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HYDROCARBONS AND ENERGY FROM PLANTS

Final Report
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Abstract

Plant hydrocarbon (isoprenoid) production was investigated as an alternative source to fossil fuels. Because of their high triterpenoid (hydrocarbon) content of 4-8%, *Euphorbia lathyris* plants were used as a model system for this study. The structure of the *E. lathyris* triterpenoids was determined, and triterpenoid biosynthesis studied to better understand the metabolic regulation of isoprenoid production.

Triterpenoid biosyntheis occurs in two distinct tissue types in *E. lathyris* plants: in the latex of the laticifer cells; and in the mesophyll cells of the leaf and stem. We have fractionated the latex by centrifugation, and have determined that the later steps of isoprenoid biosynthesis, the conversion of mevalonic acid to the triterpenes, are compartmentized within a vacuole. We have also identified the conversion of hydroxymethyl glutaryl-CoA to mevalonic acid, catalyzed by the enzyme Hydroxymethyl glutaryl-CoA Reductase, as a key rate limiting step in isoprenoid biosynthesis. At least two isozymes of this enzyme, one in the latex and another in the leaf plastids, have been identified.

Environmental stress has been applied to plants to study changes in carbon allocation. Salinity stress caused a large decrease in growth, smaller decreases in photosynthesis, resulting in a larger allocation of carbon to both hydrocarbon and sugar production. An increase in Hydroxymethyl glutaryl-CoA Reductase activity was also observed when isoprenoid production increased.

Other species where also screened for the production of hydrogen rich products such as isoprenoids and glycerides, and their hydrocarbon composition was determined.

Executive Summary

Because the availablity of fossil fuels is finite, and because their combustion leads to increases in atmospheric carbon dioxide, we have searched for alternative sources of liquid fuels. Our search has centered on the energy-rich, photosynthetically-derived, secondary products of plants, specifically the terpenes. We have screened many species for hydrocarbon content, and have selected the *Euphorbia lathyris* plant for our studies.

Euphorbia lathyris grows wild in California, completing its life cycle in one to two years. It is adapted to semi-arid environments, and produces high levels of triterpenes (4-9% of its dry weight) while still in the vegetative stage. About 20% of these hydrocarbons are found in the milky latex of the laticifer cell. The latex maintains biological activity after its removal from the plant, and can be used to study the mechanism and regulation of triterpene biosynthesis. It is the goal of our program to develop our understanding of the terpene biosynthetic pathway to the point that we can genetically manipulate the plants and increase hydrocarbon yields, thus producing an economically viable crop.

Metabolic control of carbon allocation to the production of hydrocarbons can occur via subcellular and tissue level compartmentation, biological feedback by other cellular components, and enzyme expression and activation. We have used the latex from the *E. lathyris* plants to study each of these systems.

The triterpene biosynthetic pathway starts with acetyl-CoA and joins three of these molecules together to form the five carbon isopentenyl pyrophosphate (IPP) molecule. The IPP molecule then acts as a building block to construct the various terpene molecules. In the case of the *E. lathyris* plants, six of the IPP molecules are combined to produce the thirty carbon triterpenes that comprise the majority of the hydrocarbon fraction. By combining rate of sedimentation and isopycnic centrifugation on Percoll gradients, we have determined that the steps of the pathway are divided between two subcellular locations. The later steps of the reaction, the conversion of mevalonic acid (MVA) into the triterpenes, can thus be physically separated from the early steps, the conversion of acetyl-CoA into hydroxymethyl glutaryl-CoA (HMG-CoA). The mevalonic to triterpene steps are sequestered within a membrane-bounded particle. With the aid of electron microscopy and marker enzymes we have identified this particle as a vacuole. We have also isolated two other latex structures: a small particle that is the storage site of the triterpenes, and a starch grain that is possibly sequestered within a plastid.

The triterpenes exist in two forms in the latex, both as the free alcohols and the fatty acid ester sof the alcohols. The ratio of the triterpene esters to the free alcohols is about 6:1. We have determined that S-adenosyl methionine is involved in the production of the fatty acid esters; production of triterpenols from mevalonic acid with the isolated vacuole results in a ester to free alcohol ratio of about 1. The re-addition of S-adenosyl methionine to the reaction mixture restores the ratio back to normal levels.

Feedback inhibition of the early steps of terpene biosynthesis by the end products has been found in mammalian systems. To determine if the triterpenes in *E. lathyris* latex play any role in the regulation of their biosynthetic pathway, we first had to separate, purify, and identify the triterpenes present in the latex. We accomplished the separation and purification by a combination of solvent extraction and partitioning, TLC, and HPLC. Identification of the compounds was done by mass spectroscopy, ^{1}H-NMR, and ^{1}H-NMR with lanthanide shift reagents. We found six major triterpenes in the latex: euphol, lanosterol, cycloartenol, 24-methylenecycloartenol, 24-methylenelanosterol, and butyrospermol. S-adenosyl methionine is utilized in the production of 24-methylene lanosterol and cycloartenol.Further work is necessary to determine if any of these compounds have allosteric effects on any of the terpene biosynthetic pathway enzymes.

We have used terpene biosynthetic intermediates as substrates to study the rates of various steps

along the pathway. By comparing these rates, we have identified the conversion of HMG-CoA to MVA as a key, rate-limiting step. We have centered our investigations of the enzymatic regulation of the pathway on the enzyme that catalyzes this step, Hydroxymethyl glutaryl-CoA Reductase (HMGR).

Our initial HMGR work was done with latex. We found both HMGR and a competing enzyme, HMG-CoA lyase in the latex. The HMGR was membrane-bound, while the lyase activity was soluble, indicating that some regulation by compartmentation occurs. The pH for optimal activity was found to be 5.8. Further purification of the enzyme was inhibited by the presence of high levels of phenol oxidases and proteases in the latex.

To obtain greater amounts of HMGR we made extracts of stem and leaf tissue. The recovery of activity was 1000-fold greater than from latex. The HMGR was localized in the plastid and exhibited optimal activity at pH 6.8; both of these characteristics are different in the latex HMGR, which indicates that at least two distinct isozymes of HMGR are present within the plant. The HMGR was membrane-bound, and could be solubilized with Brij detergent. The inclusion of the thiol protease inhibitor leupeptin or the serine protease inhibitor PMSF in the incubation mixture increased the recoverable activity of the enzyme. We are currently using the leaf extract to obtain HMGR for purification.

We are also interested in the physiological mechanism that controls carbon allocation to the terpene biosynthetic pathway. We have utilized environmental stress to change the patterns of carbon allocation within our plants, and to monitor the physiological and biochemical adjustments that accompany these changes. We have found that low levels of salinity stress (< 50 mM NaCl) will significantly reduce plant growth while photosynthesis remains high. This stress resulted in a condition of reduced demand for both fixed carbon and chemical reductants, while their production remained high. The plants responded to this condition by increasing their carbon flux into both hydrocarbons and sugars. Concurrent increases were also measured in HMGR activity, providing further evidence that the activity of this enzyme limits the production of the hydrocarbons.

Water stress also caused increases in hydrocarbon production, though plant growth remained unchanged. This result indicated that the control of carbon allocation is not simply a response to demands by the various carbon sinks.

We screened other species in addition to *Euphorbia lathyris* for hydrocarbon production. We examined sesquiterpene biosynthesis in *Copaifera*; the sap of these Brazilian trees has a potential use as a diesel substitute. We characterized the composition of the fruit of the *Pittosporum resiniferum* tree of the Philippines. The hydrocarbon component of this fruit consists of both monoterpenes and alkanes, and could be used as a gasoline substitute or additive. We also determined the structures of the lignins of the *Myristica otoba* fruits, and determined the fatty acid composition of the seed oils from both the *M. otoba* fruits and from Macuaba Palm oil.

Figures

Tables

Abbreviations

Ac	acetate
AcAc	acetoacetate
AS	ammonium sulfate
DTE	dithioerythritol
FID	flame ionization detector
FPP	farnesyl pyrophosphate
F16BP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
F26BP	fructose 2,6-bisphosphate
HMG-CoA	ß-hydroxymethyl glutaryl-Coenzyme A
HMGR	ß-hydroxymethyl glutaryl-Coenzyme A reductase
IPA	isopropyl alcohol
IPP	isopentenyl pyrophosphate
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MVA	mevalonic acid
MVAL	mevalonic acid lactone
OA	organic acid
ODS	octa decyl silyl
PEG	polyethylene glycol
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TP	triterpenol
TPE	triterpenol-fatty acid ester

Chapter 1

INTRODUCTION

Scott Taylor
Melvin Calvin

This report covers the work performed at the Lawrence Berkeley Laboratory from April 1, 1984 thru December 31, 1987 on plant-derived hydrocarbons as an alternative energy source to fossil fuels. In addition to this introduction, it is comprised of twelve chapters which are individual manuscripts that have already been published, have been submitted for publication, derived from a graduate thesis, or are comprised of data that has not been previously prepared in manuscript form.

We have subdivided this work into six sections. The first section is an introduction to the field of liquid fuels from plants, and includes a paper on plant-derived fuels and a report on general method of determining biomass energy content from elemental analysis. We have also prepared additional reviews on plant-derived fuels, and these are listed in the final section of this report.

The second section consists of two papers concerned with isoprenoid biosynthesis in the latex of the *Euphorbia lathyris* plant. We have chosen this plant because it grows wild in California, it is able to withstand arid conditions, it has a high hydrocarbon content (4-8% of the dry weight), and its life cycle is completed in one to two years. In addition, it provides an ideal model system for the study of isopreoid biosynthesis: we can tap hydrocarbon-containing latex from the plant and it retains its isoprenoid biosynthetic capacity. In the two chapters we report on isoprenoid biosynthesis and the structure-function relations of particles isolated from the latex, and on the chemical structures of the major isoprenoids found in the latex. In addition, we have included an addendum containing important data concerning latex activity that was not included in the manuscripts.

The next section is concerned with a specific enzyme: Hydroxy- methylglutaryl Coenzyme A Reductase (HMGR), which we have found to catalyze a key rate-limiting step in the biosynthesis of isoprenoids. The two chapters in this section are abridged versions of chapters from Cynthia L. Skrukrud's doctoral thesis, and are concerned with HMGR in latex and in shoots. This work will be submitted for publication in scientific journals.

The fourth section deals with the effects of the environmental stress on carbon allocation to hydrocarbon production. The application of stress can be used to study the regulation of plant metabolism, and may also lead to agronomic techniques were controlled applications of stress can be used to increase yields. This section consists of a chapter dealing with the effects of salinity on hydrocarbon production, and an addendum containing further salinity stress data and water stress data not included in the chapter.

In the fifth section we report on our work in screening species other than E. lathyris for production of high levels of hydrocarbon for potential exploitation as energy alternatives. This section consists of a published paper on *Myristica* lignans, a chapter out of C.L. Skrukrud's thesis on *Copeifera* isoprenoids, and an addendum containing analyses of other species.

The final section contains a list of personnel associated with this project, and a list of publications supported by this grant.

SECTION 1

Overview of Plant Hydrocarbons as Fuels

Chapter 2

FUEL OILS FROM EUPHORBS AND OTHER PLANTS

Melvin Calvin

ABSTRACT

The increasing energy costs of finding petroleum, together with the sure knowledge that its supply is finite, has prompted us to seek other sources of liquid hydrocarbon for both fuel and material. We have turned to annually renewable plant sources such as seed oils, an obvious source, with palm oil as the most productive. Sugar cane used to produce ethanol is another fuel source already in use.

We have examined non-food plants which can be grown on marginal soil for their productivity, particularly the genus *Euphorbia* . All species of this genus produce a latex which can be converted into useful fuel and other material, including precursors for what might be a valuable anti-tumor agent. Euphorbias and other similar plants require repeated planting and harvesting of the entire plant, which constitutes a drain on the soil. Trees can be long-term sources for hydrocarbon-like materials with a single planting. Examples are; the genus *Copaifera* which can be tapped for sesquiterpenes; the genus *Pittosporum* which bears fruits rich in terpenes and can be harvested annually. Finally, there are algae whose oil productivity is already of interest.

It seems possible to modify genetically the terpene biosynthetic pathways in plants to improve both the quality and quantity of the oils produced from them.

appeared in: *Botanical Journal of the Linnean Society* **94**: 97 (1987).

INTRODUCTION

With the oil embargo of the early 1970s we were forced to concern ourselves with matters of fuel resources (Calvin 1976, 1977, 1979, 1980, 1983a, 1984, Lipinsky 1981, Calvin et al. 1983, McLaughlin et al. 1983). The price of oil today is an aberration which will also not last very long and should not divert us from developing domestic sources along renewable avenues.

The first thing suggested by many economists is that we should turn our attention to using coal in a more efficient and environmentally satisfactory manner. There is an environmental consequence of the use of any fossil fuel, but especially coal, which cannot be eliminated: that is the fact that when fossil carbon is burned enormous amounts of carbon dioxide are produced. Even within the last 50 years when most of our fossil combustion has not been carbon (coal) but hydrocarbon (oil/gas) the carbon dioxide level in the atmosphere has risen. This is a consequence which would accelerate if coal was used as a major fuel source, either indirectly in the form of a liquid fuel, or directly. Burning coal produces approximately twice the carbon dioxide per unit energy as hydrocarbon, where both carbon and hydrogen are burned.

In the last few years we have been dominated by hydrocarbons rather than coal, and even under those circumstances the rate of production of carbon dioxide in the atmosphere has been roughly twice as great as the rate at which it is being removed by both the oceans and the biosphere (Sundquist & Broecker 1985, MacCracken & Luther 1985). This global trend is illustrated in Figure 1, which shows the variation of carbon dioxide concentration in the atmosphere during the past geological time scale as well as a modern human one. One hundred years ago the carbon dioxide concentration in the atmosphere was approximately 290 ppm and the concentration today is 315 ppm. The expectation is that the concentration will continue to rise.

It is possible to detect the warming consequences of rising carbon dioxide concentration by using a device which integrates the temperature fluctuation over a long period of time. There are two ways this can be done, one by examining the size of the polar ice-caps and glaciers from satelite pictures, and also from surface measurements: these indicate that the Antartic ice-cap has decreased substantially in the last 100 years. If substantial quantities of ice have melted from the polar ice-caps and glaciers, there should be a rise in sea-level (Figure 2) which would thus be related to increased combustion of fossil fuel (Gormitz et al. 1982). The evidence is available that the earth temperature is increasing and the consequences of that increase are measurable. As a result of the increased temperature and loss of coastal areas due to higher ocean levels, there will be very severe world-wide consequences for agriculture: the agricultural pattern across the surface of the earth will change markedly. For example, the plains of the United States of America might no longer be capable of growing grain; nothern Canada and the Soviet Union would become the chielf sources of grain for the world. It will be very difficult for the human race to adjust to such an enormous change in agricultural patterns in the 20-30 years which will elapse for this rise in sea-level to manifest itself catastrophically.

HYDROCARBON-PRODUCING HERBS

We must look for our liquid fuel (hydrocarbons) in the form of a renewable resource that can be grown each year. There is one plant whose cultivation for the purpose of renewable fuels and materials is already underway, i.e. the sugar cane in Brazil (Geller 1985). In 1975, the Brazilians produced 700 million liters of ethanol from sugar cane and in 1985 the production was 7 billion liters. The Brazilians are producing more than 20% of their total liquid energy needs from alcohol and they are beginning to create a chemical industry, the sucrochemical industry, based on this energy source.

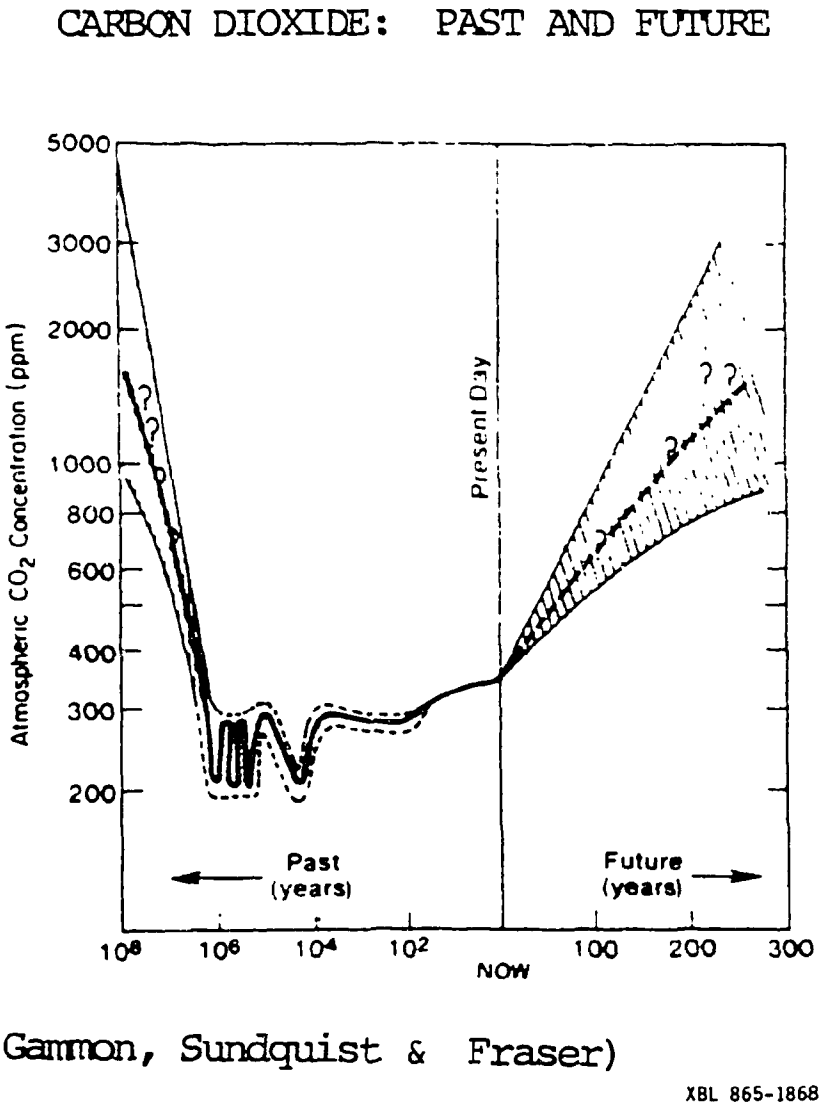


Figure 1. Concentration of CO_2 in the earth's atmosphere, past and future (redrawn, with permission, see Kerr 1986).

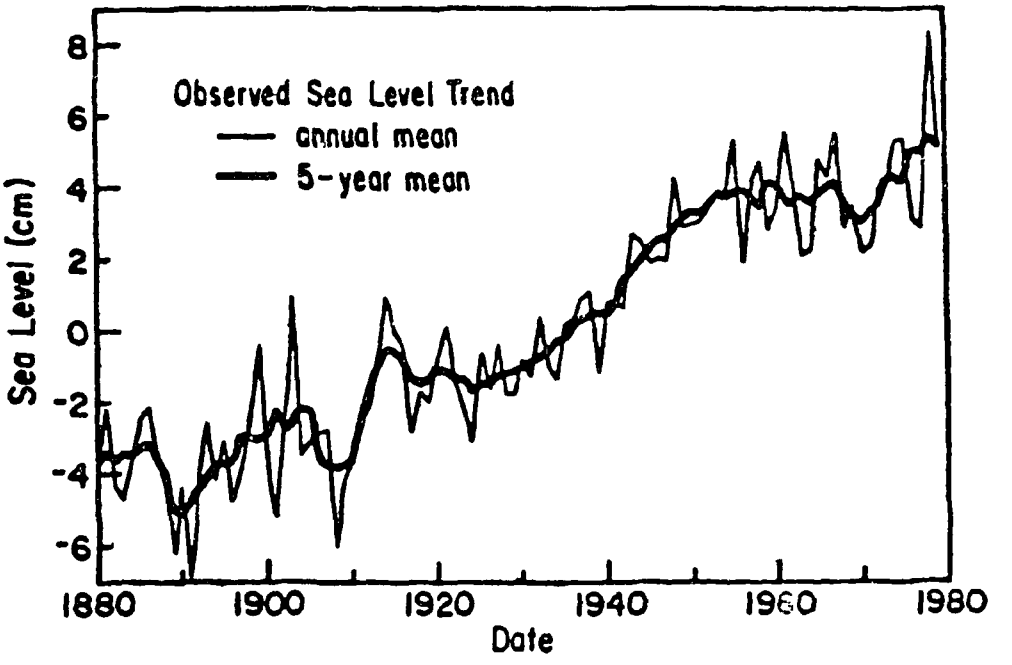


Figure 2. Global mean sea level trend based on tide guage measurements.

It seemed worthwhile to look for plants that would carry out the reduction of carbon dioxide all the way to hydrocarbon instaed of half-way to carbohydrate (Calvin 1977, 1979, 1980, 1983a, 1984, 1985; Lipinsky 1981, McLaughlin et al. 1983, Nemethy 1984). This process is exemplified in the seed oils which have substantial possibilities. The oil content and average oil yield for some oil crops is shown in Table I, with palm oil the most productive. Peanuts, safflower and sunflower are the most important seed crops in the United States of America, and all of them produce triglycerides. The oil content of the seeds represents a reasonable opportunity for renewable fuels (American Society of Agricultural Engineers 1982, Princen 1983, Schultz and Morgan 1984, Harrington 1986). Sunflower cultivation has been expanded in the United States as a real possibility to provide diesel fuel for farm machinery. The oil itself, a triglyceride (glycerine with three fatty acid chains attached to it), as it comes from the seed is not a very satisfactory diesel fuel, but by treating it with methanol the fatty acids can be transesterified so the byproduct is free glycerine and the methyl esters of the fatty acids. This latter material can be used directly as a diesel fuel.

There are also plants which take the initially produced carbohydrate and instead of converting it to fatty acids and glycerides (such as the seed oils) convert it into terpenes. The most important commercial plant of this type is *Hevea brasiliensis* (Willd. ex Adr. Juss.) Muell. Arg., a member of the Euphorbiaceae which makes polyisoprene rubber. Other members of the Euphorbiaceae produce hydrocarbons, especially the genus *Euphorbia* which has 2000 species of all sizes which grow throughout the world. Every *Euphorbia* species contains a latex which is an emulsion of about 30% terpenes in water. The latex hydrocarbon is largely a C_{30} triterpenoid which can be cracked like oil to make high octane gasoline (Weisz & Marshall 1979). The zeolite catalyst cracking of the crude oil from *Euphorbia lathyris* L. resulted in the usual group of products, similar to those obtained from standard cracking of petroleum, such as olefins, paraffin, aromatics and nonaromatics. This information confirms the desirability of the products of *E. lathyris* as possible raw materials to substitute for crude oil.

We started experimental cultivation of Euphorbias in California about 10 years ago and *E. lathyris* was the first hydrocarbon-producing plant studied to test the hypothesis that plants could be grown for fuel and chemical content in marginally suitable land (Calvin 1977, 1978, 1979, Nielsen et al. 1977). The entire *E. lathyris* plant is harvested, and from the harvest up to 8% of the dry weight of the plant is extracted as terpenes (oil), 20% of the dry weight as fermentable sugars, which leaves a residue of about 65% as lignocellulose. The terpenes can be cracked like crude oil and the sugar can be fermented like sucrose, while the lignocellulose can be used in a way similar to the bagasse of sugar cane. The products of the extraction of *E. lathyris* represent a new possibility for a future energy and materials source. The conceptual processing sequence to recover terpenoids and sugars is shown in Figure 3 (Nemethy et al. 1979, 1981ab). The oil from *E. lathyris* is black and tarry and resembles crude oil and consists mostly of triterpenes which are steroids and steroid esters (C_{30} compounds).

Some of the latex sterols of the *E. lathyris* latex are important in the pharmaceutical industry and could conceivably be of more value than the actual crude oil obtained. A very small percenatge of the latex hydrocarbons consist of C_{20} compounds, some of which are related to ingenol (Figure 4). Certain ingenol esters are potent stimulants for cell division and also have an irritant property which is harmful to the skin, mucuous membranes of the nose, eyes, etc. (Adolf & Hecker 1975, Bissell et al. 1981, Furstenberger & Hecker 1985). If ingenol is converted into the dibenzoate ester it becomes an anti-tumor agent (Kupchan et al. 1976). There is a definite relationship between the two biological effects. The stimulant molecule has a long chain on it which stimulates cell division. The binding sites are presumably the same for the dibenzoate dibenzoate ester, probably determined by the structure of the C_{20} skeleton. For the dibenzoates of the basic structure of ingenol there is no surface-active component and presumably the material binds to the binding site and reverses the stimulation of cell growth and thus has an effect on leukemic mice.

Table I

Oil content and average oil yield for some oil crops

Oil Crop (Location)	Oil Content (wt %)	Average Oil Yield (kg/ ha)
Palm Oil (Malaysia)	20	3475
Copra (Phillippines)	65-68	800
Peanuts (U.S.A.)	45-50	790
Safflower (U.S.A.)	30-35	762
Sunflower (U.S.A.)	40-45	589
Rapeseed (Canada)	40-45	409
Soybean (U.S.A.)	18-19	319
Corn kernel (U.S.A.)	4-8	254
Flaxseed (U.S.A.)	35-42	230
Sesame (India)	45-50	220
Cottonseed (U.S.A.)	18-20	140

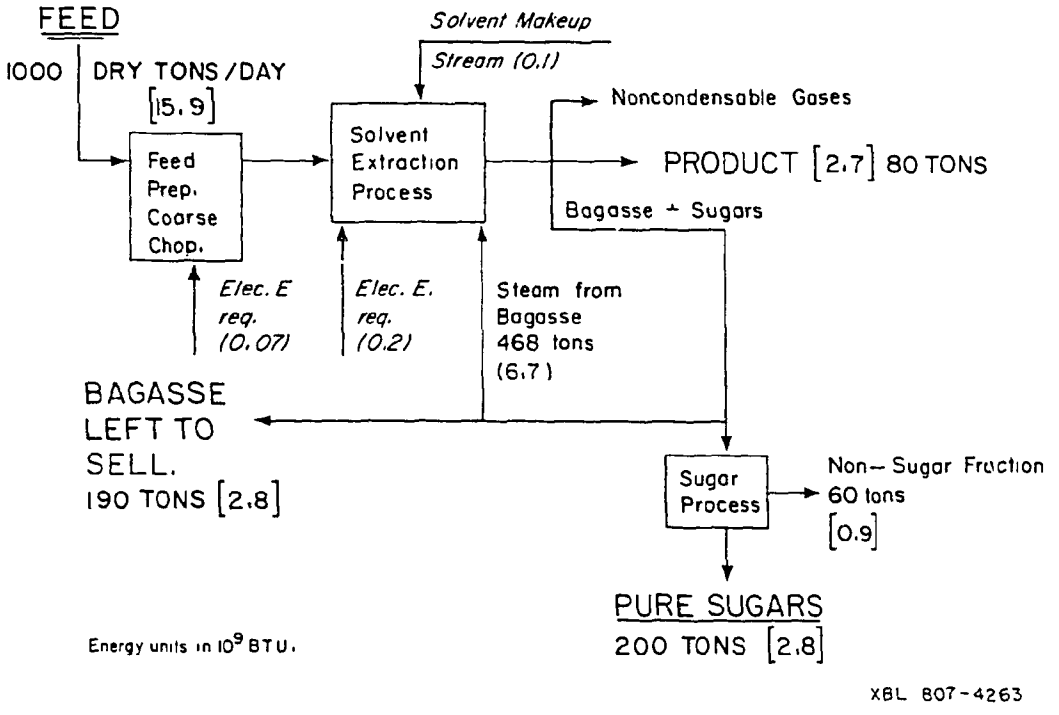
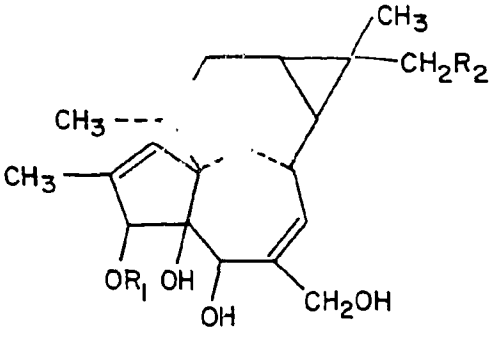


Figure 3. Conceptual processing sequence to recover terpenoids and sugars from *Euphorbia lathyris*.



