acid caused a corresponding loss of

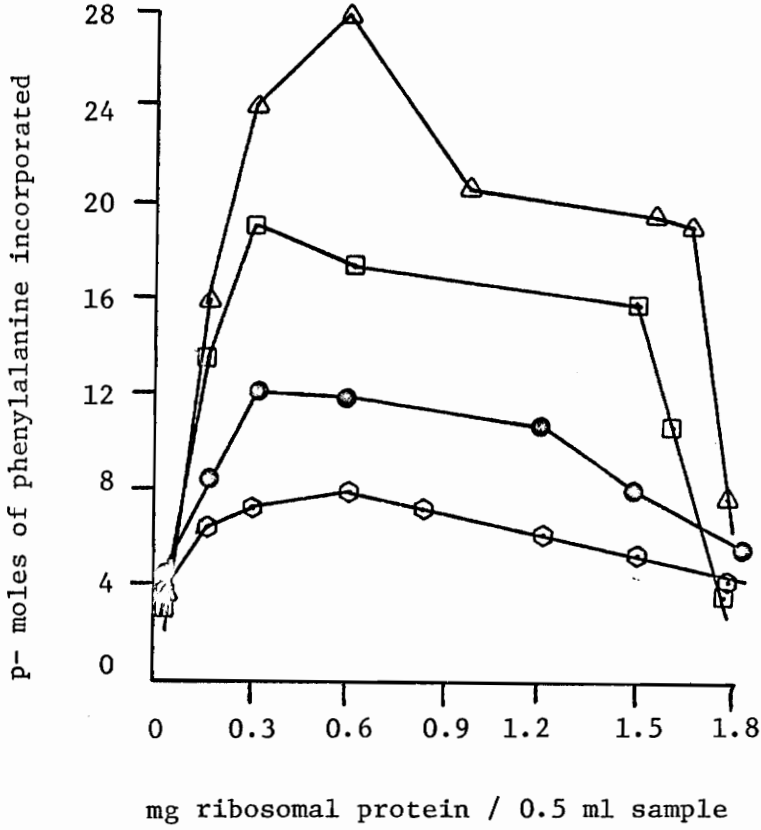


Figure 3. The effect of variation of ribosomal protein in concentration on the amount of phenylalanine incorporated in the sugar pine embryo in vitro system.

	<u>Source of Supernatant</u>	<u>Source of Ribosomes</u>
○—○	Stratified	Stratified
△—△	Stratified	Dry
◇—◇	Dry	Stratified
□—□	Dry	Dry

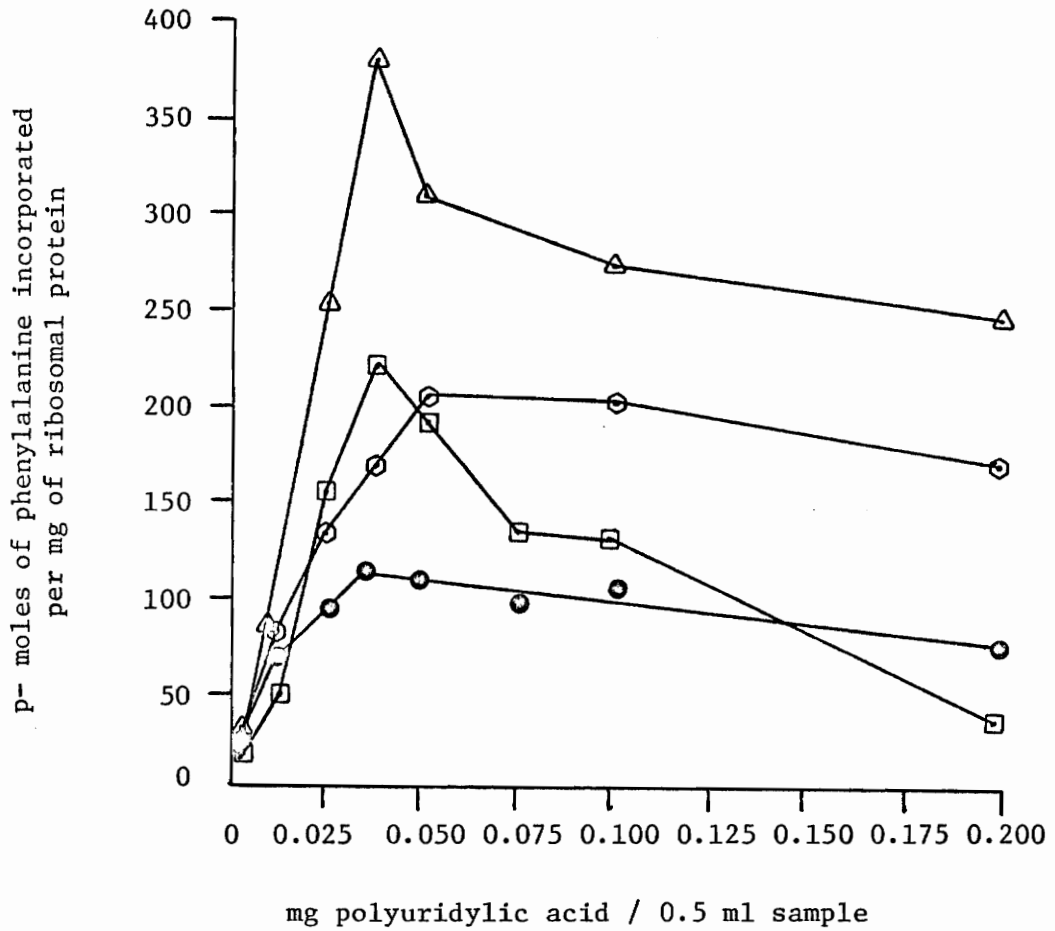


Figure 4. The effect of variation of polyuridylic acid concentration on the amount of phenylalanine incorporated in the sugar pine embryo in vitro system.

	<u>Source of Supernatant</u>	<u>Source of Ribosomes</u>
○—○	Stratified	Stratified
△—△	Stratified	Dry
○—○	Dry	Stratified
□—□	Dry	Dry

activity. Figure 5 shows that 0.075 ml of the ATP mixture yielded maximum incorporation with this system. This corresponds to 0.795 μ moles ATP, 1.8 μ moles PEP, 13.2 μ gms pyruvate kinase, and 0.090 μ moles GTP included in each 0.5 ml incubation mixture.

Figure 6 shows that maximum incorporation was measured after 60 minutes of incubation with all systems. A lag of approximately 10 minutes was noticed before significant incorporation began. Optimum temperature for incubation (Figure 7) was 37°C, except where ribosomes and supernatant fraction from dry seeds were used and 40°C was optimum. The difference is not considered meaningful. These temperatures are higher than the 25°C-30°C which is optimum for germination of sugar pine seeds (cf. Stone, 1957b; Adams, 1972).

Table 3 presents the general characteristics of this system. Deletion of the supernatant or ribosome fractions, ATP, or polyuridylic acid resulted in a substantial loss in incorporation activity. RNase and protease were also effective inhibitors of the system.

Activity of Hydrolytic Enzymes

Table 4 presents activity data for ribonuclease in the supernatant fractions of dry and stratified sugar pine embryos. Purification of the supernatant by gel-filtration significantly (analysis of variance) increased the RNase activity. This could be due to the decrease in total protein in the supernatant with purification rather than an actual increase in total RNase since RNase activity is based on the amount of total protein. The enzyme activity in the gel-filtered fraction from stratified seed embryos was significantly higher (99%

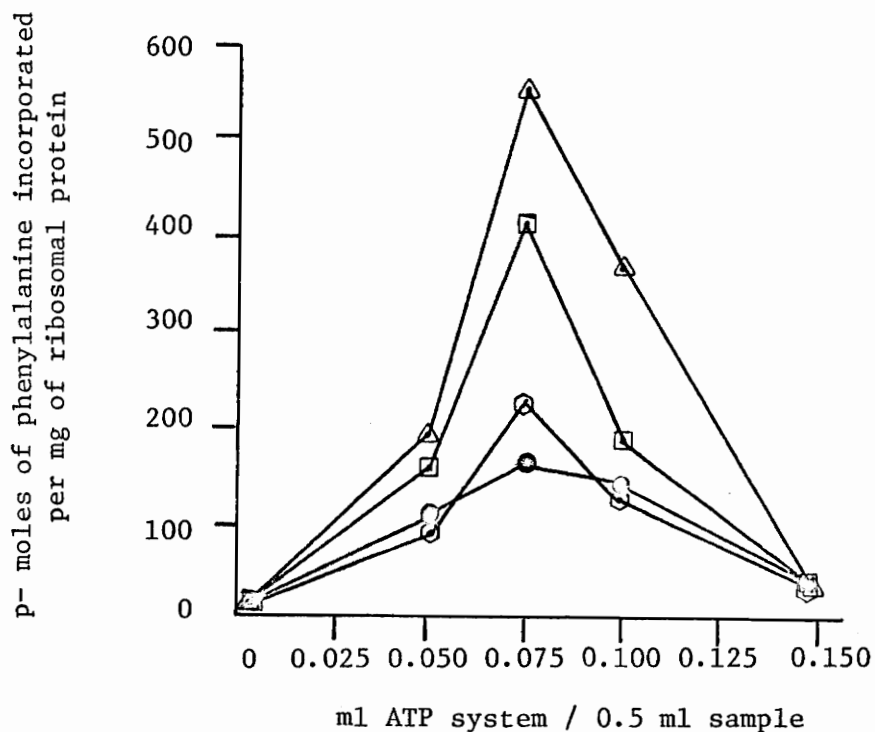


Figure 5. The effect of variation of the ATP system concentration on the amount of phenylalanine incorporated in the sugar pine embryo in vitro system.

