Title
TERPENOID BIOSYNTHESIS IN EUPHORBIA LATHYRIS AND COPAIFERA SPP

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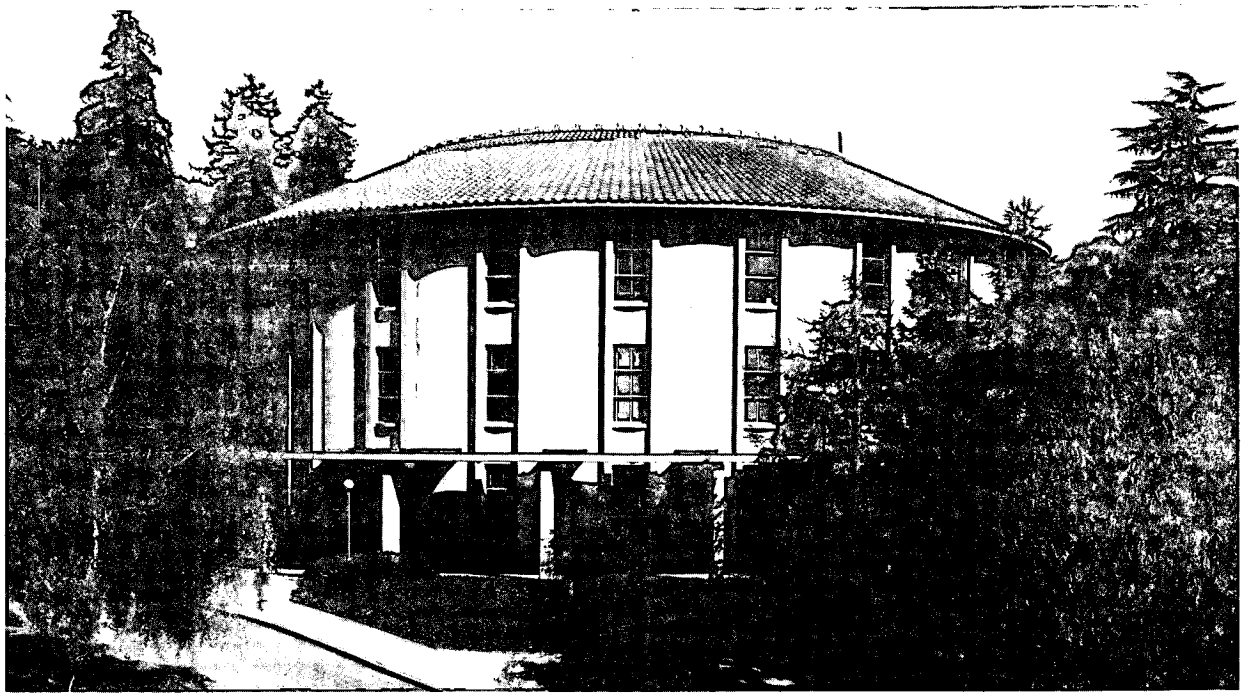
C.L. Skrukrud
(Ph.D. Thesis)

July 1987



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DISCLAIMER

Terpenoid Biosynthesis in *Euphorbia lathyris* and *Copaifera* ssp.

Cynthia Louise Skrukrud

Ph.D. Thesis

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ABSTRACT

Biosynthesis of triterpenoids (triterpene esters and triterpenols) by isolated latex of *Euphorbia lathyris* was investigated. The rate of *in vitro* incorporation of mevalonic acid (0.55 nmol 100 µl latex^{-1} h^{-1}) into triterpenoids was thirty times greater than acetate incorporation (0.02 nmol 100 µl latex^{-1} h^{-1}), indicating that the rate-limiting step in the pathway occurs prior to mevalonate. A particulate fraction, capable of converting mevalonate but not acetate into triterpenoids (15,000*g* pellet), showed a linear rate of triterpenoid biosynthesis over a period of four hours. No evidence was found to indicate that soluble latex proteins had an effect on either the biosynthesis or the removal of triterpenoids from this fraction. Electron micrographs of isolated *E. lathyris* latex showed the presence of latex particles and rod-shaped starch grains as well as a single-membrane-bounded structure which comigrated on Percoll gradients with the mevalonate to triterpenoids converting activity.

Both HMG-CoA reductase (EC 1.1.1.34) and HMG-CoA lyase (EC 4.1.3.4) activities were detected in isolated latex. HMG-CoA reductase was localized to a membrane-bound fraction of a 5000*g* pellet of latex. The rate of conversion of HMG-CoA to mevalonate by this enzyme (0.02 nmol 100 µl latex^{-1} h^{-1}) is comparable to the overall rate of acetate incorporation into the triterpenoids suggesting that this enzyme is rate-determining in the biosynthesis of triterpenoids in *E. lathyris* latex.

HMG-CoA reductase of *E. lathyris* vegetative tissue was localized to the membrane-bound portion of a particulate fraction (18,000*g*), and was solubilized by treatment with 2% polyoxyethylene ether W-1. Differences in the optimal pH for activity of HMG-CoA reductase from the latex and vegetative tissue suggest that isozymes of the enzyme may be present in the two

tissue types.

Studies of the incorporation of various precursors into leaf discs and cuttings taken from *Copaifera* spp. show differences in the rate of incorporation into *Copaifera* sesquiterpenes suggesting that the site of sesquiterpene biosynthesis may differ in its accessability to the different substrates and/or reflecting the metabolic controls on carbon allocation to the terpenes. Mevalonate incorporation by *Copaifera langsdorfii* cuttings into sesquiterpenes was a hundred-fold greater than either acetate or glucose incorporation, however, its incorporation into squalene and triterpenoids was also a hundred-fold greater than the incorporation into sesquiterpenes.

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Nothing I could write would be sufficient to thank Tom von Geldern for his love, friendship, support and patience over the last 5 years. I can only promise that I will try to be a more sane person A.T. (after thesis)!

Lastly I would like to thank the members of my family, Dad, Mom, Dori and Elizabeth for putting up with me these 30-odd years. They are me, so this thesis is as much a product of them as it is of me. Being Skrukruds, they understand my belief that it is certainly not good enough. With that apology, I especially want to note the role my mother often forgets she has played in the accomplishments of her family; this work is dedicated to her--Beverly Beryl Bowersox Skrukrud.

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INTRODUCTION

Among the many aftershocks of the OPEC oil embargo of 1973 and 1974 was renewed interest in biomass as a source of energy and chemicals. The main attraction of biomass is that it is a renewable source of energy. It has the advantage over other forms of renewable energy, such as wind and solar, that it is stored energy. In addition, it is the only substitute for petroleum in its role as a feedstock for a multitude of products such as plastics and polymers.

The concept of biomass as a source of fuel is neither new nor novel; after all, oil and coal are nothing but very old biomass. The reliance of industrialized nations on fossil fuels is a relatively recent occurrence, within the last two hundred years. Before that wood was the main fuel supply for centuries; it was the major fuel source in the United States up until 1875 (12). Even today with the seeming utter dependence of the world on fossil fuels, wood still accounts for 10% of worldwide fuel consumption, primarily among the less developed countries where it provides over 50% of the consumed energy (23).

The shift of industrialized nations to a petroleum-based energy structure was due in part to the relative ease of obtaining fossil fuels. The convenience of transportation of petroleum products has also been one of the hallmarks of the oil and gas era. A third strong point for these fossil fuels as well as for coal is their low oxygen content giving them the high energy content that makes them excellent fuels. While it was cheap and abundant, petroleum easily fueled modern society. Today with it becoming more scarce and difficult, politically, environmentally, and economically, to obtain, fossil fuel does not stand as high above the alternatives as it once did. Biomass, the original source of petroleum, is a logical choice for investigation as a substitute.

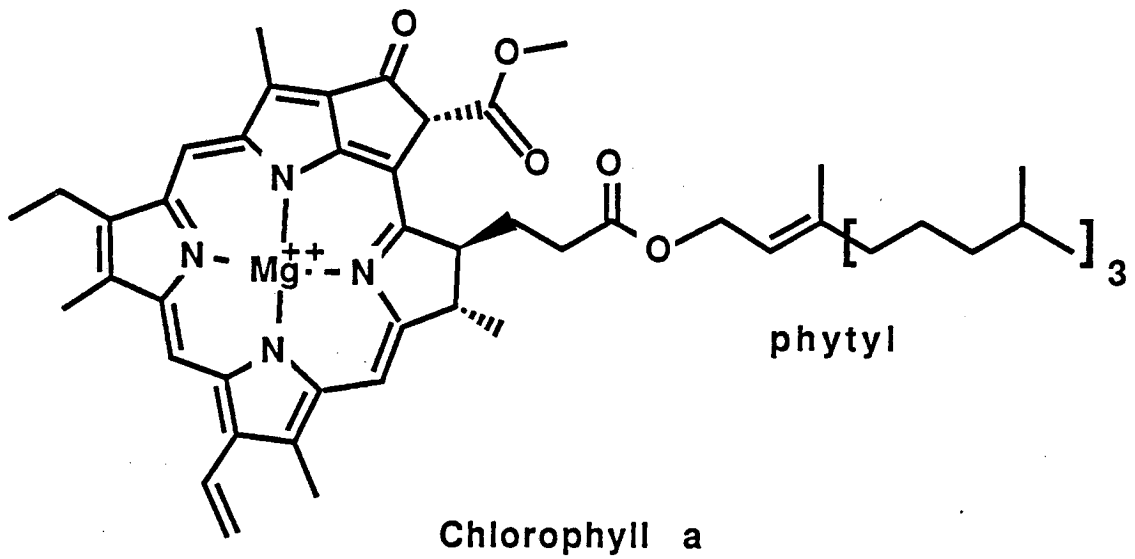
In recent years, different avenues of research have explored possibilities for developing biomass as a fuel for industrialized countries and for the 21st century. Notable examples among these are the development of "energy cane," high biomass sugar cane(2), and the use of fermentation technology to produce ethanol from high biomass crops such as energy cane and corn. These projects exemplify two different approaches to biomass development. The first is

directed at increasing the output of total biomass of a crop; the second is concerned with a process to convert the biomass into the more convenient alcohol fuel.

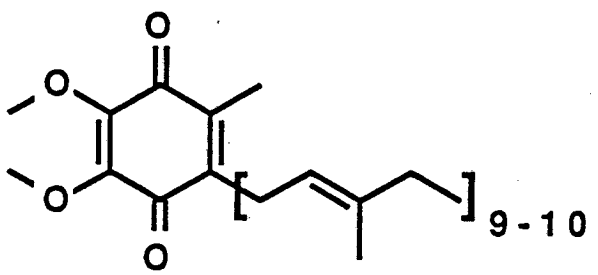
A third approach to the use of biomass as a substitute for fossil fuels emphasizes not only the quantity but quality of the biomass product. Some plants contain a greater percentage of more highly reduced compounds which like petrochemicals have greater fuel value, making them more valuable for use as fuel stock. Many plants contain unique chemical constituents that could be utilized instead of petrochemical-derived substances, both as final products and as precursors for the manufacture of synthetics. As an example, the use of both vegetable oils and waxes has been limited by the availability of cheap petroleum-derived synthetics but could conceivably play a more prominent role in the future (21).

Among those plants high in hydrocarbon content, it is often their terpenoid compounds that are the constituents of interest. In terms of energy content, terpenoids are highly desirable. Their heat of combustion of *ca* 40 GJ t^{-1} make them as energy-rich as gasoline, higher than oils, fats, and lignins at *ca* 35 GJ t^{-1}, biomass-derived alcohols and ketones at 20 to 30 GJ t^{-1}, and celluloses, starch, and sugars at 15 GJ t^{-1} (18). Plant resins of the Pinaceae and the essential oils of various plant families are examples of isoprenoids that also have the potential to replace petroleum-based feedstocks as industrial raw materials (21).

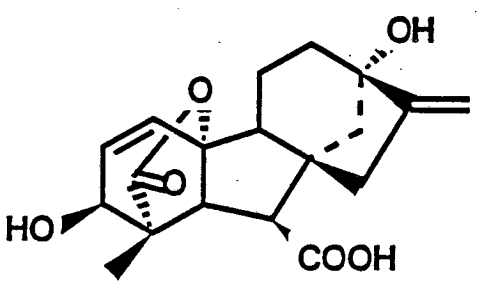
Terpenoids are commonly considered secondary plant products because the majority of these compounds appear to play no role in the primary processes essential to plant life. Important exceptions include the phytyl side chain of chlorophyll, ubiquinone involved in mitochondrial electron transport, and the hormonal gibberellins and abscisic acid (Fig. 1). Members of the terpenoid class of compounds are recognized as related by their shared structural feature, the isoprene unit (Fig. 2). The common route of their biosynthesis has been established in both animals and plants (Schemes 1-3). It is the 5-carbon isopentenyl pyrophosphate that is the building block of all terpenes, but the formation of its 6-carbon precursor mevalonic acid is generally considered to be the point on the isoprenoid pathway at which the committment to terpenoid biosynthesis is made.



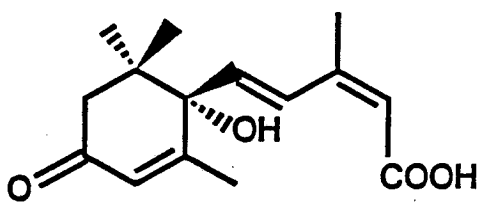
Chlorophyll a



Ubiquinone



Gibberellic Acid
(GA_3)



Abscisic Acid

Fig. 1. Terpenoid compounds important in primary plant metabolism.

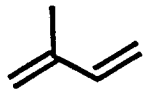


Fig. 2. Isoprene, the basic unit of terpenoid compounds.

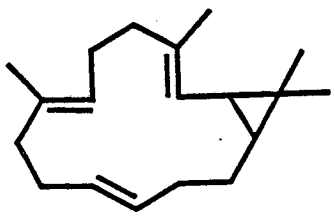
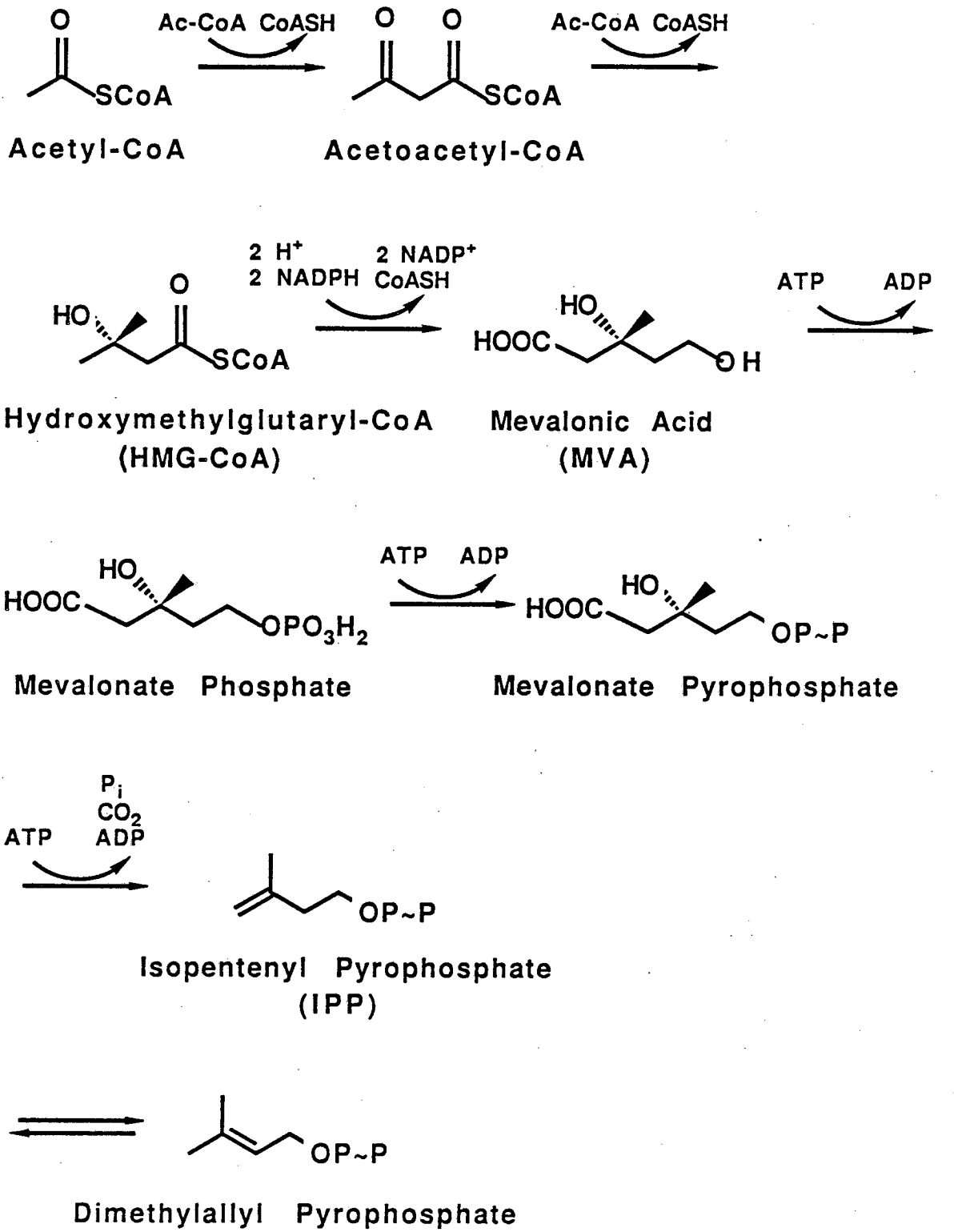
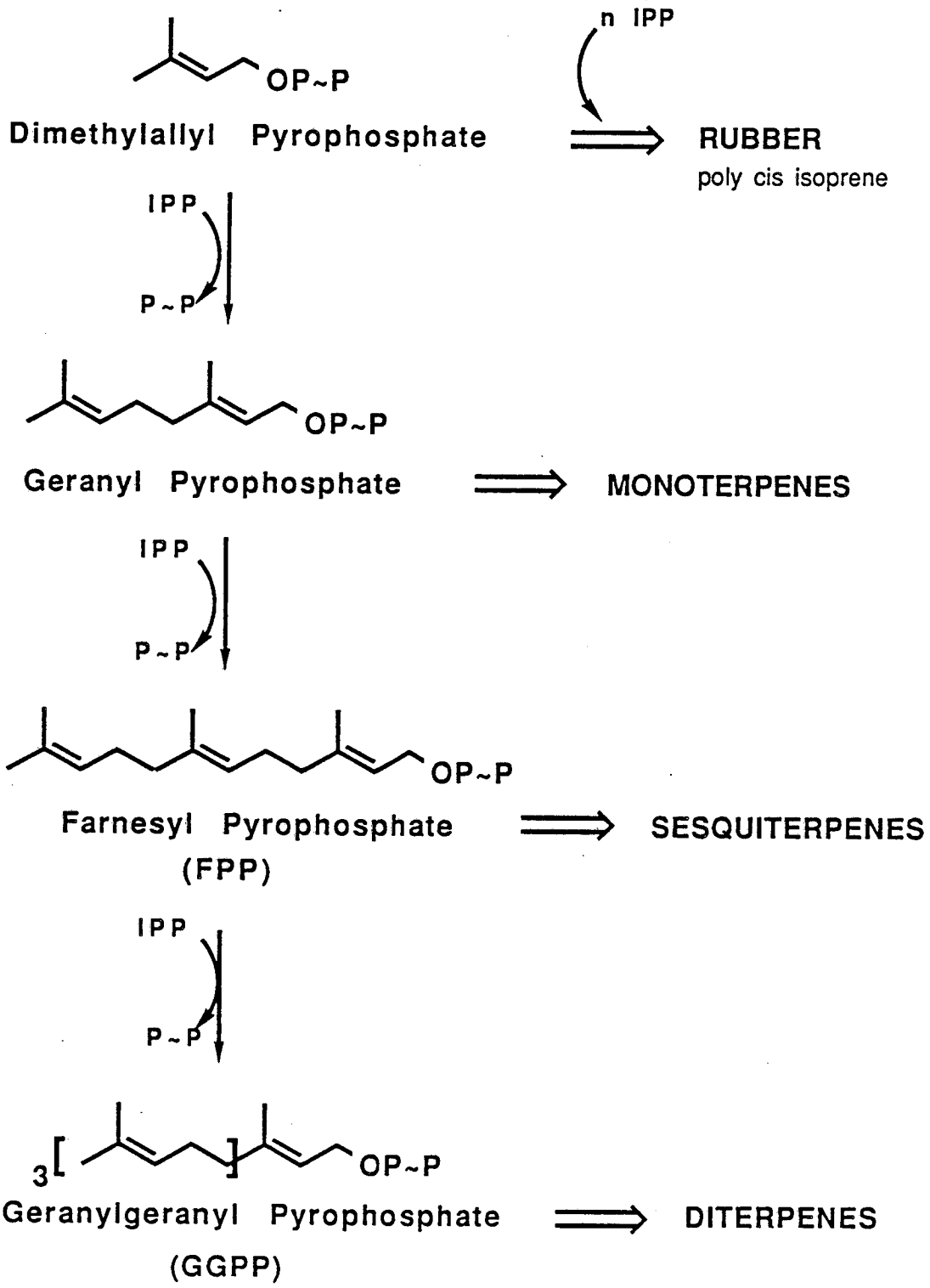


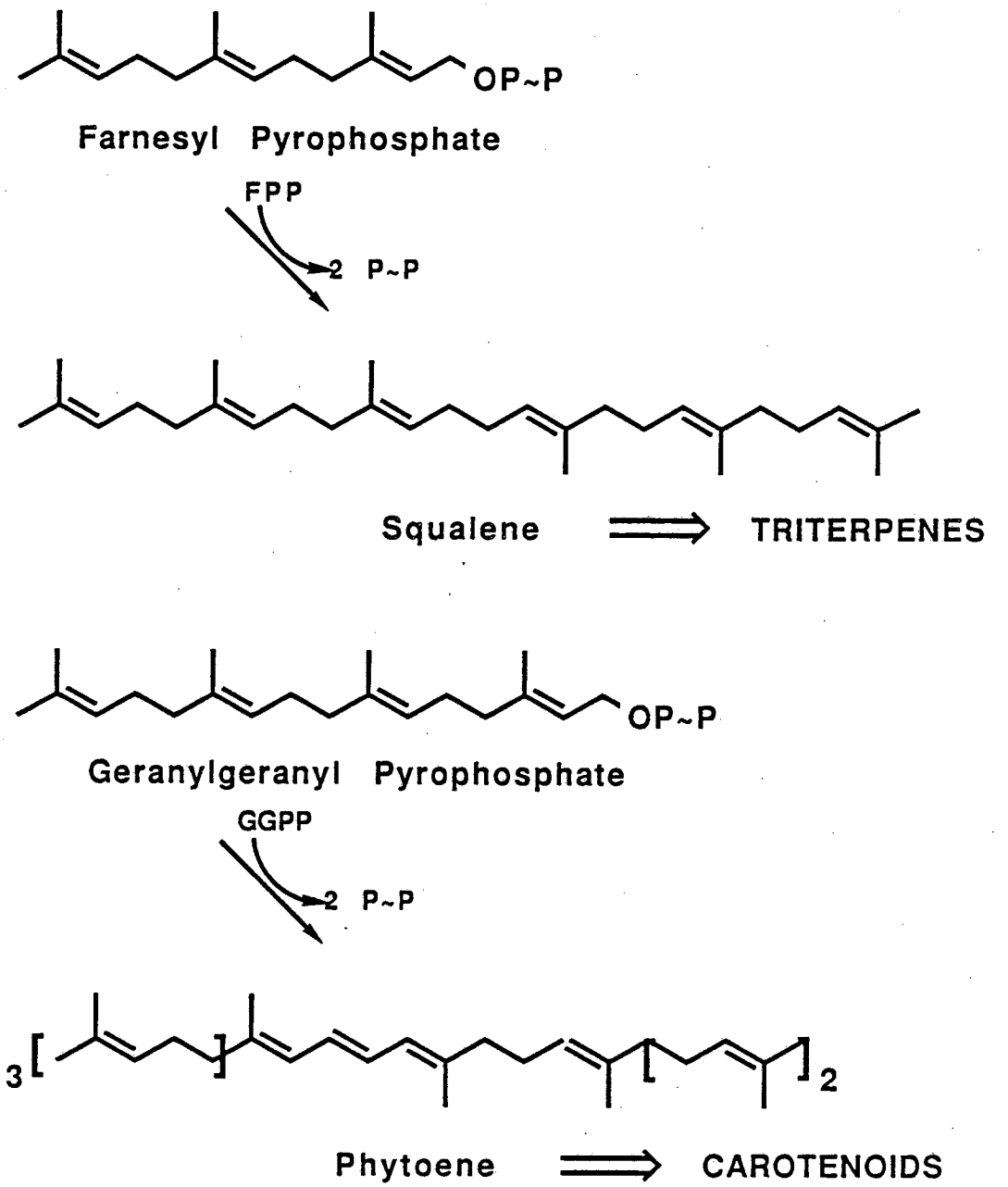
Fig. 3. Casbene, a diterpenoid phytoalexin.



Scheme 1. Pathway of terpenoid biosynthesis: formation of 5-carbon building block IPP.



Scheme 2. Pathway of terpenoid biosynthesis: formation of C_{10}, C_{15}, and C_{20} compounds and polyisoprene rubber.



Scheme 3. Pathway of terpenoid biosynthesis: formation of C_{30} and C_{40} compounds.

Large quantities of terpenoids are found in certain plants, making them desirable as a biomass resource for fuels and chemicals. Often the terpenes are stored in specialized cells such as the rubber-containing laticifers of *Hevea brasiliensis* and the glands or ducts containing the mono and sesquiterpene essential oils found in herbs of the families Labiatae and Umbelliferae (7). The large quantity and the sequestration of these pools of terpenoids led to speculation that they were non-essential waste products, yet the energy required to manufacture these highly reduced compounds from fixed CO_2 is an argument against such a squanderous process (10). Discovery of the role of some terpenoids such as the diterpene casbene (Fig. 3), a phytoalexin anti-fungal agent whose production is enhanced in castor bean seedlings by attack by potentially pathogenic fungi, has promoted the view that defense is a major role of terpenes in plants (19), but the carotenoids and essential oils of flowers serve respectively as color and odor attractants for pollinators(9). Clearly no single role for so diverse a group of compounds as isoprenoids can be ascribed, yet many seem to be important in a plant's interactions with other plant and animal species. The function of each of the multitude of structures that have been found and the explanation for the quantities stored in some species remain unclear.

Development of plants high in hydrocarbon content for use as biomass can be approached in a number of ways. Potential hydrocarbon-producing crops have been identified through screening programs (3), and improvement of yields in species such as *Grindelia camporum* are being pursued by classical agronomic practices and genetic selection of outstanding strains (13). The promise of this plan of action is substantiated by the six-fold increase in natural rubber production achieved by Malaysian growers of *Hevea* over a period of 35 years of selection and cultivational improvements (8).

A different focus is required to take advantage of the recent advances in genetic manipulation that is the basis of the emerging field of biotechnology. The idea here is to alter the plant or microbe directly at the gene level to increase the yield of a desired product. However, while the development of the techniques required to perform this type of genetic engineering has flourished, the understanding of what should be altered in a plant is scant. In order to use

either genetic or chemical technology rationally to improve plant products for biomass, Rabson and Rogers have urged support of projects involved in fundamental biological research designed to improve understanding of the distribution of fixed energy within plants (18). Primary among the knowledge that is hoped to be gained is insight into the signals and controls that govern the metabolic partitioning of photosynthetically-fixed carbon into various classes of compounds. This involves an understanding of the factors that regulate the flux of metabolites through a given pathway, including any compartmentation of pathway enzymes, rate-limiting steps, end-product inhibition, and environmental or metabolic regulation. Further sophistication will result from insight into the branch point controls of carbon allocation, the transport of assimilates, and the genetic controls of these processes. With this foundation of knowledge, manipulation of the plant genome to increase yields of desired products can be pursued in a logical manner.

Euphorbia lathyris was one of the first species proposed as an energy crop (4). It is a member of the Euphorbiaceae, subfamily Euphorboideae, a group of plants characterized by the presence of laticifer cells which contain a milky sap, the latex. Heptane extraction yields a hydrocarbon-like fraction, comprising 4 to 5 % of the dry weight of the plant, composed almost exclusively of triterpenoids (14). This fraction has a heat value of *ca* 42 GJ t dry weight^{-1} and has been catalytically converted on a zeolite catalyst to yield a product similar to gasoline in both fuel value and product size distribution (11).

Triterpenoids are also the major constituents of the latex; it has been estimated that latex isoprenoids account for 15 to 20% of the whole plant heptane extract (15). Fifty percent of the dry weight of the latex is made up of six major triterpenols and their fatty acid esters (16,20) (Fig. 4). Tapped latex is capable of biosynthesis of triterpenoids from acetate indicating that laticifer cells are active in the production of these compounds as well as in their storage (17). Consequently, the latex is a convenient *in vitro* system in which to study factors affecting the biosynthesis of the triterpenoids.

A second potential biomass producer of large quantities of terpenes is the genus *Copaifera*, whose member leguminous tropical trees produce the oleoresin copaiba balsam. Up

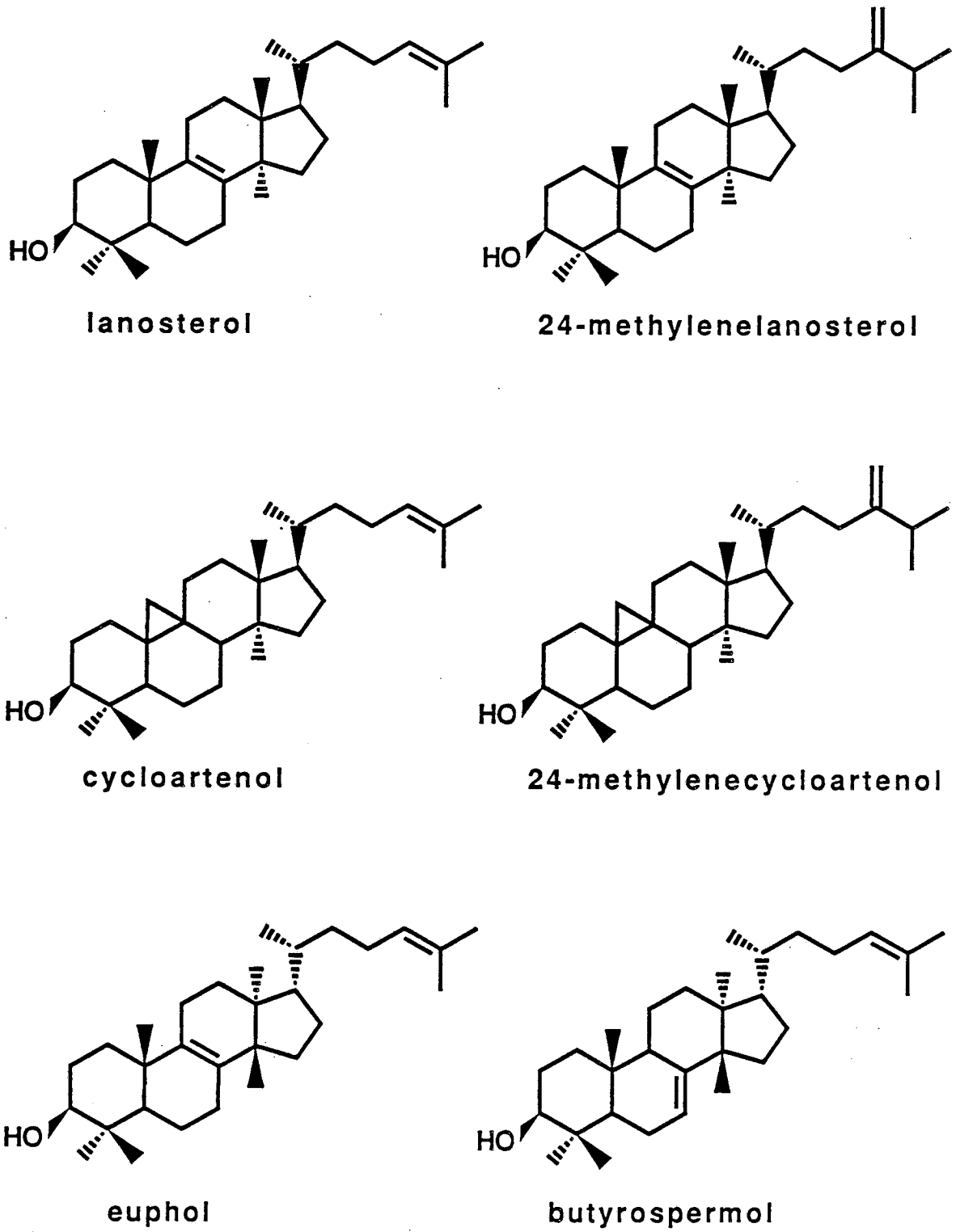


Fig. 4. Triterpenoids of *Euphorbia lathyris* latex.

to 90% of this oil, obtained by tapping the tree trunk, consists of sesquiterpene hydrocarbons (22). A mature tree can yield 40 to 60 liters of oil annually, which has been used directly as fuel in diesel engines (1,5). Both the wood and leaf resins contain a suite of sesquiterpene hydrocarbons whose biosynthetic interrelationships have yet to be fully understood (6).

The focus of the work described here was to gain understanding of biosynthesis of terpenes in plants. As the enzymes of the isoprenoid biosynthetic pathway have been detected in various other plants, it was accepted that these same steps are involved in biosynthesis of the terpenes found in the plants studied, although not all enzymes of the pathway have been analyzed in these specific plants. Rather, this investigation focused on elucidation of levels of organization and regulation of this pathway. Specifically, the work outlined in Chapter 1 presents a model of the biosynthesis of triterpenoids in *E. lathyris* latex based on the metabolism of exogenously-supplied terpenoid pathway intermediates. This includes the current level of understanding of the grouping of the enzymes of the pathway in subcellular structures as well as evidence suggesting that the enzyme β-hydroxymethylglutaryl-coenzyme A reductase catalyzes the key rate-limiting step in the pathway. The presence of β-hydroxymethylglutaryl- coenzyme A lyase in latex is also described, and evidence indicating the absence in the latex of the soluble protein factors found in mammalian systems that enhance but do not catalyze the final steps in the conversion of farnesyl pyrophosphate to triterpenoids is presented.

Chapter 2 details work exploring the location, solubilization, and partial purification of β-hydroxymethylglutaryl-coenzyme A reductase from whole plant extracts. In Chapter 3 electron microscopic studies undertaken to identify the subcellular structure in latex involved in the biosynthesis of triterpenoids are described. Experiments investigating the incorporation of various substrates into the hexane extracts of *Copaifera* leaf tissue are presented in Chapter 4.

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Chapter I. METABOLISM OF TERPENOID PATHWAY INTERMEDIATES BY *EUPHORBIA LATHYRIS* LATEX

Latex is the cell sap of specialized laticifer cells found in 12,500 species of plants, many of which are members of the families Apocynaceae, Euphorbiaceae, Asclepiadaceae, Compositae, Papaveraceae, and Sapotaceae. Laticifer cells penetrate through other cellular tissues and are often found associated with the phloem. They may be of two types: articulated and non-articulated. Articulated laticifers are a series of elongated cells whose end walls often become porous or disappear forming a laticiferous vessel. Non-articulated laticifers develop from a single cell which elongates with the growth of the plant. Both types of laticifers are multinucleate and may be simple tubes or branched. Since the contents of laticifer cells are under high turgor pressure, latex is expelled when an incision is made in plant tissue (5, 29).

Because of its importance as the source of natural rubber, the latex of *Hevea brasiliensis* is the best-studied plant latex. *Hevea* latex is capable of rubber biosynthesis *in vitro*. Numerous investigators have tested the ability of *Hevea* latex to convert various precursors into rubber. By comparing the activities of all the enzymes needed to convert acetate to rubber, Lynen suggested that HMG-CoA reductase might represent the physiological bottleneck of rubber biosynthesis since the activity of that enzyme was much lower than that of any of the other enzymes (17).

In vitro biosynthesis of the components of other plant latices has also been demonstrated. Morphinan alkaloid biosynthesis and storage occur in the latex of *Papaver somniferum* (6). Ponsinet and Ourisson demonstrated the ability of the latex of various species of *Euphorbia* to convert labelled acetate into their respective triterpenoid constituents and showed that it was the particulate fraction that was responsible for determining the structure of newly-synthesized triterpenoids when the supernatant from a ten-minute centrifugation at 5000*g* of one latex sample was mixed with the pellet of a like centrifugation of another latex containing a different suite of triterpenoid structures (25, 26).

The first investigation of the metabolism of *Euphorbia lathyris* latex *in vitro* was reported by Ponsinet and Ourisson in 1968 who found cycloartenol, 24-methylene cycloartenol, and lanosterol were labelled upon incubation of latex with [1-^{14}C] sodium acetate(26). In 1976, Groeneveld described the *in vitro* incorporation of [2-^{14}C]acetate into triterpenoids and triterpene esters of latex as well as the labelling of these components of latex by [U-^{14}C]glucose injected into the hollow stem of the plant followed by analysis of the latex after a 24 h incubation(10). The ratio of labelled triterpenols to triterpene esters differed with the different treatments, 1:10 for the *in vitro* acetate incorporation and 2:1 for the *"in vivo"* glucose labelling. The second ratio more closely matched the measured triterpenoid composition of latex: 47 mg ml^{-1}, 73% triterpenols, 27% triterpene esters. Groeneveld interpreted this as indicating that the tapped latex was incomplete, lacking part of the biosynthetic capacity of the laticifer cell *in vivo*. More likely, this difference reflects the influence of controls working on steps involved in the metabolism of glucose before it or its product enters the laticifer and is incorporated into triterpenoids.

