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SEASONAL CHANGES OF SELECTED SECONDARY PLANT PRODUCTS IN *CHRYSOTHAMNUS NAUSEOSUS* SSP. *TURBINATUS*

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ABSTRACT.—Previously, physiological studies of rubber and resin production during the growing season of *Chrysothamnus nauseosus* ssp. *turbinatus* indicated a negative correlation between rubber and resin content. The resin was highest in the spring and lowest in the summer, whereas rubber was highest in the summer and lowest in the spring. Individual compounds were followed during the growing season to see if they correlated with the rubber or resin trend. The total compounds in the cyclohexane fraction followed the resin pattern. Individual compounds varied in their changes during the growing season. Limonene, for example, was negatively correlated with rubber production, whereas β cubebene was positively correlated. The possible metabolic pathways between resin and rubber are discussed.

Recently there has been increased interest with respect to the availability and possible commercialization of rubber rabbitbrush (*Chrysothamnus nauseosus*) for natural rubber (Pierson 1975, Weber et al. 1985, Ostler et al. 1986). Some of the subspecies contain levels of natural rubber similar to those of guayule (Ostler et al. 1986, Hegerhorst et al., Resin and rubber, 1987). Additional uses of rabbitbrush, such as a winter forage and as a revegetation shrub, increase its potential as a commercially beneficial crop (McArthur et al. 1979, Weber et al. 1985). Because of the vast environmental and geographical latitude in which rubber rabbitbrush grows, the potential advantages of growing rubber rabbitbrush for a commercial source of natural rubber and other plant products become more obvious. *Chrysothamnus nauseosus* ssp. *turbinatus* is of particular interest because levels of natural rubber reported exceed 6.5% (dry wt) (Hegerhorst et al., Resin and rubber, 1987). Commercialization of rabbitbrush relies on understanding plant habitat, genetic variability, rubber accumulation in the plant, seedling characteristics, and many other aspects of plant development (Hegerhorst et al., Chemical analysis, 1987). A deeper understanding of the parameters influencing rubber synthesis will help us possibly control its accumulation. Recent results (Hegerhorst et al., Seasonal changes, 1987) show that a strong negative correlation exists between rubber and resin production in selected subspecies of rubber rabbitbrush. The purpose of this study is to better understand the relationship between resin and rubber production within the plants by following seasonal changes in selected secondary plant products.

MATERIALS AND METHODS

Plant material from rubber rabbitbrush (*Chrysothamnus nauseosus* ssp. *turbinatus* [Jones] Hall & Clem.) was collected 1.6 km west of Goshen, Utah (USA), during the

