EMBRYONIC CONTROL OF ISOCITRATE LYASE ACTIVITY IN THE MEGAGAMETOPHYTE OF PONDEROSA PINE SEEDS

by

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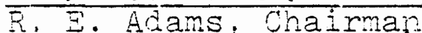
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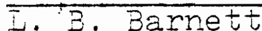
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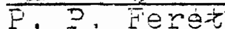
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INTRODUCTION

Exogenously applied gibberellins stimulate the germination of dormant seeds of some species (Simpson, 1965; Frankland and Wareing, 1966; Bradbeer and Pinfield, 1967) and induce the production of diverse enzymes in the seeds of a variety of plant species (Jacobsen and Varner, 1967; Jarvis, *et al.*, 1968; Pinfield, 1968b; Bennett and Chrispeels, 1972; Simmonds and Simpson, 1971; Locker and Ilan, 1975; Doig, *et al.*, 1975). Changes in levels of endogenous gibberellins have been correlated with release from dormancy and enzyme synthesis (Yomo, 1960; Frankland and Wareing, 1962; Simpson, 1966). Several experiments have implicated the embryo as the source for active gibberellins (Paleg, 1960; Doig, *et al.*, 1975). Researchers have postulated that gibberellin regulates metabolism, and seed germination of some plant species. The supporting evidence has been largely circumstantial. No rigorous proof for the occurrence of endogenous gibberellin control of metabolism has been presented.

The metabolic role of gibberellins and other growth regulators has been extensively studied in angiosperms. The effects of plant hormones on growth, development and sexual differentiation of a number of conifer species have also been studied (Pharis and Kuo, 1976). Unfortunately, there is a paucity of information concerning the effects of growth regulators on metabolism in gymnosperm seeds.

Consequently any research on the metabolic effects of growth regulators in gymnosperms would substantially reduce our ignorance and provide a foothold for future research.

Bilderback (1974) reported evidence which suggested that an activator diffused from the embryos of germinating ponderosa pine seeds (Pinus ponderosa Laws. var. ponderosa) and stimulated the synthesis of isocitrate lyase (threo-L_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) in the megagametophyte. He showed reduced enzyme activity in the absence of the embryo, and stimulation of activity in the presence of an embryo diffusate. However, he was unable to show stimulation by exogenous growth regulators. Gibberellic acid (GA_3), benzyladenine (BA) and abscisic acid (ABA) caused reduced enzyme activity.

The current study was initially planned to identify the activator reported by Bilderback (1974). It was necessary to repeat Bilderback's work to determine if the activator could be detected and to measure success of the activator extraction from the embryo. Some of the results of the repeated experiments conflicted with the results of Bilderback's experiments. Consequently the initial experiments designed to identify the activator were abandoned in favor of experiments formulated to clarify the differences between Bilderback's work and the work presented in the current study.

The research encompassed by this thesis partially refutes Bilderback's results by showing inhibition of enzyme activity to be associated with the presence of the embryo, and by casting doubt on the purported stimulating qualities of the embryo diffusate. Work designed to explain the discrepancies between these two studies is also included.

LITERATURE REVIEW

Plant Growth Regulators and the Embryonic Axis: Their Affect on Enzyme Activity

The activities of many enzymes increase substantially during the process of germination. The embryo exerts an active role in the control of the development of enzyme activity in seed storage tissue of some species (Paleg, 1960; Doig, *et al.*, 1975). Some enzymes are controlled by a chemical signal from the embryo in seeds where endosperm or the megagametophyte is the main storage tissue. In seeds with cotyledons functioning as the primary nutritive tissue, the chemical signal originates specifically in the axial tissue of the embryo.

Embryo control has been demonstrated for proteolytic activity in squash (*Cucurbita maxima* D.) cotyledons (Penner and Ashton, 1966) and bean (*Phaseolus vulgaris*) cotyledons (Gepstain and Ilan, 1970). Likewise, isocitrate lyase activity is dependent on the embryonic axis in squash and peanut (*Arachis hypogaea*) cotyledons (Penner and Ashton, 1967), and it is subject to embryonic control in wheat (*Triticum vulgare*) aleurone (Doig, *et al.*, 1975). An analogous situation occurs in pea (*Pisum sativum* L.) cotyledons where amylase (Locker and Ilan, 1975) and protease (Yomo and Varner, 1973; Chin, *et al.*, 1972) levels were lower in the absence of axial tissue.

Cytokinins were shown to be effective substitutes for the embryonic axis in stimulating the alpha amylase and protease activity mentioned in the previous paragraph. Enzymes in the seeds of other plants appear to be induced by gibberellins (GA) which can successfully supplant control by the embryo. Classical evidence for this effect was demonstrated using barley (Hordeum vulgare). Kirsop and Pollock (1958), showed that a diffusable substance which moves from the embryo into the endosperm of germinating barley caused the synthesis of hydrolytic enzymes in the aleurone. Hayashi (1940) and Paleg (1960) demonstrated that the hydrolytic enzymes are not produced in embryoless seeds except when first treated with gibberellin. Endogenous gibberellin-like substances were shown by Yomo (1960) and Radley (1959) to be released by germinating barley seed. Subsequently, Yomo and Iinuma (1966) provided evidence indicating biosynthesis of gibberellin-like substances by the germinating embryo. Since then, the pathway by which GA induces substrate mobilization has been outlined. Gibberellin produced in the embryonic axis is transported to the aleurone during the initial hours of germination (MacLeod, et al., 1966; MacLeod and Palmer, 1967; 1969; Radley, 1967; 1969). In the aleurone, gibberellin stimulates the synthesis and release (Jones, 1971) of hydrolytic enzymes which diffuse into the endosperm where stored substrates are subsequently hydrolysed (Kirsop and Pollock, 1958); Paleg, et al., 1960).

Induction of enzyme synthesis does not necessarily precede germination (as measured by radicle protrusion). Using dormant and nondormant oats, Avena fatua and Avena sativa respectively, GA was shown to induce embryo germination prior to stimulating the formation of hydrolases in the aleurone (Drennan and Berrie, 1962; Chen and Chang, 1972). Results compiled by Drennan and Berrie (1962) indicate that both species contain amylases. They showed that nondormant seeds germinated after four days when exposed to suitable conditions; however, these seeds did not produce additional amylase activity until after the sixth day. In addition, Chen and Chang (1972) presented evidence that GA_3 counteracted dormancy of Avena fatua two days after treatment. Amylase could not be detected two days after treatment and showed no activity until four days after treatment. Furthermore, they established in other time course studies that Himalaya barley (Hordeum vulgare L.) began to germinate after seven hours of soaking, yet showed no increase in amylase activity until 16 hours had elapsed.

Simpson (1965) reported that excised embryos from nondormant wild oats could be cultured on sucrose/amino acid medium. However, he found that GA_3 was an essential additive to the medium for the germination of dormant embryos. Apparently GA plays a role in the germination of dormant seeds that is distinct from its function of substrate mobilization.

Clearly GA exerts an effect on the embryo as well as the aleurone. This does not mean that amylase is not required for the initiation of germination. Investigators have established that residual amounts of GA-independent amylases are present prior to germination (Chen and Chang, 1972). These may be present in sufficient quantities to allow initiation of radicle protrusion. Large quantities are probably not necessary until the embryo has emerged and is ready for rapid growth.

Seeds of many woody plants are dormant and require low temperature stratification before they will germinate. Cold stratification can be substituted by GA treatment to promote germination of some species. Frankland and Wareing (1966) used gibberellic acid to stimulate the germination of dormant hazel (Corylus avellana L.) and beech (Fagus sylvatica L.) seed. Also detected were endogenous gibberellins in stratified hazel seed that were not found in unstratified seed (Frankland and Wareing, 1962). Bradbeer (1968) supported these data, but added that the chilling prepared the seed for gibberellin synthesis which subsequently occurred at temperatures suitable for germination.

Acetate metabolism occupies a central position in the metabolism of fat-storing seed such as hazel seed. Studies utilizing (2 - ^{14}C) acetate have indicated a wide range of metabolic activity in dormant seeds. This work showed that imbibed but dormant cotyledons have an active TCA cycle as

well as systems for the synthesis of lipid, sucrose and other compounds (Bradbeer and Colman, 1967). Gibberellic acid was shown to bring about changes in fat metabolism of dormant hazel seed cotyledons. Pretreatment with gibberellin reduced incorporation of (2 - ^{14}C) acetate into lipid reserves (Bradbeer and Pinfield, 1967) and caused the accumulation of radioactivity in sucrose (Pinfield, 1968a). Simultaneously, increased activity of isocitrate lyase was observed (Pinfield, 1968b) while TCA activity appeared to remain constant (Bradbeer and Pinfield, 1967). These data indicate a change from the predominantly lipid synthesizing metabolism of maturing seed to the actively lipid utilizing metabolism of the germinating seed. Bradbeer and Pinfield (1967) pointed out the similarities between this metabolic shift and the mobilization of substrates in barley aleurone by stimulation of alpha-amylase synthesis. They deduced that the action of gibberellin may initiate a shift from catabolic processes to anabolic metabolism by inducing the synthesis of a series of enzymes required for beta oxidation of fatty acids to acetyl CoA, conversion of acetyl CoA to malate in the glyoxylate cycle, followed by reversal of glycolysis.

Control of Isocitrate Lyase Synthesis in Seeds

Isocitrate lyase (L_s-isocitrate glyoxylate-lyase, EC 4.1.3.1), localized in the glyoxysome, is active in the

glyoxylate cycle which brings about the synthesis of isocitrate from glyoxylate and succinate (Carpenter and Beevers, 1958). Its role in the conversion of fats to carbohydrates in the storage tissues of seeds has been well documented (Carpenter and Beevers, 1958; Abraham and Velasco, 1960; Abrahamsen and Sudra, 1966; Bradbeer and Colman, 1967; Bradbeer and Pinfield, 1967; Pinfield, 1968b). In higher plants isocitrate lyase is species specific and activity is limited to those tissues capable of active fat catabolism (Carpenter and Beevers, 1958). Typically, germinating seedlings show early increases in activity of the enzyme. Enzyme activity decreases later as fat storage material decreases (Carpenter and Beevers, 1958; Abraham and Velasco, 1960; Ching, 1966; 1970; Firenzuoli, et al., 1968b).

Little has been known about the control of isocitrate lyase until recently. Major activity seems to be triggered by the embryonic axis in most seeds. Synthesis of isocitrate lyase in germinating seeds is dependent on the presence of the axial tissue in a fashion similar to regulation of synthesis of a multitude of other enzymes (Yomo and Iinuma, 1966; Penner and Ashton, 1967; Gepstain and Ilan, 1970; Yomo and Varner, 1973; Locker and Ilan, 1975). A cytokinin may supplant the axis stimulus in seeds of squash (Cucurbita maxima) and peanuts (Arachis hypogaea) (Penner and Ashton, 1967). It was noted earlier that

gibberellin causes a substantial increase in isocitrate lyase activity in dormant hazel (*Corylus avellana*) seeds (Pinfield, 1968b). Cyclic-AMP promotes similar induction of lyase in hazel (Protempa and Galsky, 1973). This suggests that c-AMP may be a mediator in GA_3 response. Other investigators have also demonstrated axial control of isocitritase (Bilderback, 1974; Smith, *et al*., 1974; Doig, *et al*., 1975; Doig and Colborne, 1975; Schrauwen and Sondheimer, 1975); however, axial control is not a universal phenomenon. Developmental changes in the endosperm of castor bean (*Ricinus communis*) are independent of the embryonic axis (Huang and Beevers, 1974).

Presley and Fowden (1965) reported that the synthesis of isocitrate lyase was not *de novo*. Using cotyledons from cucumber (*Cucumis sativus*) seedlings, and working with azetidine -2- carboxylic acid (a proline analogue), they concluded that the activity observed resulted from activation of preformed macromolecules such as zymogens. However, they failed to show that azetidine -2- carboxylic acid actually entered the cotyledons or that it was incorporated into protein. More recently isocitrate lyase has been shown to be synthesized *de novo* in cotton (*Gossypium hirsutum*) cotyledons (Ihle and Dure, 1972; Smith, *et al*., 1974), though the mechanism is still ill-defined.

Firenzuoli *et al*., (1968a) demonstrated the presence of the glyoxylate cycle in the systematic group of conifers.

They studied the role of isocitrate lyase in the germinating seeds of these species and correlated its occurrence with the prevalence of lipid as the main storage compound in the female gametophyte of some conifers. Gymnosperm seed which did not contain fat were devoid of enzymes specific for the glyoxylate bypass. Also, the correlation between activity of the bypass and lipid breakdown (Ching, 1966) is similar to that found by investigators of angiosperm seed (Firenzuoli, et al., 1968a). Ching (1970) has since confirmed these observations using glyoxysomes from the megagametophyte of germinating ponderosa pine seeds. Firenzuoli et al., (1968a) provided further proof that isocitrate lyase was synthesized de novo.