	<u>Source of Supernatant</u>	<u>Source of Ribosomes</u>
○—○	Stratified	Stratified
△—△	Stratified	Dry
◇—◇	Dry	Stratified
□—□	Dry	Dry

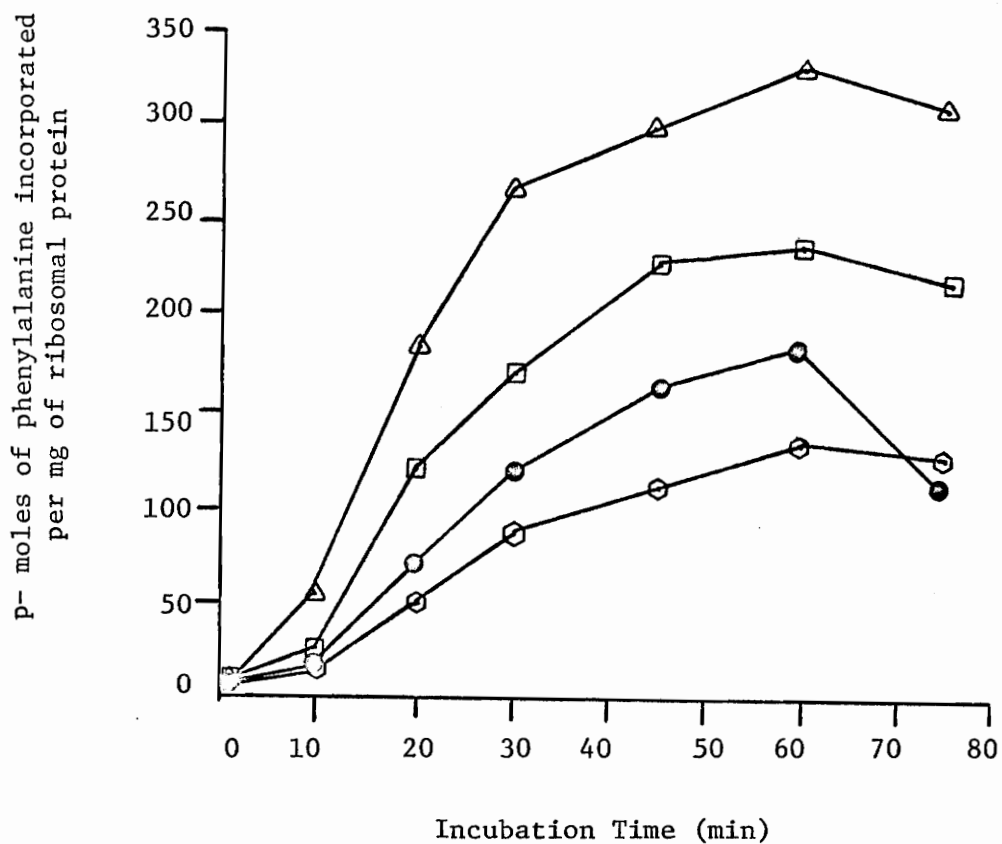


Figure 6. The effect of variation in the time of incubation on the amount of phenylalanine incorporated in the sugar pine in vitro system.

	Source of Supernatant	Source of Ribosomes
○—○	Stratified	Stratified
△—△	Stratified	Dry
◇—◇	Dry	Stratified
□—□	Dry	Dry

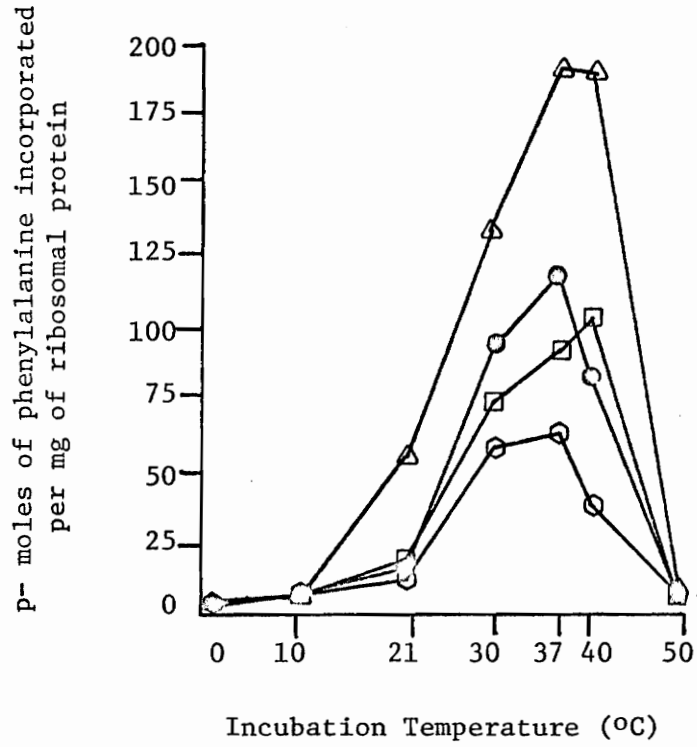


Figure 7. The effect of variation of incubation temperature on the amount of phenylalanine incorporated in the in vitro sugar pine embryo system.

	<u>Source of Supernatant</u>	<u>Source of Ribosomes</u>
○—○	Stratified	Stratified
△—△	Stratified	Dry
◇—◇	Dry	Stratified
□—□	Dry	Dry

Table 3. Requirements and inhibitors of the sugar pine embryo in vitro phenylalanine incorporating system.*

Source of supernatant:	Stratified	Stratified	Dry	Dry
Source of ribosomes:	Stratified	Dry	Stratified	Dry
<u>Systems components used</u>				
Total	1708	3470	1448	2077
- Supernatant	449	276	511	283
- Ribosomes	347	293	220	400
- ATP	328	509	272	459
- Polyuridylic acid	463	429	322	379
+ 100 μ g RNase	388	425	301	297
+ 50 μ g Protease	173	147	254	216

*Data expressed as detected counts per minute in 0.1 ml of sample on paper discs.

Table 4. Ribonuclease activity* in the various stages of purification of the sugar pine embryo supernatant from dry seeds and from 90-day stratified seeds.

Purification Step	Source of Supernatant	
	Dry	Stratified
27,000 xg	1.47 ± 0.30**	2.38 ± 0.23
100,000 xg	1.80 ± 0.81	2.79 ± 0.73
Gel-filtered	3.90 ± 0.10	4.87 ± 0.15

* Activity expressed in 10^{-5} mg of standard RNase per mg of protein.

**Averages ± standard deviation.

confidence level) than that from dry seed embryos.

The activity of protease enzymes in the sugar pine embryo supernatant and ribosome fractions is presented in Table 5. The activity decreased in the supernatant fraction with purification and appeared to be carried with the ribosome precipitate during the first 105,000 xg centrifugation, although much of this was lost when the ribosomes were washed and resedimented. Analysis of variance showed that at no stage of purification of the supernatant was there a significant difference between the protease activity in the dry and stratified seed embryos. There were insufficient data to run statistical tests on the ribosome fraction.

Protease from Streptomyces griseus (Sigma Chemical Co.) was added to the incorporating assay mixture after 60 minute incubation at 37°C. Buffer was added as a control and 50, 100 and 200 µg of protease were used in the test solution. The results are presented in Table 6. Incubation of the samples with 50 µg protease for 15 minutes at 37°C after normal incubation caused a 20-25% decrease in the amount of hot TCA insoluble material and increasing the amount of protease had no additional significant effect.

Effect of Seed Imbibition on Protein Synthesizing Activity

To determine the effect of imbibition of the seed on the protein synthesizing system isolated from the sugar pine embryos, tests were run using the supernatant and ribosome from dry, imbibed and stratified seeds. Figure 8 presents the results of varying supernatant protein

Table 5. Protease Activity* in the in vitro components from the embryos of dry and from 90-day stratified sugar pine seeds.

Fraction	Stratified Embryos	Dry Embryos
27,000 <u>xg</u> supernatant	0.264 ± 0.039**	0.254 ± 0.016
100,000 <u>xg</u> supernatant	0.070 ± 0.019	0.076 ± 0.023
Gel-filtered supernatant	0.060 ± 0.018	0.036 ± 0.008
Unwashed Ribosomes	0.405	0.643
Washed Ribosomes	0.128	0.143

* Activity expressed as the change in absorbance at 280 nm per milligram of protein per milliliter of sample.

**Average value ± 1 standard deviation.

Table 6. Variation in the amount of ^{14}C -labeled hot-TCA insoluble material* with post incubation** addition of protease to the sugar pine embryo in vitro phenylalanine incorporating system.

Source of Supernatant	Stratified	Stratified	Dry	Dry
Source of Ribosomes	Stratified	Dry	Stratified	Dry
Addition to System				
Normal***	100****	186	82	102
Buffer	103	129	71	102
50 μg Protease	79	106	53	85
100 μg Protease	85	102	60	97
200 μg Protease	84	107	69	78

*Expressed in p-moles of phenylalanine incorporated per mg of ribosomal protein.

