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PHYTOHORMONAL REGULATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN PLANT CELL CULTURES

The University of Arizona M.S. 1986

PHYTOHORMONAL REGULATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN PLANT CELL CULTURES

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN AGRONOMY AND PLANT GENETICS

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Frank Katterman, Dr David Stumpf and Dr. Paul Bartels for their helpful suggestions.

I would also like to recognize my fellow lab partners, Jay Thomas, Ken Helm and Kamel Saka for their comradeship and for providing a lighter side to the quest for knowledge.

Most importantly, I thank my wife, Sachiko, and my daughter, Tina Mariko, for their love and patience. Without their help and support, this thesis would have not been possible.

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ABSTRACT

The membrane bound enzyme, 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase (HMGR), catalyzes the rate limiting reaction and committed step in the biosynthesis of the isoprenoids. The isoprenoid plant hormone, abscisic acid (ABA) was investigated for a possible interaction with the enzyme. A 24 h treatment of Euphorbia lathyris suspension cultures with 0.2 uM ABA in vivo was found to change the properties of the enzyme. The apparent K_m of microsomal HMGR with respect to the substrate, HMG-CoA, was changed from 7.8 uM to 19.0 uM. The maximal velocity of the enzyme however, did not change with the in vivo ABA treatment. In vitro additions of ABA did not change the kinetic properties of microsomal HMGR.

In vivo application of a competitive inhibitor of HMGR, mevinolin, to E. lathyris suspension cultures for 24 h resulted in a two-fold increase in the specific activity of the enzyme.

INTRODUCTION

Plant isoprenoids comprise a large and diverse class of important biological and commercial compounds. These isoprenoid compounds fulfill many important physiological functions such as plant growth regulators, photosynthetic accessory pigments, phytotoxins, phytoalexins, insect anti-feedants and membrane stablilzers. Other isoprenoids from plants have been commercially exploited for rubber products, chemical feedstocks, and pharmaceutical compounds.

Included in this important class of chemicals are abscisic acid (ABA), the gibberellins (GA), the carotenoids, sterols, steroids, and beta-carotene. Other important compounds have isoprenoid side chains such as the cytokinins, ubiquinone, plastoquinone and chlorophyll.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) Reductase (HMGR) catalyzses the reduction of HMG-CoA into mevalonic acid (MVA), the common precursor to this important class of chemicals. This enzyme is the key link from acetyl Coenzyme A to the active 5 carbon isoprene unit which is the building unit for all of the isoprenoids (Fig.1). This enzymatic reaction is a major control point of animal and human cholesterolgenesis and has attracted a great deal of interest (Sabine 1984, Brown and Rodwell 1980). HMGR also appears as a key regulating enzyme in

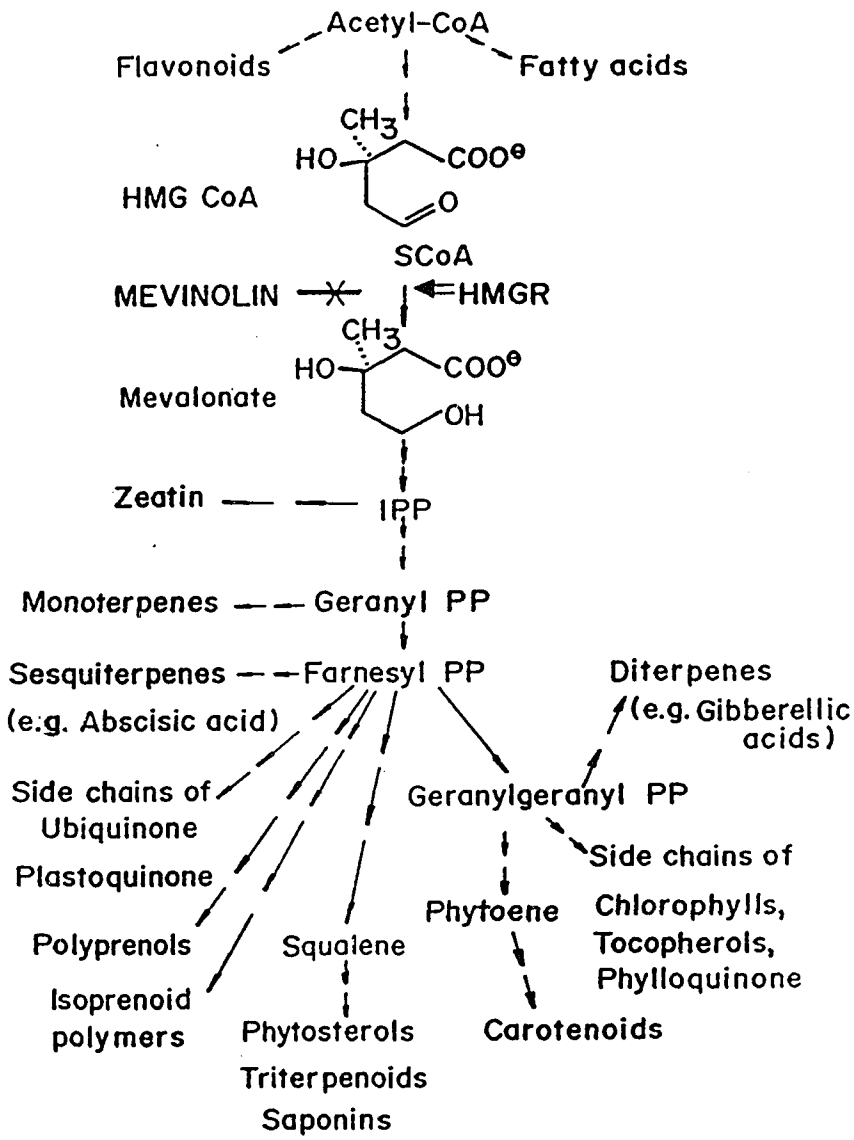


Figure 1. Biosynthetic Pathway from Acetyl-CoA to the Isoprenoids.

isoprenoid biosynthesis and as one of the major key regulating enzymes that influence growth in both plants and yeasts (Garg and Douglas 1983).