Recently, Bilderback (1974) reported evidence suggesting a two-phase development of isocitrate lyase in ponderosa pine seeds. Isocitrate lyase activity in the megagametophyte develops prior to germination and is stimulated by the process of cold stratification. This activity is independent of embryo control. Further synthesis of isocitrate lyase depends on a factor which diffuses into the storage tissue from the embryo after germination occurs. Nyman (1971) concluded that amylase activity was dependent on the presence of the embryo in Scots pine (Pinus sylvestris) seed. Neither of these investigators could replace the effect of the embryo with GA_3; however, the development of the enzymes was inhibited by abscisic

acid (ABA). Antagonism of GA action is a well recognized function of ABA. It is possible that the GA_3 was not mobile and that a glycoside linkage is necessary for transport into the target tissue. Also, a different gibberellin may be required to elicit a response. For example, GA_4 and GA_7 have been shown to be much more active (at lower concentrations) than other gibberellins in stimulating germination of Grand Rapids lettuce (*Lactuca sativa*) seeds and tobacco (*Nicotiana tabacum*) seeds (Ikuma and Thimann, 1963). Another hormone or combination of hormones may be required to generate an increase in isocitrate lyase activity.

In their review of seed germination, Mayer and Shain (1974) stated that the glyoxylate cycle was clearly not one of the early metabolic events of germination. The biphasic synthesis of isocitrate lyase mentioned above would tend to dispute this contention. Early workers with alpha-amylase of barley adhered to the hypothesis that substrate mobilization was an essential ingredient for germination to take place. The hypothesis has since been disproved for barley (Ching, 1970; 1974), but in ponderosa pine it would seem that substrate mobilization is at least a possible factor necessary for germination to commence.

Two-phase development was also shown for catalase and isocitritase in glyoxysomes in embryos of *Fraxinus americana* seeds (Schrauwen and Sondheimer, 1975). Isocitritase,

catalase and fumarase in the mitochondria increased when dormant ash embryos were incubated at 22° C. The increase occurred before the embryos were capable of germinating. After germination, a second increase was noted. Enzyme activity was shown to be regulated by zeatin, GA_3, and ABA. Gibberellic acid did not increase the activity of isocitrate lyase beyond that observed for dormant embryos but it did result in germination. This is not surprising since Harvey and Oaks (1975), comparing dwarf mutant and normal *Zea mays*, clearly showed that some tissue already contains sufficient gibberellin to stimulate enzyme production. The development of isocitrate lyase activity in ash embryos was suppressed by ABA and enhanced by zeatin. Zeatin may function in an antagonistic role against inhibitors (Kahn, 1971). Schrauwen and Sondheimer (1975) suggested the first appearance of activity is brought on by cold stratification and the second surge of activity may be the result of growth of the root tip.

Chen and Chang (1972) developed a scheme for cereal grains which may apply here. They postulated that after-ripening or stratification generates gibberellin which triggers events in the embryo that initiate germination, then the embryo synthesizes gibberellins which are secreted to the nutritive tissue stimulating synthesis of certain enzymes.

Glyoxysomes have been shown to possess some degree of autonomy (Ching, 1970). They contain DNA and RNA and can synthesize protein from labeled amino acids. Thus, any factor controlling the development of the organelle membrane would necessarily affect synthesis of enzymes known to be associated with the glyoxysomes. Johnson and Kende (1971) have shown that enzymes of the cytidine diphosphate pathway of lecithin biosynthesis are stimulated by GA_3. Evins and Varner (1971) have shown ^{14}C -choline incorporation into endoplasmic reticulum (ER) prior to amylase synthesis in barley, (*Hordeum vulgare*). Kagawa, *et al*., (1973) reported the possible sequential incorporation of ^{14}C -choline from light membranous material to membranes of glyoxysomes and mitochondria in castor bean endosperm. More recently, Doig, *et al*., (1975) demonstrated that glyoxysomes are present in the quiescent wheat (*Triticum vulgare*) grain but contain no enzymes usually found associated with glyoxysomes. During germination the number of glyoxysomes increased. Increases paralleled the appearance and increase in associated enzymes. Furthermore, they showed that activities of glyoxysomal enzymes are either embryo-dependent or GA_3-dependent in embryoless seeds. Enzymes found exclusively in the cytosol are under no such control. Also, enzymes such as citrate synthetase and malate dehydrogenase, normally found in both the cytosol and cellular compartments, were regulated accordingly. Their

GA_3-independent activities were located in the cytosol or the mitochondria while their GA_3-dependent activities were limited to the glyoxysomes.

It may be concluded from these experiments that the synthesis of new membrane - specifically glyoxysomes - is essential to the synthesis of glyoxysomal enzymes such as isocitrate lyase. Membrane synthesis may be controlled by the embryo through the action of gibberellin and/or other plant hormones.

Actual mechanisms for the control of isocitrate lyase and other enzymes of the glyoxysomes have been only partially defined. Much work is necessary to clarify the mechanisms which are probably as varied as the seeds used in the various experiments. However, several factors seem reasonably clear: 1.) Isocitrate lyase is synthesized _de novo_ after a hormonal signal (probably gibberellin) from the embryo (or axis) is received. 2.) Synthesis may also precede germination in which case it is partially independent from the embryo. 3.) Activity depends on the prior synthesis of new membrane elements.

In Vitro Analysis of Isocitrate Lyase

Isocitrate lyase cleavage of isocitrate to glyoxylate and succinate is favored thermodynamically at physiological concentrations of reactants and products (Smith and Gunsalus, 1957). Therefore, many calculations for the catalytic

activity of isocitrate lyase are based on the quantitative measurement of glyoxylate produced.

The activity of isocitrate lyase has been shown to be stimulated by both EDTA (Kennedy and Dilworth, 1963) and sulfhydryl compounds (Smith and Gunsalus, 1954). Jacks and Alldridge (1967) found that when a β-aminomercaptan such as cysteine was used to enhance the activity of isocitrate lyase, it interfered with the determination of enzyme activity by condensing with glyoxylate to form thiazolidines. They subsequently developed a method, used by Bilderback (1974), to correct this problem.

Bilderback (1974) used an assay for isocitrate lyase which depended on the measurement of glyoxylate formed during the reaction. Unfortunately, his technique limited him to a single measurement, because the reaction had to be stopped in order to determine the amount of glyoxylate produced. Also, his assay procedures required several steps for the elimination of the cysteine interference discussed above. In addition, he treated the enzyme extracts with activated charcoal to remove nicotinamide adenine dinucleotide phosphate ($NADP^+$) which could result in the formation of αketoglutarate by the action of isocitrate dehydrogenase; αketoglutarate also interferes with the determination of glyoxylate.

A continuous recording spectrophotometric assay was developed by Dixon and Kornberg (1959). This procedure

allowed considerable flexibility by permitting instant monitoring for immediate determination of enzyme activity and for correction of possible aberrations in the assay procedures. Also, it condensed the assay into a simple single step operation. Dixon and Kornberg (1959) also used cysteine, but they were unaware of its interference with the assay.

TECHNIQUES AND PROCEDURES

Unstratified ponderosa pine seeds were obtained in 1976 from a single seed source near McCloud, California (United States Department of Agriculture seed zone 741) at an elevation of approximately 4000 feet. Seeds were stored at 0-4° C in a sealed container until used. Moisture content was 7.2 percent (ODW).

Stratification Procedure

Unstratified seeds were washed and allowed to imbibe in cold water (0-4° C) for 15 hours. The water was drained and seeds were stratified in polyethylene freezer bags at 0-4° C for 15 days.

Seed Germination and Incubation

The integuments and nucellar membranes of stratified seeds were removed and the gametophyte with enclosed embryo was sterilized in one percent sodium hypochlorite solution for ten minutes followed by a ten minute soak in sterile distilled water. Seeds were germinated in the dark at 26° C under sterile conditions in 10.0 cm petri dishes containing two 10.0 cm No. 3 Whatman filters and 4.0 ml of treatment solution or distilled water. For experiments which required embryo removal, gametophyte halves were subjected to the same sterilization and incubation conditions after the embryo was excised (unless otherwise specified).

Isocitrate Lyase Stimulating Factor Determination

A slight modification of Bilderback's method (Bilderback, 1974) was used for this determination. At germination, as determined by radicle protrusion, 50 or 100 intact embryos were removed from the seeds and incubated in 10.0 ml of sterile distilled water for 24 hours with shaking. The resulting diffusate was filter sterilized in a 0.2-micron plastic filter unit. For some experiments, the diffusate was subjected to a five-minute low-speed (10,000 x *g*) centrifugation to remove cell debris prior to filter sterilization. Twenty newly isolated half-gametophytes (ten seeds) were incubated with the embryo diffusate from 50 embryos for five hours with continuous shaking on a Burrell model 75-777 wrist action shaker (rheostat setting No. 3). A more rapid shaking speed (rheostat setting No. 7) was used in later experiments. Simultaneously, other half-gametophytes were prepared and incubated in distilled water as controls. Various plant growth regulators were used in place of the water in separate experiments. The gametophytes prepared in this fashion were then incubated on moist filter paper as previously described.

Enzyme Extract Preparation

Enzyme extracts were prepared by grinding megagametophytes in a Duvall tissue homogenizer with 1.0 ml per seed of Tris · HCl buffer, 0.1 M, pH 7.4, containing 10 mM

2-mercaptoethanol (2-ME), 1 mM disodium ethylenedinitrilotetraacetate (EDTA), and Tween 80, 1% (v/v). This slurry was centrifuged at 27,000 g for 15 minutes; the supernatant was removed from between the pellet and lipid layer then recentrifuged. The top layer of fat was again discarded and the remaining "soluble" fraction decanted and assayed for isocitrate lyase.

Isocitrate Lyase Assay

A method similar to that designed by Dixon and Kornberg (1959) was utilized to assay isocitrate lyase. The enzyme was assayed in a total volume of 1.0 ml containing 50 mM Tris · HCl, 10 mM 2-ME, 10 mM $MgCl_2 \cdot 6H_2O$, 13 mM phenylhydrazine · HCl, 20 mM trisodium D,L-isocitrate (allo-free), 1 mM EDTA and 50 μl of enzyme extract (0.15 - 0.25 mg protein), final pH = 7.4. The reaction was started with the addition of substrate, and the formation of glyoxylic acid phenylhydrazone from glyoxylate and phenylhydrazine was continuously followed spectrophotometrically by examining the rate of increase in absorbance at 324 nm. The absorbtivity ($a^{1\%}_{1cm}$) for glyoxylic acid phenylhydrazone is 1.7×10^4. The enzyme assay was performed at a constant temperature of 25° C.

Protein Analysis

Protein concentration of enzyme extracts was measured by the method of Folin-Ciocalteau, with crystalline bovine serum albumin as a standard (Lowry, *et al*., 1951).

Data Analysis

Data were grouped into a one-way classification and an analysis of variance was completed (Snedecor and Cochran, 1967). Individual comparisons among treatment means were performed to determine if the differences between means were significant.

Linear regression techniques were utilized to calculate protein content.

RESULTS

Characteristics of the Isocitrate Lyase Enzyme Assay System

The assay developed by Dixon and Kornberg (1959) was adapted to the specific crude enzyme extract used in this research. Mercaptoethanol was substituted for cysteine because it did not produce the interference described in the Literature Review. Also, the activated charcoal treatment was found to be unnecessary for an accurate determination of glyoxylate.

Dixon and Kornberg (1959) used a pH of 6.85, since the formation of phenylhydrazones is best accomplished in slightly acid conditions; however a pH of 7.4 was observed to be most favorable for our assay procedure. This was probably the result of an antagonism between the high pH optimum reported for the enzyme *per se* (McFadden, and Howes, 1963) and the acid conditions necessary for the condensation to phenylhydrazone. Although a complete pH profile was not performed, the pH optimum was found to be very broad, as shown for the pseudomonad enzyme by McFadden, and Howes (1963).

