Acta Bot. Neerl. 25(3), June 1976, p. 227–250.

BIOSYNTHESIS OF PHYTOSTEROLS AND LATEX TRITERPENES IN HOYA AUSTRALIS R.Br. EX TRAILL AND HOYA CARNOSA R.Br.

H. W. GROENEVELD and J. KONING

Botanisch Laboratorium, Utrecht

SUMMARY

Young plant parts of *Hoya* species incorporate exogenously supplied acetic acid and mevalonic acid mainly into phytosterols; triterpenes are synthesized mainly in older plant tissues. Sugars are the only efficient substrates in biosynthesis of latex triterpenes. This synthesis occurs in all laticifer containing parts of the plant and is not related to sterol and triterpene biosynthesis of laticifer surrounding tissue.

1. INTRODUCTION

1.1 Freshly tapped latex of several plant species is able to synthesize latex specific compounds from naturally precursors

The in vitro biosynthesis of rubber in *Hevea brasiliensis* from acetate (BANDURSKY & TEAS 1957), from mevalonate and isopentenyl pyrophosphate (ARCHER et al. 1963), triterpenol biosynthesis from acetate in *Euphorbia helioscopia* and *E. cyparissias* (PONSINET & OURISSON 1967), and alkaloid biosynthesis from tyrosine in latex from *Papaver somniferum* (FAIRBAIRN et al. 1968, BÖHM et al. 1972) lead to the view that expelled latex in several cases is more than vacuolar sap only (FAIRBAIRN et al. 1968).

It may not be concluded that all latices are capable to in vitro synthesis of their constituents. Isolated latex of *Euphorbia helioscopia* and *E. lathyris* were incapable in converting m-tyrosine into m-hydroxyphenylglycine and 3,4 dihydroxyphenylalanine, both occurring in the latex of these species (LISS 1961, MÜLLER & SCHÜTTE 1971). Incorporation of amino acids into latex proteins could neither be stated in isolated latex of *Strophantus scandens*, *Asclepias curassiva* and ten *Euphorbia* species (MEISSNER 1966), nor was pipecolic acid converted to 4-hydroxy pipecolic acid and 4-amino pipecolic acid by isolated latex of *Strophantus scandens* (SCHENK et al. 1962). Attempts to achieve in vitro rubber formation with *Cryptostegia grandiflora* latex were uniformly completely negative (STEWART 1948).

POPOVICI (1926) described synthesis of latex particles in a parietal cytoplasm of *Ficus carica* and their subsequent excretion into a large central vacuole, as did Trécul in *Musa* (TRÉCUL 1867). More recently Pfänder observed a ribosomerich wall lining cytoplasm in *Euphorbia lathyris* laticifers (Pfänder, quoted

by MORITZ 1967). MARTY (1968) supplied electron optical evidence for a polyterpene particle synthesis in the parietal plasmatic layer of *Euphorbia characias* laticifers. This plasmatic layer is considered to be retained at tapping (BOBILIOFF 1925, LISS 1961, MORITZ 1967, GROENEVELD 1976b) and therefore, in accord with Bobilioff's view the expelled latex may also be regarded as a vacuolar fluid.

In latices triterpenes occur together with usually small amounts of polyterpenes in latex particles. As their biosynthesis from acetate or isopentenyl pyrophosphate could not be achieved in tapped latex of several *Hoya*, *Dischidia* and *Euphorbia* species (GROENEVELD 1976b), latex triterpene biosynthesis was assumed to occur within the parietal cytoplasm of the laticifers. In the present study, therefore, triterpene synthesis was studied by incubating stem parts with labelled precursors.

Besides in latices, triterpenes have a widespread occurrence in the plant kingdom (BOITEAU 1965) and their biosynthesis has been investigated by many workers (e.g. AEXEL et al. 1967, KASPRZIJK 1969, BAISTED 1971, REES et al. 1966, GOODWIN 1971). In the present work two *Hoya* species were investigated: *H. australis* R.Br. ex Traill having white latex with triterpene cinnamates as the main constituent of the latex particles (GROENEVELD 1976a) and *H. carnosa* R.Br. having a translucent latex, without triterpene particles.

1.2 General description of the plants and the laticiferous system

H. australis and *H. carnosa* are epiphytic climbers. Their root system is poorly developed. Leaves arise in pairs and their growth starts normally at the 4th internode (counting from apex to base), which means that the end of the stem over a length of about three internodes is leafless. A typical growth pattern is presented in *fig. 1* which demonstrates a growth of up to 2 cm a day. In the third internode the vascular cambium develops and secundary xylem and phloem become visible in the fourth internode. Except in the roots, the laticiferous system occurs in all parts of the plant. Like that of other Asclepiadaceae as *Cryptostegia grandiflora* (BLASER 1945, ARTSCHWAGER 1946), it is of the non-articulated, branched type also characteristic of many Apocynaceae, Moraceae and Euphorbiaceae (SCHMALHAUSEN 1877, SCHAFFSTEIN 1932, VREEDE 1949).

In the internodes laticifers occur in very large numbers in the cortex, pith and phloem regions of the bicollateral vascular system. Just below the nodal plane the latex tubes branch freely in all directions, and connections between pith and cortex laticifers by radial latex cells could be regularly observed, as has been reported for *Beaumontia grandiflora* (WOODWORTH 1932), *Ficus* species (VREEDE 1949) and Asclepiadaceae in general (TREIBER 1891). In the leaf they occur mostly in the midrib region and near the veinlets. In longitudinal sections the laticifers appear as thin walled elements completely filled with uniformly sized particles (GROENEVELD 1976 a, b). Light optically no parietal cytoplasm is visible. Non-articulated laticifers are multinucleated (TREUB 1879, MAHLBERG 1959, RAO & MALAVIYA 1966a, b); several nucleï were observed

in the young laticifers of *Hoya carnosa*. Lutoids occurring to a considerably degree in *Hevea* (RUINEN 1950) and several other latices (SOUTHORN 1964) could not be detected in *Hoya* latex.

2. PLANTS AND METHODS

Plants. Vegetatively propagated *Hoya*, plants were cultivated in the greenhouse on a gravel culture and parts of well growing plants were used in all incorporation experiments.

Incubation. For incubation with radioactive substrates parts of internodes of 0.5 grams fresh weight were sliced into discs of about 0.5–1.0 mm thickness. These discs were transferred to 5 ml incubation vials and moistened with 0.3–0.4 ml of water containing 5–10 μCi 2-^{14}C sodium acetate (58 mCi/mmol) or 5–10 μCi DL – 2-^{14}C mevalonic acid (10.9 mCi/mmol). These vials were placed in a covered beaker, water was added to maintain the viability of the plant tissue and incubated at room temperature with illumination by two 40 Watt TL-tubes placed 30 cm above the vials.

In other experiments more intact internodes were placed in 40–150 μl aqueous isotope solutions which were completely absorbed within 6 hours.

The incubation of chopped stem tissue was terminated by adding 2 ml ethanol. They were subsequently saponified as described earlier (GROENEVELD 1976b), extracted with petroleum ether (40–60) yielding the unsaponifiable components. Total terpenoid extracts were made according to a slightly modified method of GRUNWALD (1970). Frozen plant parts were chopped and extracted with acetone in a soxhlet apparatus during 2 hours. Acetone was evaporated and the residue dissolved in a 10 ml mixture of equal parts of ethanol 96% and petroleum ether 40–60. Water was added to obtain two layers and the petroleum ether fraction was cleaned by extracting once with ethanol 90% and subsequently evaporated.

Latex tapping. After incision with a razor blade expelled latex was taken up in 20 μl micro-capillaries and suspended in a 1 ml K_2HPO_4 solution resulting in a homogenous stable particle suspension (GROENEVELD 1976a, b). The first incision was made within 2 cm from the site of isotope application. Triterpenoids were extracted completely with petroleum ether (40–60) after adding an equal volume of acetone, followed by vigorous shaking. Petroleum ether extracts were chromatographed on 5 g alumina columns (Brockman, grade II-III) yielding separate fractions containing (a) hydrocarbons (squalene) and triterpenyl/steryl esters, (b) 4,4-dimethyl sterols (and triterpenes), (c) 4α-methylsterols and (d) sterols (GOAD & GOODWIN 1966) as described earlier (GROENEVELD 1976b).

