STUDIES ON PHENYLALANINE AMMONIA-LYASE ACTIVITY IN CULTURES OF PINUS ELLIOTTII

by

Yiu-Lam Lau

A THESIS

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in partial fulfillment of the
requirement for the
degree of

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In memory of my mother
who gave me things that I am thankful for.

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) activity increased 10- to 15-fold in pine callus tissue upon subculturing to new medium. The stimulation of PAL activity was maximum 2 days after transfer. The increase in PAL activity was followed by a subsequent decrease in activity. PAL activity in the callus tissue also was stimulated by premature subculturing if the subculturing was done after PAL activity had begun to decline. The stimulation of PAL activity was only moderately affected by cycloheximide, whereas, the decay in PAL activity was sensitive to cycloheximide. The increase in PAL activity was sensitive to temperature with low temperatures delaying the stimulation of PAL activity. Low temperatures also prevented the decay of activity. Sucrose was the principle nutrient that affected PAL activity. Under laboratory conditions physical injury, light and dark conditions did not affect PAL activity.

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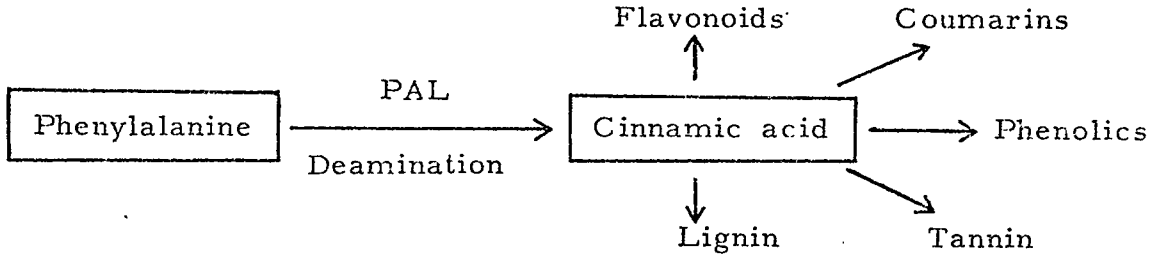
INTRODUCTION

Since its discovery in 1961 by Koukol and Conn (69) phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5) has been shown to be a key enzyme in secondary plant metabolism. The product formed from the reaction, cinnamic acid, is a key branch compound in the secondary metabolism of plants



In addition, PAL activity has been shown to greatly influence protein synthesis in leukemic cells (1). Therefore, the enzyme may influence not only aromatic biosynthesis but also protein synthesis in plants. PAL activity has been shown to exhibit drastic changes in plants, often in different plant parts, during a considerably short time. This rapid change can be brought about by physical or environmental changes and is of great interest to plant physiologists as well as biochemists.

The interest in PAL activity in Dr. Joe R. Cowles' laboratory was derived from the key position of this enzyme in relation to

protein and aromatic biosynthesis. Several reports (45, 56, 89, 112) have correlated PAL activity with the synthesis of various aromatic end-products including lignin. Lignin is of particular interest in the laboratory because the synthesis of this structural polymer is influenced by gravity (94). Since PAL activity has been shown to be related to aromatic biosynthesis then it probably influences gravity-mediated lignin synthesis. We are interested in determining as much about the regulation of PAL activity in slash pine (a gymnosperm) as possible especially since the regulation of aromatic biosynthesis in this major plant genus is essentially unknown.

Tissue cultures were used to investigate PAL activity in slash pine as a model for subsequent research in pine seedlings. In many cases the physical and chemical manipulation of callus tissue is easier than in whole plants. Also there is less possible interference from microorganisms due to the asceptic condition required for tissue culture experimentation.

The present study involves the effect of temperature, light, physical injury, cycloheximide, sucrose, and other medium constituents on the change in PAL activity in pine cultures. Cycloheximide is chosen for the investigation of de novo protein

synthesis in PAL induction (11, 15, 19). Sucrose is chosen because it is a major carbohydrate transported and metabolized by higher plants and it has been shown to enhance PAL activity in various tissues (15, 17, 19, 109). The data illustrate the influence of these factors on PAL activity. Since PAL and secondary metabolism can be affected by the same physical and chemical parameters the results provide insight on the regulation of secondary metabolism in a gymnosperm tissue. The data will be useful for further studies on this system and on the intact seedlings.

REVIEW OF LITERATURE

Occurrence in organisms

The first demonstration of phenylalanine ammonia-lyase (PAL) activity was by Koukol and Conn (69) in Hordeum vulgare in 1961. Since then PAL has been reported in many higher plant tissues including bamboo (56), gherkin (26), sorghum (95, 96), potato tuber (115), mustard (92), maize (73), buckwheat (6), soybean (45), tobacco (80), grapefruit (87), castor bean (34), pea (38), strawberry (109), parsley (47), and dwarf pea (16). PAL has been reported in higher crytogams (111) and recently in the algae, Porphyridium (15). PAL also has been studied in the fungi Ustilago hordei (98) and Rhizoctonia solani (65) and reported in other basiodiomycetous fungi (recently reviewed by Vance, Bandon, and Towers, 103). PAL activity has not been established in Ascomycetes although Aspergillus candidus has been shown to contain flavonoids (72). In some cases the inability to demonstrate PAL activity may be due to the presence of endogenous inhibitors in the extracts as was demonstrated in Chrysanthemum extracts (110).

Enzymological properties

PAL catalyzes the deamination of L-phenylalanine yielding

trans-cinnamic acid and ammonia. The enzyme does not require a cofactor for activity and is generally specific for phenylalanine. In some plants, however, the enzyme can also deaminate L-tyrosine to trans-p-coumaric acid. The enzyme which catalyzes the latter reaction, tyrosine ammonia-lyase (TAL), is considered to be a separate enzyme since PAL and TAL activities have been separated by purification (66, 77, 79). In addition, PAL and TAL exhibited different pH curves (79). In wheat, the PAL / TAL ratio varied from 4 to 20 (110) and in Sporobolomyces roseus from 1.35 to 5 (14) during purification. In maize, however, a constant ratio of PAL/TAL activity was found during purification and both activities were lost at the same rate on treatment with $NaBH_4$ (55). In Sporobolomyces pararoseus, TAL activity co-purified with PAL activity throughout a 450-fold purification of PAL (82). In view of the reports on PAL and TAL co-purification one can generally conclude that some PAL preparations display catalytic activity towards tyrosine.

One caution concerning the use of acetone powder as a source of enzyme for PAL assay is that it may give a positive test in the absence of added substrate due to endogenous phenylalanine (84). These extracts also were a good source of bound cinnamic acid

which could be released resulting in pseudo PAL activity especially in the spectrophotometric assay.

Based on studies using stereospecifically labelled phenylalanine it has been established that during the PAL reaction a pro-S proton along with ammonia is eliminated from C-3 of L-phenylalanine in an anti-periplanar fashion to generate trans-cinnamic acid (51, 52, 60). The enzyme reaction has been demonstrated to be reversible *in vitro* (97) but not *in vivo*.

Molecular weights reported for PAL include: 330,000 daltons, potato (53), 306,000 daltons, maize (73), 300,000 daltons, mustard (92), and 226,000 daltons, *Streptomyces verticillatus* (10, 26). In mustard (92) and potato (53) aggregate forms also have been reported. The aggregated form had a molecular weight twice that of the unaggregated form.

PAL isozymes have been reported (13, 78) which have different sensitivities to phenolic inhibitors. In general, however, isozymes have not been demonstrated as illustrated with PAL studies on dark and light-grown buckwheat plants (6), mustard (92), and *Glycine max* tissue cultures (45). These systems have been examined electrophoretically and demonstrated to contain a single enzyme.

In potato (54), maize (55), and tobacco (80), cyanide and $NaBH_4$ irreversibly inhibit PAL activity. Tritiated alanine was produced when the hydrolysis of purified potato or maize PAL was treated with tritiated $NaBH_4$ (50, 54). This supports the presence of a dehydroalanyl residue at the active site.

The Km values for phenylalanine in the PAL reaction range from 0.3×10^{-4} M to 1.5×10^{-2} M (54, 73, 78, 79). When D-phenylalanine, a competitive inhibitor, was added the Vmax was not reduced and the reaction maintained Michaelis-Menten kinetics. This behavior is best explained by an allosteric interaction and the presence of different conformations of the enzyme . When D-phenylalanine binds to the enzyme a single conformation predominated which , in turn, exhibits simplified kinetics. Cinnamic acid also is a strong inhibitor of PA L activity and is most effective at pH 7.0 in potato (54), and tobacco (80) which have pH optima of 8 and above. PAL prepared from barley (69) but not from potato (54) and maize (73)was sensitive to sulfhydryl inhibitors. Flavonoids were also inhibitory to the enzyme.

Synthesis and Decay of PAL

In many experiments involving light induction of PAL activity a lag in increased PAL activity is observed . Rissland and Mohr

(88) attempted to explain this in terms of gene activation. They suggested that part of the lag phase was needed in order to make the potentially active genes accessible for the action of phytochrome. They proposed that the initial activation (1.5 hr in mustard seedlings) the "potentially active genes (P_{730})" were being made and became accessible for the action of P_{730}. These workers also have shown that a second far-red illumination (6 to 12 hours after the first) was effective in increasing PAL activity without a lag, indicating that the gene was still active from the first far-red illumination.

In many PAL induction systems there is a decrease in PAL activity from the induced maximum even in continuous light. This indicates a decay in PAL activity and has been shown to be prevented by cycloheximide. Cycloheximide also prevented the induction of PAL activity and the loss of induced activity. Based on these results, PAL activation and inactivation appears to be dependent upon protein synthesis. Studies on Xanthium (114) showed that placing leaf discs in the dark quickly decreased PAL activity while the uptake of ^{14}C-labelled amino acid into PAL continued. Therefore, it appears that in the dark there is still synthesis of the PAL protein despite the decrease (inactivation) in PAL activity.

Blondel and others (11) have shown the presence of inactive and active forms of PAL in etiolated and light-grown radish cotyledons, respectively. The two forms were isolated by affinity chromatography and showed complete antigenic identity. French and Smith (33) have isolated a factor capable of inactivating PAL reversibly in vitro in extracts of gherkin hypocotyls. The kinetic results showed the formation of a freely reversible complex between the inactivator and the enzyme.

The control of PAL activity in gherkin seedlings (29, 30) appears to be more complex than in potato. There is the typical increase in PAL production after irradiation of dark-grown seedlings followed by a decrease in activity. Then if the seedlings were given a cold treatment (4 °C) in the dark followed by a treatment at 25 °C, a second maximum in PAL activity appeared. Cycloheximide did not inhibit this response when applied prior to transfer to higher temperature. From the above observation, Engelsma (30) postulated a cold shock release of PAL from an inactive enzyme-inhibitor complex which was predominant at higher-temperatures. This postulation is not as satisfactory as the inactivating system mentioned by Zucker (114) since one would expect an increase in enzyme activity in time (24 hours) at low temperature (4 °C) which does not appear

to be the case.

Localization of PAL

Recently more interest has been focused on the localization of PAL in plant tissues. In most of the systems studied so far PAL is considered to be a soluble enzyme. The presence of PAL isozymes, however, suggests the possible compartmentalization and localization of the enzyme. In addition, the differential sensitivity of PAL towards different C_6 - C_1 and C_6-C_3 compounds suggests different forms of PAL controlling different pathways and possibly located in different organelles.

Saunders and Mc Clure (91) showed that PAL was present in intact barley plastids. From a continuous sucrose gradient centrifugation they showed that PAL activity was correlated with chlorophyll content. Additional support of plastids localization of PAL activity was through a positive phytochrome effect on PAL activity in the plastids.

Czichi and Kindl (20) have shown that PAL is associated with a microsomal fraction isolated from potato tuber. This fraction contains a membrane-bound PAL activity and cinnamate p-hydroxylase activity as demonstrated by the conversion of o- and p-coumaric acid from phenylalalnine. This report suggests

a possible multiple enzyme complex as postulated by Stafford (96). Recently Gregor (35) demonstrated the distribution of PAL activity in castor bean endosperm. These studies have shown that up to 13% of the PAL activity is found in the particulate fraction. The PAL activity was shown to be associated with endoplasmic reticulum, mitochondria, and glyoxysomes using enzyme markers. The association of PAL with more than one cellular organelle could be general adsorption of PAL to membranes. However, according to Stafford (96) the presence of PAL in more than one organelle appears likely.

Light Effect

In general, light has a pronounced effect on PAL activity in plant seedlings as well as tissue culture. This effect was first shown in a classic study by Zucker (112) who demonstrated that light induced the production of PAL in sliced potato tuber tissue. Although the light effect is rather common, there are exceptions. For example, PAL activity in excised axes of Phaseolus vulgaris (105), grapefruit peel (87), and roots of several plants (85) is insensitive to light.

Although white light is effective in PAL induction there are specific regions of the light spectrum which are responsible for effecting PAL activity. The most effective regions are the red,

far-red, blue, and ultraviolet region. The phytochrome control of PAL activity has been shown in gherkin seedlings (28), parsley cell suspension (108), barley shoot (75), and isolated barley plastids (91). In the barley shoot system (75) McClure demonstrated that 30 sec. of 1.1 kerg.cm.$^{-2}$.sec^{-1} red light was sufficient to saturate the response. Further illumination gave similar but no addition stimulation. The action spectra for PAL activity in barley shoots (76) show peaks at 420, 620, and 660nm.

Even in the same system there are discrepancies in the action of phytochrome in the induction of PAL activity. For example, Bellini and Hillman (8) working with mustard and radish seedlings were unable to show a correlation between PAL activity and far-red form of phytochrome (Pfr). Schopfer and Mohr (93), however, claim that 5 min. irradiation of red light induced PAL formation. They state that Pfr (ground state) cannot be distinguished from the effective form, P·fr (excited state).