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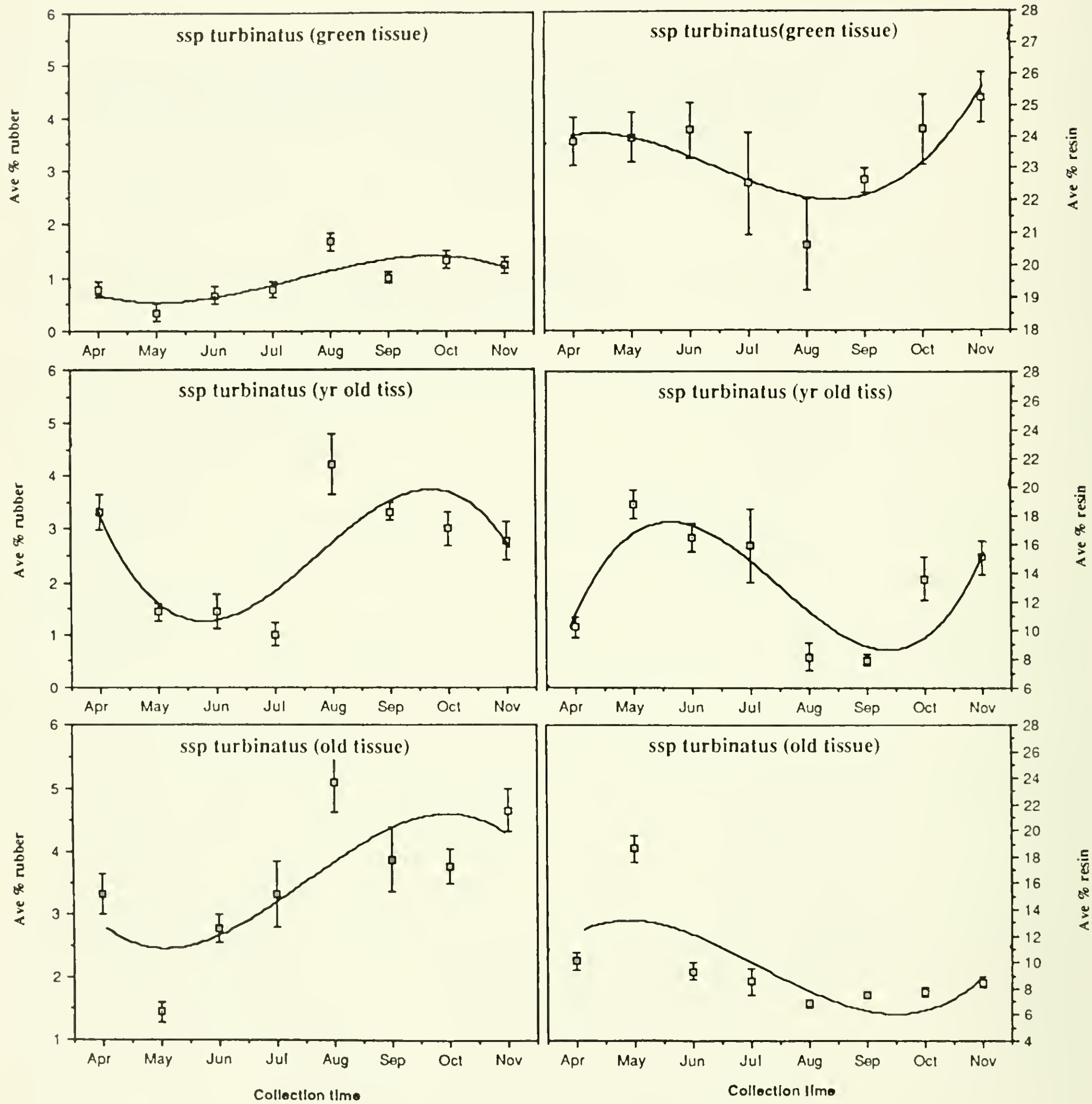

Fig. 1. Left: The average percentage of rubber in green tissue, year-old tissue, and several-years-old tissue of *Chrysothamnus nauseosus* ssp. *turbinatus* during the growing season at Goshen, Utah. The vertical lines are standard error bars. Right: The average percentage of resin in green tissue, year-old-tissue, and several-years-old tissue of *Chrysothamnus nauseosus* ssp. *turbinatus* during the growing season at the Goshen, Utah, site. The vertical lines are standard error bars (Hegerhorst et al., Seasonal changes, 1987).

months of April through November 1986. The plants are in a dormant state during the winter months because of the cold, and often freezing, temperatures. The temperature and precipitation were recorded by the state weather station of Goshen, Utah. Random samples of new growth (current growth), one-year-old tissue, and tissue older than one year were clipped from three different plants each month. The samples were bagged and taken to the laboratory where they were frozen at −20 C. Following the last collection period, all of the samples were ground in liquid nitrogen using a motorized steel mortar and pestle. Liquid nitrogen grinding was used to prevent the loss of volatile compounds due to enzyme hydrolysis during the grinding process. Compounds of interest were extracted from 2 g of tissue using three portions of cyclohexane. The tissue was extracted in a 125-ml

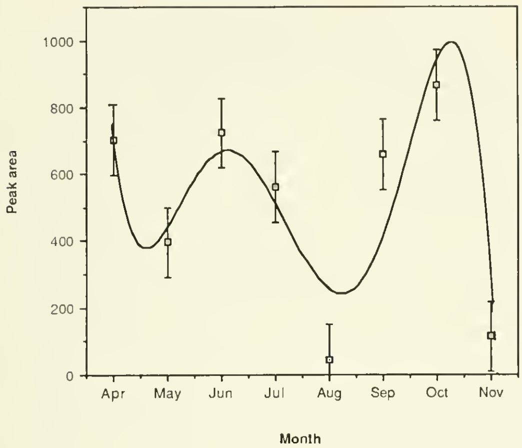

Fig. 2. Total compounds from *Chrysothamnus nauseosus* ssp. *turbinatus* during the growing season at Goshen, Utah. The vertical lines are standard error bars (Hegerhorst et al., Seasonal changes, 1987).