Thin layer chromatography. TLC of samples (7500 dpm minimum) for radiochromatographic scanning purposes was carried out on 0.25 mm silica gel G and developed twice in cyclohexane-ethylacetate (9:1, v/v) (BENVENISTE 1966) separating sterols, 4α-methylsterols, 4,4-dimethylsterols (and triterpenes), triterpenyl/steryl acetates, triterpenyl cinnamates, triterpenyl/steryl palmitates,

carotenes and squalene. Squalene, β-amyrin palmitate, β-amyrin cinnamate, -acetate, β-amyrin, sitosteryl acetate and sitosterol were used as reference compounds. The ester fractions and acetylated triterpenes and sterols were separated on silica gel G thin layer plates impregnated with 15% $AgNO_3$ (w/w) and developed in benzene-petroleum ether 40–60 (3 : 2, v/v) (BARNA & DUTTA 1967). β-amyrin cinnamate, β-amyrin acetate, cycloartenyl acetate, 24-methylene cycloartenyl acetate and sitosteryl acetate were used as references. Spots were detected by spraying with chlorosulfonic acid-glacial acetic acid (1/2, v/v) and heating at 120°C for 5–10 minutes. After hydrolysis of the ester fractions and extraction of the unsaponifiable lipids the pH was adjusted to 1 with HCl and long chain fatty acids were extracted with petroleum ether and aromatic acids with ethylacetate containing 2% cinnamic acid.

Radioactive counting. Samples of lipid extracts were dissolved in 10 ml of scintillator containing 4.5 g PPO-POPOP Premix P/1. of toluene (A), aliquots of diluted latex particle suspensions were dissolved in 10 ml of scintillator solution consisting of 5 g permablend I (Packard), 800 ml dioxane, 48 g naphtalene and 110 ml cellosolve, and essayed in a liquid scintillation spectrometer. After thin layer chromatography radioactive spots were detected with a radio chromatogram scanner, scraped from the sheets and counted in scintillator (A).

Isolation of cell organelles. After slicing into 1 mm discs 3 grams of stem tissue was homogenized with an Ultra Turrax mixer for 20 seconds in 15 ml 0.02 M glycylglycine buffer at pH 7.5 containing 0.5 M sucrose. The homogenate was filtered through four layers of cheese cloth to remove the cell debris; the pH was adjusted to pH 7.5 and the extract was centrifuged according to the following scheme (TOLBERT et al. 1969): 30 minutes at 700g removing whole chloroplasts; broken chloroplasts and peroxisomes were precipitated at 6000g during 20 minutes, and the mitochrondrium fraction was precipitated at 39,000g during 30 minutes. The 6000g and 39,000g pellets were resuspended with a Potter tube in 1.5 ml 0.5 M sucrose containing glycylglycine buffer and layered on a discontinuous sucrose gradient in the same buffer. The 2.5–2.3–1.8–1.3–0.5–0.35 M sucrose gradient was centrifuged for 2 hours in a SW 50 L Spinco rotor at 35,000 rpm. All operations were conducted at 2–4°C. After the run the gradient was frozen and separate fractions were excised, thawed and the lipids were extracted as described for the latex particles.

Identification of triterpenes and sterols in Hoya australis. In *Hoya australis* the triterpene cinnamates and acetates are the major compounds of the latex particles. Chromatography of the triterpene esters on silica gel G thin layer plates impregnated with $AgNO_3$ and developed in benzene-petroleum ether (40:60, v/v) (BARNA & DUTTA 1967) revealed three spots cochromatographing with samples of authentic β-amyrin cinnamate (Rf 0.67), β-amyrin acetate (Rf 0.54) and cycloartenyl acetate (Rf 0.40). After saponification and subsequent acetylation the latex triterpenyl cinnamates revealed triterpenyl acetates which cochromatographed with authentic samples of β-amyrin acetate (major compound), cycloartenyl acetate and 24-methylene cycloartenyl acetate. After acetylation the free triterpenes present in latex also cochromatographed

with these references. Gaschromatographically the presence of cycloartenol, 24-methylene cycloartenol and β-amyrin could be demonstrated and α-amyrin cochromatographing with β-amyrin in argentum thin layer chromatography appeared to be present in the free and esterified triterpenols. β-amyrin was found the major triterpenol of the esters, 24-methylene cycloartenol in the free triterpenols.

Fragmentation patterns in gaschromatography-mass spectrometry of the *H. australis* latex triterpenols mentioned were in agreement with those of authentic samples and identical to the patterns described in the literature (DJERASSI et al. 1962, APLIN & HORNBY 1966, AUDIER et al. 1966).

In latex phytosterols are present as traces only (GROENEVELD 1976b). In extracts of plant parts the sterols were separated into three spots (after saponification and acetylation) on $AgNO_3$-impregnated thin layer plates. The major compound cochromatographed with the common phytosterols, sitosterol, stigmasterol and campesterol (Rf 0.45). Two other spots with Rf <0.45 have not been identified and are stated with a Rf value relative to sitosterol. The 4α-methylsterols, absent in latex, were not investigated in detail, neither the 4,4-dimethylsterols of plant tissue as the pentacyclic and tetracyclic latex triterpenes cochromatograph with this fraction. In *Hoya carnosa* only appreciable amounts of sterols were found in the unsaponifiable fraction. The presence of sitosterol in this plant species was already reported by KERN & HASELBECK (1942). 4,4-dimethylsterols and triterpenes and their esters could be detected as trace components only in thin layer chromatography.

3. RESULTS

3.1 Incorporation of acetic acid-2-^{14}C and mevalonic acid 2-^{14}C into the 4,4-dimethylsterols and sterols by chopped internodes of *H. australis*

Preliminary investigation showed that no qualitative differences within the radioactive unsaponifiable lipids were obtained in chopped leaves and stem after 24 hours' incorporation of acetate and mevalonate. In the present work stem parts were used because they can be sliced more uniformly resulting in more reproducible quantitative data. In all experiments the internodes used were numbered from the stem apex which means the youngest internode numbers one *(fig. 1)*. Chopped parts of internodes (up to 0.5 g fresh weight) were incorporated with 2-^{14}C acetate during 24 hours and radioactivity in the unsaponifiable lipids was measured. Results of typical acetate incorporation in *H. australis* are presented in *fig. 2*. The data were obtained by separating the unsaponifiable lipids on thin layer plates and after scanning of t.l.c. sheets, zones corresponding to the appropriate standards and radioactivity were scraped from the plates and measured in a scintillation counter. The results were in agreement with those obtained after chromatography on alumina columns. In general a percentage of incorporation of 0.9–2.7% was found. Similar experiments were carried out with 2-^{14}C mevalonic acid. For this

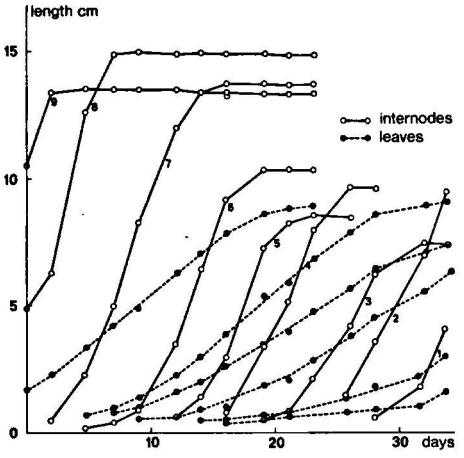

Fig. 1. Growth curves of internode and leaves of *Hoya australis* cultivated on a gravel culture: average temperature at day 24°C, at night 20°C, relative humidity 40–60%. The leaf length is represented by the average length of leaf laminas in each pair of leaves. The first growth curve of leaves corresponds with the apical leaf pair at the 9th internode. The numbers at the internodes growth curves correspond with those of the first 2-^{14}C acetate incorporation experiment (fig. 2).