In addition to phytochrome responses, wavelengths in the blue region also can induce PAL activity. Engelsma (26, 27) found that gherkin seedlings responded to blue light and red and far-red light differently. A higher level of PAL activity could be induced in blue light than in red and far-red light. Also high

intensity blue light induced a new maximum of PAL activity in plant that were insensitive to further red and far-red irradiation. The reverse sequence of irradiation (high intensity far-red after high intensity blue light) did not give rise to a second maximum. In pea pods (38), psoralen compounds increased PAL activity 6-fold when the pods were activated by 366 nm light but not with psoralen compounds or 366 nm light alone.

Recently, Engelsma (31) suggested a possible role of ultraviolet and blue light in changing PAL activity in gherkin hypocotyl. This involved the conversion of trans-hydroxycinnamic acid to the less inhibitory cis-hydroxycinnamic acid. The effect of UV light was direct in that a trans-cis conversion of hydroxycinnamic acid could be observed in the absorption spectra. The blue light effect could be indirect since a photoreceptor was required for photoisomerization of hydroxycinnamic acid. Riboflavin, a possible photoreceptor, at a concentration of 2×10^{-6}M was effective in the photoconversion. Since both riboflavin and hydroxycinnamic acid may not be present in the same cell compartments, the effect of blue light may not be observed in all tissues. In the gherkin seedlings, however, a slight shift to lower wavelengths near the 250 nm peak in seedlings irradiated with blue light may be an indication of photoconversion. In

addition, 5% of the p-coumaric acid present in the hypocotyl treated with blue light was in the cis-form which was absent in dark-grown seedling or in seedlings irradiated with red light only.

In strawberry leaf discs (109), the action spectrum for PAL induction resembled that of photosynthesis, and therefore was an exception to the typical system. The effective wavelengths were 475 nm and 625 nm. In this system ultraviolet and far-red light failed to induce PAL activity. In the presence of 25 um 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) the light effect on PAL was absent.

Effect of Wounding and Infection

Increased PAL activity is commonly associated with wounding and infection. The effect of wounding on stimulation of PAL activity has been demonstrated in several plant species including gherkin (28), buckwheat (4), potato tuber (59), sweet potato (74), and strawberry leaf discs (109). A lag period after wound induction was present in all systems. The agent responsible for increased PAL activity may be ethylene since it is known to be produced as a result of wounding and has been shown to enhance PAL activity.

Another type of wound inductor is high energy radiation. Gamma radiation was shown to induce PAL in potato (buds only)

(83) and citrus fruit peel (86). UV light could be considered as another type of wound inducing radiation. The effect of UV light on PAL activity has been demonstrated in pea (41) and other systems as discussed in a previous section on light effects.

Infection of plants by pathogens also induces PAL activity. The infection of pinto bean leaves with alfalfa mosaic virus (104) resulted in stimulation of PAL activity that peaked around 2 days. Induction of PAL activity in Bramley's seedling apples infected with Nectria galligena Bres. peaked 8 days after infection (100). Interestingly, the PAL activity peaked in 5 days after infusion of a protease extracted from rotten apples. The protease then seemed to be an inducing agent. Penicillium expansum Thom. which does not produce proteolytic enzymes failed to induce PAL activity. In addition, when sensitive and resistant tomato plants were infected with Verticillium albo-atrum (37) only the resistant tomato plant exhibited a fast stimulation of PAL activity (peaking at 1 hour after infection). From the above evidence, the increased PAL activity appears to be related to a defense mechanism and may be induced via a protease. Ethylene, however, cannot be ruled out as a possible inducing agent associated with the wounding response.

Effect of hormone, growth modifiers, and other compounds on PAL

Plant hormones have been reported to have an effect on secondary metabolism. Gibberellic acid which is known to promote elongation in dwarf pea also was shown to affect PAL activity and lignification (16). The percentage of lignin in stems of light-grown dwarf peas was increased by gibberellic acid treatment while that of tall (normal) pea was not. In addition, PAL activity was higher in gibberellin-treated dwarf plants grown under white or red light than in untreated dwarf plants. Gibberellic acid, however, had no effect on PAL activity or lignification in plants which were grown in the dark. Therefore, in gibberellin-deficient pea plants (dwarf peas) PAL activity seems to be one of the limiting factors involved in lignification. Abscisin II , which is a gibberellin antagonist caused an increase in PAL activity in excised bean axes (105). The possible action could be the synthesis of a PAL species less affected by the endogenous inhibitor since abscisin II stimulates PAL activity before the period in which the inhibitor is presumably synthesized . Ten and 100 μM gibberellic acid has been shown to inhibit *in vitro* PAL activity 18 and 27% respectively using an acetone extract from corn (64). In the same report (64) abscissic acid showed different effects on *in vivo* PAL activity levels: 103% in *Setaria viridis*, -74% in

Cyperus esculentus, and -42% in Cyperus rotundus. Abscisic acid (100μM) inhibited in vitro PAL activity 28% in acetone preparations of corn (64).

Indole acetic acid (IAA) and tryptophan have been shown to inhibit PAL activity in tobacco tissue cultures (63). 2,4-Dichlorophenoxyacetic acid (2,4-D) ($5x10^{-6}$M and $1x10^{-4}$M) retarded the development of PAL activity with a concomitant delay in polyphenol accumulation in dark-grown Paul's scarlet rose suspension cultures (21). PAL activity in the suspension cultures became less sensitive to 2,4-D if the inhibitor was added after PAL activity had begun to increase. Polyphenol accumulation, however, was still sensitive to high levels of 2,4-D. It appears then that 2,4-D may adversely affect other components of the biosynthetic pathway in addition to PAL activity.

Pyranyl benzyladenine (PBA), a cytokinin, was demonstrated to increase in vivo PAL activity 4.5-fold in pigweed extracts (64) 2 days after treatment. In a corn acetone preparation, however, PBA inhibited in vitro activity 23% and 27% at 10 and 100 μM concentrations, respectively. In another example (112) 10^{-4}M kinetin had no effect on PAL activity of excised radish cotyledons. Therefore, the effect of cytokinins on PAL activity varies greatly and appears to be tissue specific.

In general, exogenous ethylene stimulates PAL activity but the effective concentration varies considerably in different plants. One hundred ppm is required for maximum induction in citrus fruit peel (87) while 4 ppm is effective in swedes (85). In gherkin, excised (but not intact) hypocotyls were sensitive to ethylene (32). Matsushita and Uritani (74) working with sweet potatoes suggest that the ethylene effect is dependent on protein synthesis and that removal of carbon dioxide further enhances the effect of ethylene on PAL activity. In rice, ethylene production was shown to be inhibited and enhanced by red light and far-red light, respectively (61). This implies that in some systems far-red light may influence PAL activity via ethylene production.

Overall there are not enough data to establish a definitive effect of plant hormones on PAL activity, especially since the systems have complex interactions through hormone interaction.

Other effectors of PAL activity

In addition to plant hormones a number of other compounds have been investigated relative to their effect on PAL activity. In Avena coleoptile segments (81) p-fluorophenylalanine, an amino acid analogue, enhanced coleoptile elongation, caused an increase in fresh weight and inhibited PAL activity. The authors suggested that decrease in the phenol production rendered the plant less

toxic and therefore stimulated growth. In radish cotyledons (67) actinomycin D, cycloheximide and D- and L- threo-chloramphenicol inhibited far-red light stimulated PAL activity. In pea pods (40), poly-L-lysine induced PAL activity. Poly-L-lysine in combination with actinomycin D and bovine pancreatic ribonuclease A (RNase) further induced PAL activity. Bovine histones and spermidine but not poly-L-arginine were moderate positive effectors of PAL induction when applied singly. When applied in combination with actinomycin D and RNase, they diminish PAL induction caused by the latter compounds. In pea pod system (39) chlorpromazine and 16 other phenothiazine derivatives, all related to quinacrine [a deoxyribose nucleic acid (DNA) intercalating agent], induced up to 11 fold increase in PAL activity. Fungicides such as benomyl, carbendazim, and carboxin (99) decreased PAL activity in barley seedlings. Herbicides such as diuron, dalapon, amiben, and chloropropham (64), tested on Setaria viridis, Amaranthus retroflexus, Cyperus esculentus, and Cyperus rotundus, were shown to reduce PAL activity. Compounds related to secondary metabolism including p-coumaric acid, trans-cinnamic acid, caffeic acid, syringic acid, sinapic acid, and tri-iodobenzoic acid showed in vitro inhibition ranging from 24-83% in acetone extracts from corn (64). In a recent report, Lamb and Rubbery (70) show showed that

exogenous supplies of phenylalanine, cinnamic acid, and p-coumaric acid could inhibit the appearance of PAL activity in potato tuber discs. The metabolic derivatives of phenylalanine were the active PAL-inactivating agents and not the amino acid per se.

Effect of Carbohydrate Level

In green leaf discs of strawberry the production of PAL required not only light but also carbon dioxide (17). The light and carbon dioxide requirements could be substituted for by floating the discs on a sucrose solution. The leaf discs responded to 5 mM sucrose up to an optimal concentration of 150 mM sucrose. In green buckwheat leaf discs, sucrose, glucose, and fructose increased the level of PAL activity more than light alone (6). In dark-grown buckwheat seedlings, however, there was a lack of correlation between the level of PAL activity and the level of endogenous sugar (5). DCMU, which inhibited the light stimulation of PAL activity in green buckwheat tissues, had no effect on PAL induction in etiolated tissue. Based on the limited data from these systems it appears that PAL activity and carbohydrate metabolism is interrelated but in an indirect fashion.

Intrinsic factors affecting the level of PAL activity and plant development

PAL activity changes drastically in some plants during certain

stages of development. PAL activity is very low or absent in dormant seeds but appears soon after germination. PAL activity reaches a maximum in etiolated buckwheat (5) and in light and dark-grown Impatiens (106) 4 days after germination, and in etiolated radish cotyledons (9) 2.5 days after germination. In light-grown parsley suspension cultures the peak PAL activity coincided with the logarithmic phase of growth (49).

Even within the same plant, various regions have their own pattern of PAL development. For example, radish seedling (9) exposed to far-red light, showed peak PAL activity in the cotyledons and hypocotyls 2.5 and 4.5 days, while PAL activity in the roots was still increasing after 4 days. In germinating buckwheat (5) PAL activity in the cotyledons and hypocotyls peaked at 4 days but activity in the cotyledons sharply declined while that in the hypocotyls decayed slowly. These variations in PAL activity patterns in different parts of the plants clearly show the intrinsic factor inherent in plant secondary metabolism.

Relationship between PAL activity and phenolic metabolism

In many plant tissues there is a good correlation between PAL activity and synthesis of phenolic compounds. Infection of Bramley's seedling apples with Nectria galligena Bres. (100),

resulted in simultaneous stimulation of PAL activity and formation of benzoic acid. Irradiation of fresh potato tissue (112) resulted in a corresponding linear stimulation of PAL activity and chlorogenic acid production. Both processes were inhibited by cycloheximide. Similar results involving PAL activity and chlorogenic acid were obtained from swede tissues (85) and ethylene-treated sweet potato (62). In gherkin seedlings the accumulation of hydroxycinnamic acid was reflected by the changes in PAL activity induced by blue light (26), wounding (28), temperature change (30), and long term red light (27).

Various compounds further removed from the point of phenylalanine deamination show a correlation between their production and the level of PAL activity. In dark-grown corn seedlings (23) the accumulation of anthocyanin in roots and mesocotyls in continuous white light was correlated with the increase in PAL activity. The same phenomenon was shown in cell cultures of Glycine max (45). McClure (70) obtained action spectra for PAL activity and flavonoid synthesis in barley shoots that were similar to each other. Hahlbrock et al (48) show that the biosynthesis of flavone glycosides in cell suspension cultures of parsley exposed to light was correlated with an increase in PAL activity. Their study also included other enzymes involved in the biosynthesis pathway

of flavone glycosides . They reported three enzymes including PAL, cinnamic acid hydroxylase, and p-coumarate: CoA ligase which fall into one group (group I) having the same kinetics and five others including chalcone-flavonone isomerase, glucosyl-transferase, apiosyltransferase, UDP-apiose synthetase, and methyltransferase which fall into another group (group II) having different kinetics from the first group. Hahlbrock et al suggested from the time course studies of enzyme activities that group I enzymes exhibit primary control over flavone glycoside synthesis while the group II enzymes exhibits secondary control. Similarly, the treatment of parsley cell suspensions with UV light (45) showed a good correlation between group I enzymes and flavonoid glycoside accumulation. In Haplopappus gracilis cell cultures exposed to UV light (107) anthocyanin formation and PAL activity were correlated.

PAL activity also has been correlated with lignification in higher plants. Higuchi (56) has shown a good correlation between the regions of highest PAL activity and those undergoing rapid lignification. Areas that were already lignified have little PAL activity. In Coleus internode slices PAL activity followed the same kinetics as the formation of lignified wound vessel members (89). In soybean callus tissue, PAL activity is higher in cultures

which produced tracheid elements than those which did not produce tracheids (90). PAL activity and lignification, however, are not always correlated. Very little if any PAL activity was demonstrated in lignifying Eucalyptus leaves (57).

Whenever one interprets the results involving the correlation between PAL activity and certain phenolic compounds one has to be careful. First, the "normal" level of PAL activity could be sufficient for the required level of carbon skeleton and secondly, there are other phenolic derivatives produced by a particular tissue. In gherkin seedlings red light increased the hydroxycinnamic acid concentration without increasing PAL activity (27). The level of PAL activity in Melilotus alba was the same in strains which contain high or low levels of hydroxycinnamic acid (68). Furthermore, if strawberry leaf discs were bathed in 2.6 mM phenylalanine significant changes in the concentration of hydroxycinnamic acid were seen without any change in PAL activity (18). Zucker (112) also has shown that potato discs exposed to phenylalanine either in light or darkness contained less than half as much enzyme activity as those supplied only with water. Although the PAL level was decreased by phenylalanine in the dark, the discs synthesized more chlorogenic acid than the water controls. In light there was no decrease in chlorogenic acid content corresponding to the decrease in PAL activity. In radish seedlings

(9) there was no correlation between light-induced PAL activity and anthocyanin formation in any part of the seedling.