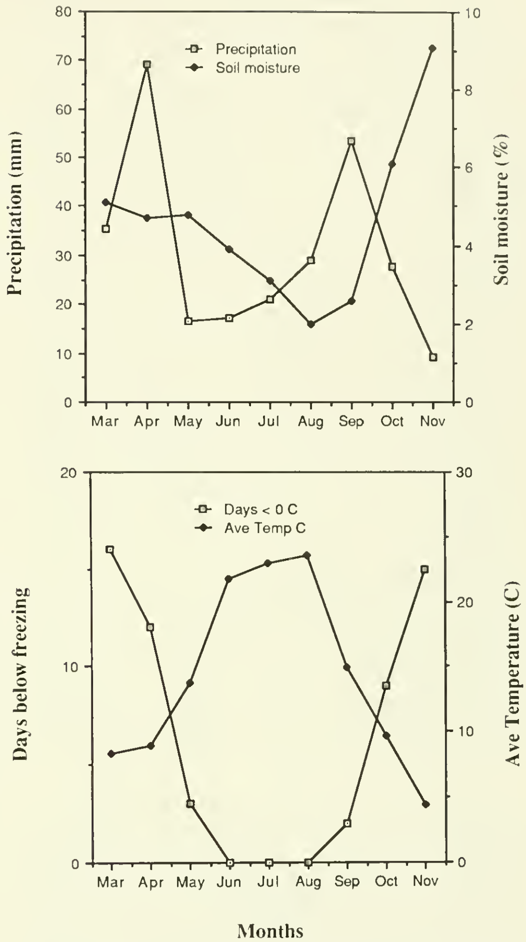

Fig. 3. The precipitation (mm) and soil moisture (upper figure), and average temperature (C) and days below freezing (lower figure) during the growing season at Goshen, Utah (Hegerhorst et al., Seasonal changes, 1987).

Erlenmeyer flask on a rotary shaker for 30 minutes at 160 rpm. The samples were filtered using a coarse, sintered glass funnel. The filtrate was reduced to 5 ml under nitrogen, and 50 μl of gamma terpinene (internal standard at 1 mg/ml) was added to 150 μl of sample. Analysis was carried out with a Hewlett Packard 5995C gas chromatograph-mass spectrometer (GC-MS). Partial identification of the compounds was done by comparing the mass spectral data to a library of about 87,000 compounds, using a Hewlett Packard HP 1000 computer linked with the GC-MS system (Hewlett Packard 1986). A cross-linked methyl silicon capillary column (0.20 mm) was used with a splitless injection system and a capillary-direct interface with the MS. Carrier flow was adjusted to 1.5 ml per minute, and the temperature gradient went from 35 to 290 C with a 10 C per minute ramp. The data from the triplicate runs were statistically analyzed. Chemical standards were also used to identify some of the compounds.

Results and Discussion

A previous study (Hegerhorst et al., Seasonal changes, 1987), using the same plants collected at the same site, found a strong negative correlation between resin and rubber in rubber rabbitbrush ($r^2 = -.64$, $p = .03$). Figure 1 represents the changing pattern of rubber and resin content through the growing season. As resin content increased, rubber content showed a decrease. This could be significant if the energy used to synthesize these compounds could be directed into rubber. In this research we investigated specific resinous compounds and their relationship to rubber production. In the cyclohexane extraction the compounds were identified by GC-MS using the large computer spectra library (Hewlett Packard 1986). Figure 2 is a graph representing the sum of all the compounds, as determined by GC-MS, for each month in the growing season. The resin pattern in Figure 1 and the total compounds in Figure 2 are not identical, but they have similar patterns, with

TABLE 1. Compounds present in *Chrysothamnus nauseosus* ssp. *turbinatus* as determined by GC-MS and their GC retention time.