purpose parts of internodes nr. 1 + 2, 3, 4, 5, 6, 8, 10 and 12 were sliced in 0.5–1.0 mm thick sections and incubated during 24 hours. Results of a typical experiment are presented in *figs. 3*, *4a* and *4b*. They demonstrated that all parts of a *Hoya* stem can incorporate acetic acid and mevalonic acid into unsaponifiable lipids. In all parts of the stem about equal amounts of label were incorporated but the distribution of radioactivity after 24 hours' incorporation appeared to be different in each internode. In the youngest internodes mainly sterols were formed, while the labelled 4,4-dimethylsterols gradually appeared in older internodes. The 4,4-dimethylsterol-to-sterol radioactivity ratio became rather constant from the 5th internode onward. The distribution of radioactivity within the sterols and triterpenes was also determined in relation to the internode number. In stem segments which had incorporated acetate or mevalonic acid the relative amount of the sitosterol fraction of the sterols diminished strongly with age (*fig. 4b*). Within the 4,4-dimethylsterols of the first internodes ± 60% of the radioactivity was localized in the α- and β-amyrin fraction, but this percentage decreased gradually to about 35% in the 5th internode and remained fairly constant at increasing internode number (*fig. 4a*). All plant parts showed some radioactivity in the first eluted hydrocarbon containing fraction in column chromatography. On silica gel G thin layer plates developed in hexane about 90–95% of this fraction cochromatographed

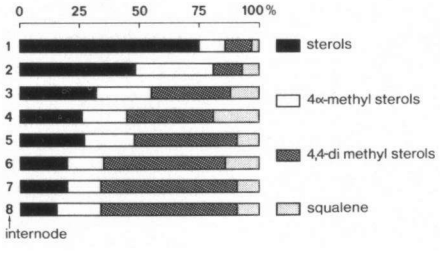

Fig. 2. Distribution of radioactivity between squalene, 4,4-dimethylsterols, 4α-methylsterols and sterols after 24 hours incorporation of 2-^{14}C acetate by various internodes of *H. australis*. The internode numbers correspond to those presented in fig. 1. Experimental details are given in the text.

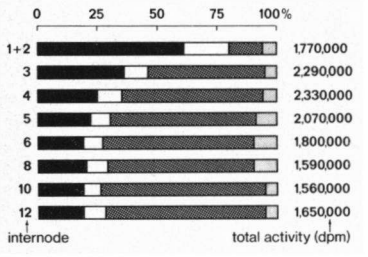

Fig. 3. Distribution of radioactivity between squalene, 4,4-dimethylsterols, 4α-methylsterols and sterols after 24 hours incorporation of 2-^{14}C mevalonate (4.5 μCi) by various internodes of *H. australis*. Experimental details are given in the text.

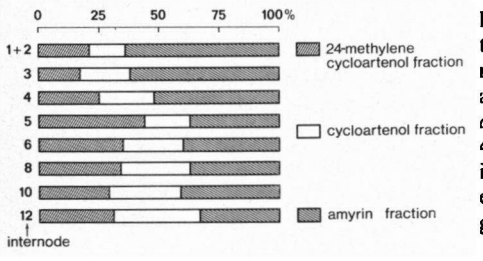

Fig. 4a. Distribution of radioactivity between the amyrin fraction, the cycloartenol fraction and the 24 methylene cycloartenol fraction of the triterpenes of *H. australis* after 24 hours incorporation of 4.5 μCi 2-^{14}C mevalonate by various internodes. Radioactive spots are indicated by their reference compounds in argention thin layer chromatography.

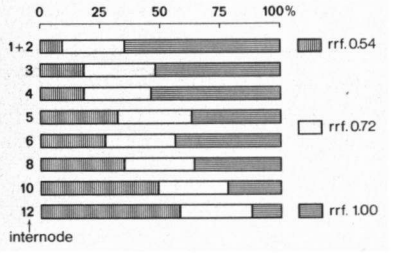

Fig. 4b. Distribution of radioactivity incorporated into the different sterol fractions of *H. australis* after 24 hours incorporation of 4.5 μCi 2-^{14}C mevalonate by various internodes. $AgNO_3$ thin layer chromatography of acetylated sterols. The sterol fractions indicated by rrf 1.00 ran coincident with sitosterol. The other fractions with mobilities smaller than sitosterol are indicated by a Rf. value relative to the Rf of sitosterol.

with squalene, in argention thin layer chromatography all radioactivity remained at the origin as did authentic squalene, and carotene. In general this fraction got little radioactivity and has therefore not further been studied.

3.2 Incorporation of 2-^{14}C mevalonic acid into sterols and 4,4-dimethylsterols of *Hoya australis* versus time

Results of these experiments are presented in *fig. 6*. The data obtained indicate that within a 24 hours' incubation period the sterol fractions steadily increased in radioactivity while the hydrocarbon(squalene)-activity decreased already after $1^1/_2$ hour. The 4α-methylsterols are fairly constant and the 4,4-dimethylsterols decreased absolutely after 6 hours. The final relative distribution after 24 hours is similar to that presented in *fig. 3*.

3.3 Incorporation of mevalonic acid into sterols and 4,4-dimethylsterols of six other *Hoya* species

In these experiments internodes were used which had just fully developed leaves,

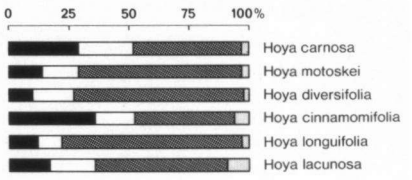

Fig. 5. Distribution of radioactivity between squalene, 4,4-dimethylsterols, 4α-methylsterols and sterols after 24 hours incorporation of 2-^{14}C mevalonate by various internodes from several *Hoya* species. Legenda as in fig. 2. Experimental details are given in the text.

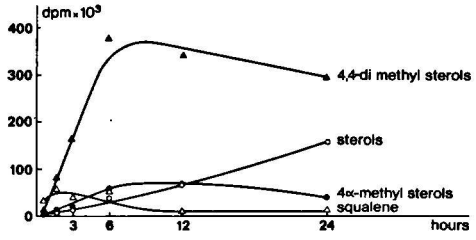

Fig. 6. Time course of labelling of squalene, 4,4-dimethyl sterols, 4α-methylsterols in *H. australis* from 2-^{14}C mevalonic acid. Data presented were from chopped 3rd and 4th internode. All fractions were determined via scintillation counting of thin layer chromatography scrapings.

Table 1. Incorporation of radioactivity from 2-^{14}C mevalonic acid (9 μCi) into the non saponifiable lipids and its distribution (%) between squalene, 4,4-dimethylsterols, 4α-methylsterols and sterols for various internodes of *H. carnosa*. Percentages are calculated as was done for *H. australis* (fig. 2).

internode	squalene %	4,4-dimethyl sterols %	4α-methyl sterols %	sterols %	total dpm recovered in unsaponifiable fraction
1+2+3	1	34	13	52	3,420,000
5	2	46	20	32	2,038,000
7	2	51	18	28	3,034,500
9	2	59	15	24	3,072,000

as is the case with the seventh internode of *H. australis*. The results, shown in *fig. 5*, do not much differ from those of *H. australis*. Remarkably, the pattern of incorporation of mevalonate and acetate into the various lipids was the same for plants as *H. carnosa* and *H. motoskeï*, both having colourless translucent latices without triterpene particles, and for *Hoya* species which have white latex. In addition internodes of different ages of *Hoya carnosa* incorporated mevalonic acid in the same way as *Hoya australis* tissue did *(table 1)*. In the youngest stem parts phytosterols of the sitosterol fraction were major compounds, while in older internodes most radioactivity of the 4,4-dimethylsterols cochromatographed with β-amyrin (*table 2a* and *2b*).