**Post incubation refers to a 15 minute treatment at 37°C .

***No post incubation treatment.

****Average of two tests.

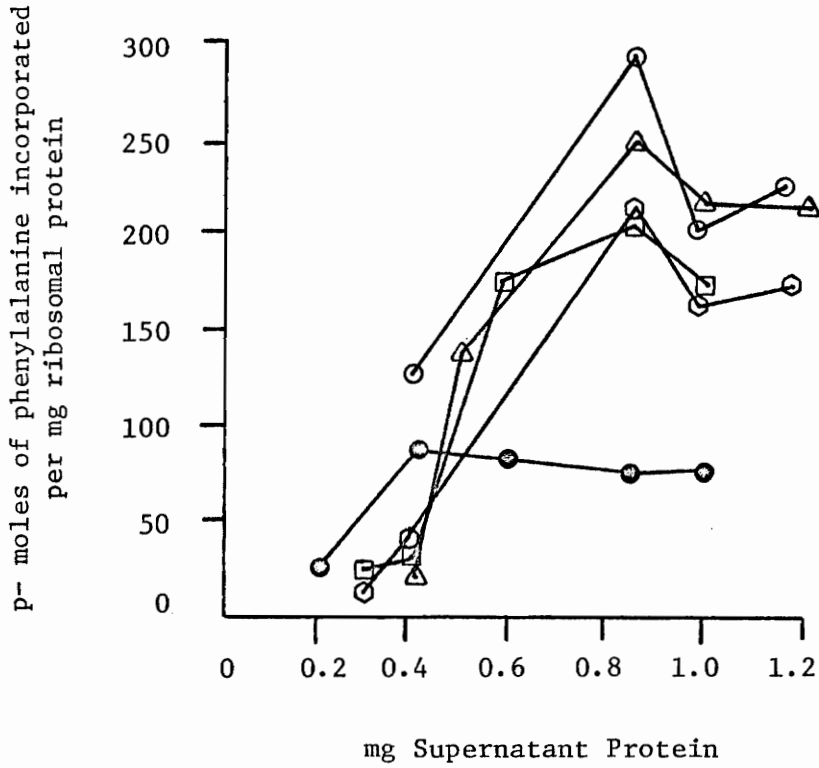


Figure 8. The effect of variation of supernatant protein concentration on the amount of phenylalanine incorporated using components from imbibed, stratified and dry seeds in the sugar pine embryo in vitro system.

	Source of Supernatant	Source of Ribosomes
○—○	Stratified	Imbibed
□—□	Dry	Imbibed
◇—◇	Imbibed	Imbibed
●—●	Imbibed	Stratified
△—△	Imbibed	Dry

concentrations using the components from imbibed seeds combined with components of the other two treatment types. It appears that the ribosomes from these embryos are similar to those from dry seed embryos in the concentrations of supernatant protein yielding maximum activity (compare Figure 8 with Figure 2). Table 7 presents the maximum incorporation activities of the system at the optimum supernatant protein concentrations. It appears that the activity is greatest using ribosomes from embryos of imbibed seeds as compared with dry and stratified seeds (imbibed > dry > stratified) and also greatest using the supernatant from the embryos of stratified seeds as compared to dry and imbibed seeds (stratified > imbibed > dry). However, it should be noted that the variability was high with systems using the components from imbibed seeds as the standard deviations often overlapped.

RNA - Ribosome Binding Study

Data from a study of the binding of polyuridylic acid to the ribosomes from dry and stratified embryos are presented in Table 8. The results of these studies allow no definite conclusions because differences between means were not significantly different by analysis of variance. Variability probably resulted from the inclusion of ^{14}C -labelled phenylalanine in the incubation mixture along with ^3H -labelled polyuridylic acid, and the subsequent sampling errors in separation of these counts. The ^{14}C -phenylalanine was used in order to duplicate the usual incubation mixtures and also to determine the difference in binding of phenylalanyl-tRNA to the ribosomes. However, the latter values were also highly variable (these data not included)

Table 7. Protein synthetic activities* of supernatant and ribosome fractions from the embryos of dry, imbibed and stratified sugar pine seeds.

Source of Ribosomes	Source of Supernatant		
	Stratified	Dry	Imbibed
Stratified	112 ± 26**	79 ± 15	87 ± 06
Dry	260 ± 19	144 ± 22	253 ± 75
Imbibed	293 ± 38	203 ± 91	227 ± 131

* Activity expressed in p-moles of ^{14}C -phenylalanine incorporated per mg of ribosomal protein.

**Average value ± standard deviation.

and no conclusions could be drawn.

If the average values in the poly U-ribosome binding study (Table 8) are generally correct (additional data are needed to determine this), it would appear that more polyuridylic acid is bound to the ribosomes from the embryos of dry seeds than from stratified seeds.

Sucrose Density Gradient Profiles

Figure 9 shows 10-40% sucrose density gradient profiles of the ribosomes from dry, imbibed and stratified sugar pine seed embryos. Profiles of ribosomes from dry and imbibed embryos are similar with almost all the ribosomes existing as monomers. The stratified seed ribosomes have a definite dimer peak and a small region that could correspond to a trimer. Figure 10 presents results from a similar experiment where 20 $\mu\text{g/ml}$ polyvinyl sulfate, a nuclease inhibitor, were added to the ribosome mixtures before application to the gradients. The stratified embryo ribosome peaks remained about the same with obvious dimer and smaller trimer peaks. However a great change was observed in the ribosomes from dry seeds. A definite dimer and trimer plus possibly larger polyribosomes were observed. It appears that nucleases are present in the ribosome fractions, especially in those from dry seeds, and that the ribosomes exist in both dry and stratified seeds as polymers, presumably associated with native messenger RNA. It should be noted that some of the absorbing material observed in the denser sucrose regions might have been removed by a second ribosome washing, similar to that used by Adams (1972), and thus this may not correspond to actual ribosomes.

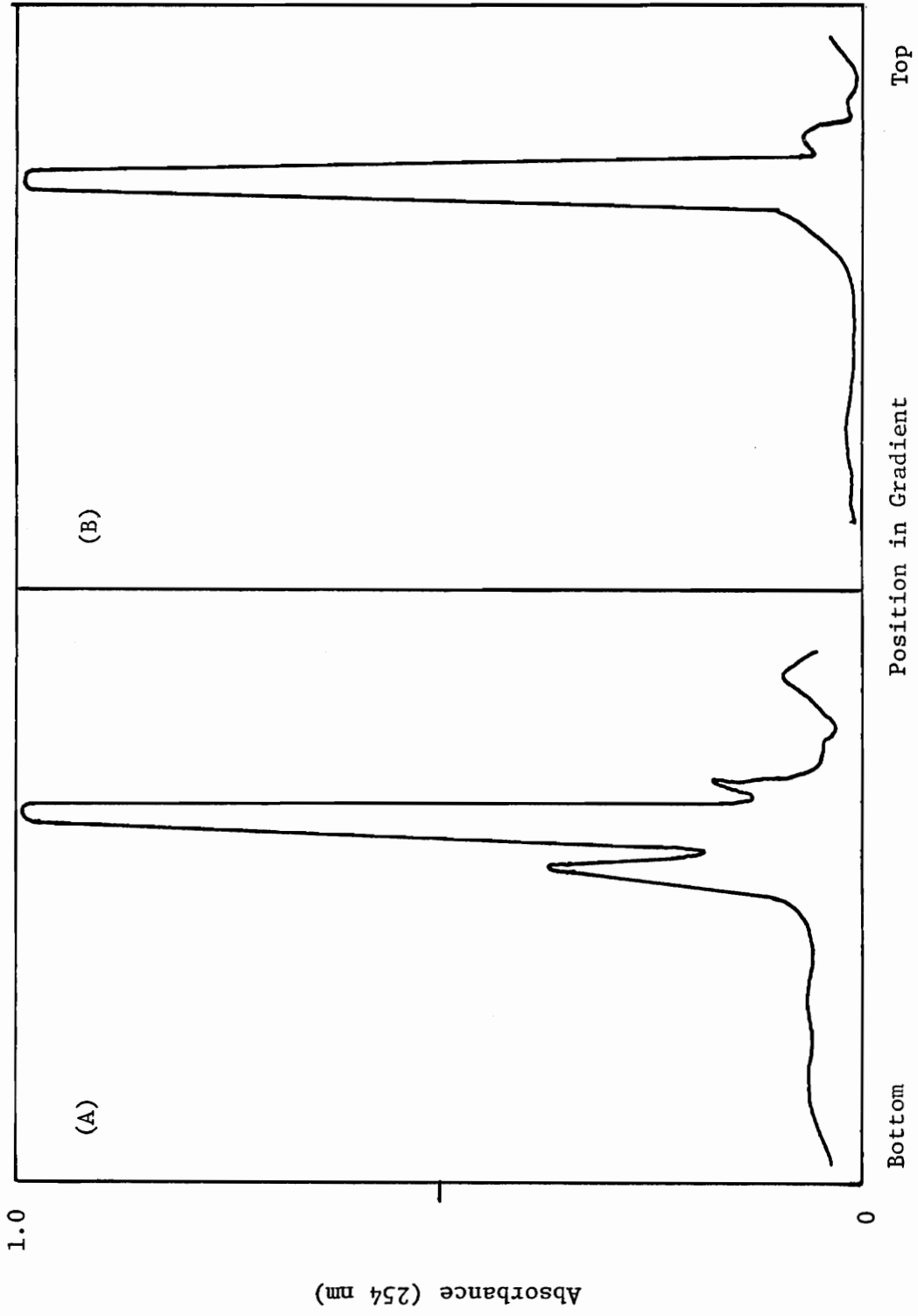
Table 8. Amount of polyuridylic acid - ribosome binding in the in vitro system using supernatant and ribosome fractions from the embryos of dry and from 90-day stratified sugar pine seeds.

Source of supernatant	Source of ribosomes	$\frac{\mu\text{g Poly U}}{0.505 \text{ mg ribosome}}$
Stratified	Stratified	$2.262 \pm 0.861^*$
Stratified	Dry	$3.086 \pm 1.075^*$
Dry	Stratified	$2.327 \pm 0.654^*$
Dry	Dry	2.817**

* Average value \pm standard deviation.

**Average of two values.

Figure 9. Profile of absorbance at 254 nm of the ribosomes from sugar pine embryos of A) 90-day stratified seeds, B) dry seeds, and C) 40-hour imbibed seeds. Ribosomes (5.0 absorbance units) were layered on a 10-40% sucrose density gradient and spun at 95,000 xg for 2 hours. The gradients were analyzed using an ISCO Gradient Analyzer.