Name of the compound	Retention times	% of total
Sabinene	6.8	7.40
Myrcene	7.1	1.60
Cymene	7.6	11.5
Limonene	7.7	15.2
β cubebene	14.5	0.76
Δ cadinene	15.1	9.80
Cyclohexanol, 3 ethenyl-3-methyl-2-(1 methylethenyl-6-)	15.7	5.20
Unknown A	15.9	0.99
Epi-bicyclosesquiphellandrene	16.4	8.80
Unknown B	16.4	2.80
Unknown C	16.6	3.20
Unknown D	17.1	7.00
Unknown E	17.5	2.40
β elemene	17.6	1.60
Unknown F	17.9	0.78
Unknown G	18.3	3.20
Unknown H	18.7	0.56
C-27 branched hydrocarbon	26.2	0.34
C-28 branched hydrocarbon	27.6	0.46
Nonacosane C-29	27.9	1.70
C-29 branched hydrocarbon	28.5	0.41
Triacontane C-30	28.7	0.25
C-30 branched hydrocarbon	29.4	0.38
Hentriacontane C-31	29.8	1.30

the highest content in June and the lowest in August. Some of the difference between the graphs can be attributed to the variation in solubility of compounds between cyclohexane used in this experiment and the acetone-water mixture used in the crude resin extraction.

The resin content and the total monthly precipitation have limited similarity, with higher values in the spring and fall (Fig. 3). In the case of precipitation and rubber content it can be inferred from the data (Figs. 1 and 3) that a decrease in precipitation resulted in an increase in rubber content. This subspecies of rubber rabbitbrush grows in a very sandy soil and would be sensitive to high temperatures and low moisture (Fig. 3), which would cause water stress in the plant.

The compounds detected and their average concentration over the growing period in the cyclohexane fraction are listed in Table 1. Six compounds, sabinene, cymene, limonene, Δ cadinene, epi-bicyclosquiphellandrene, and 3 ethenyl-3-methyl-2-(1 methylethenyl-6-) cyclohexanol, represented 58% of the concentration of compounds detected. Limonene was the highest with 15.2% of the total.

There are also some interesting patterns that can be seen with specific compounds as compared with the rubber and resin content in rubber rabbitbrush during the growing season. For example, Figure 4a is the graph of a typical hydrocarbon. It is high at the beginning of the growth season (April) and increases more in May. The compound then decreases steadily through the remainder of the season. When we used a similar extraction and GC-MS analyses of callus tissue from rubber rabbitbrush, we found that the major secondary plant products being produced in the rapidly dividing callus cells were simple hydrocarbons. This seems to suggest that early in the season, as new shoots and leaves are rapidly expanding, the major secondary plant products are predominantly simple hydrocarbons.

The terpenes extracted in our analysis show a varied correlation in relation to the pattern for rubber content shown in Figure 1. Limonene is an example of a terpene that is negatively correlated with that of rubber production (Fig. 4b). On the other hand, β cubebene is an example of a terpene that is positively correlated with rubber production (Fig. 4d). Sabinene was low in the spring, had a peak in June, decreased in August, and was high in November, which was similar to resin production (Fig. 4e).

The changes in the individual compounds from rubber rabbitbrush for each month were rated and compared with the changing pattern of rubber and resin content. A similarity value was calculated using Statview 512+ (1986), and the compounds were grouped in relationship to rubber (Table 2) or resin (Table 3). The rubber pattern had the greatest similarity to the sesquiterpene cyclohexanol, 3 ethenyl-3-methyl-2-(1-methylethenyl-6-), which was also the compound that was the least similar to the resin pattern. In contrast, compounds that had a high similarity to the resin pattern were sabinene, limonene, unknown C, and unknown E (Table 3). All of these compounds were rated as having little similarity to the rubber pattern (Table 2). A rather interesting observation is that four of the patterns of the straight-chained and branched-chained hydrocarbons had little similarity to either rubber or resin (Tables 2 and 3). These compounds could be associated with cuticle and wax formation of the leaves.