3.4 Incorporation of 2-^{14}C mevalonic acid into sterol, 4,4-dimethylsterols and the corresponding ester fractions of *Hoya australis* by different internodes

Slices of different internodes were incubated with 2-^{14}C mevalonic acid and processed after 24 hours. The petroleum ether extracts of these internodes were

Table 2a. Distribution of radioactivity between the amyrin fraction, the cycloartenol fraction and the 24-methylene cycloartenol fraction of the 4,4 dimethylsterols of *H. carnosa* after 24 hours incorporation of 2-^{14}C mevalonate by various internodes.

internode	Amyrin fraction %	cycloartenol fraction %	24-methylene cycloartenol fraction %
1, 2, 3	21	52	27
5	50	41	9
7	52	36	12
9	48	42	10

Table 2b. Distribution of ^{14}C incorporated into the different sterol fractions of *H. carnosa* after 24 hours incorporation of 2-^{14}C mevalonate by various internodes. The sterol fractions indicated by rrf 1.00 ran coincident with sitosterol, the other sterol fractions are indicated by a Rf-value relative to those of sitosterol.

internode	rrf 1.00	rrf .88	rrf .67
1, 2, 3	62	11	27
5	41	24	35
7	37	22	41
9	33	25	42

separated on silica gel G thin layer plates and in column chromatography. Labelled hydrocarbons, esters, 4,4-dimethylsterols and sterols appeared to be present. The ester fraction was saponified and submitted to column chromatography again to obtain the 4,4-dimethylsterols and sterols of the ester fraction. Complete distribution of radioactivity is given in *table 3*. With increasing internode number the ester fraction exhibited a relative increase which was apparently caused by 4,4-dimethylsterolester synthesis. The relative amount of radioactivity in the free sterols and esterified sterols diminished in older internodes and is in agreement with the first incorporation experiments. The ester fractions of older internodes, which contained mainly 4,4-dimethylsterol esters cochromatographed with authentic β-amyrin palmitate on silica gel G thin layer plates but, subjected to argention chromatography only about 10% of this fraction still did so. Other spots of radioactivity with mobilities smaller than β-amyrin palmitate were present but they did not run coincident with β-amyrin cinnamate and β-amyrin acetate.

3.5 Incorporation of isotopes by more intact plant parts

Most apical stem parts above the first internode (up to 0.7 cm length) and stem parts consisting of the first and second internode were incorporated with 2-^{14}C mevalonic acid. 50 μl isotope solutions were administered and absorbed within five hours by the internode parts. Radioactivity was found after 24 hours in the hydrocarbon (squalene), 4,4-dimethylsterols, sterols and their esters.

Table 3. Distribution of ^{14}C between squalene, sterols and sterolesters in different internodes of *H. australis* after 2-^{14}C mevalonic acid incorporation during 24 hours.

internode	2 %	3 %	5 %	7 %
squalene	1.5	2.8	0.7	1.6
4,4-dimethylsterol esters	15.8	13.1	32.6	37.8
sterol esters	6.6	5.9	2.7	2.3
4,4-dimethylsterols	18.1	19.4	31.7	29.6
4α-methylsterols	8.5	5.7	8.7	12.3
sterols	49.5	52.9	23.3	16.6

Table 4. Distribution of ^{14}C between squalene, 4,4-dimethylsterols, sterols and their esters after 24 hours incorporation of 2-^{14}C mevalonic acid by excised stem parts of *H. australis*.

	stem apices	internode 1 + 2
	%	%
squalene	33.4	7.1
4,4-dimethylsterol esters	9.9	10.0
4,4-dimethylsterols	23.4	12.3
sterol esters	3.3	5.7
sterols	29.2	65.5

From the data presented in *table 4* may be concluded that as far as the intact internodes are concerned mevalonic acid yields the same incorporation pattern as obtained in chopped plant tissue. The stem apices incorporated a remarkable amount of ^{14}C into the hydrocarbons. After addition of squalene and formation of squalene hexahydrochloride by the method of GOAD & GOODWIN (1966) 70% of the original radioactivity remained after two recristallisations, which indicates that squalene is the main labelled component of this hydrocarbon fraction.

To obtain latex from incubated plant tissue 10 cm long apical stem parts were used. From the basic parts of these stem parts thin slices were cut off until no latex exuded and a clear wound surface was obtained. These stem parts were placed immediately in different 50 µl isotope solutions which were absorbed within six hours. The turgor pressure was restored after 24 hours and latex could be tapped again from these stem parts. Data presented in *table 5* show that only the latex obtained from stem parts that had absorbed labelled sugars had incorporated radioactivity into the petroleum ether soluble latex compounds. After the latex had been tapped, lipids were extracted from the remaining stem tissue and submitted to thin layer chromatography. In all cases radioactivity in the 4,4-dimethylsterols and sterols was measured, but radioactive triterpenyl cinnamates could only be established in stem parts which had absorbed labelled sugars. Very much of the radioactivity of the

Table 5. Recovery of ^{14}C in latex, petroleum ether extracts of latex and the rest of the plant after absorption of solution of isotopes by 10 cm apical stem parts of *H. australis*. After incorporation during 24 hours, stem parts were cut into two parts of equal length and processed separately as was the expelled latex.

		latex	petroleum ether extracts		
			latex	apical stem part	basal stem part
		dpm	dpm	dpm	dpm
2-^{14}C acetic acid	7.5 μCi	2,600	–	1,080	1,765,000
2-^{14}C mevalonic acid	1.9 μCi	5,100	–	615	325,000
u-^{14}C leucine	1.5 μCi	1,850	–	4,800	67,000
u-^{14}C glucose	7.5 μCi	32,500	14,600	9,100	132,000
u-^{14}C fructose	8.0 μCi	21,300	11,400	5,600	91,700
u-^{14}C sucrose	3.5 μCi	14,600	7,200	3,400	70,500

mevalonic acid and acetic acid fed stem parts was retained by the internode to which the isotope solution was applied.

Summarizing these incorporation experiments it may be stated that exogenously supplied acetate or mevalonate are not incorporated into latex triterpenes. Regarding the time course study of the incorporation of mevalonic acid into sterols it may be assumed that these precursors are incorporated into the sterols of plant tissue besides some non-latex triterpenes (β-amyrine fraction). Other possible triterpene precursors as leucine and malonic acid are not incorporated into latex triterpenes. Only sucrose, glucose and fructose appeared to be efficient substrates in biosynthesis of latex triterpenes.

3.6 Incorporation of u-^{14}C sucrose (8.1 mCi/mmol) into triterpenoids of *Hoya carnosa* and *H. australis* latex

U-^{14}C sucrose solution was absorbed by apical stem parts of 20 cm length, which were subsequently incubated for 40 h at 27°C in a moist atmosphere after which the latex was tapped. Latex and the tissue from which it was obtained were extracted and radioactivity of the triterpene-sterol fractions and their esters were measured. The data in *table 6* show that in *H. australis* the major part of the incorporated carbon-14 occurred in the triterpene esters of the tapped latex (acetates and cinnamates). The synthesis of sterols (which are virtually absent in latex) is apparently of minor importance. In *H. carnosa* latex no radioactive triterpenes and sterols could be detected. The rest of this plant showed a very low incorporation rate into the triterpene-sterol fraction which is undoubtedly due to absence of latex particles in this species. In the triterpene ester fraction of *H. australis* latex both the acid (cinnamic acid) and the triterpenol component were labelled. Radioactive cinnamic acid was demonstrated by radio-gaschromatography of the prepared methyl cinnamate (WARNAAR 1976), while the labelled acetylated triterpenols of the ester fraction were demonstrated in argentum thin layer chromatography and radio-

gaschromatography. The radio-gaschromatogram presented in *fig. 7a* shows a roughly equal specific activity of the triterpenols in the latex triterpene esters, which is in agreement with the quantitative data obtained in argentum thin layer chromatography of the same fraction *(table 6)*. Identical results were obtained with the free triterpenols *(fig. 7b, table 6)*. Gelfiltration of labelled latex particle suspensions on 35 cm sephadex G–100 columns as described earlier (GROENEVELD 1976a, b) showed that 97.5–100% of the radioactive triterpenes was eluted in the particle fraction. The distribution of radioactivity within the ester fraction of the remaining stem tissue was similar to that measured in latex esters. Synthesis of triterpenes esterified with long chain fatty acids could not be established as in stem parts to which ^{14}C-acetate or ^{14}C-mevalonate

Table 6. Incorporation of ^{14}C from u-^{14}C sucrose into (latex) triterpenes of *H. australis* (33.8 μCi) and *H. carnosa* (22.5 μCi). Experimental details are given in the text. Figures derived after: 1) separation on alumina column, 2) separation on argentated thin layer sheets. 3) saponification and extraction with ethylacetate.
a) consist of α- and β-amyrin, cycloartenyl and 24-methylene cycloartenyl cinnamate.
b) may contain labelled long chain fatty acids.