In view of the importance of isoprenoid compounds in plants, early and irreversible regulation of HMGR would have a dramatic effect on plant growth and development. Thus an investigation of the regulation of this enzyme could help to understand the importance of isoprenoid biosynthesis and its relation to plant growth and development. Such an understanding may also allow the direct manipulation of HMGR to increase the production of commercially valuable isoprenoids.

This study will investigate the possible regulatory role of an isoprenoid phytohormone, ABA , on the microsomal bound HMGR activity. ABA will be applied in vivo at a specific time of cell growth to plant cell cultures before assaying the enzyme. In addition, the phytohormone will also be applied in vitro during the assay of the HMGR enzyme.

Plant tissue culture systems will be used in this investigation because the growth conditions can be easily controlled and exogenous compounds can also be easily introduced. In addition, the results of this investigation may be applicable towards the increased production of secondary metabolites from plant cell cultures.

LITERATURE REVIEW

HMGR activity was first demonstrated in yeast extracts in 1958 (Ferguson, Durr, and Rudney 1958). HMGR has since been found in various organisms including mammals, insects, birds, fishes, microorganisms and plants (Sabine 1984). Since isoprenoids are ubiquitous in nature, HMGR probably occurs in all life forms and tissues which synthesize isoprenoids. Until the mid 1970's, however, evidence of the widespread distribution of HMGR was limited and in part inferential. Since then, improved assay techniques and increased interest in HMGR have demonstrated the widespread occurence of HMGR in nature (Brown and Rodwell 1980).

Indirect evidence of the presence of HMGR in higher plants was provided by experiments showing the incorporation of radiolabeled precursors into isoprenoid compounds. The labeled compounds $^{14}CO_2$, [2-^{14}C]acetate and [2-^{14}C]-mevalonate were all incorporated into beta-carotene in maize seedlings (Goodwin 1958). Later, rubber synthesis from [3-^{14}C]HMG was demonstrated in Hevea brasilensis latex (Hepper and Audley 1969). These radiolabeling experiments provided evidence that the isoprenoid pathway in plants is similar to that in animals and microorganisms.

Brooker and Russell (1975) were the first to assay and study the properties of HMGR in a higher plant, Pisum sativum. All species of plants tested since then have shown the presence of HMGR. These include sweet potato root (Suzuki, Oba and Uritani 1974), radish seedlings (Bach, Lichtenthaler, and Rêtey 1980), tobacco seedlings (Douglas and Paleg 1980), sycamore tissue cultures (Ryder and Goad 1980), catnip leaf tissue (Arebalo 1978), anise suspension culture (Humber and Rüdiger 1978), Hevea brasilensis latex (Sipat 1982), carrot cell cultures (Nishi and Tsuritani 1983), and spinach leaves (Kreuz and Kleinig 1984).

Subcellular Distribution of HMGR

HMGR is a membrane bound enzyme localized in the plastids, microsomes and the mitochondria within the plant cell. In both pea seedlings and radish seedlings, the microsomal fraction contains about 80% of the total HMGR activity, with the plastids and the mitochondria each having 10% of the HMGR activity. (Brooker and Russell 1975, Bach, Lichtenthaler and Retey 1980). In fresh sweet potato root tissue, HMGR activity is only found in the mitochondria and not in the microsomal fraction. When the sweet potato is infected with the black rot fungus, Ceratocystis fimbriata, HMGR activity is then found in both the microsomes and in the mitochondria. Induction of furanoterpene biosynthesis in

the sweet potato root corresponds with the infection by the fungus. (Ito, Oba and Uritani 1979).

The different subcellular locations of HMGR raises the possibility that isoprenoid biosynthesis might be compartmentalized in the cell. Work with radish seedlings showed that low levels of mevinolin, a HMGR inhibitor, reduced the elongation of roots by a rate limiting effect of inhibiting phytosterol biosynthesis whereas other endpoints of the multibranched pathway were not affected (Bach and Lichtenthaler 1983a). Plastoquinone, phylloquinone, and alpha-tocopherol synthesis in the plastids was not inhibited by the in vivo addition of 2.5×10^{-7}M Mevinolin and ubiquinone synthesis in the mitochondria was only slightly affected. The plastid and mitochondrial membranes may be impermeable to mevinolin which would allow isoprenoid biosynthesis within the organelles to proceed while mevinolin inhibits the microsomal HMGR.

Work with HMGR from pea seedlings indicate that microsomal HMGR has different properties than those of the plastid HMGR. The microsomal HMGR has an apparent K_m of 80 uM for HMG CoA and a pH optimum of 6.9 (Brooker and Russell 1975), whereas the plastid HMGR has values of 0.34uM and 7.9 respectively (Wong, McCormack, and Russell 1982). In addition, exposure of the dark grown seedlings to red light decreased the HMGR activity in the microsomal fraction by up

to 50% (Brooker and Russell 1979) but increased the plastid HMGR activity by 40% (Wong, McCormack, and Russell 1982). They postulated that there are HMGR isozymes in the plant.