Phenylhydrazine concentration is a critical factor in this assay as shown in Figure 1, the plot of initial velocity versus concentration. A relatively small change in concentration from 13.0 mM causes a loss in activity. Enzyme

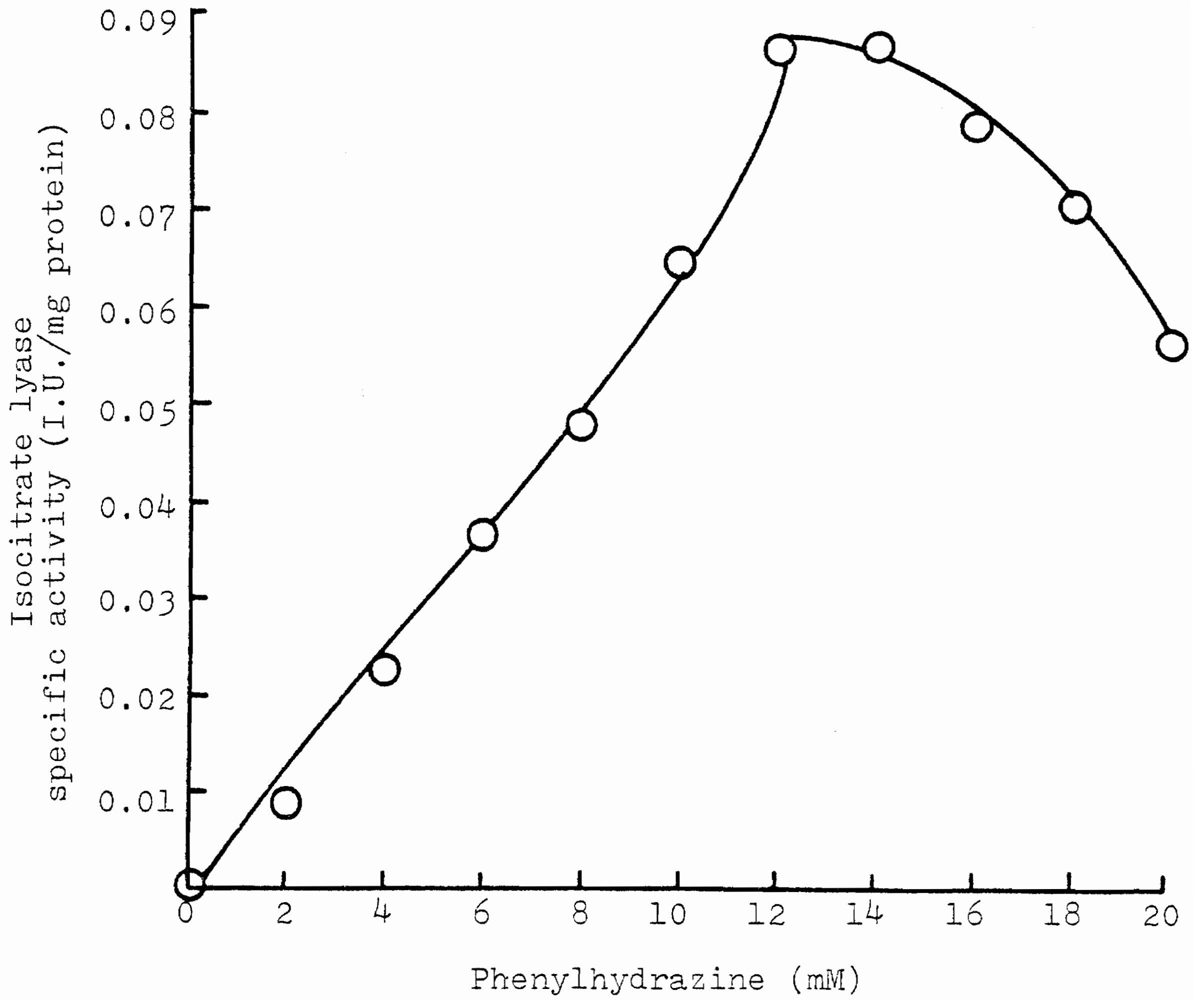


Figure 1. Plot of initial reaction velocity of isocitrate lyase versus phenylhydrazine · HCl concentration.

activity reaches a sharp peak then quickly tails off parroting a temperature or pH profile of activity. Since the buffering system was found to be adequate, it can only be concluded that the graph indicates an apparent concentration optimum resulting from two processes. An increasing rate of reaction is antagonized by an increasing rate of deactivation above a critical concentration. This was probably caused by the phenylhydrazine combining with side groups of the isocitrate lyase. Operating with this hypothesis, the protein content was maintained from one extract to another as uniformly as possible since the phenylhydrazine would probably affect a larger proportion of the isocitrate lyase in a less concentrated protein extract than in an extract with higher protein concentration. At 13.0 mM, the optimum concentration for phenylhydrazine was four times more than the concentration used by Dixon and Kornberg (1959). The optimum concentration was determined from measurements of initial velocity whereas Dixon and Kornberg (1959) measured velocity after a one minute lag to achieve a linear reaction rate. It is, of course, important to record initial kinetics since crude extracts could contain other enzymes which may ultimately interfere by utilizing the substrates produced or by producing compounds which also condense with phenylhydrazine.

Figure 2 shows the Lineweaver-Burk plot of saturation kinetics. The enzyme appeared to obey classical Michaelis-Menten steady state kinetics. The V_{max} was calculated to be 0.093 μmoles/min/mg and the Km = 0.348 mM. Actually, the Km should be halved, since isocitrate lyase has been shown to be specific for L_s-isocitrate in peanuts and castor bean (Marcus and Velasco, 1960); however, this determination was not made for the ponderosa pine enzyme. Isocitrate lyase was saturated with 15 mM D,L - isocitrate, so 20 mM D,L - isocitrate was used to assure saturation in the assay mixture. An allo-free form of D,L - isocitrate was used to avoid the inhibitory effects demonstrated for erythro- D,L - isocitrate (allo-isocitrate) on the yeast isocitritase (Olson, 1959).

A Km was not determined for Mg^{++} yet it was determined to be essential to the reaction as were other components of the assay (Table 1). Figures in Table 1 show very little activity was observed when $MgCl_2$ was excluded. It should also be noted from this table that there was some minimal activity in the absence of D,L - isocitrate. This endogenous activity of the enzyme extract was not always a problem, yet when it occurred, two minutes was sufficient for it to be completely exhausted.

Figure 3 shows that the concentration of extract protein in the reaction mixture is critical. A concentration greater than 0.30 mg protein/ml of reaction mixture resulted in severely reduced efficiency of the assay to

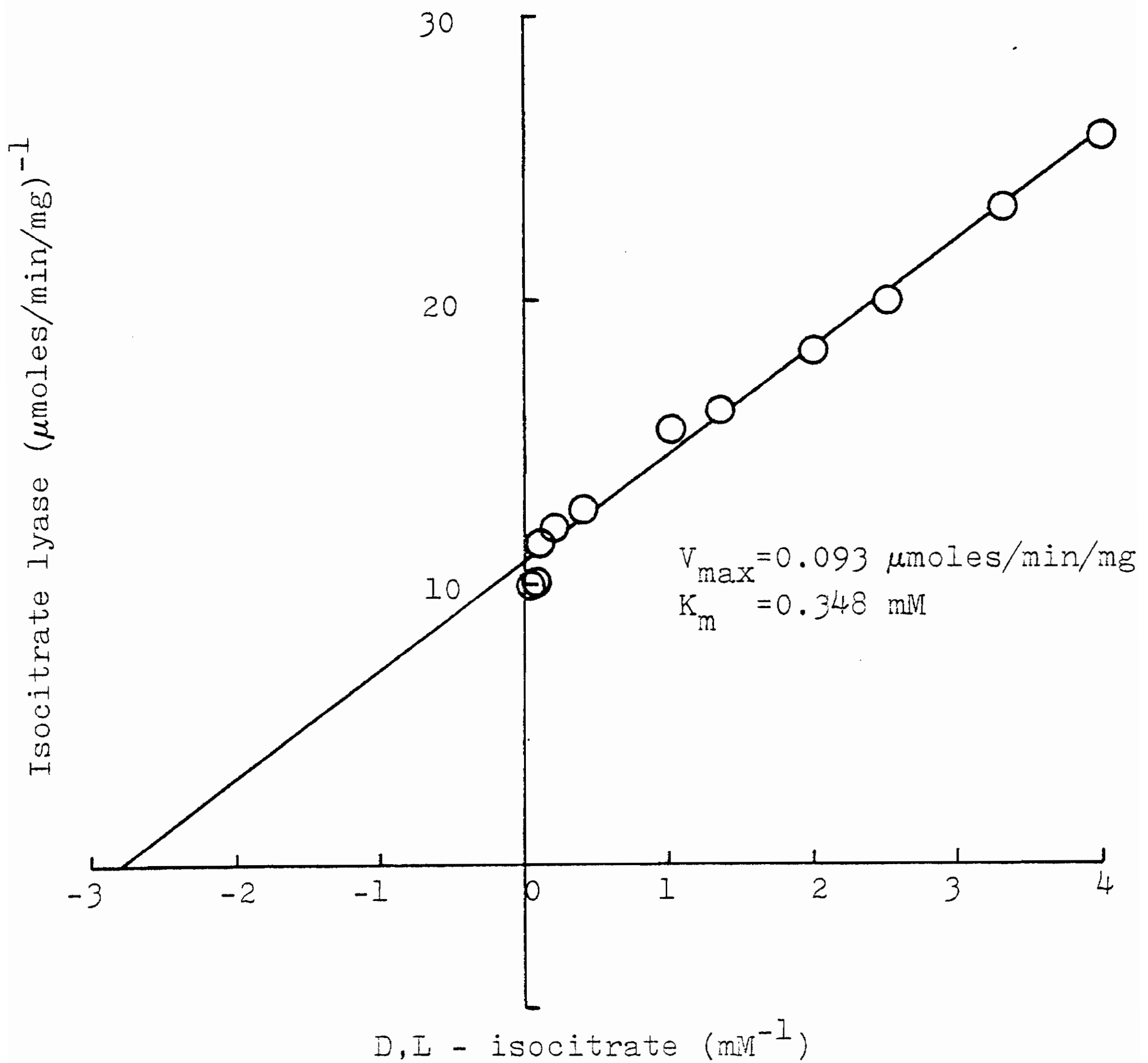


Figure 2. Lineweaver-Burk plot showing D,L - isocitrate saturation kinetics of isocitrate lyase.

Table 1. Dependence of isocitrate lyase activity[1] on individual requirements of assay mixture.

Assay Conditions	Specific Activity
Complete	0.101
No Isocitrate	0.009
No Phenylhydrazine	0.0
No $MgCl_2$	0.006
No Extract	0.0

[1]Specific activity of enzyme expressed as I.U./mg protein.

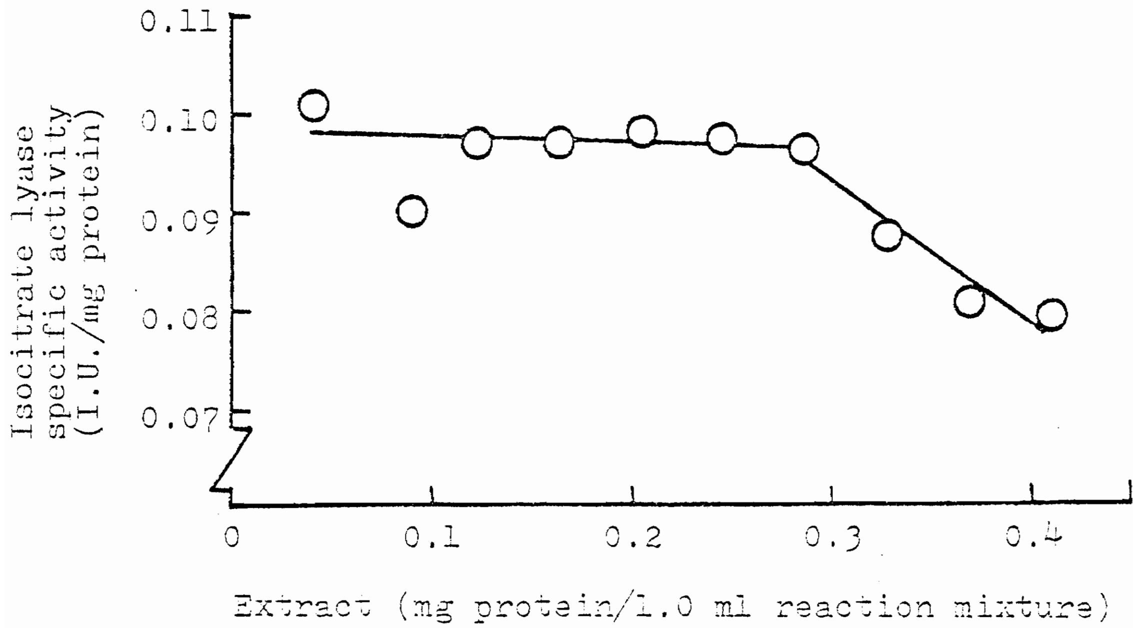


Figure 3. The effect of increasing the extract protein concentration on the specific activity of isocitrate lyase.

reflect the total activity. To accommodate this characteristic, enzyme extract added to the reaction was kept within a range of 0.15 to 0.25 mg protein/ml of reaction volume. At times, the enzyme extract was stored frozen overnight, Several aliquots of the same extract were frozen and periodically assayed over a 13-day span to determine the effect of freezing and thawing on the enzyme activity. This treatment had no significant effect (Table 2).

Effect of Embryo Excision on Isocitrate Lyase Activity of Megagametophytes

Two experiments (Figure 4, and Appendix Table 1) resulted in activity data similar to that shown by Bilderback (1974). The peak activity for each treatment is only about half that obtained by Bilderback (1974), but the relationship of one treatment to another and the general pattern over time is approximately the same. Seeds incubated without embryos from day zero reached a lower activity at all points than seeds germinated with their embryos. Activity was higher if the embryos were left *in situ* for the first two days to allow germination to occur prior to excision. However, this activity diminished more rapidly than the activity of seeds with embryos remaining in place for the full incubation period.

During attempts to replicate the experiment described above and to originate new experiments, considerable

Table 2. Isocitrate lyase activity[1] of enzyme extract[2] stored frozen at -20° C.

Days Stored	Specific Activity
0	0.106
1	0.119
2	0.119
3	0.101
4	0.122
5	0.107
6	0.106
10	0.107
12	0.111
13	0.114

[1]Specific activity (I.U./mg protein).

[2]27,000 x g supernatant from homogenized cells. Protein content = 3.2 mg/ml.