	Latex *Hoya australis*	Tissue *H. austr.* 2184 mg	Tissue *H. carnosa* 1632 mg
	dpm × 10^3	dpm × 10^3	dpm × 10^3
total activity	1485		
petroleum ether extract	1098		
hydrocarbons[1])	3	31	47.6
triterpene and sterol esters[1])	744	1019	12.4
free triterpenols[1])	139	192.4	19.7
free sterols[1])	3.4	19.7	10.8
esters:[2])			
cycloartenyl acetate	98.5 (13%)	149.6 (14%)	
α- and β-amyrin acetate	242 (33%)	314 (30%)	
triterpenyl cinnamates[a])	404 (54%)	585 (56%)	
triterpenols of the esters[2])	503.5	664	5.1
24-methylene cycloartenol	61.5 (12%)	94.2 (14%)	
cycloartenol	90.5 (18%)	110 (17%)	
α- and β-amyrin	351 (70%)	459 (69%)	
sterols of the esters	5.3	10	.9
cinnamic acid[3])	117.2	163.4	6.4[b])
free triterpenols:			
24-methylene cycloartenol	66.6 (48%)	80.5 (42%)	
cycloartenol	41.4 (30%)	33.8 (18%)	
α- and β-amyrin	31.6 (23%)	78 (41%)	
ratio triterpene esters/free triterpenols	5.35	5.3	
ratio triterpenol of the esters/free triterpenol	3.6	3.46	
^{14}C recovery in triterpenes (% of supplied ^{14}C)	1.2%	1.5%	

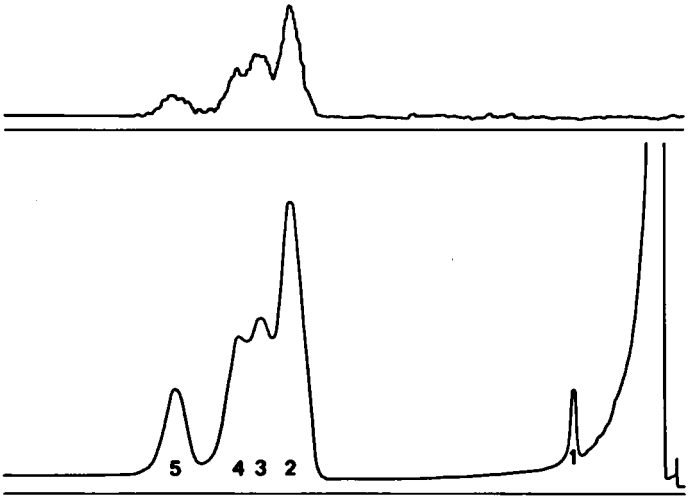

Fig. 7a. Radio gas chromatogram of the triterpene esters of *H. australis* latex separated as acetates on 3% SE-30 at 245°C. 5α cholestane (1) was used as internal standard; 2. β-amyrin acetate. 3. α-amyrin acetate 4. cycloartenyl acetate and 5. 24-methylenecycloartenyl acetate. upper curve: activity trace. lower curve: trace of mass.

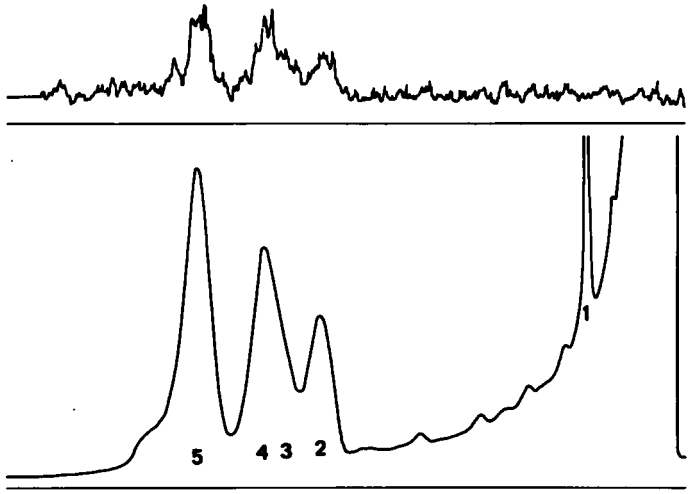

Fig. 7b. Radio gas chromatogram of the triterpenols of *H. australis* latex separated as triterpenyl acetates on 3% SE-30 at 245°C with 5α cholestane as internal standard. 2. β-amyrin acetate, 3 α-amyrin acetate 4, cycloartenyl acetate 5, 24-methylene cycloartenyl acetate lower curve: trace of mass. upper curve: activity trace.

had been administered. Gaschromatographically the acetylated triterpenols of both fractions were identical. Very probably these radioactive compounds were localized in latex constituents which were not expelled at tapping. The very low incorporation of carbon-14 into the triterpene and sterol esters (even less than into the saturated hydrocarbons) in *H. carnosa* tissue supports this view.

From the results obtained it was concluded that the labelled ester fraction or particularly the triterpenyl cinnamates synthesized after sucrose feeding could be used to investigate quantitatively latex triterpene biosynthesis. The distribution patterns of radioacitivity of the free triterpenols of latex and stem tissue were not identical. Probably triterpenols of non-latex origin may be synthesized simultaneously to an unknown extent, or latex triterpenols prior to their esterification are present in this fraction. On the other hand sterols are synthesized and intermediates in this synthesis may contribute to the radioactivity of the triterpenols. The locus of radioactive latex triterpenols which remained after tapping in u-^{14}C sucrose incorporation experiments was investigated by means of cell fractionating.

A 20 cm apical stem part was incubated with u-^{14}C sucrose during 20 hours. After tapping the tissue was homogenized and cellular fractions were prepared according to GRUNWALD (1970) in a phosphate buffer. As all fractions, cell debris, nucleï, chloroplasts, mitochondria and microsomal fractions contained radioactive triterpenyl cinnamates a contamination with latex particles was assumed. Adding some labelled latex to non-incubated stem material resulted in a similar distribution of radioactive triterpene esters over different cell fractions. The isolation procedure for peroxisomes described by TOLBERT et al. (1969), revealed a number of fractions in discontinuous gradient centrifugation of the 6000g and 39,000g fractions in which radioactivity was present and latex specific organelle fractions could be shown. (*H. carnosa* was used for comparison.) In density gradients of both fractions at the top a very thin film of latex particles was present and at the upper half of the 0.5 M sucrose layer (density $\pm$1.07), distinctly separated from the chloroplasts at interface between 1.3 M and 1.5 M sucrose, a particulate fraction was present in which latex triterpene esters occurred. The latex triterpene esters containing particle fraction with density of 1.07 was present in freshly tapped latex as a trace only. The distribution of radioactivity within the triterpene esters was similar to those in latex particles of the top layer with a density of 1.008–1.045 (GROENEVELD 1976a).

As in *H. carnosa* a particulate suspension was present at the interface between the 1.8 and 2.3 M sucrose layers, corresponding with the peroxisomes locus described by TOLBERT. This fraction contained no neutral lipids and was most abundant in stem tissue compared with equal quantities of leave tissue (both in *H. carnosa* as in *H. australis*).