Bach and Lichtenthaler's (1984) work with radish seedlings indicate that the apparent K_m of microsomal HMGR is between 2.0 and 2.9 uM. Purified plastid HMGR has an apparent K_m of 1.5 (Bach 1986). Other reported K_m values include 6.5 uM for mitochondrial HMGR and 21.0 uM for microsomal HMGR from sweet potato (Suzuki, Uritani, and Oba 1974), and 28 uM for the HMGR from the latex of Hevea brasiliensis.

HMGR Activity in Different Tissues

Within the plant, the level of HMGR activity varies widely with the location and the age of the tissue. Microsomal HMGR activity is twice as high in etiolated pea seedlings as in light grown seedlings. (Brooker and Russell 1975). This same difference between etiolated and light grown seedlings was also observed in radish seedlings (Bach, Lichtenthaler and Retey 1980). Within the green seedlings, the microsomal HMGR activity declines drastically with the age of the tissue. The semi-mature leaves contain 66% of the HMGR activity found in the apical shoots and the fully mature leaves exhibit only 7% of the activity.

This correlates with the findings that sterol levels are higher in etiolated tissues as compared to light grown

tissues (Guens 1973). Sterol levels are also higher in young and developing tissues than in mature tissues. (Grunwald 1975). Apparently, high levels of microsomal HMGR activity are correlated with young and developing tissues which have a high demand for sterols.

While the decrease of microsomal HMGR activity with age may hold for plants in general, it may not be so for those plants which produce large amounts of sterols and isoprenoids as secondary products. In these plants the HMGR activity may be high in the differentiated cells where these isoprenoids are produced.

The specific activity of Hevea brasilensis HMGR from latex is reported to be about 28nmol MVA/mg protein/hr (Sipat 1982) which is about half of the value reported for etiolated pea seedling microsomal HMGR (Brooker and Russell 1975)

Isoprenoid Hormones

Abscisic acid (ABA) is a sesquiterpenoid hormone which may either suppress or activate the activity of certain physiological processes in plant tissues. ABA can also both suppress and activate a particular process depending on the concentration of the hormone. Barley seed germination, for example, is inhibited by 10^{-4} to 10^{-5}M ABA but is enhanced at 10^{-7} to 10^{-8}M ABA (Ho 1984). ABA may affect membrane related processes such as ion transport as

in the case of the rapid induction of stomatal closure by causing the efflux of K^+ from the guard cells. The presence of binding proteins specific for ABA have been shown in the guard cells of Vicia faba (Hornberg and Weiler 1984). ABA may also control the formation of nucleic acids and proteins and may redirect the biosynthesis of other proteins. ABA antagonizes GA_3 in the barley aleurone by reversing all GA_3 promoted changes in protein synthesis or mRNA levels whether they be increases or decreases (Jacobsen and Beach 1985). The synthesis of ABA from MVA has been demonstrated in radiolabeling experments (Millborrow 1975).

Gibberellins are diterpenoid compounds which exert a wide range of effects on plant tissues. They play an important role in seed germination, cell elongation, induce flowering and parthenocarpy among other effects (Hedden, McMillan, Phinney 1978). GA_3 promotes the synthesis of alpha-amylase in the aleurone layer of barley seeds to hydrolyze the stored reserves in the endosperm. GA_3 probably regulates specific gene transcription and thus mRNA formation leading to alpha amylase synthesis (Jacobsen and Beach 1985). Gibberellins have been shown to be biosynthesized from radiolabeled MVA (Graebe et al 1974).

HMGR and Phytohormones

ABA applied in vivo to etiolated pea seedlings at 2umol per 200 seedlings caused a 17% decrease in HMG-CoA

reductase activity within 1.5h. This reduction was still evident at 4h after application. Millimolar applications of ABA *in vitro* did not affect the activity of the enzyme (Brooker and Russell 1979). In a later study , Russell and Davidson (1982) applied 75 umol ABA per 200 pea seedlings and obtained a 39% reduction in HMG-CoA reductase activity. When equilmolar amounts of ABA and GA_3 were applied *in vivo* simultaneously , there was no reduction in the enzyme activity implying that GA_3 blocked the action of ABA.

HMGR from the apical tips of pea seedlings were assayed with the *in vitro* addition of up to 0.2mM GA_3. The hormone had no effect on the enzyme activity (Brooker and Russell 1975). Later Brooker and Russell (1979) assayed the enzyme after the *in vivo* application of GA_3 and again found no changes in the microsomal HMGR activity. In experiments studying the inhibition of radish root growth by Mevinolin (Bach and Litchenthaler 1983a), applications of GA_3 up to 30 uM was unable to reverse the essentially complete inhibition of radish root growth by 2.5 uM Mevinolin. The same amounts of GA_3 also had no effect at 0.25uM Mevinolin where a 50% inhibition of root elongation occured.

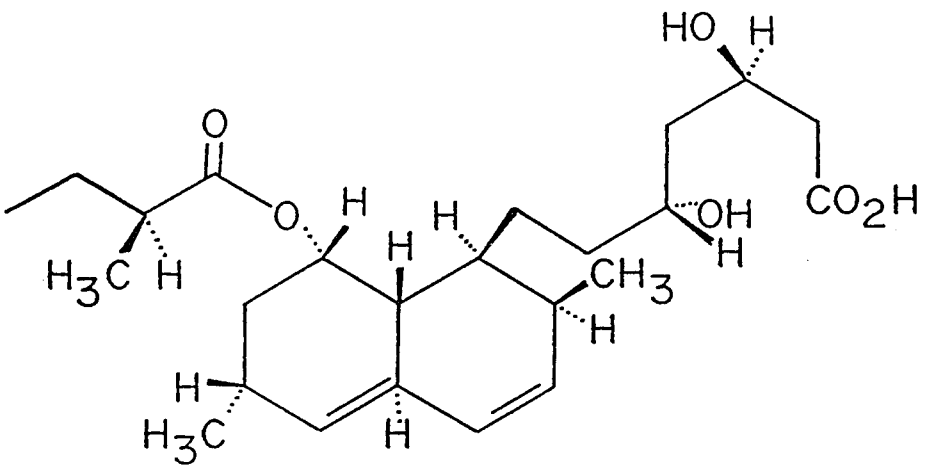
When stigmasterol was applied to the apical tips of pea seedlings, there was a 35% reduction in HMGR extracted after the application (Russell and Davidson 1982). Cholesterol also caused a 29% decrease in HMGR when applied *in vivo*. The application of sitosterol, which is a