Active Esters of Ingenol:

R_1 = hexadecanoate
R_2 = H

R_1 = 3-tetradeca-2, 4, 6, 8, 10-pentaenoate
R_2 = H

Inactive:

16-hydroxy ingenol: R_2 = OH

W. Adolf and E. Hecker Z. Krebsforsch, 1975

XBL 815-4637

Figure 4. Ingenol esters found in *Euphorbia lathyris* (reproduced, with permission, from Adolf and Hecker, 1975).

BIOSYNTHETIC PATHWAYS

It might be possible to modify the products of the Euphorbias by modifying their biosynthetic routes to terpenes. The terpenes in hydrocarbon-producing plants and trees are probably made by a well known biosynthetic pathway: sugar to pyruvic acid to mevalonic acid to isopentenylpyrophosphate (IPP). A portion of the unsaturated IPP is isomerized to dimethylallylpyrophosphate (DMAP), and the two isomers are combined. The allylic pyrophosphate comes off DMAP and the resulting carbonium ion attacks the double bond of IPP, followed by a proton loss, which results in the same allylic structure as before. Eventually, if the process continues, rubber is the result.

A comparison of the biosynthetic routes might be useful. In the case of *E. lathyris* , the sequence produces C_{15} compounds which dimerize to C_{30} followed by cyclization to produce triterpene steroids. However, in *Pittosporum* the route is to the C_{10} compounds at which cyclization occurs to create monoterpenes in the fruit. The biosynthetic method by which the "diesel" oil from *Copaifera* is made is the same as that used by *E. lathyris* up to the C_{15} step. *Copaifera* cyclizes the C_{15} farnesyl pyrophosphate, producing cyclic C_{15} compounds.

HYDROCARBON-PRODUCING TREES

One problem with using herbaceous plants as sources of hydrocarbons is soil erosion. It seemed better agronomic practice to grow trees (Seibert et al. 1986), and either harvest the fruit or tap the tree for oil.

A tree that seems to be a likely candidate is *Pittosporum resiniferum* Hemsl. (Figure 5), a member of the Pittosporaceae which grows in the Philippines. The fruit of this particular tree is rich in light oil, containing about 30% terpenes. The fruits can be picked and distilled and thus a terpene-like fuel extracted. The composition of the steam distilled oil contains four major components, the most predominate being -pinene and ß-pinene, both C_{10} compounds (Nemethy & Calvin 1982). The results indicate that the fuel properties of steam distilled oil from *P. resiniferum* fruits after hydrogenation are quite comparable with those of gasoline.

The *Copaifera multijuga* Hayne (a member of Leguminosae) grows in the Amazon region of Brazil and produces C_{15} hydrocarbons. The oil obtained by tapping is light yellow, very similar in appearance to olive oil (Alencar 1982). A hole is bored horizontally in the trunk of a *C. multijuga* tree, into the heartwood (Figure 6) and a bung is placed in the hole. The bung is removed at certain times of the year and the oil flows directly into a container. A single hole in a large tree may yield about 25 liters of oil in 24 h. The bung is reinserted and 6 months to a year later another 25 liters is drained out.

The main components of the Copaiba oil, as it is commonly called, are caryophyllene, bergamotene and copaene, all cyclic C_{15} compounds (Wenninger et al. 1967). This oil has also been subjected to the Mobil zeolite catalyst process, as was the oil from *E. lathyris*, and the results indicate that it can be cracked into a useful suite of compounds. The characteristic that makes this species so attractive is the fact that the material from the tree can be used directly in a diesel engine without further processing.

OIL PRODUCTION BY ALGAE

There is another candidate for oil production which grows in many parts of the world where it occurs primarily in fresh waters. This is the unicellular green alga *Botryococcus braunii* Kuetz. which produces terpenoid oils (Wolf, 1983; Wolf et al. 1985). Colonies are often observed floating on the surface of undisturbed waters and this bouyancy is due to large amounts of accumulated oil in the alga. The

Figure 5. *Pittosporum resiniferum*, the Phillippines.

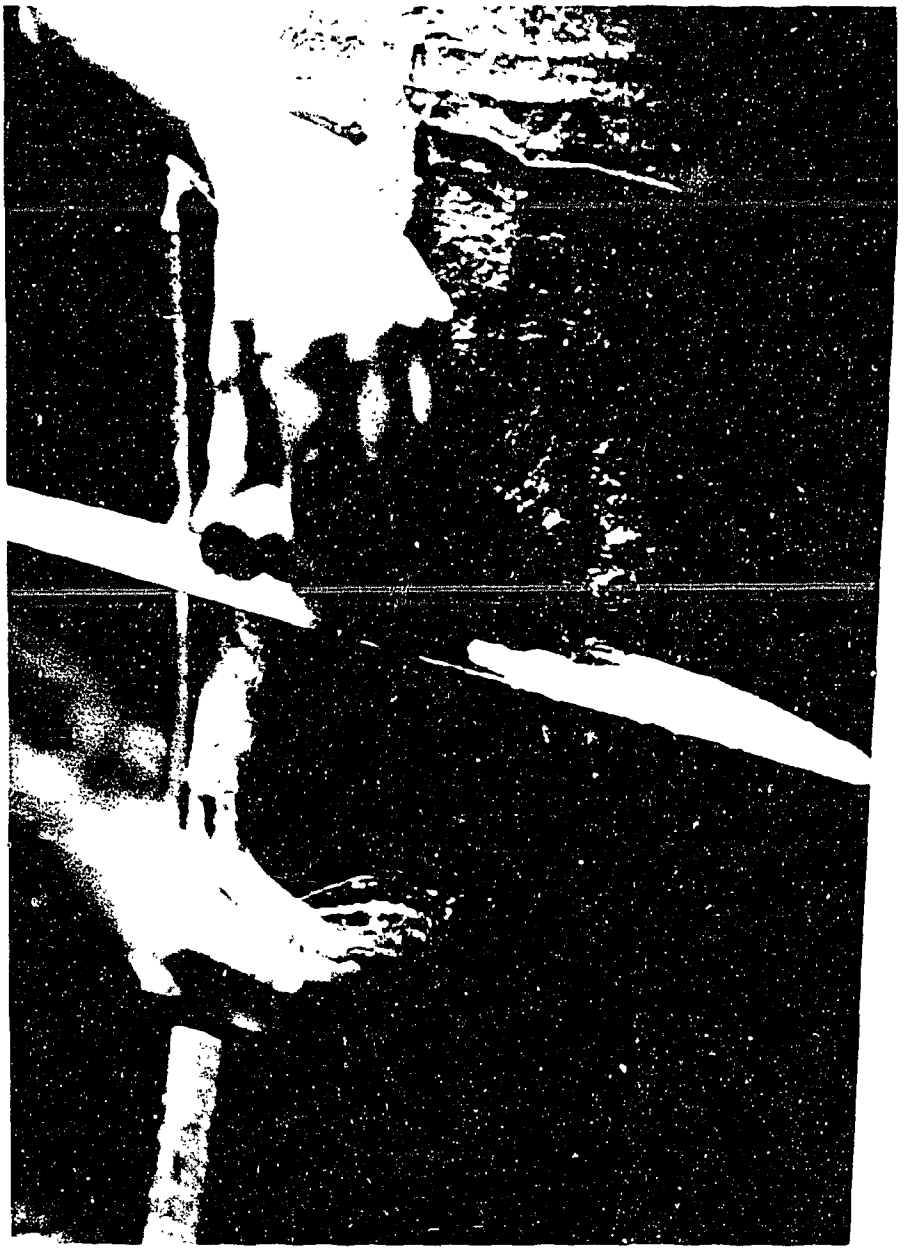


Figure 6. *Copaifera multijuga* showing oil flow, Manaus Brazil.

hexane-extracted *Botryococcus* oil is orange as a result of the presence of carotenoids. After removal of the pigments a clear oil is obtained that contains a homologous series of unusual isoprenoids (Hillen et al. 1984). The structure of the C_{34} component (Botryococcene)(Cox et al. 1973) has been determined, and more recently the structure of the C_{36} compound (Darwinene)(Galbraigh et al. 1983) has also been elucidated (Figure 7). This alga is a very real candidate. We have learned how to grow it in the laboratory but we do not yet know how to regulate the algal metabolism to produce large amounts of oil.

GENETIC ENGINEERING

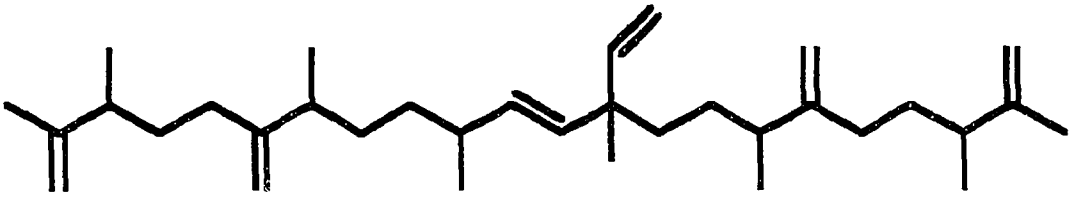
It would be very useful to be able to obtain from the Euphorbias a product similar to the Copiaba oil (Calvin et al. 1982). To achieve this it seems that a single gene would need to be transferred from the plant that cyclizes the C_{15} from farnesyl pyrophosphate into a plant species such as a *Euphorbia*, which has all the other enzymes already, but which goes on to create C_{30} materials. It appears that only one class of enzyme, the farnesyl pyrophosphate cyclase, is needed for this purpose. In other words, a single gene transplant from a donor cell of *Copaifera* to the acceptor cell of *Euphorbia* would be required. To perform this operation it would first be necessary to extract the required gene from a plant which has it and insert it into a plant that does not have it. One relevant possibility would be to take protoplasts from the two plants (*Copaifera* and *Euphorbia*) and have a fusion/selection procedure for plant regeneration (Figure 8)(Redenbaugh, personal communication, Calvin 1983b). We have been able to reproduce a Euphorbia plant from a protoplast. A single protoplast in culture aggregates very quickly into a callus; the callus is adjusted with various hormones to form shoots and roots and thus regenerates a cloned plantlet.

CONCLUSION

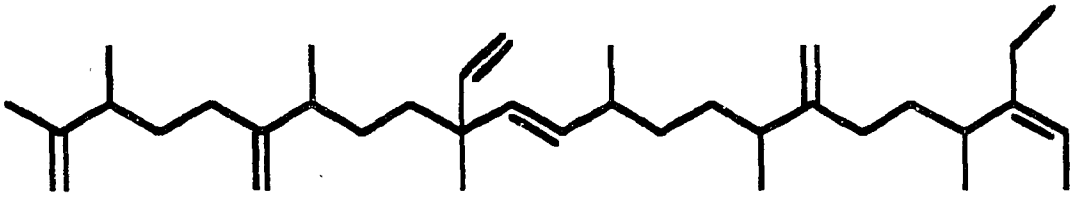
A summary of energy yields from different hydrocarbon producing plants is shown in Table II in terms of yield of liquid fuel/acre/year/inch of water. This indicates the work that is underway with different plants of different types to develop the idea of using biomass a a significant component of total energy use, particularly in the United States. It has been predicted that, by the year 2000, biomass will represent approximately 6% of the total energy used in the United States. The idea of using plants to create hydrocarbon-like materials as a substitute for our current fuel and materials sources will become more important, especially in some of the less developed areas of the world which have a great deal of land not suitable for food production (Calvin 1985). Various efforts are being made toward this end in Okinawa, Thailand, Australia and Spain, and attempts are underway to improve agronomic yields, develop small scale extraction plants, learn more about the composition of the plant oils and study possible ways of modifying the biosynthetic routes (particularly with cyanobacteria) to produce more desirable end products.

The idea of growing hydrocarbons is not new. It was first promulgated by the Italians in 1936 in Ethiopia (Frick 1938): they were running out of oil and thought they would grow plants such as *Euphorbia abyssinica* J. F. Guel., extracting the oil from these plants and using it for their vehicles. This development was not pursued because of the war. Similarly, the French in Morocco in 1940 developed plantations for growing *Euphorbia resinifera* Betg over a period of several years; however, this was also abandoned because of World War II (de Steinheil 1940).

What is needed now is an effort on the part of the agricultural and energy community to commit itself to an energy agriculture which would have long term benefits for the entire world.



Botryococcene



Darwinene

Figure 7. Structures of botryococcene and darwinene.

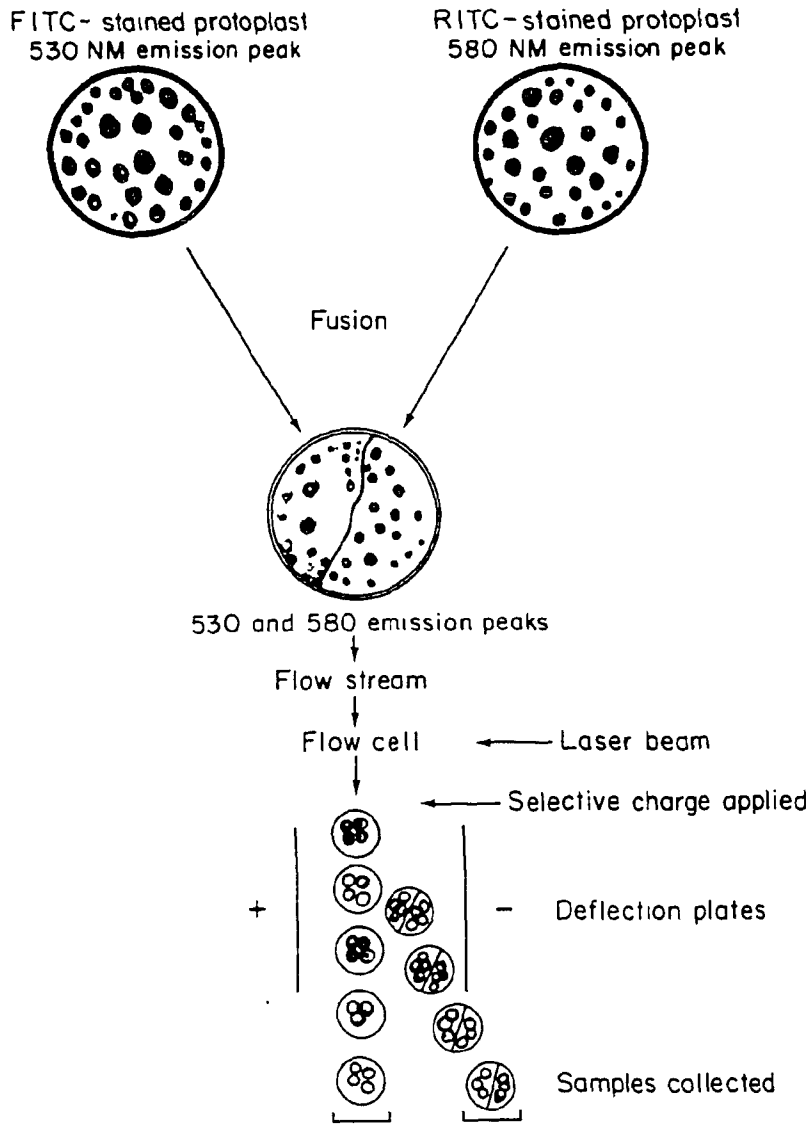


Figure 8. Protoplast selection by flow cytometry.

Table II

Comparison of Energy Yields for Different Crops

PROCESS	DRY BIOMASS YIELD TONS ACRE^{-1}YR^{-1}	LIQ FUEL YIELD/ACRE YR^{-1}	WATER REQ IN YR^{-1}	ENERGY IN LIQ FUEL (10^6 BTU) ACRE^{-1}YR^{-1} PER INCH OF WATER	CELLULOSIC RESIDUE ACRE^{-1} YR.	ENERGY IN CELLULOSE (10^6 BTU) PER ACRE YR^{-1} PER INCH
CORN TO ETHANOL	5	16×10^6 BTU (0.64 tons)	25	0.65	44.2×10^6 BTU (3.4 tons)	1.77
SUGAR CANE TO ETHANOL	30	60×10^6 BTU (2.4 tons)	78	0.78	312×10^6 BTU (24 tons)	4
ENERGY CANE TO ETHANOL	35-50	65×10^6 BTU (2.56 tons)	48	0.35	400×10^6 BTU (31 tons)	8.2
EUPHORBIA LATHYRIS TO HYDROCARBON AND ETHANOL	8.5	20×10^6 BTU (0.58 tons) 17.3×10^6 BTU (0.68 tons)	25	0.82 0.78	79.6×10^6 BTU (6.12 tons)	3.2
PITTOSPORUM RESINIFERUM (FRUIT ONLY) to HYDROCARBONS	7.8	50×10^6 BTU (1.5 mtons)	~25	2.0	101×10^6 BTU (7.8 mtons)	4.0
JATROPHA CURCAS (SEED ONLY) to HYDROCARBONS	5.0	92×10^6 BTU (2.2 mtons)	~25	3.6	36×10^6 BTU (2.8 mtons)	1.45

XBL 849-3892

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Chapter 3

ENERGY CONTENT OF BIOMASS: CALCULATION FROM ELEMENTAL COMPOSITION

John W. Otvos

ABSTRACT

The heat of combustion, or energy content, of an organic compound or mixture can be estimated with reasonable accuracy from the elemental composition alone. The R-value of the fuel, which equals the number of grams of oxygen needed to burn a gram of fuel, times the factor 3.34 gives the specific heat of combustion in kilocalories per gram.

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INTRODUCTION

In dealing with biomass as a renewable energy source it is useful to be able to estimate the "degree of reduction" of the total organic matter of the plant material. This quantity, which we will call R, is proportional to the specific heat of combustion (Thorton 1917). It is an expression of the energy content of the biomass and can be calculated from the elemental composition alone. Such an approximation is possible and so successful because heats of combustion of organic compounds are large compared to the differences in value among isomers. Consequently, elemental composition is much more important than molecular structure. Also, since heats of combustion of mixtures are additive, the R-value can be used to estimate relative abundances of carbohydrate and lipid,, whose R-values are quite different from one another.

R-VALUE AND HEAT OF COMBUSTION

The quantity, R, is defined as the number of grams of oxygen required to burn completely one gram of fuel material to CO_2, H_2O and N_2. The required oxygen is calculated stoichiometrically from the amounts of carbon and hydrogen present less the amount of oxygen already in the fuel. The nitrogen present merely acts as a diluent since no oxygen is needed to convert it to N2. Thus, the expression for R in terms of the elemental composition is given by

$$R = [(2.67 \times \%C) + (8 \times \%H) - \%O] \times 10^{-2} \qquad (1)$$

where 2.67 is the weight of O required to convert 1 g of C to CO_2 while 8 is a like factor for the conversion of H to H_2O. Figure 9 shows a plot of specific heats of combustion (kcal per gram) of a series of 21 organic compounds versus R (see also Table III). The range of values extends over a factor greater than 20, yet all of the points can be well represented by a straight line through the origin. The slope of this line corresponds to a single value of 3.34 kcal eveolved per gram of O_2 used for combustion. Thus the heat of combustion, ΔH_C, of a mixture of organic compounds, or biomass, can be expressed as

$$\Delta H_C \text{ (kcal/g)} = 3.34R \qquad (2)$$

DISCUSSION

This constancy of the heat of combustion based on oxygen consumed rather than fuel consumed is remarkable and, at first, rather surprising when it is noted that elemental carbon and hydrogen have such different values for that quantitiy (kcal per gram of O_2 consumed): 2.95 and 4.27, respectively. However, even in a wide variety of organic compounds the %w of carbon in the CH portion of the molecule varies only between about 80% and 92%. Therefore, the kcal per gram of O_2 consumed varies only between 3.5 and 3.2 for the extreme H/C ratios of 3/1 and 1/1. Most compounds of interest in biomass lie near the middle of that range, corresponding to the value of 3.34 mentioned above. Therefore, a calculation for the heat of combustion of an unknown from its R-value [Equation (1)] should be good to within 3%.

Just as N acts as a diluent in the combustion process so also does ash. Therefore the actual elemental composition of the whole sample must be used in Equation (1) and not the composition expressed on an ash-free basis. The presence of moisture in the sample causes no problem either. The additional amounts of H and O cancel each other out in Equation (1) and water thus becomes another diluent. Ash and nitrogen must be determined, however, so that oxygen can be computed by difference.

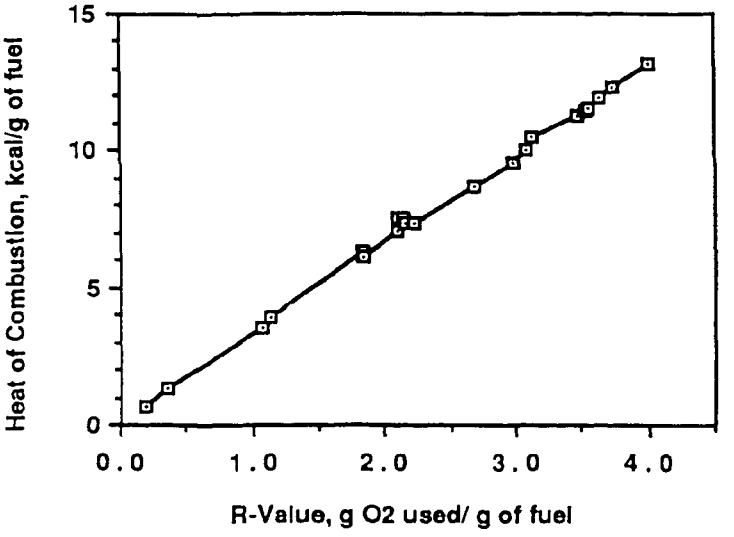

Figure 9. Specific heats of combustion of organic compounds.

Table III

R-Values and Specific Heats of Combustion of Organic Compounds

Compound	Formula	R	Heat of Combustion (kcal/g)
Methane	CH_4	4.00	13.18
Ethane	C_2H_6	3.73	12.28
Propane	C_3H_8	3.64	11.96
n-Pentane	C_5H_{12}	3.56	11.58
n-Hexane	C_6H_{14}	3.53	11.51
n-Hexadecane	$C_{16}H_{34}$	3.47	11.32
Cetyl Palmitate	$C_{32}H_{64}O_2$	3.13	10.50
Benzene	C_6H_6	3.08	10.03
Anthracene	$C_{14}H_{10}$	2.97	9.55
Aniline	C_6H_7N	2.67	8.73
Acetone	C_3H_6O	2.21	7.36
Acetonitrile	C_2H_3N	2.15	7.38
Ethylenediamine	$C_2H_8N_2$	2.13	7.54
Ethanol	C_2H_6O	2.09	7.10
Dimethyl ether	C_2H_6O	2.09	7.56
Acetaldehyde	C_2H_4O	1.82	6.34
Ethyl acetate	$C_4H_8O_2$	1.82	6.10
Sucrose	$C_{12}H_{22}O_{11}$	1.12	3.95
Acetic acid	$C_2H_4O_2$	1.07	3.49
Formic acid	CH_2O_2	0.35	1.37
Oxalic acid	$C_2H_2O_4$	0.18	0.67

APPLICATION TO MIXTURES OF BIOLOGICAL COMPOUND TYPES

Given the constancy of the heat of combustion based on oxygen consumed and also the near constancy of elemental composition of compound types such as protein, carbohydrate, and lipid, Spoehr and Milner (1949) were able to determine the percentage of these three compound classes in *Chlorella* grown under different conditions from their R-values and nitrogen contents. From %N they obtained a protein value by assuming that all the nitrogen was in protein whose N content was 16%. Then, assuming additivity in R-value and average R-values for protein, carbohydrate, and lipid of 1.68, 1.12, 1.70, respectively, they calculated carbohydrate and lipid content. Table IV, taken from their paper, gives an example of their results. They state: " The calculated percentages of lipid agreed closely with the values which were obtained by means of solvent extraction".

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Table IV

Constituents of *Chlorella* Calculated from the R-Value*

R-Value	Protein(%)	Carbohydrate(%)	Lipid(%)
1.52	58.0	37.5	4.5
1.68	50.0	32.2	17.7
2.00	28.3	26.2	45.5
2.24	15.7	19.0	65.3
2.52	8.7	5.7	85.6

*from Spoehr & Milner 1949

Section 2

Biochemistry of *Euphorbia lathyris* Latex

Chapter 4

TRITERPENOID BIOSYNTHESIS IN *EUPHORBIA LATHYRIS* LATEX ASSOCIATED WITH A VACUOLE

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Douglas R. Hawkins
Esther K. Nemethy
Melvin Calvin

ABSTRACT

Latex isolated from *Euphorbia lathyris* laticifer cells maintains its ability to synthesize triterpenols (and their esters) from acetate. When the latex is centrifugated at 5000 xg for 15 min., this biosynthetic activity can be subdivided into two separate fractions: the acetate to mevalonic acid activity remains in the supernatant, while the mevalonic acid to triterpenol activity is pelleted. Further purification of the pellet by isopycnic centrifugation on Percoll gradients yielded a particle responsible for the conversion of mevalonic acid to triterpenol. Electron microscopy of this particle and comparison with marker enzyme activity indicated that this organelle is a vacuole.

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INTRODUCTION

The laticifer cells of *Euphorbia* species allocate a significant amount of their carbon to the production of triterpenoids; triterpenols and their fatty acid esters constitute up to 50% of the dry weight of Euphorbia lathyris latex (Nemethy et al. 1983). These energy-rich compounds may provide a renewable alternative to petroleum for both fuel and chemical feedstocks. Before isoprenoid-producing plants can become an economically viable alternative to fossil fuels, their isoprenoid yields must be increased. This will require an understanding of the physiological processes that control carbon allocation to isoprenoid production. Since subcellular compartmentation is one form of biological control, we have attempted to isolate the various particles involved in the biosynthesis of these isoprenoids so that we may further study their role in the regulation of hydrocarbon production.

Many previous studies of the subcellular organization of the laticifer cell have been done with *Hevea brasiliensis.* Ultrastructural studies have shown that in addition to the normal cellular structures, including nuclei, mitochondria, endoplasmic reticulum and ribosomes, *Hevea* laticifers contain three unique particles (for review, see Archer 1980). The major unique component, called a lutoid, is a single-membrane-bounded structure with a diameter from 0.5 to 5 μm. A second, less abundant particle is the Frey-Wyssling complex, a double-membrane-bounded particle 4 to 6 μm in diameter. This organelle contains lipid globules and various membrane structures, and is thought to be a plastid. The third structures are minute polyisoprene (rubber) particles.

When Gomez (1975) tapped latex from *H. brasiliensis* plants he found lutoids, Frey-Wyssling complexes and polyisoprene particles, but few mitochondria or nuclei were observed. The lutoids were separated by centrifugation, and found to comprise about 20% of the total latex volume. The biochemistry of these organelles has been well studied. They have been found to contain acid hydrolases, peroxidase, lysozyme, and a-mannosidase, and accordingly are thought to be lysosomal vacuoles (D'Auzac et al. 1982).