One criticism on the correlation between PAL activity and phenolic synthesis is that most of the assay involved grinding the tissue and assaying for PAL activity. However, this may not represent the actual *in vivo* enzyme activity. Recently, Amrhein *et al* (2, 3) working with buckwheat hypocotyl presented an assay for PAL in intact tissue. This assay relies on the liberation of $^{3}H\text{-}NH_2$ from L-(3-^{3}H) phenylalanine which was equilibrated with tissue water to yield ^{3}HOH. The ^{3}HOH is recovered by sublimation and counted by liquid scintillation. In the buckwheat system there was a difference between the intact tissue PAL activity and *in vitro* PAL activity in terms of the time of peak PAL activity after germination. This assay procedure may give a more accurate picture of the amount of enzyme functioning within the cells. When this assay is applied to other systems it may help to clear up some discrepancies between PAL activity and phenolic synthesis.

In conclusion, PAL is widespread in plants including lower plants. PAL is sensitive to the physiology of the plant and external physical and chemical parameters. Red, far-red, blue and UV light has pronounced effect on PAL activity in most systems.

The wounding and infection of plant tissue induces PAL activity. In addition, a number of chemicals including hormones, growth modifiers, phenolics and carbohydrate affect PAL activity to various extents. Moreover, there is an intrinsic factor governing the development of PAL activity in each system. In general, PAL is considered to be a soluble enzyme, however, recent reports indicate a part of the total PAL activity is located in cell organelles. Overall, there is good correlation between PAL activity and synthesis of various phenolic compounds.

MATERIALS AND METHODS

Preparation and growth of seedlings

Seeds of slash pine (Pinus elliottii, Engelm.) were germinated on moist Ottawa sand (deionized water) in shallow stainless steel trays covered with plastic film. The seeded trays were maintained at 28°C under Duro-Test Vita-Lite (TM), fluorescent tubes (1.77 mwatt. cm^{-2}, 400-725 nm, at tray level). Germinated seeds with radicle length of 2 to 4 mm were removed daily and planted in Petri dishes.

In preparation for planting, plastic Petri dishes were filled with Ottawa sand, covered with polyvinylidene film, and watered with deionized water. The germinated seeds were inserted, radicle first into the 16 holes inserted in the sand at evenly spaced intervals around the circumference of the dish. Subsequently, the seedlings were grown in a Percival I-35 environment chamber under Duro-Test fluorescent tubes (1.77 mwatt. cm^{-2}, 400-725 nm, at the sand level). The chamber was maintained on a light:dark cycle of 14:10 hr at a temperature cycle of 28:23 °C in the light and dark phases, respectively.

Initiation of pine tissue culture

One-week old pine seedlings (6 to 7 cm long) were used

for callus initiation. The pine hypocotyls were cut into three sections of approximately 2 cm each and surface sterilized (10 min) with a sterilant consisting of 20 ml of commercial Clorox and 2 ml of Triton X-100 in 200 ml of distilled water. The tissues were then rinsed four to five times in sterile distilled water and transferred to jars containing the growth medium. Three or four hypocotyl sections were transferred to each mason jar containing 30 ml of modified Murashige and Skoog medium containing 20% coconut milk (MM_1 2XCM) (Table I). The mason jars were covered with a sterile sheet of polypropylene and fastened into position with a rubber band. The callus tissues normally began initiation within one week and were ready for subculturing in four weeks. Once the callus was initiated the cultures were subcultured on 14 day intervals. Transfers were made to new MM_1 2XCM medium using approximately 2 gm of inoculum per jar. The surface sterilization and tissue transfers were carried out asceptically under a Laminar flow chamber.

Establishment of shake cultures

In certain experiments the pine callus tissue were grown in a liquid medium prior to extraction and subsequent analysis for PAL activity. In these experiments the callus tissue was

cut into small sections (4 mm cubes) and placed in a liquid medium. The cultures were gently shaken (approximately 75 rpm) for the required amount of time and then harvested by filtering on Whatman #1 filter paper.

Tissue extraction

An appropriate amount of pine tissue (typically 3 to 4 gm) was weighed and placed in a glass tissue homogenizer. An homogenizing buffer, approximately 2 times the tissue weight (volume to weight), was added to the homogenizer. The buffer contained 5 mmoles of borate buffer (pH 8.8), 1 gm of polyvinylpyrrolidone (PVP)-40 (soluble) and 5.5 gm of sucrose in a volume of 50 ml. The tissue was homogenized at full speed for 2 to 3 min using a teflon pestel attached to an electric drill. The resulting homogenate was allowed to stand in an ice bath for 30 min and then centrifuged at 12,000 xg for 15 min. The homogenization and centrifugation was carried out at approximately 4°C.

Batch purification

Phenylalanine ammonia-lyase (PAL) was purified for some experiments using saturated ammonia sulfate in 0.1 M borate, pH 8.8. The purification was carried out by slowly adding (dropwise) the ammonia sulfate solution to the crude extract with continuous stirring in an ice bath. The first ammonia sul-

fate fractionation was at 30% saturation and the resultant solution was stirred for an additional 30 min before centrifuging at 10,000 xg for 10 min. The supernatant was increased to 55% $(NH_4)_2SO_4$ saturation, stirred and centrifuged as before. The pellet (30 - 55% fraction) was redissolved in a minimum amount of 0.1 M borate (pH 8.8) buffer.

PAL assay

PAL activity was determined by a radioactive assay utilizing ^{14}C-labelled phenylalanine (21). A typical assay mixture contained 100 μmoles of borate buffer (pH 8.8), 100 nmoles of L-phenylalanine, 0.2 μCi of ^{14}C(U)-L-phenylalanine (460 mCi/mmole) (New England Nuclear), 1 μmole of mercaptoethanol and 0.1 ml of extract (0.1 to 0.2 mg of protein) in a volume of 1 ml. The assay mixtures were incubated at 37 °C for 1 hr and then terminated with the addition of 0.4 ml of 50% trichloroacetic acid (TCA). Cinnamic acid (10 μl of 1% cinnamic acid in ethanol) was added to the mixtures as a carrier. Then 5 ml of toluene (21) was added and the mixtures vigorously vortexed for 1 min. The mixtures were allowed to equilibrate for 30 min and then centrifuged at approximately 5,000 xg for 10 min. One ml aliquots of the upper phase were added to 5 ml of standard scintillation fluid containing 0.3 gm phenyl-oxazolyl-phenyl-oxazolyl-phenyl (POPOP) and 5 gm 2,5-diphenyloxazole

(PPO) per liter of toluene. Radioactivity was determined by a Tricarb (Model 3003) liquid scintillation spectrometer. PAL activity is typically expressed as nmole cinnamic acid produced/ mg protein· hr. The data points represent the average values for at least three separate experiments.

Peroxidase assay

Peroxidase activity was determined by measuring colorimetrically the rate of hydrogen peroxide decomposition (Worthington Enzyme Manual, Worthington Chemical Co). The assay mixture consisted of 3 ml of hydrogen peroxide solution (1 ml of 30% hydrogen peroxide in 100 ml of water) diluted 100-fold in 0.01 M phosphate buffer pH 6.0 (prepared fresh daily), 25 ul of 1% o-dianisidine in methanol and 0.1 ml of extract. The reaction mixture minus the extract was added to a test cuvette (2.9 ml) and a blank cuvette (3.0 ml). At zero time 0.1 ml of enzyme extract was added to the test cuvette, the cuvette inverted to mix and placed in a Gilford spectrophotometer. The absorbance (A_{460}) was recorded at 15 second intervals for 1 to 2 min. Peroxidase activity was expressed in Δ O. D. /mg protein· min. The data points represent an average of two separate experiments.

Paper chromatography

Reaction products and standards were spotted on Whatman

#3 filter paper and chromatographed (descending) in a glass chamber at room temperature. The chromatography solvent consisted of n-butanol-acetic acid-water (4: 1 : 1.8) (69). The chromatogram was air-dried and scanned with a UV mineral lamp (short-wavelength). The UV fluorescent spots were circled and identified. For samples containing radioactivity, the chromatogram was cut into squares (1 cm^2), the squares placed into scintillation vials containing 10 ml of the standard toluene scintillation fluid and radioactivity determined by a Tricarb liquid scintillation spectrometer.

Sucrose determination

Sucrose concentrations were determined by invertase hydrolysis followed by reacting the sample with Worthington's glucostat reagent. The assay mixture consisted of 0.25 ml acetate buffer, pH 4.6, 0.25 ml invertase (20 mg/ml acetate buffer) and 0.5 ml extract in a total volume of 1 ml. The resulting glucose (break-down product plus glucose present in the original extract) was determined by the addition of 2 ml of Worthington's glucostat reagent and subsequent incubation at room temperature for 10 min. The same procedure was repeated for 1 ml of untreated extract. The reaction was terminated by the addition of 1 drop (50 µl) of 4 N HCl. The absorbance (A_{450}) was determined for each sample in a Gilford spectrophotometer. The

standard curve was determined over the range of 20 to 200 ug of glucose. The difference between the breakdown products and glucose content of untreated extract was taken as the sucrose content of the extract.

Protein determination

The quantity of protein was determined after precipitation of the extract with TCA. One-tenth ml of the extract was precipitated by the addition of 0.1 ml distilled water and 0.1 ml of 50% TCA. The mixture was centrifuged at 10,000 xg for 10 min and the precipitate resolubilized in 1 ml of 0.1 N NaOH. The protein concentration was determined on 0.1 ml of the alkaline sample by a modified Lowry method (71). The standard curve was determined with 20 to 200 ug of bovine serum albumen (BSA) by reading the absorbance (A_{660}) in a Gilford spectrophotometer.

TABLE I

Modified Murashige and Skoog Medium (MM-1)

Component	Final concentration (mg/liter)
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
KH_2PO_4	170.00
Na_2-EDTA (Ethylenediaminetetraacetic acid)	37.30
$FeSO_4.7H_2O$	27.80
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Kinetin	0.5
2,4-dichlorophenoxyacetic acid	5.0

TABLE I continued

Inositol	100.00
Asparagine	100.00
Ascorbic acid	50.00
Sucrose	3%
Coconut milk*	200.00
Yeast extract	1%

pH is adjusted to pH 5.7 - 5.8 with NaOH

For semi-solid medium Bactor-agar is added to the liquid medium to make a 1% agar medium.

* The coconut milk used in this medium was obtained from coconuts purchased from local distributors. The milk was filtered twice through Whatman #1 paper and stored in freezer.

RESULTS

Pine callus cultures were routinely transferred to new MM_1 2XCM medium every 2 weeks in order to maintain a viable growing tissue. Phenylalanine ammonia-lyase (PAL) activity was measured in the pine cultures throughout the 2 week growth period. PAL activity in 12 to14 day-old tissues is relatively low toward the end of the growth cycle, but is stimulated 10 to 15-fold 2 days after transfer of the tissue to new medium (Fig. 1). Descernable stimulation was observed six hours after tissue transfer (Fig. 2). Twelve hours after subculturing PAL activity had increased about 3 fold. The enzyme activity increased linearly between 12 and 36 hours and then began to level off. Once the maximum activity was obtained PAL activity begun to decline to a pre-stimulated level. The stimulation and subsequent decline of PAL activity in pine callus cultures follows a similar pattern whether the data were expressed as specific activity (Fig. 1) or on a fresh tissue weight bases (Fig. 3). The callus cultures also were assayed for peroxidase activity. The activity of this enzyme was highest in 14 day-old tissues and declined to about 40% of that activity 12 hours after transfer and to about 15% in 48 hours (Fig. 4).

The growth pattern of the callus tissue is shown in Fig. 5.

The tissue had only slightly increased in weight during the period when PAL activity was the highest.

Stimulation of PAL activity also occurred if the callus tissue was placed in liquid MM_1 2XCM medium (shaking culture). Instead of the peak PAL activity occurring at day 2 a broader peak appeared at day 4 (Fig. 6).

Subculturing Effect

The effect on PAL activity of pre-maturely subculturing the pine callus is shown in Fig. 7. If the tissue was subcultured two days after the initial transfer (a point of maximum PAL activity) there was no further stimulation of PAL activity. The typical decline in PAL activity, however, was postponed two days. If the tissue transfer was made at day 4 or day 10, PAL activity was stimulated to the level obatined at day 2.

Effect of Inoculum Size and Tissue Injury

The size of the tissue inoculum also influenced the rate of PAL stimulation. Maximum PAL activity was obtained at day 2 with inocula of 1.0 gm or greater and at day 4 with inocula of 0.5 to 0.7 gm.

The effect of imposing additional physical injury to the pine tissue at the time of subculturing was tested on day 14 tissue. The tissue was chopped into cubes of less than 4 mm and placed

back onto the original medium. PAL activity did not increase in these tissues 2 days after the injury treatment.

Effect of Cycloheximide

Cycloheximide, an inhibitor of polypeptide translation, was used to study the stimulation of PAL activity. The standard growth medium was supplemented during preparation with 1, 10, and 100 µg/ml of cycloheximide. Tissues were transferred to the cycloheximide conatining medium and assayed for PAL activity on day 2 and 4. Cycloheximide caused a 15 to 40% decrease in PAL activity at day 2 in comparison to the control (Fig. 8). However, at day 4 PAL activity was 40 to 64 % higher than the activity obtained in the control tissue.