In 1983, a paper by Nemethy et al. reported the first biosynthetic studies performed on *E. lathyris* latex by this lab (20). Experiments testing the incorporation of various precursors showed that latex has the ability to convert pyruvate, acetate (Ac), and mevalonate (MVA) but not glucose, glucose-6-phosphate, acetyl-CoA, hydroxymethylglutarate(HMG), HMG-CoA, mevalonolactone (MVAL), or isopentenylpyrophosphate (IPP) into triterpenoids. Time course incorporations of Ac and/or MVA indicated no precursor/product relationship between the triterpenols and their fatty acid esters since no lag in the labelling of either component was seen, even with a 15 sec incubation of latex with [^{3}H]Ac. The presence of mitochondria in latex was indicated by the detection of labelled TCA cycle acids when latex was incubated with radiolabelled Ac and is substantiated by ultrastructural work of Groeneveld et al. demonstrating the regular occurrence of these organelles in laticifers of the cotyledons and hypocotyl of young seedlings(11). Dilution of latex with buffer containing various concentrations of sorbitol showed the sensitivity of triterpenoid biosynthesis to changes in osmolality. A 10-fold dilution of latex with 0.4 M sorbitol

restored triterpenol biosynthesis to its undiluted level but only 60% of triterpene ester biosynthesis was recovered. This result, as well as the inability of various intermediates along the established terpenoid pathway (Ac-CoA, HMG-CoA, IPP) to serve as precursors for triterpenoid biosynthesis, suggests compartmentation of terpenoid biosynthesis in latex. George Piazza was able to show that a particulate fraction was capable of converting MVA, but not acetate, into triterpenoids (24). Experimental results supporting this finding are reported in this chapter.

The rest of the experiments described were intended to further elucidate the organization and regulation of triterpenoid biosynthesis in latex. Much of the work focused on the metabolism of HMG-CoA since the principle of comparative biochemistry would suggest that this could be an important control site since its reduction to MVA is the rate-limiting step in cholesterol biosynthesis in mammals and the activity of HMG-CoA reductase was significantly lower than the other enzymes of the terpenoid pathway in *Hevea* latex (17).

MATERIAL AND METHODS

Plant Material. *Euphorbia lathyris* L. plants were propagated from seed collected from wild plants growing near Healdsburg, Sonoma Co., CA. Plants were grown in a soil mixture of peat, sand, and Perlite in 6 inch clay pots in a growth chamber under conditions of a 16 h day at 600 μE m^{-2} s^{-1} provided by a combination of fluorescent and incandescent lights, 27°C day, 18°C night temperature. Latex was obtained by the collection of droplets expelled when shallow incisions were made with a razor blade at the bases of petioles. The latex was stored briefly on ice until it was used.

Materials. DL-3-[glutaryl-3-^{14}C]hydroxy-3-methylglutaryl coenzyme A; R-[5-^{3}H] mevalonic acid, triethylammonium salt; and Aquassure were purchased from New England Nuclear. [^{3}H]acetic acid, sodium salt was obtained from ICN Radiochemicals. Bradford dye reagent was purchased from Bio-Rad. Silica gel plates were obtained from Analtech. All other biochemicals were from Sigma.

Solutions. The potassium salt of mevalonate was formed from mevalonolactone by incubating the lactone with 1.2 eq KOH for 30 min at 40°C; the solution was diluted to 1 mM and kept frozen. REACTION BUFFER contained 10 mM potassium phthalate, pH 5.5, 0.4 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, and 30 mM $CaCl_2$. 95% PERCOLL was prepared by mixing 9.5 ml Percoll that had been dialysed against distilled water with 10 ml REACTION BUFFER that had been concentrated to 0.5 ml under a stream of N_2.

Instruments. Any UV or visible absorbance of a sample was measured using a Hewlett-Packard 8450-A UV/VIS spectrophotometer. HPLC was performed using a Beckman 322 HPLC system equipped with a Hitachi 100-10 variable wavelength spectrophotometer detector and a Waters R 410 differential refractometer. Centrifugations were done either using a 50Ti or Type 21 rotor in a Beckman model L ultracentrifuge or, for samples of less than 1.5 ml and *g* values less than 12,000*g*, in a Beckman microfuge 11. Fractions were collected using a LKB 2112 redirac fraction collector. All counting of radioactive samples was done using a Packard

640-C scintillation counter.

Protein Analysis. PROTEIN ASSAY 1-- One part 4% $CuSO_4 \cdot 5H_2O$ was diluted 100-fold with a solution of 2% Na_2CO_3, 0.16% sodium tartrate, and 1% SDS to give MIX 1. One hundred microliters 1.2 N NaOH was added to a volume of 0.5 ml sample. If the sample turned cloudy or geled, it was boiled briefly to clear the solution, then 2.5 ml MIX 1 was added. The sample was allowed to sit for 10 to 30 min at which time 125 µl 1:1 Sigma Folin-Ciocalteu 2N phenol reagent: water was added, and the solution was rapidly mixed. The sample was allowed to develop for exactly 1 h and then its A_{420} and A_{750} were measured. A standard curve was developed using known amounts of a protein standard. This assay was developed based on the method of Folin and Ciocalteu (7).

PROTEIN ASSAY 2-- This assay was based on the method of Bradford (2) as modified by Vincent and Nadeau (31). A 30 µl aliquot of sample and buffer in a total volume of 300 µl was added to 120 µl 0.1% Triton X-100. An aliquot of 50 µl of this mixture was added to 1 ml 5-fold dilute Bradford dye reagent, and the A_{594} of the solution was measured. A standard curve was constructed using the same procedure with a protein standard.

Analysis for labelled triterpenoids. TLC isolation of triterpenoids-- The general method for determination of triterpenoid labelling involved extraction of a dried incubation mixture by stirring with acetone overnight. Incubations were quenched with *ca* 3 ml methanol and were dried under a stream of N_2. The dried sample was washed four times with 3-ml volumes of water then extracted with the acetone. In some experiments incubations were quenched by placing the sample in a boiling water bath for a few minutes; the sample was then centrifuged, and the pellet was extracted with acetone. The acetone extract was spotted as a band and a marker spot on a 20 x 20 cm silica gel G plate which was developed in a solvent mixture of 3:1 ether: petroleum ether. The triterpenols and triterpene esters have R_fs of 0.60 and 0.85, respectively, in this system. Marker spots were visualized with sulfuric acid spray and heat. The bands corresponding to the markers were scraped into scintillation vials,.sonicated with 5 ml water, shaken with 15 ml

Aquassure to form sols, and counted.

HPLC analysis of triterpenols-- Triterpenols were first chromatographed by TLC and then eluted from the silica gel with acetone. The solvent was removed by evaporation under a stream of N_2, and the triterpenols were redissolved in methanol. HPLC analysis was performed on two 4.6 mm x 25 cm ODS columns (Altex) in 100% methanol at a flow rate of 1 ml min^{-1}. The eluate was monitored by its absorbance at 214 nm. Fractions were collected directly into scintillation vials, were mixed with 15 ml Aquassure, and were counted.

Analysis for labelled MVA. TLC system 1– Sample was spotted on a silica gel G plate and developed in $CHCl_3$:acetone, 2:1. In this system, MVAL has a R_f of 0.38 to 0.43 (3).

TLC system 2– Sample is eluted with a 1:1 mixture of acetone and benzene on silica gel G plates where the R_f of MVAL is 0.42 (28).

Organic acid HPLC– Sample is chromatographed on an HPLC column designed for organic acid analysis (Bio-Rad Laboratories HPX-87H, 300 x 7.8 mm) equipped with a guard column (Bio-Rad 125-0129) in 0.0025 N H_2SO_4 at a flow rate of 0.3 ml min^{-1}. The eluant is monitored by its absorbance at 214 nm. The retention times of HMG, acetoacetate, Ac, and MVA are 22, 30, 31.5, and 41 min in this system.

ODS HPLC– In this system, a sample is chromatographed on a 250 x 4.6 mm ODS column (Altex) in 10 mM potassium phosphate pH 2.6 at a flow rate of 1 ml min^{-1} where the retention times for acetic acid, acetoacetic acid, mevalonic acid, HMG, and mevalonolactone are 5.5, 7.75, 11, 14, and 15 min, respectively. The elution of sample components was determined by changes in the eluant's A_{214}.

Substrate Saturation Experiments. A series of experiments were performed in order to determine the concentrations of Ac and MVA that were saturating for the biosynthesis of triterpenoids in latex. In each experiment latex was incubated with DTE and various concentrations of either radiolabelled Ac or MVA, then the triterpenols and triterpene esters were isolated, and the amount of radiolabel that had been incorporated into each fraction was

determined.

Ac incorporation 1-- One hundred microliter volumes of latex were incubated with a combination of 10 µl 0.1 M DTE and various amounts of [^{3}H]Ac, Na salt (5, 10, 20, 40, and 60 µl; Ci mmol^{-1}; 10 mCi ml^{-1}) that had been dried under a stream of N_2. The final concentrations in the incubation mixture were 10 mM DTE and varied from 0.25 to 3 mM Ac.

Samples were incubated for 1 h at room temperature (20 to 22°C) then were quenched by the addition of *ca* 3 ml MeOH and dried under a stream of N_2. The remaining residue was washed three times with water then extracted overnight with stirred acetone. The water wash was extracted with hexane; the hexane and acetone extracts were combined, and solvent was removed by evaporation under a stream of N_2. The residue was redissolved in 0.5 ml acetone, sonicated, and spotted onto a 20 x 20 cm 250 µm layer silica gel G plate as a band and an isolated marker spot. An acetone rinse of the spotting vial was also applied to the plate which was developed in a solvent mixture of 3:1 ether: petroleum ether. The region containing the marker spot was visualized with sulfuric acid spray and heat, and the bands containing the triterpenols and triterpene esters were scraped from the rest of the plate. Five ml water was added to the silica gel of each fraction; the mixture was sonicated 5 min; 15 ml Aquassure was added, and the mixture was shaken to form an uniform gel. Samples were scintillation-counted.

Ac Incorporation 2-- A second experiment was performed exactly as the first with one exception- the final concentration of Ac in the incubations ranged from 50 µM to 1.5 mM (1, 2, 10, 20, and 30 µl [^{3}H]Ac; 2 Ci mmol^{-1}; 10 mCi ml^{-1}).

MVA Incorporation 1-- One ml latex was placed on top of 4 ml 95% PERCOLL and centrifuged at 130*g* for 5 min. The top layer was removed and diluted with an equal volume of REACTION BUFFER. The resulting solution was centrifuged at 100,000*g* for 10 min; supernatant and pellet were mixed, and 400 µl of the mixture was added to the incubation vial. Each incubation contained 50 µl 3 mM NADPH, 50 µl 104 mM DTE, 50 µl 20 mM ATP, and 50 µl 1% BSA added to the mixtures of unlabelled and [^{3}H]MVA (30 µl [^{3}H]MVA, 0.5 mCi ml^{-1}, 10.3 Ci

mmol^{-1} + 50, 75, 100, 150, or 300 µl 1 mM MVA, potassium salt) that had been dried in the vial. The final concentrations of the incubation components were 0.25 mM NADPH, 8.7 mM DTE, 1.7 mM ATP, 0.08% BSA, and from 86 to 502 µM MVA. Samples were incubated at room temperature for 7.5 h then quenched with 2.5 ml MeOH. An additional volume of MeOH was used to rinse the vial. The samples were dried under a stream of N_2 overnight and extracted three times with 2.5 ml acetone, the last volume containing 0.5 ml of acetone extract of latex as a source of carrier triterpenoids. The acetone extracts were concentrated and applied to 20 x 20 cm 500 µm silica gel G plates which were developed, scraped, and counted as in the acetate incorporation experiments.

MVA Incorporation 2-- A second substrate saturation curve for MVA was determined as in incorporation 1 with the following exceptions: the final MVA concentration in the incubations varied from 0.077 to 1.06 mM (30 µl [^{3}H]MVA (0.5 mCi ml^{-1}, 10.3 Ci mmol^{-1}) + 25, 100, 150, 250, 250, or 350 µl 1.8 mM MVA, potassium salt); the incubation time was 6 h; the dried samples were extracted twice with acetone.

MVA Incorporation 3-- Ten microliters 10 mM DTE and a combination of unlabelled and [^{3}H]MVA (µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}) + µl 20 mM MVA, potassium salt were 6+5, 12+10, 18+15, 18+15, 24+20, and 30+25) were dried under a stream of N_2 in each incubation vial. To each vial, 100 µl latex was added giving final concentrations of 10 mM DTE and MVA from 1 to 5 mM with a specific activity of 30 mCi mmol^{-1}. The samples were incubated at room temperature for 1 h, quenched with *ca* 2 ml MeOH, and dried overnight under a stream of N_2. The residue was extracted, chromatographed, and counted as in Ac Incorporation 1.

Ac and MVA Incorporation in the same latex sample-- One hundred microliters aliquots of a latex sample were added to incubations vials containing DTE and either Ac or MVA that had been dried under a stream of N_2. To each vial was added 10 µl 100 mM DTE + 1, 2, 5, 10, 15, 20, or 3 µl [^{3}H]Ac (2 Ci mmol^{-1}, 10 mCi ml^{-1}) or a combination of unlabelled + [^{3}H]MVA to give a final specific activity of 50 mCi mmol^{-1} as shown in Table I.

Table I. *Quantity of MVA used as Substrate for Latex Incorporation Experiment*

Incubation	Final Concentration (mM MVA]	[^{3}H]MVA 10.3 Ci mmol^{-1}, 0.5 mCi ml^{-1} µl	unlabelled MVA µl	
			2 mM	20mM
1	0.1	1	5	-
2	0.25	2.5	12.5	-
3	0.5	5	-	2.5
4	1	10	-	5
5	2	20	-	10
6	3	30	-	15

In each incubation the final concentration of DTE was 10 mM, and the Ac concentration varied from 0.05 to 1.5 mM. Samples were incubated for 1 h then quenched and analyzed as in Ac Incorporation 1.

Effect of Percoll on triterpenoid biosynthesis. Dr. George Piazza was using Percoll gradients to try to isolate the organelle responsible for the biosynthesis of triterpenoids in latex. This experiment looked at the effect of Percoll and centrifugation on this biosynthesis. Aliquots of 0.4 ml were taken from a single sample of latex and subjected to five different treatments before they were incubated with [^{3}H]MVA and analyzed for labelled triterpenoids. The five treatments were:

1. latex mixed with 4 ml 95% Percoll
2. latex placed on 4 ml 95% Percoll, centrifuged at 400*g* for 5 min then mixed
3. latex placed on 4 ml 95% Percoll and centrifuged at 400*g* for 5 min. The supernatant was removed and mixed with 0.5 ml 95% Percoll, replacing the 0.5 ml pellet left behind.

4. latex placed on 4 ml 95% Percoll and centrifuged at 130*g* for 5 min. The white latex layer remaining on top of the Percoll was removed and mixed with 4 ml 95% Percoll.
5. latex mixed with 4 ml REACTION BUFFER

Each treatment was incubated with 50 µl 20 mM ATP pH 5.5, 50 µl 100 mM DTE, 50 µl 1% BSA, and 50 µl 3 mM NADPH, all solutions were made up in reaction buffer. The final concentration of MVA was 11.4 µM with a specific activity of 380 mCi $mmol^{-1}$ derived from a combination of 50 µl 1 mM MVA, potassium salt and 40 µl [^{3}H]MVA (10.3 Ci $mmol^{-1}$, 0.5 mCi ml^{-1}). All substrates were combined and dried in the incubation tube under a stream of N_2 before the addition of the sample; the volume of each incubation was 4.4 ml. Samples were incubated 7 h and quenched with 3 ml methanol. The dried samples were analyzed for labelled triterpenoids by TLC.

Recovery of site of triterpenoid biosynthetic activity from Percoll gradient. A 1.5 ml sample of latex was collected, and 200 µl was incubated with [^{3}H]MVA. The remainder was placed in a test tube on 5 ml 95% Percoll and centrifuged at 130*g* for 5 min. The white layer remaining on top of the Percoll was removed, and 250 µl was incubated with [^{3}H]MVA. The Percoll layer was removed and diluted 4-fold with REACTION BUFFER. Five 3-ml aliquots were taken, and each was centrifuged for 10 min at a different *g* value (1000, 3000, 5000, 10,000, and 15,000*g*). The supernatant of each was removed, and the bottom 300 µl pellet was incubated together with 250 µl of the top white layer and [^{3}H]MVA.

Each incubation contained 50 µl 20 mM ATP pH 5.5, 50 µl 100 mM DTE, 50 µl 1% BSA, 50 µl 3 mM NADPH, 50 µl 1 mM MVA, potassium salt, and 30 µl [^{3}H]MVA (10.3 Ci $mmol^{-1}$, 0.5 mCi ml^{-1}). These substrates and cofactors were dried under a stream of N_2 in the incubation tube. Three hundred microliters of 95% Percoll diluted 4-fold with REACTION BUFFER was added to the whole latex and top layer incubations to substitute for the pellet volume present in the other incubations. The concentration of MVA in the incubations was 94 µM at a specific activity of 290 mCi $mmol^{-1}$. Incubations were quenched after 11.75 h with methanol and dried under a stream of

N_2. The incubations were analyzed for labelled triterpenoids by TLC.

Distribution of site of triterpenoid biosynthesis from top layer of Percoll gradient. Latex was centrifuged at 130*g* for 5 min on 5 ml 95% Percoll, and the layer that remained on top of the Percoll was removed, diluted 2-fold with REACTION BUFFER, and centrifuged at 100,000*g* for 10 min. Four incubations were performed: one containing 105 µl of the 100,000*g* pellet with 350 µl REACTION BUFFER, a second of 350 µl 100,000*g* supernatant with 105 µl REACTION BUFFER, and duplicate incubations of 105 µl 100,000*g* pellet with 350 µl 100,000*g* supernatant. The latex samples were added to 25 µl 1.8 mM MVA, potassium salt and 30 µl [^{3}H]MVA (10.3 Ci mmol^{-1}, 0.5 mCi ml^{-1}) that had been dried under N_2 in the incubation tubes. Fifty microliters 20 mM ATP pH 5.5, 50 µl 100 mM DTE, 50 µl 1% BSA, 50 µl 3 mM NADPH were added to each incubation, giving a final volume of each incubation of 655 µl with a MVA concentration of 70 µM at a specific activity of 0.32 Ci mmol^{-1}. The samples were incubated for 5 h, then quenched with methanol, and analyzed for labelled triterpenoids by TLC.

Distribution of triterpenoid-biosynthetic activity between supernatant and pellet of latex centrifuged at different *g* values. A sample of latex was divided into 3 200-µl aliquots; two aliquots were diluted to 1.5 ml with REACTION BUFFER. The diluted samples were centrifuged for 15 min, one at 30,000*g* and one at 60,000*g*. The supernatants were removed and diluted to a total volume of 1.5 ml with REACTION BUFFER. The pellets were resuspended in 0.5 ml REACTION BUFFER. Nine hundred microliters of each supernatant was incubated with 40 µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}) and 150 µl 1 mM MVA, potassium salt that had been previously dried under a stream of N_2 in the incubation tube. One hundred-fifty microliters each of 1% BSA, 104 mM DTE, 20 mM ATP pH 5.5, and 3 mM NADPH were added to each incubation. A 300 µl aliquot of each pellet and 200 µl whole latex diluted to 300 µl with REACTION BUFFER were incubated with 30 µl [^{3}H]MVA and 50 µl 1 mM MVA, potassium salt that had been previously dried under a stream of N_2 in the incubation tube. Fifty microliters each of 1% BSA, 104 mM DTE, 20 mM ATP pH 5.5, and 3 mM NADPH were added to these three incubations. The final volume

of the supernatant incubations was 1.5 ml; the volume of the pellet and whole latex incubations were each 0.5 ml. The final concentrations of cofactors and MVA were the same in all incubations, but the specific activity of MVA was 0.13 Ci $mmol^{-1}$ and 0.29 Ci $mmol^{-1}$ in the supernatant and pellet (& whole latex) incubations, respectively. The samples were incubated for 3 h then diluted with water and quenched by placing in a boiling water bath for 5 to 10 min. The samples were centrifuged for 15 min at 180,000*g* to obtain a firm pellet. The supernatant was removed; the pellet was dried under a stream of N_2, extracted with acetone, and analyzed for labelled triterpenoids by TLC.

A second experiment was performed following the same procedures. Here three samples were centrifuged at 5000*g*, 15000*g*, and 20,000*g*, and the pellets and supernatants were incubated as described above. A whole latex sample was incubated as before.

Acetate versus MVA metabolism by 15,000*g* pellet. Two 200 µl aliquots were each diluted to 1.5 ml with REACTION BUFFER and centrifuged at 15,000*g* for 15 min. The supernatant was removed, and the pellet was resuspended in 1.5 ml REACTION BUFFER and recentrifuged. The pellets were then each resuspended in 50 µl 20 mM ATP, 50 µl 104 mM DTE, 50 µl 1% BSA, and 50 µl 3 mM NADPH. The suspensions were transfered to incubation tubes in which the labelled substrate had been dried under a stream of N_2. One tube contained 30 µl [^{3}H]MVA (10.3 Ci $mmol^{-1}$, 0.5 mCi ml^{-1}) and 25 µl 2 mM MVA, potassium salt, giving a final incubation concentration of 0.25 mM mevalonate at a specific activity of 0.29 Ci $mmol^{-1}$. The second incubation had an acetate concentration of 0.25 mM provided by 10 µl of [^{3}H]acetate (2 Ci $mmol^{-1}$, 10 mCi ml^{-1}). The samples were incubated for 3 h and quenched in a boiling water bath. Each was analyzed for labelled triterpenoids by TLC.

Lifetime of 15,000*g* pellet. The lifetime of the triterpenoid-biosynthetic capability of the pellet was tested. A ml of latex was divided into 5 200 µl aliquots which were each diluted to 1.5 ml with REACTION BUFFER and centrifuged at 15,000*g* for 15 min. The supernatants were removed, and the pellets were each resuspended in 0.5 ml REACTION BUFFER. Each pellet was added to a reaction tube in which 50 µl 1 mM MVA, potassium salt and 30 µl [^{3}H]MVA (10.3 Ci

mmol^{-1}, 0.5 mCi ml^{-1}) had been dried under a stream of N_2. Fifty microliters 20 mM ATP pH 5.5, 50 µl 100 mM DTE, 50 µl 1% BSA, 50 µl 3 mM NADPH were added to each incubation, giving a final volume of each incubation of 700 µl with a MVA concentration of 0.1 mM at a specific activity of 0.29 Ci mmol^{-1}. Each sample was incubated for a different period of time (0.5, 1, 2, 3, and 4.5 h) then quenched in a boiling water bath. The sample was centrifuged at 180,000*g* for 15 min, and the pellet was analyzed for labelled triterpenoids by TLC.

Comparison of triterpenoid biosynthesis by whole latex, 15,000*g* pellet, and washed 15,000*g* pellet. Some experiments require that whole latex be incubated for a period of time and then centrifuged. The recovered pellet is incubated for an additional period of time. Four 175 µl samples of latex were incubated for 2 h with 25 µl [^{14}C]MVA (50.1 mCi mmol^{-1}, 0.1 mCi ml^{-1}) that had been dried in the incubation tube. Fifteen microliters each of 145 mM DTE and 58 mM $MgCl_2$ were added to each latex sample. The final concentrations of DTE, $MgCl_2$, and MVA were 9.5, 3.8, and 0.22 mM, respectively.

At the end of the initial incubation period, one sample (205 µl) was transfered to a tube in which 30 µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}) had been taken to dryness under a stream of N_2. Fifty microliters each of 20 mM ATP pH 5.5, 1% BSA,and 3 mM NADPH were added, and the sample was incubated an additional 2 h. With the addition of the [^{3}H]MVA, the specific activity of [^{14}C]MVA changed little, from 50.1 to 48.6 mCi mmol^{-1}; the specific activity of [^{3}H]MVA was 291 mCi mmol^{-1}.

The remaining three incubations were diluted to 1.5 ml with REACTION BUFFER and centrifuged at 15,000*g* for 15 min. The supernatants and pellets were separated in each case. One set was immediately quenched in a boiling water bath. All supernatants were quenched. The pellet of the next sample was resuspended in 200 µl REACTION BUFFER and placed in a tube in which 30 µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}) and 50 µl 1 mM MVA, potassium salt had been dried under a stream of N_2. Fifty microliters 20 mM ATP pH 5.5, 50 µl 100 mM DTE, 50 µl 1% BSA, and 50 µl 3 mM NADPH were added, and the sample was incubated for 2 h. The final

pellet was resuspended in 1.5 ml REACTION BUFFER and recentrifuged as an additional wash of the pellet. The washed pellet was resuspended in 200 µl REACTION BUFFER and incubated for 2 h with the same components as the third sample.

At the end of the second 2 h incubation period, all samples were quenched in a boiling water bath, and all samples were centrifuged to pellet the water-insoluble terpenes. The pellets were extracted with acetone and analyzed for labelled triterpenoids by TLC.

Lifetime of a preincubated, washed 15,000*g* pellet. Four 200 µl latex samples were incubated for 2 h with 25 µl 2 mM MVA, potassium salt and 30 µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}), dried in the incubation tube, and 15 µl 145 mM DTE and 15 µl 58 mM $MgCl_2$. The samples were diluted to 1.5 ml with REACTION BUFFER and centrifuged at 15,000*g* for 15 min. The supernatants were removed, and the pellet was resuspended in 1.5 ml REACTION BUFFER, was transfered to a second tube, and recentrifuged. The final pellet was resuspended in 50 µl 20 mM ATP pH 5.5, 50 µl 104 mM DTE, 50 µl 1% BSA, and 50 µl 3 mM NADPH and transfered to an incubation tube containing a dried sample of 25 µl [^{14}C]MVA (50.1 mCi mmol^{-1}, 0.1 mCi ml^{-1}). The incubations were quenched in a boiling water bath after 0.75, 1.5, 2.5, and 4 h and analyzed for labelled triterpenoids by TLC.

Distribution of labelled triterpenoids among fractions of latex first incubated as whole latex with [^{3}H]acetate. Two 250 µl aliquots of latex were each incubated with 20 µl 58 mM $MgCl_2$, 20 µl 145 mM DTE, and 10 µl [^{3}H]acetate (2 Ci mmol^{-1}, 10 mCi ml^{-1}), giving final concentrations of 4 mM $MgCl_2$, 10 mM DTE, and 0.17 mM acetate. After 3 h incubation, one of the samples was fractionated as shown in Fig. 1, and at 3.5 h all samples were quenched with methanol. Each fraction was analyzed for labelled triterpenoids by TLC.

Test for soluble protein effectors of triterpenoid biosynthesis in latex. A 250 µl sample of latex was incubated with 20 µl 58 mM $MgCl_2$, 20 µl 145 mM DTE, and MVA. The 30 µl [^{3}H]MVA (10.3 mCi mmol^{-1}, 0.5 mCi ml^{-1}), and 50 µl 1 mM MVA, potassium salt had been dried in the incubation tube before the addition of latex and cofactors; the final concentration of MVA was

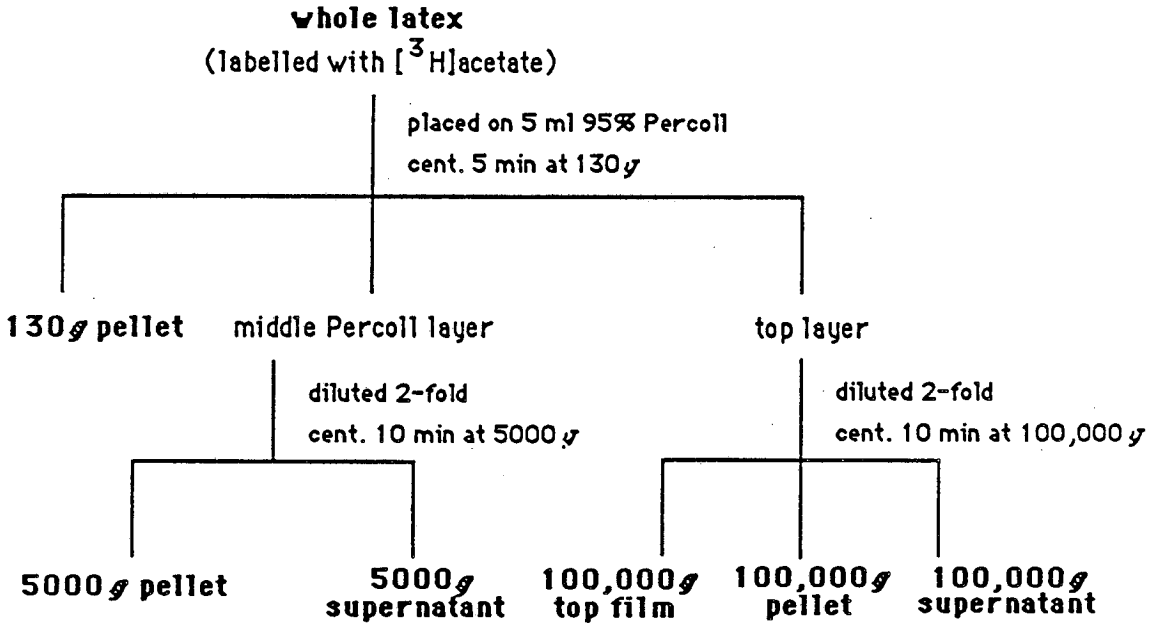

Fig. 1. Preparation of latex fractions for analysis of distribution of labelled triterpenoids.

177 µM at a specific activity of 0.29 Ci $mmol^{-1}$. At 2 h a second 300 to 400 µl of latex was diluted to *ca* 3 ml with REACTION BUFFER. The latex incubation was also diluted to *ca* 3 ml with the buffer, and both samples were centrifuged at 100,000 *g* for 10 min. The pellet from the labelled sample (0.8 ml) was divided among four incubation tubes containing 50 µl 1 mM MVA, potassium salt. One milliliter of supernatant from the unlabelled sample was added to two tubes; the third tube received 1 ml REACTION BUFFER, and 1 ml REACTION BUFFER containing 30 mg ml^{-1} BSA was added to the fourth. The first sample containing supernatant was immediately diluted to 3 ml with REACTION BUFFER and centrifuged at 100,000*g* for 10 min, and the supernatant and pellet were separated. One milliliter of the supernatant and the pellet were quenched with methanol and analyzed by TLC for labelled triterpenoids. The remaining incubations were diluted, centrifuged, quenched, and analyzed as the first was after 2 h of incubation.

In a second experiment testing the effect of supernatant on the biosynthesis and distribution of labelled triterpenoids, a 250 µl sample of latex was incubated with 20 µl 58 mM $MgCl_2$, 20 µl 145 mM DTE, and MVA. The 30 µl [^{3}H]MVA (10.3 mCi $mmol^{-1}$, 0.5 mCi ml^{-1}), and 50 µl 1 mM MVA, potassium salt had been dried in the incubation tube before the addition of latex and cofactors; the final concentration of MVA was 177 µM at a specific activity of 0.29 Ci $mmol^{-1}$. After 3 h incubation, the latex incubation and a second 250 µl of latex were diluted to 1.5 ml with REACTION BUFFER. Both samples were centrifuged at 100,000 *g* for 15 min. The pellet from the labelled sample was resuspended in 1 ml REACTION BUFFER, and 200 µl aliquots were subjected to four different treatments:

1. + 300 µl unlabelled 100,000*g* supernatant + 108 µl 1 mM MVA, 2 h
2. + 300 µl unlabelled 100,000*g* supernatant + 108 µl REACTION BUFFER, 2 h
3. + 300 µl 30 mg ml^{-1} BSA in REACTION BUFFER + 108 µl 1 mM MVA, 2 h
4. + 300 µl unlabelled 100,000*g* supernatant + 108 µl 1 mM MVA, 0 h

At the end of each incubation period, the sample was diluted to 1.5 ml with REACTION BUFFER and centrifuged at 100,000*g* for 10 min. The supernatants and pellets were separated and quenched in a boiling water bath. Each sample was centrifuged at 100,000*g* for 10 min, and

the pellet was extracted with acetone and analyzed for labelled triterpenoids by TLC.

Time course of effect of 100,000*g* supernatant on triterpenoid biosynthesis by 15,000*g* pellet. Eight 200 µl samples of latex were added together with 15 µl 145 mM DTE and 15 µl 58 mM $MgCl_2$ to incubation tubes containing 25 µl [^{14}C]MVA (50.1 mCi mmol^{-1}, 0.1 mCi ml^{-1}) that had been taken to dryness under a stream of N_2. The samples were incubated 2 h, diluted to 1.5 ml with REACTION BUFFER then centrifuged at 15,000*g* for 15 min. The supernatant of each sample was saved; the pellet was resuspended in 1.5 ml REACTION BUFFER and recentrifuged. Each supernatant was again removed and combined with the first supernatant. The pellets were resuspended in a solution of 50 µl 20 mM ATP pH 5.5, 50 µl 104 mM DTE, 50 µl 1% BSA, and 50 µl 3 mM NADPH and were transfered to a tube in which 25 µl 2 mM MVA, potassium salt and 30 µl [^{3}H]MVA (10.3 Ci mmol^{-1}, 0.5 mCi ml^{-1}) had been dried. Five hundred microliters REACTION BUFFER were added to four tubes designated as controls. The other four tubes received 500 µl of supernatant obtained by centrifuging latex at 100,000*g* for 15 min.

At time points of 0.5, 1, 1.5, and 2 h, a control incubation and a 100,000*g* supernatant incubation were each diluted to 1.5 ml with REACTION BUFFER and centrifuged at 15,000*g* for 15 min. The supernatant and pellet were separated, and each sample was quenched in a boiling water bath. Analysis of an acetone extract of each sample for labelled triterpenoids was done by TLC.

Effect of concentration of 100,000*g* supernatant on triterpenoid biosynthesis by a 15,000*g* pellet. One milliliter latex was diluted to 1.5 ml with REACTION BUFFER and centrifuged for 15 min at 100,000*g*, providing the 100,000*g* supernatant for this experiment. A second milliliter of latex was divided among 5 tubes; each sample was diluted to 1.5 ml with REACTION BUFFER and centrifuged for 15 min at 15,000*g*. The supernatant was removed, and the pellet was resuspended in 1.5 ml REACTION BUFFER and recentrifuged. Each of these washed pellets was resuspended in a different combination of REACTION BUFFER and 100,000*g* supernatant (Table II). Fifty microliters 20 mM ATP pH 5.5, 50 µl 104 mM DTE, 50 µl 1% BSA, and 50 µl 2.75 mM NADPH were also added to each pellet, and each sample was placed in an incubation tube in

which 25 µl 2 mM MVA, potassium salt and 30 µl [^{3}H]MVA (10.3 Ci mmol^{-1}, 0.5 mCi ml^{-1}) had been dried. After 1.5 h incubation, the samples were quenched by boiling and were analyzed for labelled triterpenoids (TLC).

Table II. *Combination of 100,000g supernatant and REACTION BUFFER incubated with 15,000g pellet*

Sample	µl 100,000*g* supernatant	µl REACTION BUFFER	-fold dilution of supernatant	-fold dilution of pellet
1	0	300	∞	2.5
2	50	250	15	2.5
3	100	200	7.5	2.5
4	200	100	3.75	2.5
5	300	0	2.5	2.5

HMG-Co A metabolism in latex. Once it was established that the conversion of MVA into triterpenoids took place in a particulate fraction of latex, a number of experiments were performed to locate the site of the enzyme HMG-CoA reductase.

HMG-CoA incubation 1-- Two hundred microliters latex was diluted to a volume of 1.5 ml with REACTION BUFFER and was centrifuged 15 min at 15,000*g*. The cloudy supernatant was removed and recentrifuged at 100,000*g* for 20 min giving a clear supernatant and a firm white pellet. The pellet was resuspended in 140 µl REACTION BUFFER. The resuspended pellet and 140 µl supernatant were each incubated with 20 µl 104 mM DTE, 40 µl 10 mM NADPH added to tube in which 10 µl [^{3}H]HMG-CoA (0.1 mCi ml^{-1}, 12.2 Ci mmol^{-1}) and 25 µl 2mM HMG-CoA had been dried under a stream of N_2. The total volume of each incubation was 200 µl containing 2 mM NADPH and 260 µM HMG-CoA with a specific activity of 19.7 mCi mol^{-1}. The samples were incubated 1.5 h, were quenched by immersion in a boiling water bath for 1 min, and were diluted

to 1.5 ml with 400 µl each of 1 mg ml^{-1} sodium acetate, 1 mg ml^{-1} HMG, 10 mM MVA, and 100 µl REACTION BUFFER. They were centrifuged to pellet the denatured protein and were filtered through a 0.45 µm filter. Fifty microliters of each sample was analyzed for MVA production by Organic Acid HPLC.

HMG-CoA incubation 2-- Two 200 µl samples of latex were each diluted to 1.5 ml with buffe one with REACTION BUFFER (pH 5.5) and second with buffer identical to REACTION BUFFER with the exception that the phthalate pH 5.5 was replaced by10 mM MOPS pH 7.2. The measured pHs of the diluted latex samples were 5.5 and 6.7, respectively. Each sample was centrifuged for 15 min at 15,000*g*; the supernatant was removed, mixed with 50 µl 104 mM DTE, and centrifuged at 100,000*g* for 1 h. The supernatant was removed, and the pellet was resuspended in 150 µl buffer. The pellet and 150 µl of the supernatant of each pH treatment were incubated with 10 µl [^{3}H]HMG-CoA (0.1 mCi ml^{-1}, 12.2 Ci $mmol^{-1}$) and 25 µl 2mM HMG-CoA had been dried under a stream of N_2. In addition, 20 µl 104 mM DTE and 40 µl 10 mM NADPH were added to the incubation. The final concentrations of NADPH and HMG-CoA were 2 mM and 254 µM at a specific activity of 20 mCi ml^{-1}. Samples were incubated 1.5 h, quenched in a boiling water bath, and diluted to 1.5 ml with with 325 µl each of 1 mg ml^{-1} sodium acetate, 1 mg ml^{-1} HMG, 1 mg ml^{-1} MVA, and 200 µl 0.25 mg ml^{-1} lithium acetoacetate. Samples were titrated to pH 11 with KOH and heated for 10 min at 55°C to hydrolyze the thiol esters. Next the pH was lowered to 3 with H_2SO_4 to allow formation of mevalonolactone and to ensure that the sample's pH was compatable with the pH of the organic acid HPLC column. Samples were filtered through a 0.45 µm filter and analyzed by organic acid HPLC.

Effect of EDTA on HMG-CoA metabolism in latex. HMG-CoA Incubation 3- Incubation of HMG-CoA with 5000*g* pellet and supernatant in the presence of 30 mM EDTA-- Five hundred microliters latex was diluted with 1 ml buffer (0.4 M sorbitol, 10 mM phthalate pH 5.5, 30 mM EDTA, 1% BSA, 10 mM DTE) and centrifuged 15 min at 5000*g*. The supernatant was removed, and the pellet was resuspended in 200 µl buffer. The pellet and 200 µl of the supernatant were each combined with 0.6 mg NADPH, giving a NADPH concentration of 3.3 mM. Each sample was

transfered to a tube in which 35 µl 2 mM HMG-CoA and 20 µl [^{3}H]HMG-CoA (12.2 mCi mmol^{-1}, 0.1 mCi ml^{-1}) had been taken to dryness under a stream of N_2. The final concentration of HMG-CoA was 350 µM at a specific activity of 28.5 mCi mmol^{-1}.