Ultrastuctural studies of *Euphorbia* species have shown that their laticifers resemble those of *H. brasiliensis.* Although the major isoprenoid components of *Euphorbia* species are triterpenoids, not rubber, these hydrocarbons are still contained in small particles (Groeneveld 1976). Fineran (1982, 1983) examined the laticifers of both mature and developing tissue of Euphorbia pulcherrima.. Carefully fixed mature tissue had a wall-lining layer of cytoplasm bounded by the plasmalemma, and a large central vacuole with an intact tonoplast, indicating that the mature laticifer was a living cell. Nuclei were observed, but mitochondria were poorly differentiated and ribosomes were scarce. Plastids containing a single large starch grain were also observed. The triterpenoid particles were contained within the central vacuole. Laticifers in the developing tissue of the sub-apical region of the stem had many smaller vacuoles each containing triterpenoid particles, along with normal appearing mitochondria and higher levels of ribosomes.

Investigation of exuded Euphorbia latex has centered in large part on the unique rod-shaped starch grains. Mahlberg and his coworkers (Mahlberg 1973, Mahlberg et al. 1983) have used starch grain morphology along with latex triterpenoid composition to determine phylogenic relationships within the genus. Some work has been done on isoprenoid production in isolated latex. Ponsinet and Ourisson (1967, 1968), Bisboer and Mahlberg (1979), Groeneveld et al. (1982, 1987), and Nemethy et al. (1983) have all reported on various aspects of isoprenoid biosynthesis in isolated *Euphorbia* latex. Groeneveld et al. (1987) reported that triterpene biosynthesis occurred in two separate sites, the amyloplast and latex particle-containing vesicles.

We have combined centifugation techniques with biochemical assays and electron microscopy to determine the structure-function relationship of the various components of *Euphorbia lathyris* latex. In this

paper we report on the assignment of a biochemical function to one of the latex subcellular particles.

MATERIALS AND METHODS

Plant Material. *Euphorbia lathyris* L. plants were propagated from seed collected in the wild near Healdsburg, California. Plants were grown in a Controlled Environment PGW-36 growth chamber, under a light regime of 16 h day at 600 µmol quanta m^{-2} s^{-1}, followed by 8 h of darkness. Light was provided by a combination of General Electric warm-white fluorescent and General Electric 52 watt incandescent lamps. The temperature was set at 27 °C day/ 18 °C night. Latex was collected from shallow incisions made by razor blade at the base of the petioles.

Centrifugation. Latex was first fractionated by differential sedimentation. Two hundred microliters of latex was combined with an equal volume of centrifugation buffer (final concentration of 10 mM MES, pH 5.5, 0.25 M sorbitol, 2 mM $MgCl_2$) in a 1.2 ml microfuge tube and spun at 5000 xg for 10 min. in a Beckman 11 microfuge. This pellet was used for much of the biochemical and structural analysis.

Further fractionation was accomplished by isopycnic centrifugation of the 5000 xg pellet on Percoll gradients. Percoll (Pharmacia) was dialysed overnight against 10 mM MES, pH 5.5. A self-generated gradient (ro=1.015-1.14) was formed by mixing 3.1 ml of the dialysed Percoll with 7.9 ml of centrifugation buffer (same final concentration as above) in a 15 ml tube, and centrifuging in a Beckman Ti50 rotor at 30,000 xg for 30 min. Density Marker Beads (Pharmacia) were used to monitor the formation of the gradient. The 5000 xg latex pellet was resuspended in I ml of the centrifugation buffer, layered on the top of the Percoll gradient, and centrifuged at 800 xg for 25 min. at 4 °C. The gradient was fractionated from the bottom of the tube into 1 ml aliquots.

Biosynthesis Assays. Triterpenol biosynthesis from acetate and MVA was monitored in whole latex, 5000 xg supernatant and pellet, and Percoll gradient fractions. Whole latex (200 µl) was combined with an equal volume of centrifugation buffer and added to a tube containing U-^{14}C-acetate (final concentration = 1.0 mM, 8.25 mcuries $mmol^{-1}$) and/or ^{3}H-MVA (final concentration = 0.75 mM, 33 mcuries $mmol^{-1}$). Samples were incubated for 2-4 h and then quenched by the addition of acetone. The triterpenols were extracted with hexane and the solvent was evaporated under a stream of nitrogen gas. The triterpenols and triterpenol esters were further purified by thin layer chromatography on silica gel G plates (Analtech), developed in ethyl ether: petroleum ether (3:1). The triterpenols and their esters were localized by comparison of a marker spot (visualized by spraying with H_2SO_4 (conc) followed by charring) with known standards. Bands were cut corresponding to the triterpenols and triterpenol esters, and radiolabel content was determined by performing liquid scintillation counting on the material scraped from half of each band. The incorporation of radiolabel into sterols was confirmed by eluting the triterpenols from the other half of these bands and separating them further by HPLC. The eluted compounds were chromatographed on an Altex ODS column with 100% methanol, monitored at 214 nm, and retention times of the peaks were compared to those of known triterpenols. These fractions were collected and analyzed by liquid scintillation counting.

The 5000 xg supernatant was assayed as above, substituting the supernatant for the whole latex/buffer mix. The 5000 xg pellet was resuspended in 400 µl of centrifugation buffer and assayed. The reconstituted latex was assayed after recombining a 5000 xg pellet with 5000 xg supernatant. Two hundred fifty microliter aliquots of Percoll gradient fractions were added to directly to the ^{3}H-MVA and assayed.

Marker Enzyme Assays. The presence of subcellular organelles in the gradient fractions was monitored using specific marker enzymes. Endoplasmic reticulum were identified by a cytochrome reductase assay (Phillips and Langdon, 1962), mitochondria by a fumarase assay (Hill & Bradshaw 1969),

peroxisomes by a catalase assay (Maehly & Chance 1954), and vacuoles by a a-mannosidase assay (Chrispeels & Boulter 1975). Protein content was determined using the procedure of Vincent and Nadeau (1983).

Electron Microscopy. All buffered solutions, unless noted otherwise, used 0.1 M Na cacodylate, pH 6.0 as the buffer.

The 5000 xg pellet was prepared for scanning electron microscopy (SEM) by dividing it up into small pieces and then adding 1 ml of fixative (2% glutaraldehyde, 1% OsO_4, in cacodylate buffer) and incubating for 1 h in the dark on ice. The sample was then centrifuged at 5000 xg for 15 min, and the supernatant removed. The pellet was washed 3x with cacodylate and incubated for 30 min at room temperature in l ml of 1% glutaraldehyde in cacodylate. Following the removal of the glutaraldehyde solution, the sample was washed 3x with buffer, postfixed by incubation with 1 ml of buffered 0.5% OsO_4 for 20 min, and then was washed 8x with buffer. The sample, broken into small pieces, was dehydrated using an ethanol series and critical point dried from liquid CO_2. The dried, powdery sample was mounted on stubs by shaking onto drying graphite glue. The samples were sputter coated with a 15 nm Pt layer using a Polaron sputter coater equipped with a quartz crystal thickness monitor.

Percoll gradient samples were prepared for SEM using the technique of Mazia et al (1975). Fractions from a gradient were applied to poly-L-lysine coated cover slips, which were allowed to sit for 20 min. on ice, and were then rinsed 4x with a buffered solution (50 mM MES, pH 6.1, 0.4 M sorbitol, 5 mM $MgCl_2$, 5 mM DTE). The samples were fixed for 1 h in 2% glutaraldehyde in cacodylate buffer, washed with buffer, and then postfixed in buffered 1% OsO_4. The samples were rinsed with distilled water and dehydrated as before with an ethanol series. The samples (in 100% ethanol) were dried from liquid CO_2, mounted on stubs and coated with 15 nm Pt. All SEM was performed with an ISI DS-130 SEM at 10 kv.

The 5000 xg pellet was prepared for transmission electron microscopy (TEM) by incubation with 5 ml of 1% glutaraldehyde and 0.5% OsO_4, in cacodylate buffer, in the dark for 1 h at 0-4 °C. The sample was centrifuged at 1000 xg for 5 min, and rinsed 3x with 0.1 M Na cacodylate, pH 7.0. The sample was then postfixed in 4 ml of 2% glutaraldehyde in 0.1M Na cacodylate, pH 7.0 for 30 min and rinsed 3x with Na cacodylate buffer. Samples were next incubated in 4 ml of 0.5% OsO_4 for 45 min, and rinsed 3x with water. The samples were dehydrated through an ethanol series to 70% ethanol, placed in a 1:1 mixture of 70% ethanol and LR White resin (Polysciences, Inc) for 30 min, and then infiltrated with three changes of LR White resin. The samples were placed in capsules, degassed for 1-2 h and hardened for 18-20 h in a vacuum oven at 60 °C. Thin sections were cut from the resin blocks using a glass knife, collected on formvar-coated 200 mesh copper grids, and poststained with 5% uranyl acetate in water for 30 min and lead citrate stain for 5 min (Reynolds 1963).

Fractions from the Percoll gradients were prepared for TEM by incubation with the fixative solution (final concentration 0.3% OsO_4 and 1% glutaraldehyde in in cacodylate buffer) for 1.5 h at 0-4 °C. The samples were then centrifuged at 5000 xg for 15 min, and the pellets washed 3x with cacodylate buffer, followed by two washes of distilled water. Samples were dehydrated using an 8 step ethanol series to 70% ethanol, then infiltrated with a 1:1 solution of 70% ethanol and LR White resin for 45 min followed by incubation with 100% LR White resin overnight. The samples were then incubated with fresh LR White resin for 1.5 h and placed in capsules which were degassed and hardened as before. Sections were cut with a glass knife, collected on naked 300 mesh copper grids, and poststained with uranyl acetate and lead citrate. All TEM was performed with a Zeiss 109 TEM at 80 kv.

RESULTS

As we have reported previously (Nemethy et al. 1983, Skrukrud et al 1987) isolated latex is capable of converting both acetate and MVA to the triterpenols and their esters (TP+TPE) (Table V, Figure 10). When latex was fractionated by differential centrifugation the ability to use both of these substrates was altered. The 5000 xg supernatant exhibited little TP+TPE biosynthetic activity. The level of acetate incorporation was reduced to 21% of that of whole latex, and the incorporation of MVA into triterpenols was negligible at 2% of whole latex rates. The 5000 xg pellet showed no acetate to triterpenol activity, while the ability to convert MVA into triterpenols remained relatively high at 38% of control levels. Recombining the supernatant and the pellet restored acetate to triterpenol activity to 45% of whole latex values, and MVA incorporation was increased slightly above that of the pellet to 41%.

The ratio of incorporation of MVA into triterpenol esters and tritepenols also changed with centrifugation. Whole latex had a TPE/TP ratio of 6.7, while the 5000 xg pellet ratio dropped to 0.8. When the supernatant and pellet were reconstituted the ratio returned to 6.8. The incorporation of MVA in the supernatant was too low to generate an accurate TPE/TP ratio.

To further purify the organelles, the 5000 xg pellet was resuspended in buffer, applied to a Percoll density gradient and centrifuged. Fractions were collected from the gradient, and aliquots from these fractions were tested for the ability to convert ^{3}H-MVA into TPE's and TP's. In addition, marker enzyme assays were performed on aliquots of the fractions to identify the presence of specific organelles, and protein levels were determined. The particle responsible for the conversion of MVA to TP+TPE banded within a narrow region in fractions 3-5 (figure 11). This corresponded to a major peak of a-mannosidase activity, indicating the presence of vacuoles within this region. The major microsomal peak, as indicated by cytochrome c reducatse activity, occurred above the band of TP+TPE synthesis. No fumarase nor catalase activity was detected, indicating that there were no intact mitochondria or microbodies in the gradient.

Scanning electron micrographs of the 5000 xg pellet were dominated by the presence of rod-shaped starch grains with lengths from 8 to 34 µm (figure 12). Other regular ovoid structures with lengths of 4 to 12 µm were also seen in this fraction (figure 13). Examination of thin sections of the 5000 xg pellet by TEM showed the presence of numerous ovoid structures with diameters from 0.5 to 4 µm (figure 14). Some of these structures had internal osmophilic structures, while others had a more dispersed and diffuse substructure. In addition, many osmophilic particles with diameters of 0.125 to 0.3 µm were found outside of the ovoid particles.

Fractions from Percoll gradients were prepared for SEM by first adhering any particles to poly-lysine coated glass coverslips and washing; this method captures negatively charged particles and allows the removal of Percoll particles. Only fractions 1 and 2, above the band of TP+TPE biosynthetic activity, exhibited structure in these SEM's. The structures, regular spheres of about 0.2 µm in diameter (figure 15), are not Percoll; Percoll particles range in size from 15 to 30 nm in diameter. No other structures were observed on the coverslips. Only starch grains were observed in the Percoll pellet (figure not shown);the fraction directly above the pellet (fraction 11) also contained a few starch grains. A structure was found in fractions from the biosynthetically active region of the gradient (fractions 3-5) when observed by TEM (figures 16 and 17) . These structures were found only in this region. They ranged in size from 2 to 9 µm and were bound by a single membrane (figure 17 insert).

DISCUSSION

The results presented in Table V show that the conversion of acetate to the isoprenoids can be physically separated into two processes: the conversion of acetate into MVA, and the conversion of that MVA into triterpenoids. The 5000 xg pellet had no measurable acetate to TP+TPE activity but retained a significant

Table V

Incorporation into Triterpenols and Triterpenol Esters
(p mol substrate incorporated/hr/ml latex)

substrate:	Acetate	MVA	Triterpenol Esters: Triterpenols
Sample			
whole latex	24.2	530	6.7
5000 xg supernatant	5.0	9.0	1.7
5000 xg pellet	0	158	0.8
reconstituted (5000 xg + 5000 xg supernatant)	10.8	217	6.6

The triterpenol ester/ triterpenol ratio was calculated from the assays using MVA as the substrate.

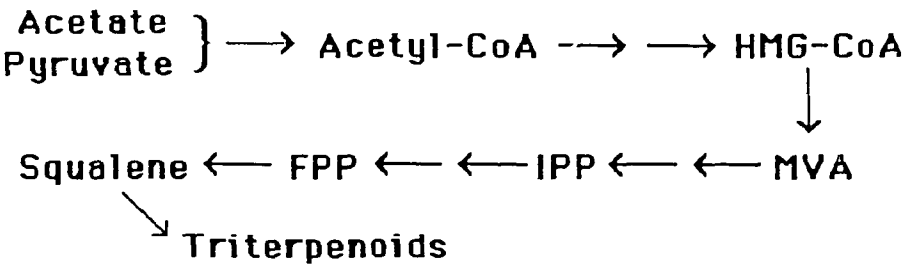

Figure 10. Triterpenoid biosynthetic pathway (HMG-CoA, ß-Hydroxymethylglutaryl-CoA; MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate).

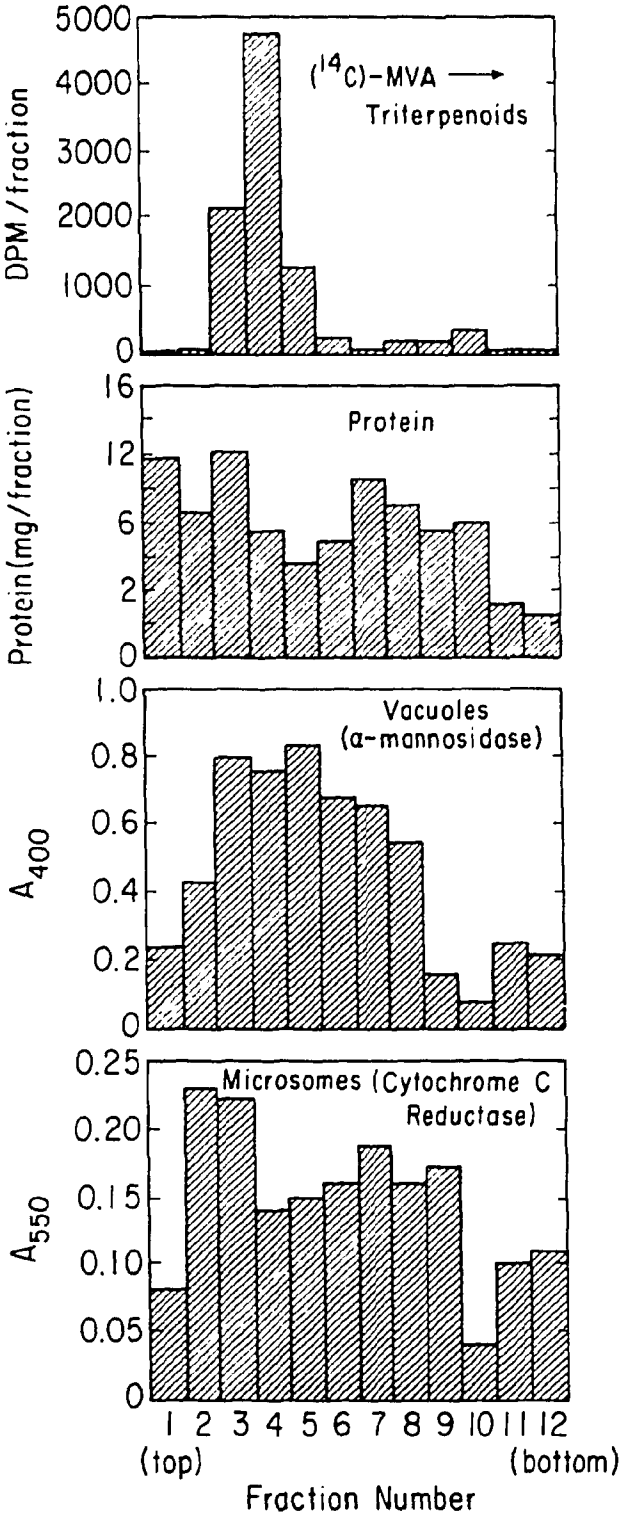

Figure 11. Activity of fractions collected from a Percoll gradient. The 5000 xg pellet was applied to the gradient and centrifuged as described in the text. Fractions were collected and assayed for triterpenoid biosynthetic activity, protein content, α-mannosidase activity, and cytochrome C reductase activity. Assays for fumarase activity (for mitochondria) and catalase activity (for microbodies) were also performed; no activity was detected ineither assay.

Figure 12. SEM of dried 5000 xg pellet of *Euphorbia lathyris* latex. Bar equals 10 µm.

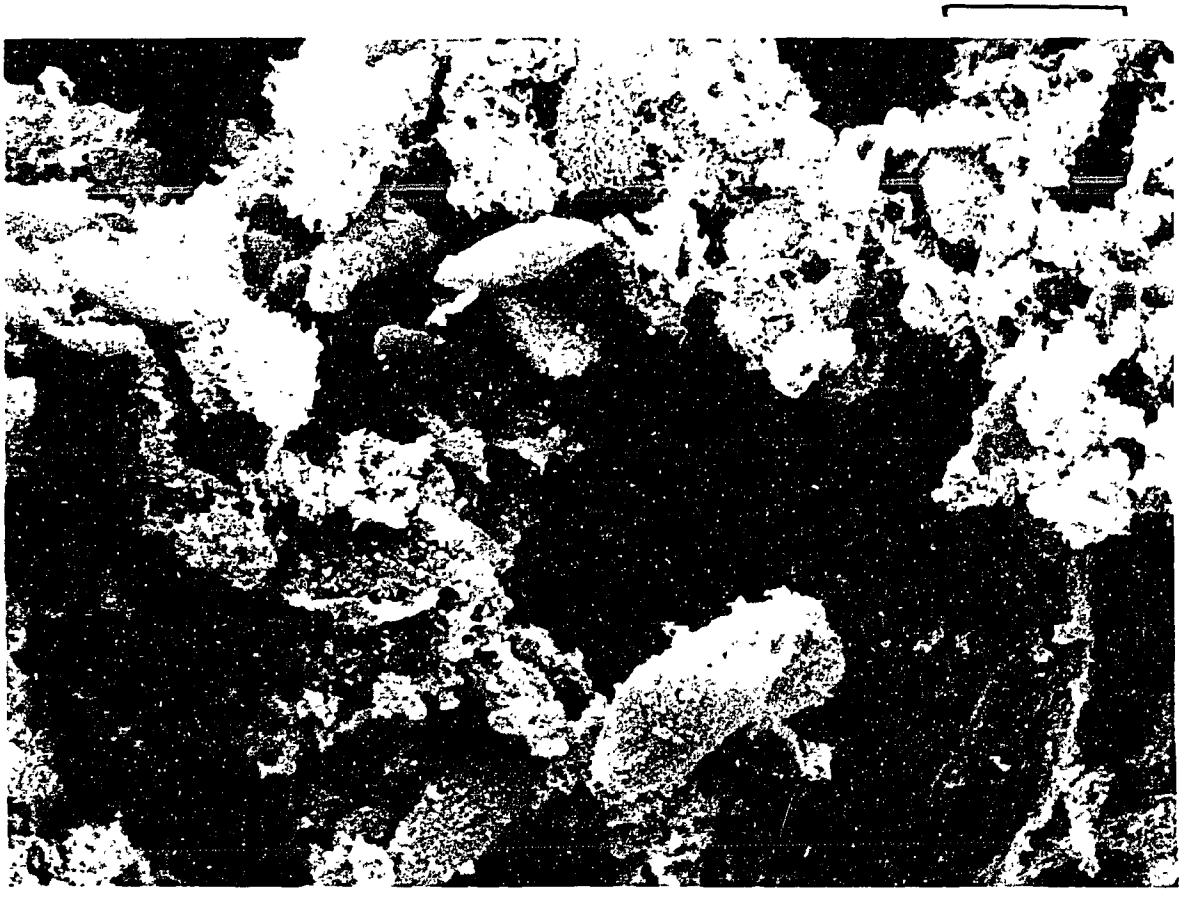

Figure 13. SEM of dried 5000 xg pellet of *Euphorbia lathyris* latex. Bar equals 10 µm.

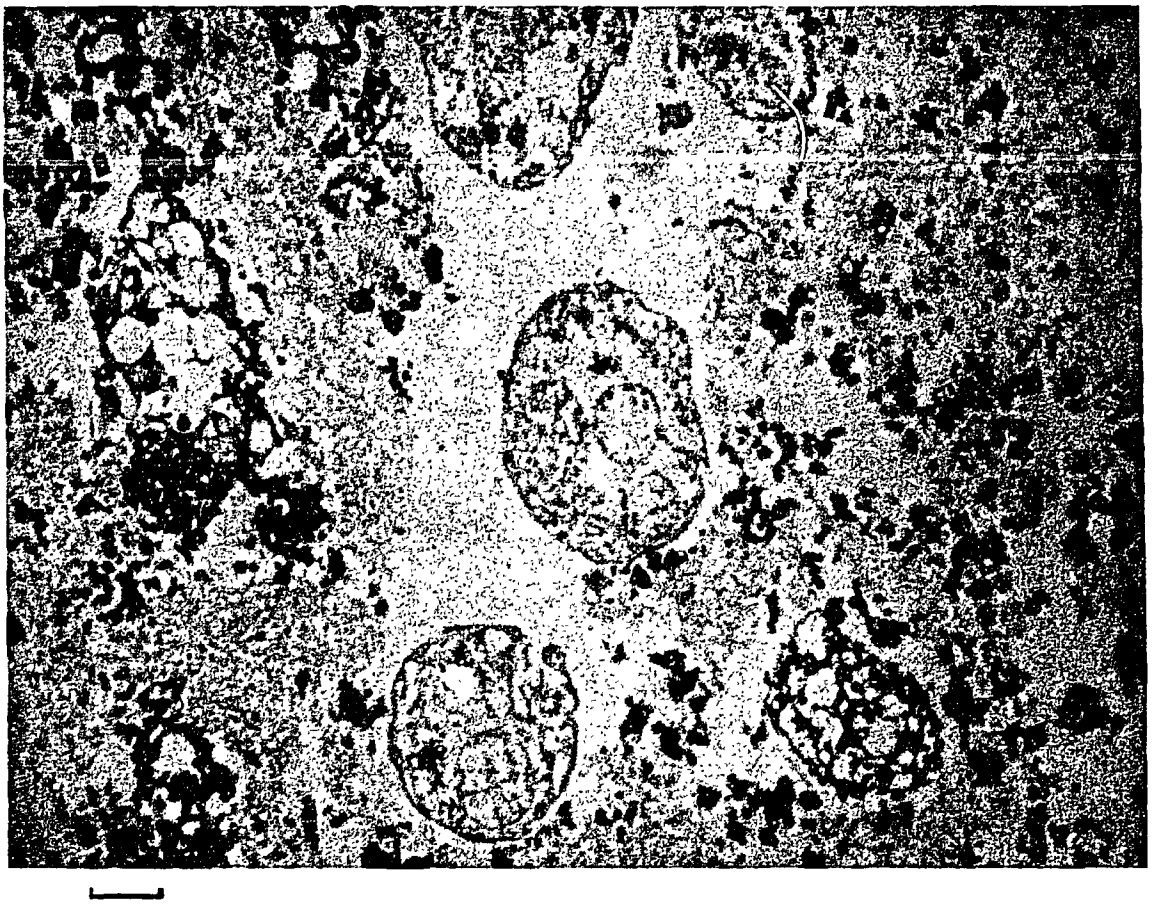

Figure 14. TEM of resin-embedded 5000 xg pellet of *Euphorbia lathyris* latex. Bar equals 1 μm.

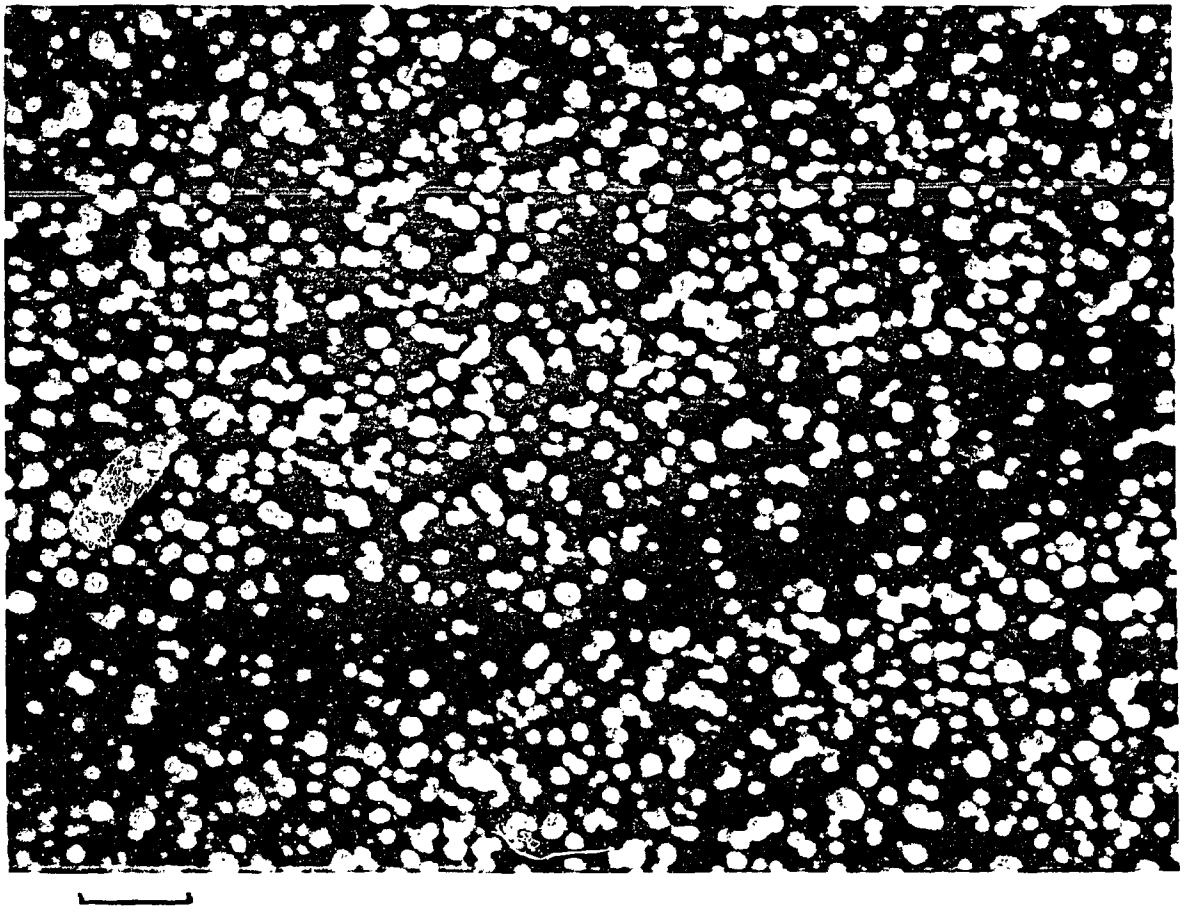

Figure 15. SEM of *Euphorbia lathyris* latex particles isolated on a Per coll gradient. Bar equals 1μm.