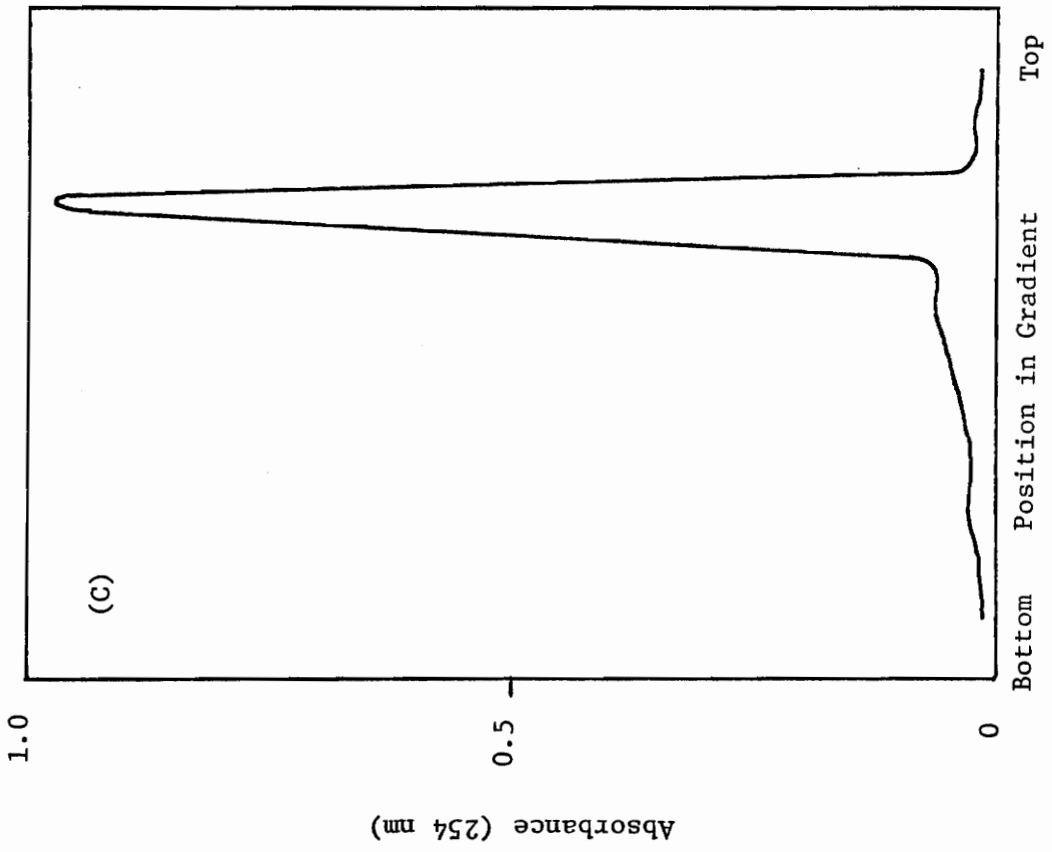
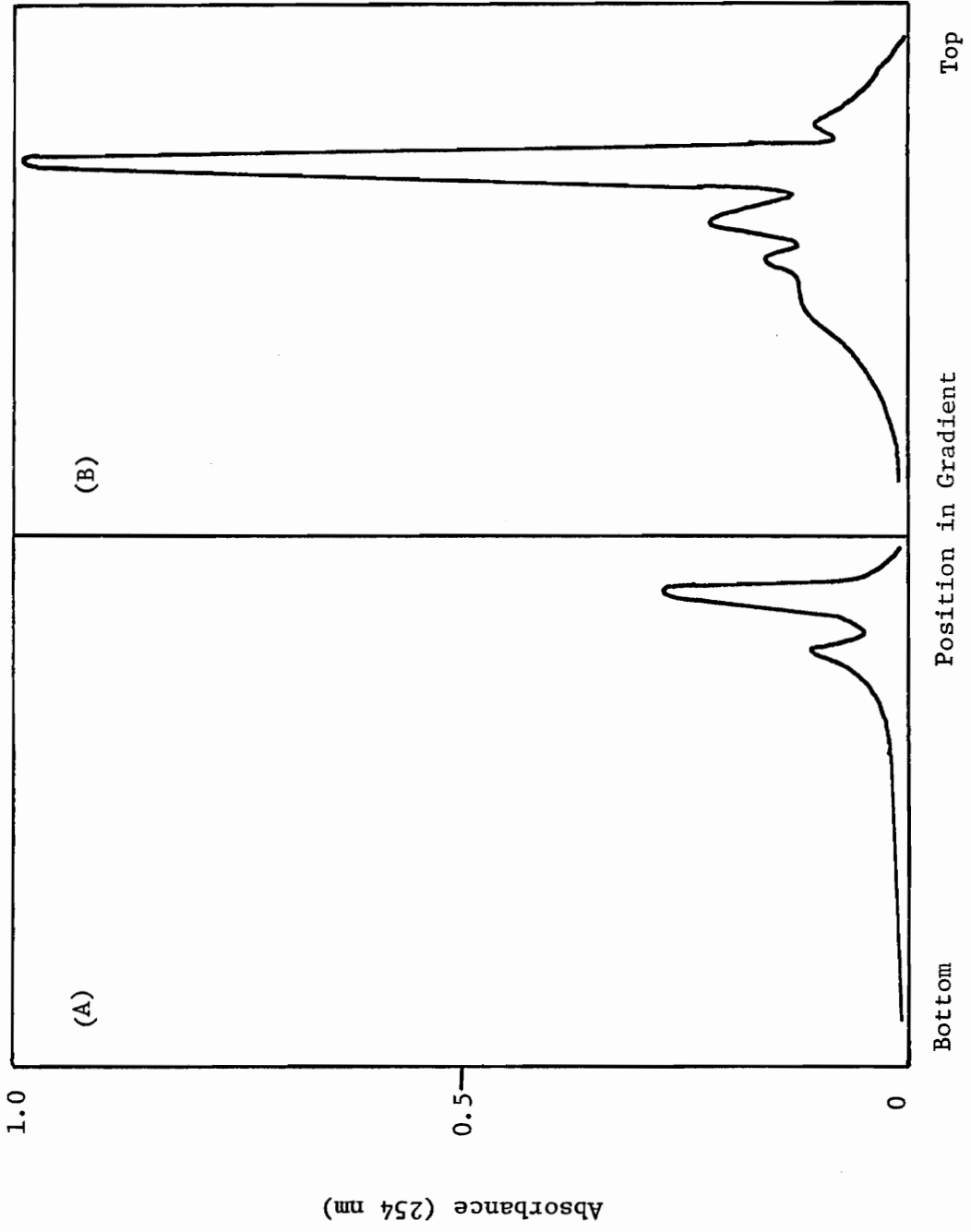


Figure 10. Profile of absorbance at 254 nm of the ribosomes from sugar pine embryos of A) 90-day stratified seeds and B) dry seeds, with 20 $\mu\text{g}/\text{ml}$ polyvinyl sulfate added. Ribosomes (5.0 absorbance units) were mixed with the polyvinyl sulfate and layered on 10-40% sucrose density gradients and spun in a Beckman SW-27 rotor at 95,000 $\times g$ for 2 hours. The gradients were analyzed using an ISCO Gradient Analyzer.



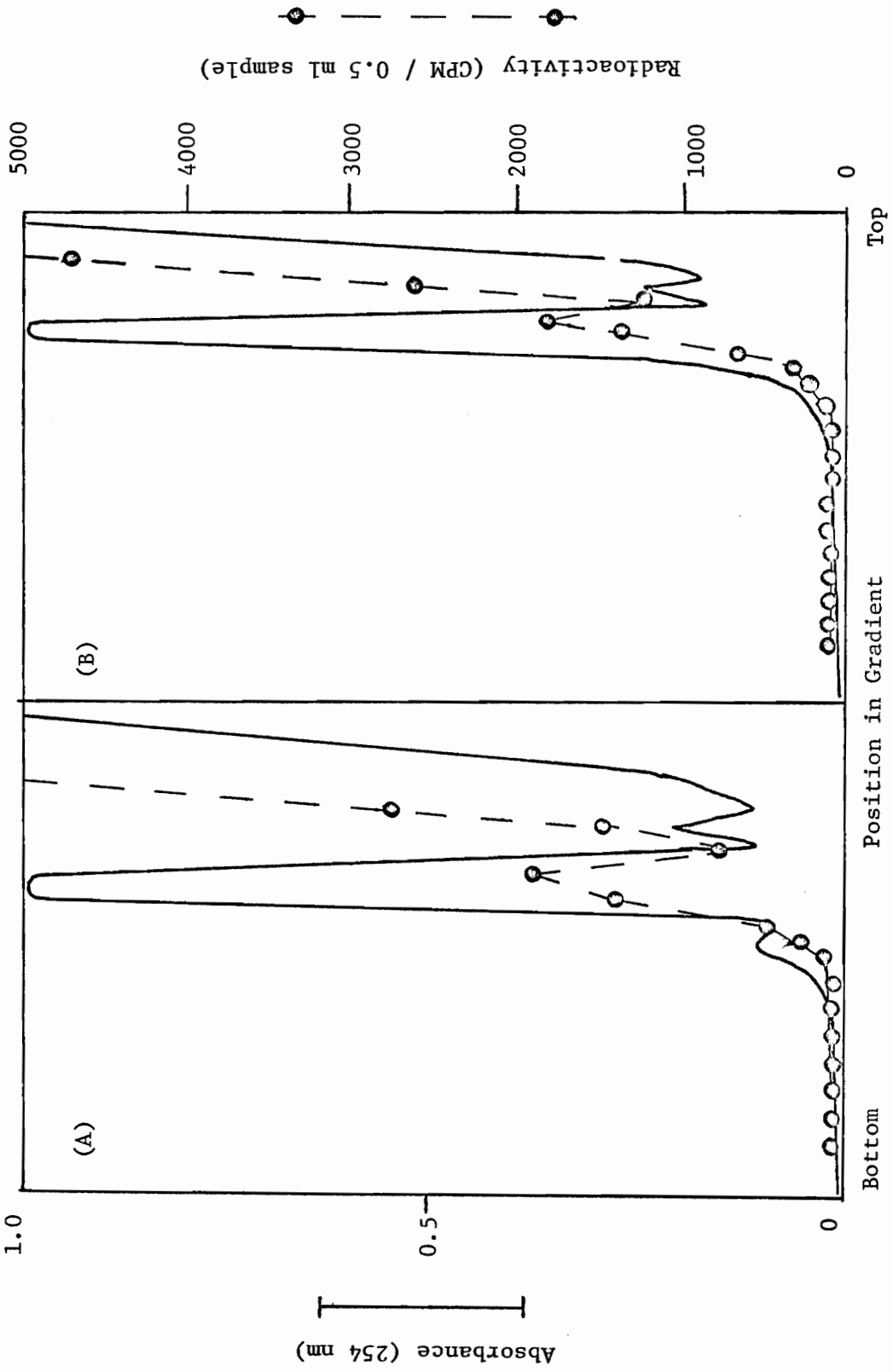
Sucrose density gradient profiles were also obtained for incubation mixtures using the four combinations of supernatant and ribosome fractions from dry and stratified embryos (see Figure 11). These profiles were similar to those of the gradients of resuspended ribosomes alone (see Figure 9). The largest amount of ^{14}C -phenylalanine attached to the ribosomes appeared under monomer peaks, with much less under the polymer peaks.