Cycloheximide also was used to study the decay of PAL activity. In these experiments the tissues were subcultured as normal and at day 2 the entire tissue was transferred to media containing cycloheximide. PAL activity was maintained at near the day 2 level for at least 2 days after transfer. Four days after transfer PAL activity in the control tissue had decreased 30 % while in the cycloheximide treated tissues PAL activity had decreased 7 to 17 % (Fig. 9). Interestingly, the cycloheximide treated tissues showed a marked difference in color over the controls. Four days after transfer, tissues in the cycloheximide

containing medium had turned brown.

Effect of Nutrient Supplement

New medium was added to day 14 cultures in an effort to establish whether only a nutrient supplement was required to re-stimulate PAL activity in these cultures. The addition of 3 ml of liquid MM_1 2XCM medium to the day 14 tissue stimulated PAL activity 3 to 4 fold over the unsupplemented tissue (Table II). PAL activity in the cultures was still about 3-fold less than the stimulation obtained by subculturing. The addition of 3 ml of 10% sucrose stimulated PAL activity as well as the complete medium.

Effect of Medium Constituents and Sucrose

The effect of MM_1 2XCM medium constituents on the stimulation of PAL activity in day 14 tissue was examined using shake cultures. The day 14 tissues were transferred to a liquid media containing the MM_1 salts and various supplements. Two days later the tissue was assayed for PAL activity. The only media in which PAL activity was stimulated were those containing complete medium or sucrose (Table III). The sucrose supplement stimulated PAL activity approximately 8-fold above the day 14 activity and was 50 % that obtained with the complete medium. Omitting sucrose or coconut milk from the complete medium did not affect the stim-

ulation of PAL activity.

The effect of different sucrose concentrations on the stimulation of PAL activity also was investigated. Increasing the sucrose concentration in the semi-solid media (above the control) did not appreciably change the stimulation of PAL activity (Table IV). Increased levels of sucrose, however, did retard the subsequent decay of PAL activity as measured in day 4 tissue. The 6-fold difference in sucrose concentrations in the shake cultures likewise had little effect on the stimulation of PAL activity.

The sucrose content within the callus tissue was measured at various periods during the growth cycle. The sucrose content in the callus increased 6- to 7-fold within 4 days after transfer to the new medium at which point the sucrose content declined sharply (Fig. 10).

Effect of Temperature

The effect of temperature on PAL activity in pine callus tissue was investigated using three different growth temperatures : 28°C (control), 19°C, and 14°C. As the growth temperature decreased the rate in which PAL activity was stimulated decreased and the period at which maximum activity was reached was delayed (Fig. 11). At 19°C and 14°C the peak PAL activity occurred in day 4 and 9, respectively. In addition, as temperature decreased the total PAL

profile became broader. The net effect was that over a 12-day growth period the cultures grown at 14°C had more t_{total} activity than those grown at higher temperatures.

Another investigation was conducted concerning the effect on PAL activity of transferring the cultures to a cold temperature (4 ± 1°C). If day 2 cultures were transferred to a cold cabinet PAL activity was maintained near the day 2 level for at least 6 days (Fig. 12). If the transfer to the cold cabinet was made 4 days after subculturing an arrest in the decay of PAL activity was obtained.

Effect of Other Factors

Various other factors were examined and shown to have minimal effect on the induction or subsequent decay of PAL activity. When the callus cultures were kept in dark after transfer to new semi-solid media there was no difference between the day 2 PAL activity in the dark- and light-grown tissue. Changing the pH of the medium from 5.6 (control) to 4.8 did not change the level of PAL activity over the control. Supplementing the medium with glutamine (10^{-3} M) gave similar results to the control.

Change in the pH of Media

The shake cultures were used to follow the pH during tissue

growth. The pH of the media decreased from pH 5.6 to 4.8 two days after subculturing and maintained the lower pH for at least 6 days after transfer (Fig. 13). If the initial pH was 4.8 the initial drop in pH was not observed. If the growth media contained only the MM_1 salts then the pH increased to approximately 7 , four days after transfer.

Figure 1 : A profile of PAL activity in pine callus tissue. The tissues were routinely transferred every 12 to 14 day. In this figure day 12 PAL activity is equivalent to day 0 activity.

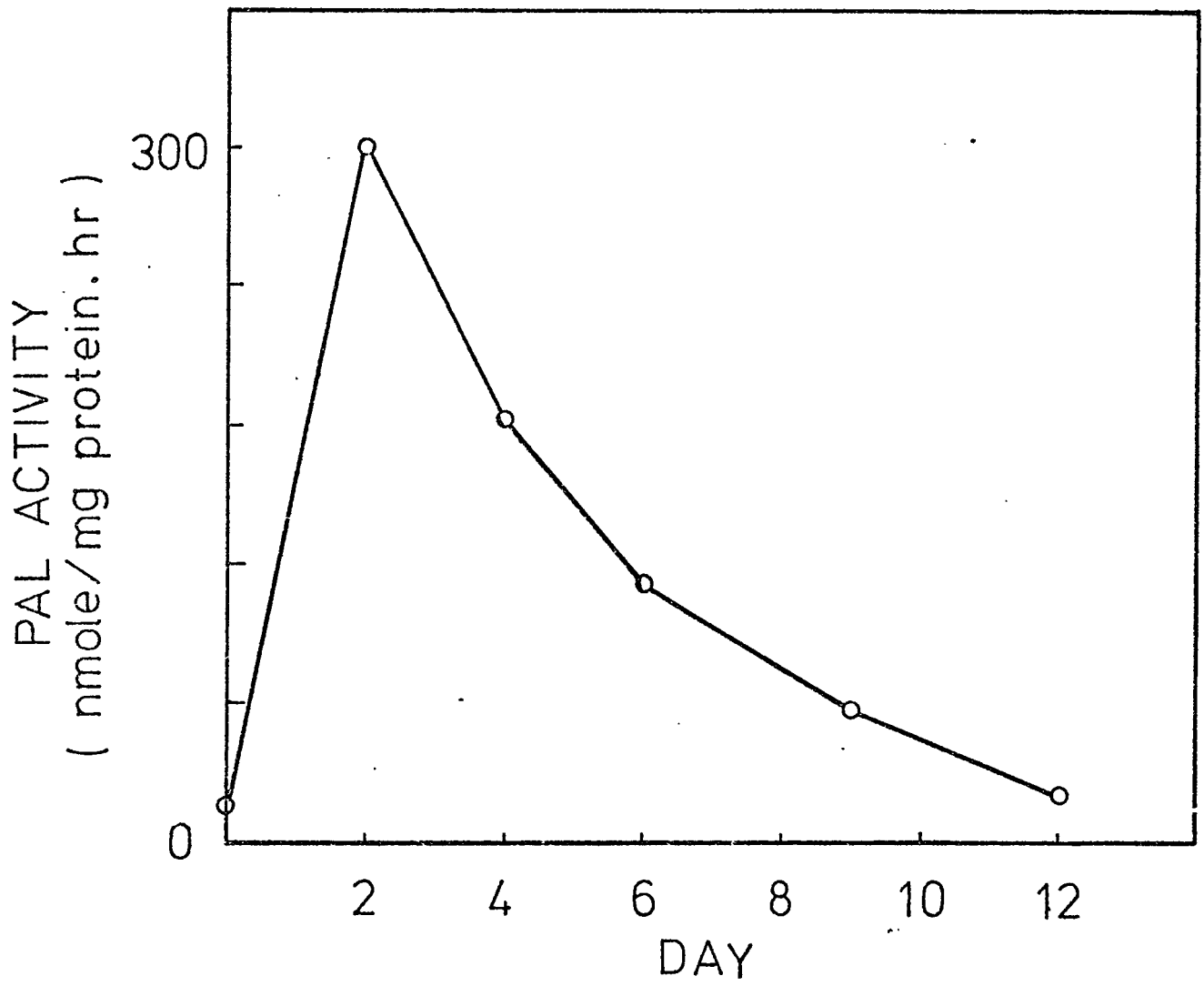

PAL ACTIVITY
(nmole/mg protein. hr)
300
0
2
4
6
8
10
12
DAY

Figure 2 : Change in PAL activity during the first 48 hr after subculturing. PAL activity is expressed in % of day 2 control activity.

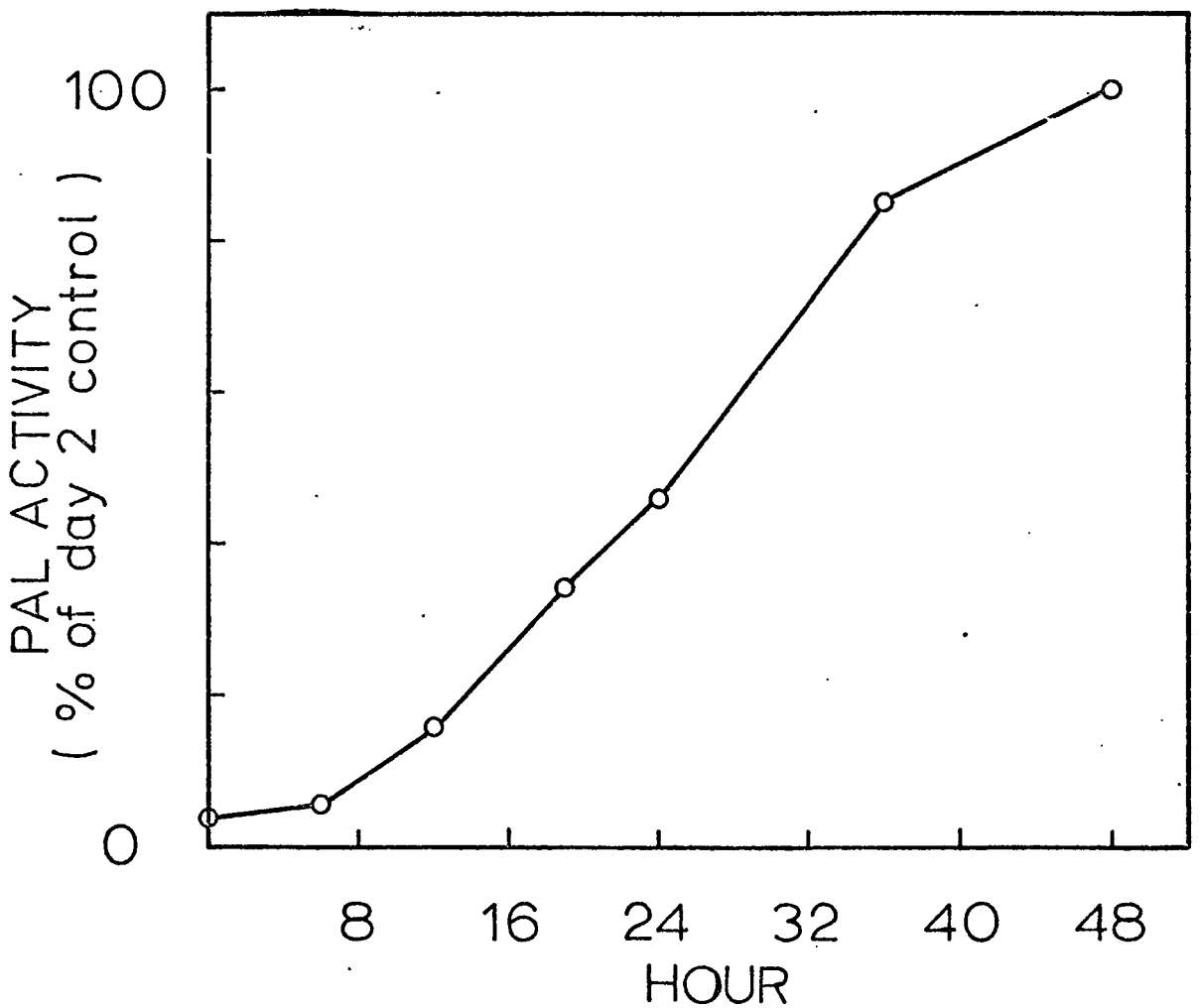

100
0
PAL ACTIVITY
(% of day 2 control)
8
16
24
32
40
48
HOUR

Figure 3 : Profile of PAL activity in pine callus tissue. PAL activity is expressed as μmoles of cinnamic acid produced per gm. fr. wt. of tissue per hr.