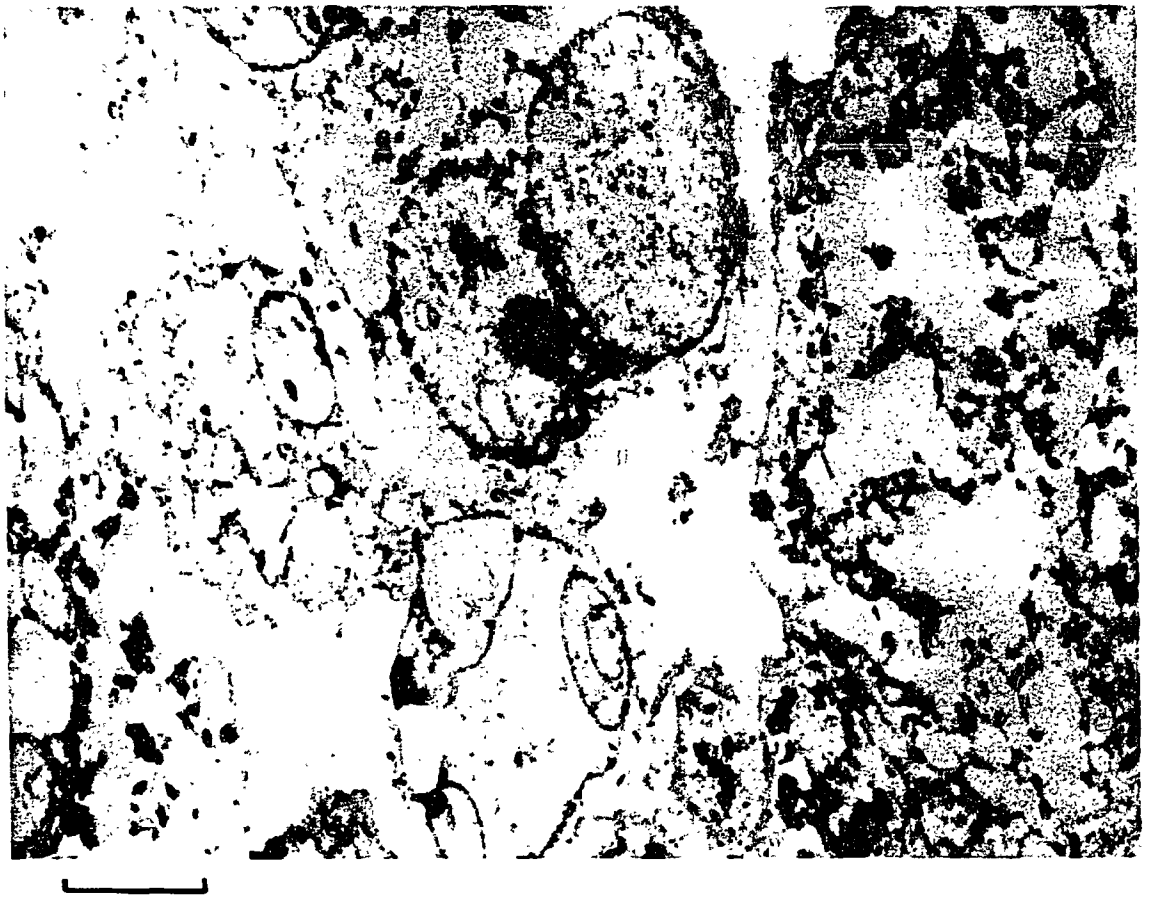

Figure 16. TEM of triterpenoid-synthesizing fraction of *Euphorbia lathyris* latex, isolated on a Percoll gradient. Bar equals 1µm .

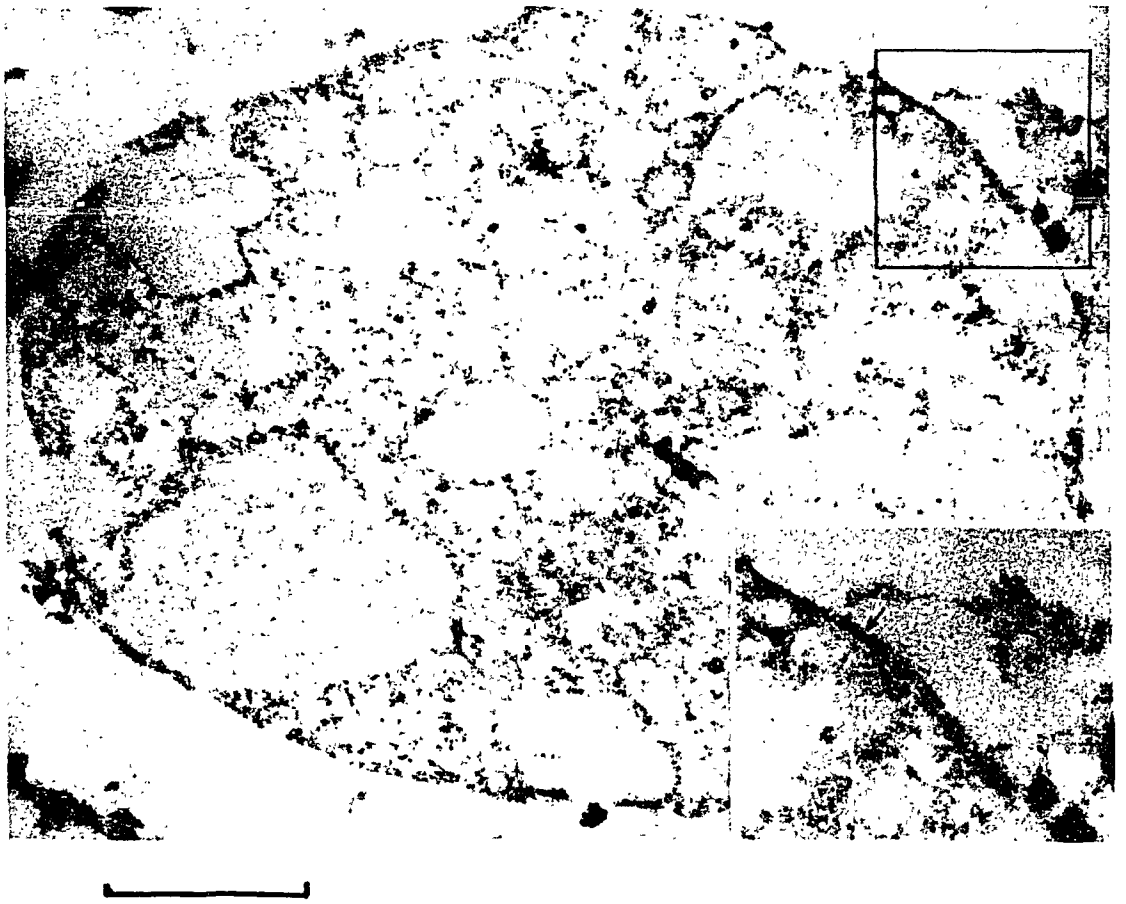

Figure 17. TEM of triterpenoid-synthesizing structure of *Euphorbia lathyris* latex, isolated on a Percoll gradient. Insert) Expansion of region showing single bounding membrane (arrow). Bar equals 1 μm.

proportion of its MVA to TP+TPE activity. When the latex was reconstituted after centrifugation, half of the acetate to TP+TPE activity was restored, showing that this activity was not lost by physical damage from centrifugation, but was actually separated from the pelleted activity.

The reconstituted latex also exhibited a small increase in MVA to TP+TPE activity over the pellet, suggesting a synergistic relationship between the supernatant and the pelleted material. One possible explanation for this observation has been reported previously (Nemethy et al. 1983): the synthesis of the TPE's requires a phospholipid as the acyl donor and the phospholipid pool may be present in the supernatant. This is further supported by a comparison of the ratio of MVA incorporation into TPE and TP. The whole latex had a TPE/TP ratio of 6.7, while the pellet had a ratio of only 0.8, due to a large decrease in TPE biosynthesis. Recombining the pellet with the supernatant restored TPE biosynthesis, and the ratio returned to whole latex levels (6.8).

The 5000 xg supernatant had little MVA to TP+TPE activity (2%), but retained some of its ability (21%) to utilize acetate. At first glance this is confusing, since it should be impossible to utilize acetate without MVA to TP+TPE activity. But the conversion of acetate to MVA is rate limiting (Skrukrud et al. 1987), and the incorporation of acetate into TP+TPE occurs at only 5% of the MVA to TP+TPE rate. Thus the MVA to TP+TPE activity that remains in the supernatant, while low, is still capable of converting any MVA produced from acetate into triterpenoids.

We were able to observe three different particles in the electron micrographs of the 5000 xg pellets and the Percoll gradient fractions: membrane-limited organelles, starch grains, and triterpenoid or latex particles. The structure of most interest, the organelle (figures 14,16 and 17), was collected from the region of MVA to TP+TPE activity. We have concluded that this vesicle is a vacuole. It is limited by a single membrane, with some organized internal structure. Its physical characteristics are that of a vacuole, and it co-migrates with the marker enzyme a-mannosidase, indicating that it is vacuolar in nature. Its structure and lack of fumarase activity both indicated that it is not mitochondrial, the absence of catalase activity eliminates the microbody, and both its structure and its having a greater bouyant density greater than the major peak of cytochrome c reductase activity rule out it being microsomal. It is not a plastid because it is limited by a single membrane. In addition, vacuoles are extremely osmosensitive, which would explain the absence of the organelles on the polylysine-coated coverslips used for SEM of the Percoll gradient fractions.

The observation that the MVA to TP+TPE activity is associated with this structure agrees with Fineran's (1983) ultrastructural determinations that indicated that *E. pulcherrima* latex particles are synthesized in tubular vacuoles found in the cytoplasm. Though the size of these vacuoles (2-9μm) is greater than that reported for the vacuolar lutoids of *Hevea brasiliensis* (1-5 μm; D'Auzac et al. 1982), we believe that this organelle is similar in both structure and function. Groeneveld et al. (1987) also reported that a vesicle was the site of triterpenol biosynthesis, but found that this vesicle contained large amounts of latex particles. In contrast to these data, we were able to physically separate the latex particles from the organelle responsible for triterpenol biosynthesis, which indicating that the site of storage and the site of synthesis differ.

The second structures we observed were starch grains (figures 12 and 13). The elongated shapes observed were characteristic of non-articulated *Euphorbia* laticifers (Mahlberg 1973). Mahlberg et al. (1983) ascribed the wrinkled surface of these grains in SEM to the membrane of the plastid. Since the site of starch synthesis in higher plants is the plastid, this is a logical conclusion, though the extrachloroplastic production of starch in latex must be ruled out before this structure can be positively identified. We also observed an irregular surface on the starch grains, and these structures did adhere to the polylysine-coated coverslips (figure not shown), indicating a negative charge on the surface. We could not

determine if this is due to the outer envelope of an amyloplast or to adhesion of smaller particles to the starch grain's surface. Unlike Groeneveld et al. (1987) we were unable to detect any MVA to TP+TPE biosynthetic activity in fractions containing starch grains. If these structures are amyloplasts, they may have lost their structural integrity during centrifugation, with a resulting loss of biosynthetic capability. Plastids are a likely site of isoprenoid biosynthesis; in whole plant extracts of *E. lathyris* a major portion of the ß-Hydroxymethyl glutaryl-CoA Reductase activity (the enzyme responsible for the synthesis of MVA) is associated with the plastids (Skrukrud, unpublished results).

The final structures observed were the latex (triterpenoid) particles (figures 14 and 15). Most of these particles were found in the 5000 xg supernatant (data not shown), but some pelleted, probably adhering to more dense particles. The lipid nature of these structures is indicated by their heavy staining by osmium and low buoyant density. They also adhered to the polylysine-coated coverslips, indicating a negative surface charge. The question of whether latex particles are encapsulated within some limiting material has been studied by many groups. Fineran (1982) observed boundary layer on some latex particles of *E. pulcherrima*, and Groeneveld (1976) reported a membrane-like film surrounding latex particles of *Hoya australis* (Asclepiadaceae), but found no membrane around the latex particles of *E. milii.* Our data suggest that *E. lathyris* latex particles may indeed be membrane-bounded.

CONCLUSION

The results indicate that the conversion of acetate to TP and TPE in latex is compartmentalized into two separate subcellular regions. The early steps, the conversion of acetate into MVA, remain in the supernatant when latex is centrifuged at 5000 xg, while the later steps, the utilization of MVA to make isoprenoids, occurr within an organelle that pellets at 5000 xg. The co-migration of this pelleted organelle with the marker enzyme a-mannosidase, and electron micrographs showing that a single membrane encloses this organelle, indicate that this structure is a vacuole.

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Chapter 5

TRITERPENOLS OF *EUPHORBIA LATHYRIS* LATEX

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ABSTRACT

The triterpenols of the latex of *Euphorbia lathyris* have been isolated and identified by spectroscopic methods as euphol, lanosterol, cycloartenol, 24-methylenecycloartenol, 24-methylenelanosterol, and butyrospermol.

submitted to: *Phytochemistry*

INTRODUCTION

Euphorbia lathyris has been considered for use as an energy crop (Nishimura 1980, Calvin et al. 1982, Calvin 1987) and its white, milky latex has been examined as a model system for triterpenoid biosynthesis in plants (Ponsinet and Ourisson 1967, 1968b, Groenveld et al. 1982, Nemethy et al. 1983, Groeneveld and Mahlberg 1986). During the course of these studies there have been several reports concerning the identity of the triterpenols in *E. lathyris* and its latex (Ponsinet and Ourisson 1968a, Neilsen et al. 1979, Nemethy et al. 1979, Conti et al. 1983, Williams and Home 1983, Nishimura et al. 1984). Most of these reports either failed to mention the presence of certain compounds or left others unidentified. We report herein the results of a rigorous structure determination of the triterpenols isolated from the latex of *E. lathyris.*

Euphorbia lathyris latex was previously reported to contain euphol(**1**), lanosterol(**2**), cycloartenol(**3**), and 24-methylenecycloartenol (**4**) (figure 18) (Ponsinet and Ourisson 1968a). We have confirmed the presence of each of these by HPLC coelution with standards, mass spectroscopy, and ^{1}H-NMR spectroscopy. In addition, the latex was reported to contain a compound which was referred to as "lanosterol isomer" (Nemethy et al. 1979, Conti et al 1983). Nishimura and coworkers (1984) identified this compound as butyrospermol(**5**) on the basis of its behavior on GC. They also reported finding euphorbol(**6**).

MATERIALS AND METHODS

Euphorbia lathyris latex was collected as described previously (Nemethy et al. 1983). The lipid fraction was extracted out with hexanes and chromatographed on preparative silica gel TLC. The triterpenols isolated from the TLC plate were then seperated by HPLC using an ODS column (4.6x500mm) eluted with methanol. In order to obtain sufficient quantities for NMR spectra a semi-preparative 10x250mm ODS column was used with multiple injections.

^{1}H-NMR spectra were recorded at 250 MHz in $CDCl_3$ with TMS as internal standard. The spectra recorded in the presence of the lanthanide shift reagent were obtained by incremental additions of $Eu(fod)_3$ to the NMR sample. MS were obtained at 70 eV; high resolution data were within 5ppm of calculated values.

RESULTS

The NMR and MS data are as follows:

Butyrospermol (**5**): ^{1}H-NMR: ∂ obs for the methyl groups: 0.742; 0.803; 0.847, d; 0.806; 0.970; 0.970; 1.683; 1.60. Other signals: 5.27, m (vinyl); 5.11, m (vinyl); 3.25, m (C3a-H). Literature values for the methyl groups (Itoh et al. 1976a): 0.75, 0.81, 0.85, 0.99, 0.99, 1.69, 1.63 (no value was reported for the C-20 methyl group). Using 7.29 mg of **5** a series of ^{1}H-NMR spectra were recorded after incremental additions of $Eu(fod)_3$. The chemical shift data were then analyzed by a least squares progression and normalized as suggested by Itoh et al. (1976a) for comparison to literature results. Chemical shifts observed (for 1:1 molar ratio of **5** to Eu): 11.00, 10.20, 4.66, 2.14, 1.53, 1.13d, 1.64, 1.67. Literature values (Itoh et al. 1976a): 11.00, 10.18, 4.51, 2.10, 1.58, 1.16d, 1.62, 1.68. $[a]_D(CHCl_3, c\ 0.52)=-11.9°$.

24-methylenelanosterol (**7**): ^{1}H-NMR ∂ obs for the methyl groups: 0.693; 0.811; 0.881; 0.982; 1.001; 1.012, d, J=6.9Hz; 1.040, d, J=6.9Hz. Other signals: 4.71, 1H, br s (vinyl); 4.66, br s (vinyl); 3.24, dd, J=11,4.8Hz (C3a -H); part of a multiplet is visible at 2.21, J=6-7Hz (C25-H). Literature values for the

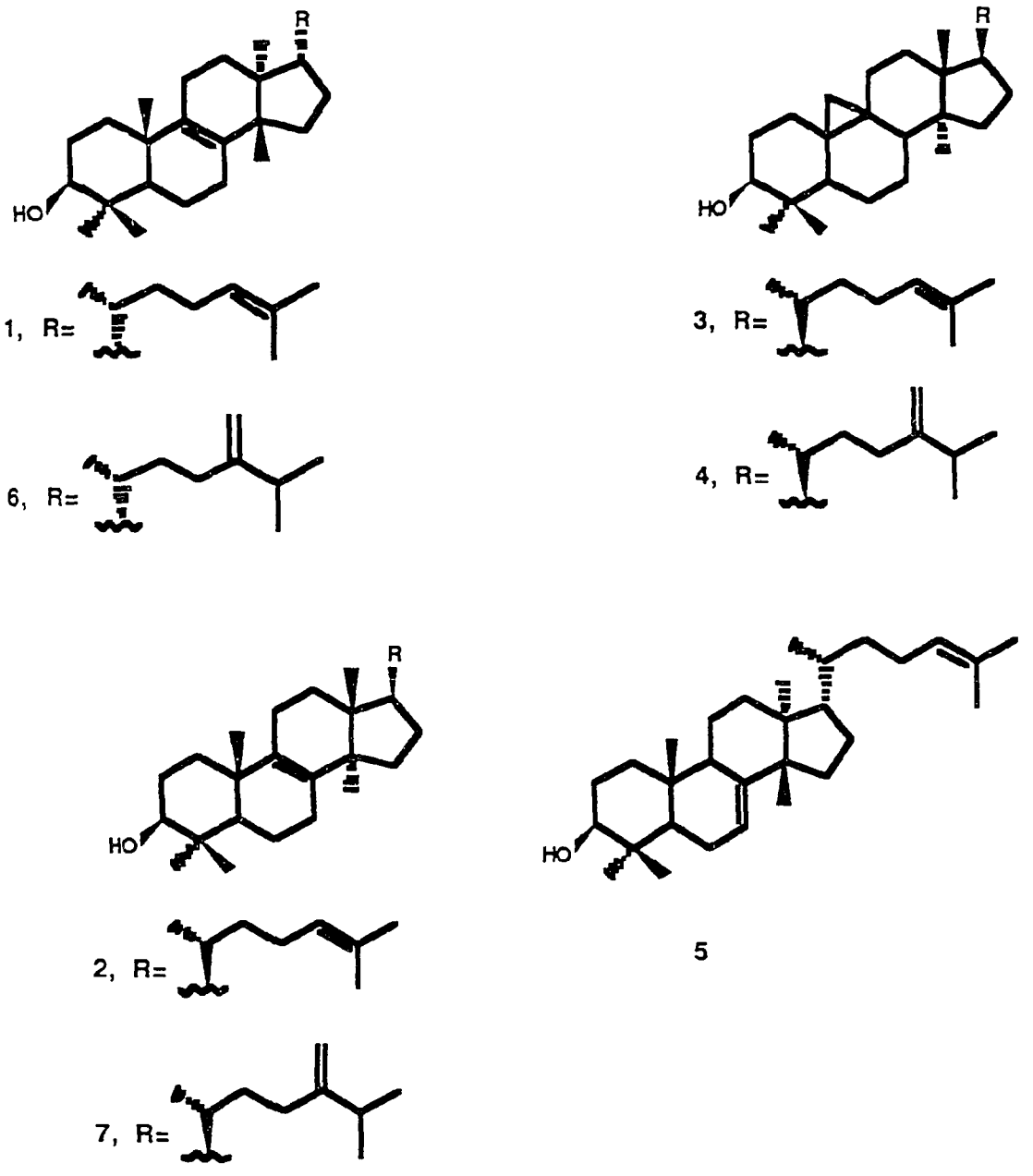

Figure 18 Structures of E. lathyris triterpenols

methyl groups (Barton et al. 1970): 0.70; 0.81; 0.88; 0.99; 0.99; 0.99, d; 1.08, d.

DISCUSSION

We have now verified the identity of butyrospermol by spectroscopic methods. On the basis of its mass and ^{1}H-NMR spectra we were able to confirm that it was a D-7 triterpenol of either the euphane, lanostane, or tirucallane series. Considerable work has been published concerning the ^{1}H-NMR of triterpenols in the presence of a lanthanide isotope shift reagent (Buckley et al. 1971, Itoh et al. 1976ab). Itoh and coworkers (1976a) have shown that in the presence of lanthanide shift reagents the allylic methyl groups (C26,C27) of tirucala-7,24-dienol are shifted downfield to 1.74 and 1.80 while in compound **5** they do not shift downfield and are observed at 1.62 and 1.68. The chemical shifts observed for the compound from *E. lathyris* latex are within experimental error of the chemical shifts reported for **5**. Examination of the spectra in the presence of Eu(fod)$_3$ thus made it clear that the compound was butyrospermol (**5**).

We then examined the compound previously identified as euphorbol. In the ^{1}H-NMR spectrum the farthest upfield chemical shift of a methyl group was 0.693. The farthest upfield shift for a methyl group of eupha-8-enol is reported to be 0.77 and that of euphorbol is also reported to be 0.77 (Itoh et al. 1976a). The farthest upfield chemical shift of a methyl group in 24-methylenelanosterol (**7**) is reported to be at 0.70 (Itoh et al. 1976a). A closer examination of the proton spectrum shows an excellent agreement with that of **7**. To further verify the identity as **7**, an authentic sample was synthesized by the method of Barton et al (1970). The natural product from latex has identical mass and proton NMR spectra to those of this synthetic standard. On this basis we believe that this compound is 24-methylenelanosterol (**7**), not euphorbol (**6**).

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Chapter 6

ADDENDUM TO SECTION 2: ADDITIONAL PHYSIOLOGICAL STUDIES OF THE LATICIFER CELL

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ABSTRACT

The role of S-adenosyl methionine, fructose 2,6-bis phosphate, and Mn^{++} in the physiology of *Euphorbia lathyris* laticifer cell has been investigated. S-adenosyl methionine was found to play a role in the biosynthesis of the fatty acid esters of the triterpenols. Addition of fructose 2,6-bis phosphate to isolated latex increased triterpenoid production from glucose. The addition of Mn^{++} to isolated latex resulted in a doubling of the rate of triterpenoid biosynthesis.

INTRODUCTION

Even though we have established that the site of MVA to triterpene biosynthesis is the 5000 xg pellet, we have observed some qualitative differences between triterpene biosynthesis in whole latex versus the 5000 xg pellet. We have reported previously (Nemethy et al. 1983) that in whole latex triterpene ester synthesis was significantly faster than that of the free triterpenols. The typical ratio in whole latex is 6:1; the corresponding ratio in the pellet is 3:1. This change in ratio is due to loss of triterpene ester synthesis only. This change in ratio is due to loss of triterpene ester synthesis only. On recombination of supernatant and pellet some increase in triterpene ester synthesis is observed, but the ratyio is never restored to that seen in whole latex. The supernatant does not have an affect on the rate of free triterpenol production.

Extensive work has been done in mammilian systems on the role of membrane-bound and soluble enzymes involved in sterol biosynthesis. The rate of cyclization of squalene to lanosterol in liver microsomes was shown to be enhanced by an order of magnitude on addition of supernatant. A stimulatory protein was isolated and characterized, and is considered to be obligatory to lanosterol biosynthesis in liver.

The existence of analogous proteins has never been established in plants, and while we have observed stimulation of triterpene synthesis on addition of supernatant in the latex system, this stimulation only occurred in the production of the triterpene esters and not for the triterpenols. Thus the stimulation did not occur at the cyclization step, and does not involve a stimulatory protein similar to that found in mammilian systems.

Examination of the synthesis of the six individual triterpenols in latex also showed a marked difference between pellet and whole latex (Table VI). The production of 24-methylene cycloartenol is decreased and the production of cycloartenol increased upon pelleting. The substrate in the conversion of cycloartenol to the 24-methylene compound is S-adenosyl methionine (SAM). As any SAM would remain in the supernatant, these data suggest the change in activity is due to a deficiency in SAM in the pellet. We tested this possibility and also investigated the possibility that SAM plays a role in the production of triterpene esters.

In previous attempts we have been unable to observe any incorporation of radiolabel from supplied glucose or sucrose into sterols in isolated latex. As these are the common forms of carbohydrate that would be translocated to the laticifer cell, this suggested an inhibition of conversion of these sugars to the precursors of isoprenoid biosynthesis (ie. acetate or pyruvate) occurred upon removal of the latex from the laticifer cell.

Fructose 2,6-bis phosphate (F26BP) has been identified as a regulator of the conversion of fructose 6-phosphate (F6P) to fructose 1,6-bis phosphate (F16BP). This reaction is a key step in the conversion of sugars to acetate. In the presence of F26BP this conversion will take place, and in its absence the back reaction is favored. Since F26BP is unstable in acid, and isolated latex has a pH of 5.5, any F26BP present in the cytoplasm of the cell would be destroyed when the latex is collected (which causes a breakage of the acidic vacuoles). We tested this possibility by providing exogenous F26BP to isolated latex.

Recent studies have indicated a role for Mn^{++} in the biosynthesis of sterols in latex (G. Piazza, personal communication). We investigated this possibility by adding Mn^{++} to isolated latex.