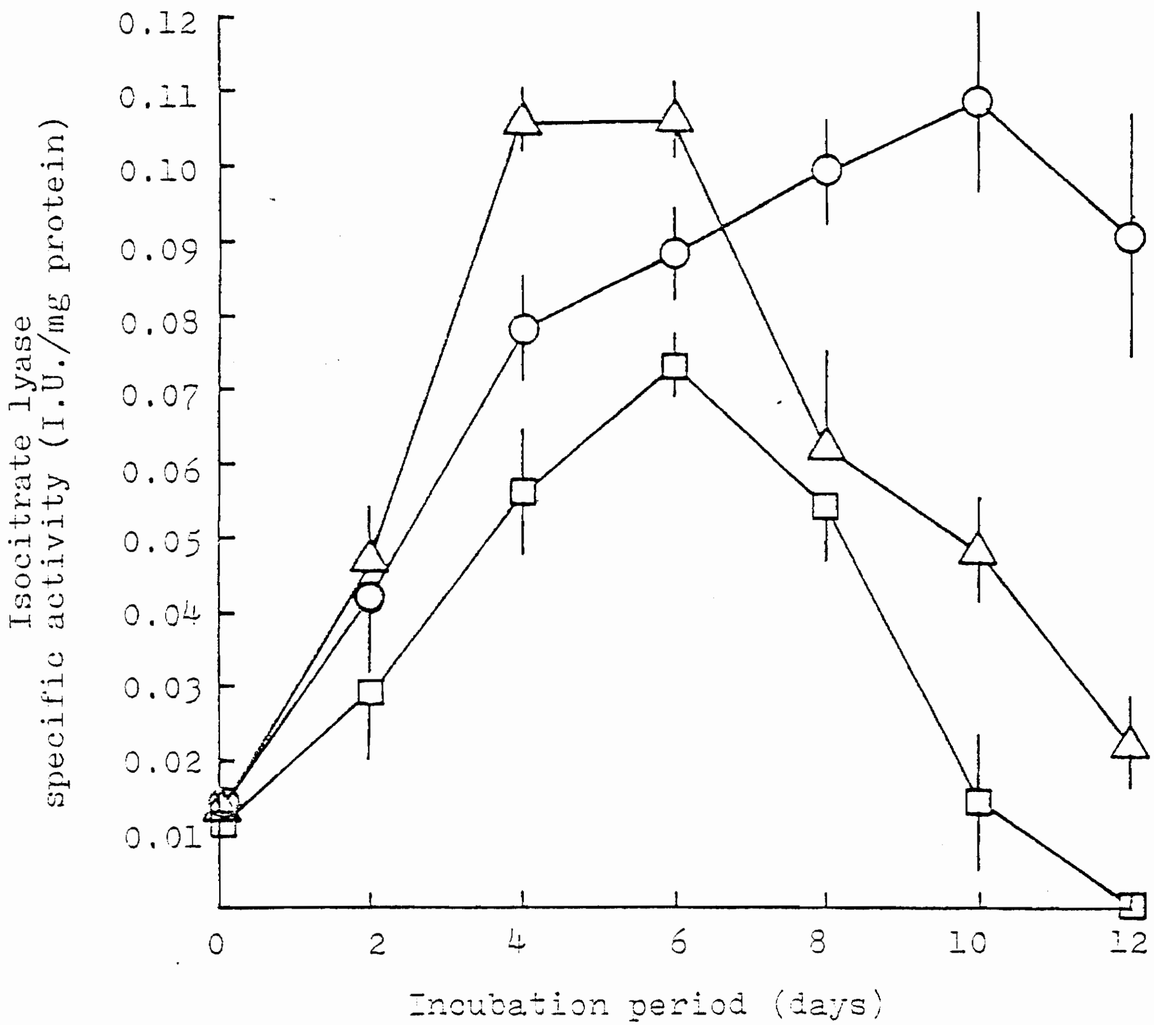


Figure 4. The effect of removal of the embryo on development of isocitrate lyase activity of gametophytes. Seeds received a 10 min. sterilization and 10 min. H_2O soak, then incubated with the embryo for a full 12 days (○), or incubated with the embryo for 2 days, after which the embryo was removed and the gametophytes incubated the remaining 10 days (△); embryos were excised, then gametophyte halves were sterilized 10 min. and soaked in H_2O for 10 min. (□). Each point is an average of three values. Vertical lines indicate ± 1 standard deviation.

variability was noticed among experiments using embryoless seeds. There seemed to be some relationship between experimental variability and the sodium hypochlorite sterilization and water soak. The sterilization and soak may have had a detrimental effect on the subsequent capacity of gametophytes to produce the enzyme because the cut surface of the seeds more readily exposed the cells to damage. In fact, preliminary experiments pointed to the seeds' extreme sensitivity to excessive moisture during germination. A second possible cause of variation was that some important regulator of activity may have been washed from the cells which were in contact with the embryo, or leached from the surface cells of the gametophyte. Experiments were designed to analyze this erratic behavior. A discussion of the results of these experiments follows.

The data in Figure 5 reveal that the sodium hypochlorite treatment did affect enzyme activity. Comparisons between treatment means for day six (Appendix Table 4) revealed that seeds sterilized prior to excision of embryos developed significantly ($P = 0.05$) higher maximum activities than gametophytes sterilized afterward. Also, a one minute water soak of presterilized gametophytes did not significantly change the subsequent enzyme activities on day six. This effectively eliminated the second possibility that a regulator was being washed from the surface cells. Later

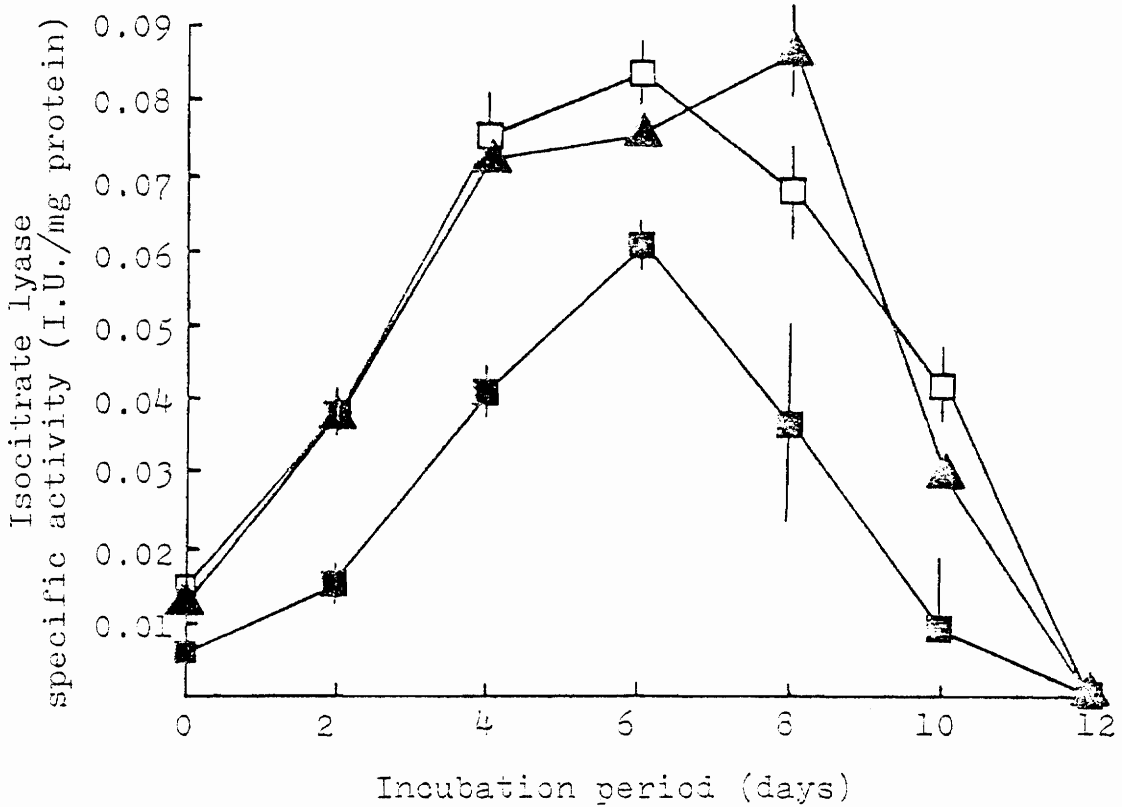


Figure 5. The effect of sodium hypochlorite sterilization on development of isocitrate lyase activity of gametophyte halves. Seeds received a 10 min. sterilization in $NaOCl_2$ and 10 min. soak in H_2O. Embryos were then excised and the gametophytes received the following treatments prior to incubation: no treatment (□); 10 min. sterilization and 10 min. H_2O soak (■); 1 min. soak in H_2O (▲). Each point is an average of three values. Vertical lines indicate ± 1 standard deviation.

experiments indicated that the sterilization process probably reduced activity by limiting gas exchange.

The initial results could now be interpreted differently. Consequently, the experiment used to determine effects of embryo excision was redesigned to avoid the earlier pitfalls. Two experiments (Figure 6, and Appendix Table 2) reflect this altered treatment and clearly indicate a different pattern of enzyme development. In seeds sterilized before embryo excision, both the embryoless gametophytes (Figure 6, (□)) and the gametophytes embryoless after the second day (Figure 6, (△)) show an initial rate of increase in enzyme activity that is higher than seeds germinated with embryos. Enhancement of activity lasts for four days after the embryos are removed then begins to decline. Furthermore, comparisons of treatment means (Appendix Table 5) show significant differences (P = 0.10) between peak activities of these two embryoless samples and the activities on the corresponding days of seeds germinated with embryos (Figure 6, (○)). The highest level of activity was observed for seeds retaining their embryos for the complete incubation period. A second set of gametophytes, with embryos left in until the end of the second day, were sterilized after excision (in addition to initial sterilization) to see if diminished activity could be observed. This was, in fact, the result (Figure 6, (▲)). The specific activity was lower for all points than with gametophytes sterilized prior to excision of embryos.

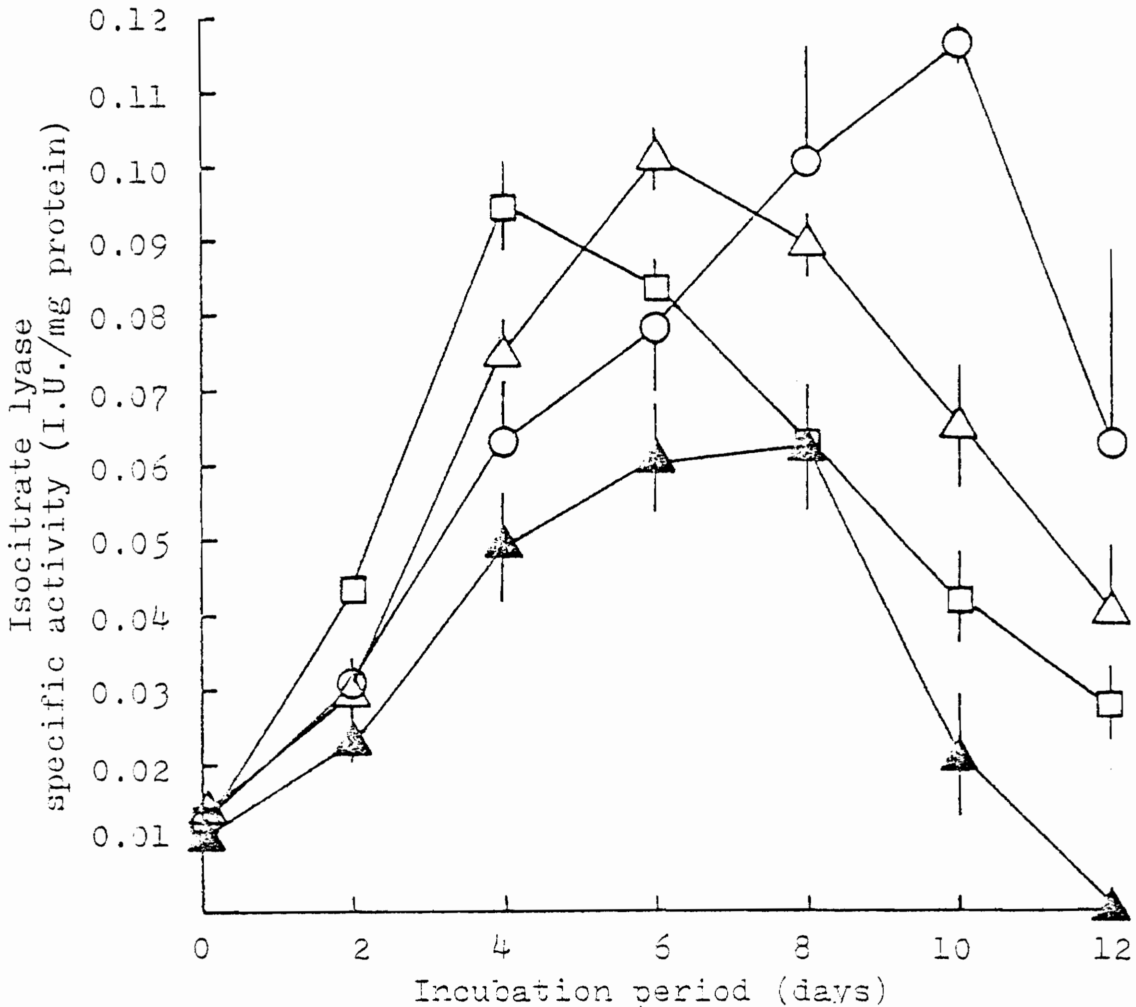


Figure 6. The effect of removing the embryo, and the effect of sterilizing after excision of the embryo on development of isocitrate lyase activity of gametophytes. Seeds were sterilized 10 min. and soaked 10 min. in H_2O then received the following treatments: seeds incubated with embryos for 12 days (○); seeds incubated 2 days with embryos, then 10 days embryoless (△); embryos were excised and gametophytes incubated (□); seeds incubated 2 days with embryos, then embryos were excised and gametophytes sterilized and soaked prior to incubation for remaining 10 days (▲). Each point is the average of three values. Vertical lines indicate ± 1 standard deviation.