In order to determine whether the time of incubation or the addition of polyvinyl sulfate affected the ribosome profiles from the incubation mixtures, gradients were run with and without polyvinyl sulfate from mixtures incubated 0, 20, and 45 minutes. Time of incubation had no effect as ribosome profiles from the 0-time incubations were similar to those after 20 and 45 minutes incubation. The addition of polyvinyl sulfate to the incubation mixtures caused polymers to appear where they were not present in samples without polyvinyl sulfate, indicating that nucleases were present and active in ribosomal fractions as well as the supernatant fractions. The ^{14}C -phenylalanine associated with the ribosomes increased as the time of incubation increased but again was associated mainly with the monomer peak.

Profiles were next prepared for all four combinations of ribosomes and supernatant fractions from dry and stratified embryos (see Figure 12) using polyvinyl sulfate in each mixture as a nuclease inhibitor and incubating the samples for 60 minutes at 37°C in order to measure ^{14}C -phenylalanine associated with the ribosomes. The addition of polyvinyl sulfate resulted in polymer peaks on the gradients but most of the ^{14}C -phenylalanine was associated with the monomer peaks.

Figure 11. Profile of absorbance at 254 nm of the ribosomes and amount of radioactivity in corresponding samples of the sugar pine embryo incubation mixtures containing ^{14}C -labelled phenylalanine:

- A) Supernatant and ribosomes from embryos of 90-day stratified seeds;
- B) Supernatant from embryos of 90-day stratified seeds and ribosomes from embryos of dry seeds;
- C) Supernatant from embryos of dry seeds and ribosomes from embryos of 90-day stratified seeds;
- D) Supernatant and ribosomes from embryos of dry seeds.



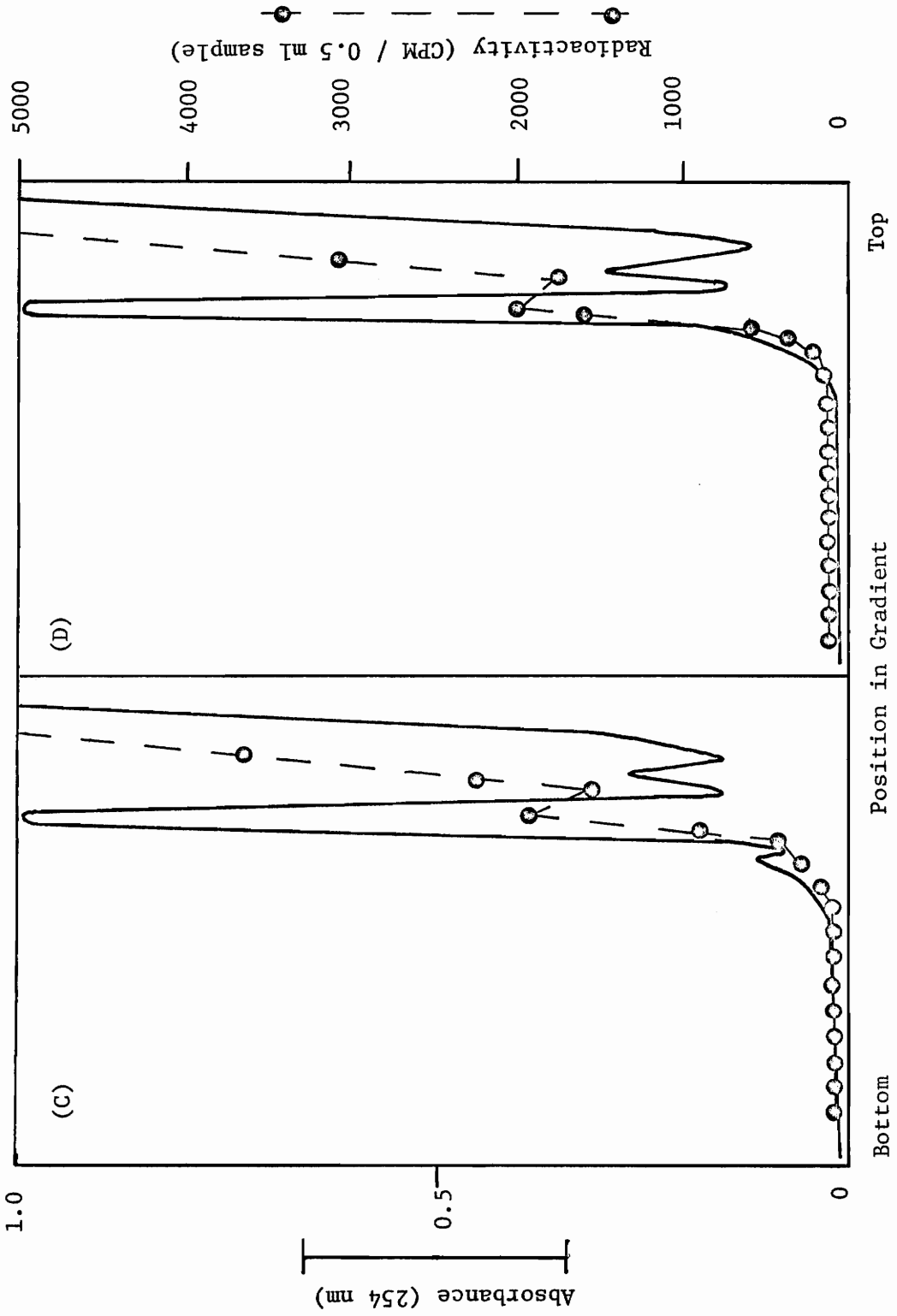
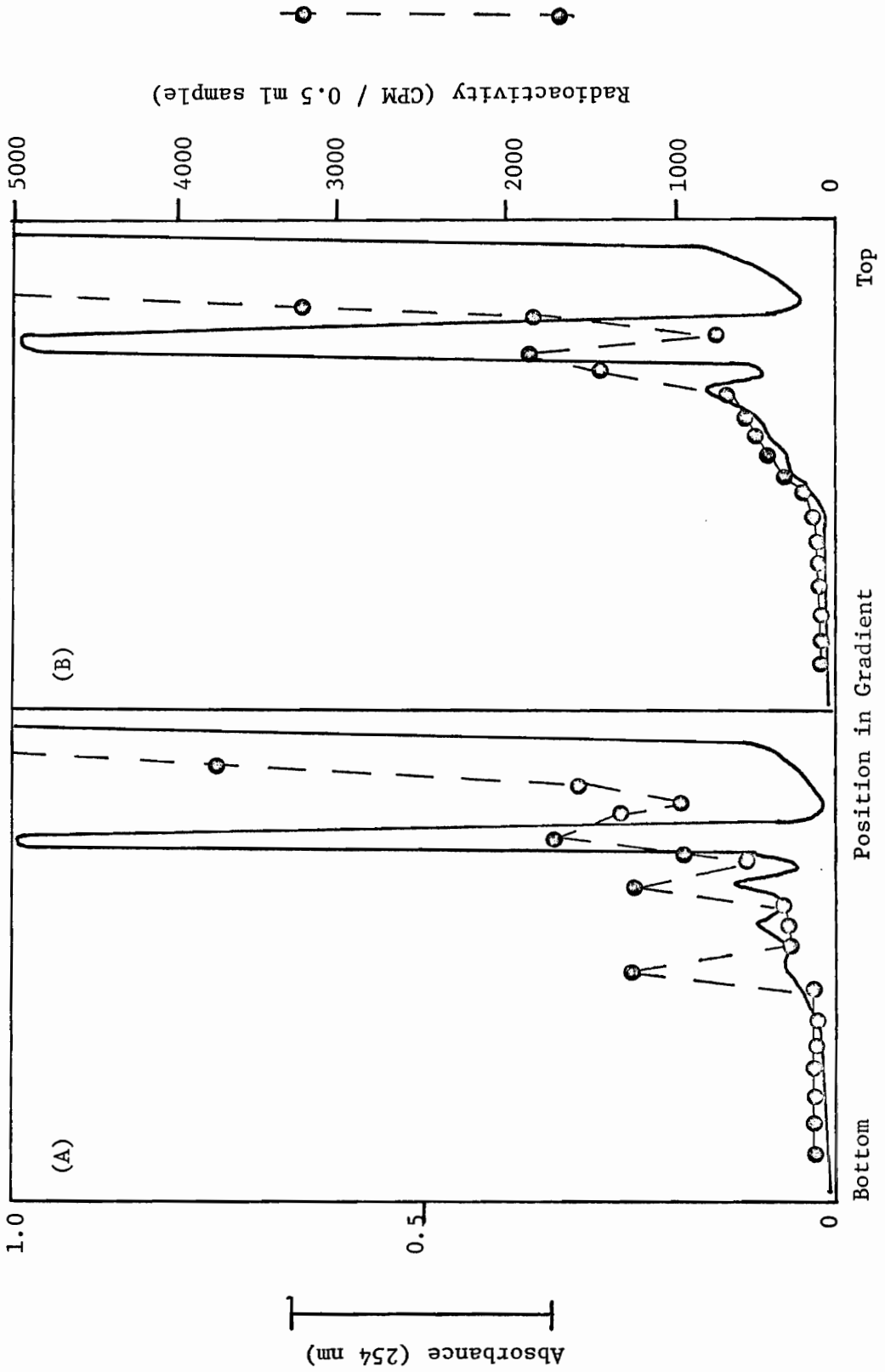
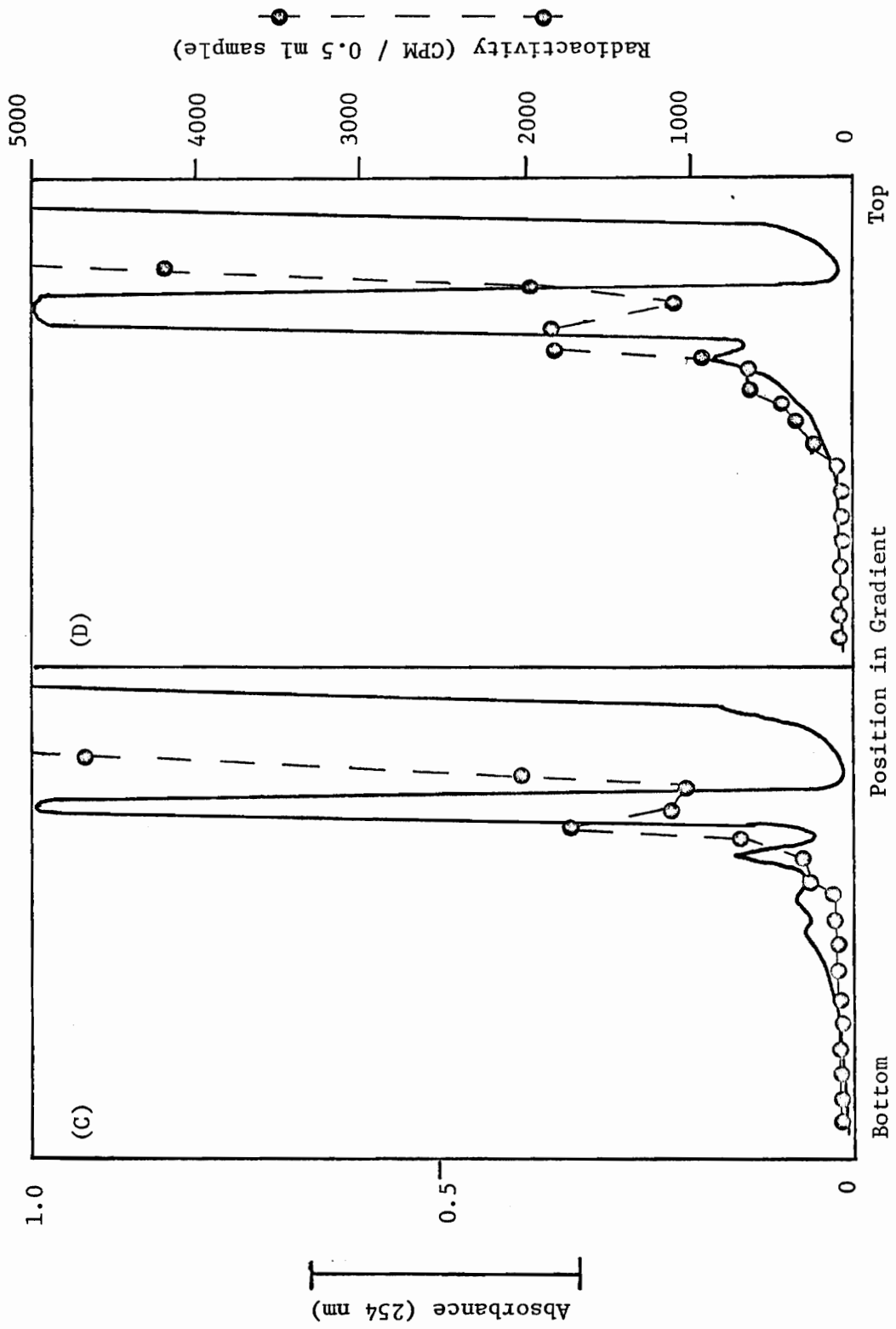