Table VI

Distribution of Radioactivity (in %) Among Latex Triterpenoids

Triterpenol	Whole Latex	Pellet	Pellet + SAM
Lanosterol	9.5	13.2	9
24-Methylene Lanosterol	6.4	4.6	7
Cycloartenol	13.2	22.2	11
24-Methylene Cycloartenol	35.2	27.2	38
Butyrospermol	28.0	29.0	28
Euphol	7.5	4.0	7

MATERIALS AND METHODS

Latex was collected and assayed with MVA as described previously (Nemethy et al. 1983, chapter 4). To test for the effect of SAM, levels from 10 to 500 µm were added. Fructose 2,6 bisphosphate studies were performed in one of two ways. The first set of assays were done with unbuffered latex, so F26BP levels of 50 mM were used to offset its destruction by the acidic latex. These values are about 100x higher than those found in leaf mesophyll cells. The product of F26BP breakdown is F6P, so this was added as a control to insure that it was notaffecting the reaction. The second set of experiments was performed with latex buffered to pH 7.0 with 100 mM MOPS buffer and at 1 mM F26BP. ^{14}C-Glucose (specific activity of 5 µc/µmol) was supplied at 2 mM. Stimulation of triterpenol production by Mn^{++} was tested by the addition o 10 mM Mn^{++} to the latex.

RESULTS

When SAM was added to the latex it resulted in an increase in triterpenol and triterpene ester synthesis from 22% of whole latex without SAM to a maximum of 50% of whole latex values at the optimal concentration of 50 µm (Figure 19). Exogenously added SAM also affects the triterpene ester to triterpenol ratio. At 50 µm SAM this ratio is restored to the value observed in whole latex (Figure 20). SAM was also found to methylate phosphatidylethanolamine (PE) to form N-monomethyl PE, a precursor of phosphatidylcholine (PC).

Fructose 2,6-bis phsophate was found to increase incorporation of radiolabel from glucose into sterols at both pH 5.5 and pH 7.0 (Table VII). Fructose 6-phosphate had not affect on synthetic rates.

The addition of 10 mM Mn++ increased triterpenoid biosynthesis from MVA by about a factor of 2 (Table VIII).

DISCUSSION

We have previously shown that the acyl donor for triterpene ester is PC. SAM is essential for the synthesis of PC; three methyl groups are donated by SAM to the nitrogen of PE to form PC. In the absence of SAM, triterpene ester synthesis is probably decreased because insufficient acyl donor is produced. SAM, therefore, has two functions in latex: methylation on the nitrogen of PE to form PC, and methylation on the carbon of the C_{30} triterpenes to form the C_{31} triterpenes (Figure 21).

The results of both sets of F26BP experiments indicate that it does play a role in the regulation of carbon allocation to the production of triterpenoids. The increase in activity at pH 7.0 probably occurred because the conversion of F6P to F16BP occurs in the cytoplasm, which is normally at a pH of 6.8. The presence of activity at pH 7.0 in the absence of added F26BP might indicate the presence of endogenous F26BP that was preserved when the latex is tapped into a buffer.

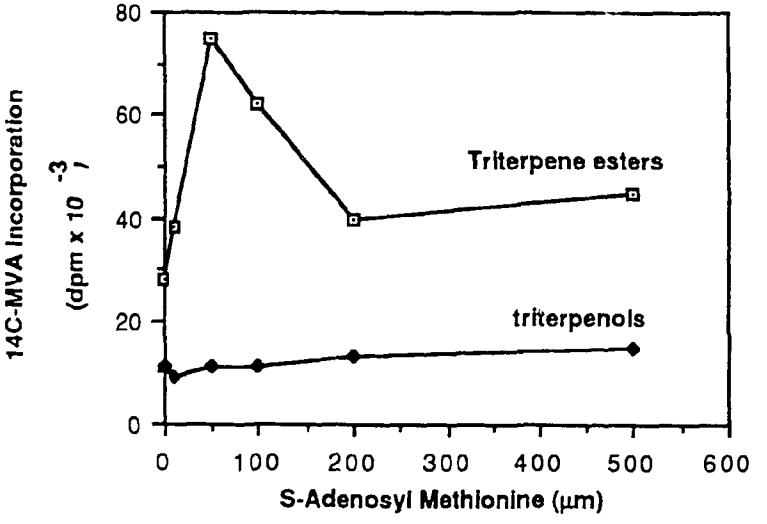

Figure 19. The effect of S-adenosyl methionine on triterpene production by *Euphorbia lathyris* latex.

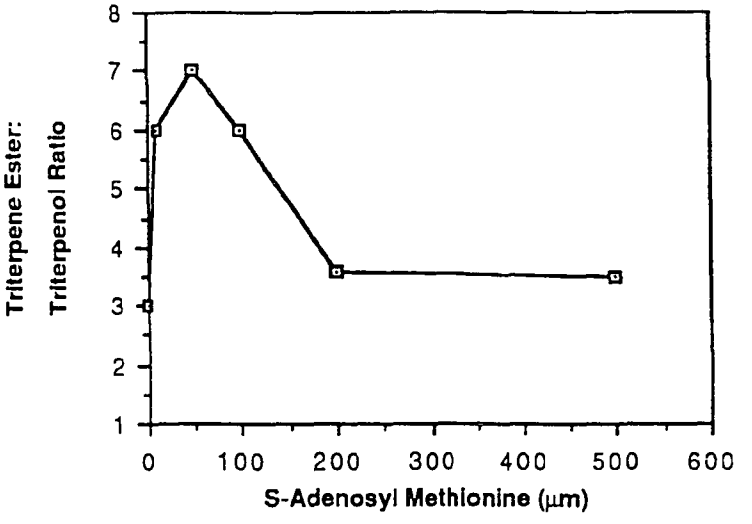

Figure 20. The effect of S-adenosyl methionine on the ratio of triterpene ester: triterpenol biosynthesis by *Euphorbia lathyris* latex.

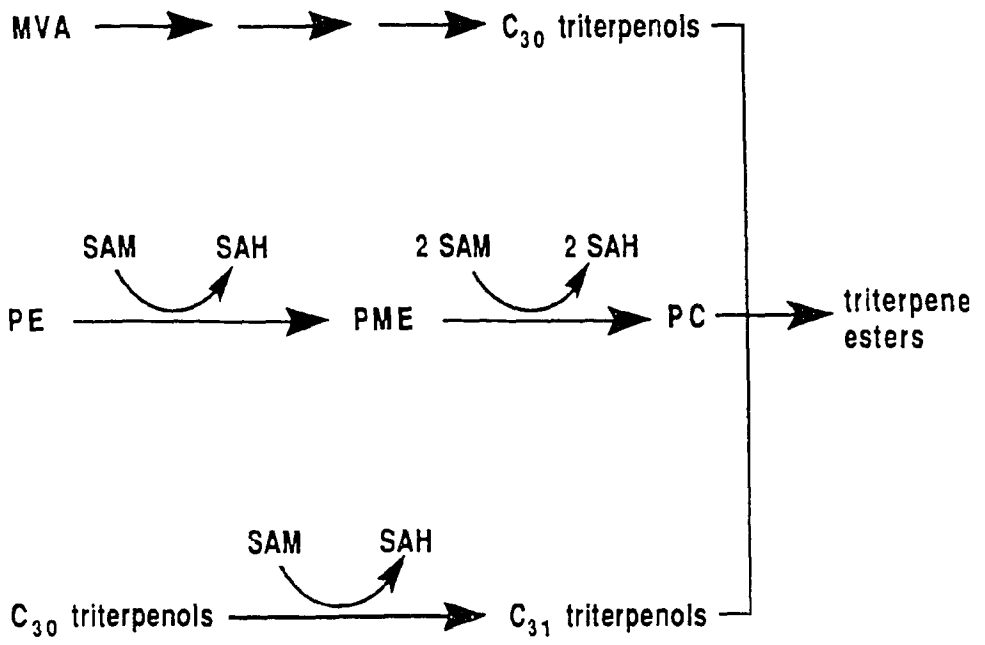

Figure 21. The role of S-adenosyl methionine in triterpenoid biosyntheis in *Euphorbia lathyris* latex.

Table VII

The Effect of Fructose 2,6-bis Phosphate on ^{14}C-Glucose Incorporation into Triterpenoids

	Triterpenoid Synthesis (dpm above background)	
treatment	latex (pH 5.5)	latex + MOPS buffer (pH 7.0)
control	0	795
+Fructose 2,6-bis Phosphate	369	1443
+Fructose 6-Phosphate	0	not performed

Table VIII

The Effect of Mn^{++} Ion on Triterpenoid Biosynthesis

treatment	Triterpenoid Biosynthesis (pmol/hr) Triterpenols	Triterpenol Esters
control	44	114
10 mM Mn^{++}	70	220

Section 3

Hydroxymethyl Glutaryl-CoA Reductase Activity in *Euphorbia lathyris*

Chapter 7

HYDROXYMETHYL GLUTARYL-COENZYME A REDUCTASE ACTIVITY IN LATEX

Cynthia L. Skrukrud
Melvin Calvin

ABSTRACT

Biosynthesis of triterpenoids (triterpene esters and triterpenols) by isolated latex of *Euphorbia lathyris* was investigated. The rate of *in vivo* incorporation of mevalonic acid into the triterpenoids was 30x greater than that of acetate incorporation (0.55 vs. 0.02 nmol 100 µl latex $^{-1}$ h^{-1}), indicating that the rate limiting step in the pathway occurs prior to mevalonate conversion.

Both HMG-CoA reductase (EC 1.1.1.34) andHMG-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 xg pellet. The rate of conversion of HMG-CoA to mevalonate by this enzyme (0.02 nmol 100µl latex^{-1} h^{-1}) was comparable to the overall rate of acetate incorporation into the triterpenoids, indicating that this enzyme is rate-determining in the biosynthesis of the latex terpenoids.

appeared in: Skrukrud, C.L. *Terpenoid Biosynthesis in Euphorbia lathyris and Copaifera spp.* (Ph.D. thesis, University of California, Berkeley, 1987).

INTRODUCTION

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 (Shukla and Krishna Murti 1971, Fahn 1979).

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 (Lynen 1967).

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* (Fairbairn and Steele 1981). Ponsinet and Ourisson (1967, 1968) 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 xg of one latex sample was mixed with the pellet of a like centrifugation of another latex containing a different suite of triterpenoid structures.

The first investigation of the metabolism of *Euphorbia lathyris* latex *in vitro* was reported by Ponsinet and Ourisson (1968) who found cycloartenol, 24-methylene cycloartenol, and lanosterol were labelled upon incubation of latex with [1-^{14}C] sodium acetate. Groeneveld (1976) 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 ^{14}C-glucose injected into the hollow stem of the plant followed by analysis of the latex after a 24 h incubation. 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.

Nemethy et al. (1983) reported the first biosynthetic studies performed on *E. lathyris* latex by our laboratory. 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, hydroxy-methylglutarate(HMG), HMG-CoA, mevalonolactone (MVAL), or isopentenylpyrophosphate (IPP) into triterpenoids.

The experiments described in this chapter 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 (Lynen 1967).

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 $\mu E\ m^{-2}\ s^{-1}$ 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 $CaCl_2$.

Instruments. All UV and 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 xg values less than 12,000 xg, 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 (1927).

PROTEIN ASSAY 2) This assay was based on the method of Bradford (1976) as modified by Vincent and Nadeau (1983). 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.

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 (Brown and Goldstein 1980).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 (Shapiro et al. 1969).

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}.

Localization of HMGR in latex fractions. HMG-CoA Incubation) Latex was subjected to the differential centrifugation scheme diagramed in Figure 22. This scheme was based on work which localized *Pisum* seedlings HMGR in the heavy microsomal fraction (P3) (Brown and Goldstein 1980). 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). Fo 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 xg. The supernatants were filtered through 0.45 µm filters, and their volume brought to 250 µl with water.

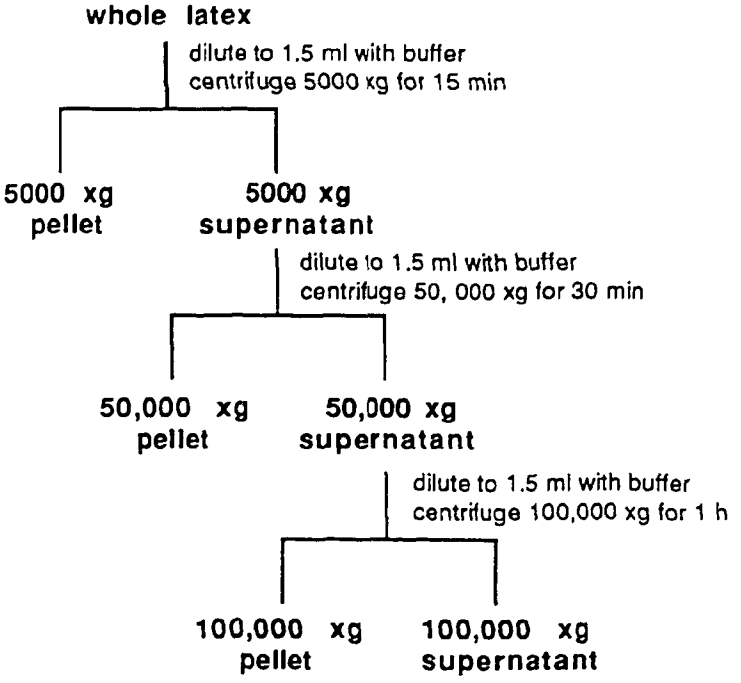

Figure 22. Preparation of latex fractions for analysis of distribution of HMG-CoA reductase activity.

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 xg Mix. A 1.5 ml sample of latex was centrifuged at 100,000 xg 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 IX).

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 xg 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 xg. Four 200 µl samples of the 100,000 xg 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 xg 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 xg. 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 xg 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 xg. 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 m

Table IX

Incubation Components for Determination of HMG-CoA Reductase Activity versus Volume of 100,000 xg Mix

Sample	1	2	3	4
µl 100,000 xg 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

$mmol^{-1}$, 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 xg 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 xg 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 xg 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 X). 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 xg 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.

RESULTS AND DISCUSSION

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. 23). 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 (Goad 1983). 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 (Brown and Goldstein 1980).

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 HMGR was a likely candidate for the rate-determining role (Fig. 24). Exogenously-supplied HMG-CoA was not incorporated into triterpenoids by whole latex, indicating that HMGR was possibly sequestered in latex (Nemethy et al. 1983). 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 (Liscum et al. 1983).

Numerous incubations of fractionated latex with radiolabelled HMG-CoA were performed; the results of these experiments are tabulated in Table XI 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

Table X

Quantity of HMG-CoA used in HMG-CoA Reductase Substrate Saturation Experiment

Incubation	Final Concentration μM HMG-CoA	[^{3}H]HMG-CoA μl	2 mM HMG-CoA 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

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

Figure 23. Comparison of rates of incorporation of MVA and Ac into latex triterpenoids with HMG-CoA reductase activity in latex.

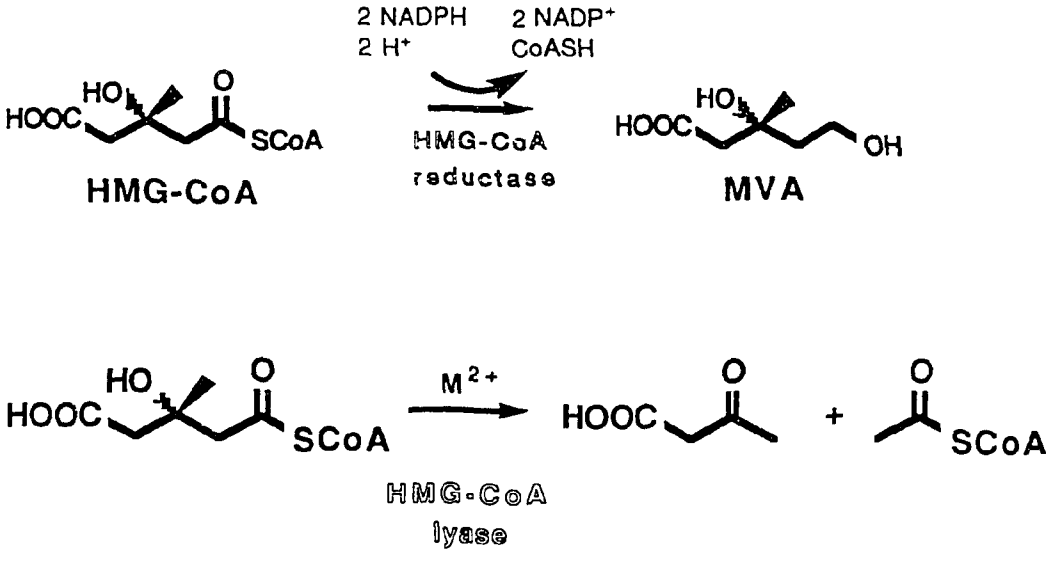

Figure 24. Reactions catalyzed by HMG-CoA Reductase and HMG-CoA Lyase

Table XI.

HMG-CoA metabolism of various latex fractions

incubation	sample	conversion rate (nmol 100 µl latex^{-1} h^{-1})	conditions
1	100,000 xg pellet		
	-> Ac + AcAc	0.22	OA analysis
	-> MVA	0.1	
	-> Ac	0.8	treated with base, OA analysis
	100,000 xg 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 xg pellet		treated with base, 0A analysis
	-> Ac + AcAc	1.5	incubated pH 5.5
	" "	1.6	incubated pH 6.7
	100,000 xg supernatant		treated with base, 0A analysis
	-> Ac + AcAc	22	incubated pH 5.5
	" "	32	incubated pH 6.7
3	5000 xg 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 xg pellet		incubated with 30 mM EDTA
	-> Ac + AcAc	0.9	OA analysis
	-> MVA	0.022	
	-> MVA	0.032	TLC analysis

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

	50,000 xg pellet -> MVA	0.001	
8 (con't)	100,000 xg supernatant -> MVA	0.016	
	100,000 xg pellet -> MVA	0.002	
9	5000 xg supernatant -> MVA	0.005	TLC + ODS analysis
	100,000 xg supernatant of sonicated 5000 xg pellet -> MVA	0.001	
	100,000 xg pellet of sonicated 5000 xg pellet -> MVA	0.026	
10	5000 xg supernatant	0	TLC + ODS analysis
	100,000 xg pellet of sonicated 5000 xg pellet -> MVA	0.019	

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 (Ness and Moffler 1978), but the product has not been identified. Washing of the microsomes removed the competing activity.

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 (Figure 24). Avian lyase is a soluble protein with a $pH_{optimum}$ of 8.9. It requires a divalent cation (Mg^{++} or Mn^{++}) for activity (Kramer and Miziorko 1980). Lyase activity has been detected in *Hevea brasiliensis* latex (Hepper and Audley 1969). 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.

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)(Sabine 1983). 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 HMGR 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 xg pellet (100,000 xg pellet of sonicated 5000 xg pellet). This location for HMGR is consistent with the results of an experiment investigating the specific activity of HMGR in latex fractions (Table XII). The 10,000 xg 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 (Ness et al. 1981). *Hevea brasiliensis* latex HMG-CoA reductase has also been localized to a particulate fraction (40,000 xg pellet) but had a higher specific activity of 17 nmol h^{-1} mg $protein^{-1}$(Sipat 1982).

The activity of HMG-CoA reductase measured in the 5000 xg pellet fraction (average of 4 experiments) is comparable to the overall rate of conversion of acetate into triterpenoids in latex (Fig. 23). This result suggests that HMG-CoA reductase is indeed the rate-determining enzyme in the pathway to

Table XII.

Distribution of HMG-CoA Reductase Activity in Latex Fractions.

	Total Activity (pmol /h)	Total Protein (mg)	Specific Activity (pmol/ h /mg protein)
10,000 xg pellet	54	0.62	87
15,000 xg pellet	7	0.67	10
50,000 xg pellet	5	0.43	12
100,000 xg pellet	5	1.5	3
100,000 xg supernatant	160	5.5	29

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 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.

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 (Chapter 8). These experiments are being reported as a reference for possible further work on latex HMGR.

The dependence of HMGR activity on the volume of latex sample and time are shown in Figures 25 and 26. HMG-CoA reductase was precipitated by bringing latex to 70% saturation in ammonium sulfate (Table XIII). 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 (Figure 27). At pH 6.1 a four-fold purification from the original HMG-CoA reductase activity measured in the 100,000 xg mix was obtained. The possible difference in $pH_{optimum}$ for latex HMGR and HMGR from the vegetative tissue (pH 6.8- Chapter 8) suggests that isozymes of the enzyme exist in *E. lathyris*. A substrate saturation curve run on the desalted $(NH_4)_2SO_4$-precipitated HMGR 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 (Sipat 1982) (Figure 28).

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 xg). 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.

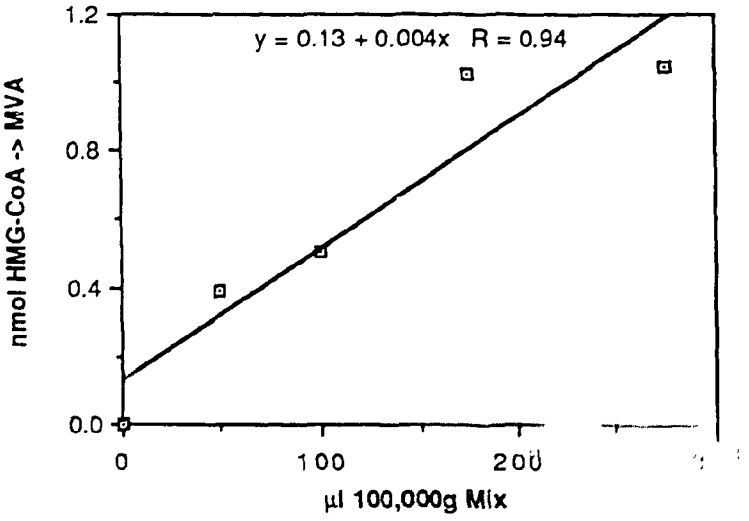

Figure 25. HMG-CoA Reductase activity versus [illegible]

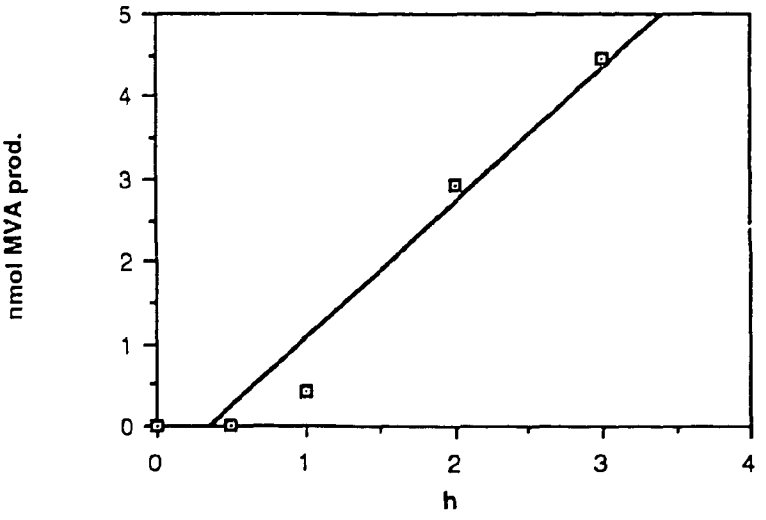

Figure 26. Time course of HMG-CoA Reductase activity in 100,000 xg supernatant.

Table XIII

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 xg 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

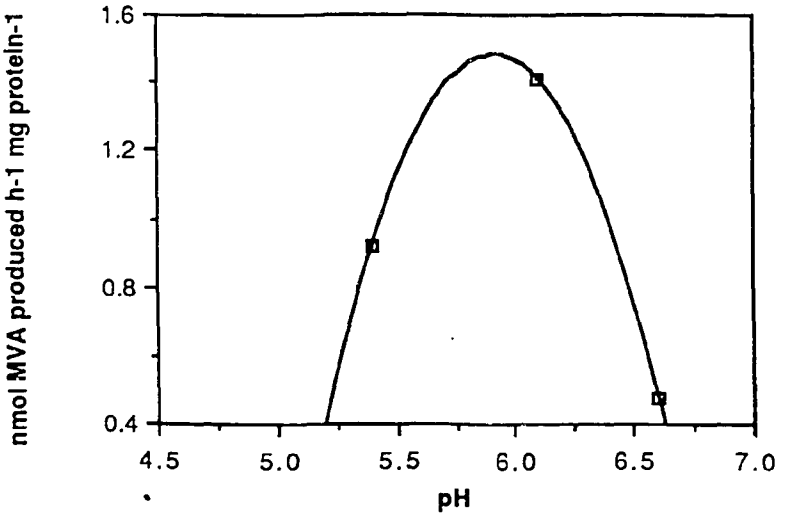

Figure 27. Effect of pH on HMG-CoA reductase activity of latex.

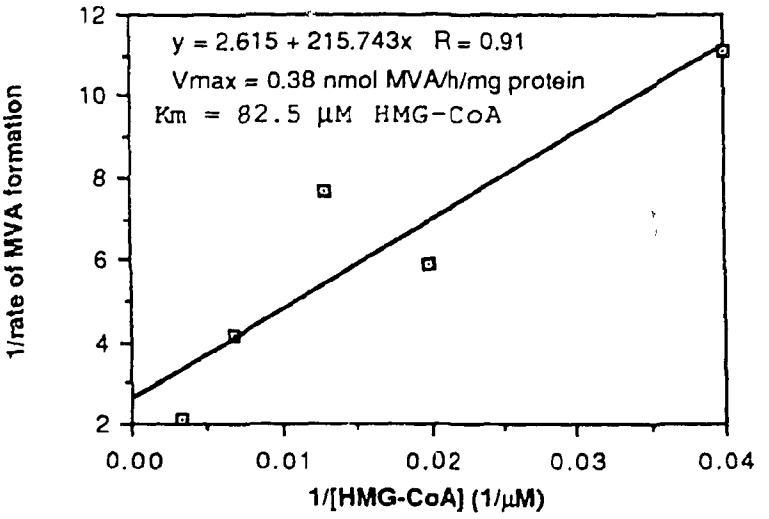

Figure 28. Double-reciprocal plot of HMG-CoA reductase activity versus [HMG-CoA].

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Chapter 8

LOCATION AND SOLUBILIZATION OF HYDROXYMETHYL GLUTARYL-COENZYME A REDUCTASE FROM EXTRACTS OF *EUPHORBIA LATHYRIS* STEM AND LEAF TISSUE

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Melvin Calvin

ABSTRACT

The major portion of the HMG-CoA reductase activity of *Euphorbia lathyris* vegetative tissue was localized to a plastid fraction, while the highest specific activity was found in the mitochondrial fraction. Differences in the optimal pH for HMG-CoA reductase activity between the vegatative and latex (Chapter 7) tissues suggests that isozymes of this enzyme may be present in the two tissue types.

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INTRODUCTION

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 (Chin et al. 1982). Mammalian HMG-CoA reductase is subject to multivalent feedback regulation both at the gene and enzyme level (Brown and Goldstein 1980). Transcription of the reductase gene is supressed by cholesterol-containing low density lipoproteins, 25-hydroxycholesterol, and mevalonate(Faust et al. 1979). 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 (Gil et al. 1985), and a phosphorylation/ dephosphorylation mechanism converts the enzyme between an active and inactive form (Beg et al. 1980).

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 (see Kreuz and Kleining 1984) 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. 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 (Brooker and Russell 1975, Cerebalo and Mitchell 1984).

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* . 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 (Garg and Douglas 1983).