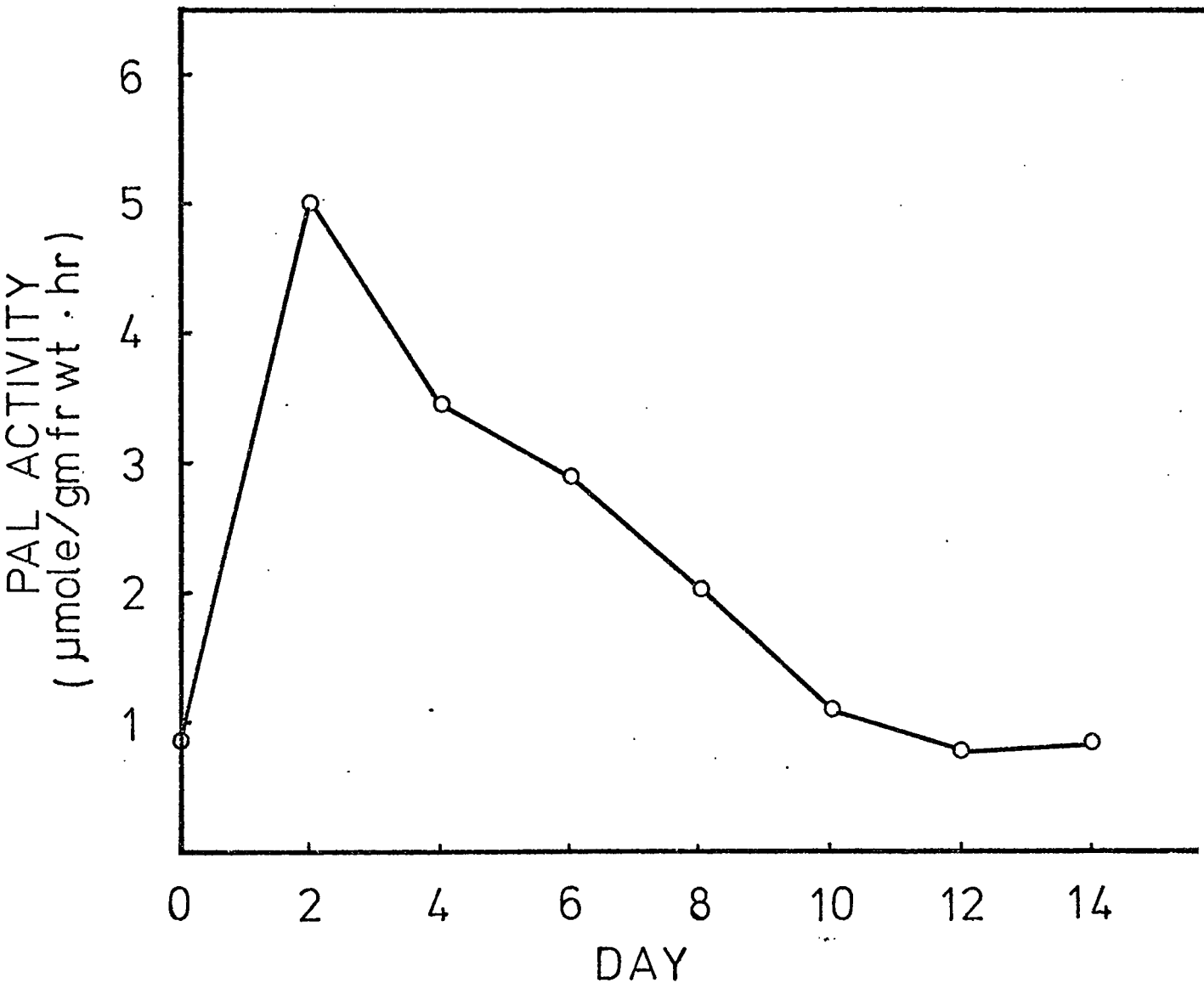

PAL ACTIVITY
(µmole/gm fr wt · hr)
1
2
3
4
5
6
0
2
4
6
8
10
12
14
DAY

Figure 4 : Profile of peroxidase activity in pine callus tissue.

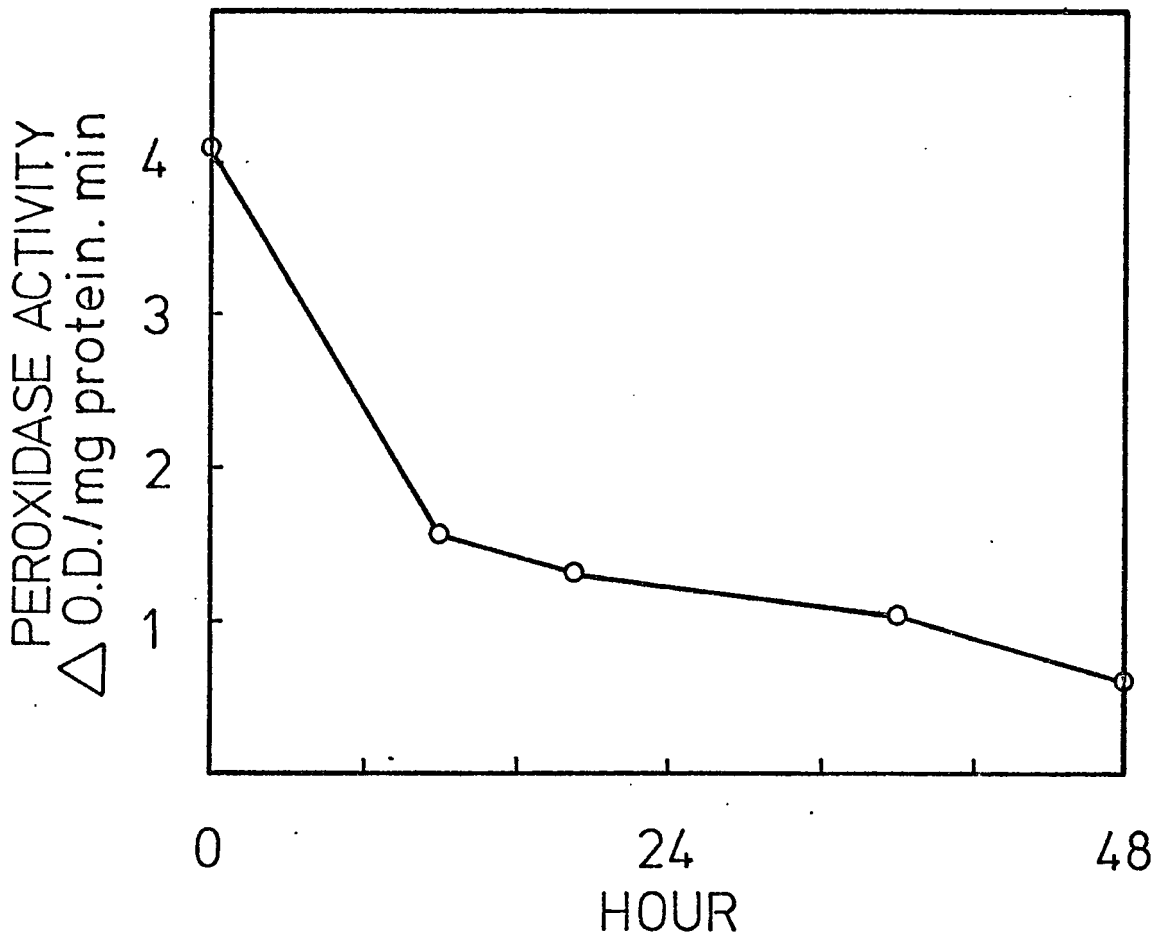

PEROXIDASE ACTIVITY
△ O.D./mg protein. min
4
3
2
1
0
24
48
HOUR

Figure 5 : Growth profile of the pine callus tissue from the point of subculturing. The change in fresh weight is expressed in % of the initial weight (0 day).

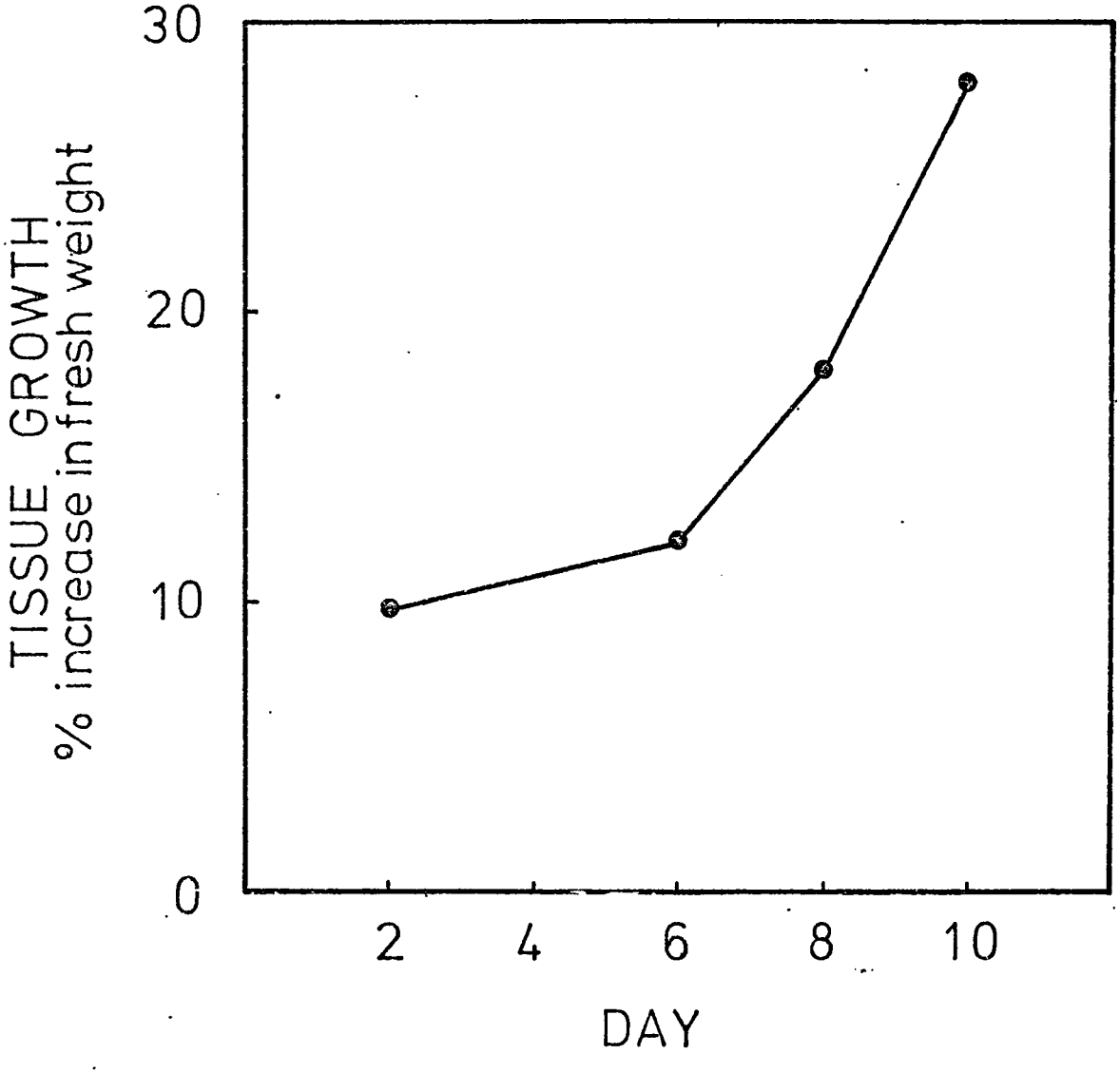

TISSUE GROWTH
% increase in fresh weight
30
20
10
0
2
4
6
8
10
DAY

Figure 6 : Profile of PAL activity in pine shake cultures.

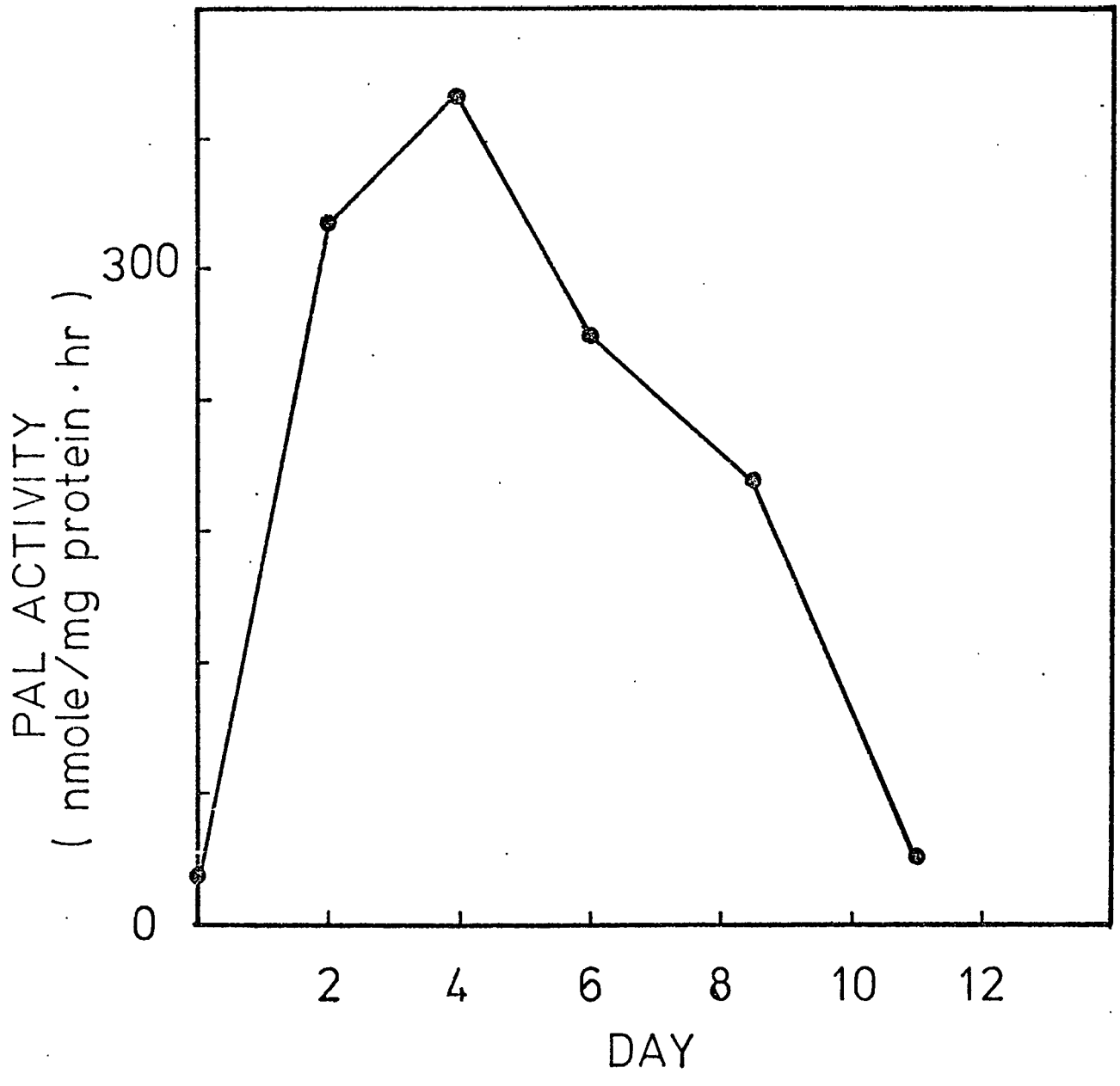
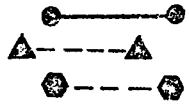

PAL ACTIVITY
(nmole/mg protein · hr)
300
0
2
4
6
8
10
12
DAY

Figure 7 : The effect on PAL activity of premature subculturing of the pine callus.


control, transfer on day 2, transfer on day 4, and transfer on day 10.