The samples were incubated 1.5 h and were quenched in a boiling water bath. One hundred microliters each of 1 mg ml^{-1} solutions of MVAL, sodium acetate, and HMG and 50 µl of 1 mg ml^{-1} lithium acetoacetate were added to the quenched incubations. Each sample was centrifuged, and the supernatants were recovered. The volume of each was brought to 1 ml, and the samples were filtered through a 0.45 µm filter before being analyzed by organic acid HPLC.

In addition, four injections of the 5000*g* sample were chromatographed on the organic acid HPLC column, and the region where MVA elutes was collected and combined. An aliquot of [^{14}C]MVA (31500 dpm) was added as carrier, and the sample was brought to pH 11 with KOH and heated at 30 to 40C for 30 min to open the lactone. The sample was divided into halves and dried under a stream of N_2. One half was redissolved in 200 µl 10 mM KH_2PO_4 pH 2.7 and was analyzed for labelled MVA by ODS-HPLC. The second half was redissolved in a mixture of 5 µl 1 M MVA, potassium salt, 25 µl concentrated HCL, and 175 µl water and allowed to sit 2 h to allow the MVA to lactonize. It was analyzed for labelled MVA by TLC method 2. A second TLC analysis was performed on the remaining sample from the ODS-HPLC analysis, after acidifying the sample to form the lactone.

The residue from the 5000*g* pellet incubation was extracted overnight with acetone and analyzed for labelled triterpenoids by TLC.

HMG-CoA Incubation 4- Incubation of HMG-CoA with 7500*g* pellet, 40,000*g* pellet and supernatant in the presence of 50 mM EDTA-- Eight hundred microliters latex was diluted to 1.5 ml with buffer (0.4 M sorbitol, 10 mM phthalate pH 5.5, 50 mM EDTA, 1% BSA, 10 mM DTE) and centrifuged 15 min at 5000*g*. The supernatant was removed and recentrifuged at 7500*g* for 30 min. The pellet was resuspended in 200 µl buffer, and the supernatant was centrifuged for a third time at 40,000*g* for 30 min. The 40,000*g* pellet was also resuspended in 200 µl buffer. The pellets and 200 µl of the 40,000*g* supernatant were each combined with 0.6 mg NADPH, giving a

NADPH concentration of 3.3 mM. Each sample was transfered to a tube in which 35 µl 2 mM HMG-CoA and 20 µl [^{3}H]HMG-CoA (12.2 mCi mmol^{-1}, 0.1 mCi ml^{-1}) had been taken to dryness under a stream of N_2. The final concentration of HMG-CoA was 350 µM at a specific activity of 28.5 mCi mmol^{-1}.

The samples were incubated 1 h and were quenched in a boiling water bath. One hundred microliters each of 1 mg ml^{-1} solutions of MVAL, sodium acetate, and HMG and 50 µl of 1 mg ml^{-1} lithium acetoacetate were added to the quenched incubations. Each sample was centrifuged, and the supernatants were recovered. The volume of each was brought to 1.2 ml, and the samples were filtered through a 0.45 µm filter before being analyzed by organic acid HPLC. The 40,000*g* supernatant was also analyzed for labelled MVA by TLC method 2.

HMG-CoA Incubations 5&6- Incubation of HMG-CoA with 40,000*g* supernatant, 100,000*g* supernatant and pellet-- One milliliter latex was diluted to 1.5 ml with buffer (0.4 M sorbitol, 10 mM phthalate pH 5.5, 50 mM EDTA, 1% BSA, 10 mM DTE) and centrifuged 30 min at 40,000*g*. The supernatant was removed; a 200 µl aliquot was taken for incubation with HMG-CoA; and the remainder was diluted to 1.5 ml with buffer and recentrifuged at 100,000*g* for 1 h. The pellet was resuspended in 200 µl buffer, and it and 200 µl of the 100,000*g* supernatant were also taken for incubation with HMG-CoA. Each of the three different samples was combined with 0.6 mg NADPH, giving a NADPH concentration of 3.3 mM. Each sample was transfered to a tube in which 35 µl 2 mM HMG-CoA and 20 µl [^{3}H]HMG-CoA (12.2 mCi mmol^{-1}, 0.1 mCi ml^{-1}) had been taken to dryness under a stream of N_2. The final concentration of HMG-CoA was 350 µM at a specific activity of 28.5 mCi mmol^{-1}.

The samples were incubated 1.5 h and were quenched in a boiling water bath. Each sample was centrifuged, and the supernatants were recovered. [^{14}C]MVAL (2380 dpm) and 1 µl 1 M MVA, potassium salt were added as a marker and carrier. The samples were acidified to pH 3 with HCl to promote formation of MVAL. Each sample was analyzed for labelled MVA by TLC method 2.

This experiment was repeated with the following exceptions: samples were incubated 1 h

then quenched with 25 µl concentrated HCl. [^{14}C]MVA (41240 dpm) and 20 µl 0.5 M MVAL were added as carrier. The samples were centrifuged to obtain a clear supernatant. The pellets were washed in 200 µl water, recentrifuged, and the wash was combined with the first supernatant. Each sample was divided in half; half was analyzed for labelled MVAL by TLC method 2. The MVAL band was eluted with ether; 10% was counted, and the remainder was dried under a stream of N_2 then resuspended in 250 µl 10 mM potassium phosphate pH 2.7. A 50 µl aliquot was analyzed by ODS HPLC.

HMG-CoA reductase activity of buffered 100,000*g* mix, supernatant, and pellet. HMG-CoA Incubation 7- One half milliliter of latex was diluted to 1 ml with buffer (20 mM MES pH 6.5, 0.4 M sorbitol, 60 mM EDTA, 20 mM DTE, 5 mM NADPH). The resulting solution was divided into two samples which were centrifuged at 100,000*g* for 30 min. The top lipid film was removed from each of the tubes; the supernatant was removed from one tube, and the pellet was gently resuspended in 0.25 ml buffer (10 mM MES pH 6.5, 0.4 M sorbitol, 30 mM EDTA, 10 mM DTE, 2.5 mM NADPH). The supernatant and pellet of the second tube were gently mixed with a brush. Two hundred fifty microliters each of the supernatant, pellet, and mix were incubated with 10 µl [^{3}H]HMG-CoA (12.2 Ci mmol^{-1}) and 37.5 µl 2 mM HMG-CoA that had been dried under a stream of N_2. The final concentration in the incubation mixture was 300 µM HMG-CoA. Samples were incubated 1.5 h and quenched with 50 µl concentrated HCl, 100 µl standards (1.25 mg ml^{-1} HMG, 0.1 mg ml^{-1} AcAc, 0.25 mg ml^{-1} Ac and 0.25 mg ml^{-1} MVA), and 100 µl ^{14}C MVA, DBEB salt (10,200 dpm). The samples were centrifuged to pellet the precipitated protein, and the supernatants were analyzed for labelled MVA by OA-HPLC.

HMG-CoA reductase activity of latex fractions. HMG-CoA Incubation 8- One milliliter latex was diluted to 1.5 ml with buffer (10 mM MES, 10 mM DTE, 30 mM EDTA, 0.4 M sorbitol, pH 6.5) and subjected to the centrifugation scheme outlined in Fig. 2. Before the next centrifugation step, 200 µl of each supernatant was removed. Of this, half was sonicated for 30 s then incubated for 1 h with [^{3}H]HMG-CoA.

The 5000*g* and 50,000*g* pellets were resuspended in 1.6 ml buffer and were sonicated 30

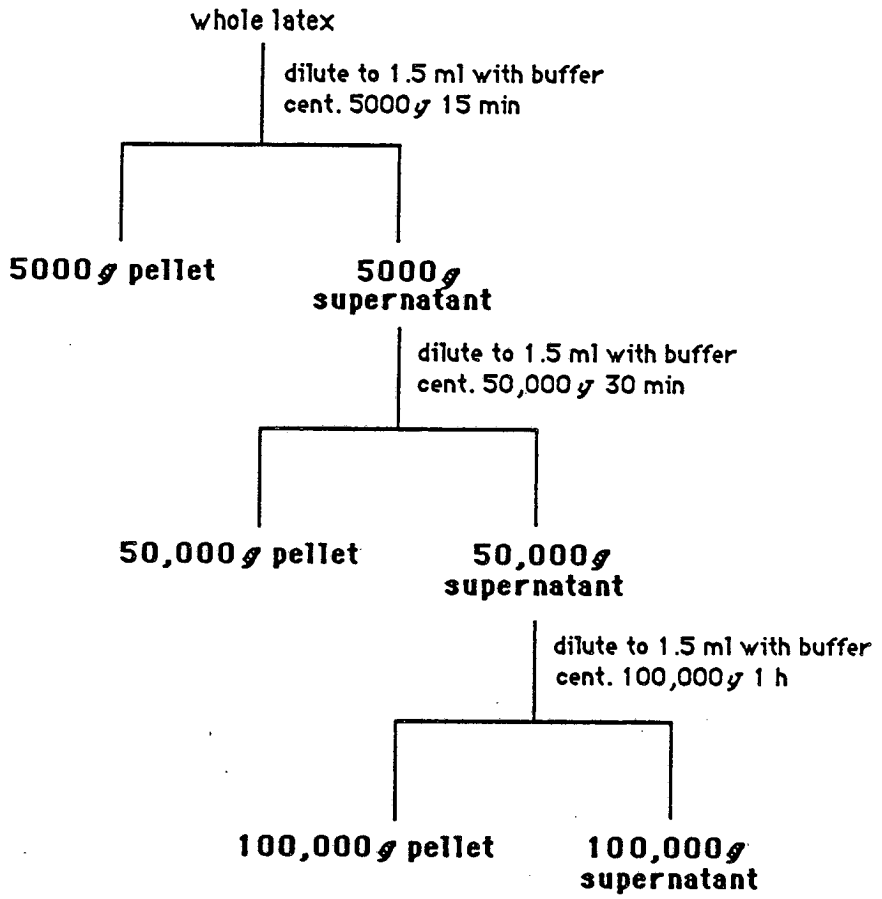

Fig. 2. Preparation of latex fractions for analysis of distribution of HMG-CoA reductase activity.

s; a 100 µl aliquot was removed, and the remaining volume was centrifuged at 100,000*g* for 1 h. The pellet was resuspended in 100 µl buffer, sonicated 30 s, and incubated 1 h with [^{3}H]HMG-CoA. The 100,000*g* pellet was resuspended in 100 µl buffer, sonicated 30 s, and incubated 1 h with [^{3}H]HMG-CoA.

The incubations were done in prepared vials containing 0.33 µmol NADPH (Sigma) in which 15 µl 2 mM HMG-CoA and 10 µl [^{3}H-HMG-CoA] (12.2 Ci mmol^{-1}, 0.1 mCi ml^{-1}) had been taken to dryness under a stream of N_2. The final concentrations were 3,3 mM NADPH and 301 µM HMG-CoA at a specific activity of 33 mCi mmol^{-1}.

The incubations were quenched with 50 µl 10 N KOH, then acidified with 75 µl concentrated HCl; 20 µl 0.5 M mevalonolactone and 20 µl [^{14}C]MVA (8230 dpm) were added as carriers. The samples were centrifuged to pellet the precipitated protein, and the supernatants were spotted onto silica gel G 500 µm 20 x 20 cm plates which were developed in 1:1 acetone: benzene. Neat MVAL was used as the marker spot; the band corresponding to the marker was scraped, and the MVAL was eluted from the silica gel with ether. A 10% aliquot of the eluant was counted, and the solvent was evaporated from the remaining sample under a stream of N_2. The precipitate was resuspended in 150 µl of 0.0025 N H_2SO_4, and a 50 µl sample was analyzed by OA -HPLC.

HMGR In 5000*g* supernatant and pellet. HMG-CoA Incubations 9&10- One milliliter latex was diluted to 1.5 ml with buffer (10 mM MES pH 6.5, 10 mM DTE, 30 mM EDTA, 0.4 M sorbitol) and was centrifuged at 5000*g* for 15 min. The supernatant was removed, and the pellet was resuspended in 1.5 ml buffer and sonicated 30 sec. The pellet suspension was centrifuged at 100,000*g* for 1 h. The 100,000*g* supernatant was removed, and the pellet was resuspended in 250 µl buffer. Four incubation vials were prepared by drying 30 µl 2 mM HMG-CoA and 20 µl [^{3}H]HMG-CoA (12.2 Ci mmol^{-1}, 0.1 mCi ml^{-1}) under a stream of N_2 in a vial containing 0.66 µmol NADPH. The four samples added to the vials were 100 µl 5000*g* supernatant + 100 µl buffer, 200 µl 5000*g* supernatant, 200 µl 100,000*g* supernatant, and 200 µl 100,000*g* pellet. The

5000*g* supernatant samples were sonicated prior to the incubation. The final concentrations were 3.3 mM NADPH and 300 µM HMG-CoA at a specific activity of 33 mCi $mmol^{-1}$.

Each sample was incubated 1 h then quenched with 50 µl 10 N KOH; after 30 min, 75 µl concentrated HCl, 10 µl 0.5 M MVAL, and 20 µl [^{14}C]MVA (2540 dpm) were added. The samples were analyzed for labelled MVAL by TLC method 2. The MVAL band was eluted with ether of which a 10% aliquot was counted. The solvent of the remainder of the eluate was removed under a stream of N_2, and the residue was redissolved in 150 µl 0.0025 N H_2SO_4 and analyzed by organic acid HPLC. Samples were also analyzed by ODS-HPLC.

A second experiment looked at the HMGR activity of the 5000*g* supernatant and 100,000*g* pellet prepared in the same manner but without EDTA in the buffer. Samples were analyzed by TLC followed by ODS-HPLC.

Localization of HMGR in latex fractions. HMG-CoA Incubation 11- Latex was subjected to the differential centrifugation scheme diagramed in Fig. 3. This scheme was based on work which localized *Pisum* seedlings HMGR in the heavy microsomal fraction (P3) (3). A volume of 1.5 ml latex was collected into a tube where 114 µl 0.4 M EDTA and 60 µl 0.25 M DTE had been dried under N_2, making the final concentrations 30 mM EDTA and 10 mM DTE, and centrifuged as shown, with each pellet being resuspended in 300 µl buffer (100 mM MES, 30 mM EDTA, 10 mM DTE, 0.4 M sorbitol, pH 5.9). For each incubation, 150 µl sample was added to 5 µl [^{3}H]HMG-CoA (0.1 mCi ml^{-1}, 11.7 Ci $mmol^{-1}$), 45 µl 2 mM HMG-CoA, and 15 µl 25 mM NADPH which had been dried under N_2 in the incubation tube. The final concentrations of the substrates were 600 µM HMG-CoA at a specific activity of 5.6 mCi $mmol^{-1}$ and 2.5 mM NADPH. The samples were incubated 2 h at 28°C then quenched by addition of 15 µl 6 M HCl with 15 µl 1 M MVA, potassium salt and 5 µl [^{14}C]MVA, DBED salt (4998 dpm). The samples were allowed to sit 10 min to promote lactonization of the MVA and were then kept frozen overnight. Seventy five microliters water was added to each, and the samples were centrifuged 30 min at 12,000*g*. The supernatants were filtered through 0.45 µm filters, and their volume brought to 250 µl with water.

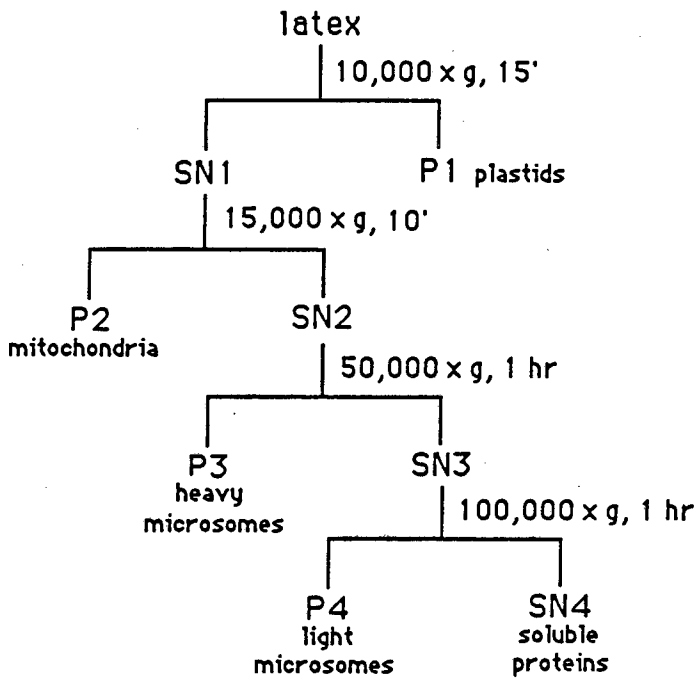

Fig. 3 Fractionation of latex for analysis of the distribution of HMG-CoA reductase activity.

Aliquots of 100 µl were analyzed for labelled MVAL by TLC ($CHCl_3$: acetone, 2:1) followed by chromatography by HPLC using the ODS-potassium phosphate system.

HMGR activity of 100,000*g* Mix. A 1.5 ml sample of latex was centrifuged at 100,000*g* for 1 h. The supernatant and pellet were mixed, and various volumes of the mixture were incubated with the HMG-CoA substrate and cofactors which had been dried in the incubation vials (Table III).

Table III. *Incubation Components for Determination of HMG-CoA Reductase Activity versus Volume of 100,000g Mix*

	Sample			
	1	2	3	4
µl 100,000*g* Mix	50	100	175	275
µl [^{3}H]HMG-CoA (12.2 Ci mmol^{-1}, 0.1 mCi ml^{-1})	2.5	5	8.75	13.75
µl 2 mM HMG-CoA	7.5	15	26.25	41.25
µl 0.1 M DTE	5	10	17.5	27.5
µl 50 mM NADPH	2.5	5	8.75	13.75

The final concentrations in all incubations were 300 µM HMG-CoA at a specific activity of 17 mCi mmol^{-1}, 10 mM DTE, 30 mM EDTA, and 2.5 mM NADPH. Each incubation was quenched after 1.5 h with concentrated HCl; [^{14}C]MVA (20520 dpm) and aliquots of a mixture containing 1.25 mg ml^{-1}each of HMG, sodium acetate, and MVAL and 0.25 mg ml^{-1} lithium acetoacetate were added as carriers. The samples were centrifuged; the supernatants were diluted to 0.5 ml, and 50 µl was analyzed by organic acid HPLC.

Time course of HMGR activity of 100,000*g* supernatant. Latex (0.5 ml) was diluted with an equal volume of buffer containing 0.4 M MOPS pH6.5, 60 mM EDTA, 0.4 M sorbitol, and 20 mM DTE and was centrifuged for 30 min at 100,000*g* . Four 200 µl samples of the 100,000*g* supernatant were each incubated with 30 µl 2 mM HMG-CoA, 10 µl [^{3}H]HMG-CoA (12.2 Ci

mmol^{-1}), and 100 µl 5 mM NADPH in methanol which had been taken to dryness under a stream of N_2. After time periods of 30 min, 1 , 2 , and 3 h, the incubations were quenched with 50 µl concentrated HCl, 100 µl ^{14}C MVA, DBED salt, and 100 µl containing standards. The samples were analyzed by OA-HPLC.

Ammonium sulfate fractionation of HMG-CoA reductase of latex. A volume of latex (1.5 ml) was diluted with an equal volume of buffer (20 mM MES pH 6.5, 60 mM EDTA, 20 mM DTE, 0.4 M sorbitol) and was centrifuged at 100,000*g* for 30 min. The supernatant was removed, and two aliquots each 150 µl were taken for HMGR and protein assays. The remaining sample was brought to 30% saturation in ammonium sulfate and was stirred for 15 min. The sample was centrifuged for 15 min at 10,000*g*. The supernatant was removed, brought to 70% saturation with ammonium sulfate, and centrifuged as before. Both pellets were resuspended in 300 µl buffer containing 10 mM MES pH 6.5, 30 mM EDTA, 10 mM DTE, and 0.4 M sorbitol. HMGR activity was measured by incubation of 150 µl of sample with 10 µl [^{3}H]HMG-CoA (12.2 Ci mmol^{-1}), 22.5 µl 2 mM HMG-CoA, and 7.5 µl 50 mM NADPH; all had been taken to dryness under a stream of N_2. After 1.5 h incubation the samples were quenched in a boiling water bath. They were analyzed for labelled MVA by OA-HPLC. Protein content was determined by PROTEIN ASSAY 2.

pH effect on latex HMGR. Latex (1.5mL) was added to 112.5 µl 0.4 M EDTA and 60 µl 0.25 M DTE that had been dried under N_2 in the centrifuge tube giving final concentrations of 30 mM EDTA and 10 mM DTE and was centrifuged at 100,000*g* for 30 min. The lipid film was removed, and the clear supernatant and white pellet were mixed giving 950 µl of a solution of pH 4.6. A 200 µl aliquot was removed, 150 µl taken for HMGR assay and 50 µl for protein determination. The mix was brought to 70% saturation with 0.49 g pulverized ammonium sulfate. The tube was rinsed with buffer (100 mM MES, 30 mM EDTA, 10 mM DTE, pH 6.0) also 70% saturated with ammonium sulfate. The mix and rinse were combined and centrifuged 15 min at 10,000*g*. The supernatant was removed, and the pellet was resuspended in 400 µl buffer. The protein fraction was desalted on a 5 ml G-25 column equilibrated with buffer. The A_{280} of the eluant was monitored, and a 1-ml

fraction containing the protein was collected. Of this fraction, 50 µl was taken for protein analysis and 150 µl was assayed for HMGR activity (pH 6.1). Two 300 µl aliquots were taken from the remaining protein solution; one was brought to pH 5.4 with 7.5 µl 1 M HCl, and the second was brought to pH 6.6 with 7.5 µl 1 M KOH. A 150 µl aliquot of each was used for the HMGR assay.

Each 150 µl sample selected for HMGR activity assay was incubated with 10 µl [^{3}H]HMG-CoA (0.1 mCi mmol^{-1}, 11.7 Ci mmol^{-1}), 22.5 µl 2 mM HMG-CoA, and 15 µl 25 mM NADPH that had been dried under a stream of N_2 in the incubation tube. The final concentrations were 300 µM HMG-CoA at a specific activity of 22 mCi mmol^{-1} and 2.5 mM NADPH. The incubations were quenched at one hour with 7.5 µl concentrated HCl, and 100 µl [^{14}C]MVA, DBEB salt (10061 dpm) and 5 µl 1 M MVA, potassium salt were added as carrier. The samples were then centrifuged at 12,000*g* for 15 min and filtered through a 0.45 µm filter to remove particulates. A 50 µl aliquot was assayed for labelled MVA by organic acid HPLC. The samples reserved for protein analysis were assayed by PROTEIN ASSAY 2.

Substrate saturation for HMGR in desalted 70% ammonium sulfate fraction. A 1.5 ml latex sample was mixed with EDTA and DTE to give a final concentration of 30 mM EDTA and 10 mM DTE. The sample was centrifuged at 100,000*g* for 30 min. The surface lipid film was removed, and the supernatant and pellet were mixed, giving a solution with a pH of 4.7. A total of 250 µl was removed from the mixture for HMGR activity and protein assays. The remaining volume was brought to 70% saturation in ammonium sulfate and centrifuged at 10,000*g* for 15 min. The pellet was resuspended in 400 µl buffer (100 mM MES, 30 mM EDTA, 10 mM DTE, pH 5.9) and desalted on a 5.4 ml G-25 column equilibrated with buffer. Out of the 1.15 ml volume which contained the protein, five 150 aliquots were incubated with various amounts of HMG-CoA and 2.5 mM NADPH for 1 h (Table IV). All incubations were quenched with 7.5 µl concentrated HCl, 5 µl 1 M MVA, potassium salt, and 100 µl [^{14}C]MVA (10,000 dpm) and were then centrifuged at 12,000*g* for 15 min. The solutions were filtered through a 0.45 µm filter and were analyzed for labelled MVA by organic acid HPLC. Protein content was determined by PROTEIN ASSAY 2.

Table IV. *Quantity of HMG-CoA used in HMG-CoA Reductase Substrate Saturation Experiment*

Incubation	Final Concentration	[^{3}H]HMG-CoA 0.1 mCi mmol^{-1}, 11.7 Ci mmol^{-1}	2 mM HMG-CoA
	µM HMG-CoA	µl	µl
1	300	10	22.5
2	150	5	11.25
3	75	2.5	5.6
4	50	1.7	3.75
5	25	0.8	1.87

RESULTS AND DISCUSSION

Effect of substrate concentration on triterpenoid biosynthesis. A series of experiments investigating the effect of the concentration of either acetate or mevalonic acid on the biosynthesis of triterpenoids in latex from these precursors were performed. The first two sets of experiments in which the concentration of acetate supplied to the latex was varied established that the maximum biosynthesis of both triterpenols and triterpene esters occured at an acetate level of 0.25 mM (Fig. 4). Above this concentration, triterpenol biosynthesis remained level up to 3 mM, the highest concentration tested. Triterpene ester biosynthesis was relatively level between acetate concentrations of 0.25 to 1 mM. Above 1 mM acetate, the incorporation of acetate into the esters dropped steadily as the concentration of acetate increased, until at 3 mM acetate, the incorporation was less than half the maximum incorporation. The maximum amount of acetate incorporated into total triterpenoids was 56 pmol 100 µl latex $^{-1}$ h^{-1} or 9.3 pmol ml^{-1}min^{-1}. This turnover rate was two orders of magnitude less than the incorporation of acetate into rubber in *Hevea* latex where an acetate concentration of greater than 890 µM was saturating (1).

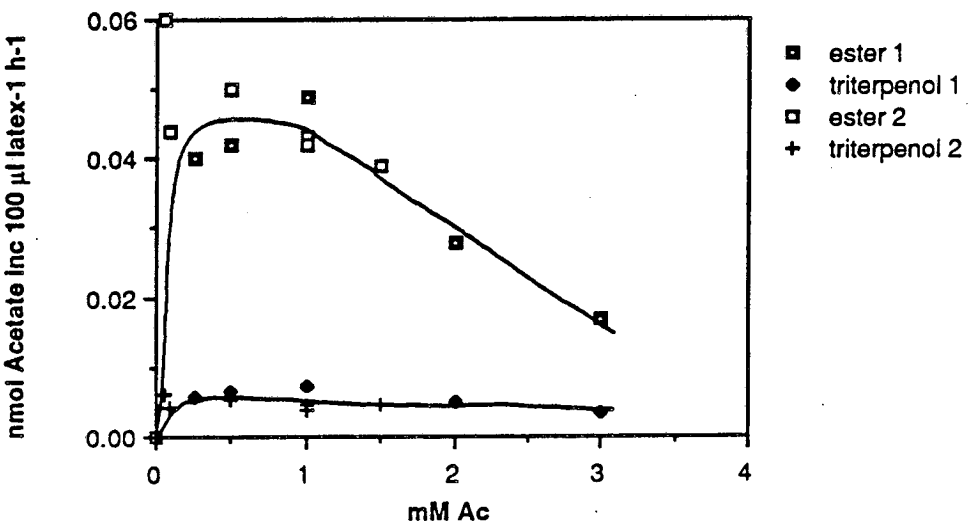

Fig. 4. Effect of concentration of substrate acetate on the biosynthesis of triterpenoids in whole latex.

The initial experiments testing the effect of MVA concentration on its incorporation into triterpenoids were done with centrifuged latex where the biosynthetic level was greatly reduced compared to later experiments performed with whole latex (Fig. 5). However, these initial experiments did show that the incorporation of MVA into triterpenoids continued to rise as the concentration of MVA was raised to the highest level of 1.1 mM. When whole latex was incubated with MVA concentrations varying from 1 to 5 mM, the incorporation of MVA into triterpenoids was constant for all levels indicating that 1 mM MVA was probably saturating.

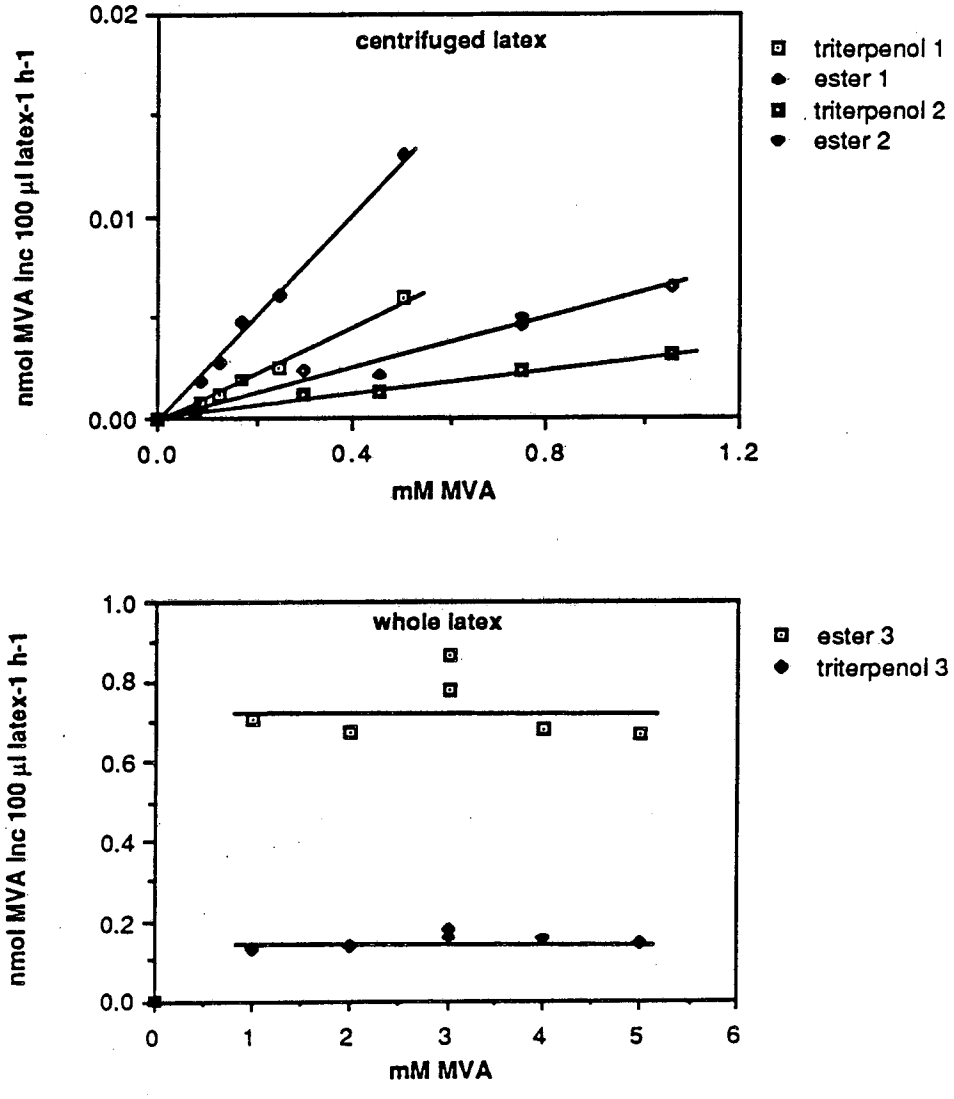

Fig. 5. Effect of concentration of substrate mevalonate on the biosynthesis of triterpenoids in centrifuged and whole latex.

This was confirmed in a final experiment where different concentrations of both acetate and MVA were incubated with aliquots of latex from the same sample (Fig. 6). MVA incorporation into both triterpenols and their esters levels at 1mM MVA. Here no acetate inhibition of triterpene ester biosynthesis was seen; incorporation began to level off at 0.25 mM acetate but still increased slowly up to 1.5 mM acetate, the highest concentration tested. Acetate incorporation into triterpenols was level from 0.25 mM acetate on up.

The maximum rate of incorporation of MVA into both the triterpenols and their esters obtained was 0.55 nmol 100 µl latex $^{-1}$ h^{-1} in the third MVA incorporation experiment. For comparison, the saturating concentration of MVA for rubber biosynthesis in *Hevea* latex is greater than 3.6 mM, and the maximum incorporation is 30 nmol ml^{-1} min $^{-1}$, around 300 times greater than the incorporation of MVA into triterpenoids in *E. lathyris* latex (1).

A comparison of the rates of incorporation of Ac and MVA into triterpenoids indicates that the rate-determining step in the pathway occurs prior to MVA. Under saturating substrate conditions, MVA incorporation is 25 times greater than Ac incorporation (Fig. 7). Taking into account that it takes three acetate molecules to form one mevalonate and that the triterpenoids are formed from six mevalonates, the rate of formation of "triterpenoid equivalents" from MVA is one hundred times that from acetate. A similar, but not as dramatic situation was seen when *Acer pseudopatinus* suspension cultures were incubated with saturating levels of Ac (1 mM) and MVA (5 mM); sterol biosynthesis was 2.3 times higher with MVA as the precursor (9). Of the four enzymes required to convert acetate into mevalonate: acetyl-CoA synthetase, thiolase, HMG-CoA synthase, and HMG-CoA reductase, the reductase has been shown to be the rate-limiting step in mammalian cholesterol biosynthesis (4).

Properties of the site of triterpenoid biosynthesis in latex. Ultimately, a particle capable of converting MVA into triterpenoids was isolated by centrifugation of latex at 5000*g* (24). Attempts to first purify this structure on Percoll gradients were unsuccessful. Although Percoll is reported to be non-toxic to cells, when latex was diluted with buffered Percoll, triterpenoid biosynthesis was reduced 10-fold compared to the control sample (Table V).

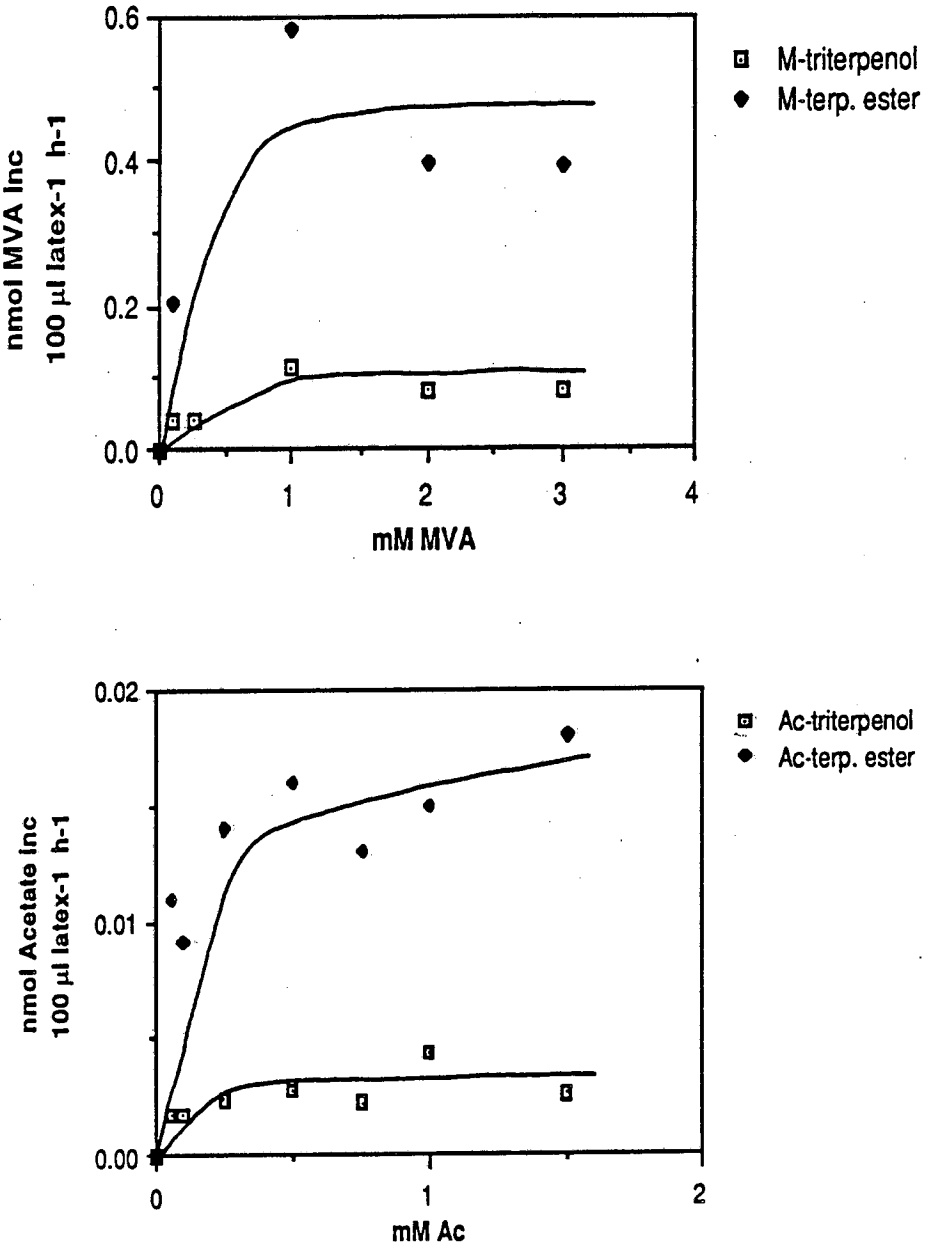

Fig. 6. Effect of concentration of substrate acetate and mevalonate on the biosynthesis of triterpenoids in aliquots of a single whole latex sample.

STEPS ALONG PATHWAY	SUBSTRATE INCORPORATION (nmol 100 µl latex^{-1}h^{-1})	TRITERPENOID EQUIVALENTS (nmol 100 µl latex^{-1}h^{-1})
Ac---------->Triterpenoids	0.02	0.001
MVA---->Triterpenoids	0.55	0.09

Fig. 7. Comparison of rates of incorporation of MVA and Ac into latex triterpenoids.

Ester biosynthesis was affected to a greater degree than that of the free alcohols. Centrifugation of latex samples placed on Percoll showed that removal of the pellet, which contained rod-shaped starch grains (Ch. 3), did not reduce the amount of biosynthesis. The layer of latex that remained on top of the Percoll after centrifugation had 20% of the biosynthetic capability of the whole sample, indicating that a large percentage of the particles responsible for the biosynthesis had a density greater than the Percoll (δ=1.0975 g ml^{-1}).