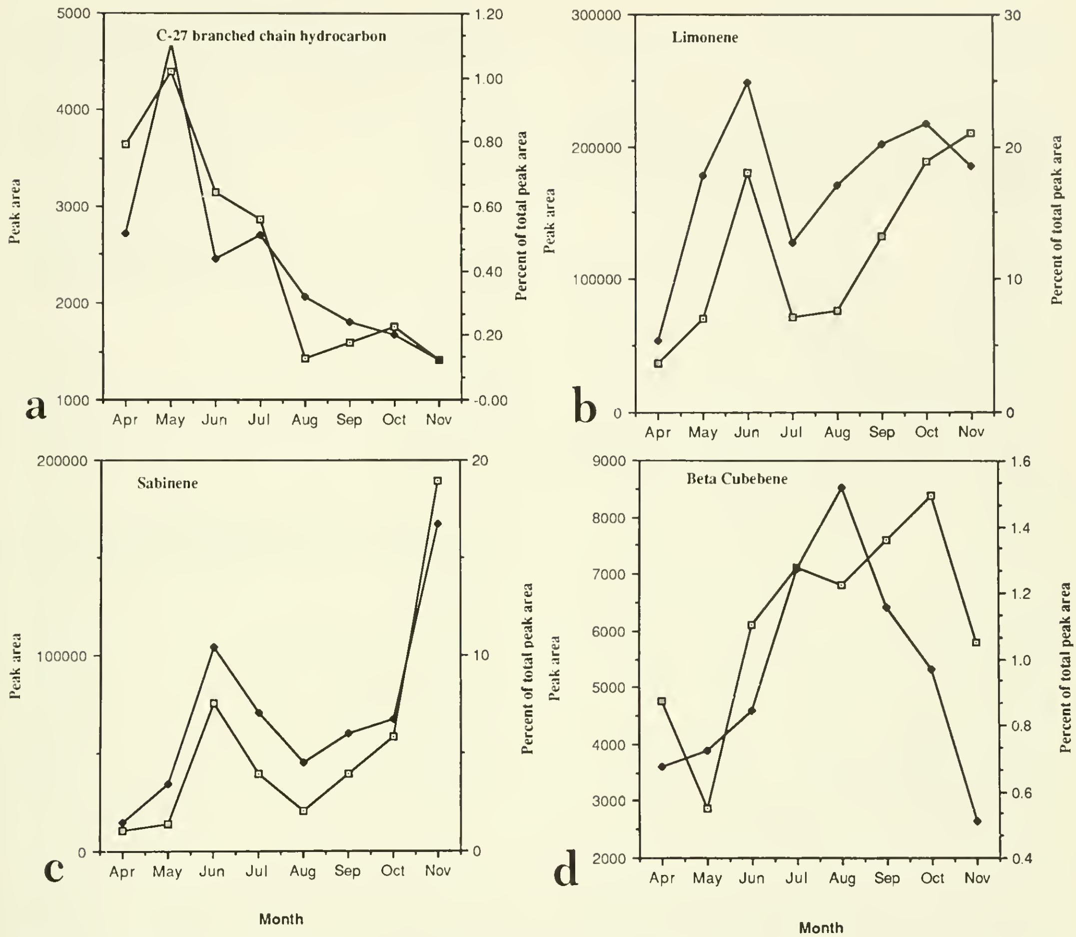

Fig. 4. Changes in C–27 branched hydrocarbon (a), limonene (b), sabinene (c), and β cubebene (d) from *Chrysothamnus nauseosus* ssp. *turbinatus* during the growing season at Goshen, Utah. The line with squares is based on the peak area, and the line with the black spots is based on the percentage of the compound in relation to the total area of the compounds.

Three of the sesquiterpenes, β cubebene, Δ cadinene, and epi-bicyclosesquiphellandrene, were common to both rubber and resin patterns at the intermediate similarity classification (Tables 2 and 3).