Comparison of treatment means (Appendix Table 5) for day six shows a significant difference (P = 0.025) between enzyme activities.

Bilderback (1974) reported that he could detect no enzyme activity in imbibed unstratified gametophyte tissue. The results shown in Figure 7 clearly contradict his finding. There is measurable activity (0.01 I.U./mg protein) after 15 hours of cold imbibition at zero days. Furthermore, the seeds germinated (Table 3) and produced a profile of isocitrate lyase activity similar to stratified seeds. In addition, dry seeds were found to produce the same amount of enzyme activity as imbibed seeds at zero days.

Activity of the Embryo Factor

Bilderback (1974) performed an experiment which showed that an embryo diffusate could increase the two day isocitrate lyase activity of gametophytes by 44 percent over water treated controls. His experiment was duplicated here and a substantial difference between the diffusate and water treatments was not noted until the sixth day. The greatest difference occurred on the eighth day (Figure 8). Neither the gametophytes incubated in the diffusate nor the gametophytes incubated in water produced enzyme activity approaching the activity of normal nontreated embryoless seeds. In an attempt to increase this activity,

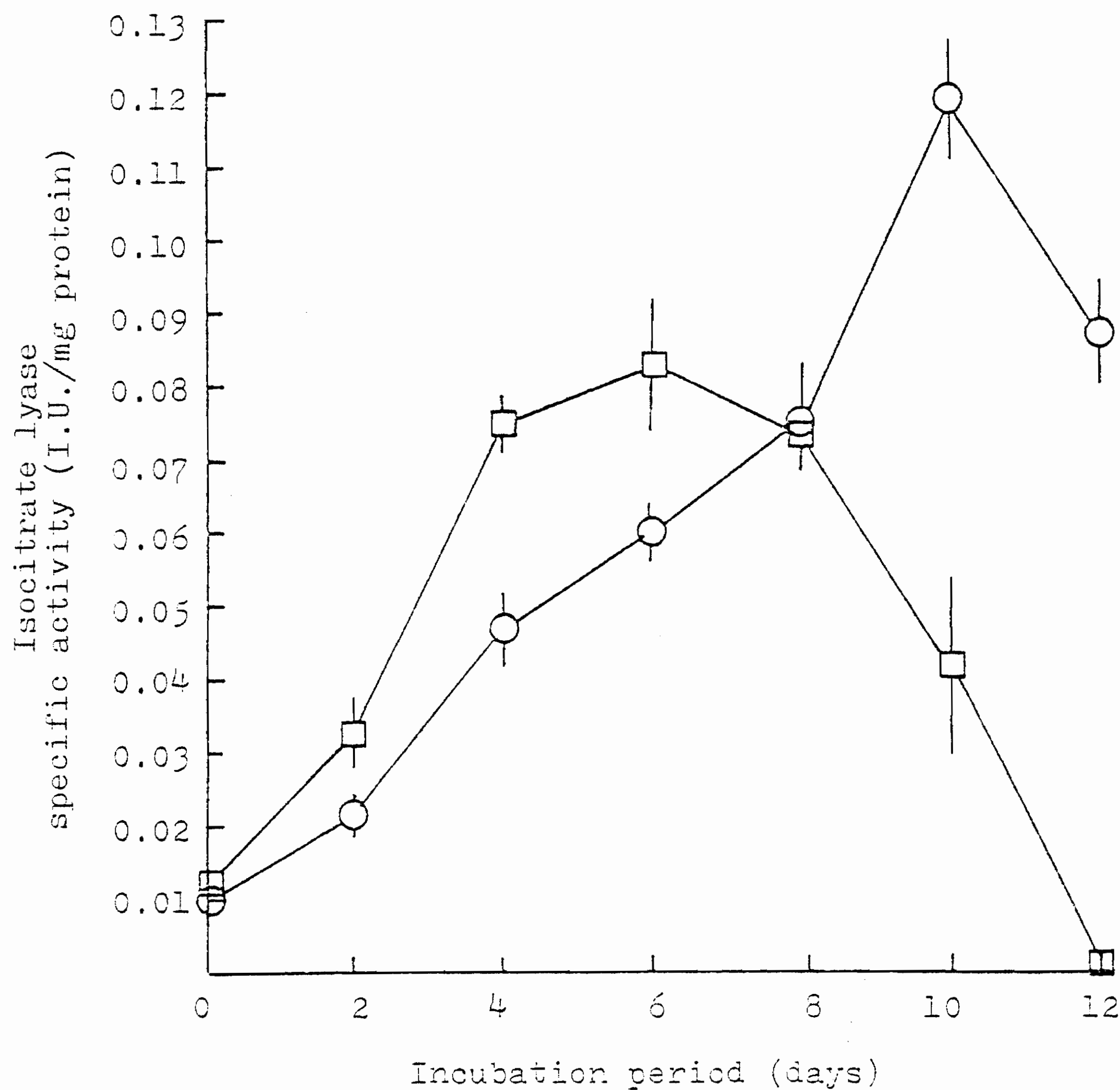


Figure 7. The effect of removal of the embryo on the development of isocitrate lyase activity of gametophytes from seeds which were imbibed only (no stratification). Seeds were imbibed 15 hrs. in cold water, then seeds were sterilized 10 min. and soaked 10 min. in H_2O; this was followed by incubation with embryo (○); or embryoless (□). Each point is an average of three values. Vertical lines indicate ± 1 standard deviation.

Table 3. Percent[1] germination of stratified[2] and nonstratified[3] ponderosa pine seeds.

Days of Incubation	Percent Germination	
	Stratified	Nonstratified
2	66 ± 3	63 ± 4
4	80 ± 4	70 ± 11
6	88 ± 5	80 ± 9
8	85 ± 3	89 ± 3

[1]Percent average of three replications of 30 seeds each ± 1 standard deviation. Seeds were germinated on moist filter paper in petri dishes in the dark at 26° C. Integuments and nucellar caps were removed.

[2]Imbibed 15 hr. in cold water, then stratified moist at 0 - 4° C in plastic bags for 15 days.

[3]Imbibed 15 hr. in cold water.

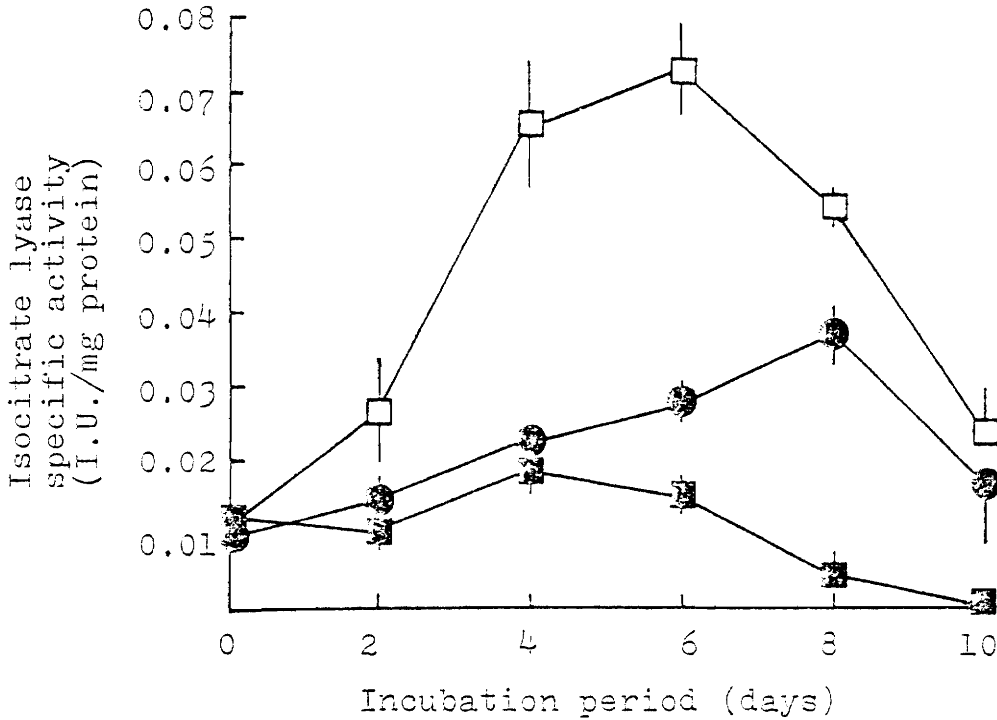


Figure 8. The effect of an embryo diffusate on the development of isocitrate lyase activity of gametophytes when pretreatment soak is accompanied by slow shaking. Gametophyte halves were sterilized 10 min. and soaked 10 min. in H_2O, then received the following treatments: no treatment (□); 5 hr. soak with slow shaking (Burrell shaker rheostat setting No. 3) in H_2O (■); 5 hr. soak with slow shaking in embryo diffusate prepared from 50 embryos (●). Each point is the average of 3 values. Vertical lines indicate ± 1 standard deviation.

the concentration of the embryo diffusate was boosted by doubling the number of embryos used while maintaining the same volume of extracting water (Table 4). Again the standard diffusate produced a maximum difference from the water control on the eighth day (Table 4), while the enriched diffusate showed a maximum on the fourth day which it maintained until the sixth day. However, the enriched diffusate did not increase the peak activity beyond the peak activity observed for the standard diffusate.

Previous experience with submersion treatments (e.g., sterilization) indicated that the gametophytes were probably not getting sufficient oxygen during the five hour soak in treatment solutions. Shaking was increased to a more vigorous rate (Burrell shaker rheostat setting No. 7) to remedy this anomaly. Two experiments (Figure 9, and Appendix Table 3) show the effects of this treatment. Notice that both sets of gametophytes receiving pretreatments have isocitrate lyase activities similar to those receiving no pretreatment. Notice also that at these levels, no difference can be detected between the embryo diffusate treatments and the controls.

Table 4. The effect of increasing the concentration of embryo diffusate on isocitrate lyase activity[1] after five-hour pretreatment[2] of megagametophyte halves prior to incubation.

Days of Incubation	Pretreatment		
	Water	Embryo Diffusate	2 x Embryo Diffusate[3]
2	0.013	0.012	0.018
4	0.019	0.021	0.038
6	0.017	0.025	0.034
8	0.008	0.034	0.013
10	0	0.008	0

[1] Specific activity (I.U./mg protein). Each number = average of two.
[2] Pretreatment refers to five hour soak with shaking (Burrell shaker rheostat setting No. 3) in solution specified.
[3] Ordinarily the diffusate is prepared by soaking 50 embryos in 10 ml of water. "2 x" refers to soaking 100 embryos in the same volume of water.

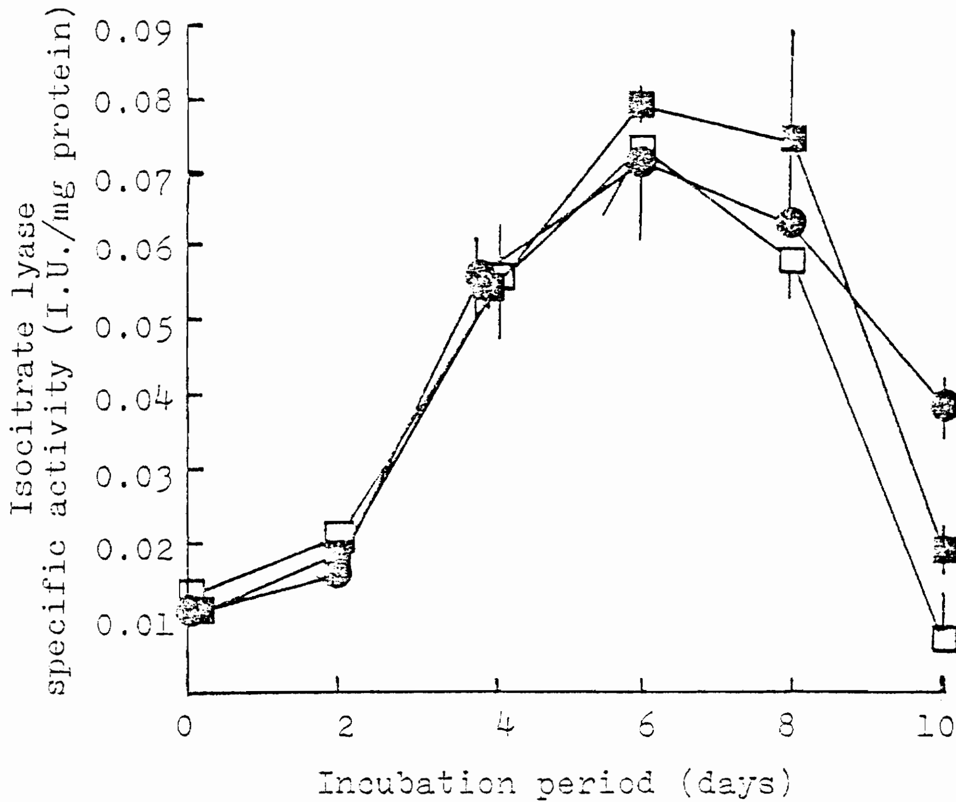


Figure 9. The effect of an embryo diffusate on the development of isocitrate lyase activity of gametophytes when the pretreatment soak includes rapid shaking. Gametophyte halves were sterilized for 10 min. and soaked in H_2O for 10 min., then they received the following treatments: no treatment (□); 5 hr. soak with rapid shaking (Burrell shaker rheostat setting No. 7) in H_2O (■); 5 hr. soak with rapid shaking in an embryo diffusate prepared from 100 embryos (●). Each point is the average of 3 values. Vertical lines indicate ± 1 standard deviation.