PAL activity is expressed in % day 2 control activity.

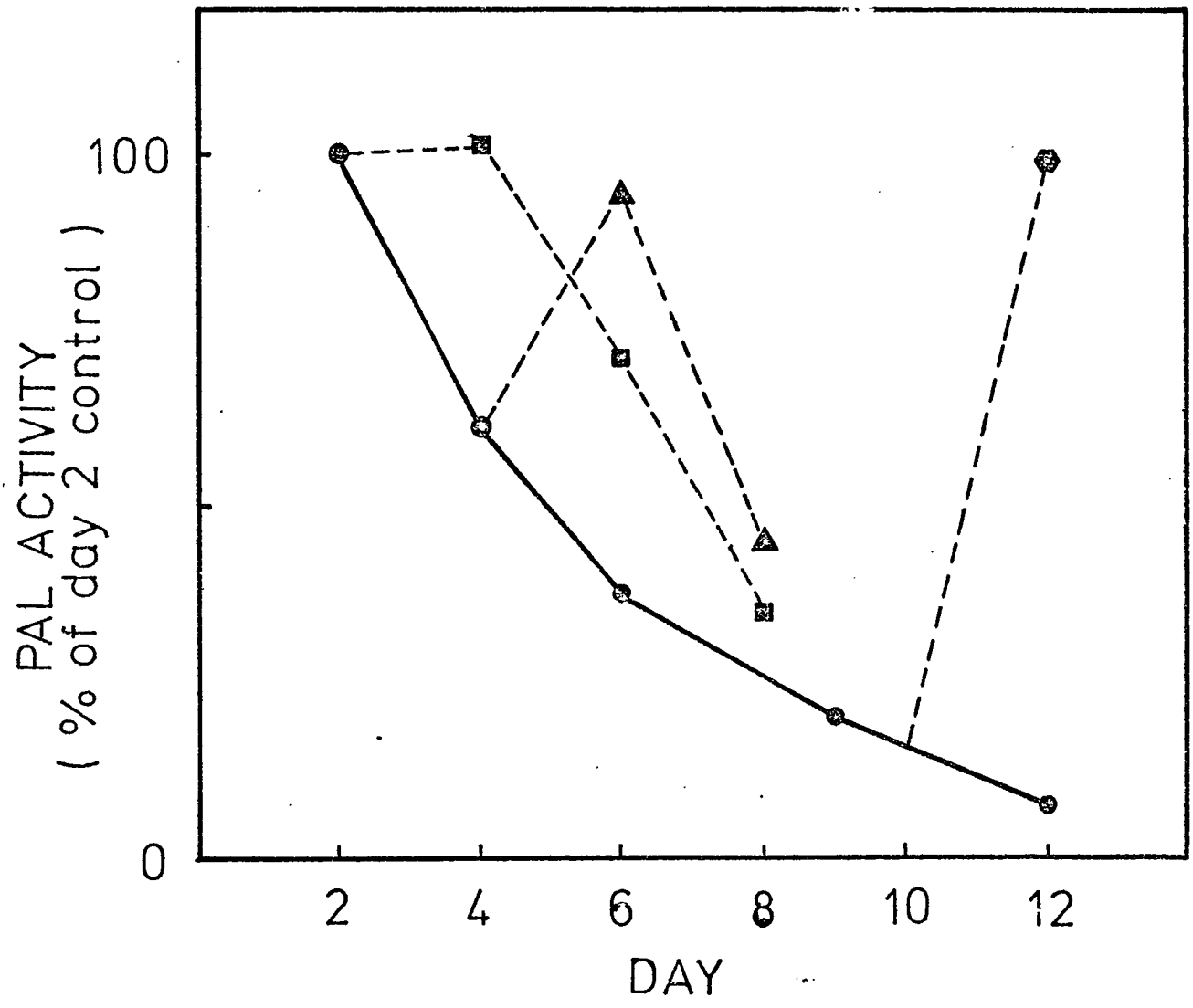
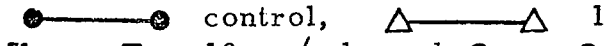
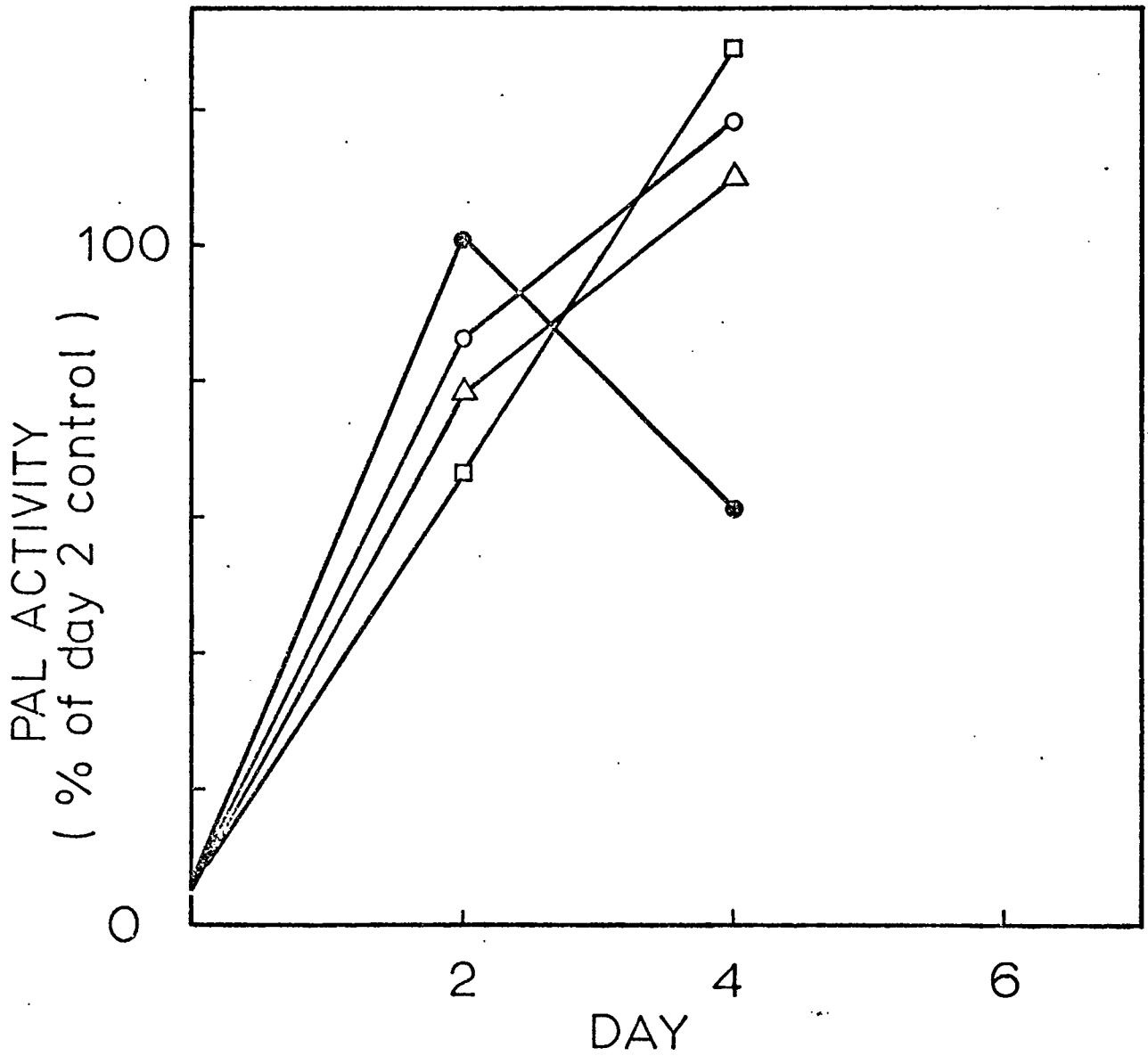

100
0
PAL ACTIVITY
(% of day 2 control)
2
4
6
8
10
12
DAY

Figure 8 : The effect of cycloheximide on the stimulation of PAL activity in pine callus tissue.



PAL activity is expressed in % day 2 control activity.


PAL ACTIVITY
(% of day 2 control)
100
0
2
4
6
DAY

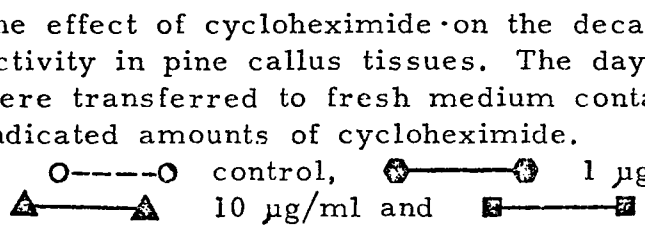


Figure 9 : The effect of cycloheximide on the decay of PAL activity in pine callus tissues. The day 2 cultures were transferred to fresh medium containing the indicated amounts of cycloheximide.
O----O control, ●——● 1 µg/ml,
▲——▲ 10 µg/ml and ■——■ 100 µg/ml.
PAL activity is expressed in % day 2 control activity.

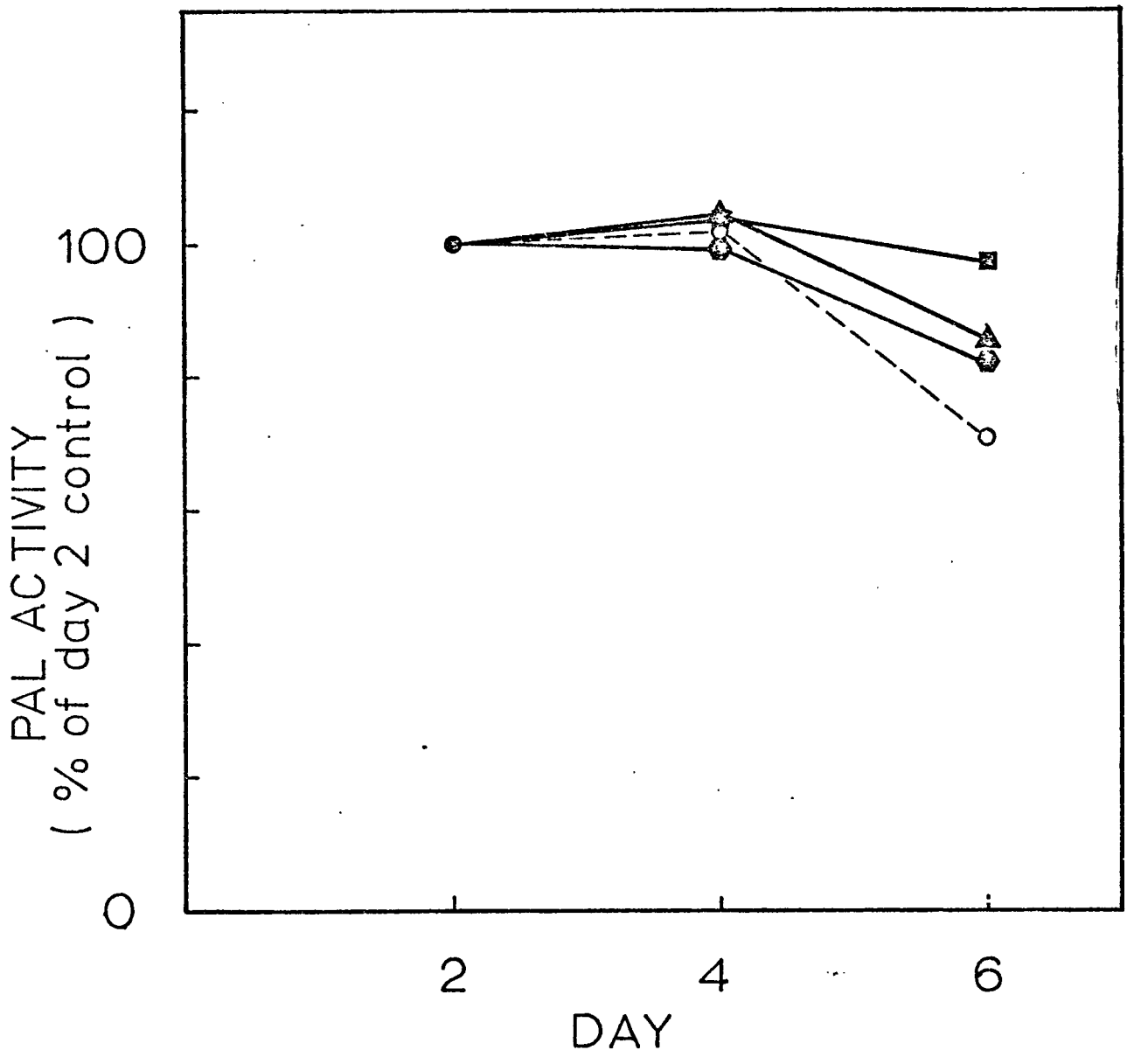

PAL ACTIVITY
(% of day 2 control)
100
0
2
4
6
DAY

Table II : The effect of nutrient supplement on PAL activity in pine callus tissue. Liquid nutrients were added to day 14 tissues. The cultures were resealed and cultured for 2 day prior to assaying for PAL activity.

SUPPLEMENT	PAL ACTIVITY (nmole/ mg protein· hr)
None	20
MM_1 2XCM (3 ml)	76
10% sucrose (3 ml)	100

Table III : The effect of various compounds on PAL activity in pine callus tissue. The day 14 tissue was transferred to liquid media containing the designated compounds and gently shaken for two days prior to assaying for PAL activity.