For comparison different internodes of a single plant were incubated for 30 hours with u-^{14}C sucrose and total lipids were extracted after freezing. Data obtained are presented in *table 7*. They show the qualitative incorporation patterns in the triterpene ester fractions are uniform in all stem parts. Most

Table 7. Distribution of radioactivity in latex triterpene esters and free triterpenols and triterpenols of the esters after u-^{14}C sucrose incorporations by 5 cm parts of various internodes of *H. australis*. Radioactivity was assayed in the esters after separation on argentated thin layer plates. Percentage of radioactivity in 24-methylene cycloartenol, cycloartenol and α- and β-amyrin in triterpene esters and free triterpenols. [1]) consists of α- and β-amyrin, cycloartenyl and 24-methylene cycloartenyl cinnamate.

		total activity	cyclo-artenyl acetate	α- and β-amyrin acetate	triterpenyl cinnamate[1])	a	b	c
		dpm	dpm	dpm	dpm	%	%	%
Apex + 1° internode	triterpene esters	73,000	11,200	20,100	41,600	25	10	65
	triterpenols	35,300				16	6	78
3° internode	triterpene esters	175,600	26,900	39,100	95,000	23	12	65
	triterpenols	30,600				33	21	45
4° internode	triterpene esters	203,000	33,800	51,700	108,000	26	10	64
	triterpenols	33,900				45	16	39
5° internodes	triterpene esters	195,000	37,600	56,500	101,000	22	17	61
	triterpenols	32,000				42	22	37
7° internode	triterpene esters	98,000	21,000	26,625	51,000	27	14	61
	triterpenols	26,630				40	18	42
9° internode	triterpene esters	83,500	16,550	23,630	43,400	24	16	59
	triterpenols	16,000				32	17	52
15° internode	triterpene esters	17,830	2,190	4,910	16,670			
	triterpenols	3,050						

a. 24-methylene cycloartenol; b. cycloartenol; c. α- and β-amyrin.

activity is found in the 4th internode, gradually decreasing in younger and older stem parts. Very old stem parts still appeared to be able to synthesize triterpenyl cinnamates. A uniform labelling pattern throughout the triterpenol fraction could not clearly be demonstrated. In most internodes the order of radioactivity was α- and β-amyrin < 24-methylene cycloartenol < cycloartenol, but in all investigated internodes the latex specific order of radioactivity (24-methylene cycloartenol < cycloartenol < α- and β-amyrin) was not observed.

As u-^{14}C sucrose incorporations into the latex triterpenes were negative in sliced stem tissue but positive in lengths of more intact internodes the relation between stem length and ability for triterpene ester biosynthesis was studied. The sixth internode was cut in parts of different lengths and to each 40 μl of ^{14}C-sucrose solution (10 μCi) was supplied, which was absorbed within 6 hours. The stem segments were subsequently incubated in a moist atmosphere during 36 hours, and radioactivity was determined in the various triterpene esters extracted from the plant parts. The values obtained, presented in *fig.8*, show that a minimal stem length of about 8 mm is required to get appreciable latex triterpene biosynthesis. In all used stem parts a uniform labelling pat-

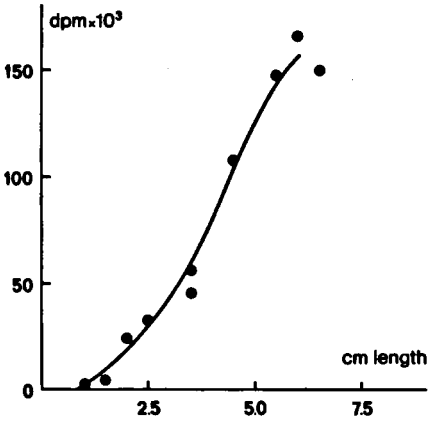

Fig. 8. Biosynthesis of latex triterpene esters from u-^{14}C sucrose by stem parts of different length (parts of 6th internode). Data represent the activity in the ester fraction obtained in column chromatography corrected for activity not associated with triterpene esters in argentum thin layer chromatography.

tern in the ester fraction was observed after argentum thin-layer chromatography. Longer internode parts resulted in irreproducible quantitative data.

When very long stem parts were employed a discontinuous distribution of radioactive triterpene esters was measured. 200 μl sucrose u-^{14}C (50 μCi) solution was absorbed by the basal part of a 52 cm long leafless *H. australis* stem; 70 minutes after the isotope was administered to the 4th internode radioactivity was detectable with a Geiger-Müller counter at the stem apex. This radioactivity increased during the subsequent 16 hours and afterwards remained constant. After 31 hours ^{14}C-incorporation the plant part was frozen, divided in 3.7 cm segments and processed. The results obtained are presented in *table 8*, which shows most radioactive triterpene esters localised in the internode to which the isotope solution was supplied. The 3rd internode showed relatively little synthesized triterpene esters. The stem apex was apparently not the principal site of latex biosynthesis. In all segments a rather uniform distribution pattern within the triterpene esters was present, only into the triterpenyl cinnamates relatively less radioactivity was incorporated by the apical stem parts.

To investigate the optimal incubation conditions and optimal yield of triterpenes, parts of the internodes were incubated in atmospheres of different humidity. This resulted in a different degree of water loss during incubation. The parts with ample water supply showed optimal ^{14}C incorporation *(table 9)*.
The data in *fig. 9* demonstrate that labelled triterpene esters synthesis starts directly after sucrose supply. Maximum incorporation is found after about 40 hours and the amount of radioactivity in the ester fraction did not decrease during another 40 hours.

Fig. 10 shows the incorporation of radioactivity into the latex triterpenes at different temperatures. Apparently latex triterpenes are not synthesized at a continuous temperature above about 33°C, and the optimum was found at about 26°C.

As laticifers are present in the pith and cortex of the stem a study was made of latex triterpene biosynthesis in both systems. A well developed secundary

Table 8. Thin layer chromatographic separation of labelled triterpene esters of a 52 cm stem part of *H. australis* after incubation with u-^{14}C sucrose. After 31 h incorporation the stem was sectioned into 14 parts of 3.7 cm each and processed. * indicates the presence of a node.

	dpm/mg fresh weight	cycloartenyl acetate	α- and β-amyrin acetate	triterpenyl cinnamates
		dpm	dpm	dpm
(apex) 1*	30		2180	2395
2	58	310	5060	5220
3*	66	2060	6470	7640
4	119	2710	9560	11270
5	120	2630	10600	11800
6	164	4440	14500	15700
7*	270	7650	21200	22500
8	51	1600	5810	6440
9	20	900	1930	2260
10	49	1790	5260	5940
11	56	2210	5800	7410
12*	176	8050	14900	25500
13	181	8400	17400	24500
14	158	8450	11800	19900

xylem is present in the fourth internode, so a separation between cortex and xylem could easily be made. Stem parts were incubated with 22.5 μCi of sucrose during 40 hours, they were subsequently frozen, cortex and xylem were separated and extracted. Results of these experiments, presented in *table 10* show a similarity in distribution of radioactivity in the various triterpenes. The

Table 9. Incorporation of ^{14}C from u-^{14}C sucrose into the latex triterpene esters of *H. australis*. Distribution between triterpenyl cinnamates, α- and β-amyrin acetate and cycloartenyl acetate. 100 μl solution of labelled sucrose was absorbed by 5 cm parts (a, b and c) of the 5th and 9th internode and incubated during 30 hours in different grades of humidities resulting in a decrease of fresh weight.

		decrease in fresh weight	total activity incorporated into triterpene esters	percentage of total activity		
			dpm	I %	II %	III %
5th internode	a	0.4%	152,500	50.1	26.6	22.4
	b	3.4%	102,000	49.2	23.4	27.4
	c	15.5%	63,000	56.8	23.0	20.2
9th internode	a	19.8%	29,000	53.1	26.0	21.0
	b	4 %	45,000	55.0	22.9	21.0
	c	0.3%	58,700	48.7	21.8	20.4

I. triterpenyl cinnamates; II. α- and β-amyrin acetate; III. cycloartenyl acetate.

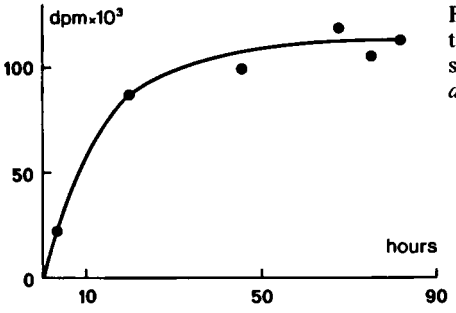

Fig. 9. Time course of labelling of latex triterpene esters from u-^{14}C sucrose by 5 cm stem parts of the 6th and 7th internode of *H. australis*.