precursor to stigmasterol, did not have any effect on HMGR activity. Stigmasterol may exert feed-back control on HMGR such as does cholesterol to mammalian liver HMGR (Kita, Brown and Goldstein 1980). When GA_3 was applied in combination with cholesterol to the apical tips of pea seedlings, there was no decrease in HMGR activity, implying that the hormone blocked the inhibition of HMGR by cholesterol.

Inhibitors of HMGR

Two chemical compounds which act as specific inhibitors of HMGR were isolated by A. Endo from different strains of ascomycetes, compactin (ML 236B) in 1976 and mevinolin (Monocalin K) in 1979. Both of these compounds contain a moiety which resembles the enzyme bound intermediate of the two step reduction of HMG-CoA, mevaldyl coenzyme A thiohemiacetal. Mevinolin (Fig. 2) is the more potent inhibitor, differing from compactin by the addition of a methyl group. Mevinolin is a highly specific inhibitor of HMG-CoA Reductase in mammals, yeasts and plants with K_i values of 0.5, 3.5, and 2.2 nM reported respectively. (Bach and Lichtenthaler 1983b) This inhibition can be completely alleviated by the addition of exogenous MVA.

Both compactin and mevinolin have been used in studies of HMG-CoA Reductase of various organisms (Sabine 1984, Brown and Goldstein 1980). In radish seedlings,



Mevinolin

Figure 2. The Chemical Structure of Mevinolin.

mevinolin does not inhibit germination but does prevent the elongation of the roots due to the rate limiting effect of decreased sterol biosynthesis and possibly due to changes in the phytohormone balance within the cell (Bach and Litchenthaler 1983a).

In view of the isoprenoid nature nature of ABA and GA_3 and their important hormone functions, there have been surprisingly limited investigations describing how these hormones may interact with HMGR.

MATERIALS AND METHODS

Initiation and Maintenance of Callus Cultures

All Euphorbia lathyris callus cultures were initiated and maintained on a basal medium with and without the addition of various phytohormones as noted. The basal medium was composed of B5 medium (Gamborg et al 1976) with 2% (w/v) sucrose and 0.8% (w/v)Bacto agar. The pH was adjusted to 5.5 before the addition of agar. Twenty ml of medium was poured into 25x150 mm glass culture tubes and then autoclaved for 15 min.

Seeds of E. lathyris variety "Chico" were surface sterilized with 10% (v/v) Chlorox bleach for 15 min followed by 3 rinses with sterile distilled:deionized water. The seeds were then placed on the basal medium in the test tubes and placed in the growth room on a 18h light, 6h dark cycle at 25 ± 2C. After 8 days, 5mm segments of the hypocotyls were excised and placed on the bsasal medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L 6-benzyladenine (6BA).

Proliferating callus arising from the cut edges of the hypocotyl segments was subcultured and maintained on the basal medium without the addition of phytohormones. The cultures were subcultured every four weeks and grown at 25 ± 2C, in the dark.

Suspension cultures were established from the callus cultures and maintained by transferring two grams of cells into 40 ml of the basal medium, without agar, in a foam stoppered 125 ml erlenmeyer flask. The suspension cultures were subcultured every two weeks and were placed in the dark at 25 ± 2C on a rotary shaker at 100 rpm.

Mevinolin Inhibition Studies

The inhibition of callus growth by mevinolin was determined by adding a range of concentrations of mevinolin (6.25nM to 62.5nM) to the basal medium. The growth of the callus on media with ABA (0.4uM) combined with the various mevinolin concentrations was also examined. Filter sterilized solutions of mevinolin and ABA were added to the media after autoclaving and prior to gelling of the agar. Callus was subcultured onto these media as described above and grown in the dark. After 28 days, the cultures were removed from the tubes with a spatula and the fresh weights were recorded. The experiments were set up as a randomized complete block design with 3 replications and the means were compared using the standard deviation.

Mevinolin (62.5nM) and ABA (0.2uM) were added separately *in vivo* to one week old suspension cultures for 24h before extracting and assaying the specific activity of HMGR. HMGR from the 24h ABA *in vivo* treatment was assayed with or without the *in vitro* addition of mevinolin(62.5nM).

Extraction of HMGR

Suspension cultures of E. lathyris hypocotyl callus were poured into 50 ml conical centrifuge tubes and centrifuged at 500xg for 5 min in a clinical centrifuge at room temperature. The packed cell volume (PCV) was then recorded. The cells were homogenized for 2 to 3 min with grinding (80 to 100 strokes/min) in a cold mortar on ice, with three volumes of cold (4C) homogenizing buffer composed of: 10 mM HEPES, 0.35 M sucrose, 0.03 M Na_2EDTA, 0.02 M mercaptoethanol (added fresh) 5g, insoluble polyvinyl-pyrrolidone (PVP) /100ml and 0.5 g of washed sea sand.