TREATMENT	PAL ACTIVITY (nmole/mg protein·hr)
MM_1 salt	< 1.0
MM_1 salt + vitamins	< 1.0
MM_1 salt + inositol	< 1.0
MM_1 salt + 2, 4-D	< 1.0
MM_1 salt + asparagine	< 1.0
MM_1 salt + ascorbic acid	< 1.0
MM_1 salt + sucrose	160.0
MM_1 salt + yeast extract	< 1.0
MM_1 salt + sucrose + yeast extract	160.0
MM_1 2XCM complete	320.0
MM_1 2XCM (minus sucrose)	328.0
MM_1 2XCM (minus coconut milk)	333.0

Table IV : The effect of sucrose on PAL activity in pine callus tissues. The effect was established in semi-solid and liquid shake cultures. The growth medium consisted of the complete MM_1 2XCM with varying amounts of sucrose.

TREATMENT	PAL ACTIVITY (nmole/mg protein·hr) Day 2	Day 4
Semi-solid (agar) MM_1 2XCM pH 5.6		
with 3% sucrose (control)	300	201
with 6% sucrose	285	258
with 9%	287	268
Liquid MM_1 2XCM pH 5.6		
with 1.5% sucrose	308	376
with 3% sucrose	320	370
with 6% sucrose	354	377
with 9% sucrose	344	371

Figure 10 : A profile of the sucrose content in pine callus tissue.

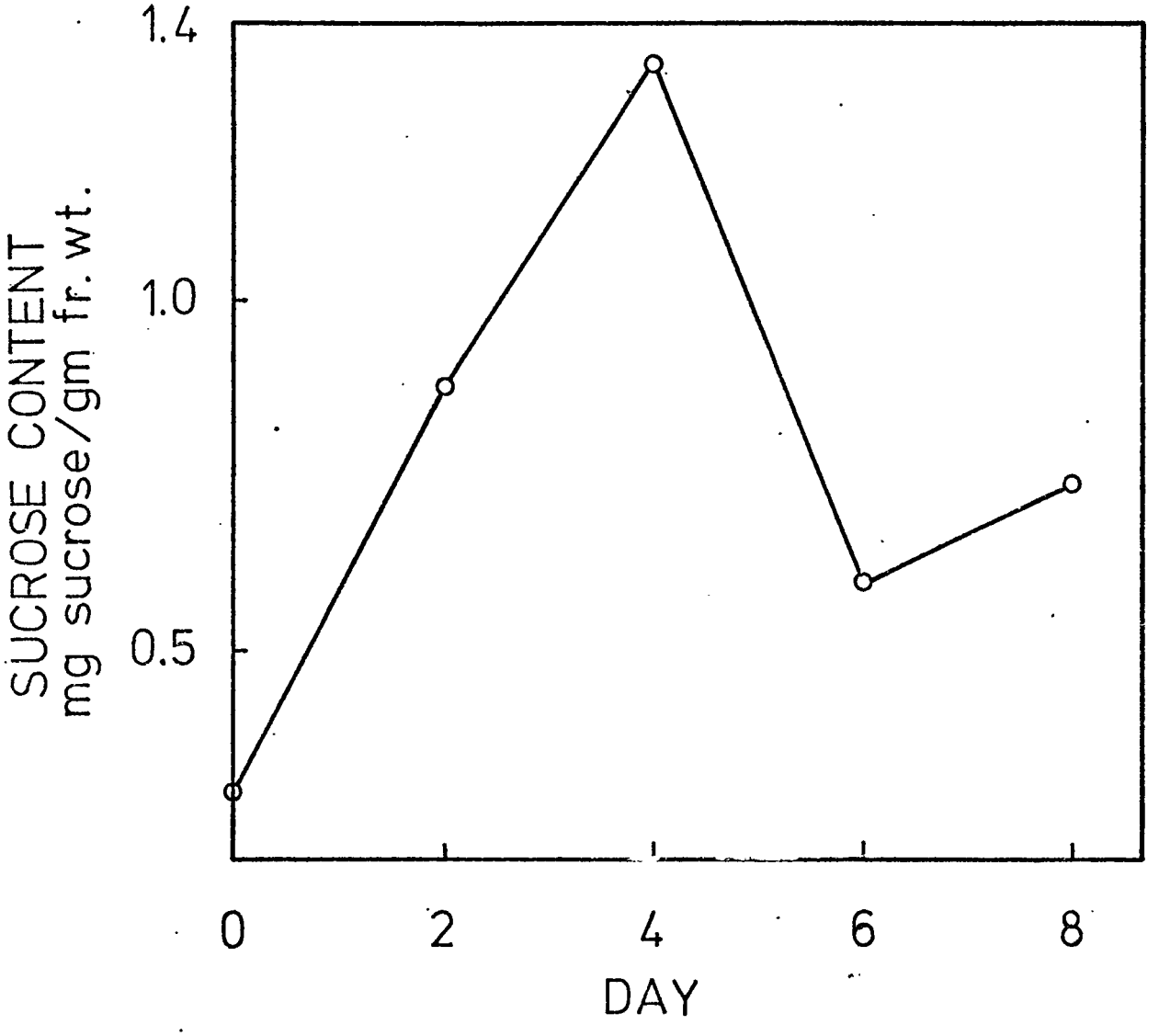
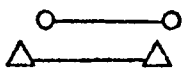

SUCROSE CONTENT
mg sucrose/gm fr. wt.
1.4
1.0
0.5
0
2
4
6
8
DAY

Figure 11 : The effect of temperature on PAL activity in pine callus tissue.

 28°C (control),  19°C and 14°C.

PAL activity is expressed in % day 2 control activity.

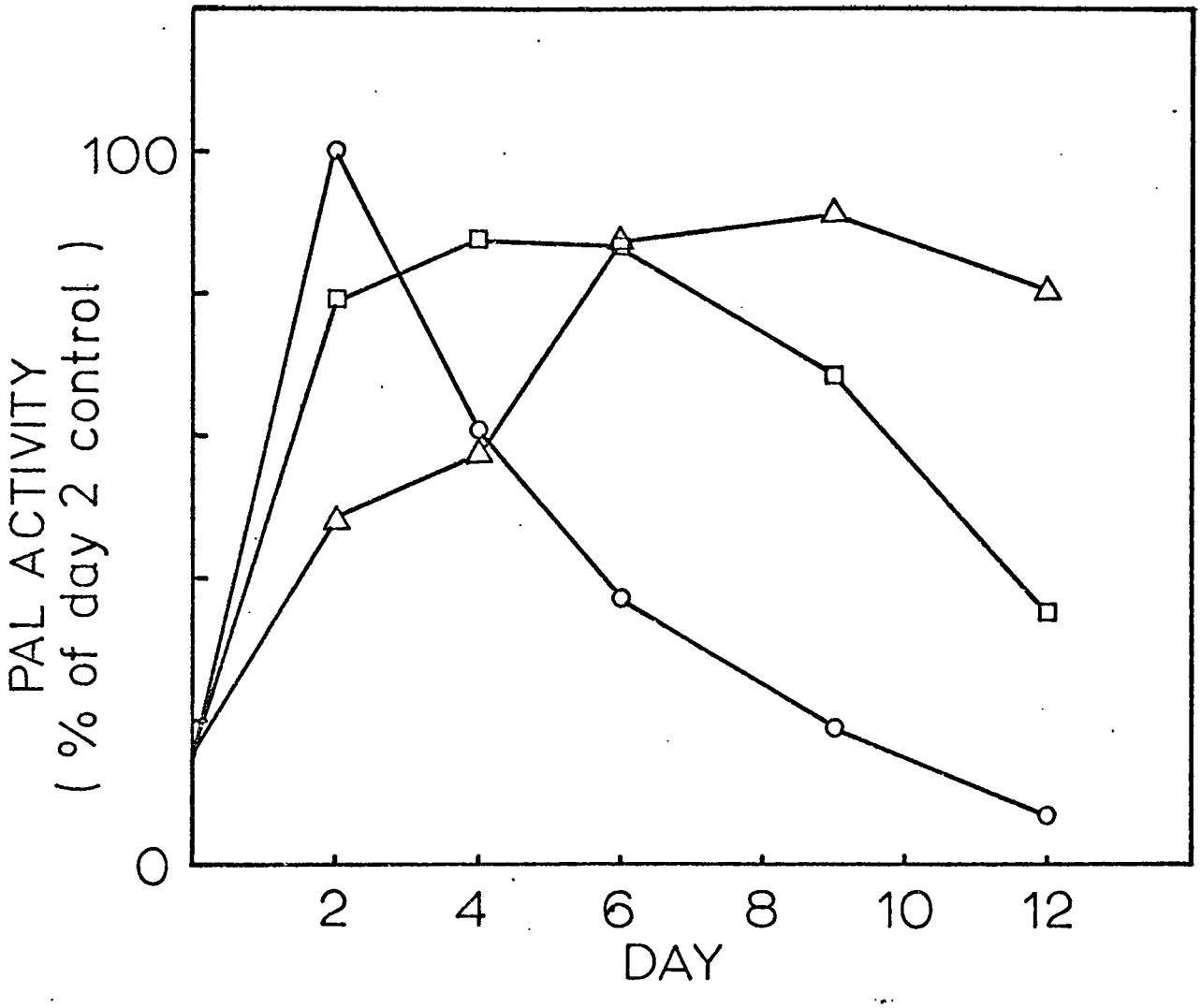
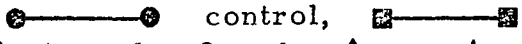
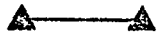

100
0
PAL ACTIVITY
(% of day 2 control)
2
4
6
8
10
12
DAY

Figure 12 : The effect of cold temperature on maintaining PAL activity in pine callus tissue.

 control, transfer to cold cabinet on day 2 and  transfer to cold cabinet on day 4.

PAL activity is expressed in % day 2 control activity.

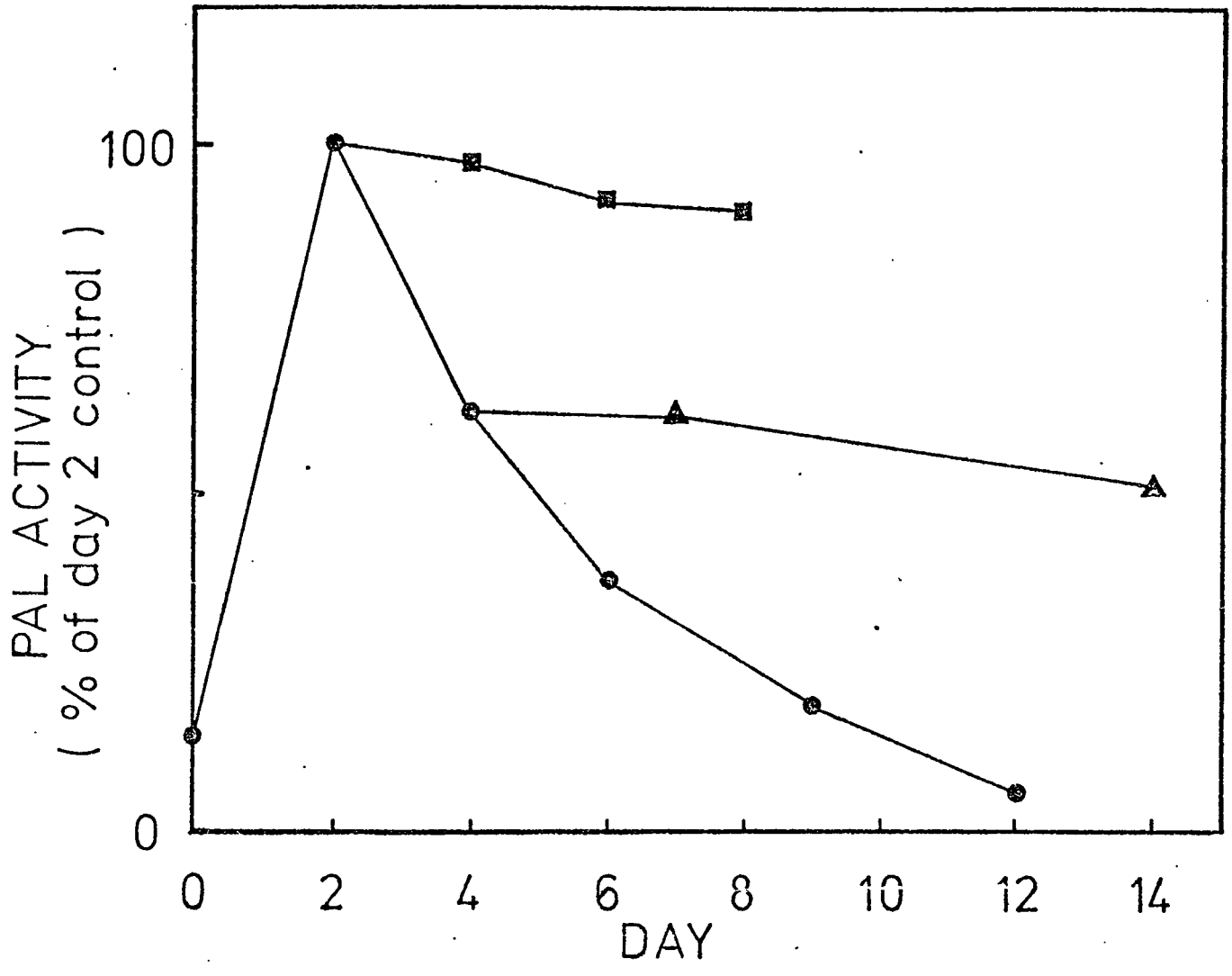
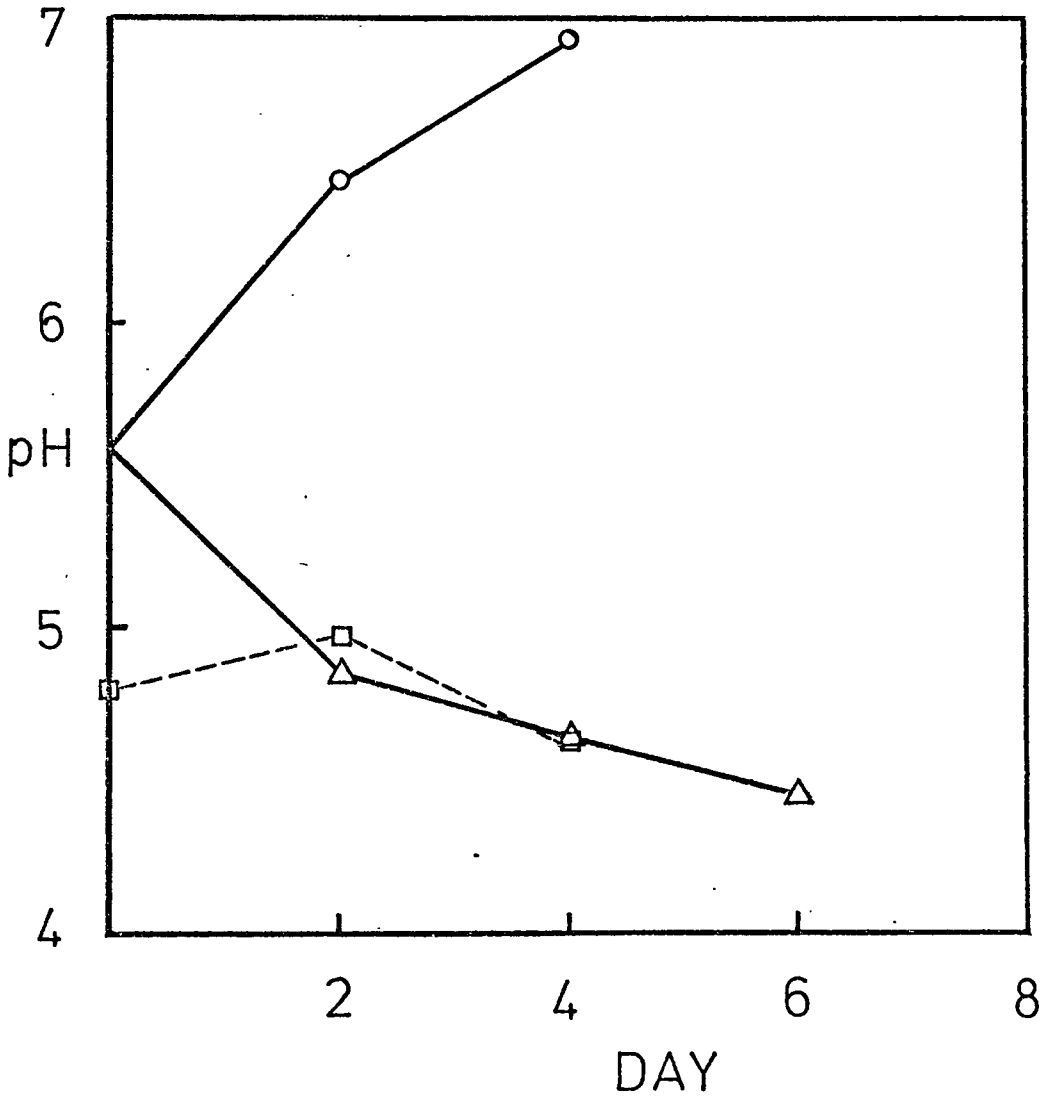