A correlation matrix and factor analysis of individual compounds to each other was determined by Statview 512+ (1986). A high correlation value indicated that the two compounds had a similar pattern during the growing season and could possibly be synthesized by the same pathway. Factor analyses of the data (Statview 512+, 1986) indicated that six factors were involved. The compounds in the six factors are listed in Table 4. Factor 1 contains long-chain hydrocarbons that form a group seemingly independent of the other compounds. The synthetic pathway to waxes is considered to be different from the rubber and resin pathways (Robinson 1983). Factor 3 had three compounds, including 3 ethenyl–3-methyl–2-(1-methylethenyl–6-) cyclohexanol, which all have a high correlation with the rubber content pattern. Factor 6 contains monoterpenes, which have a high correlation with the resin synthesis. The outlined pathway (Robinson 1983) in Figure 5 shows a pathway to monoterpenes. There is a branch point at isopentenyl pyrophosphate between monoterpenes and the pathways to the sesquiterpenoids, diterpenoids, and rubber. Rubber synthesis is considered to involve isopentenyl pyrophosphate and perhaps diterpenoids or similar compounds (Robinson

TABLE 2. Similarity of individual compounds over the growing season in *Chrysothamnus nauseosus* ssp. *turbinatus* in relation to total rubber content as determined by Statview 512+.

Compounds that are very similar to rubber pattern
Cyclohexanol, 3 ethenyl-3-methyl-2-(1 methylethenyl-6-)

Compounds that have some similarity to rubber pattern
Myrcene
β cubebene
Δ cadinene
Unknown A
Epi-bicyclosesquiphellandrene
Unknown G
Unknown D
C-28 branched hydrocarbon
C-30 branched hydrocarbon
Hentriacontane C-31

Compounds that have little similarity to rubber compounds
Sabinene
Cymene
Limonene
Unknown B
Unknown C
Unknown E
β elemene
Unknown F
Unknown H
C-27 branched hydrocarbon
Nonacosane C-29
C-29 branched hydrocarbon
Triacontane C-30

TABLE 3. Similarity of individual compounds over the growing season in *Chrysothamnus nauseosus* ssp. *turbinatus* in relation to total resin content as determined by Statview 512+.

Compounds that have high similarity to resin pattern
Sabinene
Limonene
Unknown C
Unknown E

Compounds that have some similarity to resin pattern
Myrcene
Cymene
β cubebene
Δ cadinene
Epi-bicyclosesquiphellandrene
Unknown D
Unknown F
Unknown G
Unknown H

Compounds not similar to resin pattern
Cyclohexanol, 3 ethenyl-3-methyl-2-(1 methylethenyl-6-)
Unknown A
Unknown B
β elemene
C-27 branched hydrocarbon
C-28 branched hydrocarbon
Nonacosane C-29
C-29 branched hydrocarbon
Triacontane C-30
C-30 branched hydrocarbon
Hentriacontane C-31

1983). Benedict (1986) used isopentenyl pyrophosphate as the substrate for rubber synthesis in guayule (*Parthenium argentatum*). While more investigations are needed to determine the synthesis pathways of the different compounds in rubber rabbitbrush, our results provide some support for the concept that rubber and resin synthesis pathways are different (Fig. 5).

Benedict (1986) found that the enzymatic synthesis of rubber was induced by cool temperatures and that the enzymatic incorporation of isopentenyl pyrophosphate occurs on the surface of subcellular rubber particles. The rubber synthesis did not begin until the cool months of October and November (Bucks et al. 1986). The enzymatic activity of cis isopolyisoprene polymerase was related to the number of hours at 13 C and below. While the process of rubber synthesis in rubber rabbitbrush has not been studied as it has been in guayule, it is obvious that regulatory genes for the cis isopolyisoprene polymerase in rubber rabbitbrush would have to function in response to heat or stress signals rather than cool temperatures. Although not clearly demonstrated, evidence suggests that the pathway from mevalonic acid to cis-isoprene is more closely tied to the formation of some terpenes than others. Figure 5 gives a diagrammatic explanation of the possible relationship between terpene formation and that of natural rubber from cis-isoprene. While the changes in rubber and resin content imply an interconversion, radioactive tracer studies are needed to verify the concept. The significance of elucidating the exact relationship that exists between the resin and rubber formation is in controlling the flow of energy from terpene production into natural rubber. If environmental signals such as high temperature, low moisture levels, and water stress could be imitated, it may be possible to favor the conversion from mevalonic acid and certain terpenes to natural rubber, rather than to monoterpenoids.