The homogenate was filtered through two layers of Miracloth and then centrifuged at 12,000xg in a Sorvall RC2-B centrifuge for 15 min at 4C to sediment cell debris, nuclei, and PVP particles. The supernatant was transferred and recentrifuged at 18,000xg in the same centrifuge for 15 min at 4C to remove any remaining mitochondria. The supernatant was then transferred with a pasteur pipette and then centrifuged at 75,000xg at 4C in a Beckman Model LM8 ultracentrifuge for 2h to obtain the microsomal pellet.

The supernatant was removed with a pasteur pipette and the pellets were resuspended in 0.1 M potassium phosphate buffer at 4C (pH7.3, 0.02ml/ml PCV). An aliquot was removed for protein determination by the Bradford method (1976) and the remainder was made to 10mM dithioerythritol (DTE). Each enzyme assay contained 50 ul of this mixture.

HMGR Assay

Both spectrophotometric and radiochemical assays were used to measure the activity of HMGR. The procedures are based on the methods of Brooker and Russell (1975). The spectrophotometric assay contained in a final volume of 0.5 ml, 50 umol potassium phosphate buffer (pH 7.3), 0.3 umol NADPH, 1 umol HMG-CoA, 5 umol DTE, and different amounts of protein up to 0.5 mg. The HMG-CoA was omitted from the blank assay. The reaction was carried out in a Beckman Model 25 double beam spectrophotometer. The activity of the enzyme was calculated from the change in absorbance by NADPH at 340 nm. After an intial lag phase of 1 min, the enzyme reaction was linear for at least 20 min (Fig 3) and the amount of product was proportional to the amount of the enzyme up to 0.38 mg (Fig 4).

For the radiochemical assay, each incubation mixture contained in a final volume of 0.1ml; 10 umol potassium phosphate buffer (pH 7.3), 0.2 umol NADPH, 1 umol DTE, 0.01umol Na-EDTA, 0.05uCi [3-^{14}C]HMG-CoA (56.8 mCi/mmol), up to 20 nmol of unlabeled HMG-CoA and 50 ul of the microsomal suspension (not exceeding 50 ug of protein). Various amounts of the substrate HMG-CoA (0.46 to 20 nmol) were used in the kinetic studies of HMGR. In the ABA *in vitro* studies, ABA from 20 nmol to 200 umol was included in the reaction mixture in combination with the different concentrations of HMG-CoA.

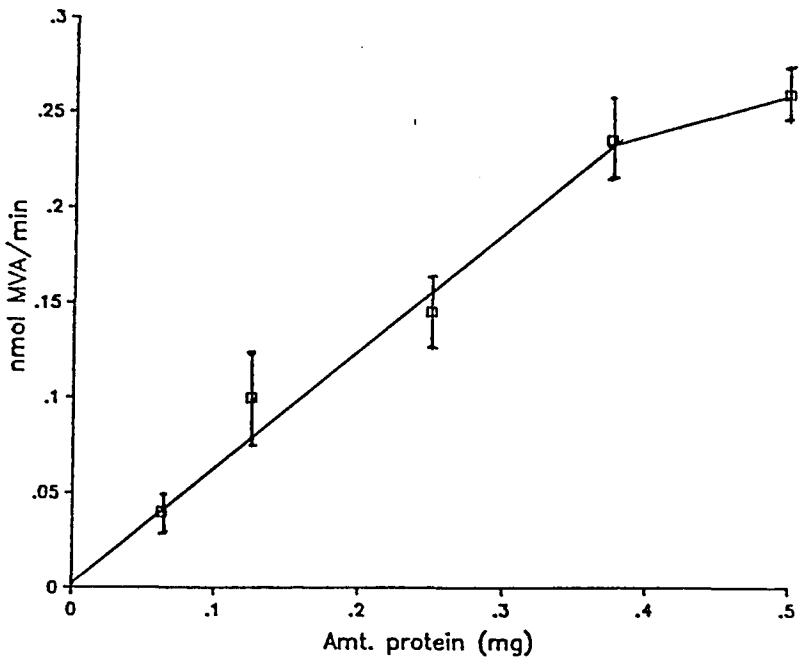


Figure 3. Amount of MVA Synthesized With Time at Different Levels of Protein.
Spectrophotometric assay was performed with different amounts of the microsomal protein in the reaction mixture. The symbols correspond in ascending order to increasing amounts of protein. The amounts of proteins are; 75, 125, 250, 375 and 500 ug.