Table V. *Effect of Percoll and Centrifugation on the Biosynthesis of Triterpenoids by Latex*

Treatment	pmol triterpenols	pmol triterpene esters	Σ pmol triterpenoids
latex+Percoll	0.9	2	2.9
latex+Percoll+ centrifugation	0.7	1.9	2.6
latex+Percoll+ centrifugation-pellet	0.9	1.9	2.8
top layer only of latex+Percoll+ centrifugation	0	0.5	0.5
latex+buffer	5.7	25.3	31

When latex was centrifuged for 5 min at 130*g* on Percoll, the greatest percentage of biosynthetic activity was recovered by combining an aliquot of the material that remained on top of

the Percoll with the 5000*g* pellet obtained from a second centrifugation of the Percoll layer (Fig. 8). Centrifugation of the Percoll fraction at higher and lower *g* forces gave lower recoveries of activity. The lower forces may not have sedimented all the particles; with the higher forces used, the particles could have been damaged. The greater amount of activity remaining on top of the Percoll in this experiment (50%) than in the previous experiment says that the density of the site of triterpenoid biosynthesis is variable.

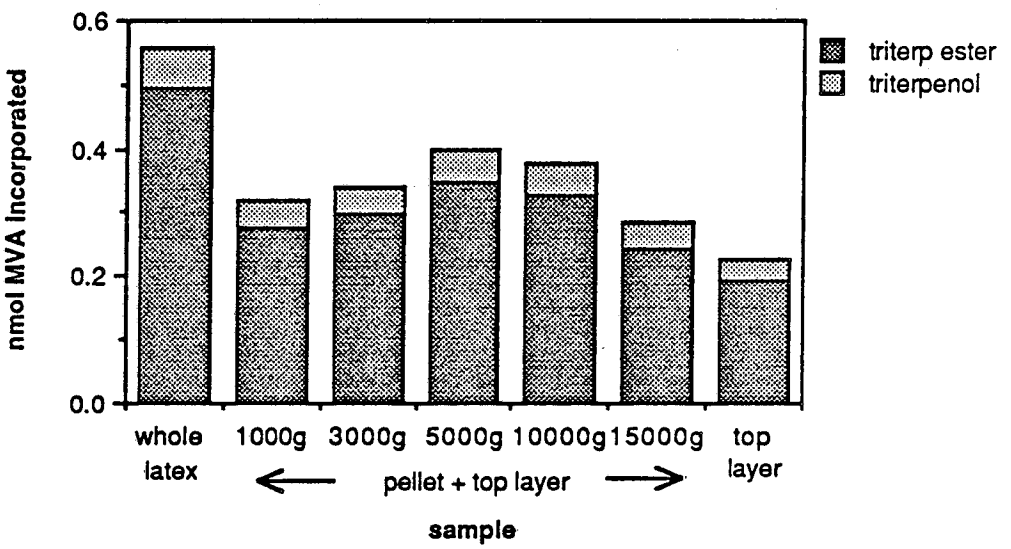

Fig. 8. Recovery of triterpenoid-synthesizing activity among fractions of latex centrifuged on Percoll.

Further validation of the particulate nature of the site of triterpenoid-synthesizing activity was obtained by a comparison of the distribution of the activity between the supernatant and pellet of the top layer from a latex sample centrifuged on Percoll which was subsequently centrifuged for 10 min at 100,000*g* (Fig. 9). The majority of the activity was associated with the pellet, in fact, incubation of the supernatant with the pellet reduced the activity. This may have been due to the inhibitory effects of Percoll which would have remained in the supernatant fraction. The duplicate incubations of supernatant with pellet show significant variability, demonstrating the difficulty of dividing pellets into equivalent samples with the same biosynthetic capability. This problem is likely due to the sticky nature of latex. In later experiments this

variability was reduced by first dividing whole latex into the number of needed fractions and then centrifuging to obtain the pellets.

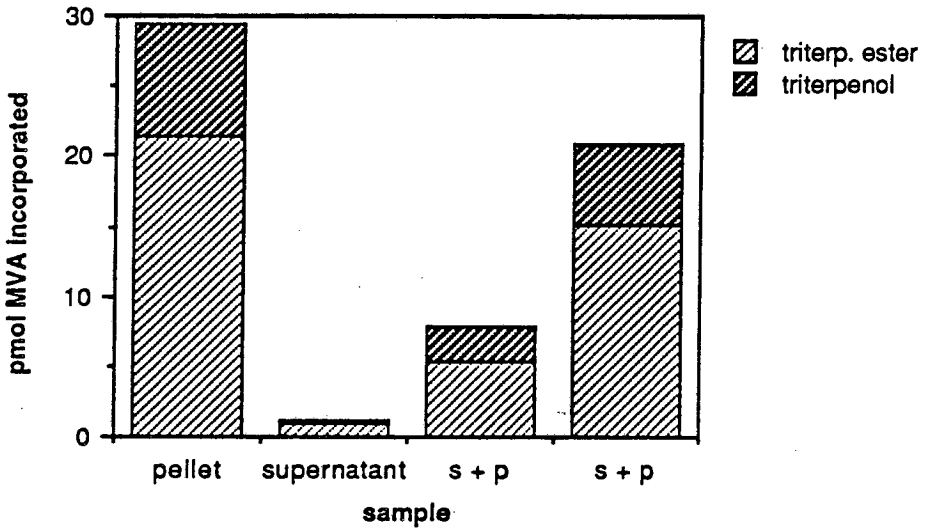

Fig. 9. Distribution of triterpenoid-synthesizing activity between particulate and soluble fractions of the top layer from latex centrifuged on Percoll.

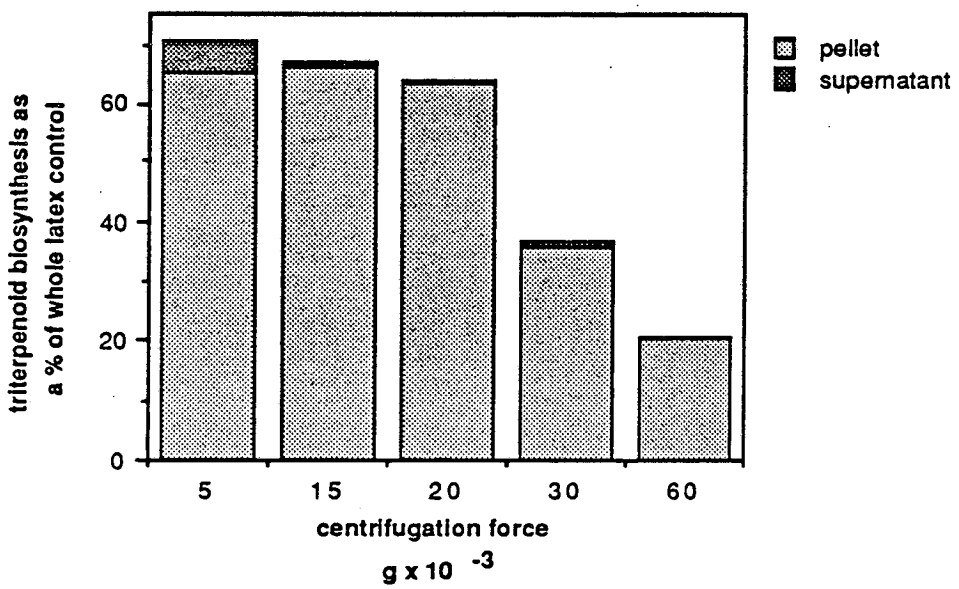

Fig. 10. Distribution of triterpenoid-biosynthetic activity between the supernatant and pellet of latex centrifuged at various forces.

When latex was centrifuged without Percoll at various *g* forces, the majority of the biosynthetic activity was recovered in the pellet fraction (Fig. 10). As in the experiment where the particle responsible for the biosynthetic activity was recovered by centrifugation of the Percoll layer, a centrifugation at 5000*g* gave the greatest recovery of activity. When latex was centrifuged at *g* forces in the range of 5000 to 20,000, the resulting pellet had 65% of the biosynthetic activity of whole latex. Slightly more activity was sedimented at 15,000*g* than at 5000*g* indicating that forces in this range are required to pellet the significant structure in the time period of 15 min. With centrifugation at higher *g* values, the recovery of activity is less, probably due to damage to the structure. Since the recovery of biosynthetic activity of in the pellet after this simple centrifugation was as good as centrifugation on Percoll, this procedure was used to obtain a fraction capable of triterpenoid biosynthesis.

When the 15,000*g* pellet was incubated with 0.25 mM [^{3}H]Ac, the incorporation of label was 4% of the incorporation when a pellet from the same latex sample was incubated with 0.25 mM [^{3}H]MVA. The rate of incorporation of MVA was 27 pmol 100 µl latex^{-1} h^{-1} into triterpene esters and 9 pmol 100 µl latex^{-1} h^{-1} into triterpenols. For Ac, the rates of incorporations were1.4 and 0.3 pmol 100 µl latex^{-1} h^{-1} respectively into triterpene esters and triterpenols. Since this is the same ratio of incorporation obtained when Ac and MVA incorporation under saturating substrate conditions into triterpenoids in whole latex was measured, it would appear that the pellet was capable of metabolizing both substrates. However, 0.25 mM MVA is not saturating for triterpenoid biosynthesis in latex. When a 5000*g* pellet was incubated with either 1 mM MVA or Ac to insure substrate saturation, Ac incorporation was negligible compared to MVA incorporation indicating that the particulate fraction is capable of metabolizing MVA but not Ac into triterpenoids (12).

Lifetime of the biosynthetic activity of the pellet. Mevalonate incorporation into triterpenoids by whole latex proceeds at a relatively uniform rate over a period of 6 h (20). The rate of biosynthesis of triterpenoids from MVA by the pellet obtained by centrifugation of latex at 15,000*g* for 15 min was measured over a period of 4.5 h and was found to be linear over that time

period (Fig. 11).

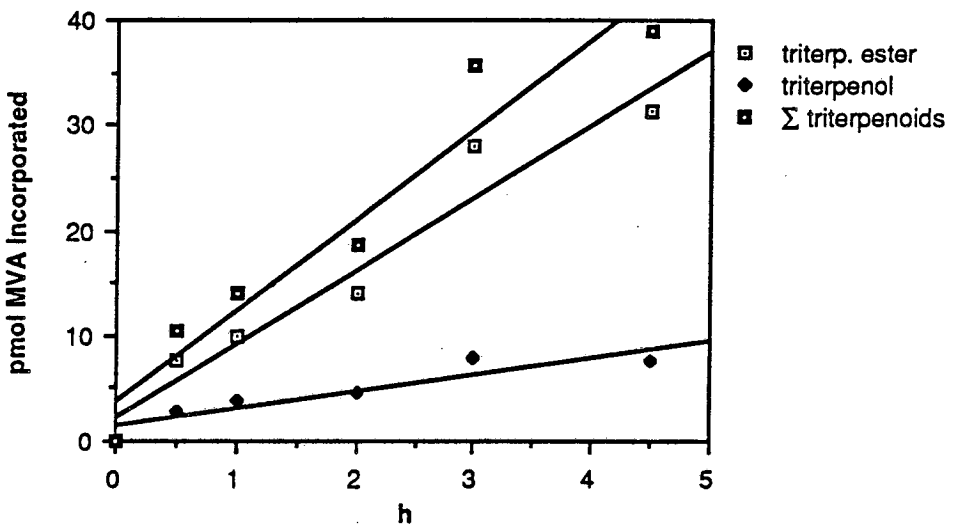

Fig. 11. Biosynthesis of triterpenoids over time by a particulate fraction obtained by centrifugation of latex at 15,000*g* for 15 min.

A second experiment looked at the incorporation of [^{3}H]MVA in the triterpenoids by a 15,000*g* pellet of latex which had first been incubated as whole latex with [^{14}C]MVA. This experiment allowed comparison of the amount of biosynthesis by whole latex and the 15,000*g* pellet of the same sample. An additional incubation was performed with a 15,000*g* pellet which had been resuspended in buffer and then resedimented by centrifugation. This process was performed as a second wash to remove [^{14}C]MVA before the tritiated substrate was added. As a control, whole latex was incubated for 2 h with [^{14}C]MVA , then [^{3}H]MVA was added . A second control was incubated 2 h with [^{14}C]MVA , separated into supernatant and pellet, and analyzed.

After a 2 h incubation with [^{14}C]MVA , labelled triterpenoids distributed about equally between the supernatant and pellet (Fig. 12). The amount of labelled triterpenoids found in the supernatant is fairly constant in the three samples analyzed. Whole latex showed twice the amount of triterpenoids labelled with ^{14}C compared to the sum of ^{14}C-labelled terpenes in the supernatants and pellets of either the sample which was quenched at 2 h or the samples whose

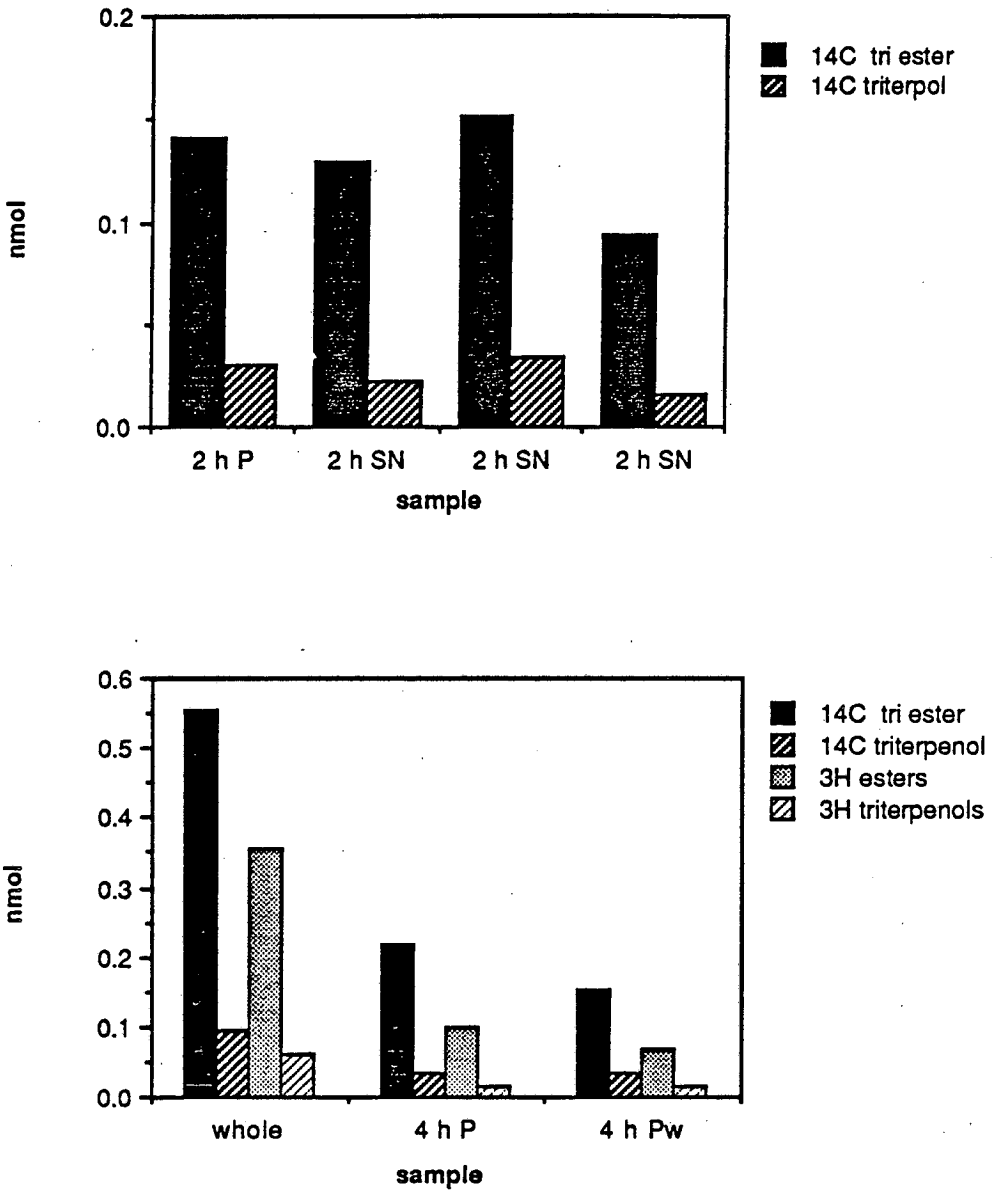

Fig. 12. Comparison of triterpenoid biosynthesis by whole and centrifuged latex.

pellets were incubated an additional 2 h; this is reasonable if the [^{14}C]MVA was removed from the pellets upon centrifugation. The pool of MVA in the pelletable structure is therefore small. Biosynthesis of triterpenoids by the pellet and the washed pellet (Pw) were 30% and 21%, respectively, of the biosynthesis in whole latex in the second 2 h incubation so centrifugation

does diminish the biosynthetic capacity of the structure. This could occur either by damage to the structure or removal of cofactor(s) present in the 15,000*g* supernatant. The second centrifugation reduced the biosynthesis of the pellet to two-thirds that of the pellet that was only centrifuged once, but as there were less ^{14}C-labelled triterpene esters in the pellet, the second wash and centrifugation may be important to insure removal of [^{14}C]MVA .

In the final experiment investigating the lifetime of triterpenoid biosynthesis by the particulate fraction, a washed, pre-incubated (2 h) pellet showed a linear rate of biosynthesis up to 4 h (Fig. 13). The amount of [^{3}H]MVA -labelled triterpenoids in the sample at each time point was nearly equal indicating that the substrate had been successfully removed by the two washes of the pellet. The rate of biosynthesis by the pellet was 32% of that of whole latex again indicating either damage to the structure or removal of cofactors by centrifugation.

Effect of soluble factors in latex on triterpenoid biosynthesis. Soluble noncatalytic proteins have been found to be involved in the transfer of phospholipids and squalene between membranes of subcellular structures(8, 14). In addition, these types of proteins have been found to stimulate the oxidation of squalene and kaurene, a step required for further metabolism of these terpenes (8, 19). The possible involvement of like factors in *E. lathyris* latex was investigated. It was thought that the reduced biosynthesis seen in the isolated particulate fraction compared to whole latex could be due to the removal of soluble cofactors which could stimulate biosynthesis. A second possible role in the removal of triterpenoids from the particulate site of synthesis was also considered since labelled triterpenoids had been found distributed equally in the pellet and supernatant when whole latex was labelled and then centrifuged (Fig.12).

In an initial experiment in which whole latex was incubated with [^{3}H]acetate then fractionated and the fractions analyzed for their labelled triterpenoid content, the majority of labelled terpenes were found in the the top lipid film and the 100,000*g* pellet (Fig. 14). The presence of labelled triterpenes in the top film which is composed of latex particles (Ch. 3) after a 3 h incubation indicates that the newly synthesized compounds are rapidly removed from the site

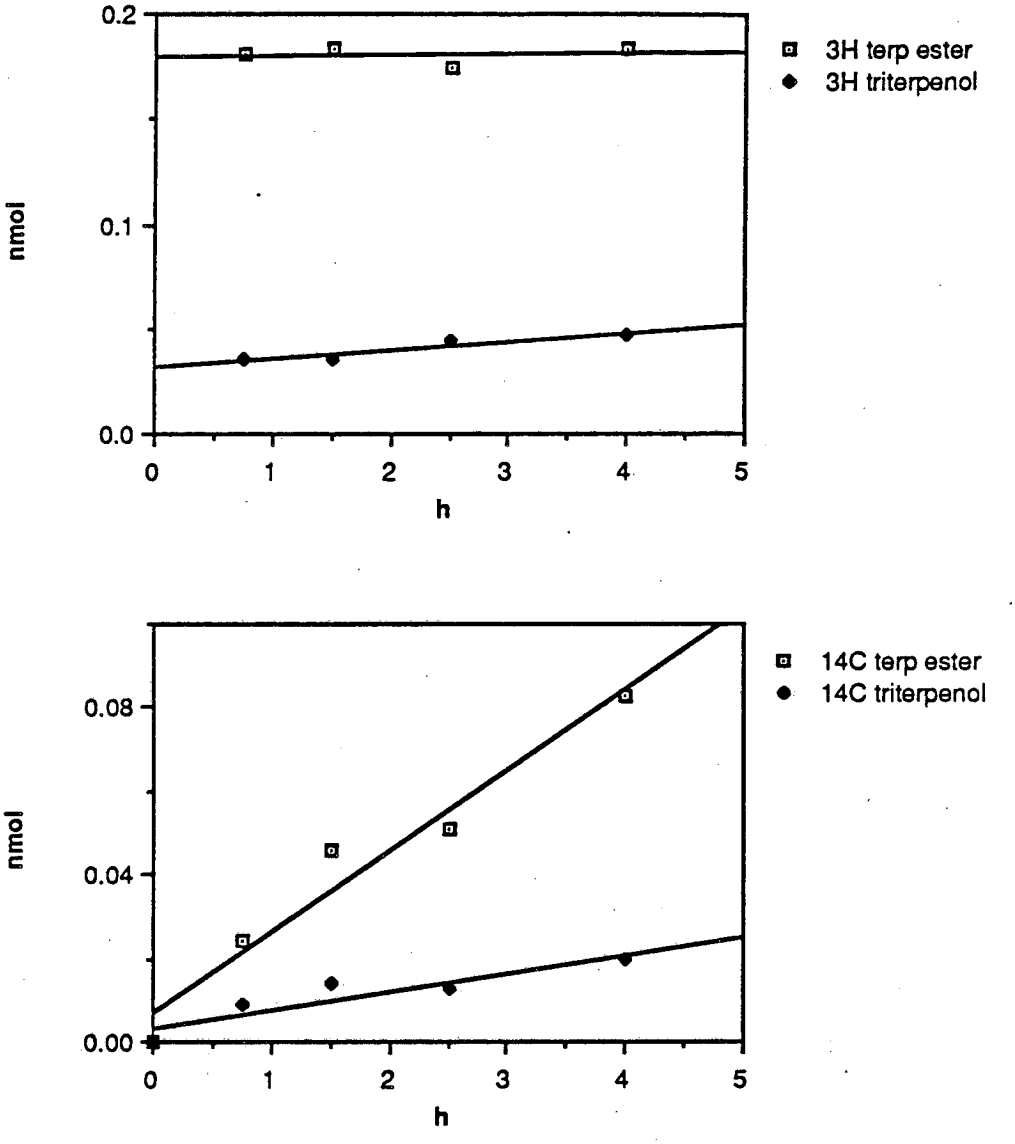

Fig. 13. Biosynthesis of triterpenoids over time by a pre-incubated, washed particulate fraction obtained by centrifugation of latex at 15,000*g* for 15 min.

of synthesis.

After a 100,000*g* pellet had been loaded with labelled triterpenoids by preincubation as whole latex with [^{3}H]MVA, incubation of it with unlabelled MVA and 100,000*g* supernatant resulted in a greater efflux of labelled triterpenoids into the supernatant than when the

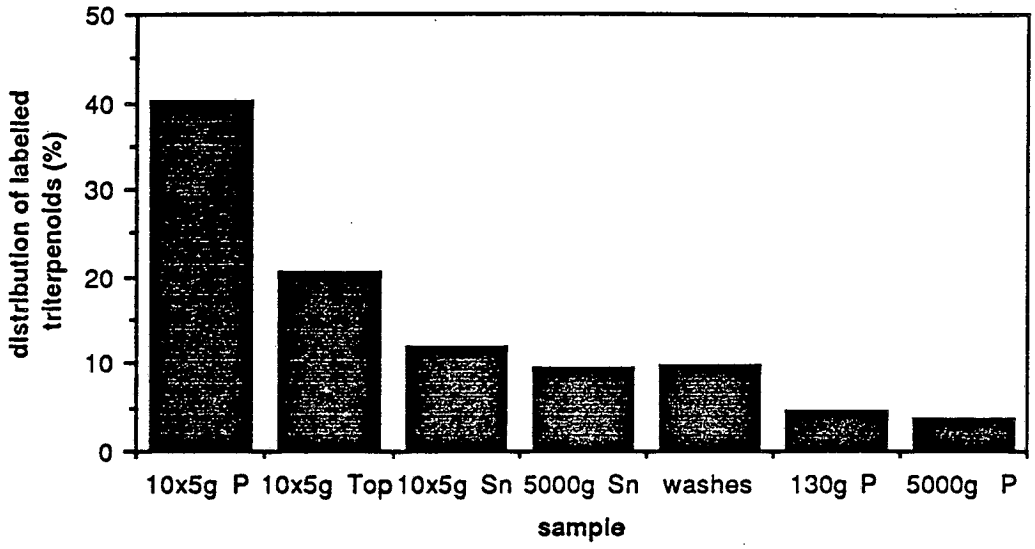

Fig. 14. Distribution of labelled triterpenoids in latex fractions.

supernatant was replaced by buffer or buffer + BSA (Fig. 15). When no unlabelled MVA was added before incubation of the 100,000*g* pellet with the 100,000*g* supernatant, more labelled triterpenoids were found in the supernatant after 2 h than when the MVA was added suggesting that newly synthesized unlabelled triterpenoids were being removed from the pellet pool as well

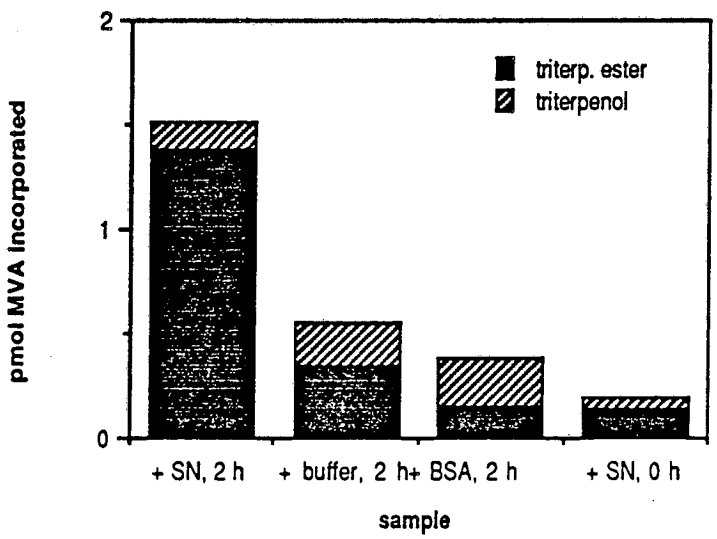

Fig. 15. Effect of soluble fractions on efflux of labelled triterpenoids from 100,000*g* pellet of latex .

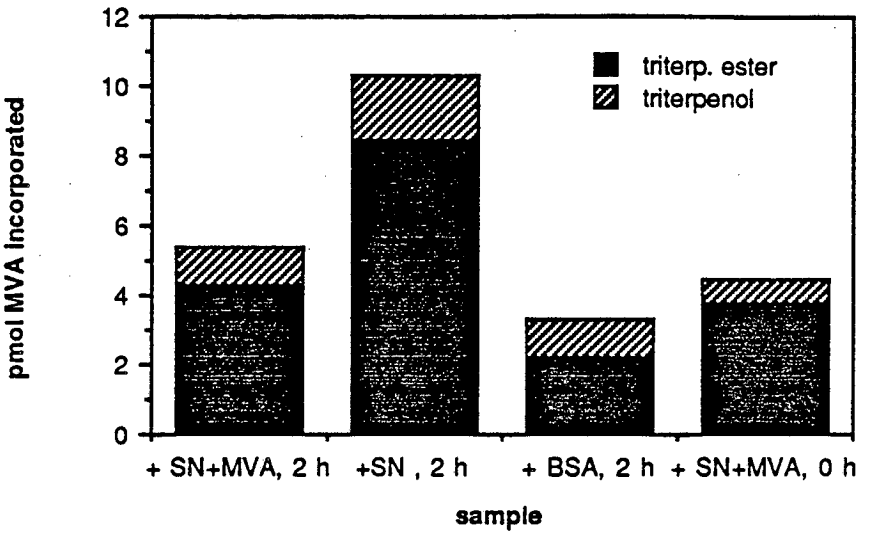

Fig. 16. Effect of soluble fractions with and without added MVA on efflux of labelled triterpenoids from 100,000*g* pellet of latex .

as the previously labelled material (Fig. 16).

When a 15,000*g* pellet which had been incubated as whole latex with [^{14}C]MVA was incubated with [^{3}H]MVA and either buffer or 100,000*g* supernatant for different time periods, no difference in the amount of [^{14}C]-labelled triterpenoids found in the soluble fraction was seen between the different time points and treatments (Fig. 17). The amount of newly synthesized, ^{3}H-labelled triterpenoids found in the soluble portion did increase with time in the presence of 100,000*g* supernatant; however, the significance of this is questionable since the amounts of labelled triterpenoids found in the soluble fraction were below picomolar levels. The amount of ^{14}C-labelled triterpenoids in the pellet remained constant over the time period indicating as seen previously that labelled MVA can be effectively removed from the pellet by two washes and centrifugations (Fig. 18). The presence of the 100,000*g* supernatant did not affect the amount of newly synthesized triterpenols (^{3}H-labelled) in the pellet which remained relatively constant over the time points assayed in both the supernatant and buffer treatments. The amount of tritium-labelled triterpene esters increased with time under both treatments, but the incubations with supernatant contained an average of 2.5 times more labelled material than their counterpart

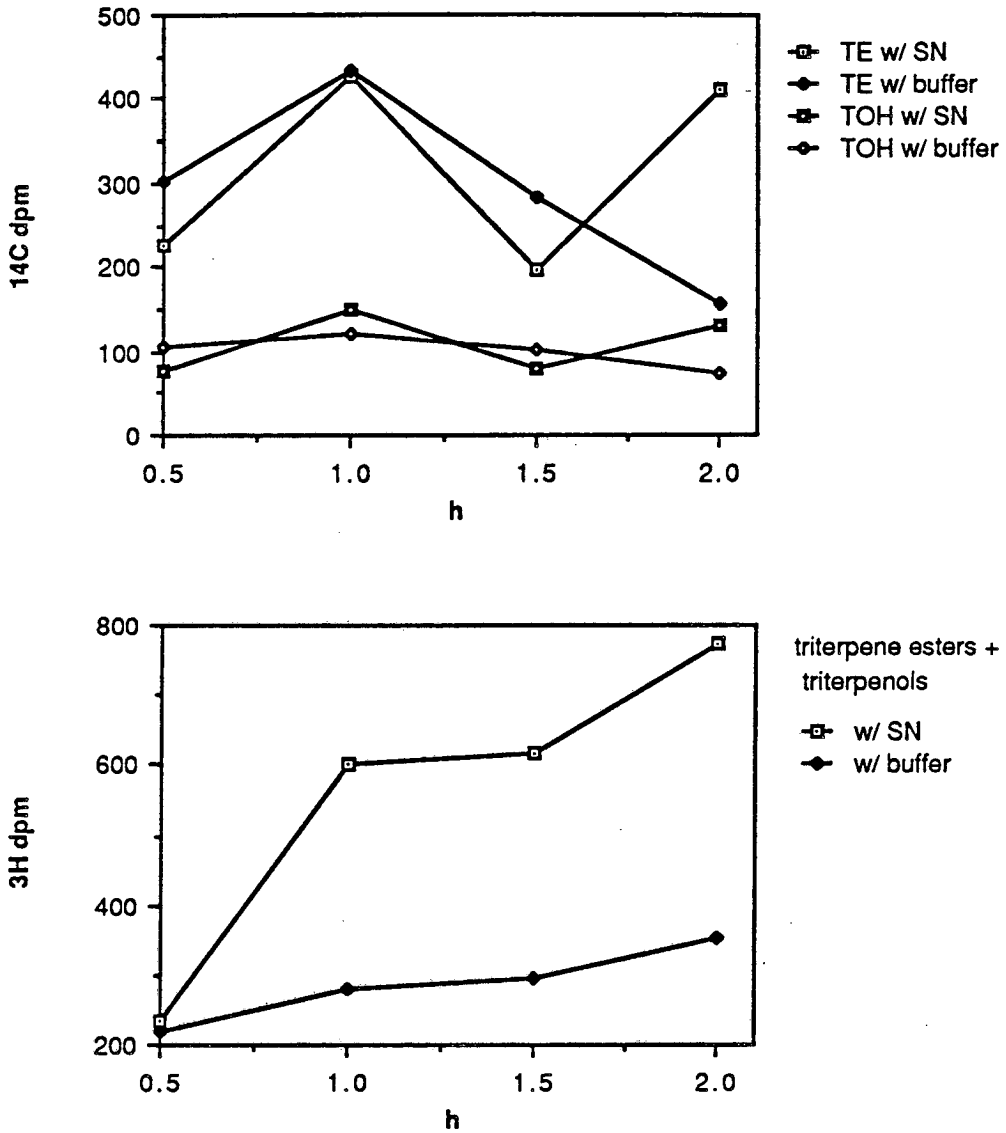

Fig. 17. Time course of effect of supernatant or buffer on efflux of labelled triterpenoids from 15,000*g* pellet of latex .

incubations with buffer.

A final experiment investigated the effect of the amount of 100,000*g* supernatant on the biosynthesis of triterpenols and triterpene esters by the 15,000*g* pellet (Fig. 19). No difference was seen in the amount of biosynthesis of either class of triterpenoid under the different treatments.

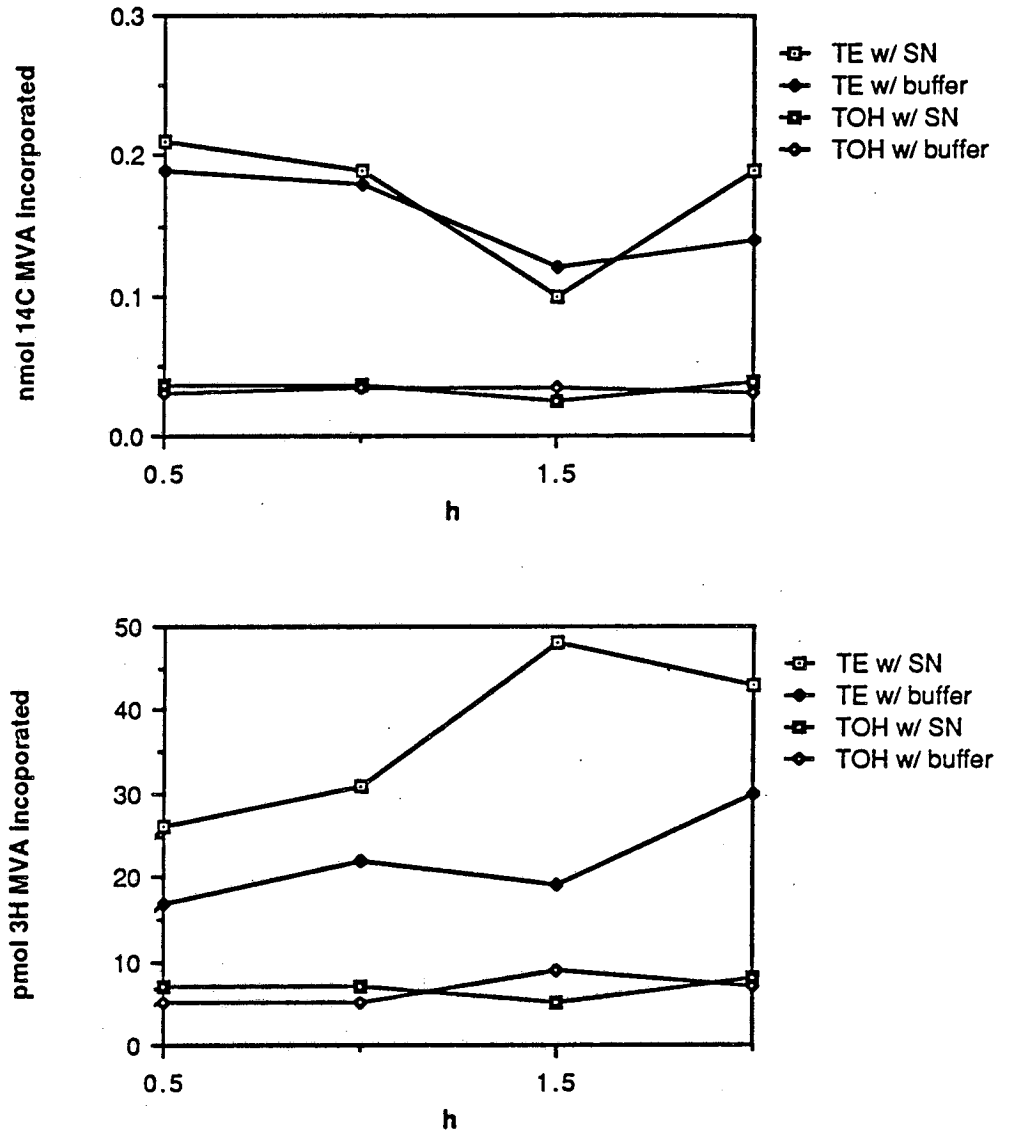

Fig. 18. Time course of effect of supernatant or buffer on labelled triterpenoid content of 15,000*g* pellet.

The most dramatic effects of supernatant on transfer of triterpenoids into the the soluble fraction were seen in the experiments performed with a 100,000*g* pellet; only an increase in the amount of newly synthesized triterpene esters in the pellet was seen when supernatant was incubated with the 15,000*g* pellet. Since centrifugation at higher *g* forces appears to disrupt the structure capable of converting MVA into triterpenoids, the effects seen with the 100,000*g* pellet

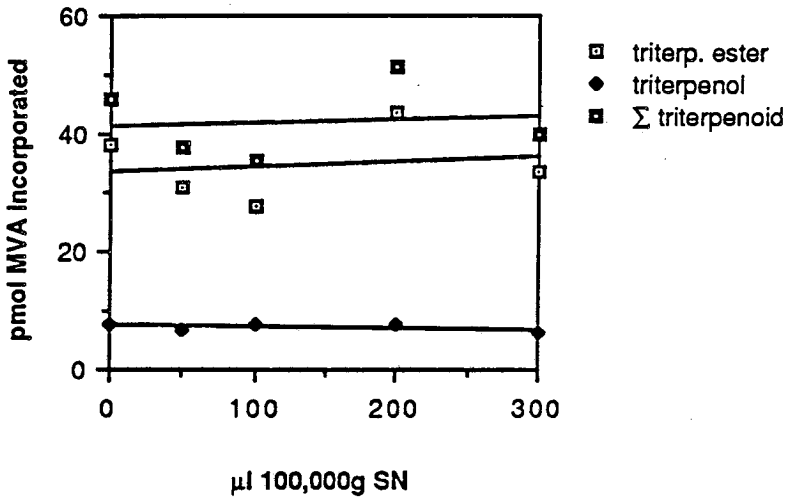

Fig. 19. Effect of 100,000*g* supernatant concentration on triterpenoid biosynthesis by 15,000*g* pellet.

may well be the result of adding back something lost from the structure due to the harsh treatment. Considering just the experiments with the 15,000*g* pellet, the supernatant does not appear to be capable of removing triterpenoids from the pellet in this *in vitro* system. Neither does it appear to enhance triterpenoid biosynthesis in general. Based on these results, further investigation into the possible role of soluble proteins in triterpenoid biosynthesis and transport was curtailed. The effect of supernatant on triterpene ester biosynthesis by the pellet was subsequently correlated with the need for phospholipids present in the supernatant to serve as donors for the fatty acid moiety of the ester (21).