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* (Chapter 7). 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 (1975). Incubations were allowed to sit at room temperature for 10+ min to ensure MVA lactonization. Samples were centrifuged 20 min at 12,000 xg 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 and 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(1976) as modified by Vincent and Nadeau(1983). A volume of 20 µl sample + buffer was added to 80 µl 0.1% Triton X-100, ther 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. (1981) for membrane proteins samples using the procedure of Bensadoun and Weinstein (1976) 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 xg 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 xg to remove cell debris, 5 min at 3000 xg to obtain a crude plastid pellet, 15 min at 18,000 xg for a crude mitochondrial pellet, and 1 h at 100,000 xg 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 xg pellet only. Each pellet fraction and the soluble protein fraction (100,000 xg 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 xg for 5 min to remove cell debris and then centrifuging at 18,000 xg for 20 min to sediment the HMGR-containing organelles (18,000 xg Pellet). In this experiment the supernatant was divided into seven fractions before the centrifugation at 18,000 xg 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 et al.(1980). The seventh fraction was centrifuged at 100,000 xgfor 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 xg Pellet was tested by incubating the pellet with the detergent in 0.5 ml BUFFER B pH 6.7 for 20 min at 0-4 C, diluting the samples to 1.5 ml with BUFFER B, centrifuging the samples at 100,000 xg for 1 h then assaying the HMGR activity and protein concentration (METHOD 1) of the resultant supernatant and pellet fractions. The 100,000 xg pellets were resuspended in 250 µl BUFFER B.

Effect of protease inhibitors on HMGR activity and solubilization. The 18,000 xg 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 xg for 1 h, and the supernatants and pellets from each incubation were analyzed for HMGR activity and protein content (METHOD B). The 100,000 xg 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 xg for 20 min, and the supernatants were removed. Two 150 µl aliquots of the 18,000 xg supernatant containing both protease inhibitors were assayed for HMGR activity. One was quenched immediately to serve as a blank. The 18,000 xg pellets were each resuspended in 1 ml solubilization buffer with differing detergent and protease inhibitor components (Table XIX); 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 xg 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.

RESULTS

Location of HMGR. The initial differential centrifugation procedure, based on that used by Brooker and Russell (1975) in their study of the subcellular location of HMGR in pea seedlings, separated the tissue homogenate into crude fractions enriched in certain subcellular components. While not being purified organellar fractions, the four fractions obtained roughly represent the chloroplastic, mitochondrial, microsomal and soluble protein components of the crude extract. The major portion of HMGR activity was found associated with the 3000 xg plastid-enriched pellet (Table XIV). HMGR activity was also found in the other fractions with the soluble protein fraction (100,000 xg supernatant) containing the next highest level of activity followed by the crude mitochondrial fraction (18,000 xg 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 (Chapter 7).

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

Properties of particulate HMGR. HMG-CoA reductase activity of the 18,000 xg pellet has a pH_{max} of 6.8 (Figure 29). Mevinolin (Figure 30) is a fungal metabolite which has been found to be a highly specific competitive inhibitor of HMGR (Endo et al. 1977). It has been shown to be effective with higher plant HMGR as well as with the mammalian enzyme (Bach and Lichtenthaler 1982). *E. lathyris* HMGR is sensitive to inhibition by mevinolin, HMGR activity being reduced by 80% in the presence of 0.5 µM mevinolin (Table XVI). HMGR localized in this heavier organellar fraction (18,000 xg 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 xg pellet was sonicated to rupture the organelles and then centrifuged for 1 h at 100,000 xg (Table XVI).

Solubilization of HMGR. In an experiment testing the effect of different detergents on the

Table XIV

Distribution of HMG-CoA Reductase Activity in Subcellular Fractions from *Euphorbia lathyris* Vegetative Tissue

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

Table XV

Concentration of HMG-CoA Reductase in a Particulate Fraction

Total Activity (nmol/ h)	Total Protein (as % of 500 xg supernatant)	Specific Activity (mg)	(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

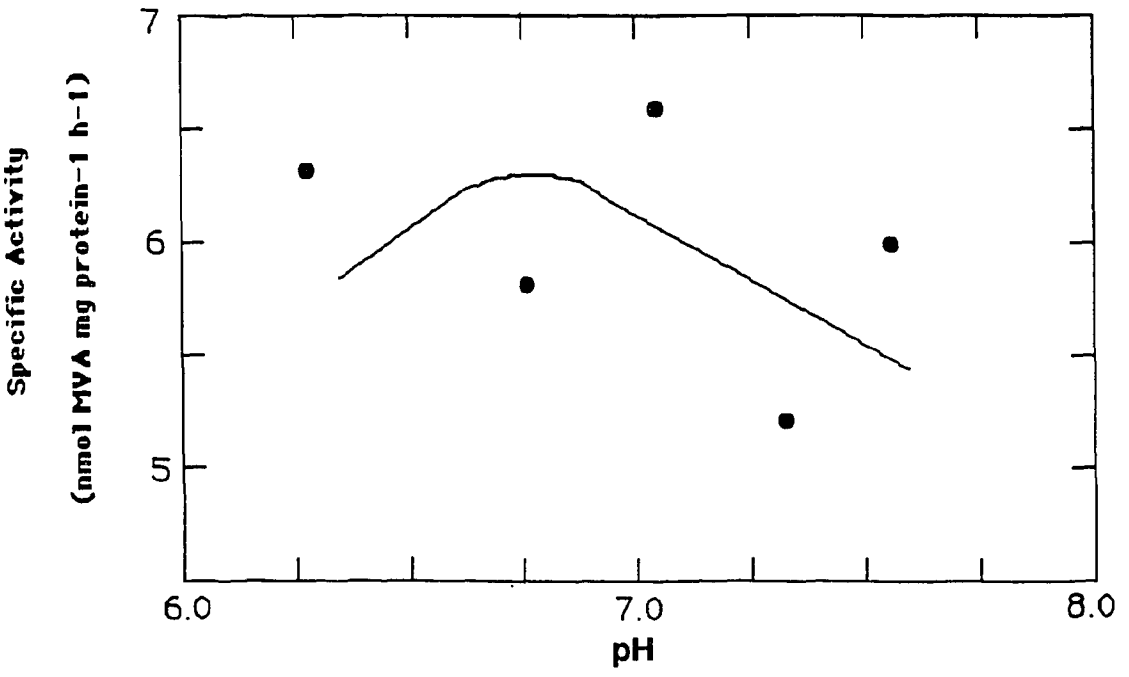

Figure 29. Effect of pH on the specific activity of particulate HMG-CoA reductase from *Euphorbia lathyris*.

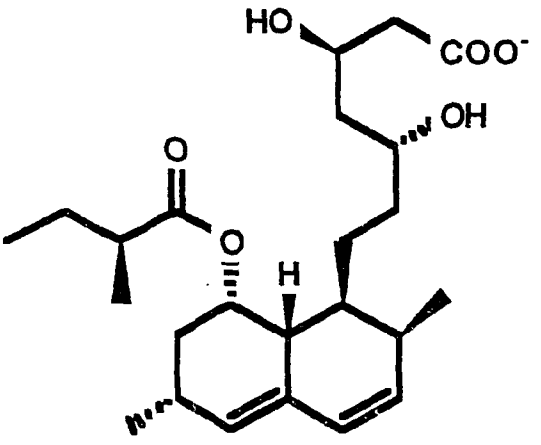

Figure 30. Structure of the fungal metabolite **mevinolin**, a competitive inhibitor of HMG-CoA Reductase.

Table XVI

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)	(% of total fractions)	(as % of 18k xg pellet)	Total Protein (mg)	Specific Activity (nmol/ mg protein/h)
18,000 xg pellet	10.5	–	–	1.6	6.6
18,000 xg pellet + 0.5µM mevinolin	2.1	20	–	1.7	1.2
from sonicated 18,000 xg pellet-					
100,000 xg supernatant	1.0	25	10	0.2	5.9
100,000 xg pellet	3.0	75	29	1.0	3.0
Σ 100,000 xg fractions supernatant	100%	39%			

solubilization of HMGR, deoxycholate treatment was the most effective of the detergents tested (Table XVII). 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 xg 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 XVI).

Effect of Protease Inhibitors on HMGR. 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 0-4°C, the HMGR activity of a 500 xg 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 XVII). While the 3000 xg and 18,000 xg 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 XIV). Treatment of the 18,000 xg 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 XIX). The PMSF-treated sample had a lower ratio of supernatant to pellet activity than the untreated sample did, while leupeptin treatment improved the ratio by a factor of three. When 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 XVIII). Here 68% of the activity was associated with the 18,000 xg supernatant, a fraction containing microsomal and soluble proteins. Treatment of the 18,000 xg pellet with both inhibitors improved the recovery of HMGR, both solubilized and membrane-bound, as compared to treatment with leupeptin alone.

DISCUSSION

Distribution of HMGR 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 xg supernatant sedimented when that fraction was centrifuged at 18,000 xg for 20 min (Table XV). 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 XIV).

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 malhamensis* 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 (Maurey et al. 1986). 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 xg supernatant (Table XVIII). 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 HMGR found associated with the organellar fractions. Based on these fractionation experiments, *E. lathyris* contains HMGR 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

Table XVII

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

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

Table XVIII

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

Table XIX

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

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

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 HMGR has been thought to be exclusively localized to the endoplasmic reticulum (Brown and Goldstein 1980), although recently its presence in the peroxisomes of rat liver cells has been demonstrated both enzymatically and immunologically (Keller et al. 1985). Yeast HMGR is located in the mitochondria (Shimizu et al. 1971). In higher plants HMGR 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 (Brooker and Russell 1975). 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 xg (Cerebalo and Mitchell 1984). In radish seedling extracts, HMGR was found in both the organellar (16,000 xg pellet) and microsomal (100,000 xg pellet) fractions, with the 16,000 xg pellet activity co-migrating on Percoll gradients with the mitochondrial marker enzyme cytochrome oxidase (Bach et al. 1980). The presence of HMGR in the 18,000 xg pellet is consistent with its location in the 5000 xg pellet of *E. lathyris* latex (Chapter 7) and in *Hevea brasiliensis* latex where it was found in a 40,000 xg pellet (Sipat 1982).

Properties of organellar HMGR. The optimum pH found for HMGR of the 18,000 xg 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 (Wong et al.1982). Purified HMGR from a heavy-membrane fraction of radish seedlings was consistently assayed at pH 7.5, presumably the optimum pH for that enzyme (Bach et al 1986). 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, Chapter 7) is a further indication of the existence of different forms of HMGR 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 HMGR. In the presence of protease inhibitors, Brij was the best solubilizing agent for HMGR of the 18,000 xg pellet, although deoxycholate treatment gave a greater recovery of total HMGR, both solubilized and still membrane-bound (Table XVII). 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 XVII). While activation of HMGR upon deoxycholate treatment has been seen with the mammalian enzyme, Bach et al. (1986) found that deoxycholate inhibited HMGR activity in radish seedlings. Maurey et al. (1986) found that microsomal HMGR activity from *O. malhamensis* was stimulated *ca* 60% in the presence of 0.5-1% Triton X-100. 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 XVII), 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 HMGR. In mammalian systems, solubilization of HMGR 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 (Ness et al. 1981). 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 (Lynn and Clevette-Radford 1983), 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* (Bach et al. 1986, Maurey et al. 1986). With *E. lathyris*, when either the thiol protease inhibitor leupeptin or the serine protease inhibitor PMSF was added to the deoxycholate solubilized HMGR from the 18,000 xg pellet, higher HMGR activity was obtained than with samples without any protease inhibitor added (Table XIX). 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* HMGR. 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 XVIII).

CONCLUSIONS

Euphorbia lathyris vegetative tissue appears to contain at least one iso-enzyme of HMGR 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.

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Section 4

Environmental Effects on Hydrocarbon Production in *Euphorbia lathyris*

Chapter 9

THE EFFECT OF SALINITY ON THE ALLOCATION OF CARBON TO ENERGY-RICH COMPOUNDS IN *EUPHORBIA LATHYRIS*

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ABSTRACT

Hydroponically-grown *Euphorbia lathyris* plants were exposed to increasing levels of NaCl to study the effect of salinity on carbon allocation within the plant. Salinization caused a decrease in overall growth, and an increase in the production of both hydrocarbons and sugars. The hydrocarbon fraction, containing mostly triterpenoids, increased by 50%, and the sugar fraction, containing mostly sucrose, was increased by 88%. This resulted in a shift of available biomass from lignocellulose to the more usable sugars and hydrocarbons.

A 2-fold increase in the activity (per leaf area) of the enzyme ß-Hydroxymethyl- glutaryl-Coenzyme A Reductase was also observed with increased salinity. This enzyme is involved in the biosynthesis of the triterpenoids, and its response to increased salinity indicates a role for this enzyme in the regulation of plant hydrocarbon productivity.

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INTRODUCTION

We are studying the possibility of using plants as a source of renewable liquid fuels to replace petroleum. Although alcohol fuels can be generated by the fermentation of plant sugars or lignocellulose, our major concern has been with the exploitation of plants naturally high in hydrocarbons (Calvin 1987).

The plant hydrocarbon fraction, made up of isoprenoids, triglycerides, and free alkanes, is lower in oxygen content than the other plant constituents, and thus has a higher energy value (on a per weight basis). The isoprenoids and free alkanes have about 2.5x and the triglycerides have about 2x the energy content of cellulose (Wang & Huffman 1981). These components are also easily converted into gasoline- or diesel-like fuels. For example, the major hydrocarbon components of the *Euphorbia lathyris* plant, the pentacyclic and tetracyclic triterpenoids, can be converted to gasoline-type fuels by cracking on a zeolite catalyst column (Figure 31).

To increase the yields of plant hydrocarbons to the point that they offer an economical alternative to petroleum fuels, we must understand the basic mechanisms that control plant hydrocarbon production. Our laboratory has been studying the biosynthesis of isoprenoids from photosynthetically reduced carbon. Specifically, we are interested a) in the conversion of acetate through the isoprenoid biosynthetic pathway to the triterpenoids, and b) in the mechanism that regulates carbon flow into this pathway. We have reason to believe that these two processes are related, and that the rate of carbon flow through the pathway will control the rate of carbon allocated to the pathway. It is possible that these two processes are related and that the rate of carbon flow through the pathway will control the rate of carbon allocation to the pathway. The experiments described in this paper were perfomed to examine this possibility.

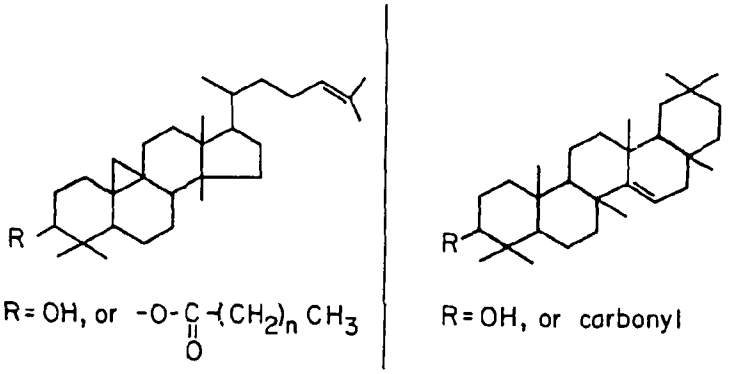
We utilize *Euphorbia lathyris* plants for most of our studies. These latex-bearing plants contain high levels of low molecular weight, reduced isoprenoids (approx. 5%), in addition to high levels of free sugars (approx. 30%). Latex can be tapped from these plants, and it maintains its biological activity, providing a convenient material for the study of isoprenoid biosynthesis. It has been proposed as a candidate for cultivation on energy farms (Calvin 1987).

We used salinity stress to study carbon allocation because of previous reports that have shown that carbon allocation to plant secondary metabolites can be increased by the application of environmental stress (Gershenzon 1981). In many stress situations, the growth processes are affected before photosynthesis is reduced. Such a condition would result in a decrease in carbon demand by the growing regions while photosynthesis remained high, making more carbon available for allocation to secondary plant products. We utilized such conditions to increase carbon allocation to the plant isoprenoids, and studied the changes in metabolism that accompanied increased hydrocarbon production. By using this approach we hoped to identify the underlying processes that control hydrocarbon biosynthesis. Such information is necessary before we can manipulate the plant genome to engineer high hydrocarbon-producing varieties.

MATERIALS AND METHODS

Growth Conditions. *Euphorbia lathyris* seeds were germinated in sand and were watered daily with half-strength Johnson's nutrient solution. The seedlings were then transferred to pots in growth chambers and were grown hydroponically in a modified Johnson's nutrient solution (the phosphate level was reduced to 0.5 mM). The plants were exposed to 16 hours of light at 25 C followed by 8 hours of darkness at 19 C. Plants were grown for 10 weeks before salinity treatments were started.

Salinity stress was applied by the addition of 25 mM increments of NaCl to the nutrient solution every



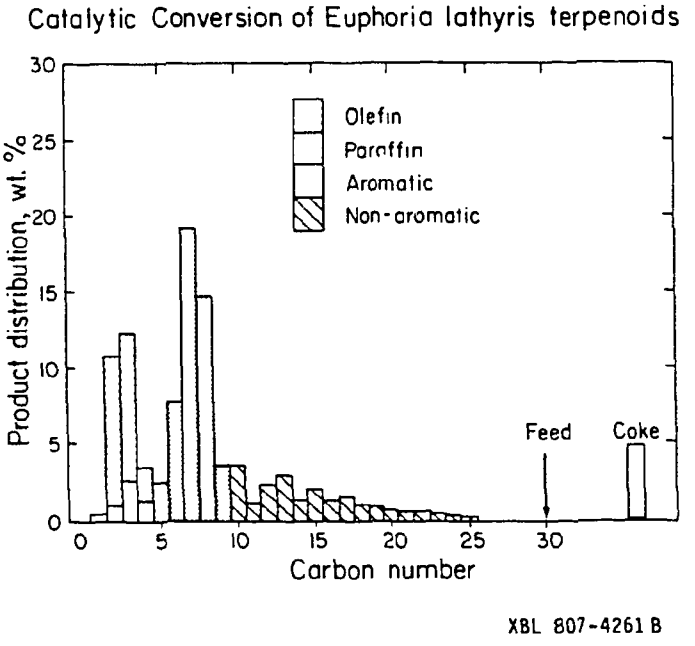


Figure 31. (top) Representative structures of tetracyclic and pentacyclic triterpenoids found in *Euphorbia lathyris*. The structures shown are cycloartenol (left) and taraxerol (right). (bottom) Catalytic conversion of triterpenoids on a zeolite column.

four days. The final concentrations of the treatments were 0 mM (control), 25 mM, 50 mM, 1000 mM and 200 mM added NaCl. Three plants were used for each treatment. The plants were harvested two weeks after the final NaCl addition.

Analysis of Plant Material . The effects of salinity on growth were determined from changes in shoot length, total fresh weight, and total and shoot dry weights. Dry weights were obtained by oven-drying plants at 70 C for four days. Changes in the levels of energy-rich compounds were determined as described previously (Nemethy et al. 1979,1981). Dried plant material was ground in a mill, and extracted in a Soxhlet appartus with boiling heptane for 24 hours to remove the hydrocarbon fraction. Remaining sugars and amino acids were then extracted with boiling methanol for 8 hours. Solvents were removed from the extracts by flash evaporation. Only the shoots were used for these extractons, as they would be the only part of the plant available for harvest.

HMGR Assay .Shoots were cut into small sections, mixed 1:5 (w/w) with cold buffer (10 mM MES, 10 mM DTE, 30 mM EDTA, 0.4 M Sorbitol, pH 6.5), and ground in a hand homogenizer. The extract was incubated for 1 hour with ^{3}H-HMG-CoA (0.3 mM, 33 mCi/mmole) and 3.3 mM NADPH, then quenched with KOH. The resulting product, mevalonic acid, was separted by silica gel TLC (acetone,benzene 1:1) and further purified by HPLC using an organic acid column in 0.0025 N H_2SO_4. Incorporation was determined by Liquid Scintillation Counting.

RESULTS AND DISCUSSION

Salinity and Growth. The results indicated that growth of *E. lathyris* plants was very sensitive to salinity, as levels as low as 25 mM NaCl resulted in reduced growth (Figure 32). Shoot length decreased about 20% and dry weights decreased 30-50% over the range of salinity treatments, while the most abrupt change occurred between 25 and 50 mM NaCl. An examination of the photosynthetic capacity of the plants showed that salinity had a much smaller effect on photosynthesis than it did on growth (unpublished data). These data suggest that the demand for carbon for growth would decrease, while photosynthetic carbon production would remain high, resulting in increased carbon available for allocation to the hydrocarbon and free sugar fractions.

Such a change in carbon allocation patterns did occur. Salinity treatments caused an increase in both the methanol (sugar and amino acid) fraction and the heptane (hydrocarbon) fraction, with a concurrent decrease in the residue, or bagasse fraction (Figure 33). These results differ from those of Ventas et al. (1983), who reported that exposure to salinity increased the sugar content but not the hydrocarbon content of *E. lathyris* plants.

Hydrocarbon Production .The heptane fraction increased by 1.5x, from 4.57% to 6.86% of the total plant dry weight. Though the actual energy increase per plant is small, these results indicate that the plant can regulate its hydrocarbon production. Nemethy et. al. (Nemethy et al. 1979, 1981) reported that the major components of the heptane fraction were triterpenoids and their fatty acid esters. We have examined the processes involved in biosynthesis of the triterpenoids to determine how production is controlled, and how salinity affects these controls.

Previous work by our laboratory (Skrukrud et al. 1987) has shown that a key rate-limiting step in triterpenoid biosynthesis is the conversion of HMG-CoA to MVA. This step is catalyzed by the enzyme HMG-CoA Reductase (HMGR). We determined the activity of this enzyme in extracts made from shoots of plants exposed to 0, 25 and 50 mM NaCl. We observed that increased salinity caused an increase in the activity of HMGR, especially between 25 and 50 mM NaCl (Table XX). We interpret these results as

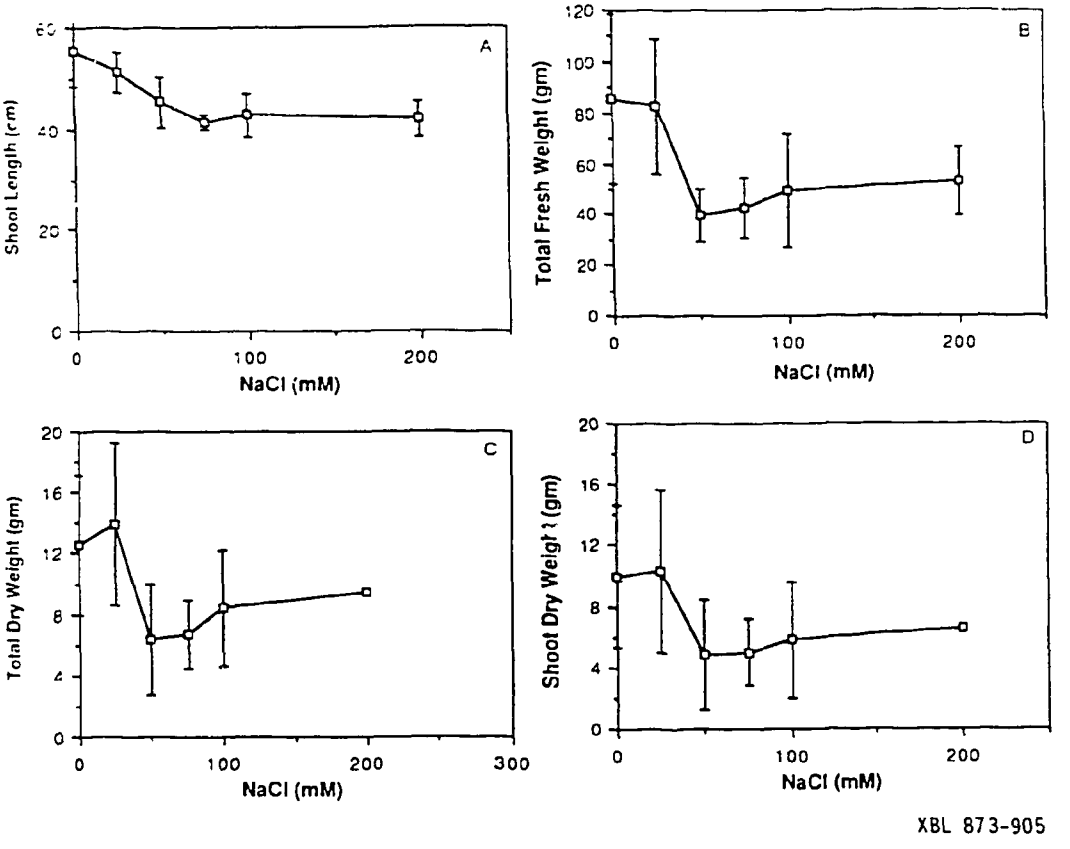


Figure 32. The affect of salinity treatments on the growth of *Euphorbia lathyris* plants. A)Shoot length; B) Total fresh weight of plants; C) Total dry weight of plants; D)Shoot dry weight, comparable to portion of the plant that would be harvested. (Error bars= standard deviation; n=3)

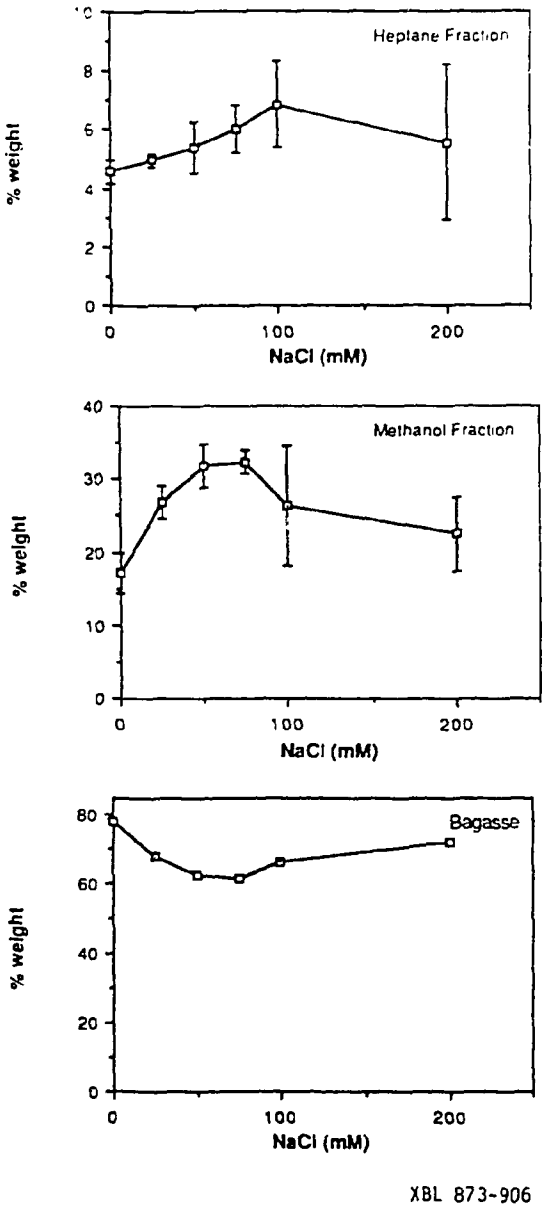


Figure 33. The affect of salinity on carbon allocation. The major constituents of the hydrocarbon fraction are hydrocarbons (isoprenoids). The major constituents of the methanol fraction are sugars and amino acids. The bagasse represents the residual.

indicating a strong correlation between the activity of HMGR and the rate of hydrocarbon production in *E. lathyris* plants.