100
0
PAL ACTIVITY
(% of day 2 control)
0
2
4
6
8
10
12
14
DAY

Figure 13 : The pH of the liquid medium during tissue growth. ○——○ MM_1 salt only, △——△ MM_1 2XCM pH 5.6 and □——□ MM_1 2XCM pH 4.8 .


7
6
pH
5
4
2
4
6
8
DAY

DISCUSSION

Stimulation and subsequent decay of PAL activity after transfer to new medium is similar to the response of several other tissues. The transfer of parsley cells to a new medium stimulated PAL activity with a maximum activity in 15 hrs (49). Cultures of citrus fruit (101) and Haplopappus gracilis (12, 35) obtained maximal activities between 1 and 2 days. In cultures of soybean (24) and Paul's scarlet rose (21), however, the maximal PAL activity was not obtained until day 6 to day 8. The lag phase observed in the pine callus (Fig. 2) is common to citrus fruit and Haplopappus gracilis culture systems. Lag periods of 2.5 hrs and 3 days were observed in parsley (49) and Paul's scarlet rose (21), respectively. The kinetics of the PAL activity shown in Fig. 1 is typical of the group I enzymes reported by Grisebach and Hahlbrock (36), where a rapid increase of enzyme activity is followed by a slower decline in activity. The peroxidase data dose not appear to be positively correlated with PAL activity. The relation obtained in pine callus tissue actually appears to be a reciprocal one. A lack of correlation between these two enzymes was shown by Vegetti et al (104) studying the infection of bean leaves with alfalfa mosaic virus. PAL activity also does not follow the growth rate of the pine tissue. This differs

from results on the Paul's scarlet rose (21), parsley (49), and soybean suspension cultures (42) where the peak in PAL activity coincides with the maximum rate of tissue growth.

The effect of cycloheximide on the stimulation of PAL activity indicated that an inactive form of PAL (11) is being activated upon transfer of the pine tissue to fresh medium. Another possibility is a dilution effect (distilled water and fresh medium) as mentioned by Hahlbrock and Schroder. These workers suggested that the stimulation of PAL activity upon subculturing was independent of the supply of certain nutrients in the medium (46). The inability of various compounds from the liquid MM_1 2XCM medium to stimulate PAL activity in the pine callus cultures suggest a more complex model for this system (Table III). Cycloheximide also significantly reduced the decay of PAL activity which indicates that the inactivation of PAL activity was dependent on protein synthesis.

Sucrose was shown to influence PAL activity in various ways. In Table III only the media containing sucrose enhance PAL activity. When only sucrose was replenished to day 14 tissues (Table I) PAL activity was stimulated. The stimulation of PAL activity upon transfer to fresh medium then can be explained

by an enhanced effect of sucrose. Increasing the concentration of sucrose in the growth media (Table IV) only delayed the decay of PAL activity. Interestingly, if sucrose was omitted from the complete medium, PAL activity was still stimulated. The coconut milk which was used in the complete medium contained 0.74% sucrose. Therefore the sucrose and probably other carbohydrates were present in sufficient quantities in the coconut milk to stimulate PAL activity and a higher level of sucrose only delayed the decay of PAL activity. This agrees with reports that sucrose enhanced PAL activity in Helianthus annus (19) and strawberry leaf discs (109).

The effect of low temperature on PAL activity may be primarily associated with the decay of PAL activity. The decay in PAL induction at reduced temperatures was expected but the greatest effect of low temperatures was on the decay of PAL activity. In addition , the effect of transferring the cultures to lower temperatures also indicated an arrest in the decay in PAL activity. Engelsma (30) had shown an increase in PAL activity upon transfer of gherkins to cold temperatures.

The lack of or low response of pine callus tissues to light, darkness, or wounding is atypical to most tissue cultures studied

thus far. Therefore intrinsic factors governing PAL activity are present in the pine tissue cultures. The lack of wounding response could be explained by the fact that the callus "tissue" is not an organized tissue *per se*.

The present study provides a baseline data for studying secondary metabolism in gymnosperm tissues. Several factors were shown not to effect PAL activity but in examples where an effect was demonstrated the most striking effect was on the decay of PAL activity. The principle exception was that subculturing the tissue stimulated PAL activity.

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APPENDIX

The product of the PAL assay was spotted and cochromatographed with ^{14}C cinnamic acid and cold cinnamic acid. The product in the toluene phase occupied the same spot as the radioactive cinnamic acid and the same UV-absorbing region as the cold cinnamic acid.

PAL activity from the callus tissue was partially purified by ammonium sulfate fractionation (Table V). The crude enzyme is brought to 30% saturation by addition of saturated ammonia sulfate in borate buffer (0.1 M, pH 8.8). The highest specific activity and purification was obtained in the 30 to 55% pellet.

Some property of the PAL in this fraction was investigated. An assay temperature curve show maximum activity at 50 °C and a considerable inhibition at 60 °C (Fig. 14). In addition the effect of different sulfhydryl groups and hydrogen peroxide was investigated (Table VI). Dithiothreitol and cysteine begin to inhibit PAL activity above 5×10^{-4} M and 10^{-5} M, respectively. Hydrogen peroxide at concentrations as high as 8.8×10^{-2} M did not completely inhibit the enzyme.

Table V : PAL activity in various fractions purified with ammonium sulfate.

FRACTION	PAL ACTIVITY (nmole/mg protein·hr)	% YIELD	PURIFICATION
Crude	123	100	1
0 - 30% pellet	98	4	0.8
30 - 55% pellet	293	74	2.4
30 - 55% supernatant	33	5	0.27

Figure 14 : The effect of the assay temperature on PAL activity. The 30 to 55% ammonium sulfate pellet was used as the enzyme source.

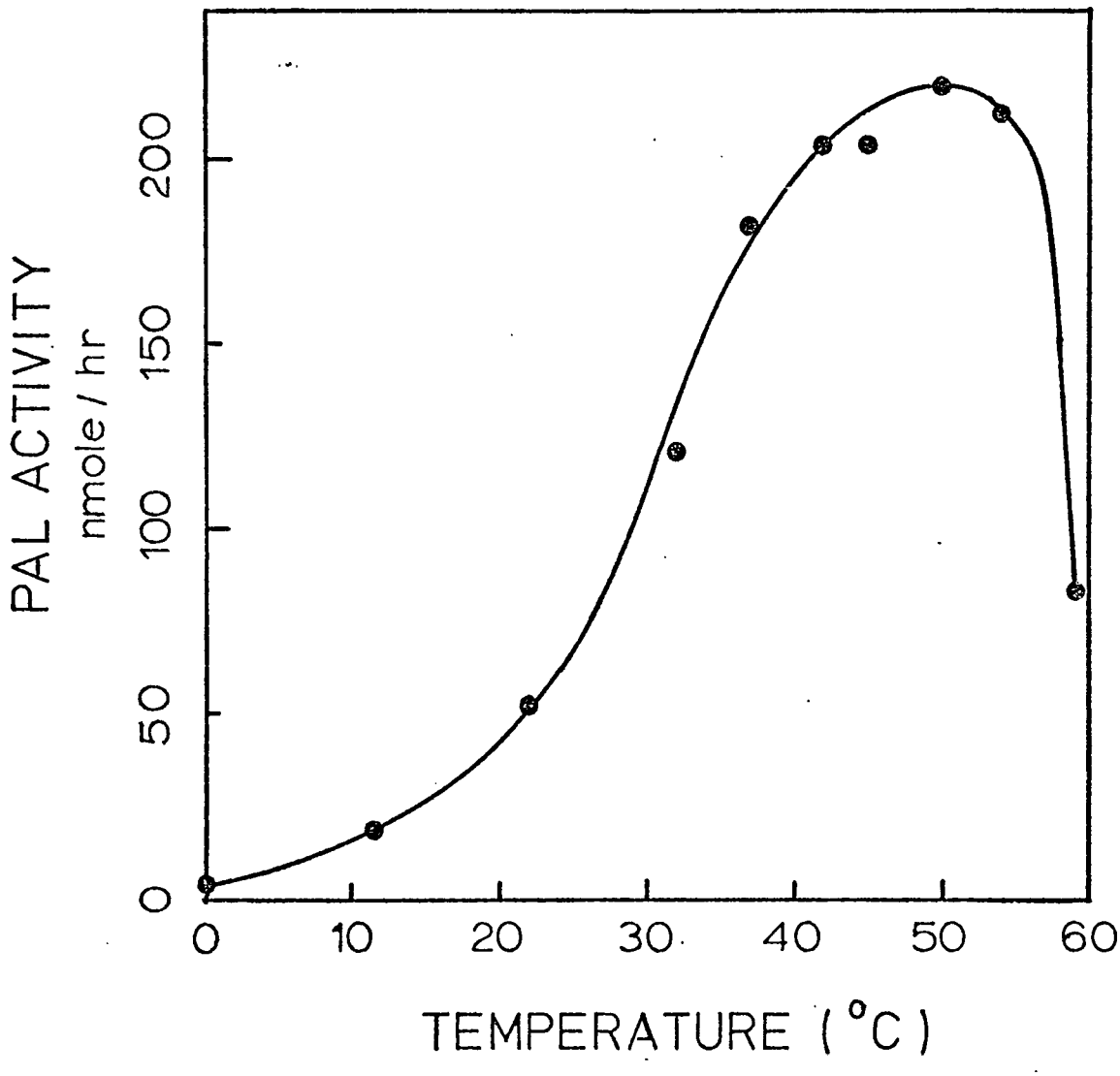
PAL ACTIVITY
nmole / hr
0
50
100
150
200
0
10
20
30
40
50
60
TEMPERATURE (°C)

Table VI : The effect of oxidant and anti-oxidants on PAL activity. The 30 to 55% ammonium sulfate pellet was used as the enzyme source.

TREATMENT	ACTIVITY (CPM)	% ACTIVITY OF CONTROL
Control	5180	100
DTT 10^{-4} M	5170	100
5×10^{-4} M	4331	84
10^{-3} M	3108	60
3×10^{-3} M	911	18
10^{-3} M	293	6
Cysteine 10^{-5} M	5101	98
5×10^{-4} M	3227	62
10^{-3} M	2264	44
3×10^{-3} M	853	16
5×10^{-3} M	611	12
Hydrogen peroxide		
8.8×10^{-2} M	3025	58