HMG-CoA metabolism in latex. The determination that the rate-limiting step in triterpenoid biosynthesis in latex occurs prior to MVA led to an investigation of HMG-CoA metabolism in latex since the enzyme HMG-CoA reductase (HMGR) was a likely candidate for the rate-determining role (Fig. 20). Exogenously-supplied HMG-CoA was not incorporated into triterpenoids by whole latex, indicating that HMGR was possibly sequestered in latex (20). Although the enzyme converts a soluble substrate to a soluble product, it has been found to be a membrane-bound

enzyme. In mammalian systems it is a transmembrane protein of the ER with its active site present on the cytoplasmic side of the membrane (16).

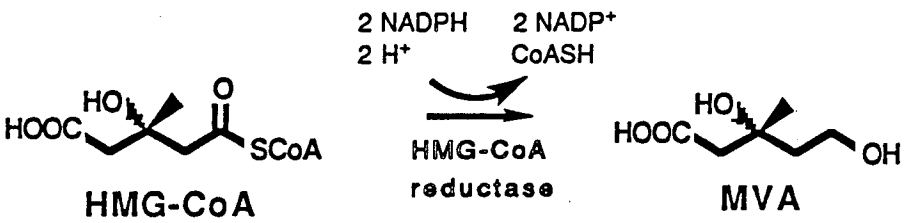

Fig. 20. Reaction catalyzed by HMG-CoA reductase.

Numerous incubations of fractionated latex with radiolabelled HMG-CoA were performed; the results of these experiments are tabulated in Table VI with individual experiments presented separately since the biosynthetic capacity of latex can vary daily. Included in the listed conditions under which each incubation was performed is the way in which the labelled MVA was detected. This is crucial since it was discovered that while a compound labelled from HMG-CoA did coelute with MVA on the HPLC organic acid column, this material did not coelute with MVAL either using TLC or on the HPLC ODS column. Analysis of HMG-CoA metabolism using the organic acid column was desirable since conversion of HMG-CoA to acids other than mevalonic acid could also be determined. Since many experiments were analyzed only using the organic acid column HPLC, the results of these experiments are not conclusive. However, since in some experiments, another form of chromatography verified the results determined by OA HPLC, these experiments are being reported so as to indicate possible routes further investigation of HMG-CoA metabolism in latex might follow. The nature of this second HMG-CoA metabolite was not determined; however, it is not acetate, acetoacetate, or mevaldic acid. An enzymatic activity in rat liver microsomes which converts HMG-CoA into a product which coelutes with MVAL by anion-exchange chromatography but not on TLC has been reported (22), but the product has not been identified. Washing of the microsomes removed the competing activity.

Table VI. *HMG-CoA metabolism of various latex fractions*

incubation	sample	conversion rate (nmol 100 µl latex^{-1} h^{-1})	conditions
1	100,000*g* pellet		
	-> Ac + AcAc	0.22	OA analysis
	-> MVA	0.1	
	-> Ac	0.8	treated with base, OA analysis
	100,000*g* supernatant		
	-> Ac	0.3	OA analysis
	-> AcAc	6	
	-> MVA	0.06	
	-> Ac	2.7	treated with base, OA analysis
	-> AcAc	1.4	
2	100,000*g* pellet		treated with base, 0A analysis
	-> Ac + AcAc	1.5	incubated pH 5.5
	"	1.6	incubated pH 6.7
	100,000*g* supernatant		treated with base, 0A analysis
	-> Ac + AcAc	22	incubated pH 5.5
	"	32	incubated pH 6.7
3	5000*g* supernatant		incubated with 30 mM EDTA
	-> Ac + AcAc	2	OA analysis
	-> MVA	0.26	
	-> MVA	0.2	ODS analysis
	-> MVA	0.13	TLC analysis
	-> MVA	0.15	TLC analysis
	5000*g* pellet		incubated with 30 mM EDTA
	-> Ac + AcAc	0.9	OA analysis
	-> MVA	0.022	
	-> MVA	0.032	TLC analysis

4	7500*g* pellet		incubated with 50 mM EDTA
	-> Ac + AcAc	0.013	OA analysis
	-> MVA	0.03	
	-> MVA	0.002	TLC + ODS analysis
	40,000*g* supernatant		incubated with 50 mM EDTA
	-> Ac + AcAc	0.35	OA analysis
	-> MVA	0.22	
	-> MVA	0.031	TLC analysis
	40,000*g* pellet		incubated with 50 mM EDTA
	-> Ac + AcAc	0.042	OA analysis
	-> MVA	0	mM EDTA
5	40,000*g* supernatant-> MVA	0.012	TLC analysis
	100,000*g* supernatant-> MVA	0.009	TLC analysis
	100,000*g* pellet-> MVA	0.003	TLC analysis
6	40,000*g* supernatant-> MVA	0.018	TLC analysis
	100,000*g* supernatant-> MVA	0.010	TLC analysis
	100,000*g* pellet-> MVA	0.002	TLC analysis
7	100,000*g* supernatant -> MVA	0.16	OA analysis
	100,000*g* pellet -> MVA	0.05	OA analysis
	100,000*g* mix -> MVA	0.24	OA analysis
8	5000*g* supernatant -> MVA	0.011	TLC + OA analysis
	5000*g* pellet -> MVA	0.009	
	50,000*g* supernatant -> MVA	0.009	
	50,000*g* pellet -> MVA	0.001	
	100,000*g* supernatant -> MVA	0.016	
	100,000*g* pellet -> MVA	0.002	

9	5000*g* supernatant -> MVA	0.005	TLC + ODS analysis
	100,000*g* supernatant of sonicated 5000*g* pellet -> MVA	0.001	
	100,000*g* pellet of sonicated 5000*g* pellet -> MVA	0.026	
10	5000*g* supernatant	0	TLC + ODS analysis
	100,000*g* pellet of sonicated 5000*g* pellet -> MVA	0.019	

HMG-CoA lyase activity in latex. When latex was incubated with radiolabelled HMG-CoA (incubations 1 & 2), the label incorporated into acetate and acetoacetate predominated over that incorporated into MVA. This activity was greatest in the 100,000*g* supernatant and increased when the pH of the incubation was raised from 5.5 to 6.7. These facts indicate that the enzyme HMG-CoA lyase (EC 4.1.3.4) is present in *E. lathyris* latex (Fig. 21). Avian lyase is a soluble protein with a $pH_{optimum}$ of 8.9. It requires a divalent cation (Mg^{2+} or Mn^{2+}) for activity (15). Lyase activity has been detected in *Hevea brasiliensis* latex (13). The significance of lyase presence in latex is uncertain. Since it serves to essentially decompose HMG-CoA to its precursors, it may play a role in the regulation of the level of HMG-CoA in latex. The detection of lyase activity in latex explains why exogenously supplied HMG-CoA was not incorporated into the triterpenoids, however, some mechanism must exist to channel endogenously produced HMG-CoA to HMG-CoA reductase since acetate is incorporated into triterpenoids by latex.

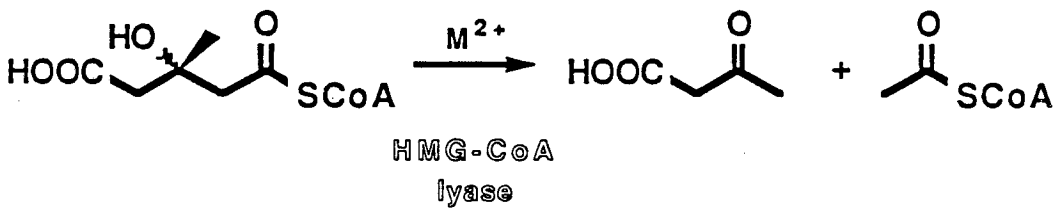

Fig. 21. Reaction catalyzed by HMG-CoA lyase.

Effect of EDTA on HMG-CoA metabolism in latex. In order to further investigate HMGR, a

mechanism was needed to inhibit lyase activity. Since lyase requires divalent cations for activity and HMG-CoA reductase does not, inclusion of EDTA in the incubation mixture to chelate the metal inhibited lyase activity without affecting HMGR (incubations 3 & 4)(27). Comparing incubations 1 and 3, with EDTA the ratio of lyase activity/ HMGR activity decreased 14-fold in the supernatant fractions. All further investigations of HMG- CoA reductase were performed by including 30 mM EDTA in the buffer.

HMG-CoA reductase activity in latex. The localization of HMG-CoA reductase to a specific latex fraction was hampered by both the presence of lyase and the second HMG-CoA metabolizing activity. HMGR was detected in both soluble and particulate fractions (incubations 5 to 10). However, in the most carefully analyzed experiments where the labelled MVA was analyzed by TLC followed by ODS-HPLC (incubations 9 & 10), HMGR was associated with the membrane fraction of the 5000*g* pellet (100,000*g* pellet of sonicated 5000*g* pellet). This location for HMGR is consistent with the results of an experiment investigating the specific activity of HMGR in latex fractions (Table VI). The 10,000*g* pellet had the highest specific activity of any fraction. The majority of HMGR activity was found in the supernatant but with a lower specific activity; it is possible that this activity is derived from the membrane-bound HMGR. In mammalian systems, it has been found that the soluble subunit of HMGR can be cleaved from the membrane-bound subunit by proteases. This soluble truncated HMG-CoA reductase retains its catalytic activity (23). *Hevea brasiliensis* latex HMG-CoA reductase has also been localized to a particulate fraction (40,000*g* pellet) but had a higher specific activity of 17 nmol h^{-1} mg $protein^{-1}$(30).

The activity of HMG-CoA reductase measured in the 5000*g* pellet fraction (average of 4 experiments) is comparable to the overall rate of conversion of acetate into triterpenoids in latex (Fig. 22). This result suggests that HMG-CoA reductase is indeed the rate-determining enzyme in the pathway to triterpenoid biosynthesis in latex. Attempts to confirm this by showing that the rate of conversion of acetate to HMG-CoA in latex was greater than HMG-CoA reductase activity

Table VI. *Distribution of HMG-CoA Reductase Activity in Latex Fractions.*

	Total Activity (pmol h^{-1})	Total Protein (mg)	Specific Activity (pmol h^{-1} mg protein^{-1})
10,000*g* pellet	54	0.62	87
15,000*g* pellet	7	0.67	10
50,000*g* pellet	5	0.43	12
100,000*g* pellet	5	1.5	3
100,000*g* supernatant	160	5.5	29

were unsuccessful since acetate incorporation into HMG-CoA was not detectable in latex. Lyase activity in latex could be the cause; since the enzymes involved in the conversion of acetate into HMG-CoA also require divalent cations for activity, EDTA could not be used to exclusively inhibit lyase activity in this case.

STEPS ALONG PATHWAY	SUBSTRATE INCORPORATION (nmol 100 µl latex^{-1}h^{-1})	TRITERPENOID EQUIVALENTS (nmol 100 µl latex^{-1}h^{-1})
Ac----------->Triterpenoids	0.02	0.001
MVA-->Triterpenoids	0.55	0.09
HMG-CoA->MVA	0.02	0.003

Fig. 22. Comparison of rates of incorporation of MVA and Ac into latex triterpenoids with HMG-CoA reductase activity in latex.

Purification and properties of latex HMG-CoA reductase. The results reported in this section were obtained by analysis of labelled HMG-CoA incorporation into MVA by organic acid HPLC only, before it was determined that another HMG-CoA-derived product coeluted with MVA on this column. The experiments were not repeated since *E. lathyris* vegetative tissue proved to

be a better source of HMG-CoA reductase for purification of the enzyme (Ch. 2). These experiments are being reported as a reference for possible further work on latex HMG-CoA reductase.

The dependence of HMG-CoA reductase activity on the volume of latex sample and time are shown in Figures 23 and 24. HMG-CoA reductase was precipitated by bringing latex to 70% saturation in ammonium sulfate (Table VII). This did not result in a purification of HMGR, but it did provide a means to remove the enzyme from endogenous latex acids. This fraction was used to determine the optimum pH for latex HMG-CoA reductase (Fig. 25). At pH 6.1 a four-fold purification from the original HMG-CoA reductase activity measured in the 100,000*g* mix was obtained. The possible difference in $pH_{optimum}$ for latex HMGR and HMG-CoA reductase from the vegetative tissue (pH 6.8- Ch. 2) suggests that isozymes of the enzyme exist in *E. lathyris.* A substrate saturation curve run on the desalted $(NH_4)_2SO_4$-precipitated HMG-CoA reductase sample gave a K_m of 82.5 µM HMG-CoA, comparable to that determined for *H. brasiliensis* latex HMGR of 56 µM HMG-CoA (30) (Fig. 26).

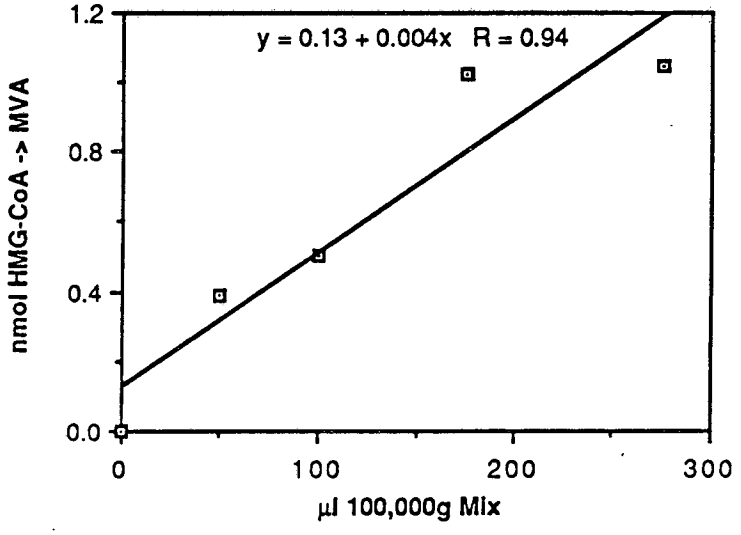

Fig. 23. HMG-CoA reductase activity versus [100,000*g* mix of latex].

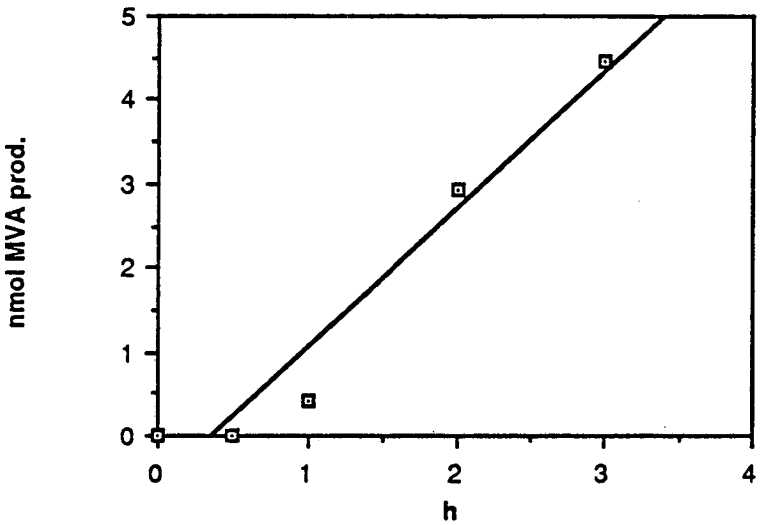

Fig. 24. Time course of HMG-CoA reductase activity in 100,000*g* supernatant.

Table VII. *Ammonium Sulfate Fractionation of Latex HMG-CoA Reductase.*

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (nmol h^{-1})	SPECIFIC ACTIVITY (nmol mg $protein^{-1}$ h^{-1})
100,000*g* supernatant	10.5	2.94	0.28
30% AS pellet	0.2	0	0
30 to 70 % AS pellet	6.3	1.7	0.27
70% AS supernatant	0.8	0	0

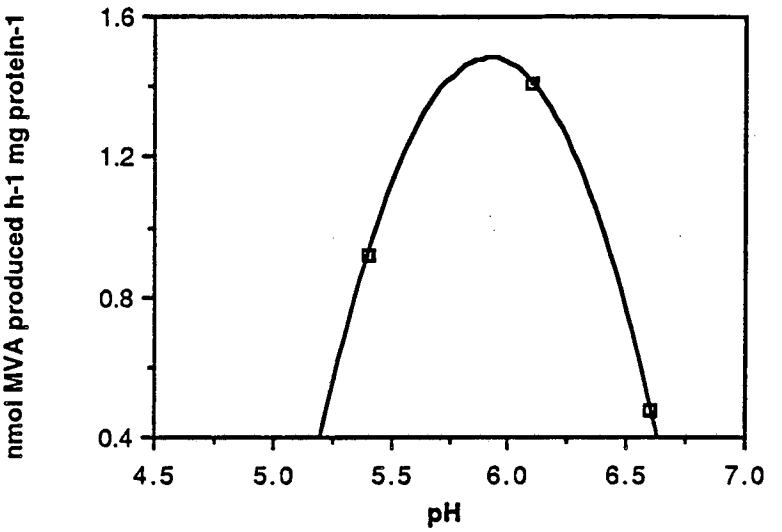

Fig. 25. Effect of pH on HMG-CoA reductase activity of latex.

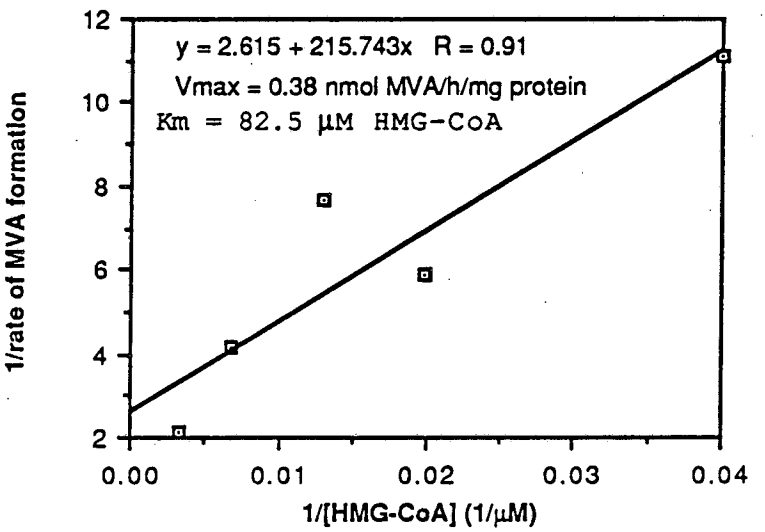

Fig. 26. Double-reciprocal plot of HMG-CoA reductase activity versus [HMG-CoA].

CONCLUSIONS

E. lathyris latex contains all the enzymes needed to convert acetate into triterpenols and their esters. The final enzymes of the pathway, which will metabolize mevalonic acid to the triterpenoids, are segregated within a pelletable structure (5000*g*). Membrane-bound HMG-CoA reductase was also localized to this fraction, but it was not established whether it was contained in the same structure. HMG-CoA lyase was present in the soluble fraction of latex, and a second enzymic activity which converted HMG-CoA to a mevalonate-like product was also detected.

Measurements of the rates of acetate and mevalonate incorporation into the triterpenoids and the rate of HMG-CoA reductase indicate that the conversion of HMG-CoA to mevalonic acid is the rate-determining step in the pathway in latex.

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Chapter II. LOCATION AND SOLUBILIZATION OF HYDROXY-METHYLGLUTARYL-COENZYME A REDUCTASE FROM EXTRACTS OF *EUPHORBIA LATHYRIS* STEM AND LEAF TISSUE

In 1985 Michael S. Brown and Joseph L. Goldstein were awarded the Nobel Prize in Physiology or Medicine for their work in uncovering factors involved in the regulation of cholesterol biosynthesis in mammalian cells. Through their work and that of many other researchers, the central role of 3-hydroxy-3-methylglutaryl coenzyme A reductase (mevalonate:NADP oxidoreductase (acylating CoA) EC 1.1.1.34) as the major rate-limiting enzyme in sterol biosynthesis was elucidated. It is a membrane-bound enzyme, located primarily in the endoplasmic reticulum, and a trans-membrane protein, glycosylated on the luminal side with a soluble catalytic subunit on the cytoplasmic side (12). Mammalian HMG-CoA reductase is subject to multivalent feedback regulation both at the gene and enzyme level (10). Transcription of the reductase gene is supressed by cholesterol-containing low density lipoproteins, 25-hydroxycholesterol, and mevalonate(17). Turnover of the protein itself in cultured mammalian cells is accelerated by addition of sterols to the media in a process that is mediated by the membrane-bound domain of the enzyme (15), and a phosphorylation/dephosphorylation mechanism converts the enzyme between an active and inactive form (5).

Within the plant kingdom the number of isoprenoid-derived products is staggering. From the ten-carbon monoterpene essential oils to polyterpene rubbers with molecular weights up to four million, plants synthesize a multitude of terpenoid compounds important as growth hormones, phytoalexins, pigments, and membrane components. Yet despite the abundance and diversity of terpenes in plants, the means by which plants control the flow of carbon into these compounds is little understood. Terpenoid compounds, including the carotenoids and the phytyl chain of chlorophyll found within the chloroplast and ubiquinone involved in electron transport in the mitochondria, are essential components of a number of subcellular structures. The question of which enzymes of the terpene pathway these individual organelles contain has become a recent subject of controversy. Work by Kleinig and coworkers with daffodil, spinach,

and potato tubers suggests that isopentenylpyrophosphate (IPP) is the central intermediate synthesized in the cytoplasm and transported into the specialized organelles where it is further metabolized to the required compounds (20). Yet the detection of HMGR and MVA kinase activity in plastid and mitochondrial fractions by other researchers supports the viewpoint that individual organelles contain their own IPP-synthesizing system (9,11).

Propelled by the discovery of the major role it plays in the regulation of cholesterol biosynthesis in mammalian systems, HMGR has become the focus of study of a number of investigations in various photosynthetic organisms. In 1975 Brooker and Russell were the first to detect HMGR activity in a higher plant *Pisum sativum* (8). Since then others have reported on the enzyme in radish seedlings, sweet potato roots, *Nepeta cataria* leaf tissue, *Hevea brasiliensis* latex, tobacco seedlings, barley seedlings, spinach, carrot cell culture, soybean, pepper, sycamore tissue culture, and anise cell suspension culture (14).

We undertook this investigation of HMG-CoA reductase from stem and leaf tissue of *Euphorbia lathyris* because the high percentage of triterpenoids found in this plant suggests that interesting differences in the regulation of this enzyme could be a factor in the greater flow of carbon into these compounds than in other plants. The data on the relative rates of incorporation of Ac and MVA into the triterpenoids of latex and the turnover activity of latex HMGR indicate that this enzyme could also be catalyzing the rate-limiting step in triterpenoid biosynthesis in *E. lathyris* (Ch. 1). In order to learn more about *E. lathyris* HMG-CoA reductase, purification of the enzyme from vegetative tissue was begun. As the first acts of this purification, the subcellular location of this enzyme was investigated and steps were taken to solubilize the membrane-bound enzyme and to protect it from endogenous protease activity.

MATERIAL AND METHODS

Plant material. *Euphorbia lathyris* L. plants were propagated from seed collected from wild plants growing near Healdsburg, Sonoma Co., CA. Plants were grown in a soil mix of peat, sand, and perlite in 6 inch clay pots under growth chamber conditions of a 16 h day at 600 $\mu Em^{-2}s^{-1}$ provided by a combination of fluorescent and incandescent lights, 27°C day/ 18°C night temperature.

Materials. DL-3-[glutaryl-3-^{14}C]-hydroxy-3-methylglutaryl coenzyme A, R-[5-^{3}H]-mevalonic acid, triethylammonium salt, and Aquassure were purchased from New England Nuclear. Polyvinylpyrrolidone MW 40,000 (PVP) was obtained from Calbiochem. Bradford dye reagent was purchased from Bio-Rad. Silica gel plates were obtained from Analtech. Mevinolin was a gift of A. W. Alberts of Merck, Sharp & Dohme. All other biochemicals were from Sigma.

Crude homogenate preparation. Approximately 15 g *E. lathyris* stem and leaf tissue (upper 10 cm of main stalk) from 4 to 6 month-old plants was quickly chopped into pieces with a razor blade then homogenized with 75 ml BUFFER A (10 mM potassium phosphate pH7.2, 0.4 M sorbitol, 30 mM EDTA, 10 mM DTE) and 1.5 g insoluble PVP using a mortar and pestle. The crude extract was obtained by filtering the homogenate through cheesecloth.

HMGR assay. Fifteen microliters of 25 mM NADPH, 42.9 µl 2 mM D,L-HMG-CoA, and 10 µl [^{14}C]HMG-CoA (47.2 mCi $mmol^{-1}$, 0.02 mCi ml^{-1}) were taken to dryness under a stream of N_2, then 150 µl of sample was added bringing the final concentrations of substrates to 2.5 mM NADPH and 0.6 mM HMG-CoA (2.2 mCi $mmol^{-1}$). Samples were incubated 1 to 2 h at 28°C then were quenched by addition of 15 µl 6 N HCl, 15 µl 1 M MVA, potassium salt and 15 µl [^{3}H]MVA, TEA salt (*ca* 20,000 dpms). Quenched incubations were stored in a freezer until their workup based on the TLC procedure described by Brooker and Russell (8). Incubations were allowed to sit at room temperature for 10+ min to ensure MVA lactonization. Samples were centrifuged 20 min at 12,000*g* using a Beckman microfuge 11 to pellet precipitated protein, and the supernatant

was removed. Two hundred microliters of water was added to the pellet; the sample was vortexed then recentrifuged. The supernatant was removed, and the extraction was repeated with 200 µl acetone. Supernatants were combined and taken to dryness under a stream of N_2. A 200 µl volume of acetone was added to the residue; the sample was sonicated 5 min then spotted on a 5 x 20 cm, 250 µm silica gel G plate along with a marker spot of 5 µl of a 1:1 mixture of 6 N HCl 1 M MVA, potassium salt. The spotting vial was rinsed with an additional 200 µl acetone and sonicated 5 min, and the acetone extract was applied to the plate. Plates were developed in a solution of 2:1 $CHCl_3$: acetone, and the marker spot was visualized by treatment with sulfuric acid spray and heat. The silica gel band was scraped, wet with methanol, and the MVAL was eluted with ether and acetone. The eluate was concentrated to 10 to 15 ml, and a 10% aliquot was mixed with 15 ml Aquassure and counted using a Packard 640-C scintillation counter. The remaining 90% of the sample was dried under a stream of N_2 then redissolved in 150 µl 10 mM potassium phosphate pH2.5. Fifty microliters was chromatographed on a Beckman 322 HPLC system using an Altex 4.6 mm x 25 cm ODS column in 10 mM potassium phosphate, pH 2.5 at a flow rate of 1 ml min^{-1} with detection at 214 nm using a Hitachi 100-10 variable wavelength spectrophotometer. One-minute fractions were collected directly into scintillation vials using a LKB 2112 redirac fraction collector; 15 ml Aquassure was added to each vial which was subsequently counted.

Protein determination. METHOD 1-- Protein was analyzed per Bradford(7) as modified by Vincent and Nadeau(28). A volume of 20 µl sample + buffer was added to 80 µl 0.1% Triton X-100, then 50 µl of the Triton mix was assayed in 1 ml 5-fold dilute Bradford reagent. Absorbance of the samples at 595 nm and also at 720 nm, where there is less interference from chlorophyll absorbance, was determined using a Hewlett-Packard 8450-A UV/VIS spectrophotometer. METHOD 2-- Protein was analyzed by the method of Markwell et al. (22) for membrane proteins samples using the procedure of Bensadoun and Weinstein (6) for removal of

interfering substances by initial trichloroacetic acid (TCA) precipitation of protein. Ten microliters 2% sodium deoxycholate was added to 1.2 ml sample and allowed to stand 15 min, then 0.4 ml 24% TCA was added and the protein was pelleted by centrifugation at 3300*g* for 30 min. The protein pellet was redissolved in 1 ml reagent C (100:1 mix of reagent A (2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% SDS) and reagent B (4% $CuSO_4 \cdot 5H_2O$)) and incubated 10 min. One hundred microliters reagent D (1:1 Sigma Folin-Ciocalteu 2N phenol reagent: H_2O) was added; the mixture was incubated 45 min, and the absorbance at 730 nm was read against a reagent blank.

Fractionation 1-- Organelle isolation by differential centrifugation. Crude extract was fractionated by a differential centrifugation scheme using a Beckman L ultracentrifuge with type 50 Ti and type 21 rotors. The centrifugation sequence was 5 min at 500*g* to remove cell debris, 5 min at 3000*g* to obtain a crude plastid pellet, 15 min at 18,000*g* for a crude mitochondrial pellet, and 1 h at 100,000*g* to obtain a microsomal pellet and a soluble protein supernatant. Pellets were resuspended in 300 µl BUFFER B (0.1 M potassium phosphate, pH 7.2 or 7.9, 30 mM EDTA, 10 mM DTE) containing 0.04% Triton X-100. BUFFER B of pH 7.9 was used to resuspend the 3000*g* pellet only. Each pellet fraction and the soluble protein fraction (100,000*g* supernatant) were assayed for HMGR activity and protein content (METHOD 1).

Fractionation 2-- Concentration of HMGR in a particulate fraction. A pellet fraction containing HMGR activity was obtained by first centrifuging the Crude extract at 500*g* for 5 min to remove cell debris and then centrifuging at 18,000*g* for 20 min to sediment the HMGR-containing organelles (18,000*g* Pellet). In this experiment the supernatant was divided into seven fractions before the centrifugation at 18,000*g* giving seven different pellets to test under various conditions. Five of the pelleted fractions were resuspended in 250 µl BUFFER B containing 0.04% Triton X-100 at various pH values to investigate the pH_{max} of HMGR; the sixth and seventh fractions were resuspended in 250 and 500 µl BUFFER B pH 7.5 containing 0.04%

Triton X-100, respectively. All fractions were sonicated for 30 s, then an 150 µl aliquot of fractions 1-6 was assayed for HMGR activity, the sixth fraction in the presence of 0.5 µM mevinolin, potassium salt. Mevinolin was converted from the lactone to its potassium salt following the procedure of Kita, Brown, and Goldstein(19). The seventh fraction was centrifuged at 100,000*g* for 1 h, and the pellet was resuspended in BUFFER B containing 0.04% Triton X-100, pH 7.5; 150 µl each of the supernatant and pellet was assayed for HMGR activity. The remainder of the fractions was saved for protein assay (METHOD 1) .

Solubilization of HMGR. The ability of three different detergent treatments (1% Triton X-100, 1% Triton X-100 + 2% SDS, 0.25% sodium deoxycholate) to solubilize HMGR from the 18,000*g* Pellet was tested by incubating the pellet with the detergent in 0.5 ml BUFFER B pH 6.7 for 20 min at 0-4C, diluting the samples to 1.5 ml with BUFFER B, centrifuging the samples at 100,000*g* for 1 h then assaying the HMGR activity and protein concentration (METHOD 1) of the resultant supernatant and pellet fractions. The 100,000*g* pellets were resuspended in 250 µl BUFFER B.

Effect of protease inhibitors on HMGR activity and solubilization. The 18,000*g* Pellet was incubated for 1.5 h with 0.25% sodium deoxycholate in 1 ml BUFFER B pH 6.8 under two protease inhibitor treatments (1 mM PMSF, 0.1 mM leupeptin) and a control treatment with no inhibitor. Samples were diluted to 1.5 ml with BUFFER B, centrifuged at 100,000*g* for 1 h, and the supernatants and pellets from each incubation were analyzed for HMGR activity and protein content (METHOD B). The 100,000*g* pellets were resuspended in 0.5 ml BUFFER B.

Further effect of protease inhibitors and detergents on HMGR activity and solubilization. A crude extract was prepared from 16.8 g *E. lathyris* tissue as described giving 75 ml homogenate. This was divided 2:1, and 0.5 ml 10 mM leupeptin and 0.5 ml 0.1 M PMSF in ethanol was added to the first part. The second part was treated with only 0.25 ml 10 mM leupeptin. The final concentrations of leupeptin and PMSF were 0.1 mM and 1 mM, respectively. An 150 µl aliquot of the crude extract containing both inhibitors was assayed for HMGR activity, and the remainder of

that 50-ml portion was divided in half. The three 25-ml samples were centrifuged at 18,000*g* for 20 min, and the supernatants were removed. Two 150 µl aliquots of the 18,000*g* supernatant containing both protease inhibitors were assayed for HMGR activity. One was quenched immediately to serve as a blank. The 18,000*g* pellets were each resuspended in 1 ml solubilization buffer with differing detergent and protease inhibitor components (Table I); the pellet from the crude extract containing leupeptin was resuspended in solubilization buffer a. The samples were treated for 1.5 h at 28°C, were diluted to 1.5 ml with BUFFER B, and were centrifuged at 100,000*g* for 1 h. The supernatants were removed, and the pellets were resuspended in 0.5 ml BUFFER B. A 150 µl aliquot of the supernatant and pellet from each treatment was assayed for HMGR activity.

Table I. *Components of Solubilization Buffers a,b, and c*

1 ml solubilization buffer--	a	b	c
0.5 ml 2X BUFFER B pH 6.8	+	+	+
0.125 ml 2% sodium deoxycholate	-	+	+
0.01 ml 10 mM leupeptin	+	+	+
0.01 ml 0.1 M PMSF in ethanol	+	+	-
0.335 ml 6% polyoxyethylene ether W-1	+	-	-
0.01 ml ethanol	-	-	+
ml water	0.145	0.355	0.355

RESULTS

Location of HMG-CoA Reductase. The initial differential centrifugation procedure, based on that used by Brooker and Russell in their study of the subcellular location of HMGR in pea seedlings, separated the tissue homogenate into crude fractions enriched in certain subcellular components (9). While not being purified organellar fractions, the four fractions obtained roughly represent the chloroplastic, mitochondrial, microsomal and soluble protein components of the crude extract (16). The major portion of HMG-CoA reductase activity was found associated with the 3000*g* plastid-enriched pellet (Table I). HMGR activity was also found in the other fractions with the soluble protein fraction (100,000*g* supernatant) containing the next highest level of activity followed by the crude mitochondrial fraction (18,000*g* pellet) which had the highest specific activity of HMGR. The microsomal pellet contained the smallest portion of the total activity. The specific activity of HMGR measured in the crude extract and the subcellular fractions was on the order of one thousand times higher than that measured in the latex alone (Ch. 1).

Concentration of HMG-CoA reductase in a particulate fraction. Further purification of HMG-CoA reductase was based on these initial findings. Consequently, it was decided to concentrate the 75% of the recovered activity found in the 3000*g* and 18,000*g* pellets together by centrifuging once at 18,000*g*. This resulted in a particulate fraction containing 38% of the activity of the 500*g* supernatant fraction with a specific activity of 6.5 nmol mg protein^{-1} h^{-1}, a 1.4-fold purification based on the 500*g* supernatant fraction (Table II). A significant portion of HMGR activity was also found associated with the 500*g* pellet, so in later experiments, this centrifugation step was eliminated.

Properties of particulate HMG-CoA reductase. HMG-CoA reductase activity of the 18,000*g* pellet has a pH_{max} of 6.8 (Fig. 1). Mevinolin (Fig. 2) is a fungal metabolite which has been found to be a highly specific competitive inhibitor of HMG-CoA reductase (13). It has been shown to be effective with higher plant HMGR as well as with the mammalian enzyme (3). *E. lathyris* HMG-CoA

Table I. *Distribution of HMG-CoA Reductase Activity in Subcellular Fractions from Euphorbia lathyris vegetative tissue*

	Total Activity (nmol h^{-1})	(% of total fractions)	(% recovered of crude extract)	Total Protein (mg)	Specific Activity (nmol mg $protein^{-1}$ h^{-1})
Crude Extract	360.			70.	5.1
3000*g* pellet	48.	62	13	18.	2.7
18,000*g* pellet	10.	14	3	1.4	7.1
100,000 pellet	5.	6	1	1.4	3.6
100,000*g* supernatant	14.	18	4	17.	0.8
Σ all fractions	76	100%	21%		

Table II. *Concentration of HMG-CoA Reductase in a Particulate Fraction*

	Total Activity (nmol h^{-1})	(as % of 500*g* supernatant)	Total Protein (mg)	Specific Activity (nmol mg $protein^{-1}$ h^{-1})
500*g* supernatant	252.		53.	4.8
500*g* pellet	35.		15.	2.3
18,000*g* supernatant	–		32.	–
18,000*g* pellet	96.	38.	15.	6.5

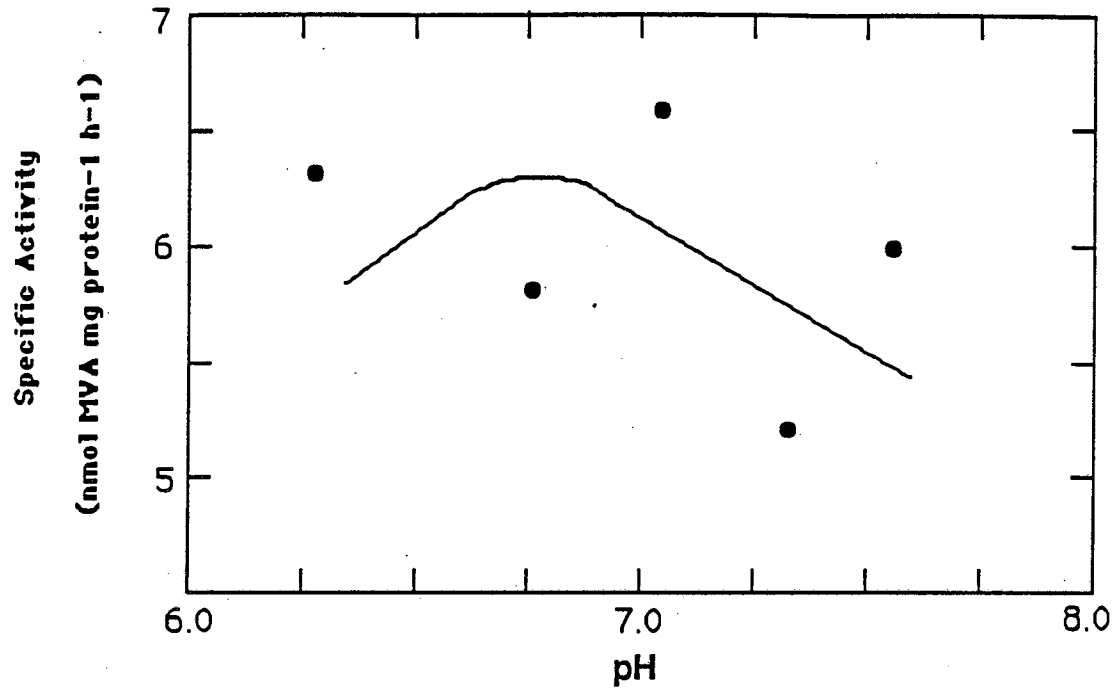

Fig.1. Effect of pH on the specific activity of particulate HMG-CoA reductase from *E. lathyris*.