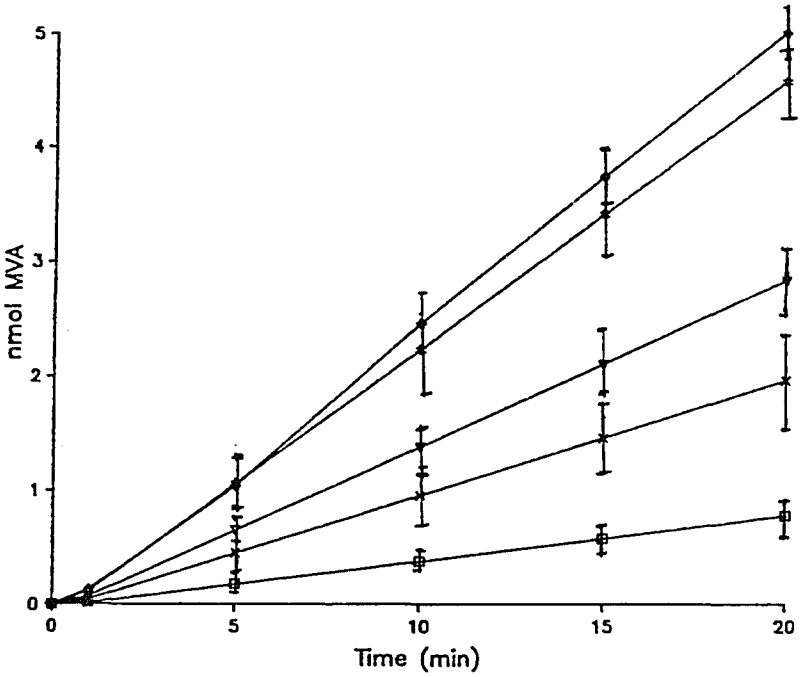


Figure 4. Rate of MVA Synthesis at Different Concentrations of Protein.
Spectrophotometric assay was performed with different amounts of proteins ranging from 75 to 500 ug. The data reflects the mean and standard deviation of triplicate assays.

The reaction was started by the addition of HMG-CoA and was incubated at 30C for 20 min. The reaction was stopped by the addition of 0.2 ml of 2N HCL. A blank assay with the 2N HCL added prior to adding the substrate, HMG-CoA, was included. A 10 ul xylene solution containing 20,000 DPM of [4-^{3}H]Mevalonic acid was added as an internal standard. The solution was then allowed to stand for 30 min to allow for lactonization of the mevalonic acid.

A chloroform extraction procedure which utilized a phase separation filter paper to separate the MVA from the unreacted HMG-CoA was used (Ackerman et al 1977). The radioactivity of each sample was measured in a Beckman LS7000 scintillation counter using a dual labeling program for ^{14}C and ^{3}H with automatic quench compensation factor.

In using the method as described, the recovery of the internal standard was in the range of 15% to 30% which is much lower than the 50% to 60% reported by Ackerman et al (1977). This low recovery may be due to retention of the labeled product on the phase separation filter paper. The low recovery of the product and the internal standard led to a higher degree of variation of the results, making it difficult to obtain significantly different results between treatments.

To increase the recovery of the internal standard, the extraction method was modified as follows. Instead of using the phase separation filter paper, a disposable 3 ml

syringe was used to carefully withdraw the bottom chloroform layer and transfer to the scintillation vial after each extraction with chloroform. This modification of the procedure increased the recovery of the internal label to the range of 55% to 70%. This modified method was employed for all the assays described in this work with the exception of the results presented in Fig 5 which were obtained by means of the phase separation filter paper. In this case the differences were great enough to overcome the large variation caused by the filter paper method.

The chemicals NADPH, HMG-CoA, DTE, MVA and PVP were obtained from Sigma Chemical Company and the radiolabeled HMG-CoA and MVA were obtained from NEN Research Products.

RESULTS AND DISCUSSION

The inhibition of E. lathyris callus growth by mevinolin is shown in Fig 5. There is a 65% decrease in the fresh weight when the callus is grown for 28 days on medium with 25 nM mevinolin. Callus grown on medium with 62.5 nM mevinolin had less than 5% of the fresh weight of the callus grown on the mevinolin free medium. The addition of 0.4 uM ABA did not significantly change the response of the callus to the different concentrations of mevinolin. Since there was greater than 95% inhibition of growth by 62.5 nM mevinolin, this concentration was used in all latter studies using mevinolin.

Initial experiments which evaluated the effects of ABA on HMGR activity indicated that in vivo ABA treatment increases the susceptibility of HMGR to the competitive inhibitor of the enzyme, mevinolin. When suspension cultures were treated with ABA (0.2 uM) for 24 h prior to the extraction of HMGR and then assayed with and without the in vitro addition of 62.5 nM mevinolin (Table 1), there was a substantially higher inhibition of HMGR from the in vivo ABA treated cultures as compared to the enzyme from the control treatment. The specific activity of the enzymes from both the control and the ABA treatments were the same when assayed without the in vitro addition of mevinolin.