Figure 12. Profile of absorbance at 254 nm of the ribosomes and amount of radioactivity in corresponding samples of the incubation mixtures containing ^{14}C -phenylalanine, 20 $\mu\text{g/ml}$ polyvinyl sulfate, and supernatant and ribosomes from embryos of sugar pine seeds:

- A) Supernatant and ribosomes from embryos of 90-day stratified seeds;
- B) Supernatant from embryos of 90-day stratified seeds and ribosomes from embryos of dry seeds;
- C) Supernatant from embryos of dry seeds and ribosomes from embryos of 90-day stratified seeds;
- D) Supernatant and ribosomes from embryos of dry seeds.





DISCUSSION

Characteristics of the In Vitro Phenylalanine Incorporating System

There have been numerous reports of cell-free, in vitro amino acid incorporating systems isolated from higher plant tissues (cf. Mans, 1967a). In addition, many systems have been isolated from other types of eucaryotic and procaryotic cells. Mans (1967a) pointed out that these incorporating systems do not significantly differ and that this fact is consistent with the view that the mechanisms of protein synthesis are universal. However, Mans said that subtle features unique to a given species may exist and should not be overlooked.

The phenylalanine incorporating system for Pinus lambertiana seeds developed by Adams, Shih and Barnett (1970) and later used by Barnett, Adams and Ramsey (1974) involved precipitation at pH5 to purify the 105,000 xg supernatant fraction. However, pH5 precipitation yields a product that often contains substances not essential for incorporation and sometimes lack compounds that are essential. Furthermore, it is difficult to control precisely the pH during preparation. For example, Mans and Novelli (1961b) reported that their pH5 precipitate from maize varied in activity with minute changes in pH values and that the pH5 supernatant contained components necessary for leucine incorporation. Mans, Purcell and Novelli (1964) used DEAE-cellulose columns to remove an "inhibitory component" from dialyzed supernatant fractions of maize and found that the purified supernatant fraction contained transfer RNA and catalyzed the aminoacylation of tRNA and the transfer of the aminoacyl moiety from RNA to protein.

Travis, Lin, and Key (1972) reported two amino-acyl transfer factors required for peptide chain elongation in a polyuridylic acid- directed maize system associated with the supernatant fraction.

The present study used Sephadex G-25 gel filtration of the sugar pine embryo 105,000 xg supernatant fraction. The gel retains in the matrix all particles of molecular weight less than 5000 Daltons (Anon., 1973). Therefore free amino acids, which might dilute the isotope in the assay mixture, nucleotides, and other low molecular weight components are removed. The technique has recently been used in preparing amino acid incorporating systems from wheat (Allende and Bravo, 1966) and maize (Ramagopal and Hsiao, 1973) and appears to be a more efficient method of isolating the required supernatant factors than pH5 precipitation (Mans and Novelli, 1961b; Mans, et al., 1964).

Figure 2 and Table 2 indicate that although more supernatant protein is required using the ribosomes from embryos of dry seeds than with those from stratified seeds, the dry-seed ribosomes are more active in promoting phenylalanine incorporation. Sturani (1968) reported that the ribosomes from castor bean endosperm decreased in activity during ripening, were lowest when the seeds were dry, and increased in activity when germination commenced. It would appear that a reverse of this process is occurring in the sugar pine embryo during stratification, i.e., a degradation or partial deactivation of the ribosome is occurring, such as that reported for sugar pine female gametophyte ribosomes (Barnett, et al., 1974). It is possible that fewer ribosomes from the embryos of stratified seeds have active

sites available for binding of the protein synthesizing components (mRNA, tRNA, initiation, elongation and transfer factors, enzymes, etc.), therefore the activity is lowest and less supernatant is required in order to reach maximum incorporation activity. With the ribosomes from the embryos of dry seeds, where more may have active sites, more supernatant is required but also more phenylalanine is incorporated into polyphenylalanine. Further data are needed to confirm this theory.

The effect of stratification on the supernatant appears to be opposite of the effect on the ribosome. Less supernatant from embryos of stratified seeds is required with a particular ribosome type than supernatant from the embryos of dry seeds but this concentration yields greater incorporation activity. Although the ribosomes may be losing activity, the supernatant appears to be increasing the ability of the system to promote amino acid incorporation during stratification. This would explain the observation that the greatest amount of phenylalanine incorporated occurred in the system using supernatant from the embryos of stratified seeds and the ribosomes from embryos of dry seeds. Tome et al. (1972) reported that the supernatant fraction from 2-day germinated Pisum sativum seeds promoted amino acid incorporation while that from 6-day germinated seeds inhibited incorporation. They found that an inhibitor was present in the 6-day supernatant fraction and this affected the ability of the ribosomes to bind mRNA and tRNA. It is possible that the supernatant fraction from dry sugar pine embryos acts in a similar manner and that some inhibitor is removed during stratification. There is no direct evidence for such an inhibitor in

sugar pine embryos however.