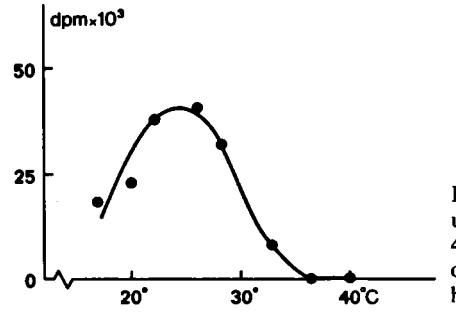

Fig. 10. Incorporations of radioactivity from u-^{14}C sucrose into latex triterpene esters by 4 cm stem parts of the 5th and 6th internode of *H. australis* versus temperature, during 18 hrs.

Table 10. Incorporation of ^{14}C from u-^{14}C sucrose into latex triterpenes in cortex and pith laticifers. 4th internode *H. australis*. a) consists of α- and β-amyrin, cycloartenyl and 24-methylene cycloartenyl cinnamate.

	PITH dpm		CORTEX dpm	
triterpene esters	140,800		123,000	
triterpenyl cinnamates[a])		50,600 (36%)		45,500 (37%)
α- and β-amyrin acetate		32,400 (23%)		34,500 (28%)
cycloartenyl acetate		54,800 (39%)		43,000 (35%)
free triterpenols	32,100		44,100	
24-methylene cycloartenol		17,050		18,300
cycloartenol		7,350		7,760
α- and β-amyrin		7,200		17,400

ratio triterpene ester triterpenol, being 4.4 in the pith is in accord with the ratio found in tapped latex. In the cortex a ratio of 2.8 is measured, probably caused by a relative large contribution of α- and/or β-amyrin of non-latex origin or contribution of labelled triterpenols prior to their esterification with acetic acid or cinnamic acid.

3.7 $^{14}CO_2$-incorporation

Apical stem parts of *H. australis* having at least four full-grown leaves were supplied with 100 μCi $^{14}CO_2$ during 2–3 hours and tapped after a few days. In

Table 11. Incorporation of $^{14}CO_2$ into latex triterpenes of *H. australis*. 100 μCi $^{14}CO_2$ was administered during 3 hours to an apical stem part having 4 full grown leaves. After 3 days latex was tapped from stem and leafstalks. Latex expelled from leaf sided petiole part is regarded as leaf latex, the remaining parts yielded stem latex.

	stem latex dpm × 10^3	leaf latex dpm × 10^3
total latex	187.2	115
petroleum ether extract	147.5	52
triterpene esters	123	43.5
free triterpenols	18	7.3
triterpene esters:		
cycloartenyl acetate	24.4	8.8
α- and β-amyrin acetate	40	12.2
triterpenyl cinnamates	58.4	31.1
triterpenols of the		
triterpene esters:	89.6	37.5
α- and β-amyrin	53.2 (59%)	24.8 (66%)
cycloartenol	20.4 (22%)	5.3 (14%)
24-methylene cycloartenol	16.1 (18%)	7.1 (19%)
cinnamic acid	21.1	10.1
free triterpenols:		
α- and β-amyrin	3.3 (18.1%)	1.3 (18%)
cycloartenol	4.1 (23%)	1.6 (22%)
24-methylene cycloartenol	10.5 (59%)	4.4 (60%)
ratio triterpene esters/free triterpenols	6.8	6.0
ratio triterpenol of the esters/free triterpenols	4.9	5.2

general these apical stem parts of well growing plants cultivated on a gravel culture yielded most radioactivity in the latex constituents after a few days compared with potted *Hoya* plants. From the results of a typical CO_2-incorporation experiment presented in *table 11* may be concluded that there is a rather uniform incorporation pattern in the latex triterpene moiety throughout the whole plant: all triterpenols and triterpene esters are labelled in quantities leading to an approximately equal activity distribution as found in the sucrose incorporated stem parts.

4. GENERAL DISCUSSION

As reported for several plant species excised stem parts of *Hoya* species can synthesize sterols and triterpenes from exogenously supplied substrates. But despite the positive results in latex triterpene biosynthesis from acetate and mevalonate (PONSINET & OURISSON 1967, GROENEVELD 1976b) in isolated latices of *Euphorbia* species and in the non-latex rubber biosynthesis in *Parthenium argentatum* cuttings (ARREGUIN & BONNER 1950, ARREGUIN et al. 1951) from acetate, no latex triterpenes could be synthesized from these supplied precursors in *Hoya* species. In addition exogenous leucine is not incorporated

into the triterpene moiety of terpenoids present in the latex particles of *Hoya australis*. Latex particle biosynthesis appears directly related to carbohydrate metabolism of the plant as sugars are the only efficient substrates in the biosynthesis of latex particle constituents. Although mevalonate is incorporated into the mono- and sesquiterpenes of *Mentha piperita* oil glands, CO_2 and glucose are the most efficient substrates in biosynthesis of lower terpenoids (LOOMIS & CROTEAU 1973). Regarding the negative results in triterpenyl cinnamate biosynthesis mevalonic acid and acetic acid apparently do not reach the site where latex triterpenes are synthesized. As only intact stem parts of sufficient size are active in latex biosynthesis the necessity of a largely intact laticifer for latex biosynthesis is suggested as was already supposed for protein biosynthesis in laticifers by MEISSNER (1966).

On the other hand, the required "degree of intactness" may be overestimated since mevalonate and acetate are incorporated to a high degree by chopped stem tissue (up to 40% in mevalonate incorporation) into terpenoids. Compared with sucrose both compounds are not easily translocated through intact stem parts and most radioactive triterpenes and sterols are found close to the site of substrate application. With increasing distance from the site of isotope application the recovered labelled triterpene moiety decreases strongly. Sucrose is easily translocated through plant tissues and the found minimal size of the internode may be required for sufficient sucrose uptake to detect triterpene biosynthesis, as sucrose is involved in many metabolic pathways in plant metabolism. *Hoya carnosa*, in which no triterpene particles are detectable in the laticifers does hardly incorporate sugars into triterpenes or sterols. Sterol synthesis from sucrose in *Hoya australis* latex is present, but negligible compared with triterpene biosynthesis. Therefore latex triterpene synthesis is not directly comparable to triterpenol/sterol biosynthesis in non-latex cells of this species. In very young tissue of *Hoya australis* triterpenes are synthesized in the laticifers and phytosterol synthesis occurs in adjacent cells as was also observed in *Euphorbia* (PONSINET & OURISSON 1968). On the other hand laticifer surrounding cells as those of meristems are supposed to determine laticifer growth (SCHAFFSTEIN 1932, ARTSCHWAGER 1946, VREEDE 1949). Carbohydrate metabolism and particularly carbohydrate translocation directly influences the triterpene synthesis in *Hoya* latex. In general this indirect relation to photosynthesis was already observed by PROKOFIEF (1939, 1944) and BLOKHINTSEVA (1940) in *Taraxacum kok-saghyz* and more recently by READ (1972) in *Lactuca sativa*.