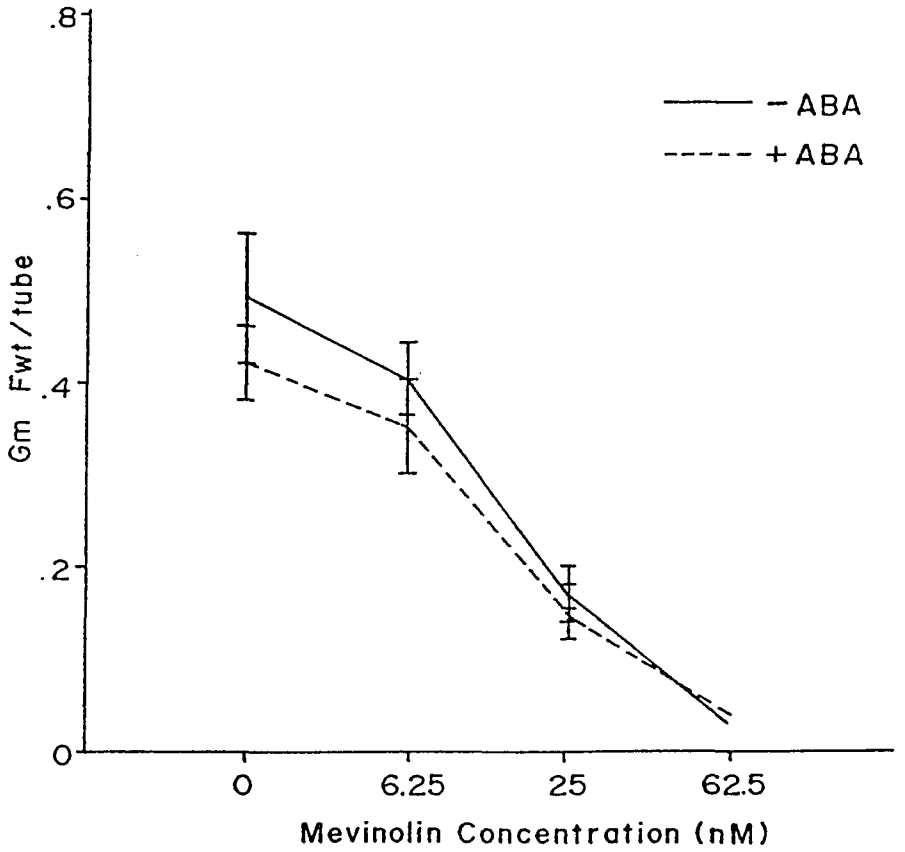


Figure 5. Inhibition of E. lathyris Callus Growth by Mevinolin.
Callus was grown on Gamborg's B5 basal medium (1976) with 0 and 0.4uM ABA. The data reflect the average fresh weight and standard deviation of 3 experiments with 20 tubes/concentration of mevinolin.

Table 1. *In Vitro* Inhibition of HMGR by Mevinolin Following *In Vivo* ABA Treatment.

E. lathyris suspension cultures were treated with the *in vivo* additions of 0 and 0.4 uM ABA 24 hr prior to the assays. Specific activity of HMGR from the suspension cultures was measured with or without the *in vitro* addition of 62.5 nM mevinolin. The data was obtained from duplicate assays using the phase separation filter paper as described in Materials and Methods.

Treatment	Specific Activity
Control	36-46 nmol MVA/mg protein/h
Control + *in vitro* mevinolin	15-18
In vivo ABA	42-47
In vivo ABA + *in vitro* mevinolin	0.5-2

The higher degree of inhibition of the ABA *in vivo* treated enzyme by the *in vitro* addition of mevinolin may be indirect evidence for an interaction of the enzyme with ABA or a metabolite of ABA. When HMGR is assayed without the *in vitro* addition of mevinolin, the activity measured is close to the maximum velocity of the enzyme since an excess of the substrate, HMG-CoA, was used. The specific activity of the enzyme is not changed by the *in vivo* ABA treatment (Tables 1 and 2).

When HMGR is assayed with the *in vitro* addition of mevinolin, the saturation of the active sites on the enzyme by the high concentration of substrate may be substantially decreased due to competition with the inhibitor. Thus, the rate of the reaction may behave as if the substrate concentration is in a range closer to the K_m of the enzyme.

That the HMGR from the ABA *in vivo* pretreatment will exhibit a much lower activity than that of enzyme from the control treatment at the same concentration of mevinolin *in vitro* may indicate that ABA or an ABA metabolite, such as phaseic acid (Millborrow 1983), may negatively modify the enzyme to decrease the rate of the reaction at physiological concentrations of the substrate HMG-CoA.

To investgate the possible modes of interaction of ABA with HMGR, the activity of the enzyme from the 24 h ABA *in vivo* treatment was assayed against various concentrations of the substrate. Figure 6 shows that the *in vivo* treatment

Table 2. Mevinolin Induced Increase in the Specific Activity of HMGR.

Specific activity of E.lathyris HMGR from suspension cultures assayed 24 hours after the in vivo addition of 62.5 nM mevinolin and 0.2uM ABA. The data shows the mean and standard deviation of triplicate assays.

Treatment	Specific Activity
Control	27.3 + 2.5 nmol MVA/mg protein/h
Mevinolin	55 + 2.6
ABA	27.8 + 1.1

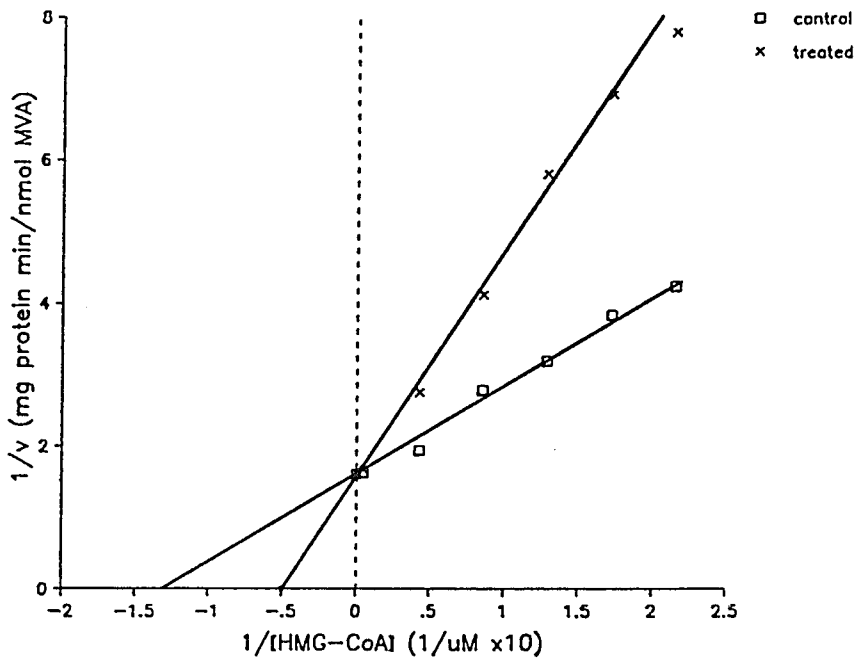


Figure 6. Lineweaver-Burk Plot for HMGR Following 24h *In Vivo* ABA Treatment.
The data reflects the mean of triplicate assays repeated 3 different times.

of the suspension cultures with ABA does cause a change in the kinetic properties of HMGR. The apparent K_m of HMGR from the ABA 24 h *in vivo* treatment is 19.0 uM, whereas the apparent K_m of the enzyme from the control treatment is 7.8 uM. The maximal velocities of the enzymes from the 2 treatments are the same. This change in the apparent K_m without a change in the V_{max} suggests a competitive inhibition by ABA on HMGR with respect to the substrate.