DISCUSSIONS AND CONCLUSIONS

There are several differences between the results presented by Bilderback (1974) and the data gathered in these experiments. Paramount among these is the difference in isocitrate lyase activity between gametophytes incubated with and without embryos. Also, there are other discrepancies which are no less important and should be resolved. Before these differences are dealt with on an individual basis, a general discussion of the problems encountered with techniques used in Bilderback's study and this study will be included as support for the interpretations to follow.

Problems Inherent in Technique

It is essential to consider differences between the enzyme assay procedures used in the two studies. Error hidden in the assays would effectively mask actual events. The underlying principles on which both assay methods were developed are essentially identical, but the means for extracting the information from them are different. Phenylhydrazine was used in both assays to capture the glyoxylate formed from the cleavage of isocitrate. The phenylhydrazone formed was measured optically. Bilderback (1974) allowed this reaction to proceed for five minutes in the absence of phenylhydrazine which was added after the reaction was stopped. One measurement of phenylhydrazone was taken after a suitable incubation. The current study

used an assay which included phenylhydrazine in the initial reaction mixture to allow immediate capture of glyoxylate and the consequent measurement of initial kinetics using a continuously recording spectrophotometer. There are advantages and disadvantages to both techniques. Excluding phenylhydrazine from the initial reaction probably eliminated its apparent deactivating effect on the enzyme (Figure 1). At the same time, its absence prevented the advantages realized from a continuous monitoring system, and it also permitted time for the glyoxylate formed to be picked up by other enzymes and converted to other products. Bilderback (1974) did not measure initial kinetics. Thus his data may have been the result of multiple reactions that may have either utilized the glyoxylate or produced other aldehydes and ketones.

While both factors could explain why Bilderback (1974) recorded higher maximal activities, the effect would appear to be offset by another difference in technique. Bilderback (1974) followed Ching's (1970) method of using a very high protein concentration in his reaction mixture. This protein content was much greater than the level of protein causing the severely reduced specific activity shown in Figure 3. Finally, Bilderback (1974) used an assay incubation temperature (30° C) higher than the temperature used in the current study (25° C). The temperature Bilderback (1974) used was reported to be conducive for maximal

isocitrate lyase activity in microbial systems (McFadden and Howes, 1963), but it has not been shown to be beneficial to the action of the plant enzyme. This determination was not made for the present study. All these factors may have had a bearing on the general level of enzyme activity observed in the two studies; however, in the final analysis, none provide the insight necessary to explain the differences in the relationship between the activities of embryoless and whole seeds of the two studies.

Analysis of sterilization and germination methods utilized in Bilderback's (1974) study and this study may provide the necessary information to explain anomalies observed. There are several treatments which can cause reduced activity of gametophyte halves. Submersion of gametophytes prior to incubation leads to lower enzyme activity. It is tempting to dismiss much of Bilderback's (1974) work as artifacts of the gametophyte's sensitivity to these types of treatments. Unfortunately, it is not certain how Bilderback (1974) prepared the gametophytes used in his study. According to him (personal communication), the seeds were sterilized prior to the removal of the integuments. Our experience indicated that bacteria and fungi were present inside the integuments. Thus, it was important to sterilize after they were removed. Seeds which were not prepared in this way would support bacterial growth unless the embryos were left intact. Tissue quickly became

contaminated and necrotic once the gametophyte was cut to excise the embryo. This decaying condition would also explain why Bilderback (1974) found that the activities of gametophyte halves were lower than those of seeds with intact embryos.

In the current study, gametophytes were incubated on moist filter paper rather than the vermiculite used by Bilderback (1974). It has been suggested that there may have been sufficient soluble carbohydrates in the filter paper to induce the enzyme to a higher level in embryoless seeds. However, only acetate has been shown to enhance the formation of isocitrate lyase while tricarboxylic acid intermediates, glucose and other complex media suppress formation of the enzyme (Kornberg, and Elsden, 1961). Therefore, it is more probable that suppressed activity would occur as a result of incubation on filter paper if indeed any compound was absorbed.

Germination or incubation was performed in the dark in the present study. In comparison, a twelve-hour light period was included in the study performed by Bilderback (1974). Firenzuoli, et al., (1968b) have shown that pine seeds germinated in the dark produced higher isocitrate lyase activity than those germinated in the light. Consequently, the lower activity observed for seeds germinated in the dark in the present study cannot be attributed to this difference.

There are a few remaining miscellaneous differences between the two studies which should be mentioned. Bilderback (1974) obtained his seeds from Washington, whereas seeds used in this research were acquired from a California source. Also, he did not report which variety he acquired; however, Boyd (1970) indicated that only *Pinus ponderosa* var. *ponderosa* grows in both these areas. Still, it is remotely possible that different varieties were used, because the ranges of var. *scopulorum* and var. *arizonica* do slightly overlap that of var. *ponderosa* (Boyd, 1970). Seed age and storage conditions may also be important factors. It was noticed, for example, that a longer storage period had no effect on the ability of the seeds to germinate, but it did cause noticeably lower enzyme activities. Actually the same maximum levels were eventually attained, but at a slower rate of increase. The seeds used in this study were obtained late in the year, so they had already been stored for several months. This alone could account for some variation if Bilderback (1974) used fresher seeds, although this is speculation.

In any event, the significance of all these divergent procedures may be minimal. Their effects may be only superficial. The fact remains that gametophyte halves in our study produced a surge in isocitrate lyase activity over seeds germinated with embryos. It is important to consider this difference as a "real" event, and to analyze it accordingly.

Proposed Models for Plant Growth Regulator Action

Many enzymes in the nutritive tissue of seeds of numerous species of angiosperms increase in activity during germination. These increases have been variously shown to be the result of *de novo* synthesis (Bennett and Chrispeels, 1972), translation of preformed mRNA (Radin and Trelease, 1976), or activation of zymogens (Presley and Fowden, 1965). These mechanisms, in turn, are affected by various plant growth regulators. Cytokinins and gibberellins activate these mechanisms and enhance enzyme activities (Yomo and Iinuma, 1966); (Yomo and Varner, 1973), while abscisic acid (ABA) prevents enzyme activities from reaching normal levels (Tester, 1976). However, the effects attributed to ABA have only been shown for exogenously applied ABA, and are often only indirect effects of inhibition of germination (Bradbeer, 1968). Although gibberellins and cytokinins (Chen and Chang, 1972) affect germination of dormant seeds, their stimulating effect on enzymes follows germination (Yomo and Iinuma, 1966; Yomo and Varner, 1973). However, cause and effect are not well established. Thus one may consider these processes as intermingled and interdependent. Control for enhancement of the enzymes resides in the embryos of the seeds of some species (Locker and Ilan, 1975). In certain species, the controlling factors have been definitely identified as specific plant growth regulators synthesized by the germinating embryo and transported

to the nutritive tissue where enzymes are affected (Paleg, *et al.*, 1960).

In view of the overwhelming evidence in support of a positive type of embryo control for angiosperms, one might expect it to be also operative in gymnosperms. Unfortunately, very little work has been completed using gymnosperms. Both Ching (1970) and Nyman (1971) concluded from their experiments with ponderosa pine and scots pine, respectively, that the embryos of conifers exert a positive influence on gametophyte enzyme activities. But, the data reported by these researchers do not justify the conclusion that the embryo controls enzyme activity.

Nevertheless, Bilderback (1974), inspired by the work completed by Ching (1970) and Nyman (1971), performed experiments on ponderosa pine that seemed to support the same conclusion. In contrast to Ching's (1970) and Nyman's (1971) work, substantial differences in enzyme activities were recorded by Bilderback (1974). In addition, Bilderback (1974) showed evidence for an embryo diffusate which could substitute for the embryo in its enhancing effect. Bilderback (1974) reported this effect as the percent of control. The actual level of enzyme activity was not indicated, and no information on statistical significance of the data was included.

The initial work performed here tended to confirm Bilderback's suppositions. Contrary to this, later experiments

clearly showed that the results from earlier experiments completed in this study were artifacts of technique. For example, the reduced activity in the absence of the embryo (Figure 4) was shown to be caused by the submersion of gametophyte halves in sterilizing and washing solutions (Figure 5). Also, although it cannot be wholly ignored, the stimulating action observed for the embryo diffusate (Figure 8) must be considered suspect because this same effect could not be demonstrated for experiments in which normal levels of activity were attained (Figure 9).

The problems with technique do not necessarily reflect error in Bilderback's (1974) work, but the fact remains that once these problems were resolved, the corrected data clearly dispute his findings. Removal of the embryo caused release from inhibition (Figure 6) rather than loss of stimulation. However, as mentioned before, the enhancing effect of the embryo diffusate must not be totally discarded and should be included in any scheme devised to explain the unusual behavior noted for embryo control over enzyme activity in ponderosa pine.

Several models can be proposed to account for the opposing inhibiting and activating aspects of the embryo. To fully understand these models, one must first have an appreciation for the concept of hormone binding to receptor proteins and how this concept can be applied to plants.

Several theories have been postulated to explain the mechanism of action through which plant growth regulators control their varied responses. The most promising theory suggests plant hormone activation of regulatory proteins that subsequently stimulate transcription by gene activation (Kende and Gardner, 1976).

Investigations of the regulatory action of animal steroid hormones have clearly revealed the presence of specific receptor proteins in cells of hormone target tissues (Jensen, et al., 1971). The sequence of events, following binding into a hormone receptor complex, has been elucidated and eloquently described by O'Malley and Schrader (1976). The progesterone receptor molecule is a dimer of distinctive subunits designated as A and B. Each subunit has a binding site for the steroid, hence the receptor binds two molecules of hormone. The progesterone alters the structure of the protein effectively activating it. This hormone-receptor complex enters the nucleus where it binds to chromatin at a nonhistone protein. Binding occurs exclusively with the B subunit, after which the dimer dissociates to liberate the A subunit for binding to naked DNA where it increases the number of initiation sites for binding by RNA polymerase and transcription of particular genes. This action causes a production of RNA, and specifically, mRNA for ovalbumin.

In choosing a theory to describe hormone regulation in plants, one must keep in mind the features shared by higher plants and animals. They are both eukaryotes possessing, among other organelles, a double nuclear membrane surrounding chromatin. Each cell possesses the same complement of genes yet produce different proteins. In these ways cells of higher plants and animals are more complex than bacteria, and they probably have systems for gene expression more closely resembling one another than the mechanism proposed for prokaryotes. Both plants and animals produce hormones which act in minuscule concentration (10^{-6} to 10^{-10} M) and selectively stimulate small populations of cells while passing through many other cells without effect. Finally, any theory accounting for hormone action must explain how relatively simple molecules, closely resembling one another, can be distinguished by target tissues. Therefore, it is most plausible to propose a system for gene expression in plants similar to the mechanism found to be operative in animal cells. This, in fact, is the basis for most studies on the regulatory role of plant growth regulators.