TABLE 4. Individual compounds present in the factors as determined by factor analyses by Statview 512+ in relation to each other over the growing season in *Chrysothamnus nauseosus* ssp. *turbinatus*.

Factor 1
C-27 branched hydrocarbon
C-28 branched hydrocarbon
Nonacosane C-29
C-29 branched hydrocarbon
Triacontane C-30
C-30 branched hydrocarbon
Hentriacontane C-31
Factor 2
Δ cadinene
Unknown D
Unknown F
Unknown B
Unknown H
Factor 3
Cyclohexanol, 3 ethenyl-3-methyl-2-(1 methylethenyl-6-)
Epi-bicyclosesquiphellandrene
Unknown C
Factor 4
Unknown A
Unknown E
β elemene
Unknown F
Factor 5
β cubebene
Factor 6
Sabinene
Myrcene
Cymene
Limonene

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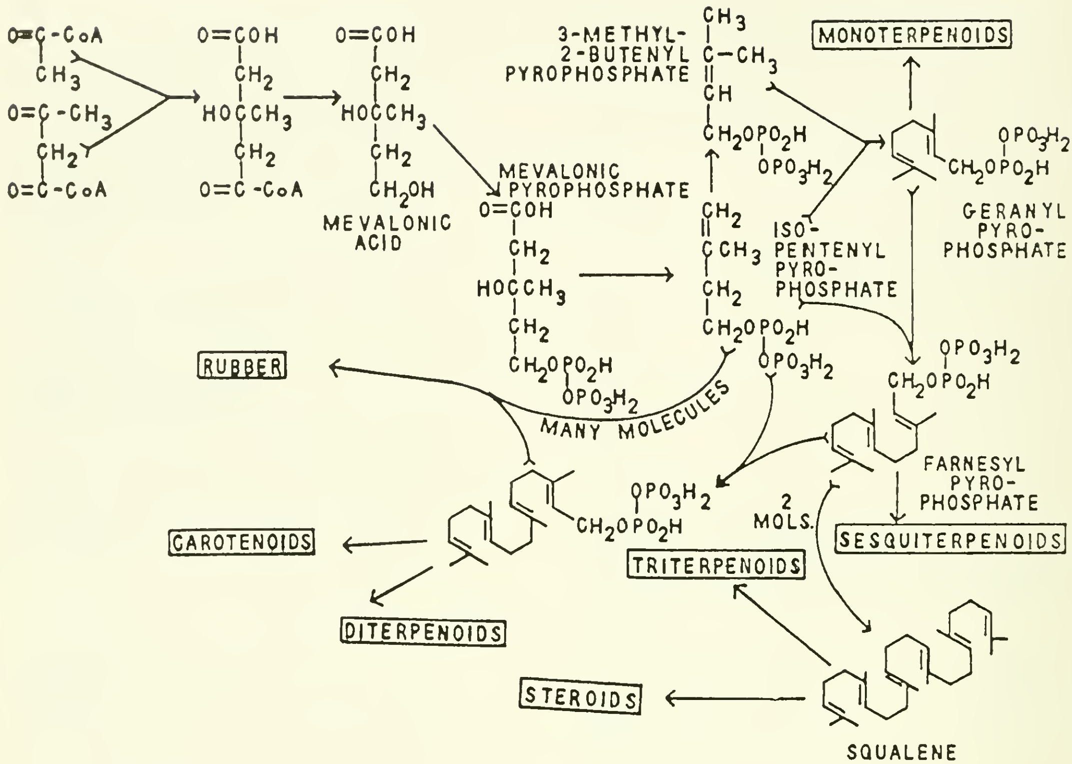

Fig. 5. Simple diagram of mevalonic acid pathways to hydrocarbons and rubber. Modified from Robinson (1983).