However this is only observed after the *in vivo* treatment with ABA and not when ABA is added in *vitro*. When the enzyme from the control treatment was assayed with different concentrations of the substrate and with the *in vitro* additions of different fixed concentrations of ABA (0.02 to 200 mM), the rates of the reactions were indistinguishable from the those of the enzyme of the control treatment (Fig 7). Thus the *in vitro* addition of ABA to the enzyme assay has no effect on the activity of the enzyme. This agrees with the results of Brooker and Russell (1979).

If ABA can modify the enzyme *in vivo* and not *in vitro*, then ABA might be metabolized into another compound which may directly modify the enzyme. Another possibility is that ABA might interact with or is bound to a necessary component of the membrane-protein complex which was not extracted with the microsomal membrane fraction.

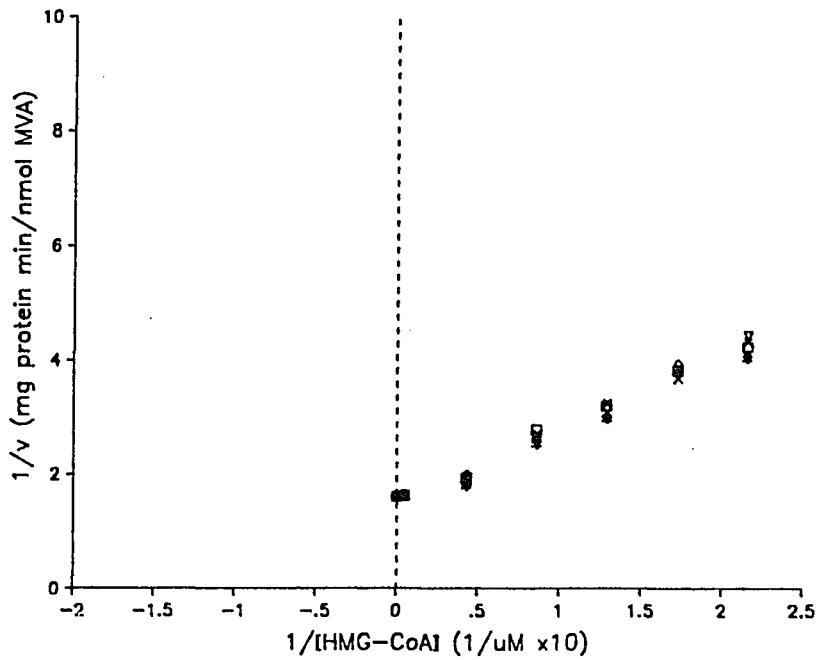


Figure 7. Lineweaver-Burk Plot for HMGR With <u>In Vitro</u> ABA. The data points are from triplicate assays repeated 2 times. The concentrations of ABA used were 0.02, 0.2, 20 and 200 mM. The open square corresponds to the control assay without <u>in vitro</u> addition of ABA.

The modification of the enzyme by ABA may be a short term and reversible effect as ABA does not cause any significant changes in the yield of the fresh weight of E. lathyris callus grown for 28 days on different levels of the HMGR inhibitor, Mevinolin (Fig 5).

Further investigations are needed to detail the mechanism of ABA modification of the enzyme with respect to dosage and time of ABA application. In view of the hormonal nature of ABA and its known antagonism to GA_3 (Jacobsen and Beach 1985), investigations of a possible interaction of HMGR with GA_3 would also be worthwhile. Preliminary results in this investigation seem to suggest that GA_3 interacts positively with HMGR.

In addition to the results reported on the interaction of ABA with HMGR, an effect of the in vivo application of mevinolin to the suspension was observed. The treatment of the suspension cultures with 62.5 nM mevinolin for 24h results in a two-fold increase in the specific activity of HMGR over the specific activity of the control (Table 2). This increase most likely results from an increase in HMGR biosynthesis due to a depletion of an endproduct, such as stigmasterol, which may regulate the enzyme. Depletion of cholesterol by mevinolin treatment in mammalian liver also causes a substantial increase in HMGR biosynthesis (Kita, Brown and Goldstein 1980).

Mevinolin might be useful to elevate levels of HMGR in plant suspension cultures to increase the production of useful secondary metabolites or to prepare larger amounts of enzyme for isolation for use in immobilized enzyme systems.

CONCLUSION

This study was undertaken to investigate a possible regulatory role of the isoprenoid hormone ABA on the synthesis of MVA from HMG-CoA. The results of this study indicate that ABA causes an *in vivo* modification of HMGR through some as yet unknown mechanism. The *in vivo* application of 0.2 uM ABA to *E. lathyris* suspension cultures for 24h changes the apparent K_m of the HMGR enzyme from 7.8 uM to 19 uM. ABA applied *in vitro* has no effect on the activity of the enzyme.

The specific activity of HMGR in plant cell cultures may be increased by the use of a competive inhibitor of the enzyme, mevinolin. Exogenous applications of mevinolin to *E. lathyris* suspension cultures for 24h results in a doubling of the specific activity of the enzyme.

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