We are currently involved in a project to isolate and purify HMGR, with an ultimate goal of isolating the gene(s) coding for it. The work presented here and the work of Maurey et. al (1986) with the alga *Ochromonas mathamensis* indicate that it may be possible to increase hydrocarbon production by increasing the expression and activity of this enzyme.

Carbohydrate Production. The methanol fraction has been found to contain 90% sugars (sucrose, galactose, glucose and fructose) and 10% amino acids (Nemethy et al. 1979, 1981). Elemental analysis of the methanol fractions isolated from the salinized plants showed that there were no significant changes in in the C/N ratio, indicating that the relative concentrations of sugars and amino acids remained unaffected by salinity. Since we have previously shown that this fraction is easily converted into ethanol with no further purification (Nemethy et al 1981), we see this shift of carbon allocation from the bagasse to the free sugars as an increase in the "useful-energy" content of the plant.

Total Energy Content . The total energy content of the plant (on a weight basis) remained unchanged. Energy content can be calculated from an elemental analysis of the plant material. A determination of the amount of oxygen required to burn a gram of sample is made (the "R" value), and this can be converted to BTU per unit weight by comparison to known standards (Otvos 1987). This was done with 0, 25, and 50 mM NaCl treated samples, and little change was seen in their energy contents (Table XXI). This occurred because most of the difference in carbon allocation resulted from a reduction in the bagasse fraction (high in lignocellulose) and an increase in the methanol fraction (high in sugars). Lignocellulose and sugars have the same energy value, though the limitations of present conversion technologies make the sugars a more valuable product than the lignocellulose.

It is difficult to apply the results obtained with plants grown in hydroponic solutions in growth chambers to field grown plants, but this system can be used as a model to demonstrate how changes in carbon allocation can result in large increases in available liquid fuel. For example, a projection of liquid fuel yields can be made for a field of control plants (0 mM NaCl) and slightly stressed plants (25 mM). Since the data indicate that little change occurs in growth between these treatments, we can use Kingsolver's (1982) values for optimum *E. lathyris* biomass yields of 15 tons of dry biomass per hectare for both treatments. We then assume that liquid fuel will be generated from only the extractable hydrocarbons and sugars. The total hydrocarbon content of the control and 25 mM treatments was determined to be 4.57% and 4.95%, or 0.686 and 0.743 metric tons per hectare, respectively. From elemental analysis of the heptane extract (Otvos 1987), we can determine that this fraction has an average energy content of 3.18 g oxygen per g fuel, or 44.4 GJ per metric ton. Thus the energy value of the hydrocarbons in the control plant will be 0.686 ton per hectare x 44.4 GJ per ton, or 30.4 GJ per hectare, and the yield from the 25 mM plants would be 33.0 GJ per hectare.

The same type of calculations can be made for the extracable sugars. The methanol extracable content of the control and 25 mM treated plants was 17.2% and 26.9%, or 2.58 and 4.03 tons per hectare, respectively. We have previously determined the efficiency of conversion of the methanol fraction to ethano, and can thus estimate the yield of ethanol production from control plants to be 0.69 tons per hectare and for the 25 mM plants to be 1.08 tons per hectare. Ethanol has an energy content of 1.96 g oxygen per g fuel, or 27.6 GJ per ton, so the ethanol yields would be 18.8 GJ per hectare for the control plants and 29.4 GJ per hectare for the 25 mM plants. The total fuel yield (hydrocarbons + ethanol) would be 49.2 GJ per hectare for the control plants and 62.4 GJ per hectare for the 25 mM plants. So if one can extend growth chamber experiments to field conditions, it should be possible to increase liquid fuel yields by the appropriate application of environmental stress.

Table XX

Effect[illegible]ity on the Activity of
β–Hydroxymethyl[illegible]Coenzyme A Reductase (HMGR)

Treatment (added NaCl)	HMGR Activity (m moles/m^2/hr)
0 mM	1.57 ± 0.10
25 mM	1.31 ± 0.04
50 mM	2.91 ± 0.52

(extracts were made from whole leaves; n=2; ± values are standard deviation)

Table XXI

The Effect of Salinity on the Energy Content of Dried Plant Shoots

Treatment (added NaCl)	"R" Value (g oxygen/ g fuel)	Agronomic Value (GJ/ ton)
0 mM	1.33 ± 0.3	18.5
25 mM	1.34 ± .03	18.6
50 mM	1.36 ± .04	18.9

(R values were determined from elemental analysis [C, H, N, and ash] as described in Otvos 1987; n=3; ± values are standard deviations)

In terms of yield per plant the increases in sugars and hydrocarbons was negated by the decrease in above ground biomass. Only the 25 mM treated plants showed an increase in the total yield of hydrocarbons and carbohydrates (Table XXII). If salinity stress were to be used as an agronomic technique to increase "useful-energy" yields in *E. lathyris*, it should be applied at low levels and probably no more than a few weeks before harvesting.

CONCLUSIONS

We have been able to alter carbon partitioning within an *E. lathyris* plant by exposure to increased NaCl levels. Growth was reduced, and carbon allocation to both hydrocarbon and sugar and amino acid production was increased. The activity of the enzyme HMGR was correlated with the change in carbon movement within the plant, indicating a role for it in the control of hydrocarbon biosynthesis.

Though there was little change in the total energy content of the plant, the shift of carbon from the bagasse fraction to the hydrocarbon and sugar fraction resulted in an increase in the "useful-energy" of the plant.

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Table XXII

Effect of Salinity on Yields per Plant of Heptane and Methanol Extracables and Residual Bagasse

Treatment (added NaCl)	Heptane Fraction gm/plant	Methanol Fraction gm/plant	Residual Bagasse gm/plant
0 mM	0.46	1.71	7.82
25 mM	0.51	2.77	7.02
50 mM	0.27	1.56	3.08
75 mM	0.30	1.61	3.09
100 mM	0.40	1.53	3.87
200 mM	0.37	1.48	4.75

(values represent the product of the shoot dry weights from Figure 32 and the percentage weights from the fractions in Figure 33)

Chapter 10

ADDENDUM TO SECTION 4: THE EFFECT OF APPLIED ENVIRONMENTAL STRESS ON GROWTH, PHOTOSYNTHESIS, CARBON ALLOCATION AND HYDROCARBON PRODUCTION IN *EUPHORBIA LATHYRIS*

Scott E. Taylor
Melvin Calvin

ABSTRACT

Photosynthetic activity was reduced by salinity stress, but it was found to be less sensitive than growth. Salinity stress also caused changes in the concentrations of specific cations. Moderate water stress had little effect on growth, but large changes in hydrocarbon production were still observed. Carbon allocation experiments with radiolabelled carbon indicated that carbon for latex production was supplied by nearby leaves, with some translocation down the stem also occurring.

INTRODUCTION

As mentioned in the previous chapter, we used environmental stress to induce changes in the plant metabolism and increases in triterpenoid biosynthesis. The three types of stress we utilized were salinity stress, water stress, and nutritional (nitrogen deficiency) stress. It has been previously found that the processes involved in plant growth (cell division, cell elongation, cellwall deposition) are more sensitive to environmental stress than are the processes involved with photosynthesis. Thus applying the proper level of stress can generate a situation in which the demand for fixed carbon and reducing power by growing regions is reduced while the photosynthetic production of these biological comodities remains high. The plant could respond to such a condition by channeling more of its carbon and reducing power to secondary products, in the case of *Euphorbia lathyris*, triterpenoids. If such an event occurred, we could use this applied stress to monitor changes in the allocation of carbon to the triterpenoids, and to identify those steps or processes that are modified by the plant to accomplish this act. Once these steps have been identified, they could be altered so these plants could produce large amounts of hydrocarbons.

The work discussed here are the additional results of the work done with water stress and salinity stress that were not reported in chapter 9. The application of nitrogen deficiency caused a rapid, uncontrollable induction of senescence in our plants, and thus could not be utilized.

MATERIALS AND METHODS

Plant Growth Conditions. *Euphorbia lathyris* plants were grown in hydroponic culture as described in the previous chapter. Salinity stress was applied as before. Watyer stress was applied by the addition of 75 mM polyethylene glycol (PEG) of an average molecular weight of 8000 g $mole^{-1}$. Plants were exposed to the PEG for a period of 6.5 weeks.

Analysis of Plant Material. Shoot dry weight was determined after drying the shoots in an oven at 70 C for 4 days. Chlorophyll levels were determined as described in Chapter 7. The levels of K^+, Na^+ and Mg^{++} were determined by atomic adsorption spectroscopy by the Analytical Chemistry Laboratory, Department of Chemistry, U.C. Berkeley. The hydrocarbon and sugar + amino acid contents were determined by hot solvent extractions in a Soxhlet apparatus. Dried plant material was ground in a mill, and extracted for 24 h with boiling heptane. This removed the hydrocarbon fraction. The remaining residue was then extracted for 24 h with boiling methanol to remove the sugars and amino acids.

Analysis of Photosynthetic Activity. Photosynthetic activity was monitored via two different techniques: measurement of *in vivo* fluorescence, and monitoring light scattering measured at a wavelength of 535 nm. For *in vivo* fluorescence measurements, leaves were removed from the stem and placed in a fluorimeter at a diagonal to the photomultiplier. After a 2 min period of dark adaptation, the leaves were exposed to photosynthetically active actinic light (intensity= 767 µmol quanta m^{-2} s^{-1}; wavelength= 410-520 nm) and fluorecence emission was measured using a red filter (transmission maximum= 650 nm) in front of the photomultiplier.

To measure light scattering, leaves were removed from the stem and placed in an Aminco DW-2 spectrophotometer diagonal to the measuring beam. After a 2 min period of dark adaptation, the leaves were exposed to photosynthetically active actinic light (intensity= 150 µmol quanta $m^{-2} \cdot s^{-1}$; wavelength= 650-810 nm). Light-generated electron transport activity in the chloroplast causes a swelling of the thylakoids, causing changes in light scattering, and this can be monitored by measuring differences in the absorbancy at 535 nm between the leaf in the dark and after exposure to the actinic light. A green filter (transmission maximum= 530 nm; half bandwidth= 50 nm) was placed in front of the photomultiplier tube to prevent measurement of the actinic light.

Carbon Allocation Measurements. The upper portions of two *E. lathyris* stems were exposed to circulating ^{14}C labelled carbon dioxide, maintained at ambient concentrations, within a closed system. The plants were exposed to the radiolabel for 5 min, then returned to a growth chamber. After 18 h one plant harvested, and samples were analysed for presence of ^{14}C by oxidation on a Packard sample oxidizer followed by counting on a liquid scintillation counter. The sample taken were: 2 leaf and 1 stem sample from the exposed section, a leaf and a stem from near the base of the plant, 1 sample from the roots, and 6 latex samples, 4 from the exposed regions and 2 from the base of the plant. The second plant was harvested after 90 h and treated the same.

RESULTS AND DISCUSSION

Salinity.The effect of salinity on growth and hydrocarbon and sugar production is reported on in the previous chapter. Salinities of 50 mM NaCl and greater affected both growth and photosynthesis, but the reduction in growth was sharper than the reduction in photosynthetic activity. The *in vivo* fluorescence patterns showed no change from the control plant to the 200 mM NaCl treatment, but the formation of the thylakoid proton gradient, monitored by measurement of light scattering, was changed by increasing salinities (Figure 34). These data, and the results reported in the previou chapter, confirm our hypothesis that photosynthesis is less sensitive than growth processes to salinity stress, which leads to a condition of increased availability of fixed carbon, and thus increased hydrocarbon production.

Salinization of the hydroponic media also brought on changes in the internal cation composition of the plant (Table XXIII). Increased NaCl salinity resulted in an increase in internal Na^+ concentrations and a decrease in Ca^{++} concentrations. The K^+ concentration increased from the control to the 25 mM NaCl treatment, and then decreased with increasing salinity. The concentration of these ions, especially the monovalent ions, has been found to control enzymatic activity, and thus may play a role in the regulation of carbon allocation within these plants, though currently we have no evidence to support such a role.

Water Stress. Plant growth seemed unaffected by water stress, at least at the low levels applied. There was no change in plant fresh weight, shoot dry weight, or leaf chlorophyll content (Table XXIV). Despite this lack of response, carbon allocation to hydrocarbon production increased by 50% (Table XXV), though there was very little change in the production of the sugars and amino acids. The increase in hydrocarbon biosynthesis may be due to the extended growth period after application of the stress (6.5 weeks); the values for both hydrocarbon and sugar + amino acid levels are high in the control plants. It is apparent from these results that the effect of salinity on growth is not simply a response to a decrease in the water potential of the hydroponic solution. The reduction in growth probably involves an interaction of the metabolic processes with the changing concentrations of specific ions.

Carbon Allocation. The plant sampled after 18 h showed that most of its fixed radiolabel was still in the upper leaves (Figure 35). Latex isolated from the region of the plant that was directly exposed to the ^{14}C-CO_2 showed significant levels of radiolabel after 18 h. Little label was observed in the lower stem, leaf, roots or latex. After 90 h, much of the carbon had been translocated out of the leaves and into the adjacent latex. Some of the labelled carbon was also translocated down the stem; radioactivity increased in the latex isolated below the region that was initially exposed to the ^{14}C-CO_2. Little activity was detected in the roots, indicating that the carbon needed for root growth and maintenance is supplied by the lower leaves.

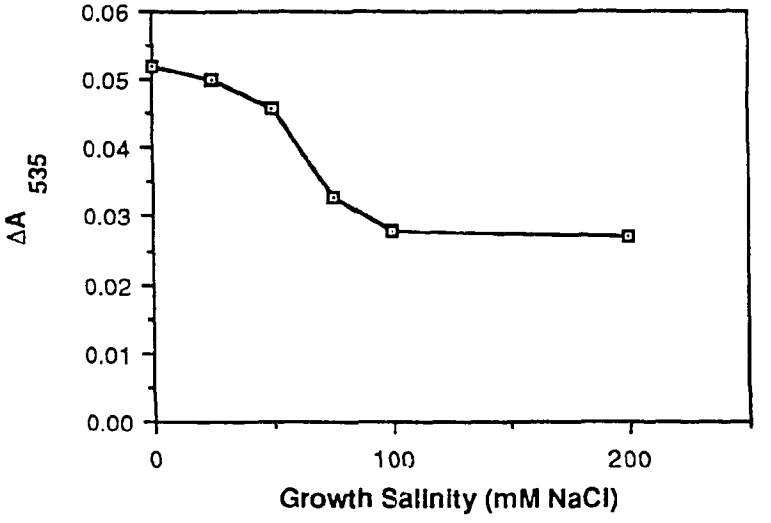

Figure 34. The effect of salinity on the proton gradient formation of thylakoids isolated from *Euphorbia lathyris*. Gradient formation monitored by measuring changes in light scattering at 535 nm.

Table XXIII

Levels of Cations in Salt-treated Plants

Treatment (added NaCl) (mM)	Content (µmoles/ g fresh wt) Na	K	Ca
0	1.9 ± 0.4	73.5 ± 2.3	31.1 ± 10.0
25	38.1 ± 9.9	90.3 ± 16.2	27.4 ± 5.5
50	40.0 ± 4.8	69.7 ± 7.7	22.3 ± 6.4

(n=3; ± values represent standard deviations)

Table XXIV

The Effect of Water Stress on Growth of *Euphorbia lathyris*

Treatment (added PEG) (mM)	Plant Fresh (Weight) (g)	Shoot Dry (Weight) (g)	Leaf Chlorophyll (mM/m^2)
0	120 ± 25	17 ± 5	0.698
75	119 ± 29	15 ± 3	0.705

(n=3; ± values represent standard deviations)

Table XXV

The Effect of Water Stress on Carbon Allocation

Treatment (added PEG) (mM)	Heptane Fraction (%)	Methanol Fraction (%)	Residue (Bagasse) (%)
0	6.0 ± 0.6	24 ± 1.0	70
75	9.0 ± 0.7	26 ± 1.1	65

(n=3; ± values represent standard deviations)

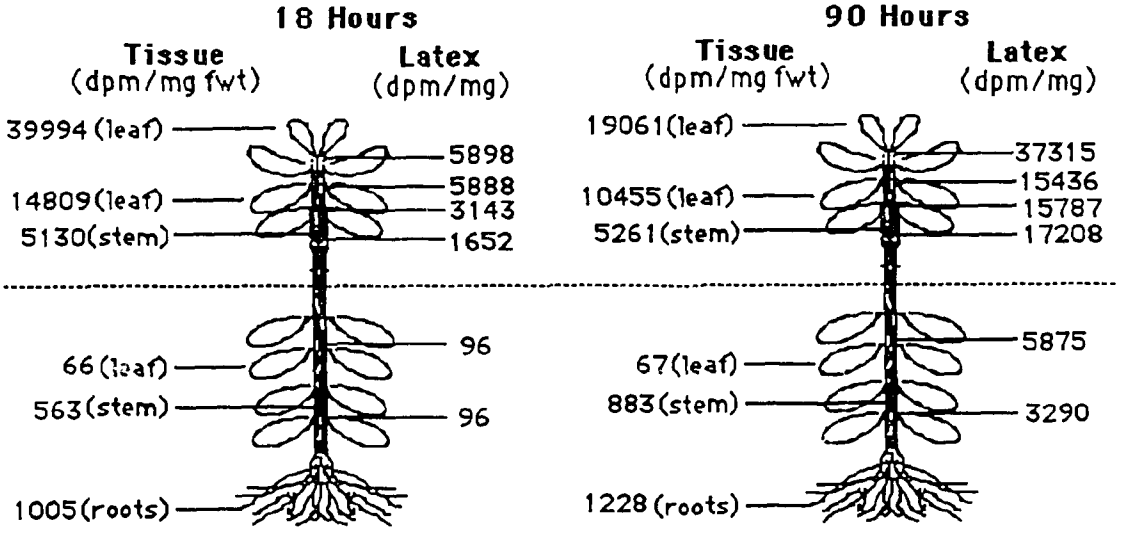

Figure 35. The translocation of carbon in *Euphorbia lathyris* plants exposed to ^{14}C-CO_2. Plants were sampled 18 h and 90 h after labelling. Only the upper portion of plant (above dotted line) was exposed to the radiolabelled carbon dioxide.

Section 5

Screening of Other Hydrocarbon-producing Species

Chapter 11

LIGNANS OF *MYRISTICA OTOBA*

Esther K. Nemethy
Regina Lago
Douglas R. Hawkins
Melvin Calvin

ABSTRACT

The neutral fraction of the oil of *Myristica otoba* fruits afforded a series of seven lignans of the aryltetralin type.

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INTRODUCTION

Lignans can be found in the neutral fraction of the oil of *Myristica otoba* fruits. Isogalactin (isootobain, **5** in Figure 36) has been identified as a component of *M. otoba* by Wallace et al. (1962). The lignan otobain [(7,8-methylenedioxy)-2,3-dimethyl-1-(3',4'- methylenedioxyphenyl)-tetralin] has been isolated from this previously, and its structure was elucidated (Abjangba and Billet, 1962, Gilchrist et al. 1962, Wallace et al. 1962, Bhacca and Stevenson 1966, Kohnen et al. 1966). The absolute configuration of otobain has been suggested by Klyne et al. (1966) based on NMR and ORD spectroscopy.

MATERIALS AND METHODS

Myristica otoba fruits were collected in Panama. The shells were removed, the fruits cut into small pieces and extracted with CH_2Cl_2 for 4 hr at room temperature. After removal of solvent, a viscous oil, 74% of the fresh weight, was obtained. Silica gel chromatography eluting with hexane, hexane-CH_2Cl_2, hexane-ethyl ether, ethyl ether and CH_3Cl-methanol (97:3) yielded a glyceride fraction (86% of the oil) and a neutral fraction (14%).

The neutral fraction was separated into two components by silica gel chromatography, eluting with hexane, hexane-ethyl ether (100:1 to 1:1), and CH_3Cl-methanol (1:1). Fraction I (20% of the neutral fraction) eluted with hexane-ethyl ether (19:1) and had a R_f = 0.41 on silica gel TLC with hexane-ethyl ether (1:1). Fraction II (40%) eluted from the column with hexane-ethyl ether (4:1), and had a Rf = 0.24 on TLC.

Fractions I and II were resolved into individual compounds by HPLC using an Altex 5 μ ODS column with a mobile phase of methanol-water (3:1). Compound **3** was further purified from a minor, isomeric compound on a 5 μ Altex SiO2 column, using hexane-ethyl ether (87:13) as the mobile phase.

The ^1H NMR spectra were recorded at 250 and 500 MHz in $CDCl_3$ with TMS as the internal standard. Coupling constants, where appropriate, were determined by decoupling experiments. The MS data were collected at 70 eV; high resolution data were within 5 ppm of calculated values. The CD spectra were taken in methanol.

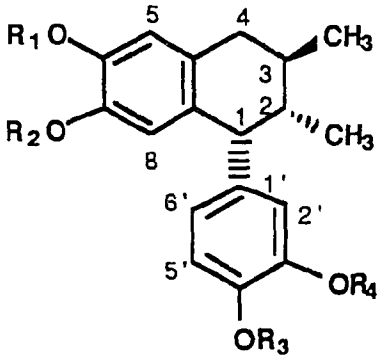
RESULTS

The ^1H NMR, MS and CD data collected are reported in Table XXVI. The absolute configurations shown in Figure 36 are based on ^1H NMR and CD data. Klyne et al. (1966) and Hulbert et al. (1981) have carried out extensive studies on the circular dichroism of aryltetralin lignans, and have concluded that the sign of the first Cotton effect reflects the configuration of the first pendant aryl group. All 4ß compounds give a negative first Cotton effect and all 4a compounds a positive one.

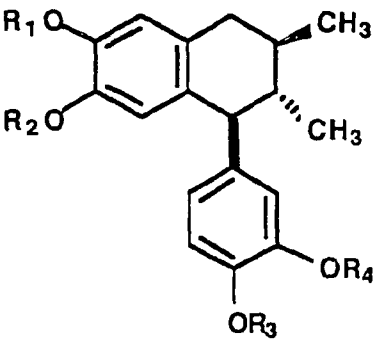
Infrared spectra of Fractions I and II showed no OH or carbonyl absorption, but did exhibit a C-O-C stretch at 1270 cm^{-1}.

DISCUSSION

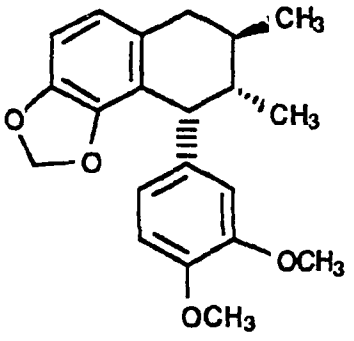
We have isolated a series of seven aryltetralin lignans (**1-7**, Figure 36) from the neutral fraction of the oil of *M. otoba* fruits. Compounds **3-5** had identical mass and ^1H NMR spectra to those reported in the literature (Adjangba 1963, Bhacca and Steveson 1963, Kohnen et al. 1966, Pelter 1968). The



	R_1	R_2	R_3	R_4
1	CH_3	CH_3	CH_3	CH_3
2	- CH_2 -		CH_3	CH_3



	R_1	R_2	R_3	R_4
3	CH_3	CH_3	CH_3	CH_3
4	- CH_2 -		CH_3	CH_3
5	CH_3	CH_3	- CH_2 -	



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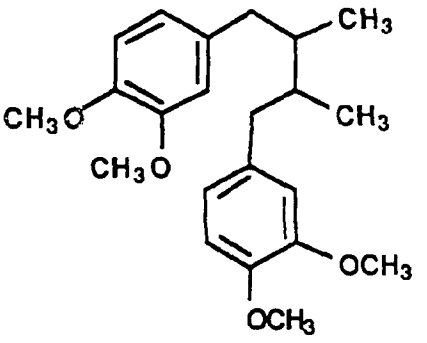

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Figure 36. Structures of the *Myristica otoba* lignins.

Table XXVI

NMR, Mass Spectra, and CD data

6,7-*Dimethoxy*-2,3-*dimethyl*-1α-(3',4'-*dimethoxyphenyl*)-*tetralin* (**1**, 37 °o of neutral fraction). ^{1}H NMR: δ6.75 (*d*, *J* = 8.2 Hz, H-5'), 6.6 (*s*, H-5), 6.57 (*d*, *J* = 2.2 Hz, H-2'), 6.52 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 6.35 (*s*, H-8), 3.87, 3.85, 3.80, 3.67 (*s*, OMe), 3.6 (*d*, *J* = 5.5 Hz, H-1), 2.85 (*dd*, *J* = 16.5, 5.4 Hz, H-4), 2.45 (*dd*, *J* = 16.5, 7 Hz, H-4), 2.18 (*m*, H-3), 1.95 (*m*, *J* = 2.3, 3 Hz, H-2), 0.9 (*d*, *J* = 6 Hz, Me), 0.9 (*d*, *J* = 6 Hz, Me). MS (*m/z*): 356 ($C_{22}H_{28}O_4$), 299, 285, 269 (C_1-H_1-O_3 base), 254, 238, 203, 165, 151, 135, 91, 69. CD: $[\theta]_{285}$ + 5520, $[\theta]_{263}$ − 6380.

6,7-*Methylenedioxy*-2α,3β-*dimethyl*-1α-(3',4'-*dimethoxyphenyl*)-*tetralin*, (**2**, 12 °o). ^{1}H NMR: δ6.75 (*d*, *J* = 8.2 Hz, H-5'), 6.6 (*s*, H-5), 6.57 (*d*, *J* = 2.2 Hz, H-2'), 6.52 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 6.35 (*s*, H-8), 5.85 (*s*, $-OCH_2O-$), 3.85 (*s*, OMe), 3.82 (*s*, OMe), 3.6 (*d*, *J* = 6 Hz, H-1), 2.9 (*dd*, *J* = 16.5, 7.3 Hz, H-4), 2.5 (*dd*, *J* = 16.5, 5.3 Hz, H-4), 1.9–2.1 (*m*, H-2, H-3), 0.9 (*d*, *J* = 6 Hz, Me), 0.9 (*d*, *J* = 6 Hz, Me). MS (*m/z*): 340 ($C_{21}H_{24}O_4$) 309, 283, 269, 253 ($C_{16}H_{13}O_3$ base), 238, 222, 209, 202, 187, 165, 152. CD: $[\theta]_{288}$ + 8600; $[\theta]_{264}$ − 5700.

6,7-*Dimethoxy*-2α,3β-*dimethyl*-1α-(3',4'-*dimethoxyphenyl*)-*tetralin* (**3**, 5 °o). ^{1}H NMR: δ6.81 (*d*, *J* = 8.2 Hz, H-5'), 6.67 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 6.57 (*s*, H-5), 6.55 (*d*, *J* = 2.2 Hz, H-2'), 6.16 (*s*, H-8), 3.89, 3.85, 3.8, 3.6 (*s*, OMe), 3.42 (*d*, *J* = 10 Hz, H-1), 2.76 (*dd*, *J* = 16.3, 4.2 Hz, H-4), 2.62 (*dd*, *J* = 16.3, 10.4 Hz, H-4), 1.45–1.65 (*m*, H-2, H-3), 1.1 (*d*, *J* = 6 Hz, Me), 0.9 (*d*, *J* = 6 Hz, Me). MS (*m/z*): 356 ($C_{22}H_{28}O_4$), 299, 269 (C_1-H_1-O_3 base), 238, 203, 165, 151, 135, 91, 69. CD: $[\theta]_{284}$ − 8040, $[\theta]_{263}$ + 4780.