The fact that increasing the ribosome concentration did not increase the amount of phenylalanine incorporated into hot TCA insoluble material is not contrary to this theory. The supernatant concentration studies were performed using 0.03 mg of ribosomal protein. When the ribosome concentration was increased, it is likely that the supernatant concentration became limiting.

Adams (1972) found that RNA synthesis increased after 3 days of germination at room temperature for stratified sugar pine seeds. Adams and Barnett (unpublished data) have also found evidence that protein synthesis in stratified sugar pine seeds increased after 3 days of germination. Therefore it seems possible that the ribosomes in 90-day stratified sugar pine seeds have low activity but this increases upon warming and the initiation of germination. This could be possibly related to the process of initiation of gibberellic acid synthesis following warming of stratified hazel seeds (Ross and Bradbeer, 1971) since gibberellic acid has been reported to increase protein synthesis (Chen and Osborne, 1970; Evins, 1971).

The studies to determine the optimum concentrations and conditions for the remaining components were run to compare the results with data from the pH5 study of Adams, et al. (1970) and also to determine if there were differences between the systems isolated from dry and stratified embryos, as was found in the supernatant concentration study.

Decrease in phenylalanine incorporation with increasing ribosome concentration has been previously reported by Allende and Bravo (1966) using a wheat embryo system, and by Perani, et al. (1968) using

ribosomes from human cells. However many other researchers have reported a continuing increase in incorporation with increasing ribosome concentration (Kobayashi and Halvorson, 1966 with Bacillus cereus; Eisenstadt and Brawermann, 1964 with Euglena gracillis; Parisi and Ciferri, 1966, with castor bean seedlings; Adams, et al., 1970 with sugar pine seeds). The decrease in phenylalanine incorporation is possibly due to corresponding increases in nucleases or an increased demand for magnesium (Allende and Bravo, 1966). Adams, et al. (1970) reported a critical magnesium concentration of 7.5mM for the sugar pine seed incorporating system, and Shih, et al. (1973) reported that lowering the magnesium concentration in this system caused dissociation of the ribosomes into their subunits. It is possible that increasing the ribosome concentration while maintaining a constant magnesium concentration would have similar effects. Spirin and Gavrilova (1971) suggested that the magnesium ions participated in protein synthesis by binding the carboxyl of an amino acid of a ribosomal protein to a phosphate group of a messenger RNA nucleotide. Therefore, a lower ribosome-magnesium ratio would result in less binding and less phenylalanine incorporation.

The development of a maximum in incorporation activity with increasing polyuridylic acid concentration is similar to that reported by Adams, et al. (1970). However, other researchers have reported a leveling off of incorporation activity (App and Gerosa, 1966 using rice embryos; Mao, 1967 using Staphylococcus aurea; Perani, et al., 1968 using human cells; Tucker and Zalik, 1973 using

wheat seedlings) or a continuing increase in incorporation with increasing polyuridylic acid concentrations (Kobayashi and Halvorsan, 1966 using Bacillus cereus). The decrease in activity using this sugar pine system cannot be presently explained.

Significant differences in optimum ATP concentrations occurred between this system and that of Adams, et al. (1970). Optimum ATP concentration in their preparations was 0.7mM while in the present study it was 1.6mM. Also they found a critical magnesium-ATP ratio of about 10:1 while in the present study it was 4.7:1. There have been several other reports of a critical magnesium-ATP ratio of 10:1 (Eisenstadt and Brawermann, 1964; Mao, 1967; Kobayashi and Halvorson, 1966). It is possible that the supernatant purification procedures of the present study resulted in the incorporating system having a greater energy requirement than in the sugar pine seed system characterized by Adams, et al. (1970).

Variation in time and temperature of incubation yielded data which were slightly different from those of Adams, et al. (1970). It was found that maximum phenylalanine incorporation occurred with 60 minutes of incubation and at 37°C. The 60 minutes is greater than the 45 minutes for the pH5 system. Also the 37°C is a slightly lower incubation temperature than the 40°C for the pH5 system, however this difference is not considered significant. The 10 minute initial lag before phenylalanine began to be incorporated possibly resulted from the time taken to warm the samples to 37°C.

Overall, this phenylalanine incorporating system from the embryos of sugar pine seeds is similar in characteristics to that reported by Adams, et al. (1970). Both systems require supernatant, ribosomes, and an ATP-energy generating system plus GTP, and polyuridylic acid, and both were inhibited by RNase (see Table 3). These characteristics are also similar to other systems (Allende and Bravo, 1966; Perani, et al. 1966; Mehta, et al., 1969; also cf. Mans, 1967a). The effect of the addition of the hydrolytic enzymes would be expected since the system is dependent on polyuridylic acid and its destruction by RNase would reduce activity. Also protease could destroy necessary enzymes and possibly the active ribosome structure. The only major difference between this system and that of Adams, et al. (1970) appears to be the differing optimum supernatant protein concentrations associated with the use of gel-filtration rather than isoelectric precipitation of the supernatant. In the earlier work, optimum supernatant protein concentrations were determined using only dry seeds. It is possible that differences similar to those reported here may occur between dry and stratified seeds using the pH5 system. However, comparison of the data suggests this would not explain the variation in results encountered using the two systems. Figure 2 shows that 0.25 mg of gel-filtered supernatant protein (the amount of pH5 protein used by Adams' group) caused no incorporation of phenylalanine with the dry embryo system and at the supernatant protein concentration yielding maximum activity with the dry embryos (1.0-1.25 mg/0.5 ml), very little phenylalanine was incorporated by the stratified embryo system. Mans

and Novelli (1961b) suggested that pH5 precipitation may not remove all active protein synthesizing components. Further research is needed to determine differences in components of pH5 and gel-filtration preparations. However it is clear that differences in protein synthesizing abilities which are associated with supernatant purification methods do occur and consideration of this fact is mandatory when applying data from an in vitro system to in vivo occurrences.

The fact that preparations from embryos of dry seeds are capable of supporting protein synthesis with polyuridylic acid supports the idea that all components involved in the translational stage of protein synthesis are present in dry seeds, with the possible exception of mRNA. This agrees with the results of Marcus and Feeley (1965) and Allende and Bravo (1966) for wheat embryo, Merlo, et al. (1972) for spores of Rhizopus stolonifer and Brambl and Van Etten (1970) for spores of Botryodiplodia theobromae, but contrasts with those of Sturani (1968) for castor bean endosperm where the dry seed preparations were inactive even in the presence of polyuridylic acid. The latter study indicated that the ribosomes were themselves inactive, i.e. the low protein synthesizing ability was not due to limiting amounts of mRNA. Therefore it appears that the sugar pine embryo ribosomes from dry seeds could be active in vivo if there is sufficient mRNA and water present.

The loss of ability of the sugar pine embryo ribosomes to support phenylalanine incorporation during stratification is similar to loss in activity of castor bean endosperm ribosomes during seed ripening

(Sturani, 1968). These phenomena may involve changes at a functional level such as alterations in rRNA and r-protein arrangements, while the structure of the ribosome remains intact.

Data from the present study are insufficient to explain the increase in ability of the supernatant fraction to promote protein synthesis as dormancy is broken. Tao and Khan (1974) found that activities of amino acyl-tRNA synthetases in pear embryos increased drastically when the dormant embryos received chilling upon stratification. Also Merlo, et al. (1972) reported both qualitative and quantitative changes in the transfer RNA's during initiation of fungal spore germination. Similar phenomena may be occurring in sugar pine embryos during stratification.

Activity of Hydrolytic Enzymes

Ribonuclease activity in the supernatant fractions (Table 4) increased significantly with purification while total protein decreased (data not included). Since the values of RNase activity were based on total protein, it appears that the RNase is soluble and not sedimented at 105,000 xg or removed by gel-filtration. Therefore the RNase activity is present in the incubation mixtures, added with the supernatant fraction, and could affect activity of the incorporating system. In fact, Table 4 shows that the RNase activity was significantly (analysis of variance) higher in the gel-filtered fraction from the embryos of stratified seeds than that from dry seeds and this could be correlated with the decrease in ribosome activity during stratification.

It would have been desirable to check RNase activity levels in the washed and unwashed ribosome fractions. Dove (1973) reported, in a review of ribonuclease in vascular plants, that 75% of the total RNase activity of pea root preparations and 80% of wheat aleurone homogenates is soluble. However, RNases are also found in the microsomal fractions of maize root homogenates, maize seedlings, wheat germ, young wheat roots, wheat seedlings, pea seeds, and tobacco leaves. Since polyvinyl sulfate, a RNase inhibitor, increased the proportion of sugar pine polyribosomes on sucrose density gradients, it would appear that ribonucleases are also associated with this microsomal or ribosomal fraction from sugar pine seeds.

Significant differences in protease activity between the dry and stratified embryo fractions never occurred (Table 5) but purification and separation of the fractions by centrifugation caused large changes. Protease was removed from the supernatant by centrifugation at 105,000 xg and therefore appears to be associated with the ribosomes or some other particles. Differences in protease activities apparently are not responsible for the differing protein synthesizing capacities of supernatant fractions from dry and stratified seeds. More data are needed for comparison of ribosome fractions.

That the addition of protease following normal incubation of the samples resulted in only a 20-25% decrease in hot TCA insoluble material (Table 6) was unexpected. These findings could be the result of 1) the labelled material counted was not protein, which is unlikely since the label was attached to an amino acid and hot TCA was used to solubilize other materials, e.g. nucleotides (cf. Mans and Novelli,

1961a), or 2) the protease was not very effective with the polyphenylalanine. The latter seems the most likely despite the fact that the enzyme was supposedly "non-specific".