As pointed out by Kende and Gardner (1976) in their review articles on hormone binding in plants, it is generally accepted that only proteins are able to distinguish the subtle differences in structural and stereospecific

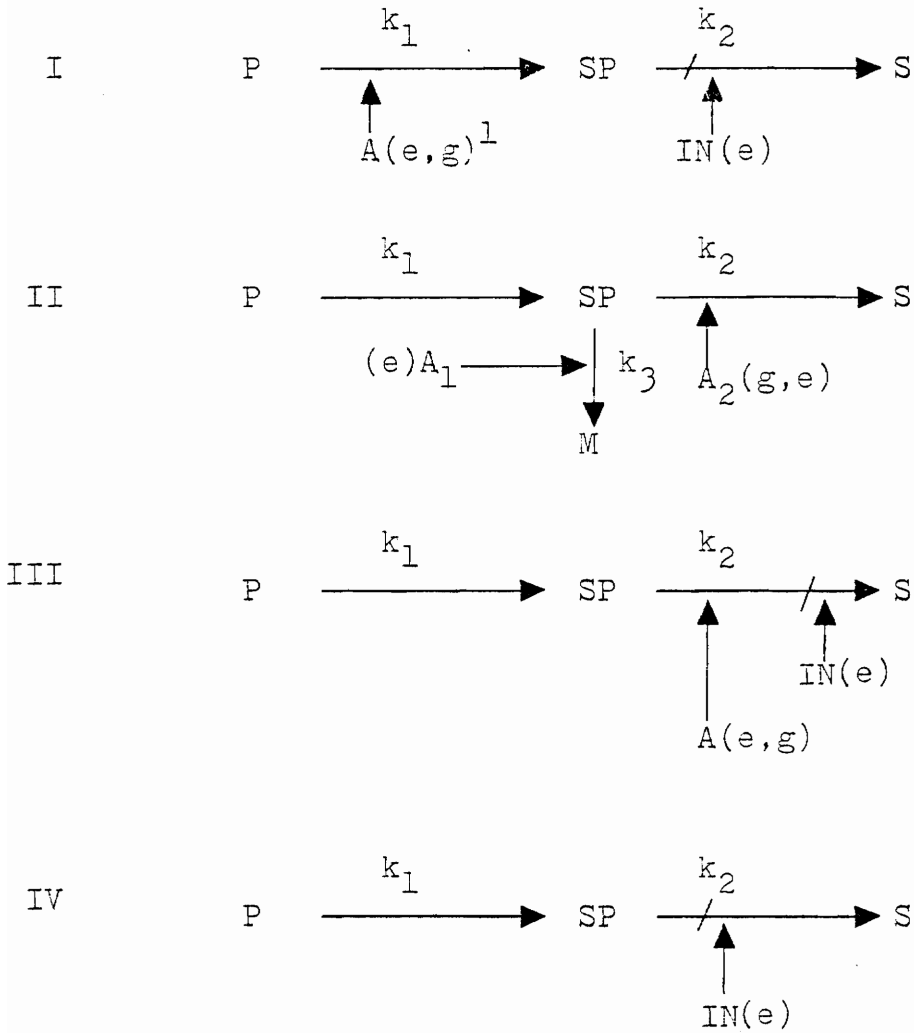
properties of simple hormones. Consequently, work has proceeded in a fashion designed to locate protein receptors for plant growth regulators. Some studies indicate that the plant growth regulators auxin (Matthysse and Phillips, 1969) and cytokinin (Matthysse and Abrams, 1970) stimulate RNA synthesis directed by pea (*Pisum sativum*) nuclei or chromatin when combined with protein mediators. Indole Acetic Acid (IAA) forms a weak noncovalent attachment to t-RNA (Bendana, *et al*., 1965), while benzyladenine is strongly incorporated into t-RNA by covalent bonds (Fox, 1966). In addition, Fox and Erion (1975) demonstrated protein facilitated binding of cytokinin to wheat germ ribosomes. Despite these findings, no evidence has been reported to link the formation of the complexes to physiological events.

The work with gibberellins (GA), though not spectacular, has been somewhat more fruitful. *In vivo* binding of GA was demonstrated for dwarf pea (Musgrave, *et al*., 1969). Sequestering occurs in GA target tissues concomitant with increasing physiological responsiveness to GA. This work was continued by Stoddard, *et al*. (1975) who were successful in showing binding of the GA to macromolecules. Konjevic (1976) showed noncovalent *in vivo* binding of GA to proteins of dwarf pea.

Admittedly, the research mentioned here has not been conclusive. Nevertheless it draws us closer to accepting the probability of protein receptors in plants.

To complete our scenario, it is necessary to borrow concepts devised by Penney (1972) and refined by Chang (1974) to explain stem elongation as stimulated by auxin and gibberellin. These researchers proposed that a growth limiting protein (GLP), a precursor, was converted to a growth protein (GP) in response to gibberellin. This growth protein or growth potential would increase in the absence of auxin. However, if auxin were present, the GP would combine with an inactive protein to form an active complex that would lead to cell elongation. If the process of enzyme synthesis is substituted for the process of growth, and the gibberellin and auxin are replaced by an activator and inhibitor, this model can be easily adapted to explain the effect of the ponderosa pine embryo on isocitrate lyase activity in the gametophyte.

Figure 10 (I and III) explains the results on the assumption that both an inhibitor (IN) and an activator (A) were present. In I, an activator supplied by the embryo and also produced by the gametophyte causes the transformation of a precursor (P) to an active complex (SP) which results in protein synthesis (S). With the embryo present, k_1 is stimulated by the activator while the Inhibitor (also from the embryo) decreases k_2. It is important to note that the inhibitor in this scheme does not completely block k_2, rather it serves as a moderator by reducing k_2. The combined action of A and IN causes the pool of SP to slowly accumulate,



[1] e indicates production of plant growth regulator by the embryo and g indicates production of plant growth regulator by the gametophyte.

Figure 10. Proposed plant growth regulator action.

and when the embryo is removed SP is rapidly converted to S causing the surge in activity evident in Figure 6. Activator action on k_1 explains why the maximum activity increased with the length of time the embryo was left <u>in situ</u>. Continuously supplied A resulted in a higher maximum as SP increased. While inclusion of the activator in the scheme is not absolutely necessary to explain the observed phenomenon, it must be included to account for the unusual results observed for the diffusate experiments. To interpret the data from the diffusate experiments, it must be assumed that A and IN diffused from the embryo at different rates and that they had unequal threshold concentrations. The shift in peak activity from eight to four days (Figure 8 and Table 4) is understandable if IN reached its critical concentration in the weak embryo diffusate and A followed suit in the strong embryo diffusate.

The success for the argument in support of model I depends on the supposition that the embryo supply both the activator and the inhibitor. A criticism of this model might cite this duality as inefficient. However, a strong case can be made in support of its efficiency. In the event that the activator had a low dissociation rate with its receptor, stopping the supply of activator would not necessarily immediately deplete the response. Also a buildup in the pool of SP would prevent rapid cessation. The addition of an inhibitor provides considerably more

flexibility by allowing the immediate limitation of response while maintaining a pool of SP for instant response once the source of inhibition is removed or decreased.

Selection II in Figure 10 provides an alternative interpretation of the results using two activators. Number IV assumes that the diffusate experiments were artifacts, and explains only the release from embryo inhibition. However number I appears to be the best selection.

All other research similar to this study has found that if the embryo exerts an influence on enzyme activity, in the nutritive tissue of seeds, it is positive. But, the research which led to these conclusions was performed using angiosperms. There are considerable differences between the seeds of angiosperms and gynmosperms. Thus, it is not reasonable to automatically assume that what is true for one must also be true for the other. It is obvious that the behavior observed for angiosperms does not fully encompass the phenomena which occurred in ponderosa pine seeds. This is the first time that the function of inhibition has been attributed to the embryo. Evidently, a novel mode of embryo control, worthy of further investigation, may be operative.

LITERATURE CITED

Abraham, M. and J. Velasco, 1960. Enzymes of the glyoxylate cycle in germinating peanuts and Castor beans. J. Biol. Chem. 235:563-567.

Abrahamsen, M. and T. W. Sudra, 1966. Studies on the soluble carbohydrates and carbohydrate precursors in germinating soybean seed. Amer. J. Bot. 53:108-114.

Bendana, F. E., A. W. Galston, R. Kaur-Sawhney, and P. J. Penny, 1965. Recovery of labeled ribonucleic acid following administration of labeled auxin to green pea stem sections. Plant Physiol. 40:977-983.

Bennett, P. A. and M. J. Chrispeels, 1972. De novo synthesis of ribonuclease and Beta-1, 3-glycanase by aleurone cells of barley. Plant Physiol. 49:445-447.

Bilderback, E. G., 1974. The regulatory role of the embryo on the development of isocitrate lyase activity during germination of ponderosa pine seeds. Physiol. Plant. 31:200-203.

Boyd, R. J., 1970. USDA Forest Service Intermt. Forest and Range Exp. Stn., Moscow, Idaho. In: Seeds of Woody Plants in the U.S. USDA handbook No. 450. U.S. Government Printing Office, Washington, D.C. 883 pp.

Bradbeer, J. W., 1968. Studies in seed dormancy. IV. The role of endogenous inhibitors and gibberellin in the dormancy and germination of *Corylus avellana* L. seeds. Planta 78:266-276.

Bradbeer, J. W. and B. Colman, 1967. Studies in seed dormancy. I. The metabolism of 2-^{14}C acetate by chilled seeds of *Corylus avellana* L. New Phytol. 66:5-15.

Bradbeer, J. W. and N. J. Pinfield, 1967. Studies in seed dormancy. III. The effects of gibberellin on dormant seeds of *Corylus avellana* L. New Phytol. 66:515-523.

Carpenter, W. D. and H. Beevers, 1958. Distribution and properties of isocitrase in plants. Plant Physiol. 34:403-409.

Chang, In-Kook, 1974. Action and interaction of plant growth hormones in seedlings of *Zea mays* L. Ph.D. thesis, University of Chicago, Chicago, Illinois.

Chen, S. S. C. and J. L. L. Chang, 1972. Does gibberellic acid stimulate seed germination via amylase synthesis? Plant Physiol. 49:441-442.

Chin, T. Y., R. Poulson, and L. Beevers, 1972. The influence of axis removal on protein metabolism in cotyledons of Pisum sativum L. Plant Physiol. 49:482-489.

Ching, T. M., 1966. Compositional changes of Douglas fir seeds during germination. Plant Physiol. 41:1313-1319.

Ching, T. M., 1970. Glyoxysomes in megagametophyte of germinating ponderosa pine seeds. Plant Physiol. 46:475-482.

Chrispeels, M. J. and J. E. Varner, 1967. Gibberellic acid-enhanced synthesis and release of alpha-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42:398-406.

Dixon, G. H. and H. L. Kornberg, 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. 72:3.

Doig, R. I., A. J. Colborne, G. Morris and L. L. Laidman, 1975. The induction of glyoxysomal enzyme activities in the aleurone cells of germinating wheat. J. Exp. Bot. 26:387-398.

Doig, R. I., A. J. Colborne, 1975. Enzymes of intermediary metabolism in the aleurone cells of germinating wheat. J. Exp. Bot. 26:399-410.

Drennan, D. S. H. and A. M. Berrie, 1962. Physiological studies of germination in the genus Avena. I. The development of amylase activity. New Phytol. 61:1-9.

Evins, W. H. and J. E. Varner, 1971. Hormone controlled synthesis of endoplasmic reticulum in barley aleurone cells. Proc. Nat. Acad. Sci. U.S.A. 68:1631-1633.

Firenzuoli, A. M., P. Vanni, E. Mastronuzzi, A. Zanobini, and V. Baccari, 1968a. Enzymes of glyoxylate cycle in conifers. Plant Physiol. 43:1125-1128.

Firenzuoli, A. M., P. Vanni, E. Mastronuzzi, A. Zanobini, and V. Baccari, 1968b. Participation on the glyoxylate cycle in the metabolism of germinating seed of Pinus pinea. Life Sciences 7:1251-1258.

Fox, J. E., 1966. Incorporation of a kinin, N,g-benzyladenine into soluble RNA. Plant Physiol. 41:75-86.

Fox, J. E. and J. L. Erion, 1975. A cytokinin binding protein from higher plant ribosomes. Biochem. Biophys. Res. Comm. 64:694-700.

Frankland, B. and P. F. Wareing, 1962. Changes in endogenous gibberellins in relation to chilling of dormant seeds. Nature 194:313-314.

Frankland, B. and P. F. Wareing, 1966. Hormonal regulation of seed dormancy in hazel (Corylus avellana L.) and beech (Fagus sylvatico L.). J. Exp. Bot. 17:596-611.

Gepstain, S. and I. Ilan, 1970. A promotive action of kinetin on amylase activity in cotyledons of Phaseolus vulgaris. Plant Cell Physiol. 11:819-822.

Harvey, B. M. R. and A. Oaks, 1975. The role of gibberellic acid in the hydrolysis of endosperm reserves in Zea mays. Planta 121:67-74.

Hayashi, T., 1940. Biochemical studies on "Bakanae" fungus of rice. VI. Effect of gibberellin on the activity of amylase in germinating cereal grains. Bull. Agr. Chem. Soc. Japan 16:531-538.

Huang, A. H. C., and H. Beevers, 1974. Developmental changes in endosperm of germinating castor bean independent of embryonic axis. Plant Physiol. 54:277-279.

Ihle, J. N. and L. S. Dure, III, 1972. The developmental biochemistry of cottonseed embryogenesis and germination. III. Regulation of the biosynthesis of enzymes utilized in germination. J. Biol. Chem. 247:5048-5055.

Ikuma, H. and K. V. Thimann, 1963. Activity of gibberellin 'D' on the germination of photosensitive lettuce seeds. Nature 197:1313-1314.

Jacks, T. J. and N. A. Alldridge, 1967. Use of mercuric acetate to remove cysteine interference in the isocitrate lyase assay. Anal. Biochem. 18:378-381.

Jacobsen, J. V. and J. E. Varner, 1967. Gibberellic acid-induced synthesis of protease by isolated aleurone layers of barley. Plant Physiol. 42:1596-1600.

Jarvis, B. C., B. Frankland and J. H. Cherry, 1968. Increased nucleic-acid synthesis in relation to the breaking of dormancy of hazel seed by gibberellic acid. Planta 83:257-266.

Jensen, E. V., M. Numata, P. I. Brecher, and E. R. DeSombre, 1971. Hormone-receptor interaction as a guide to biochemical mechanism. In: R. Smellie, ed., The Biochemistry of Steroid Hormone Action. Academic Press, New York. pp 133-159.

Johnson, K. D. and H. Kende, 1971. Hormonal control of lecithin synthesis in barley aleurone cells: Regulation of the CDP-choline pathway by gibberellin. Proc. Nat. Acad. Sci. U.S. 68:2674-2677.

Jones, R. L., 1971. Gibberellic acid-enhanced release of Beta-1, 3-glucanase from barley aleurone cells. Plant Physiol. 47:412-416.