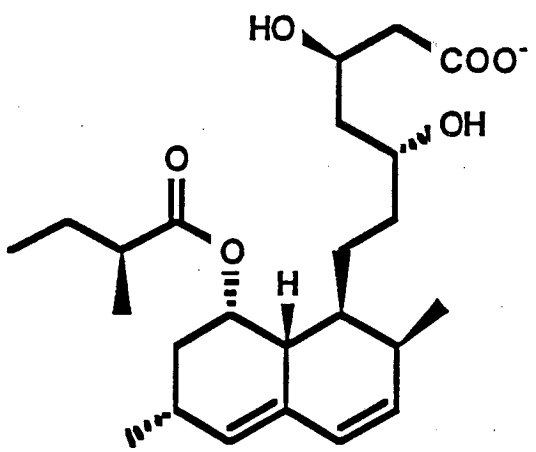

Fig. 2. Structure of the fungal metabolite **mevinolin**, a competitive inhibitor of HMG-CoA reductase.

reductase is sensitive to inhibition by mevinolin, HMGR activity being reduced by 80% in the presence of 0.5 μM mevinolin (Table III). HMG-CoA reductase localized in this heavier organellar fraction (18,000*g* pellet) is membrane-bound as demonstrated by the fact that 75% of the HMGR activity was found to be associated with the particulate fraction after a 18,000*g* pellet was sonicated to rupture the organelles and then centrifuged for 1 h at 100,000*g* (Table III).

Solubilization of HMG-CoA Reductase. In an experiment testing the effect of different detergents on the solubilization of HMGR, deoxycholate treatment was the most effective of the detergents tested (Table IV). In addition to showing the highest ratio of HMGR activity in the supernatant versus in the particulate fraction, the combined activity measured in the 100,000*g* supernatant and pellet with this treatment was an order of magnitude greater than that measured for the two other treatments, 1% Triton X-100 and 1% Triton X-100 + 2% SDS. In a second experiment comparing deoxycholate treatment with polyoxyethylene ether W1 (Brij) in the presence of protease inhibitors, total recovery from the deoxycholate treatment was higher, but the ratio of solubilized to particulate HMGR activity was higher in the case of the Brij treatment since the activity detected in the pellet was so low (Table VI).

Effect of Protease Inhibitors on HMG-CoA reductase. The effect of protease inhibitors was investigated to assess the role that proteolytic enzymes might play in the recovery of activity and the solubilization of HMGR from *E. lathyris*. Upon sitting for 140 min at 0-4C, the HMGR activity of a 500*g* supernatant decreased from 2.6 nmol mg protein^{-1} h^{-1} to 1.8 nmol mg protein^{-1} h^{-1}, a 31% loss in activity, indicating the possible action of proteases (Table IV). While the 3000*g* and 18,000*g* pellets of the original fractionation of the crude extract did represent 75% of the recovered activity, only 21% of the total activity of the crude extract was recovered among all the fractions (Table I). Treatment of the 18,000*g* pellet with either serine or thiol protease inhibitors improved the recovery of HMGR activity in both the soluble and particulate fractions ; leupeptin treatment gave the greatest improvement (Table V). The PMSF-treated sample had a lower ratio of supernatant to pellet activity than untreated sample did, while leupeptin treatment

Table III. *Effect of Mevinolin on Activity and Distribution of Activity between Soluble and Membrane-Bound Fractions of HMG-CoA Reductase from a Particulate Fraction*

	Total Activity (nmol h^{-1})	(% of total fractions)	(as % of 18,000*g* pellet)	Total Protein (mg)	Specific Activity (nmol mg $protein^{-1}$ h^{-1})
18,000*g* pellet	10.5			1.6	6.6
18,000*g* pellet + 0.5μM mevinolin	2.1		20	1.7	1.2
from sonicated 18,000g pellet-					
100,000*g* supernatant	1.	25	10	0.2	5.9
100,000*g* pellet	3.	75	29	1.0	3.0
Σ 100,000*g* fractions	100%	39%			

Table IV. *Effect of Detergent Treatment on Solubilization and Activity of HMG-CoA Reductase from a Particulate Fraction*

	Total Activity (nmol h^{-1})	Total Activity (as % recovered from 500*g* supernatant)	Ratio of Solubilized to Particulate Activity (supernatant activity/ pellet activity)
500*g* supernatant	183		
500*g* pellet	41		
18,000*g* pellet- each treatment containing one-third of this fraction:			
+ 1% Triton X-100			
100,000*g* supernatant	0.6	0.3	
100,000*g* pellet	0.65	0.4	0.9
+ 1% Triton X-100 + 2% SDS			
100,000*g* supernatant	1.3	0.7	
100,000*g* pellet	0.4	0.2	3.1
+0.25% deoxycholate			
100,000*g* supernatant	8.7	5	
100,000*g* pellet	11.2	6	0.8
500*g* supernatant- after sitting 140 min at 0-4°C:	126	69	

Table V. *Effect of Protease Inhibitors on Recovery and Solubilization of HMG-CoA Reductase from a Particulate Fraction*

	Specific Activity (supernatant+pellet nmol mg protein^{-1}h^{-1})	Total Activity (nmol h^{-1})(as % of crude extract)		Ratio of Solubilized to Particulate Activity (supernatant activity/ pellet activity
crude extract	1.6	198		
18,000*g* pellet- each treatment containing one-third of this fraction:				
+ 0.25% deoxycholate				
100,000*g* supernatant		3.9	2	
100,000*g* pellet		5.8	3	
	1.8			0.67
+ 0.25% deoxycholate, 1mM PMSF				
100,000*g* supernatant		6.1	3	
100,000*g* pellet		12.8	6	
	3.9			0.48
+ 0.25% deoxycholate, 0.1 mM leupeptin				
100,000*g* supernatant		21.7	11	
100,000*g* pellet		10.6	5	
	7.1			2.05

Table VI. *Effect of Protease Inhibitors on Recovery of HMG-CoA Reductase Activity and Comparison of Solubilization of HMG-CoA Reductase by Deoxycholate or Polyoxyethylene Ether (Brij)*

	Total Activity (nmol h^{-1})	Total Activity (as % recovered from crude extract)	Ratio of Solubilized to Particulate Activity (supernatant activity/ pellet activity)
crude extract	278		
18,000*g* supernatant	189	68	
18,000*g* pellet- each treatment containing one-third of this fraction:			
+ 2% Brij, 1mM PMSF, 0.1 mM leupeptin			
100,000*g* supernatant	28	10	
100,000*g* pellet	0.05	0.02	560
+0.25% deoxycholate, 1mM PMSF, 0.1 mM leupeptin			
100,000*g* supernatant	22	8	
100,000*g* pellet	11	4	2
+0.25% deoxycholate, 0.1 mM leupeptin			
100,000*g* supernatant	12	4	
100,000*g* pellet	8	3	1.5
Σ all fractions	270	97%	

improved the ratio by a factor of three. In a later experiment where both protease inhibitors were added to the crude extract, the total recovery of the activity of the crude extract among the subsequent fractions was 97% (Table VI.). Here 68% of the activity was associated with the 18,000*g* supernatant, a fraction containing microsomal and soluble proteins. Treatment of the 18,000*g* pellet with both inhibitors improved the recovery of HMGR, both solubilized and membrane-bound, as compared to treatment with leupeptin alone.

DISCUSSION

Distribution of HMG-CoA reductase activity among subcellular fractions of *E. lathyris* tissue. Based on the initial differential centrifugation fractionation of the *E. lathyris* tissue homogenate, it appeared that the major portion of HMGR was located in the heavy, organellar fraction. Thirty-eight percent of the activity of a 500*g* supernatant sedimented when that fraction was centrifuged at 18,000*g* for 20 min (Table 2). Both plastids and mitochondria appear to contain their own HMG-CoA reductase since the plastid-enriched fraction had the highest level of activity and the mitochondrial pellet had the highest specific activity of any fraction (Table 1).

The paucity of HMGR activity in the microsomal fraction is most interesting since the endoplasmic reticulum is the putative site of triterpenoid biosynthesis. Since only 21% of the HMGR activity of the crude extract was recovered among the differential centrifugation fractions, the low activity of the microsomal fraction could be a result of isolation conditions. In the chrysophycean alga *Ochromonas nalhamensis* which contains 1% dry weight of poriferasterol, 90% of the HMGR activity was localized in the microsomal pellet, but HMGR activity was only detectable in the presence of >1% (w/v) BSA (23). In a later experiment with *E. lathyris*, it was demonstrated that the inclusion of protease inhibitors during the fractionation improved the recovery of the activity of the crude extract to 97%; the majority of this activity (70%) was in the 18,000*g* supernatant (Table VI). Whether this activity was microsomal or soluble is not known,

however, the HMGR in the supernatant appears much more susceptable to proteolytic inactivation than the HMG-CoA reductase found associated with the organellar fractions. Based on these fractionation experiments, *E. lathyris* contains HMG-CoA reductase in a number of subcellular locations with up to 40% of the activity associated with the plastids and mitochondria. The remainder of the activity is microsomal or soluble. The distribution of HMGR among various organelles suggests the possibility of different isozymes of the enzyme and does not support the belief that IPP is synthesized in the cytoplasm and distributed to the different subcellular structures for further metabolism.

These findings can be compared with results of investigations into the subcellular location of HMGR in other organisms. In mammalian systems HMG-CoA reductase has been thought to be exclusively localized to the endoplasmic reticulum (10), although recently its presence in the peroxisomes of rat liver cells has been demonstrated both enzymatically and immunologically (18). Yeast HMGR is located in the mitochondria (26). In higher plants HMG-CoA reductase has been localized in a number of different subcellular sites. In pea seedlings 80% of the HMGR activity was found to be associated with the microsomal fraction with the remaining activity equally divided between the plastid and mitochondrial fractions (9). Two separate HMGR activities were located in *Nepeta cataria* leaf tissue, one activity associated with the chloroplast and a second activity that could be sedimented by centrifugation at 100,000*g* (11). In radish seedling extracts, HMG-CoA reductase was found in both the organellar (16,000*g* pellet) and microsomal (100,000*g* pellet) fractions, with the 16,000*g* pellet activity co-migrating on Percoll gradients with the mitochondrial marker enzyme cytochrome oxidase (2). The presence of HMG-CoA reductase in the 18,000*g* pellet is consistent with its location in the 5000*g* pellet of *E. lathyris* latex (Ch. 1) and in *Hevea brasiliensis* latex where it was found in a 40,000*g* pellet (27).

Properties of organellar HMG-CoA reductase. The optimum pH found for HMG-CoA reductase of the 18,000*g* pellet of 6.8 is comparable to that found for HMGR in other vegetative tissue. In pea seedlings, the plastid HMGR has a pH optimum of 7.9 while the microsomal enzyme

has a pH_{max} of 6.9 (29). Purified HMG-CoA reductase from a heavy-membrane fraction of radish seedlings was consistently assayed at pH 7.5, presumably the optimum pH for that enzyme (4). The difference in the optimal pH measured for HMGR from stem and leaf tissue (pH 6.8) from that measured in latex alone (pH 5.9, Ch. 1) is a further indication of the existence of different forms of HMG-CoA reductase from the two tissue types in *E. lathyris*, although a more detailed analysis of the effect of pH on purified enzyme is needed to confirm this.

Solubilization of HMG-CoA reductase. In the presence of protease inhibitors, Brij was the best solubilizing agent for HMGR of the 18,000*g* pellet, although deoxycholate treatment gave a greater recovery of total HMG-CoA reductase, both solubilized and still membrane-bound (Table VI). Since the recovery of the activity of the crude extract among all fractions in that experiment totaled 97%, no activation of HMGR was evident. In the absence of protease inhibitors, deoxycholate treatment increased total HMGR activity compared to treatment with Triton X-100 or Triton-X 100 + SDS (Table IV). While activation of HMGR upon deoxycholate treatment has been seen with the mammalian enzyme, Bach found that deoxycholate inhibited HMGR activity in radish seedlings (4). Maurey et al. found that microsomal HMG-CoA reductase activity from *O. malhamensis* was stimulated *ca* 60% in the presence of 0.5-1% Triton X-100 (23). These differences may represent variability in the makeup of the membranes containing HMGR in the various organisms. In the case of the initial experiment comparing the effects of different detergents on *E. lathyris* HMGR activity (Table IV), what appears to be an activation by the deoxycholate treatment may well instead represent the ability of deoxycholate to better protect HMGR from proteolytic inactivation than the other detergents tried.

Effect of protease inhibitors on organellar HMG-CoA reductase. In mammalian systems, solubilization of HMG-CoA reductase has been achieved with detergents and also through the action of proteolytic enzymes which have been shown to cleave the soluble catalytic domain from the membrane-bound domain (24). The truncated soluble portion of the enzyme retains its catalytic activity. Whether this kind of action was affecting the solubilization of *E. lathyris*

organellar HMGR was investigated. While *E. lathyris* latex has been reported to contain serine proteases (21), it is thiol proteases that are responsible for the solubilization of HMGR in mammalian systems. The presence of leupeptin or PMSF during homogenization did not affect HMGR recovery in either radish or *Ochromonas* (4,23). With *E. lathyris*, when either the thiol protease inhibitor leupeptin or the serine protease inhibitor PMSF was added to the deoxycholate treatment for the solubilization of HMG-CoA reductase from the 18,000*g* pellet, higher HMGR activity was obtained than without any protease inhibitor added (Table V). Neither inhibitor significantly decreased the ratio of solubilized HMGR activity to particulate activity indicating that proteolytic action is not responsible for the solubilization of *E. lathyris* HMG-CoA reductase. In fact, leupeptin treatment increased the ratio probably because the detergent-solubilized HMGR is more susceptable to protease action than the protein that is still membrane-bound, and the protease inhibitor is reducing the inactivation of the solubilized enzyme. Both endogenous serine and thiol proteases are responsible for the inactivation of *E. lathyris* HMGR since the addition of both inhibitors together improved the recovery of HMGR activity as compared to treatment with leupeptin alone (Table VI).

CONCLUSIONS

Euphorbia lathyris vegetative tissue appears to contain at least one iso-enzyme of HMG-CoA reductase that differs in its optimum pH from the HMGR detected in the latex. In addition, it is likely that there exist different forms of the enzyme associated with the plastids, mitochondria, and endoplasmic reticulum. The enzyme(s) located in the organellar fraction (plastids + mitochondria) is membrane-bound and is best solubilized by treatment with Brij. Endogenous proteases are capable of inactivation of this organellar enzyme but do not act to solubilize HMGR activity.

Inclusion of protease inhibitors in the homogenization and solubilization media is an

important requirement for further purification work. As further purification is achieved and HMG-CoA reductase is separated from the proteolytic enzymes, the inhibitors can be removed from the preparation. The first steps in purification of this enzyme, concentration of the organellar HMGR in a particulate fraction by centrifugation at 18,000*g* and solubilization of the enzyme from the membrane with 2% Brij have been delineated here. Possibilities for additional purification steps present themselves. An ammonium sulfate precipitation of the enzyme is a good next step. A HMG-CoA affinity column has been used with great success in a number of purifications of this enzyme (25). If the plant enzyme is glycosylated as is its mammalian counterpart, a concavalin A column would be useful in its ability to bind glycoproteins.

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Chapter III. ULTRASTRUCTURE OF *EUPHORBIA LATHYRIS* LATEX

While the extrusion of latex produced upon the cutting of laticifer-bearing plant tissue has been beneficial to the study of the metabolism of these cells, it can hamper ultrastructural investigations. Due to the tube-like structure and high turgor pressure of laticifer cells, often these cells can empty of their contents, leaving no observable structures within the cells in micrographs of the tissue. If rupture of the tonoplast occurs, allowing the contents of the cytoplasm and vacuole to mix, the true location of any structure within the cell is difficult to assess. The latex itself poses an unique problem in botanical microscopy; it is essentially a mix of the vacuolar contents and the cytoplasm of the laticifer, a solution of subcellular structures without the benefit of a cell wall to contain them.

Despite these difficulties, the cytology of the laticifer cell has been detailed. Once again, *Hevea* is the prototype. *Hevea* laticifers contain the normal cellular substructures such as nuclei, mitochondria, endoplasmic reticulum, and ribosomes, but two other particles were also observed in these cells. Lutoids are structures bound by a single membrane, with a diameter of 0.5 to 5 μm. The second, less abundant component unique to *Hevea* latex is the Frey-Wyssling complex, a particle of diameter 4 to 6 μm bounded by a double membrane and containing lipid globules and various membranous structures; it is thought to be a type of plastid. In addition, numerous minute polyisoprene latex particles constitute the rubber phase of the laticifer and distinguish these cells (1).

In tapped latex Gomez found rubber particles, lutoids, and Frey-Wyssling complexes; mitochondria and nuclei were rarely seen (7). Upon centrifugation the lutoids sediment; they can comprise up to 20% of the latex volume (2). The biochemistry of these organelles has been well-studied, and they are considered to be lysosomal vacuoles. They contain acid hydrolases as well as peroxidase, lysozyme, and α-mannosidase (2). The lutoid membrane is composed primarily of phosphatidic acid which accounts for greater than 80% of the total lipid (4).

Studies of *Euphorbia* species have shown that their laticifers resemble those of *Hevea.*

Although the major terpenoid components of the latices of these plants are triterpenoid compounds, not rubber as in *Hevea*, their latexes are also characterized by the presence of small particles. Groeneveld used gel filtration chromatography to purify particles from numerous species including a number of Euphorbias and showed that the particles were indeed composed of triterpenoids (8). Fineran examined the laticifers in both mature and developing tissue of *Euphorbia pulcherrima* (5,6). Laticifers in mature tissue had a wall-lining layer of cytoplasm with a large central vacuole, indicating that the mature laticifer is still a living cell. The latex particles were contained within the vacuole. Nuclei and plastids with single starch grains were present in the cytoplasm, but mitochondria were poorly differentiated, and ribosomes were scarce. Based on his observations of the presence of abundant vacuoles containing latex particles in the cytoplasm of the sub-apical region of developing laticifers, he hypothesized a developmental scheme for the production of the latex particles within these vacuoles. Mitochondria and ribosomes are present in the cytoplasm of these laticifers. Groeneveld et al. noted the regular occurence of mitochondria in the laticifers of the cotyledons and hypocotyl of etiolated *Euphorbia lathyris* seedlings (9).

Investigation of the exuded latex of Euphorbias has centered in a large part on their unique rod-shaped starch grains. Mahlberg has used the starch grain morphology along with the triterpene composition of the latex of succulent African Euphorbias to determine phylogenetic relationships within the genus (12). In thin sections of fixed *E. pulcherrima* latex, latex particles have no discernible membrane but do show a boundary-type layer (5).

While there is ample understanding of the structure of laticifer cells and some information on the nature of expelled latex, the view on how latex is formed is still speculative. The theories that the latex particles are synthesized in the parietal cytoplasm and then transfered into the central vacuole are based purely on structural studies. The structural work described here was undertaken in order to compliment the information obtained on the biosynthesis of the triterpenoids in isolated latex(Ch. 1). The goal was to correlate structure with function.

MATERIAL AND METHODS

Plant Material. *Euphorbia lathyris* L. plants were propagated from seed collected from wild plants growing near Healdsburg, Sonoma Co., CA. Plants were grown in a soil mixture of peat, sand, and Perlite in 6 inch clay pots in a growth chamber under conditions of a 16 h day at 600 μE m^{-2} s^{-1} provided by a combination of fluorescent and incandescent lights, 27°C day, 18°C night temperature. Latex was obtained by the collection of droplets expelled when shallow incisions were made with a razor blade at the bases of petioles. The latex was stored briefly on ice until it was used.

Processing of 5000*g* pellet for SEM. A 0.75 ml sample of latex was centrifuged at 5000*g* for 15 min, and the supernatant was removed. The pellet was gently resuspended in 1.5 ml buffer (50 mM MES, 0.4 M sorbitol, 5 mM $MgCl_2$, 5 mM DTE, pH 5.5) using a soft paintbrush. The sample was recentrifuged, and the supernatant was removed. Buffer was added to just cover the pellet, and the pellet was broken into 1-mm pieces. A 1-ml volume of fixative (2% glutaraldehyde, 1% OsO_4 in 0.1 M cacodylate pH 6) was added to the pellet, and the sample tube was incubated for 1 h in the dark on ice. At that time the sample was again centrifuged at 5000*g* for 15 min, and the supernatant was removed. The pellet was washed three times with buffer and was then incubated for 30 min at room temperature with 1 ml of 1% glutaraldehyde in 0.1 M cacodylate, pH 6. The glutaraldehyde solution was removed, and the sample was rinsed three times with buffer for a period of 10 min each. Next the sample was fixed for 20 min in 1 ml 0.5% OsO_4 in 0.1 M cacodylate pH 6. The osmium solution was removed, and the sample was rinsed three times with distilled water. The sample was stored overnight in the refrigerator.

Before beginning the dehydration scheme, the sample was rinsed with an additional five changes of water and broken up into 1-mm pieces. The sample was dehydrated using an ethanol series: 30%, 50%, 70%, and 90% ethanol, each for 10 min. Next the sample was incubated with two changes of 100% ethanol for 20 min each.

The sample was placed in a envelope of filter paper in ethanol and dried from liquid CO_2 in a critical point apparatus. The dried sample was stored over dessicant under vacuum and then mounted on stubs by shaking the powdery sample onto drying graphite glue. The samples were sputter-coated with a 15 nm layer of Pt using a Polaron sputter coater equipped with a quartz crystal thickness monitor. The sample was viewed with a ISI DS-130 SEM.

Processing of 5000*g* pellet for TEM. Latex (0.5 ml) was centrifuged at 5000*g* for 15 min, and the pellet was resuspended in 250 µl of the supernatant of latex that had been centrifuged at 100,000*g* for 1 h. The pellet was fixed in the dark for 1 h at 0 to 4 °C in 1% glutaraldehyde, 0.5% OsO_4 in 0.1 M cacodylate pH 6. The sample was centrifuged for 5 min at 1000*g*, and the pellet was rinsed in 0.1 M cacodylate pH 7. The pellet was postfixed in 2% glutaraldehyde in 0.1 M cacodylate pH 7 for 0.5 h and was then rinsed three times with 0.1 M cacodylate pH 7. Next the sample was fixed for 45 min in 0.5% OsO_4 and rinsed three times with distilled water. The sample was dehydrated for 15 min each in 30% ethanol then in 50% ethanol. The sample was next subjected to two changes of 70% ethanol for 30 min each. The sample was placed in a 1:1 mixture of 70% ethanol and LR White resin (Polysciences, Inc.) for 30 min. The sample was infiltrated with three changes of LR White for 45 min, 1 h, and then 2.5 h before being placed in BEEM capsules. The capsules were degassed for 1 h and then hardened for 18 h in a vacuum oven at 60°C.

Thin sections were cut from the resin blocks using a Porter-Blum MT-2 microtome and a freshly-made glass knife. Sections were collected on formvar-coated 200 mesh copper grids. Sections were poststained with 5% UrAc in water for 20 to 30 min and Reynolds's lead citrate stain for 5 min. Specimens were examined using a Zeiss 109 TEM.

Processing of percoll gradient samples for SEM. Poly-L-lysine-coated coverslips were prepared as mounts for the samples (14). Glass coverslips were broken into quarters, cleaned by sonication in a detergent solution, rinsed with distilled water, and placed in ethanol. Each was dried in an alcohol flame, then immediately after the slip had cooled, a small drop of poly-L-lysine

solution (1 μg ml^{-1}) was placed on the coverslip and was spread over the surface with a wooden applicator. The coverslips were coated no more than a few hours before the sample was to be applied.

Ten fractions from a sample of latex centrifuged on a Percoll gradient were obtained from Dr. Scott Taylor. Each sample was placed on a separate coverslip, and a blank was made by placing a drop of the Percoll solution alone on an eleventh coverslip. The coverslips were allowed to sit 20 min over ice to allow particles in the samples to sediment and were then rinsed 3 to 4 times with buffer (50 mM MES, 0.4 M sorbitol, 5 mM $MgCl_2$, 5 mM DTE, pH 6.1). Next the samples were fixed for 1 h in 2% glutaraldehyde in 0.1 M cacodylate, pH 6, were rinsed with 0.1 M cacodylate, pH 6, and were postfixed in 1% OsO_4 in 0.1 M cacodylate, pH 6.

The samples were rinsed with distilled water and dehydrated using a graded ethanol series. The samples, now in 100% ethanol, were dried from liquid CO_2 using a critical point apparatus; each coverslip was contained in a mesh basket. Each dried coverslip with sample was mounted on a stub with silver glue. The samples were coated with 15nm Pt.

Processing of Percoll gradient samples for TEM. A total of 11 1-ml fractions of a Percoll gradient of latex was obtained from Dr. Scott Taylor. Each fraction was fixed at 0-4 C for 1.5 h with 0.5 ml of a solution of 1% OsO_4 and 3% glutaraldehyde in 0.3 M cacodylate pH 6 giving a final concentration in 1.5 ml of 0.3% OsO_4 and 1% glutaraldehyde in 0.1 M cacodylate pH 6. The samples were next centrifuged for 15 min at 5000*g*, and the supernatant was removed. The pellets were washed three times with 0.1 M cacodylate pH 6, followed by two washes of distilled water. Each sample was dehydrated to 70% ethanol using a graded series of 5, 10, 20, 30, 40, 50, 60 and 70% ethanol. The samples remained in each solution for 15 to 20 min to allow the particles to sediment. Next the samples were infiltrated with a 1:1 solution of 70% ethanol and LR White resin for 45 min and with 100% LR White overnight. The next day the samples were incubated with fresh LR White for 1.5 h and were then placed in BEEM capsules. The capsules were evacuated for 2 h to remove gases from the resin, and the resin was hardened in a 60°C

vacuum oven for 20 h.

Thin sections were cut from the resin blocks using a Porter-Blum MT-2 microtome and a freshly-made glass knife. Sections were collected on naked 300 mesh copper grids. Sections were poststained with 5% UrAc in water for 20 to 30 min and Reynolds's lead citrate stain for 5 min. Specimens were examined using a Zeiss 109 TEM.

RESULTS

SEMs of the 5000*g* pellet of latex, which is the fraction capable of incorporating MVA into triterpenoids, are dominated by the presence of elongated starch grains of lengths 8 to 34 μm (Fig. 1). These rod-shaped grains resemble those of other *Euphorbia* species characterized by Mahlberg (11). The grains are coated by an amorphous material, possibly lipid in nature. Upon closer examination, other regular ovoid structures (length 8 to 12 μm) are seen in this fraction (Fig. 2). SEM does not allow characterization of these structures as the nature of any possible limiting membrane(s) and internal structures is not discernable. The heterogeneous nature of the fraction prevents correlation of the terpenoid-synthesizing activity with a particular structure.

Examination of thin sections of the 5000*g* pellet by TEM shows numerous ovoid structures with diameters in the range of 0.5 to 4 μm (Figs. 3 & 4). Some have internal osmophilic substructures; other substructures have a more dispersed matrix. Many small osmophilic particles are found external to these more well-defined structures. They are found singly and also clumped. Their size is in the range of 0.125 to 0.3 μm, consistent with that of other *Euphorbia* species triterpenoid latex particles (8).

Latex was fractionated by density gradient centrifugation using Percoll in order to isolate the triterpenoid-synthesizing structure from other latex components. SEMs of fractions taken from the gradient showed that the starch grains were found exclusively at the bottom of the gradient (Fig. 5). In contrast, the latex particles moved centripetally and were found in the top four fractions (Fig. 6). The relatively uniform size of the latex particles can be seen; their average diameter was 0.2 μm. These structures are latex particles not Percoll; Percoll particles are much smaller, ranging in size from 15 to 30 nm. No other structure was seen in any of the latex fractions. The triterpenoid-synthesizing activity was localized in fractions 3 to 6 (15). Although latex particles were found in these fractions, they were most abundant in fractions nearer the top of the gradient so their presence did not correlate with the biosynthetic activity. The absence of a structure that corresponded to the detected enzymatic activity does not mean that such a

structure does not exist. The preparation of samples for SEM involved adhering the material to polylysine-coated coverslips; this method requires that the structures of interest have a negative surface charge. If the terpenoid-synthesizing structure did not have an overall negative charge, it would not stick to the coverslip and would be rinsed off when the coverslip was washed in buffer before the fixation step. In addition, structures may not survive the drying process required in preparing SEM samples.

In order to insure that no structure was lost during the preparation of Percoll gradient samples for oberservation, the samples were fixed and embedded for TEM. Under these conditions, numerous structures resembling those seen in the TEMs of the 5000*g* pellet were observed in the fraction containing the biosynthetic activity (Figs. 7-9). No such structures were observed in other fractions. They ranged in length from 2 to 9 μm and were bounded by a single membrane (Fig. 10).

DISCUSSION

In this study, three different types of structures were observed in samples of *Euphorbia lathyris* latex. The first were the unusual rod-like starch grains that concentrated in the pellet upon differential and density gradient centrifugation. High density is a general characteristic of starch grains, and these elongated shapes are distinctive of grains of non-articulated *Euphorbia* laticifers. Rod-shaped starch grains characterize leafy taxa of the genus (12). The adhesion of the grains to polylysine-coated coverslips indicates that they have a negative surface charge. Mahlberg ascribed the wrinkled surface of grains he observed by SEM to the limiting membrane of the amyloplast (11); plastid envelopes contain a high percentage of galactolipids and phosphotidylcholine, making them not strongly negatively-charged(3). Either some change has occured in the composition of the membrane of latex starch grains giving them an overall negative charge or the density of the grains prevented them from being washed from the coverslips. The biochemical nature of laticifer starch grains, specifically, the existance and composition of any

limiting membrane(s) has not been explored; perhaps they differ in this regard from starch grains of parenchyma tissue as well as in their shape.

The second structure seen in the micrographs was the latex particle. The lipid nature of this structure was indicated by its heavy staining by osmium as well as its low density. Like latex starch grains, latex particles adhered to the polylysine-coated coverslips, indicating a negative surface charge. The question of whether latex particles are naked or have a boundary layer has been asked by a number of investigators; membrane-like boundaries have been seen in micrographs of particles from various species. Fineran observed a membrane-like boundary on some latex particles of *Euphorbia pulcherrima* (5), but Groeneveld saw no envelope on particles from *Euphorbia milii* (8). The best chemically characterized latex particles are the triterpene particles of *Hoya australis* (Asclepiadaceae) which have a protein content of 5.2% of particle dry weight and an isoelectric point of 3.2; the particles are surrounded by a membrane-like film (8). *E. lathyris* latex particles, with their negative surface charge, would have a similarly low isoelectric point, indicating that they may be bounded by a membrane. Chemical analysis of purified latex particles is needed to discern the composition of this layer.

The final structure observed in latex associated with the triterpenoid-synthesizing activity and the vacuolar marker enzyme α-mannosidase on Percoll density gradients (15). Its physical characteristics collaborate the biochemical evidence indicating that this structure is vacuolar; it is bounded by a single membrane and is of variable size. The observation that the *in vitro* triterpenoid biosynthetic activity of *E. lathyris* latex is associated with a vacuolar structure agrees with Fineran's ultrastructural observations indicating that latex particles are synthesized in tubular vacuoles in the peripheral cytoplasm of developing *E. pulcherrima* laticifers (6).

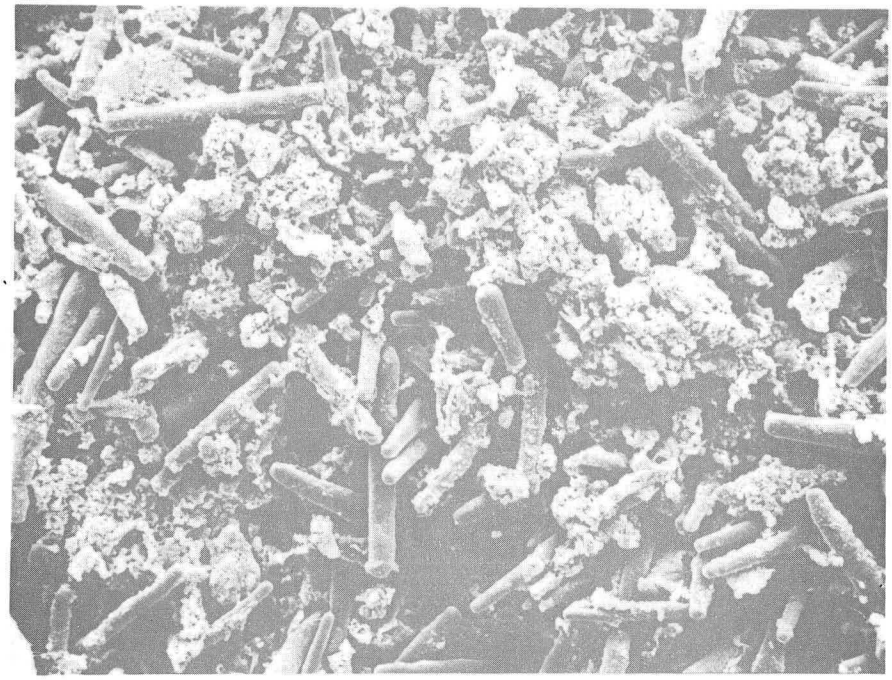
The relationship of this structure to the well-characterized vacuolar "lutoid" of *Hevea brasiliensis* latex should also be considered. The size range of 2 to 9 μm in diameter of the *E. lathyris* structure is larger than that reported for lutoids which are spherical particles ranging from 1 to 5 μm in diameter (2). The inability of the *E. lathyris* structure to adher to the polylysine surface may also be indicative of differences between this particle and the lutoid. Lutoids are

distinguished by the high content of phosphatidic acid of their membranes which would afford them a high negative surface charge and presumably insure their adherence to the cationic surface (4). However, lutoids are also characterized by their extreme osmosensitivity, and the absence of the *E. lathyris* structure on the coated coverslips may be due to breakage of the organelle during sample preparation rather than an inherent inability to adher to the surface.

The observation that triterpenoid biosynthesis occurs in vacuoles of latex contrasts with what is known about sterol biosynthesis in animals and other plants. Mammalian sterol biosynthesis is considered to be a process which occurs exclusively in the endoplasmic reticulum, and experiments with *Zea mays* support the view that this is also the location of sterol biosynthesis in plants (10). This dichotomy may be resolved by consideration of work by Marty on vacuole biogenesis in *Euphorbia characias* roots (13). Based on ultrastructural studies, he concludes that the vacuolar membrane arises from the ER membrane through the fusing of provacuoles which are formed in the region where the smooth ER is associated with the Golgi. This common origin of ER and vacuolar membranes reconciles any incongruity regarding triterpenoid biosynthesis occuring in the two sites.

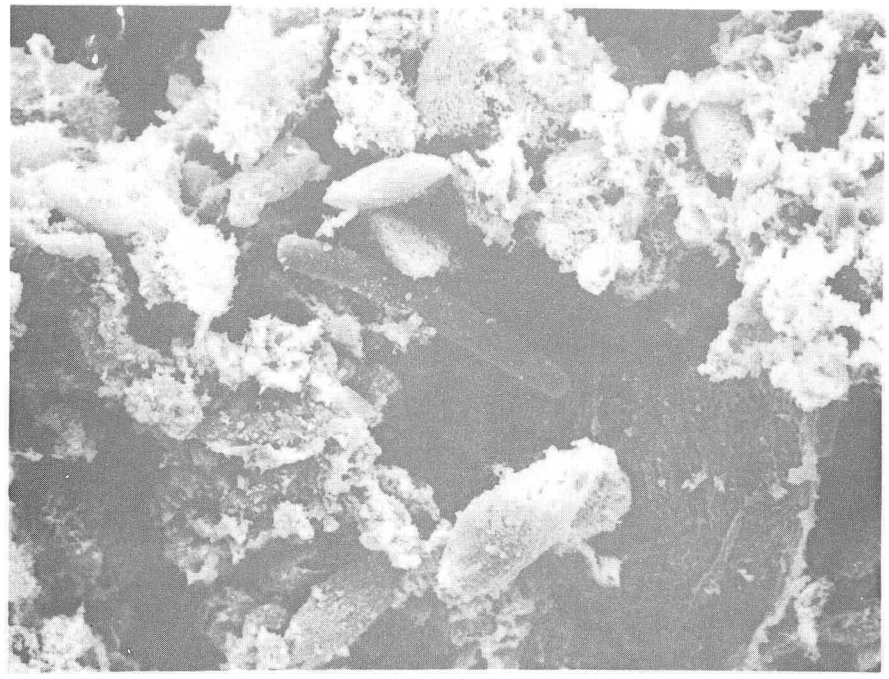
CONCLUSIONS

Ultrastructural observations of *Euphorbia lathyris* latex fractions allowed characterization of three latex structures. Rod-shaped starch grains and terpenoid latex particles, similar to those described in other species of *Euphorbia*, were observed. A third structure was associated with the mevalonate to triterpenoid converting activity of the latex and was physically and biochemically identified as a vacuole. This is the first time that the site of isoprenoid biosynthesis in latex has been linked to a specific subcellular organelle.