Effects of Pine Seed Imbibition
on Protein Synthesizing Activity

Imbibition was accompanied by an increase in protein synthesizing activity of the embryo ribosomes as compared with those from dry seeds. Also the supernatant fraction from the embryos of imbibed seeds was more active than that from the embryos of dry seeds but not as active as that from the embryos of stratified seeds. These data, along with the data in Figure 8 which indicate that similar concentrations of supernatant protein from dry and imbibed seeds yield maximum incorporation, suggest that imbibition aids in activation of some process associated with protein synthesis. Ribosomes from imbibed embryos were generally more active than ribosomes from dry seed embryos. Activity of the supernatant fraction from imbibed embryos was not as high as that from stratified embryos, however, and further changes may occur during stratification. The large standard deviations associated with experiments involving imbibed seeds should be noted when assessing the significance of these differences however.

Marcus and Feeley (1965) reported that imbibition increased the activity of peanut cotyledon and wheat embryo ribosomes to a maximum after 24-40 hours and 1 - 3 1/2 hours, respectively. However these experiments used native messenger RNA and the seeds were not dormant. Also ribosomes from dry seeds in their study showed very low incorporation activity whereas those from the sugar pine seeds were

active. It is possible that imbibition is accompanied by the association of some factor(s) in the ribosome and/or supernatant fractions that accounts for the increase in activity of the components from embryos of imbibed seeds. Further research is needed in this area.

Polyuridylic Acid-Ribosome Binding and Sucrose Density Gradient Profiles

The data from Table 8, if accepted as correct, indicate that the ribosomes from dry embryos bind more polyuridylic acid than do those from stratified embryos. Since the same amount of ribosomes were used in each analysis (5 absorbance units), this supports the theory that the dry embryo ribosomes are more active because they are more structurally and functionally intact than the stratified embryo ribosomes. Sucrose density gradient profiles (Figure 12) of the incubation mixtures indicate however that both dry and stratified embryo ribosomes form polysomes during the in vitro assay. Figure 10 also shows that resuspended ribosomes from the embryos of both dry and stratified seeds have relatively large numbers of polysomes in vivo.

Data from Figure 12 also indicate that the ^{14}C -labelled phenylalanine (tRNA) is mostly bound to monoribosomes with smaller amounts bound to dimers and trimers. This is not unique since Mao (1967) found the same occurrence with extracts from Staphylococcus aureus and suggested that this was due to a high ratio of poly-U to ribosomes which was not favorable to polyribosome formation. Takanami and Okamoto (1963) had earlier reported that the poly U-ribosome ratio was critical to polysome formation. However, Marcus and Feeley (1965) reported that the only ribosome fractions capable of supporting

protein synthesis in a wheat embryo system, after separation on a sucrose density gradient, were those ribosome that sedimented fastest, i.e. polyribosomes. They also reported that with long incubation times, when incorporation assays were placed on sucrose density gradients, some labelling of the monoribosome peak was noticed, possibly due to polysome breakdown.

Mans (1967b) reported that 90% of the newly synthesized polypeptides were attached to ribosomes in a poly U-directed system. If the same is true with this sugar pine embryo system, the results indicate that protein synthesis is associated to a great extent with monoribosomes.

Protein Synthesis during Stratification of Sugar Pine Seeds

The fact that the ribosomes are more active in dry seed embryos than stratified seed embryos suggests either a structural or functional breakdown during stratification. It is possible that ribosome synthesis is active during maturation of the seed but ceases when the seed enters dormancy. As the moisture content of the seed decreases, so does the activity of degrading enzymes and processes, thus the ribosomes from the embryos of dry seeds remain in their previous active state. Once imbibition occurs, at the beginning of stratification, these ribosomes are still capable of supporting protein synthesis but the degrading enzymes and/or processes are once again active and the ribosomes are slowly rendered non-functional. This would coincide with a slow or non-existent synthesis of new, functional ribosomes. This is only a hypothesis and further research is needed to better

understand these changes in ribosome activity.

The change in activity of the supernatant fraction during stratification is also apparent but very possibly only a result of seed imbibition (see Table 7). It is possible that imbibition causes the activation of amino acyl-transfer RNA synthetases or some other factor associated with the supernatant fraction and this activation is very rapid (less than 40 hours). Therefore it appears that changes occurring in the embryos during stratification of the sugar pine seeds are related to the ribosome fraction and not the supernatant fraction, although the possibility exists that some factor in the supernatant fraction that affects the activity of the ribosomes changes during stratification.

The mechanism of ribosome deactivation has not yet become clear. One possibility is that soluble ribonuclease activity increases in the cells of the pine embryo during stratification and thereby causes ribosome degradation. Table 5 shows that there is a significantly higher amount of RNase activity in the supernatant from the embryos of stratified seeds than from dry seeds. The result of this increase in RNase activity could be fewer functional ribosomes. The dry embryo ribosomes resulted in greater phenylalanine incorporation than did the stratified embryo ribosomes. This could be related to the RNase activity levels. If data from the poly U-ribosome binding assay can be accepted (see Table 8), it would appear that the dry embryo ribosomes bind more poly U than do those from 90 day stratified embryos, further supporting this theory.

It is difficult to relate the present data with the breaking of dormancy in pine seeds at the present time because physiological studies for this genus are rare in the literature. No references to change in abscissic acid or gibberellic acid concentrations could be located. However, if hormones act in controlling dormancy in pine seeds with a chilling requirement, it would be of great interest to study the effects of application of each to dormant and stratified seeds, to determine endogenous levels, and to note their effects, if any, on protein synthesis. Since each have been implicated as a possible translation regulator (cf. Ihle and Dure, 1972; Bonnafous, et al., 1973; Chin and Osborne, 1970; Evins, 1971), they may be involved, directly or indirectly, with the control of protein synthesis and may aid in explaining the phenomena noted in this research.

SUMMARY AND CONCLUSIONS

The in vitro protein synthesizing system using components isolated from embryos of sugar pine seeds and using Sephadex G-25 gel-filtration to purify the 105,000 xg supernatant fraction indicated that there was a difference in resulting data from this system and a similar system from the same seeds but using pH5 precipitation to purify the 105,000 xg supernatant fraction. There were also indications that the ribosomes from embryos of sugar pine seeds decrease in activity during stratification while the gel-filtered supernatant fraction increases in its ability to promote protein synthesis. The latter change is possibly a result of seed imbibition, however.

Except for the supernatant concentration, the component concentrations and environmental conditions yielding maximum phenylalanine incorporation were similar for all systems using the four possible combinations of supernatant and ribosomes from dry and stratified embryos. The optimal concentrations in the 0.5 ml samples were: 0.3-0.6 mg of ribosomal protein, 0.0375-0.050 mg of polyuridylic acid, and 0.075 ml of the ATP generating system mixture. Supernatant protein concentration yielding optimum phenylalanine incorporation varied with the source of supernatant and ribosome fraction, being greatest for dry embryo ribosomes and dry embryo supernatant. Optimum incubation procedures were 37°C for 60 minutes. RNase and protease inhibited the incorporation of phenylalanine.

Ribonuclease activity increased in the supernatant with purification. The activity was also significantly higher in the Sephadex

G-25 gel-filtered fraction from stratified embryos than from dry embryos. Protease activity decreased in the supernatant fraction during purification but was high in the ribosome fraction. There was no significant difference between protease activities in the supernatant of dry and stratified embryos at any stage of purification.

Addition of 50 μg of protease to incubation mixtures caused a 20-25% decrease in hot TCA insoluble material but increasing amounts of this enzyme caused no significant increase in the percentage decrease.

The ribosomes from dry embryos appeared to bind more polyuridylic acid than did those from stratified seeds, however this difference is questionable due to large standard deviations of the values.

Sucrose density gradient profiles showed that the resuspended ribosomes contained small polyribosomes, however the majority of the ribosomes existed as monoribosomes. Profiles of the incubation mixtures yielded similar results. Carbon-14 labelled phenylalanine showed that the majority of the label was associated with monoribosomes while a smaller percentage was associated with the polyribosomes.

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VITA

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Carl George Dury, II

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EFFECTS OF STRATIFICATION ON IN VITRO PROTEIN SYNTHESIS
USING COMPONENTS FROM THE EMBRYOS OF PINUS LAMBERTIANA DOUGL.

by

Carl George Dury II

(ABSTRACT)

An in vitro protein synthesizing system using Sephadex G-25 gel filtration for supernatant purification was used to determine the ability of extracts from embryos of dormant and stratified sugar pine seeds to promote protein synthesis. It was found that the ribosomes from the embryos of dormant seeds were more active than those from stratified seeds but they also required a greater quantity of supernatant protein to produce this activity. The supernatant fraction from stratified embryos was more active than that from dormant embryos but there were indications that this increase was due to seed imbibition.

Concentrations of other essential components of the system were the same for both dormant and stratified embryo systems. In each 0.5 ml sample, maximum incorporation occurred using 0.03-0.06 mg ribosomal protein, 0.795 μ moles ATP, 1.8 μ moles PEP, 13.2 μ gms pyruvate kinase, 0.090 μ moles GTP, and 0.0375 mg polyuridylic acid. Optimum incubation conditions were at 37°C for 60 minutes. Ribonuclease and protease inhibited phenylalanine incorporation.

Ribonuclease activity in the supernatant fraction increased with purification and was significantly higher in the dormant embryos than in the stratified. Protease activity decreased with purification of

the supernatant and there was no significant difference between activity in the dormant and stratified embryo supernatant fractions. Protease activity was high in the ribosomal fraction.

Ribosomes from dormant embryos appeared to bind more polyuridylic acid than did those from stratified embryos. Resedimented ribosomes consisted primarily of monoribosomes but some polyribosomes were present in both dormant and stratified embryos. Analysis of incubation mixtures produced similar results. The majority of the labelled polyphenylalanine was associated with the monoribosomes.