6,7-*Methylenedioxy*-2α,3β-*dimethyl*-1β-(3',4'-*dimethoxyphenyl*)-*tetralin* (**4**, 4 °o). ^{1}H NMR: δ6.75 (*d*, *J* = 8 Hz, H-5'), 6.7 (*d*, *J* = 2 Hz, H-2'), 6.55 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 6.5 (*s*, H-5), 6.25 (*s*, H-8), 5.8 (*s*, $-OCH_2O-$), 3.87, 3.83 (*s*, OMe), 3.4 (*d*, *J* = 9.8 Hz, H-1), 2.75 (*dd*, *J* = 16.5, 4.1 Hz, H-4), 2.6 (*dd*, *J* = 16.5, 10 Hz, H-4), 1.7–1.5 (*m*, H-2, H-3), 1.08 (*d*, *J* = 6 Hz, Me), 0.9 (*d*, *J* = 6 Hz, Me). MS (*m/z*): 340 ($C_{21}H_{24}O_4$), 309, 283, 253 ($C_{16}H_{13}O_3$ base), 239, 223, 187, 151. CD: $[\theta]_{288}$ − 15 100; $[\theta]_{265}$ + 5920.

6,7-*Dimethoxy*-2α,3β-*dimethyl*-1β-(3',4'-*methylenedioxyphenyl*)-*tetralin* (**5**, 2 °o). ^{1}H NMR: δ6.8 (*d*, *J* = 8.2 Hz, H-5'), 6.72 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 6.6 (*d*, *J* = 2.2 Hz, H-2'), 6.57 (*s*, H-5), 6.3 (*s*, H-8), 5.95 (*s*, $-OCH_2O-$), 3.87, 3.8 (*s*, OMe), 3.4 (*d*, *J* = 9.8 Hz, H-1), 2.75 (*dd*, *J* = 16.5, 4.1 Hz, H-4), 2.6 (*dd*, *J* = 16.5, 10 Hz, H-4), 1.7–1.5 (*m*, H-2, H-3), 1.08 (*d*, *J* = 6 H-2, Me), 0.9 (*d*, *J* = 6 Hz, Me). MS (*m/z*) 340 ($C_{21}H_{24}O_4$), 309, 283, 253 ($C_{16}H_{13}O_3$ base), 239, 223, 187, 151. CD: $[\theta]_{288}$ − 11 600; $[\theta]_{267}$ + 4930.

7,8-*Methylenedioxy*-2α,3β-*dimethyl*-1α-(3',4'-*dimethoxyphenyl*)-*tetralin* (**6**, 1.2 °o). ^{1}H NMR: δ6.72 (*d*, *J* = 8.3 Hz, H-5'), 6.70 (*d*, *J* = 8.0 H_2, H-6), 6.67 (*d*, *J* = 8.0 Hz, H-5), 6.52 (*d*, *J* = 2.2 Hz, H-2'), 6.43 (*dd*, *J* = 8.2, 2.2 Hz, H-6'), 5.77 (*d*, *J* = 1.4 Hz, $-OCH_2O-$), 3.9 (*d*, *J* = 3 Hz, H-1), 3.84, 3.82 (*s*, OMe), 2.77 (*dd*, *J* = 16.5, 12 Hz, H-4), 2.5 (*dd*, *J* = 16.5, 5.8 Hz, H-4), 1.7–1.5 (*m*, H-2, H-3), 1.0 (*d*, *J* = 6 Hz, Me), 0.9 (*d*, *J* = 6 Hz, Me). CD: $[\theta]_{285}$ + 12 250; $[\theta]_{262}$ + 3500. MS (*m/z*): 340 ($C_{21}H_{24}O_4$ base), 309, 283, 269, 253 ($C_{16}H_{13}O_3$), 239, 223, 202, 187, 151.

2,3-*Dimethyl*-1,4-*bis*-(3,4-*dimethoxyphenyl*)-*butane* (**7**, 0.2 °o). ^{1}H NMR: δ6.82–6.6 (*m*, ArH); 3.9, 3.85 (*s*, OMe), 2.76 (*dd*, *J* = 16.3, 4.1 Hz, H-1, H-4) 2.3 (*dd*, *J* = 16.3, 7.8 Hz, H-1, H-4), 1.8–1.6 (*m*, H-2, H-3), 0.9 (*d*, Me). MS (*m/z*): 358 ($C_{22}H_{30}O_4$), 269, 206, 179, 165, 151 (base).

substitution pattern on the phenyl rings was confirmed by the observed spectrum showing two singlets for H-5 and H-8 on ring A and two doublets with coupling constants 8.2 (*ortho*) and 2.2 (*meta*) for H-5' and H-2' respectively, as well as an AB pattern for H-6' with J= 8.2 and 2.2 Hz. The assignment of the methylenedioxy groups in **4** and **5** was based on the known chemical shift difference between ring A or ring C substitution (Bhacca and Stevenson 1963).The observed coupling constants, $J_{1,2}$= 10 Hz and $J_{3,4}$= 10Hz for compounds **3-5** confirm all *trans* stereochemistry with the methyl groups and the pendant phenyl substituent all pseudo-equatorial. The minor constituents galbulin (**3**) and galactin (**4**) have been isolated from other sources (Hughes and Ritchie 1954, Hobbs and King 1960, Gottlieb et al. 1976), but have not been identified as components of *M. otoba* oil.

The aromatic region of the NMR spectrum of **1** and **2** was identical to that of **3-5**, indicating the same substitution pattern. However, the small coupling constants for H-1-2 (5.5 Hz) as well as for H-3-4 and H-4 (7 and 5 Hz) indicated a different steriochemistry from that of compounds **3-5**. By decoupling experiments at 500 MHz (irradiation of the methyl), $J_{2,3}$ was determined to be 3 Hz, indicating that H-2 and H-3 are psuedo-equatorial. In the spectra of both **1** and **2** the two methyl resonances occurred at d0.9. No deshielding of either methyl groups by phenyl ring C was observed as in the spectra of compounds **3-5**. Examination of all possible conformers indicated *cis -trans* stereochemistry for **1** and **2** as shown in the formulae.

The ^{1}H NMR of compound **6** showed a different aromatic region for ring A protons. Instead of two singlets, two doublets with J= 8.2 Hz was observed, indicating the substitution pattern as shown. The methylenedioxy protons were nonequivalent (J= 1.4 Hz), due to the proximity of the phenyl C ring. The observed coupling constants for H-1-2 (3 Hz) and for H-3-$4_{a,b}$ (12 and 6 Hz) indicated the relative stereochemistry.

The mass spectrum of compound **7** showed a $[M]^+$ at *m/z* 358, two mu higher than those in **1** and **3**, but the same number of aromatic protons in the NMR. Examination of the aromatic region indicated a substitution pattern as shown.

Two additional minor components of the aryltetralin lignan type were detected by HPLC, but these were present in insufficient quantities for NMR analysis. Mass spectra indicated that one is isomeric with compound **1**, the other with compound **3**.

CONCLUSIONS

Gilchrist et al. (1962) have reported the presence of a phenolic mixture in otoba fat, from which he has isolated one compound, otobain. By HPLC we have successfully resolved this mixture and identified new lignans of *M. otoba* . We could not detect otobain in this mixture, which may be due to a different state of ripeness of the fruit, or to the fact that we have used a fresh fruit extract, whereas other workers have used commercial otoba fat.

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Chapter 12

BIOSYNTHESIS OF TERPENOIDS IN *COPAIFERA*

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Melvin Calvin

ABSTRACT

Studies of the incorporation of various precursors into leaf discs and cuttings show differences in the rate of incorporation into sesquiterpenes and the other isoprenoids, suggesting either a controlling mechanism or parallel pathways.

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INTRODUCTION

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 oin Brazil. Trees in near Manaus, Brazil are tapped biannually, yielding 20 to 30 liters of the oil in 2 to 3 hours (Calvin 1980).

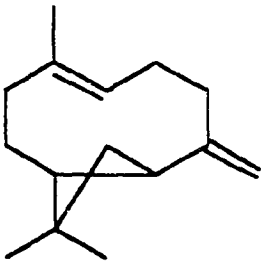
The genus *Copaifera* is a member of the Leguminosae family, subfamily Caesalpinioideae, and the tribe Detarieae. It is represented by *ca* 30 species in the New World and 4 in Western Africa (Arrhenius et al. 1983). These trees are prominent in the lowland rainforest ecosystem but are also found under drier, more-open thorn forest habitats (Langenheim et al. 1981).

Of four commercial samples of the oleoresin, 72 to 90% of the oil was found to be sesquiterpene hydrocarbons (Wenninger 1967). 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 (Loomis and Croteau 1980). 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 (Wenninger 1967). In addition to the wood resin, species of *Copaifera* also contain a leaf resin. The sesquiterpene hydrocarbons caryophyllene, g-cadinene, cyperene, and â a-copaene have been isolated and identified from leaves of *Copaifera officinalis* L. and *C. venezuelana* var. *laxa* (Figure 37). The leaf resin is secreted from specialized epithelial cells which line small, ovoid, schizogenous pockets in the mesophyll tissue (Arrhenius et al. 1983). In the wood, the oleoresin is found in concentric rings of canals in the primary stem tissue and secondary tissue (Langenheim 1981).

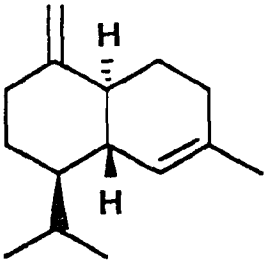
In the course of structural determinations of sesquiterpenes, Ruzicka (1953) developed the "farnesol rule" and the broader "biogenetic isoprene rule" which hypothesized that all terpenoids could be formed biologically by accepted reaction mechanisms from such simple precursors as farnesol, geraniol, geranyl-geraniol, and squalene. 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 (Cane 1981).

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 (Parker et al. 1967). 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 b-humulene as well as d-cadinene with γ-muurolene and the possible intermediate role of germacrene (Martin et al. 1976).

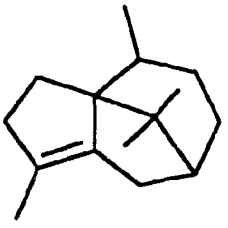
Besides interest in the relationship between individual compounds, there is the question of the turnover of sesquiterpenes in general. Essentially nothing is known about their catabolism. However, an investigation of the time course of labelling of sesquiterpenes from ^{14}C-mevalonate in peppermint 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 ^{14}C-sucrose in the sesquiterpenes a-guaiene, a-patchoulene, caryophyllene, a-bulnesene, and patchouli alcohol when incubated for 4.5 h as opposed to 17 h (Francis 1972). These results indicate that sesquiterpenes are metabolically active. Sesquiterpenes of *Copaifera* seedlings labelled with $^{14}CO_2$



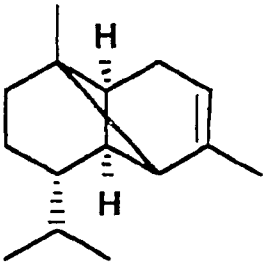
Caryophyllene



γ -Cadinene



Cyperene



α- Copaene

Figure 37. Sesquiterpene hydrocarbons of *Copaifera* leaf resin.

for a period of two weeks were turned over in 8 to 10 days, but metabolism over a shorter time period was not investigated (Langenheim et al. 1981).

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 4 parts sand, 4 parts Canadian sphagnum peat, and 3 parts perlite and 1 part redwood compost 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]glucoal 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 ^{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. 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 and Jork (1979) 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 ^{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. 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 previously.

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 x 10^{-12} and 2 x 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. The leaf tissue was extracted with hexane, and the residue subsequently extracted with 95% ethanol. The ethanol extract was centrifuged, and total chlorophyll content was calculated using the formulas of Wintermans and De Mots (1965).

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 XXVII).

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. 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.

^{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-acetate (1.6 Ci $mmol^{-1}$, 10 mC 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 oxidized in a Packard Automatic Combustion Apparatus, and the radiolabel was collected in Carbo-Sorb. Permafluor was added and the sample was counted.

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:

Table XXVII

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 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

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 ^{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 mevalonic acid (MVA) in 0.825 ml water (63 µM, 0.43 Ci $mmol^{-1}$), 10 µl ^{3}H-acetate (50 nmol, 60.6 µM, 2 Ci $mmol^{-1}$), 10 µl ^{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 ^{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 mark spot along with a second marker 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.

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.

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 (Figure 38).

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 (Figure 39). 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.

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^{-} 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% 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} (Figure 40).

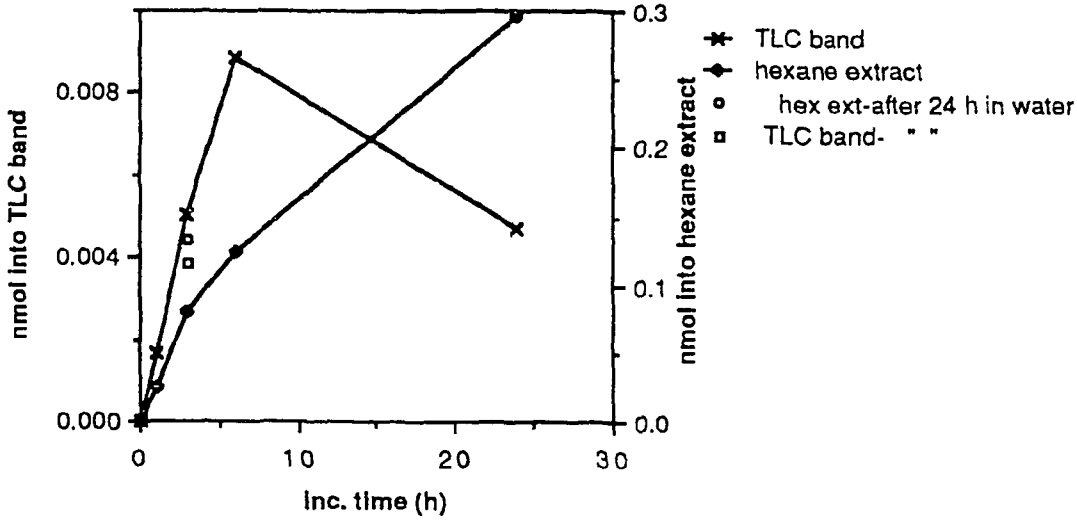


Figure 38. Time course of acetate incorporation into *Copaifera* leaf discs.

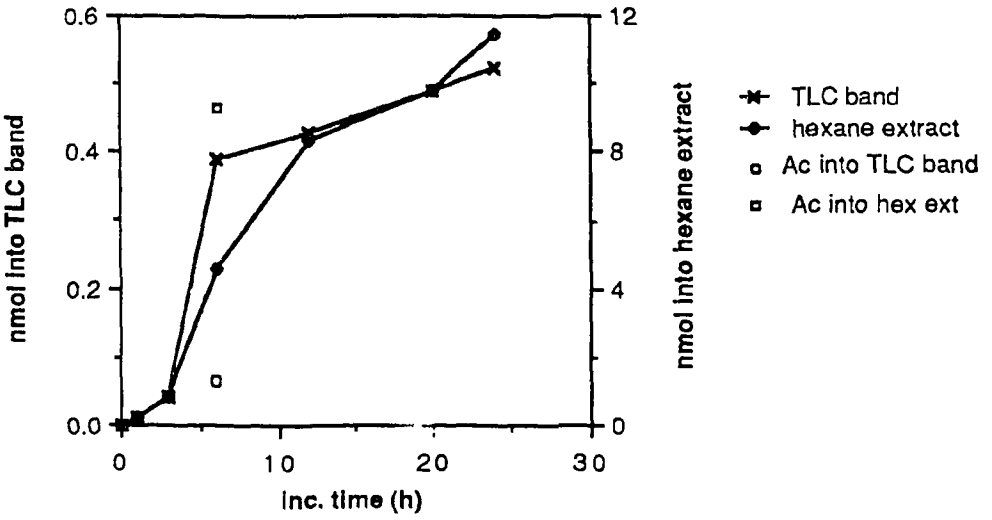


Figure 39. Time course of bicarbonate incorporation into *C. officinalis* leaf discs suggesting that the majority of the label had been incorporated into more nonpolar compounds which remained on the column.

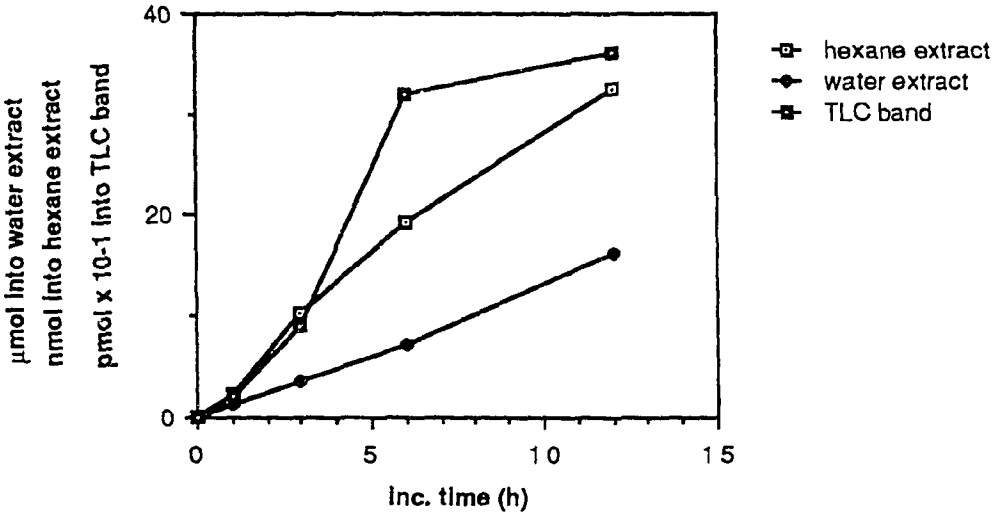


Figure 40. Time course of bicarbonate incorporation into *C. multijuga* leaf discs.

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 XXVIII). 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 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.

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 (1972)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.

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 (Croteau and Loomis 1972, Francis 1972)(Figure 38). 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 (Figure 39). 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 (Croteau et al. 1972a). 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 (Croteau and Loomis 1972, Croteau et al. 1972a). 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 (Figure 40).

The experiments with leaf cuttings further showed that incorporation of exogenous precursors into squalene and triterpenoids predominates over incorporation into sesquiterpenes (Table XXVII). This

Table XXVIII

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 pmol	1.8 pmol	7.9 pmol	
Acetate + sucrose		2.6 pmol			

same situation was seen when ^{14}C-mevalonate was fed to peppermint leaf cuttings (Croteau and Loomis 1973). 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 (Langenheim 1981).

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 (ie. alternative, parallel pathways).

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 (Croteau and Loomis 1972). 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 (Gleizes et al. 1984). 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 XXVIII). In contrast Croteau et al. (1972b) 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. 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*-b-farnesene in sesquiterpene biosynthesis in maritime pine needles was determined in this manner (Gleizes et al. 1984).

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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Chapter 13

ADDENDUM TO SECTION 5: COMPOSITION OF THE STEAM DISTILLATE OF *PITTOSPORUM RESINIFERUM* AND OF THE SEED OILS OF *MYRISTICA OTOBA* AND MACAUBA PALM (*ACROCOMIA SCLEROCARPA*)

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ABSTRACT

A steam distillate of *Pittosporum resiniferum* fruits was found to contain alkanes and monoterpenes. Seed oils were collected from *Myristica otoba* and Macauba Palm fruits and were fractionated into the triglyceride and non-saponifiable components. The fatty acid composition of the triglycerides was then determined.

INTRODUCTION

Glycerides and isoprenoids represent the two major classes of highly reduced organic chemicals that photosynthetic organisms produce in substantial quantities. Glycerides are usually concentrated in fruits and seeds. Wheras some isoprenoids can be found in the seeds, these compounds are produced and stored in other parts of the plant as well.

We have previously suggested the use of isoprenoids as an alternative to fossil fuels. The use of seed oils as substitutes or extenders of diesel oil has often been proposed. The suitability of a specific seed oil for energy or industrial uses depends upon its chemical composition, acidity and other physical properties. We analysed the seed oils of two potential energy-plants, *Myristica otoba* and Macuaba palm (*Acrocomia sclerocarpa*) for their fatty acid composition.

MATERIALS AND METHODS

A hydrocarbon fraction was isolated from *Pittosporum resiniferum* fruits by steam distillation. The composition of the distillate was then determined by gas chromatography-mass spectroscopy (GC-MS) as well as by comparison of the retention times on GC with standard compounds.

Myristica otoba fruits were extracted with CH_2Cl_2 at ambient temperature. This crude oil was separated into two fractions by silica gel chromatography, generating a triglyceride fraction and a neutral, non-saponifiable fraction. The triglycerides were hydrolyzed, and the resultant fatty acids were methylated. The fatty acid composition was then determined GC-MS as well as by comparison of the retention times on GC with standard compounds.

Macuaba palm fruit yields two different types of oil: the pulp oil and the kernal oil. Both of these oils were separated into a solid and liquid fraction by crystallization at low temperature. The resulting four fractions (a solid and liquid fraction of both pulp and kernal oils) were further purified by silica gel chromatography, which removed minor components and yielded pure glycerides. The fatty acid composition of these fractions was then determined as described for *M. otoba* fruits.

RESULTS

Pittosporum resiniferum fruits yielded about 2% of their fresh weight as a hydrocarbon fraction. This fraction contained both the free alkanes heptane and nonane as well as monoterpenes. The major monoterpenes were found to be α-pinene, ß-pinene, and myrcene (Figure 41).

Myristica otoba fruit yielded 75% of their fresh weight as oil. When separated by silica gel chromatography, 65% of the applied oil was collected in the triglyceride fraction, and 12% in the neutral fraction. The fatty acid composition is given in Table XXIX.

Macauba palm fruit yielded 10% of their dry weight as oil. This was distributed evenly between the pulp and kernal oil, each consisting of 50% of the total. Over 90% of both oils was composed of triglycerides; however the pulp oil is much more acidic than the kernel oil. Table XXX shows the fatty acid composition of the solid and liquid fractions of both the pulp and kernal oils.

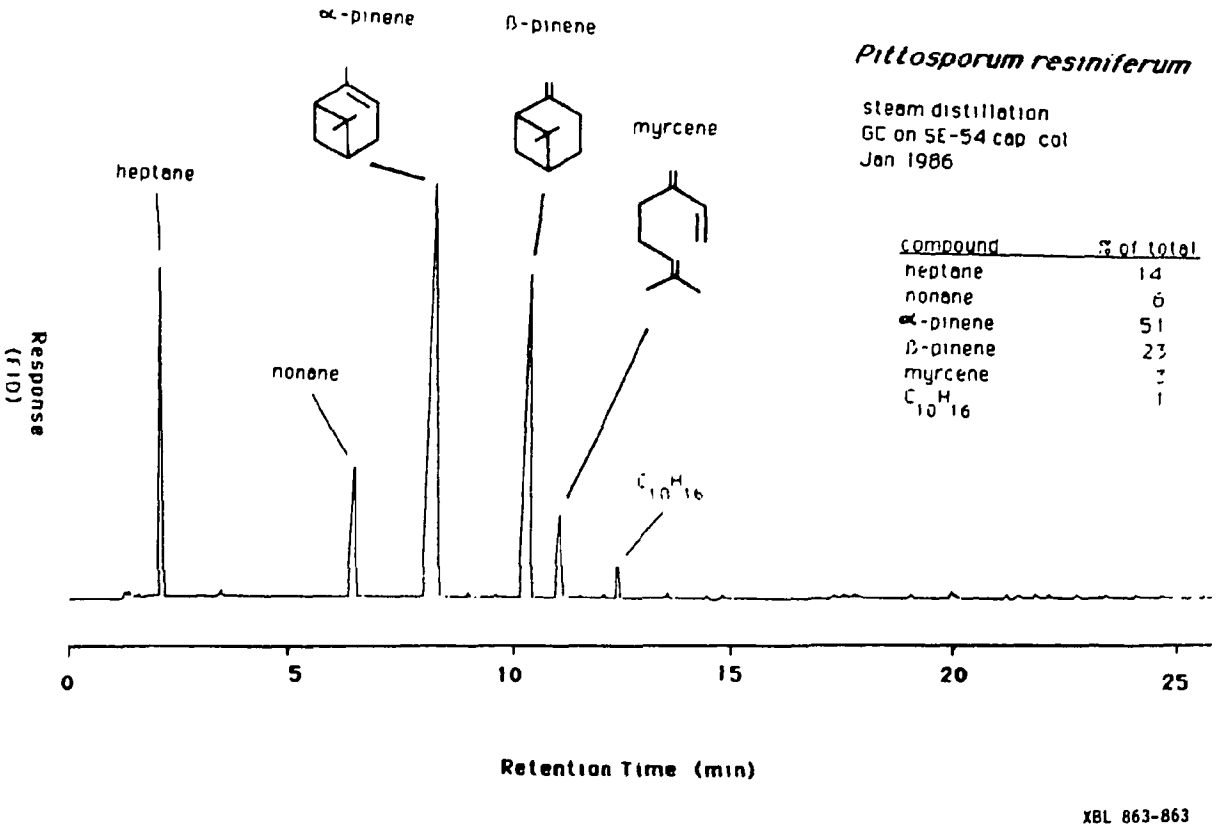

Figure 41. Gas chromatograph of a steam distillate from the fruits of *Pittosporum resiniferum*.

Table XXIX

Fatty Acid Composition of *Myristica otoba* Oil

Fatty Acid	Composition (% by weight)
capric (10:0)	0.1
lauric (12:0)	6.6
tridecanoic (13:0)	0.1
myristic (14:0)	81.5
myristoleic (14:1)	3.0
palmitic (16:0)	4.5
palmitoleic (16:1)	0.4
stearic (18:0)	0.4
oleic (18:1)	2.7
linoleic (18:2)	0.3

Table XXX

Fatty Acid Composition of Macauba Pulp and Kernel Oils

fatty acid	Macauba Pulp			Macauba Kernel		
	total	solid	liquid	total	solid	liquid
	(% by weight)			(% by weight)		
caprilic (8:0)	–	–	–	6.9	6.0	0.9
capric (10:0)	0.02	0.01	0.01	6.7	5.9	0.8
lauric (12:0)	0.05	0.02	0.03	46.2	41.6	4.6
myristoleic (14:1)	0.15	0.07	0.08	8.6	7.9	0.7
palmitic (16:0)	17.6	11.9	5.7	5.5	4.7	0.8
palmitoleic (16:1)	1.2	0.6	0.6	–	–	–
stearic (18:0)	2.2	1.2	1.0	2.3	2.0	0.4
oleic (18:1)	64.4	28.1	36.3	20.3	13	7.3
linoleic (18:2)	12.3	5.1	7.3	3.0	1.8	1.2
linolenic (18:3)	1.04	0.42	0.57	0.1	–	0.1
arachidic (20:0)	0.05	0.03	0.02	–	–	–

DISCUSSION

There are significant differences in both the quantity and the nature of the oils of *M. otoba* and the Macuaba. The *M. otoba* is richer in oil, and myristic acid (14:0) is the predominate fatty acid, comprising over 81% of the oil. Both the pulp and kernal oils of the Macuaba fruit contain only minor quantities of the myristic acid; they are rich in the longer chain fatty acids- palmitic, oleic and linoleic. As a fuel source, the *M. otoba* fruits would be a better choice, because of the higher yield of oil, the smaller fatty acid molecules, and the lower degree of unsaturation of the fatty acids.

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PUBLICATIONS

1. Calvin, M. Renewable Fuels for the Future. *Journal of Applied Biochemistry* 6: 3 (1984).

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