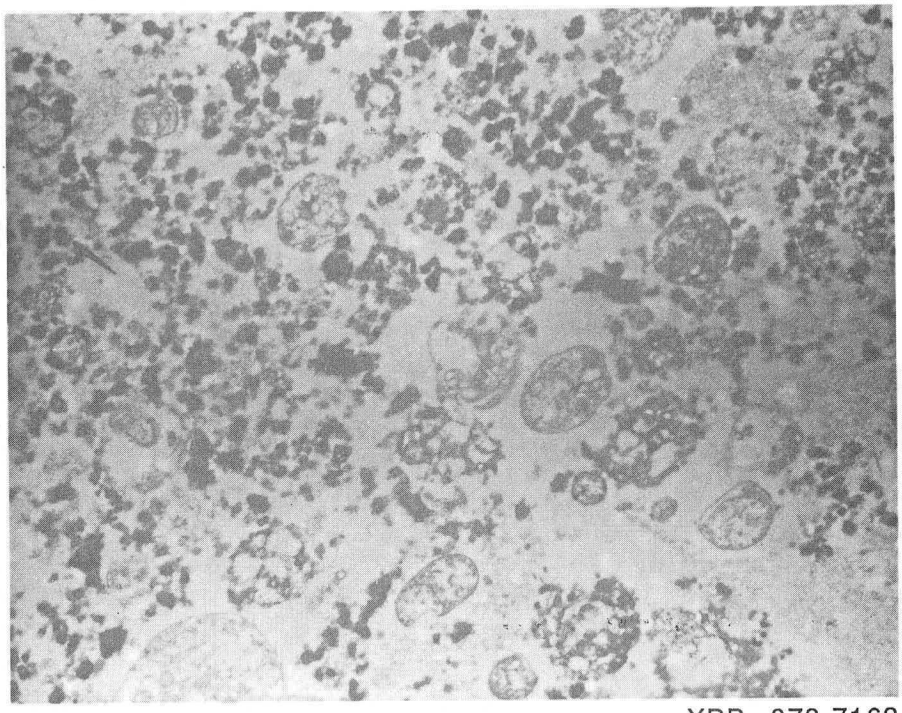
XBB 873-1889

Fig. 1. SEM of dried 5000*g* pellet of *E. lathyris* latex. Bar equals 10 μm.



XBB 873-1888

Fig. 2. SEM of dried 5000*g* pellet of *E. lathyris* latex. Bar is 10 μm.



XBB 878-7163

Fig. 3. TEM of resin-embedded 5000*g* pellet of *E. lathyris* latex. Bar equals 1 μm.

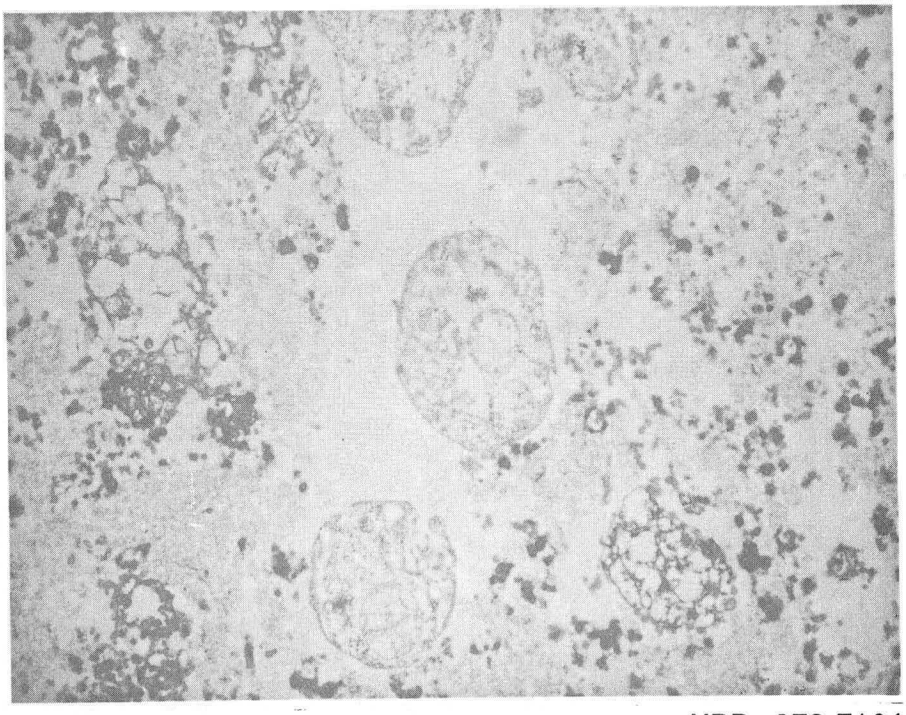


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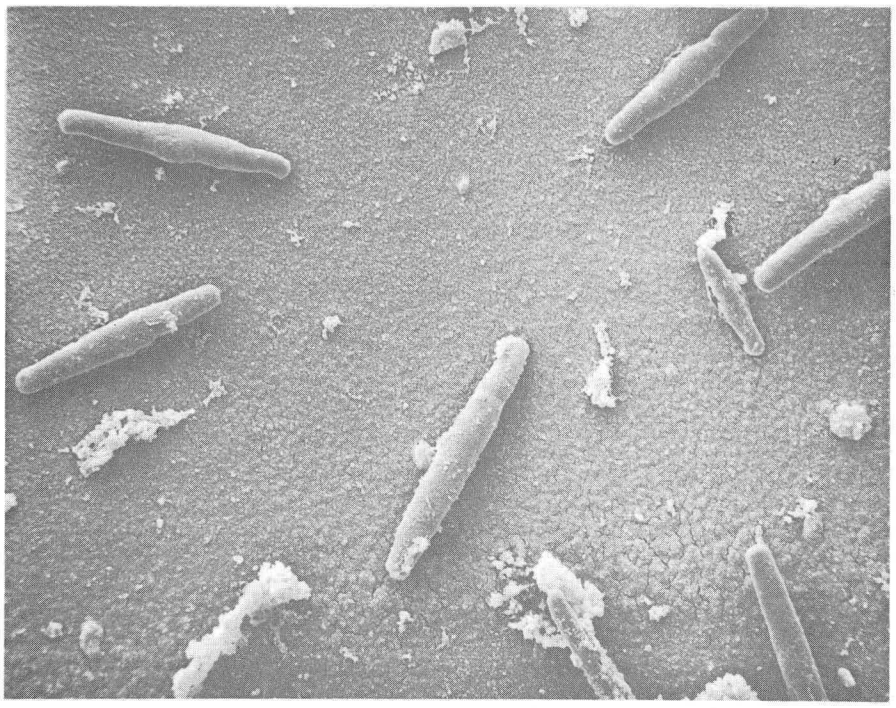
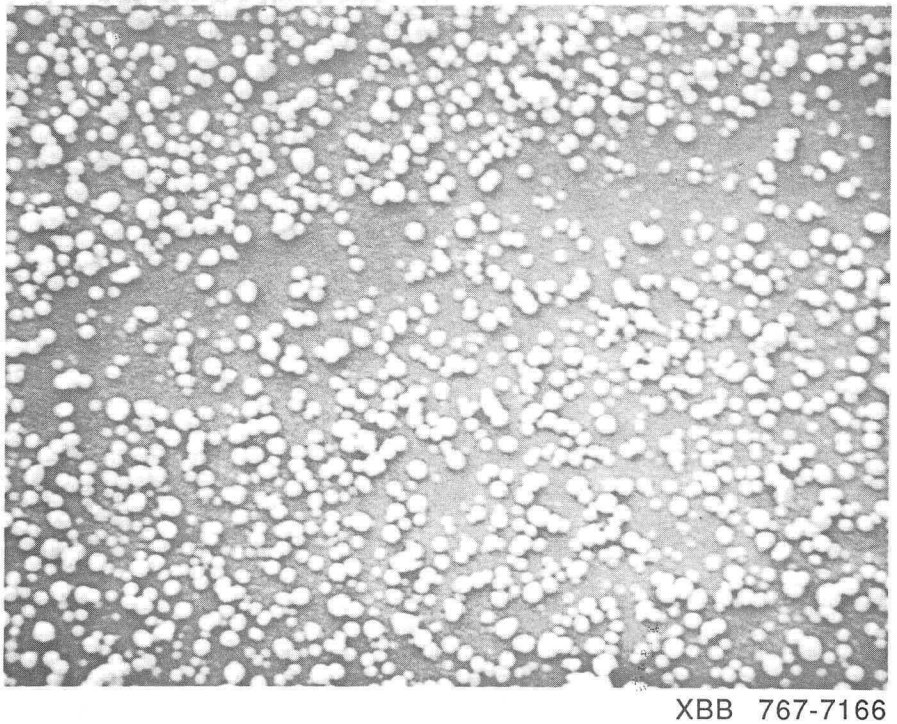
Fig. 4. TEM of resin-embedded 5000*g* pellet of *E. lathyris* latex. Bar is 1 μm.



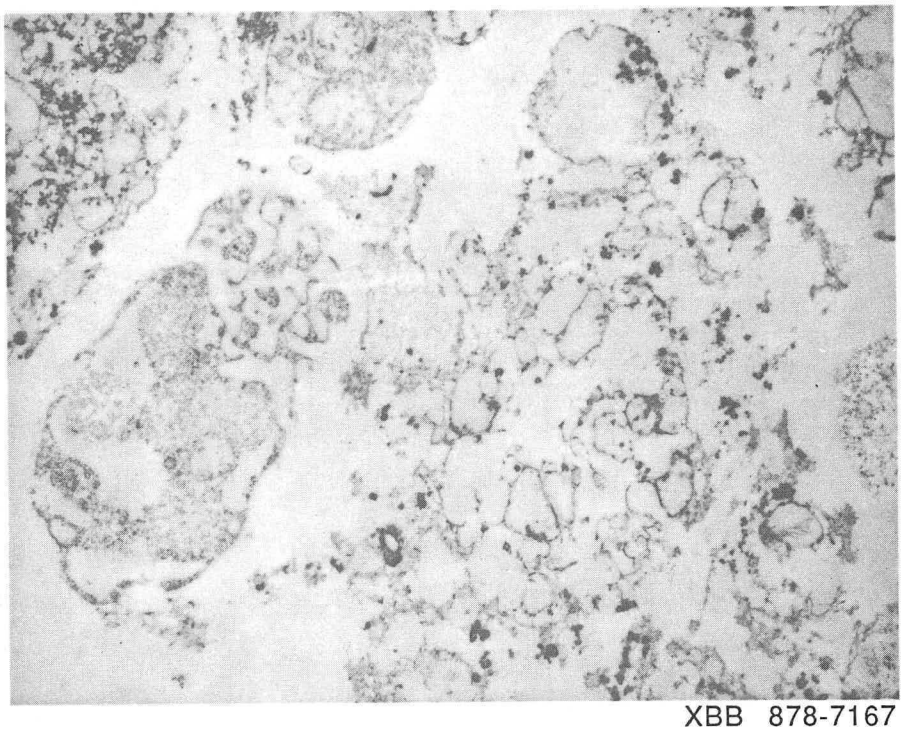
XBB 878-7165

Fig. 5. SEM of pellet from Percoll gradient of *E. lathyris* latex. Bar equals 10 μm.



XBB 767-7166

Fig. 6. SEM of latex particles from Percoll gradient of *E. lathyris* latex. Bar is 1 μm.



XBB 878-7167

Fig. 7. TEM of triterpenoid-synthesizing fraction of *E. lathyris* latex. Bar equals 1 μm.

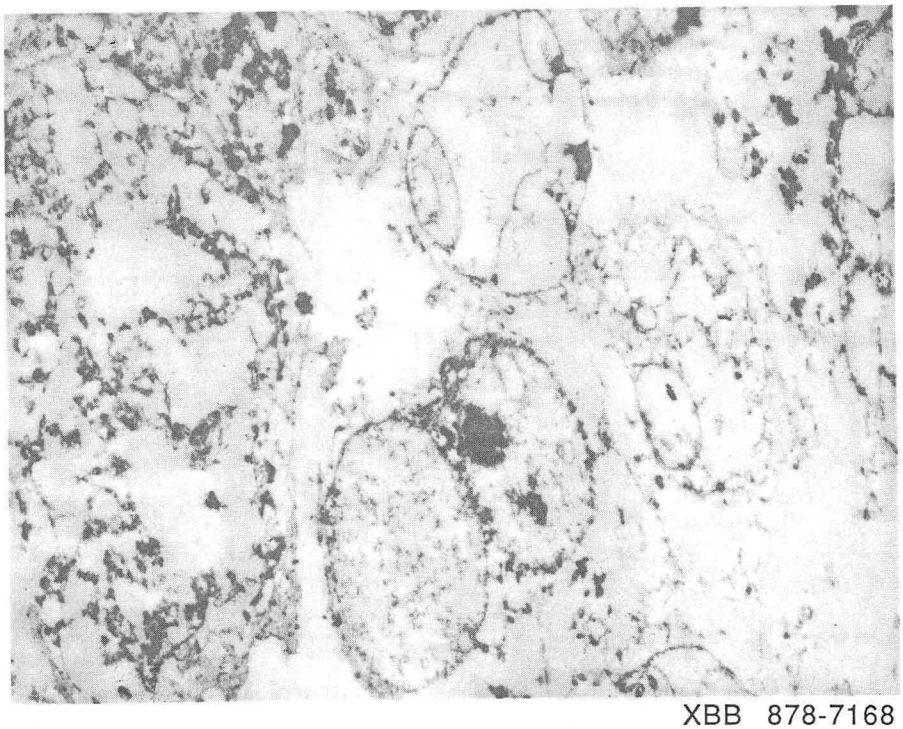


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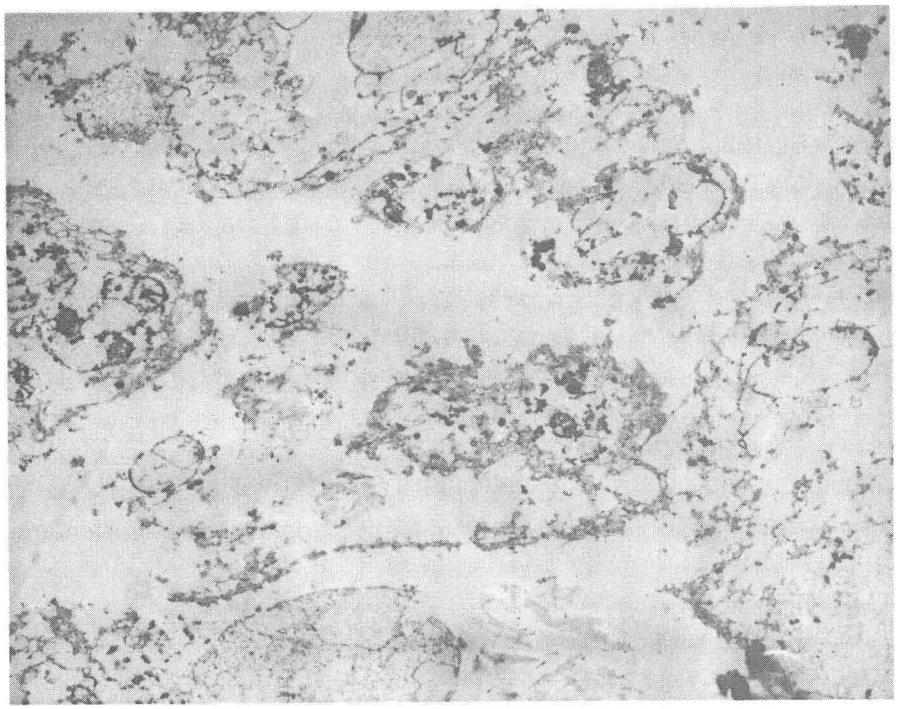
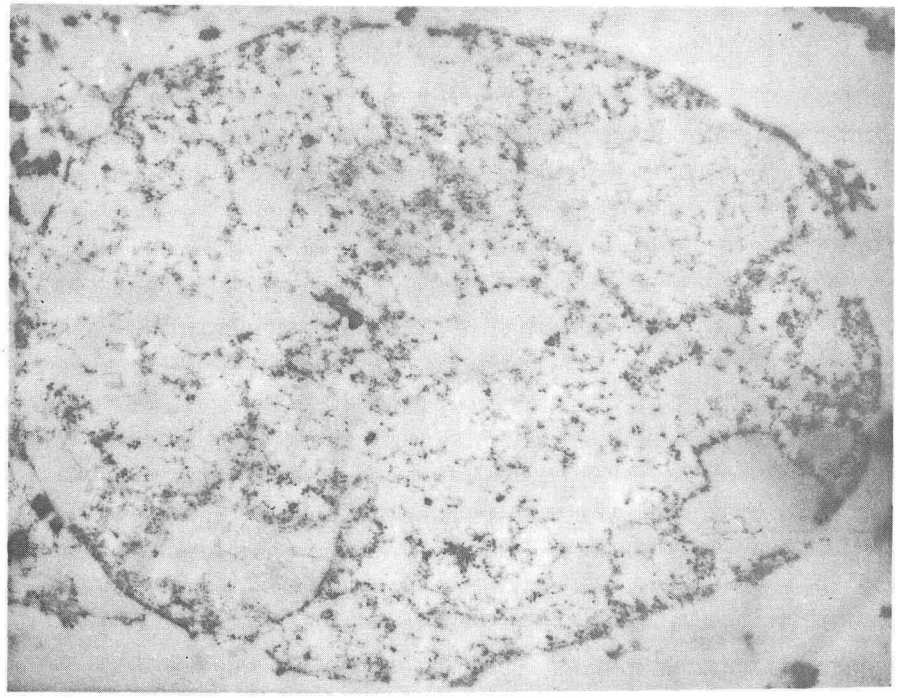
Fig. 8. TEM of triterpenoid-synthesizing fraction of *E. lathyris* latex. Bar is 1 μm.



XBB 878-7169

Fig. 9. TEM of triterpenoid-synthesizing fraction of *E. lathyris* latex. Bar equals 1 μm.



XBB 878-7170

Fig. 10. TEM of triterpenoid-synthesizing structure showing single membrane. Bar is 1 μm.

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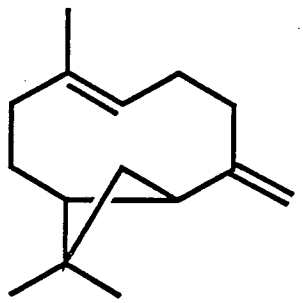
Chapter IV. BIOSYNTHESIS OF TERPENOIDS IN *COPAIFERA*

A most attractive candidate for use as a source of hydrocarbons is the tropical tree genus *Copaifera* which is the source of the oleoresin copaiba balsam. This oil, obtained by tapping the tree trunk, has been used directly as fuel in diesel-engined trucks of the Instituto Nacional du Pesquisas da Amazônia of Brazil. Trees in Ducke Reserve near Manaus are tapped biannually, yielding 20 to 30 liters of the oil in 2 to 3 hours (3).

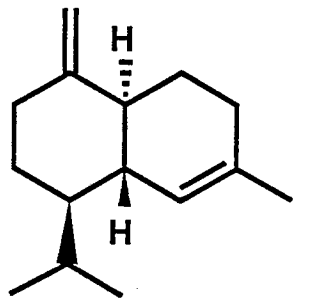
The genus *Copaifera* is a member of the Leguminosae family, subfamily Caesalpinioideae, and the tribe Detarieae. It is represented by *ca* 30 species in the New World and 4 in Western Africa (2). These trees are prominent in the lowland rainforest ecosystem but are also found under drier, more-open thorn forest habitats (12).

Of four commercial samples of the oleoresin, 72 to 90% of the oil was found to be sesquiterpene hydrocarbons (20). Sesquiterpenes are 15-carbon terpenoids constructed of three isoprene units. They compose the largest class of terpenoids with several thousands of individual compounds of *ca* 200 different carbon skeleton types having been identified (15). Both pure hydrocarbon and oxygenated structures exist. Of the 24 sesquiterpene hydrocarbons separated by GLC of copaiba balsam oil, 18 have been identified, with caryophyllene and copaene the major components (20). In addition to the wood resin, species of *Copaifera* also contain a leaf resin. The sesquiterpene hydrocarbons caryophyllene, γ-cadinene, cyperene, and α-copaene have been isolated and identified from leaves of *Copaifera officinalis* L. and *C. venezuelana* var. *laxa* (2) (Fig. 1). The leaf resin is secreted from specialized epithelial cells which line small, ovoid, schizogenous pockets in the mesophyll tissue (2). In the wood, the oleoresin is found in concentric rings of canals in the primary stem tissue and secondary tissue (11).

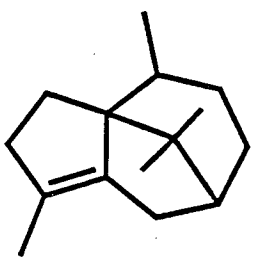
Mortality of the lepidopteran *Spodoptera exigua* in feeding experiments with different resin compositions incorporated into an artifical diet was correlated with the concentration of caryophyllene (13), and caryophyllene oxide was the constituent of various leaf resins tested which inhibited the growth of the associated leaf fungus *Pestalotia subcuticularis* (1). These



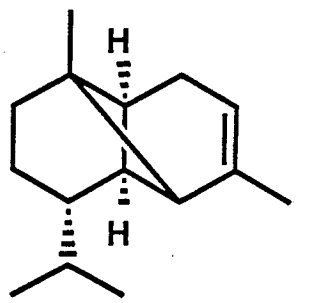
Caryophyllene



γ -Cadinene



Cyperene



α- Copaene

Fig. 1. Sesquiterpene hydrocarbons of *Copaifera* leaf resin.

observations support the view that these resins play a role in the defense of the plant against predators.

In the course of structural determinations of sesquiterpenes, Ruzicka developed the "farnesol rule" and the broader "biogenetic isoprene rule" which hypothesized that all terpenoids could be formed biologically by accepted reaction mechanisms from such simple precursors as farnesol, geraniol, geranyl-geraniol, and squalene (18). Subsequently, extensive biogenetic schemes have been formulated to account for the variety of sesquiterpene skeletons represented in nature. Experimental verification of these proposed routes is still in its infancy, but investigations using cell-free preparations and ^{13}C or radiolabelled precursors such as acetate, mevalonate, and farnesol have established the mechanism of formation of a number of sesquiterpenes. Much of this work has been carried out with fungi which have proven more amenable to uptake and metabolism of the exogenous substrates than plants (4).

The mixture of sesquiterpene hydrocarbons in *Copaifera* resin makes it an ideal system in which to study the interrelationships between the various structures. Proposed biogenetic schemes suggest that certain families of sesquiterpene skeletons are of related origin, but in many cases, a number of logical routes to the same structure exist (17). Analysis of the quantitative co-occurence of different sesquiterpenes in the leaf pocket resins of species of the closely related genus *Hymenaea* suggests the close biosynthetic linkage of caryophyllene and β-humulene as well as δ-cadinene with γ-muurolene and the possible intermediate role of germacrene (16).

Besides interest in the relationship between individual compounds, there is the question of the turnover of sesquiterpenes in general. Essentially nothing is known about their catabolism. However, an investigation of the time course of labelling of sesquiterpenes from [2-^{14}C]mevalonate in peppermint cuttings showed that the maximum incorporation was achieved at 6 h, after which time the amount of label in the sesquiterpenes decreased (5). Likewise, *Pogostemon cablin* leaf discs contained 14 times the label from [U-^{14}C]sucrose in the sesquiterpenes α-guaiene, α-patchoulene, caryophyllene, α-bulnesene, and patchouli alcohol

when incubated for 4.5 h as opposed to 17 h (9). These results indicate that sesquiterpenes are metabolically active. Sesquiterpenes of *Copaifera* seedlings labelled with $^{14}CO_2$ for a period of two weeks were turned over in 8 to 10 days, but metabolism over a shorter time period was not investigated (12).

This preliminary investigation of the incorporation of various exogenous precursors into the sesquiterpenes of *Copaifera* species was undertaken to see if the radiolabelled substrates would be metabolized sufficiently to allow study of the turnover of these compounds as a group and of the interelationships between individual sesquiterpenes. The results indicate that although the incorporation of terpenoid precursors was low, there was adequate labelling of sesquiterpenes to warrant further investigation of their metabolism in this species. In addition, the even greater labelling of squalene and triterpenoids than the sesquiterpenes from exogenous precursors suggests that *Copaifera* could be a good system in which to study the controls of the allocation of carbon to the different classes of terpenoids.

MATERIAL AND METHODS

Plant Material. All species of *Copaifera* were grown in pots in a porous soil mixture of 1 part UC Davis mix (1:1:1 sand: peat: redwood compost), 1 part sand, 1 part Canadian sphagnum peat, and 1 part perlite under greenhouse conditions at the UC Berkeley Botanical Garden.

Materials. Aquassure, R-[5-^{3}H]mevalonic acid, triethylammonium salt, sodium[^{3}H]acetate, and D-[U-^{14}C]glucose were purchased from New England Nuclear. Additional sodium[^{3}H]acetate was procured from ICN Radiochemicals. Sodium[2-^{14}C]acetate was obtained from Amersham. Silica gel plates were purchased from Analtech. Carbo-Sorb and Permafluor were obtained from Packard Instruments Co.

[^{14}C]Acetate Incorporation by *Copaifera officinalis* leaf. A leaf with two pairs of leaflets was cut from a year-old *C. officinalis* seedling and immediately placed in water. The end of the petiole was cut off under water, and the cutting was placed in the incubation solution containing 50 µl water and 50 µl sodium[2-^{14}C]acetate (57.5 mCi mmol^{-1}, 0.1 mCi ml^{-1}). The cutting was incubated for 20 h at room temperature until the acetate solution was taken up, then the plant material was pulverized with anhydrous Na_2SO_4 and hexane using a mortar and pestle. The hexane was removed and the process was repeated three more times to give *ca* 15 ml of a combined clear, bright-yellow hexane extract. Next the plant residue was ground twice with acetone, giving *ca* 20 ml clear, bright-green acetone extract. Lastly, the residue was washed with water and then removed from the mortar giving water extracts 1 to 4. One to two drops of concentrated sulfuric acid were added to the water extracts to prevent microbial growth. Aliquots of ten percent were taken from each sample; the hexane aliquot was decolorized with activated charcoal; 15 ml Aquassure was added to each aliquot; and the samples were counted. The remaining hexane extract was divided into two halves, and one was concentrated under a stream of N_2. The concentrated hexane extract (45% of total) was spotted on a 20 x 20 cm 500 µm layer silica gel G plate as a band and an isolated marker spot. The plate was developed in a solvent

mixture of 3:1 petroleum ether: CCl_4 as recommended by Stahl (19) for the separation of the sesquiterpenes humulene, caryophyllene, and isocaryophyllene. The marker spot region was visualized with sulfuric acid spray and heat, and the remainder of the plate was divided into 14 bands which were scraped from the plate, mixed with 5 ml water and sonicated 5 min, and then shaken with 15 ml Aquassure to form an uniform gel. The samples were counted using a Packard 640-C scintillation counter.

Time course of [^{3}H]Acetate incorporation by *C. officinalis* leaf discs. Fifty microliters of sodium[^{3}H]acetate in ethanol (1.6 Ci mmol^{-1}, 10 mCi ml^{-1}) was dried under a stream of N_2 overnight then redissolved in 5.1 ml sterile water giving a solution 60 μM in acetate. A 10 μl aliquot was mixed with 15 ml Aquassure and counted to confirm the concentration of the solution. Ten *C. officinalis* leaflets were collected, surface sterilized with a solution of 10% bleach, and rinsed in sterile water. Nine millimeter leaf-discs were cut from the leaf tissue with a cork-borer, and groups of 30 discs were incubated with 1 ml of the acetate solution for 1, 3, 6, and 24 h. A fifth set of discs was incubated in 1 ml pure water, then at 24 h the water was removed and substituted with 1 ml of the acetate solution. The sample was allowed to incubate an additional 3 h. At the end of each incubation period the acetate solution was removed, and the discs were washed four times with water which was then combined with the acetate solution. The discs were then killed by immersion in liquid N_2 and ground with hexane using a mortar and pestle. The hexane extract and remaining leaf-residue were removed from the mortar and further extracted by stirring overnight with Na_2SO_4 added as a drying agent. The mortar was next washed 4 times with water to give Washes 1 to 4. The hexane extract was removed from the residue; the residue was rinsed once with hexane; and the two hexane fractions were combined. The hexane extract was then backwashed three times with a total volume of *ca* 15 ml water. A 5% aliquot of each hexane sample was taken, mixed with 15 ml Aquassure, and counted. Half of the remaining hexane extract (47.5%) was concentrated, chromatographed, isolated, and counted as described for "[^{14}C]Acetate incorporation by *Copaifera officinalis* leaf."

Separation and identification of hexane extractables of *Copaifera* leaf tissue. *C. officinalis* leaf tissue (2.35 g fresh wt) was pulverized with hexane and anhydrous Na_2SO_4 using a mortar and pestle then stirred overnight with hexane. The residue was allowed to settle, and the hexane was decanted. A second hexane wash of the residue was combined with the first extract, and the combined hexane extract was backextracted three times with water. The hexane extract was concentrated under a stream of N_2 and applied to two 20 x 20 cm 500 µm layer silica gel G plates which were developed in 3:1 petroleum ether: CCl_4. A marker spot on each plate was visualized with sulfuric acid spray and heat. The region of the plate where the sesquiterpene hydrocarbon markers eluted was scraped, and the material was recovered by elution with *ca* 50 ml CH_2Cl_2. The fraction was further analyzed by gas chromatography on a SP2250 capillary column (0.5 mm x 40 m) using a Varian model 3700 gas chromatograph. The column was maintained at 100°C with an injection temperature of 130°C. Detection was done with an FID set at 300°C. The split ratio was 10:1, and the attenuation was 16×10^{-12} and 2×10^{-12} for fractions 2 and 3, respectively. One microliter aliquots out of a total volume of 1 ml sample were analyzed. A blank was made by developing two silica gel plates in the petroleum ether/ CCl_4 solvent mixture, eluting the same region of the plates with *ca* 50 ml CH_2Cl_2, combining the extracts from the two plates, and concentrating the sample volume to 1 ml under a stream of N_2. The sample and blank were given to the UC Berkeley College of Chemistry Mass Spectroscopy Lab for GC/MS analysis.

[^{14}C]Bicarbonate and [^{14}C]Acetate Incorporation by *Copaifera* Leaf discs. Leaflets were collected from 17-month old *C. officinalis* seedlings, were surface sterilized with 10% bleach, and were rinsed in sterile water. Discs of 8 mm diameter were cut from the leaf tissue, and 20 randomized discs were used for each incubation. The 20 discs were incubated with 1 ml of solution in 25-ml Erlenmeyer flasks capped with serum caps to promote a CO_2-saturated atmosphere. With this set-up each disc floated freely on the liquid surface. A time course of bicarbonate incorporation was determined by incubating sets of leaf discs with the [^{14}C]H_2CO_3

solution (58.6 mCi $mmol^{-1}$, 0.02 mCi ml^{-1}) for 1, 3, 6, 12, 20, and 24 h. A seventh set was incubated for 3 h with the acetate solution (57.5 mCi $mmol^{-1}$, 0.1 mCi ml^{-1}). The incubations were stopped and extracted as described above. The hexane extracts were divided into halves and concentrated to 1-ml volume under a stream of N_2. Both halves were spotted on 20 x 20 cm 500 μm layer silica gel G plates and were developed in 3:1 petroleum ether: CCl_4. The origin, the region of R_f .43 to .69, and the region of R_f .74 to .94 where material was visualized were scraped from the plates. Compounds were eluted from each silica gel sample from one of the two plates with a 50-ml volume of CH_2Cl_2 which was subsequently concentrated under a stream of N_2 to 20 ml. A 10% aliquot was mixed with 15 ml Aquassure and counted. To check if any volatile compounds were being lost during the elution and concentration steps of the workup, silica gel samples from the second plate were mixed with 5 ml water, sonicated 5 min, shaken with 15 ml Aquassure, and counted as silica sols.

All water fractions from the 20 h incubation were acidified with HCl to pH 2 and dried under a stream of N_2. A volume of CH_3OH was added to each vial, and the fractions were stirred overnight. The methanol extracts were combined and dried under a stream of N_2. The residue was dissolved in water and was washed three times with ether. The resulting clear water solution was concentrated under a stream of N_2 to 0.62 ml; a 10% aliquot was counted. The remaining solution was filtered through a 0.45 μm filter, and a 5% aliquot was counted. An aliquot was diluted and chromatographed by HPLC together with fructose, glucose, and sucrose standards to determine the amount of sugars labelled.

Analysis for labelled sugars in water extract. An aliquot of water extract of leaf tissue was added to a solution containing 1.25 mg ml^{-1} each of sucrose, glucose, and fructose adjusted so that 50 μl of the resulting mixture contained *ca* 10,000 dpm. Fifty microliters was chromatographed on a 4.6 mm x 25 cm NH_2 column (Brownlee Labs) in CH_3CN:H_2O, 80:20 at a flow rate of 1 ml min^{-1} on an Beckman Model 322 HPLC; detection was by refractive index using a

Waters Differential Refractometer R401.

Determination of chlorophyll content of leaf tissue. A measured weight or surface area of leaf tissue was extracted with hexane, and the residue subsequently extracted with 95% ethanol. The ethanol extract was centrifuged to give a clear green supernatant and a white pellet. The ethanol extract was kept cold and in the dark until its absorbance was measured using a Hewlett-Packard 8450A UV/VIS spectrophotometer. Total chlorophyll content was calculated using the formulas of Wintermans and De Mots (21). The equations used were Chl_{a+b} ($\mu g\ ml^{-1}$) = $6.10(A_{665}) + 20.04(A_{649})$ and Chl_{a+b} ($\mu g\ ml^{-1}$) = $1000\ (A_{654})/39.8$. An average of the two values was taken as the total chlorophyll content.

Incorporation of [^{14}C]bicarbonate by *Copaifera* leaf discs under buffered conditions. Eight 7-mm leaf discs cut from a *C. officinalis* seedling were incubated for 20 h on the surface of 10 ml incubation medium containing 25 mM sodium PIPES pH 6.8, 0.48 mM $NaHCO_3$, and 2 mM [^{14}C]H_2CO_3 (52.5 mCi $mmol^{-1}$) giving a final concentration of [^{14}C]H_2CO_3 of 50 mM at a specific activity of 2.22 mCi $mmol^{-1}$. The solution was bubbled with N_2 for 45 min to remove O_2 and CO_2 prior to the addition of the bicarbonate solutions and leaf discs. The incubation was performed in a 25-ml Erlenmeyer flask capped with a serum stopper under eight 32W cool white incandescent lights and was stirred just to the point of swirling the discs on the liquid surface. At the completion of the incubation period, the discs were washed with water, ground up and extracted with hexane overnight. The hexane extract was removed from the remaining plant residue and backextracted three times with water. It was divided into two halves; one was concentrated under a stream of N_2 and spotted on a 500 μm 20 x 20 silica gel G plate and developed in 3:1, petroleum ether: CCl_4. The plate was divided into 12 bands which were scraped, mixed with 5 ml water, sonicated 5 min, combined with 15 ml Aquassure, and counted. The second half was chromatographed as with the first, but the two regions of the TLC plate containing peaks in radioactivity were eluted with CH_2Cl_2. The eluates were concentrated under a stream of N_2, were filtered through a 0.45 μm

filter, and were chromatographed by HPLC using a 4.6 mm i.d. x 25 cm ODS column (Altex) in 100% CH_3CN at a flow rate of 1 ml min^{-1}. The column effluent's absorbance was monitored at 228 nm using a Hitachi 100-10 spectrophotometer. The incubation medium, the water wash, the backextract water, and the residue were acidified and dried under a stream of N_2; then they were extracted overnight twice with water. One aliquot of the combined water extracts was counted, and a second was analyzed by HPLC for labelled sugars as described previously.

Time course of [^{14}C]bicarbonate incorporation into *C. multijuga* leaf discs. Eight-mm leaf discs were cut from a *C. multijuga* seedling and were kept under N_2, in 50 mM MOPS, 10 mM glutathione pH 6.5 in ambient light until placed in reaction flasks. Each 25-ml Erlenmeyer reaction flask contained 6.5 ml of 77 mM MOPS pH 6.5 and15 mM glutathione that had been bubbled with N_2 to remove dissolved CO_2 and O_2. Eight leaf discs were floated on the liquid surface, and each flask was capped with a serum stopper. Bicarbonate solutions ($NaHCO_3$ (0.141 M) and [^{14}C]H_2CO_3 (52.5 mCi $mmol^{-1}$, 7.4 mCi ml^{-1})) were added to each flask via a syringe to bring the total volume of solution to 10 ml and the final concentrations to 50 mM bicarbonate, 50 mM MOPS, and 10 mM glutathione. The specific activities of the incubation media ranged from 0.6 to 12 mCi $mmol^{-1}$. Different specific activities were used to give managable levels of radioactivity incorporated over the range of time points of incorporation sampled (Table I).

The samples were incubated for the designated time under eight 32W cool white lights. At the appropriate time points, the incubation medium was removed from the leaf discs, and the discs were washed three times with 5 ml water. The discs were then ground with *ca* 15 ml hexane, giving a light-yellow solution. The tissue residue was removed form the mortar by washing with *ca* 10 ml water. The solution was centrifuged, and the pelleted residue was further extracted by stirring overnight with *ca* 15 ml hexane. The hexane extracts were combined and backwashed three times with 5 ml water, then they were concentrated under a stream of N_2 and applied to a 500 μm 20 x 20 cm silica gel G plate which was developed in 3:1, petroleum

ether:CCl_4. The plates were divided into 9 bands which were scraped, mixed with 5 ml water; sonicated, and mixed with 15 ml Aquassure to form silica sols that were counted by liquid scintillation.

Table I. *Volume of Bicarbonate Solutions added to Each Incubation.*

Sample	Vol $NaHCO_3$ ml	Vol $[^{14}C]H_2CO_3$ µl	Specific Activity mCi $mmol^{-1}$
1 h	2.74	810	12
3 h	3.28	270	4
6 h	3.41	135	2
12 h	3.475	68	1
24 h	3.5	40	0.6

All aqueous fractions and the tissue residue were acidified with HCl and dried under a stream of N_2. The remaining material was then extracted with CH_3OH to obtain an estimate of the total bicarbonate incorporation.

Determination of light saturation level of *C. multijuga* leaf discs. A *C. multijuga* seedling was incubated 5 to 6 h at a light level of 500 µE m^{-2} s^{-1}. For each experimental point, an 8-mm leaf disc was cut out under water from the seedling and was placed in 200 µl incubation medium in a grinding vial. The disc was incubated in the light for 2 min; the $[^{14}C]$bicarbonate was added, and the disc was incubated for 3 additional min. The incubation was stopped by pouring liquid N_2 into the grinding tube which was kept in liquid N_2 until extracted.