Kagawa, T., J. M. Lord, and H. Beevers, 1973. The origin and turnover of organelle membranes in castor bean endosperm. Plant Physiol. 51:61-65.

Kahn, A. A., 1971. Cytokinins: Permissive role in seed germination. Science 171:853-859.

Kende, H. and G. Gardner, 1976. Hormone binding in plants. Ann. Rev. Plant Physiol. 27:267-290.

Kennedy, I. R. and M. J. Dilworth, 1963. Activation of isocitrate lyase and triosephosphate dehydrogenase in Azotobacter vinelandii extracts. Biochim. Biophys. Acta 67:226-234.

Kirsop, B. H. and J. R. A. Pollock, 1958. Studies in barley and malt. XII. Interaction between the embryo and the endosperm of barley during malting. J. Inst. Brew. 64:227-233.

Konjevic, R., D. Grubisic, R. Markovic, and J. Petrovic, 1976. Gibberellic acid-binding proteins from pea stems. Planta 131:125-128.

Kornberg, H. L. and S. R. Elsden, 1961. Metabolism of new carbon compounds by microbes. Advan. Enzymol. 23:401-465.

Locker, A. and I. Ilan, 1975. On the nature of the hormonal regulation of amylase activity in cotyledons of germinating peas. Plant and Cell Physiol. 16:449-454.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

MacLeod, A. M., J. H. Duffus, and D. J. L. Horsfall, 1966. The embryo as an activator of gibberellic acid induced alpha-amylase. J. Inst. Brew. 72:36.

MacLeod, A. M. and G. H. Palmer, 1967. Gibberellin from barley embryos. Nature 216:1342-1343.

MacLeod, A. M. and G. H. Palmer, 1969. Interaction of indole acetic acid and gibberellic acid in the synthesis of alpha-amylase by barley aleurone. New Phytol. 68:295-304.

Marcus, A. and J. Velasco, 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. J. Biol. Chem. 235:563-567.

Matthysse, A. G. and M. Abrams, 1970. A factor mediating interaction of kinins with the genetic material. Biochim. Biophys. Acta. 199:511-518.

Matthysse, A. G. and C. Phillips, 1969. A protein intermediary in the interaction of a hormone with the genome. Proc. Natl. Acad. Sci. (U.S.) 63:897-903.

Mayer, A. M. and Y. Shain, 1974. Control of seed germination. Ann. Rev. Plant Physiol. 25:167-193.

McFadden, B. A. and W. V. Howes, 1963. Crystallization and some properties of isocitrate lyase from _Pseudomonas indigofora_. J. Biol. Chem. 238:1737-1742.

Musgrave, A., S. E. Kays, and H. Kende, 1969. _In vivo_ binding of radioactive gibberellins in dwarf pea shoots. Planta 89:165-177.

Nyman, B., 1971. Light, seed coat and gibberellic acid in relation to the amylase activity in germinating scots pine seeds (_Pinus sylvestris_). Physiol. Plant. 25:112-117.

Olson, J. A., 1959. Purification and properties of yeast isocitrate lyase. J. Biol. Chem. 234:5-10.

O'Malley, B. and W. T. Shrader, 1976. The receptors of steroid hormones. Science 234:32-43.

Paleg, L. G., 1960. Physiological effects of gibberellic acid: I. on carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. 35:293-299.

Penner, D. and F. M. Ashton, 1966. Proteolytic enzyme control in squash cotyledons. Nature 212:935-936.

Penner, D. and F. M. Ashton, 1967. Hormonal control of isocitrate lyase synthesis. Biochim. Biophys. Acta 148: 481-485.

Penney, D., 1972. Multicompartment analysis of the response of lupin hypocotyl segments to the addition and removal of indole-3-acetic acid. Can. J. Bot. 50:2015-2021.

Pharis, R. P. and C. C. Kuo, 1976. Physiology of gibberellins. Can. J. For. Res. 36:(in press).

Pinfield, N. J., 1968a. The effects of gibberellin on the metabolism of ethanol-soluble constituents in the cotyledons of hazel seeds (Corylus avellana L.). J. Exp. Bot. 19:452-459.

Pinfield, N. J., 1968b. The promotion of isocitrate lyase activity in hazel cotyledons by exogenous gibberellin. Planta 82:337-341.

Presley, H. J. and L. Fowden, 1965. Acid phosphatase and isocitratase production during seed germination. Phytochem. 4:169-176.

Protempa, L. A. and A. G. Galsky, 1973. The action of cyclic-AMP on GA_3 controlled responses. V. Similarities in the induction of isocitrate lyase in hazel seeds by giberellic acid and cyclic-3', 5' -adenosine monophosphate. Plant Cell Physiol. 14:989-995.

Radin, J. W. and R. N. Trelease, 1976. Control of enzyme activities in cotton cotyledons during maturation and germination. I. Nitrate reductase and isocitrate lyase. 57:902-905.

Radley, M., 1959. The occurrence of gibberellin-like substances in barley and malt. Chem. and Ind. 27:877-878.

Radley, M., 1967. Site of production of gibberellin-like substances in germinating barley embryos. Planta 75: 164-171.

Radley, M., 1969. The effect of the endosperm on the formation of gibberellin by barley embryos. Planta 86: 218-223.

Schrauwen, J. A. M. and E. Sondheimer, 1975. The development of organelles in embryos of Fraxinus americana. Z. Pflanzenphysiol. Bd. 75.S.67-77.

Simmonds, J. A. and G. M. Simpson, 1971. Increased participation of pentose phosphate pathway in response to afterripening and gibberellic acid treatment in caryopses of Avena fatua. Can. J. Bot. 49:1833-1840.

Simpson, G. M., 1965. Dormancy studies in seed of Avena fatua. 4. The role of gibberellin in embryo dormancy. Can. J. Botany 43:793-816.

Simpson, G. M., 1966. The suppression by (2-chloroethyl) trimethylammoniumchloride of synthesis of a gibberellin-like substance by embryos of Avena fatua. Can. J. Bot. 44:115-117.

Smith, R. A. and I. C. Gunsalus, 1954. Isocitratase: A new tricarboxylic acid cleavage system. J. Amer. Chem. Soc. 76:5002-5003.

Smith, R. H., A. M. Schubert, and C. R. Benedict, 1974. The development of isocitrate lyase activity in germinating cotton seed. Plant Physiol. 54:197-200.

Snedecor, G. W. and W. G. Cochran, 1967. One-way classifications. Analysis of variance. In: Statistical Methods. Iowa State University Press, Iowa. pp 258-298.

Stoddard, J., W. Breidenbach, R. Nadeau, and L. Rappaport, 1975. Selective binding of ^3H gibberellin A_1 by protein fractions from dwarf pea epicotyls. Proc. Natl. Acad. Sci. (U.S.) 71:3255-3259.

Tester, C. F., 1976. Control of the formation of isocitrate lyase in soybean cotyledons. Plant Sci. Lett. 6:325-333.

Yomo, H., 1960. Amylase-activating substance. II. Amylase-activating substance in the culture solution of barley embryo and the extraction of barley green malt. Hokko Kyokaishi 18:494.

Yomo, H. and H. Iinuma, 1966. Production of gibberellin-like substance in the embryo of barley during germination. Planta (Berl.) 71:113-118.

Yomo, H. and J. E. Varner, 1973. Control of the formation of amylases and proteases in the cotyledons of germinating peas. Plant Physiol. 51:708-713.

APPENDIX

Table 1. The effect of removal of the embryo on development of isocitrate lyase activity[1] of gametophytes.

Days of Incubation	Seed Treatment		
	With Embryos	Without Embryos[2]	With Embryo 2 Days
2	0.051 ± 0.012	0.029 ± 0.009	0.047 ± 0.007
4	0.088 ± 0.014	0.067 ± 0.008	0.099 ± 0.004
6	0.094 ± 0.009	0.080 ± 0.017	0.097 ± 0.005
8	0.127 ± 0.006	0.057 ± 0.007	0.053 ± 0.009

[1]Specific activity (I.U./mg protein). Average of three values ±1 standard deviation.

[2]Sterilized gametophyte halves after embryo removed.

Table 2. The effect of removing the embryo, and the effect of sterilizing after excision of the embryo on the development of isocitrate lyase activity[1] of gametophytes.

Days of Incubation	Seed Treatment	
	With Embryo	Without Embryos[2]
2	.038	.038
4	.049	.073
6	.065	.081
8	.089	.072

[1]Specific activity (I.U./mg protein). Each number is an average of two values.

[2]Sterilized before embryo excised.

Table 3. The effect of an embryo diffusate on the development of isocitrate lyase[1] activity of gametophytes when the pretreatment[2] soak includes rapid shaking.

Days of Incubation	Pretreatment	
	Water	Embryo Diffusate
2	0.019 ± 0.002	0.022 ± 0.004
4	0.052 ± 0.002	0.057 ± 0.001
6	0.060 ± 0.017	0.073 ± 0.007
8	0.058 ± 0.006	0.069 ± 0.007
10	0.045 ± 0.015	0.046 ± 0.012

[1]Specific activity expressed as I.U./mg protein. Each number is the average of three values ±1 standard deviation.

[2]Pretreatment refers to a five hour soak with rapid shaking (Burrell shaker rheostat setting No. 7) in solution.

Table 4. Analysis of variance for day six of experiment showing effects of sterilization on isocitrate lyase activity (graphically represented in Figure 5).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Treatments[1]	2	8.18×10^{-4}	4.09×10^{-4}	38.33[2]
Error	6	0.64×10^{-4}	1.07×10^{-5}	
Total	8	8.82×10^{-4}		
T_1 versus T_3	1	1.07×10^{-5}	1.07×10^{-5}	1.0
T_1 versus T_2	1	8.82×10^{-5}	8.82×10^{-5}	8.27[3]

[1] T_1, sterilized before embryo excision (□); T_2, sterilized after embryo excision (■); T_3, sterilized before embryo excision with 1 min. H_2O soak after excision (▲).

[2] Significant at P = 0.005.

[3] Significant at P = 0.05.

Table 5. Analysis of variance for day four and day six of experiment showing effects of embryo excision on isocitrate lyase activity (graphically represented in Figure 6).

	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Day 4	Treatments[1]	3	3.43×10^{-3}	1.14×10^{-3}	25.28[2]
	Error	8	3.62×10^{-4}	4.53×10^{-5}	
	Total	11	3.79×10^{-3}		
	T_2 versus T_1	1	1.71×10^{-4}	1.71×10^{-4}	3.77[3]
	T_3 versus T_4	1	1.57×10^{-4}	1.57×10^{-4}	3.46[3]
Day 6	Treatments	3	2.49×10^{-4}	8.31×10^{-5}	27.15[2]
	Error	8	2.45×10^{-4}	3.06×10^{-5}	
	Total	11	4.94×10^{-4}		
	T_3 versus T_1	1	1.07×10^{-4}	1.07×10^{-4}	3.49[3]
	T_3 versus T_4	1	2.67×10^{-4}	2.67×10^{-4}	8.71[4]

[1] T_1, germinated with embryo (○); T_2, germinated without embryo (□); T_3, embryo excised after 2 days (△); T_4, embryo excised after 2 days and gametophyte halves sterilized (▲).

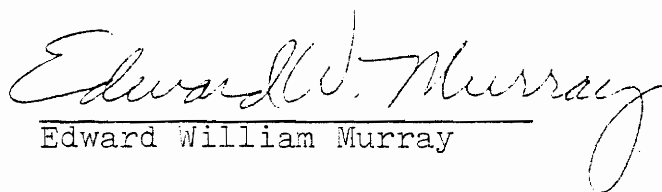
[2] Significant at $P = 0.005$.

[3] Significant at $P = 0.10$.

[4] Significant at $P = 0.025$.

VITA

Edward William Murray, born in Morgantown, West Virginia on January 19, 1947, received a Bachelor of Science in Biology on June 3, 1972 from George Mason University in Fairfax, Virginia. He worked for two years as a research technologist in microbiology at the Medical College of Virginia, Richmond before returning to school. In January, 1975, he entered Virginia Polytechnic Institute and State University to work toward a Master of Science in Forestry and Forest Products. He is a member of Phi Sigma National Biological Society and the Society of American Foresters.



Edward William Murray

EMBRYONIC CONTROL OF ISOCITRATE LYASE
ACTIVITY IN THE MEGAGAMETOPHYTE
OF PONDEROSA PINE SEEDS

by

Edward William Murray

(ABSTRACT)

An earlier publication presented evidence for enhancement of isocitrate lyase (threo-L_s isocitrate glyoxylate lyase, EC 4.1.3.1) activity by a factor that diffuses into the megagametophyte from the embryo of stratified germinating *Pinus ponderosa* seeds. In contrast, the data reported here indicate the rate of increase in specific activity of embryoless seeds was greater during the first four days than in seeds with embryos left *in situ*. A similar pattern of activity was observed in seeds which retained their embryos for two days to allow germination to occur prior to excision, but the peak activity was slightly higher. The highest enzyme activity was achieved in seeds which retained their embryos the longest. A model is included to resolve these apparently diverging effects of the embryo on isocitrate lyase activity.

A diffusate prepared from two day germinated embryos had no significant influence on the enzyme activity of embryoless seeds.

The phenomena reported here were not dependent on stratification. Imbibition was the only prerequisite for germination and development of enzyme activity to occur.