Incubation media contained 50mM MOPS, pH 7.2, 40 units ml^{-1} carbonic anhydrase added just before experiment commenced, and 4 mM $NaHCO_3$. Ten microliters $[^{14}C]H_2CO_3$ (52. 5 µCi $mmol^{-1}$, 87 µM) were added to the 200 µl of media containing each leaf disc, resulting in a final specific activity of available CO_2 of 26.25 mCi $mmol^{-1}$.

The light level, provided by a GE 500W photolamp DXB, was varied by placing various

combinations of nuetral density screens in front of the lamp. Heat was absorbed by a flask of water placed between the lamp and the grinding tube. Light intensity was measured both in front of and behind the grinding tube.

Each leaf disc was ground successively with 1 ml 80% ethanol (0.8 ml 95% CH_3CH_2OH added to 0.2 ml buffer already in tube), 1 ml 20% ethanol, and 1 ml water. The extracts were combined and centrifuged; the supernatant and a 1-ml water wash of the the grinding tube were combined, acidfied, and dried under a stream of N_2. The residue was resuspended in 2 ml 50:50 95% CH_3CH_2OH: H_2O, and a 10% aliquot was taken, mixed with 15 ml Aquassure, and counted. The pellet from the centrifugation was washed from the tube with 5 drops 95% ethanol and 5 drops 10% HCl onto a paper pad and was evacuated overnight. The pellet was oxidized in a Packard Automatic Combustion Apparatus, and the $^{14}CO_2$ was collected in Carbo-Sorb. Permafluor was added and the sample was counted.

[^{3}H]acetate and [^{14}C]bicarbonate incorporation by *Copaifera* leaf discs under saturating light conditions. Leaf discs (7 mm) were cut under water from *C. multijuga* and *C. officinalis* leaf tissue using a #3 cork borer. They were vacuum infiltrated three times with 50 mM MOPS, pH 7 and 10 mM glutathione. A set of 8 *C. multijuga* leaf discs was incubated in 2 ml acetate solution, and a set of 8 leaf discs of each species was incubated with 2 ml bicarbonate solution. The bicarbonate solution contained 50 mM MOPS pH 7, 10 mM glutathione, 90 µl 0.1 M $NaHCO_3$, and 10 µl [^{14}C]H_2CO_3 (52.5 mCi mmol^{-1}, 0.394 M) giving a final solution of 6.5 mM bicarbonate at 16 mCi mmol^{-1}, pH 5.75. The acetate solution contained 50 mM MOPS pH 7, 10 mM glutathione, 94 µl 0.1 M sodium acetate, and 100 µl [^{3}H]sodium acetate (1.6 Ci mmol^{-1}, 10 mCi ml^{-1}) giving a final concentration of 5 mM acetate at 100 mCi mmol^{-1}, pH 5.5.

The leaf discs were place in 5-ml Fernbach flasks, capped with serum stoppers, with the underside of the leaf on the surface of the liquid. They were illuminated from underneath with a GE 500W DXB photo lamp. A water-cooled IR filter was placed between the lamp and the incubations in order to reduce heating of the flasks. The light intensity at the flasks was kept at

1500 μE m^{-2} s^{-1} by checking the light intensity during the course of the experiment and moving the vials closer to the lamp as it aged. The incubation media was periodically stirred using 1/2 x 1/8 inch stir bars in each flask and a magnetic stirrer placed above the vials.

After 5 h the incubations were stopped by removing the incubation media, washing the discs 3 times with 3-ml volumes of water, and then grinding the discs with 15 ml hexane. The residue was then ground with 5 ml 95% ethanol, followed by two 5-ml volumes of water. All water and ethanol extracts as well as the residue were acidified; the residue was separated from the liquid by centrifugation and was extracted overnight with hexane. All acidified samples were dried under a stream of N_2. The hexane extracts were combined then divided into halves. The remaining residue was resuspended in water and centrifuged. The supernatant was acidified and dried under a stream of N_2. The pellet was applied to a combustion pad, acidified, and dried overnight under vacuum. All dried fractions were resuspended in water, and an aliquot was taken and counted. The residues were combusted.

A 50 μl aliquot of a 11 mg ml^{-1} mix of sesquiterpene hydrocarbon standards in hexane was added to each hexane extract before chromatography on 20 x 20 cm silica gel G plates in 3:1, petroleum ether: CCL_4. A spot of the mix of sesquiterpenes was used as a marker spot which was visualized with sulfuric acid spray and heat. The plate was divided into bands, scraped, and counted as silica sols.

Incorporation of various substrates by *C. langsdorfii* cuttings. Four matched leaf cuttings were taken from a 2.5 year-old *C. langsdorfii*, cutting the petioles underwater. Four different substrate treatments were prepared, dried under a stream of N_2, redissolved in 0.25 ml water, and added to each cutting in 0.6 ml water in an 1-ml vial. The four treatments were 45 μl R-[5-^{3}H]mevalonic acid, triethylammonium salt (0.5 mCi ml^{-1}, 10.3 Ci $mmol^{-1}$) + 50 μl 1 mM MVA, potassium salt giving a final concentration of 52 nmol MVA in 0.825 ml water (63 μM, 0.43 Ci $mmol^{-1}$), 10 μl sodium[^{3}H]acetate (50 nmol, 60.6 μM, 2 Ci $mmol^{-1}$), 10 μl sodium[^{3}H]acetate (50 nmol, 60.6 μM, 2 Ci $mmol^{-1}$) + 50 μl 10 mM sucrose (0.5 μmol, 0.61 mM), and 75 μl

D-[U-^{14}C]glucose (50 nmol, 60.6 µM, 296 mCi mmol^{-1}). The cuttings were incubated 24 h in natural light. Water was added to the vials as needed to keep the petioles immersed. Each leaf was frozen in liquid N_2 and then extracted by grinding with hexane. The residue was further extracted by stirring with volumes of hexane until the extracts were no longer yellow. The hexane extracts were combined and concentrated to 8 ml then backextracted with water. The hexane extract was further concentrated to 1-ml volume, and a sample from each treatment was spotted on a 20 x 20 cm 1000 µm silica gel G plate as a band and a marker spot along with a second m spot containing a mixture of caryophyllene, cedrene, and cyperene. The plates were developed in petroleum ether: CCl_4, 3:1. The region containing the sesquiterpenes (R_f 0.49 to 0.8) was scraped, the silica gel was wet with methanol, and the compounds eluted with CH_2Cl_2. A 10% aliquot of each eluted fraction was mixed with 15 ml Aquassure and counted. Bands above and below the sesquiterpene band were scraped, mixed with 5 ml water and 15 ml Aquassure, and counted as silica sols.

In the case of the MVA incorporation, the remaining sesquiterpene band eluate was concentrated and chromatographed by HPLC on 2 4.6 mm x 25 cm ODS columns (Altex) in either 100% methanol or 95:5 CH_3CN: H_2O at a flow rate of 1 ml min^{-1} and on a 10 mm x 25 cm C8 column (Altex) in 70:30 CH_3CN: IPA at a flow rate of 2 ml min^{-1}. The eluate was monitored by its A_{214}, and fractions were collected and counted. In addition, the origin of the plate was eluted with acetone, and half was rechromatographed on a 20 x 20 cm 500 µm silica gel G plate developed in 3:1 ether: petroleum ether, a system for resolving triterpenoids. The plate was divided into bands which were scraped and counted as silica sols. The remainder of the plate on which the hexane extract was spotted was also divided into bands which were scraped and counted.

The squalene and sesquiterpene containing fraction from the glucose-fed cutting was chromatographed by HPLC on 2 4.6 mm x 25 cm ODS columns in 100% methanol at a flow rate of 1 ml min^{-1}. Fractions were collected and counted.

***C. langsdorfii* cutting incubation with unlabelled MVA.** An analogous incubation to the one where cuttings were incubated with various substrates was performed. A leaf was cut from a 2.5 year-old *C. lansdorfii* under water, placed in a 1-ml vial containing 52.2 nmol MVA, potassium salt, and incubated in natural light from 9:50 am until 10:30 am the next day. The leaf tissue was ground with hexane and extracted by stirring for 6 h. The residue was reextracted overnight with hexane. All hexane extracts were combined and concentrated under a stream of N_2. The concentrated hexane extract was spotted on a 20 x 20 cm 1000 μm silica gel G plate and developed in 3:1 petroleum ether: CCl_4. The region encompassing the sesquiterpenes and squalene was scraped and eluted with CH_2Cl_2. The eluate was concentrated, filtered through a 0.5 μm Millipore filter, and chromatographed by HPLC in 100% methanol on 2 4.6 mm x 25 cm ODS columns at a flow rate of 1 ml min^{-1}. Successive injections were made in order to chromatograph all the material recovered by TLC. The putative squalene peak was collected from each injection, combined, reinjected to confirm its purity, and sent for MS analysis. Since the obtained mass spectrograph was weak, a sample of commercial squalene was sent for MS analysis to allow comparison of the two.

Analysis for endogenous pool of squalene in *C. langsdorfii*. A *C. langsdorfii* leaf of fresh weight 1.06 g was ground with hexane, and the residue was extracted repeatedly with hexane until it was clear. The hexane extract was spotted on a 20 x 20 cm 1000 μm silica gel G plate which was developed in 3:1 petroleum ether: CCl_4. The region containing the sesquiterpenes and squalene was scraped and eluted with CH_2Cl_2. The eluate was concentrated to the same volume as in the previous experiments with cuttings, and an aliquot was analyzed for squalene content by HPLC in 100% methanol on on 2 4.6 mm x 25 cm ODS columns at a flow rate of 1 ml min^{-1}.

RESULTS

Identification of sesquiterpenes in *Copaifera* tissue. TLC of hexane extracts made from leaf, stem, and petiole tissue from a seedling showed material coeluting with sesquiterpene hydrocarbon standards. GC-MS analysis of material recovered from the region of the TLC where the sesquiterpene standards coelute (R_f 0.44-0.72) indicated that sesquiterpene hydrocarbons are the predominant components.

Chlorophyll content of *Copaifera* tissue. Leaf tissue from a 1.5 year-old *C. officinalis* plant had a chlorophyll content of 0.1895 ± 0.0049 g m^{-2} (n=2). The chlorophyll content of a *C. multijuga* seedling was 0.283 g m^{-2}.

Incorporation of acetate by *Copaifera officinalis* cutting. In the initial experiment with *Copaifera* tissue, after 20 hours a *C. officinalis* leaf had incorporated 0.61 nmol acetate into hexane-extractable compounds, based on the total recovery of radioactivity from the 45% hexane extract that was chromatographed by TLC. The 10% aliquot of the hexane extract that was counted directly gave a lower value of incorporation of 0.28 nmol acetate indicating that the charcoal used to decolorize the solution before counting had adsorbed labelled material. Of the total hexane extract, 0.8% or 5 pmol of acetate was incorporated into compounds that coeluted with sesquiterpenes by TLC. R_f values for various standard compounds are listed in Table II, indicating that a number of different compounds coelute with the sesquiterpenes in this system.

Metabolite incorporation by *Copaifera* leaf discs. In the experiment following the time course of incorporation of [^{3}H]acetate into *C. officinalis* leaf discs, a maximum of 0.3 nmol acetate was incorporated into the hexane-extractables after 24 h. However, the maximum incorporation of 9 pmol acetate into the sesquiterpene TLC band occured at 6 h; the amount of radioactivity in that fraction decreased to 50% of the maximum after 24 h incubation (Fig. 2).

Table II. *R_f Values of Standard Compounds chromatographed by TLC in 3:1 petroleum ether: CCl_4*

Compound	R_f
distearin	0.735
cedrene (C_{15})	0.63-0.73
2-CH_3-pentadecane	0.71
eicosane (C_{20})	0.71
nonacosane (C_{29})	0.71
cyperene (C_{15})	0.66-0.71
pentadecane (C_{15})	0.68
caryophyllene (C_{15})	0.49-0.65
squalene (C_{30})	0.44-0.55
β-carotene (C_{40})	0.47-0.51
palmitic acid (C_{16})	0.09
farnesol (C_{15})	0.06
oleic acid (C_{18})	0.03
lanosterol (C_{30})	0.01

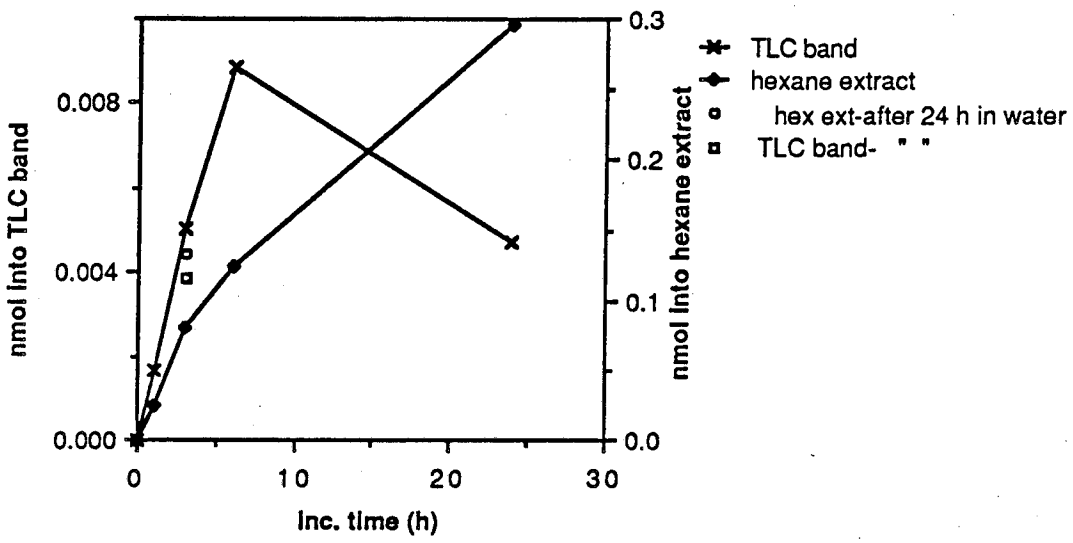


Fig. 2. Time course of acetate incorporation into *Copaifera* leaf discs.

In the first experiment testing the time course of incorporation of [^{14}C]bicarbonate, an aliquot of the hexane extract of each incubation was not counted as in previous experiments, but an idea of the total incorporation can be obtained by summing the incorporation into the three TLC bands analyzed. The whole plate was scraped from the 20 h incubation giving a value of 9.97 nmol bicarbonate incorporated into the hexane fraction, demonstrating that a good approximation of the total incorporation into the hexane extract is given by the sum of the scraped bands which gave a value of 9.76 nmol incorporated. Over the course of the incubation, the incorporation into total hexane-extractables was twenty times the incorporation into the sesquiterpene TLC band (Fig. 3). At 6 h the incorporation of acetate into the TLC band was a factor of 6 less than the incorporation of bicarbonate into that band, although acetate incorporation into the total hexane extract was twice the bicarbonate incorporation. A count of the methanol-soluble radioactivity remaining in the water fractions from the 20 h incubation after acidification and drying accounted for all the radioactivity added, indicating that all 340 nmol added bicarbonate was metabolized. Analysis of the water soluble components of the methanol extract by HPLC showed that 82 nmol bicarbonate, 24% of the total incorporation, went into

sugars.

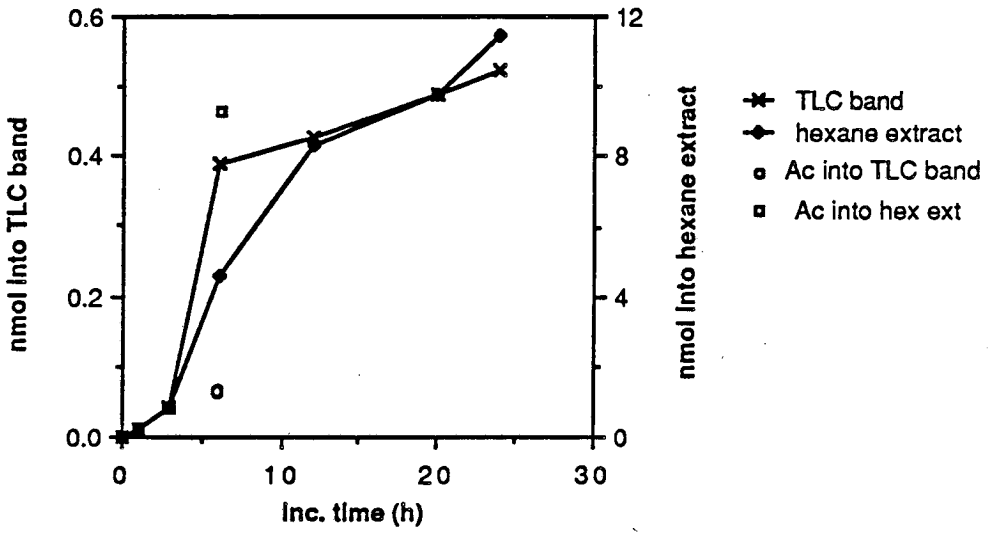


Fig. 3. Time course of bicarbonate incorporation into *C. officinalis* leaf discs.

A single 20 h incubation of [^{14}C]bicarbonate into *C. officinalis* leaf discs was repeated using buffered media and a higher concentration of bicarbonate since all 340 nmol were incorporated in the previous experiment. A total of 16 µmol bicarbonate was incorporated into the water-extractable fraction out of the 500 µmol supplied. This equals a carbon incorporation rate of 0.71 µmol m^{-2} s^{-1} or 3.8 nmol mg Chl $^{-1}$ s^{-1}. Sugars accounted for 35% of the incorporated radioactivity in the water extract or 5.6 µmol total with glucose and fructose being more heavily labelled than sucrose. The incorporation into the hexane extract was 71 nmol with 32% of that radioactivity eluting in the region of R_f 0.36 to 0.83. HPLC analysis of material recovered from the region of R_f 0.33 to 0.63 of a second TLC plate showed that only 10% of the radioactivity of that fraction or 0.15 nmol total bicarbonate was incorporated into the region where the sesquiterpene hydrocarbons elute. The recovery of radioactivity applied to the column was 37% suggesting that the majority of the label had been incorporated into more nonpolar compounds which remained on the column.

A second time course of bicarbonate incorporation into *C. multijuga* leaf discs showed incorporation on the pmolar level into the sesquiterpene band with a linear incorporation rate of 3.3 nmol carbon mg Chl^{-1} s^{-1} or 0.93 µmol carbon m^{-2} s^{-1} into methanol-extractables. Incorporation into the hexane extract was also linear at a rate of 7 pmol carbon mg Chl^{-1} s^{-1} or 1.9 nmol carbon m^{-2} s^{-1} (Fig. 4).

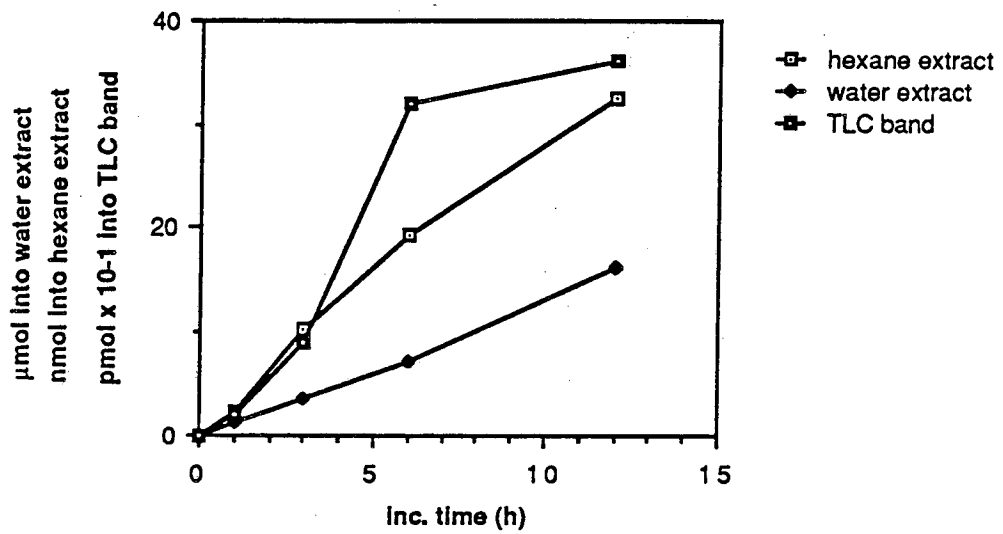


Fig. 4. Time course of bicarbonate incorporation into *C. multijuga* leaf discs.

Light saturation of *Copaifera multijuga* leaf discs. The photosynthetic rate of *C. multijuga* leaf discs saturated at a value of 0.175 µmol carbon incorporated m^{-2} s^{-1} at a light level of 1500 µE m^{-2} s^{-1} (Fig. 5).

A substrate incorporation experiment was subsequently performed at the saturating light level of 1500 µE m^{-2} s^{-1} comparing bicarbonate incorporation into *C. officinalis* and *C. multijuga* leaf discs as well as acetate incorporation by the *C. multijuga* leaf discs. The total bicarbonate incorporation (0.9 µmol C m^{-2} s^{-1}) of the *C. multijuga* leaf discs was slightly less than that of the *C. officinalis* tissue (1.2 µmol C m^{-2} s^{-1}) with total acetate incorporation being an order of magnitude lower (Fig. 6). Hexane-extractables accounted for 0.2 to 0.6 % of the total substrate incorporation; acetate-labelling gave the highest percentage of label in hexane-extractables,

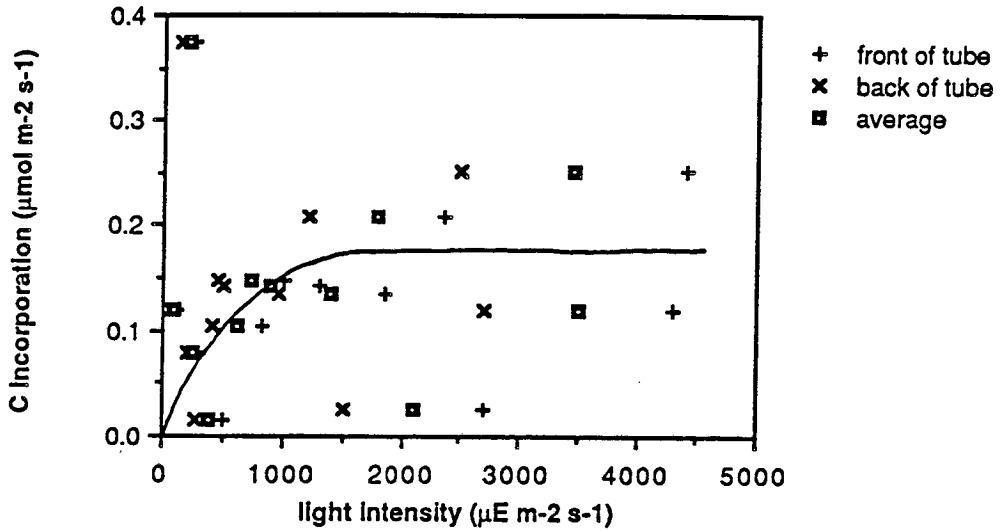


Fig. 5. Light saturation of *Copaifera* leaf discs.

twice that when bicarbonate was used as substrate. TLC analysis of the hexane extracts showed radioactivity coincident with both the sesquiterpene hydrocarbon and squalene bands. In all three incubations, squalene was more heavily labelled than the sesquiterpenes, accounting for 4 to 5 % of the label in the hexane extract. A greater percentage of the label in the hexane extract from acetate than from bicarbonate was incorporated into both the sesquiterpene and squalene bands by the *C. multijuga* leaf discs. Incorporation of bicarbonate into "squalene" by the two *Copaifera* species was comparable, but the incorporation into "sesquiterpenes" was five times higher by *C. multijuga* than by *C. officinalis.*

Incorporation of various substrates into *C. langsdorfii* cuttings. Incorporation of various substrates supplied for 24 h to *C. langsdorfii* cuttings into the sesquiterpene hydrocarbon TLC band (R_f 0.49 to 0.8) varied greatly (Table III). The best substrate was mevalonic acid with 5.8 nmol incorporated. Acetate and glucose incorporation was on the pmolar level. Acetate incorporation was 8.5 pmol, lowered to 2.6 pmol in the incubation where 0.5 µmol sucrose was also supplied to the cutting. Glucose incorporation was 13 pmol. HPLC analysis of the MVA-labelled fraction showed that 1.9% of the total label elutes with the sesquiterpene

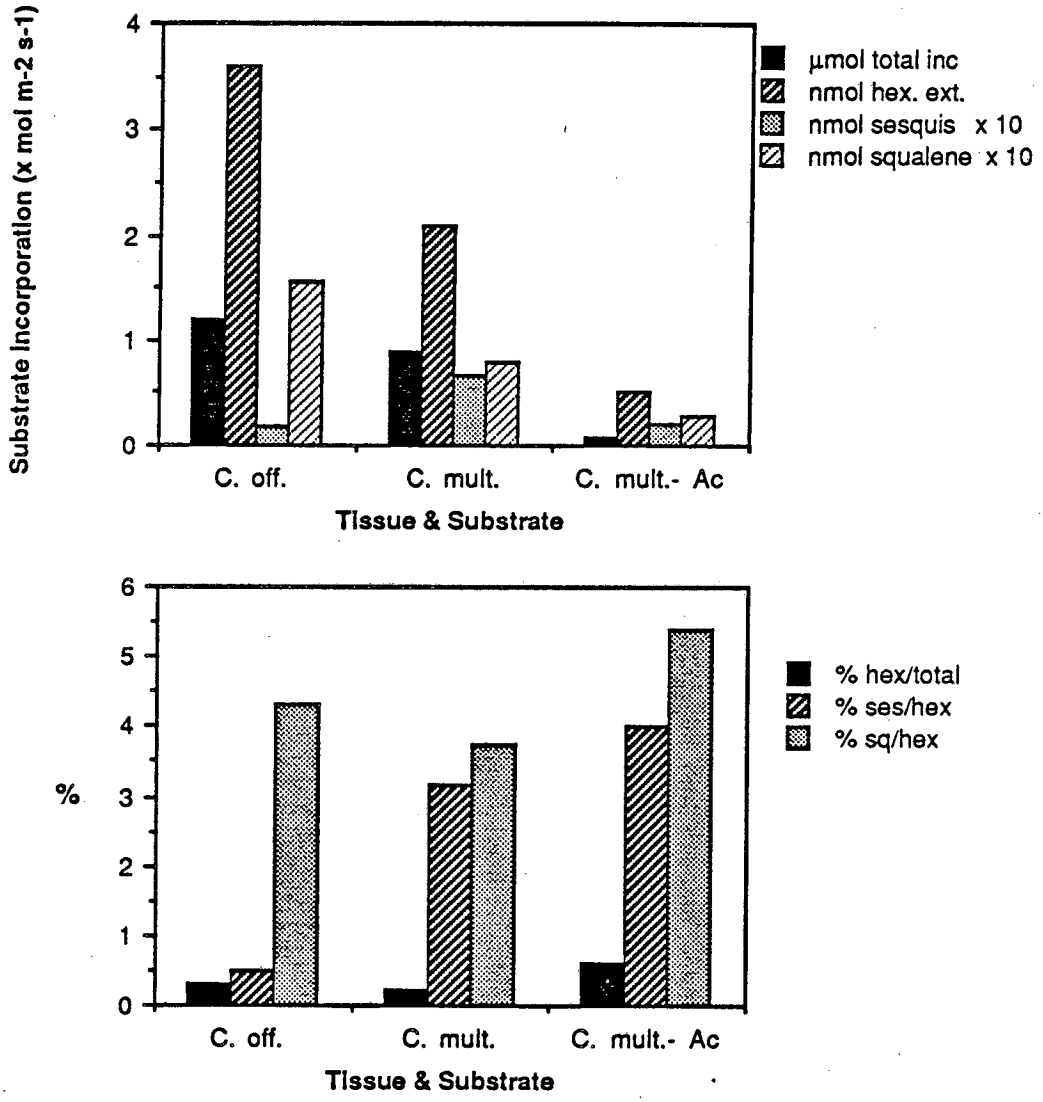


Fig. 6. Comparison of bicarbonate and acetate incorporation into total hexane extractables, sesquiterpene hydrocarbons, and squalene by *C. officinalis* and *C. multijuga* leaf discs in 5 h.

hydrocarbons; 0.11 nmol MVA had been incorporated into these compounds. Further analysis showed that 92% of the label in the fraction was coincident with a single peak which coeluted with a squalene standard. Similar HPLC analysis of the acetate-labelled fraction established that 55%

and 22% of the radioactivity was associated with squalene and the sesquiterpenes, respectively; for the glucose-labelled sample, the distribution was 61% and 13.5%. Collection of the putative squalene for MS analysis verified that the material was squalene. Quantification using HPLC established that the endogenous squalene content of a *C. langsdorfii* leaf was 0.2 mg g fresh wt^{-1}. The origin from the TLC system used to elute the sesquiterpenes and squalene was recovered and rechromatographed by a TLC system designed for isolation of triterpenoids. Based on this, 7 nmol MVA was incorporated into triterpenes and their esters. The total incorporation into the hexane extract was 19 nmol MVA.

Table III. *Distribution of incorporated substrates among various fractions and components of Copaifera langsdorfii cuttings.*

Substrate	hexane extract	TLC fraction	Sesquiterpenes	Squalene	Triterpenoids
MVA	19 nmol	5.8 nmol	0.11 nmol	5.3 nmol	7 nmol
Acetate		8.5 pmol	1.9 pmol	4.7 pmol	
Glucose		13 " "	1.8 " "	7.9 " "	
Acetate + sucrose		2.6 " "			

DISCUSSION

The two forms of *Copaifera* tissue (leaf cuttings and leaf discs) used in these experiments require different means for the substrates to reach the site of terpenoid biosynthesis. With cuttings, uptake of the metabolites is required; with leaf discs, the substrate is infiltrated into the

leaf tissue. Francis promoted the use of leaf discs to study essential oil biosynthesis because this technique could reduce the problems involved in getting the metabolites to the site of terpenoid biosynthesis and allows easier manipulation of the conditions under which the incubations are made (9). In the case of these experiments with *Copaifera* leaf discs, the overall incorporation of [^{14}C]bicarbonate was taken as a measure of the appropriateness of this system. Under saturating light levels of 1500 μE m^{-2} s^{-1}, *C. officinalis* leaf discs incorporated 1.2 μmol C m^{-2} s^{-1} which, although lower than the average photosynthetic rate of C_3 plants of 10 to 25 μmol C m^{-2} s^{-1}, is in the range of the rate measured by Langenheim of *Copaifera* in the field of 7 to 8 μmol C m^{-2} s^{-1}(14). The light level that was found to be saturating for bicarbonate incorporation agrees with Langenheim's data that *Copaifera* is a sun plant. On the basis of overall metabolism, the leaf discs appear to mimic well the response of the plant under normal field conditions.

The time course of acetate incorporation into the "sesquiterpene" TLC band by *C. officinalis* leaf discs indicates that the same short term turnover of sesquiterpenes is occuring in *Copaifera* as was seen with peppermint cuttings and *P. cablin* leaf discs (5,9)(Fig. 2). At the same time, acetate incorporation into total hexane-extractables continued to increase over the course of the experiment. Since the incubation where the leaf discs sat 24 h in water before the acetate was added and were then incubated an additional 3 h showed incorporation similar to the initial 3 h time point, the view that this turnover is a natural occurence, rather than due to senescence of the leaf material after 24 h incubation, is substantiated. That no such turnover, rather a leveling-off of incorporation into the "sesquiterpene" TLC band after 6 h incubation, was seen when bicarbonate was the added precursor is probably due to the long path to sesquiterpenes from this precursor and the conditions of continuous feeding of the substrate (Fig. 3). In experiments with peppermint cuttings, a maximum of $^{14}CO_2$ incorporation into sesquiterpenes was seen at 9 h after a 1 h pulse of the labelled substrate was given to the plant material (6). In this experiment with the *C. officinalis* leaf discs, acetate incorporation at 6 h was six times less than the bicarbonate incorporation into the "sesquiterpene" band indicating that there was some factor

preventing acetate from reaching the site of sesquiterpene biosynthesis. With peppermint cuttings, it was found that glucose and CO_2 served as much better precursors for monoterpene biosynthesis than for sesquiterpene synthesis with the accessability of MVA to sesquiterpene biosynthesis being greater than to the site of monoterpene synthesis (5,6). In a second bicarbonate incorporation time course experiment, again a leveling-off of incorporation into the "sesquiterpene" TLC band was seen even as the incorporation into the hexane and water extracts continued to rise linearly (Fig. 4).

Both the age and species differences of the plant material may account for the results of the experiment comparing bicarbonate incorporation into leaf discs of *C. officinalis* and *C. multijuga* with an acetate incorporation into *C. multijuga* leaf discs over a period of 5 h (Fig. 6). That acetate incorporation was again lower than bicarbonate incorporation into terpenoid compounds supports the view that acetate was not able to reach the site of synthesis as easily as bicarbonate. The greater incorporation of bicarbonate into sesquiterpenes of *C. multijuga* than in *C. officinalis* can be attributed to either the species or age differences of the plants. Leaf discs were cut from a 1.5 year-old *C. officinalis* and a 5 month-old *C. multijuga* seedling. The younger tissue may be more actively synthesizing sesquiterpenes.

The experiments with leaf cuttings further showed what the final leaf disc experiment indicated, that incorporation of exogenous precursors into squalene and triterpenoids predominates over incorporation into sesquiterpenes (Table III). This same situation was seen when [^{14}C]mevalonate was fed to peppermint leaf cuttings (8). The preferential labelling of squalene seen from all three precursors tested: glucose, acetate, and mevalonate, can be described most simply as a difference in the accessability of the site of synthesis of squalene and triterpenoids from the site of sesquiterpene biosynthesis. Even with the leaf disc experiments where the need for some mode of transport of the metabolites to the leaf tissue has been eliminated, incorporation into squalene of both bicarbonate and acetate appears to override incorporation into sesquiterpenes. One possible explanation is that the site of sesquiterpene

synthesis is truly physically compartmentalized which is supported by anatomical data showing that the resin is secreted by specialized epithelial cells into the leaf pockets which hold the resin (11). A second possibility is the flow of carbon into the various terpenes is regulated physiologically. Since the sesquiterpenoids and triterpenoids share the common precursor farnesyl pyrophosphate, regulation may be at this point; this would fit with these results which showed that all precursors tested, from bicarbonate to mevalonate, labelled squalene more heavily than the sesquiterpenes. However, while the ratio of incorporation of acetate and glucose into squalene as compared to incorporation into sesquiterpenes was 2.5 and 4.4 to 1, respectively, fifty times as much MVA was incorporated into squalene as into sesquiterpenes, suggesting that some control of the flow of carbon into the two sets of compounds may lie on the isoprenoid pathway between acetate and mevalonate.

Mevalonate incorporation into the sesquiterpenes of a *Copaifera* cutting was 100-fold greater than the incorporation of either acetate or glucose. This again raises the question of the accessability of substrates to the site of sesquiterpene synthesis. *Copaifera* appears like *Mentha* in that mevalonate was also the best exogenous precursor for sesquiterpene biosynthesis in that plant (5). Incorporation of [^{14}C]mevalonate was also ten times greater than $^{14}CO_2$ incorporation into the sesquiterpene hydrocarbons of maritime pine needles, but [^{14}C]acetate incorporation was nearly as efficient as mevalonate's (10). These differences suggest that there is no single answer to the nature of the site of sesquiterpene biosynthesis in plants.

Unlike in peppermint cuttings, these results indicate that sesquiterpene biosynthesis in *Copaifera* does not appear to require sugars as an energy source. When unlabelled sucrose was supplied along with [^{3}H]acetate to a *C. langsdorfii* leaf cutting, acetate incorporation into the "sesquiterpene + squalene" TLC band was less than when a cutting was incubated with acetate alone (Table III). In contrast Croteau, Burbott, and Loomis found that added sucrose promoted [^{14}C]mevalonate incorporation into sesquiterpenes of peppermint cuttings, leading them to suggest that sesquiterpene biosynthesis occured in isolated energy-deficient sites and is a

fermentative process (7). In *Copaifera* it appears that sucrose can compete with acetate in terpenoid biosynthesis thus diluting the [^{3}H]acetate label incorporated. This is further substantiated in that glucose is incorporated into sesquiterpenes as readily as acetate is. However, a direct comparison between the experiments with *Copaifera* and *Mentha* cannot be made since sucrose was incubated with different terpenoid precursors in the two cases; while it may be that the incorporation of mevalonate into terpenoids in *Copaifera* is energy-deficient, with supplied acetate there is sufficient available energy. This would suggest that the energy for the process is achieved through acetate metabolism by the citric acid cycle as opposed to from sugars through glycolysis or the pentose phosphate pathway.

CONCLUSIONS

The ability of *Copaifera* tissue to incorporate terpenoid precursors, especially mevalonate, into sesquiterpenes makes further study of this system possible. Because different sesquiterpene-producing plants show different precursor preferences, it appears that there exist significant differences in the nature of the site of sesquiterpene biosynthesis in various plants. Since it is unique in its mass production of sesquiterpene hydrocarbons, *Copaifera* continues to be of interest. Although tracer incorporation into the sesquiterpenes is on the 0.1 nanomolar level, it would be possible to continue the initial aim of this work to look at turnover of these compounds. This work has shown that turnover of sesquiterpenes in *Copaifera* does occur in leaf discs labelled with acetate or bicarbonate. Since mevalonate incorporation in cuttings was 100-fold greater than acetate incorporation, a time course of mevalonate incorporation should be investigated. Individual sesquiterpene labelling patterns can be analyzed by HPLC or RGLC. The intermediate role of acyclic *trans*-β-farnesene in sesquiterpene biosynthesis in maritime pine needles was determined in this manner (10).

The discovery that mevalonate is selectively channeled to squalene and triterpenoid biosynthesis in *Copaifera* presents an opportunity to study the controls of the flow of carbon to

two sets of isoprenoids, sesquiterpenes and triterpenes. Further work comparing the incorporation of various precursors, especially farnesyl pyrophosphate, the immediate precursor to both classes of terpenoids, into the two groups of compounds could provide insight into the nature of the regulation of the distribution of carbon.

The differences in incorporation by different species of *Copaifera* require that further experiments be performed with as uniform plant material as possible. It would be interesting to compare incorporation into sesquiterpenes of plant material of different ages. This could best be achieved by using material from the same plant, but of different ages, since the available species and ages of *Copaifera* are limited.

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