Very probably in sucrose incorporation experiments this compound is not primarily translocated by the phloem, but at least initially by the xylem. The relative high rate of sucrose incorporation into latex triterpenes seems mainly caused by an easy supply of this substance to the laticifer. The distribution of radioactivity in the triterpene moiety after $^{14}CO_2$ administration is identical to those obtained after sucrose-^{14}C absorption. Therefore sucrose-^{14}C incorporation by isolated stem parts into latex triterpenes may be regarded as a physiological approach in latex particle biosynthesis. Latex triterpenes may be

formed throughout the whole laticifer system but as far as the stem laticifers are concerned maximal activity is found in the third and fourth internode, where secundary growth starts.

Squalene seems a relatively important compound synthesized from mevalonate, in the stem tip of *Hoya australis* possibly as a result of partly anaerobic conditions already demonstrated in meristematic tissue in general by RUHLAND & RAMSHORN (1938). With respect to the oxygen poor monoterpenes it is suggested that they are the products of a partially anaerobic metabolism (SCHMIDT & VON GUTTENBERG 1953, BURMEISTER & VON GUTTENBERG 1960). As triterpene biosynthesis is not restricted to the apical parts of the laticifers near meristematic tissues where laticifer growth takes place, triterpene biosynthesis in *Hoya* laticifers needs not to be the result of (partial) anaerobic conditions as BEALING (1965) suggested for rubber biosynthesis in *Hevea*. Environmental factors, as light, temperature and water balance (hydratation) may influence quantitatively the biosynthesis of rubber in *Parthenium argentatum* (BONNER 1943), qualitatively the biosynthesis of triterpenes and sterols (BUSH & GRUNWALD 1973, KASPRZIJK 1971, DAVIS & FINKNER 1972), and monoterpenes (SCHMIDT & VON GUTTENBERG 1953). With respect to *Hoya australis* latex triterpene ester biosynthesis no qualitative effects could be demonstrated; quantitatively optimal latex particle biosynthesis was measured at 27°C.

POPOVICI (1926) already suggested the latex particle initiation in a parietal cytoplasm and its subsequent secretion into the vacuole of *Ficus carica* (POPOVICI 1926, c.f. TRÉCUL 1867). Which type of cell organelle is involved in latex particle biosynthesis is still not clear.

MARTY (1970) supposed that the peroxisomes play an important role in *Euphorbia characias* latex particle biosynthesis just as VASILEV (1970) has suggested for terpene biosynthesis An autophagic uptake of rubber particles into the large central vacuole, present in many non-articulated laticifers (BOBILIOFF 1925, HEINRICH 1970, SCHULZE et al. 1967) is supported with electronmicroscopical evidence of MARTY (1971). The normal plant cell is capable of sterol biosynthesis from acetate or mevalonate. These precursors do not enter the laticifers, but sucrose and several hexoses apparently easily do. The compartmentalized biosynthesis of latex triterpenes from sucrose found in the present work may be seen as a physiological support of such a mechanism of particle biosynthesis.

The triterpenols and their esters occurring in large amounts in the laticifers, however, are present in particles. The mutual ratios of the various compounds in such particles are genetically determined (WILDMAN et al. 1946). From a recent study of WARNAAR & KNOLLEMA (1976) it may be inferred that for example the particles of *Hoya australis* contain ten different acids esterified in a species specific manner with at least four different triterpenols. In addition latex particles contain species specific amounts of rubber hydrocarbon with a characteristic average chain length (VAN DIE 1955) and are coated with protein (GROENEVELD 1976a). The synthesis of such complexely built particles

probably can only take place in a structurally largely intact laticifer, of which consequently a minimum length would be required to ensure the degree of intactness needed.

ACKNOWLEDGEMENTS

We are much indebted to Prof. dr. J. van Die for valuable suggestions and critical reading of the manuscript and we thank our colleagues F. Warnaar for radio-gaschromatographical analysis, W. J. Baas for mass spectroscopical identification of latex triterpenes and H. Gruter for his skilful technical assistance.

REFERENCES

AEXEL, R., S. EVANS, M. KELLEY & H. J. NICHOLAS (1967): Observations on the biosynthesis and metabolism of sitosterol, β-amyrine and related methylsterols. *Phytochemistry* **6**: 511–524.

APLIN, R. T. & G. M. HORNBY (1966): Application of mass spectrometry to the structural investigation of 9, 19-cyclosterols and triterpenes. *J. Chem. Soc. (B):* 1078–1079.

ARCHER, B. L., B. G. AUDLEY, E. G. COCKBAIN & G. P. MCSWEENEY (1963): The biosynthesis of rubber. Incorporation of mevalonate and isopentenyl pyrophosphate into rubber by Hevea brasiliensis latex fractions. *Biochem. J.* **89**: 565–574.

ARREGUIN, B & J. BONNER (1950): The biochemistry of rubber formation in the guayule. Rubber formation in aseptic tissue cultures. *Arch. Biochem.* **26**: 178–186.

—, —, & B. J. WOOD (1951): Studies on the mechanism of rubber formation in the guayule. Experiments with isotopic carbon. *Arch. Biochem.* **31**: 234–247.

ARTSCHWAGER, E. (1946): Contribution to the morphology and anatomy of Cryptostegia grandiflora. *Techn. Bulletin* **915**: 1–40.

AUDIER, H. E., R. BEUGELMANS & B. C. DAS (1966): Mass spectrometry of tetracyclic triterpenes. *Tetrahedron Lett.* **36**: 4341–4347.

BAISTED, D. J. (1971): Sterol and triterpene synthesis in the developing and germinating pea seed. *Biochem. J.* **124**: 375–383.

BANDURSKI, R. S. & H. J. TEAS (1957): Rubber biosynthesis in latex of Hevea brasiliensis. *Plant Physiol.* **32**: 643–648.

BARNA, A. K. & S. P. DUTTA (1967): Thin layer chromatography of pentacyclic triterpenes. *J. Chromatogr.* **31**: 569–571.

BEALING, F. J. (1965): Role of rubber and other terpenoids in plant metabolism. *Proc. Nat. Rubber Res. Ass:* 113–122.

BENVENISTE, P. (1966): *La biosynthèse des stérols dans les tissus de tabac cultivés in vitro.* Thesis, Strassbourg.

BLASER, H. W. (1945): Anatomy of Cryptostegia grandiflora with special reference to the latex system. *Amer. J. Bot.* **32**: 135–141.

BLOKHINTSEVA, I. I. (1940): Formation of rubber in kok-saghyz. Vestnik Sel'sko-kohz. *Nauki, Tekhn. Kul'tury* **3**: 50–56.

BOBILIOFF, W. (1925): Waarnemingen aan melksapvaten in levenden toestand. *Arch. Rubber Cultuur Ned. Indië* **9**: 313–324.

BÖHM, H., B. OLESCH & CH. SCHULZE (1972): Weitere Untersuchungen über die Biosynthese von Alkaloiden in isoliertem Milchsaft des Schlafmohns Papaver somniferum. *Biochem. Physiol. Pflanzen* **163**: 126–136.

BOITEAU, P. (1964): *Les triterpenoides en physiologie végétale et animale.* Gauthiers-Villars ed. Paris.

BONNER, J. (1943): Effects of temperature on rubber accumulation by the guayule plant. *Bot. Gaz.* **105**: 233–243.

BURMEISTER, J. & H. VON GUTTENBERG (1960): Die ätherischen Öle als Produkt eines partiell anaeroben Stoffwechsels. *Planta Med.* **8**: 1–33.

BUSH, P. B. & C. GRUNWALD (1973): Effect of light on mevalonic acid incorporation into the phytosterols of Nicotiana tabacum L. seedlings. *Plant Physiol.* **51**: 110–114.

DAVIS, D. L. & V. C. FINKNER (1972): Influence of temperature on sterol biosynthesis in Triticum aestivum. *Plant Physiol.* **52**: 324–326.

DIE, J. VAN (1955): A comparative study of the particle fractions from Apocynaceae latices. *Ann. Bogor.* **2** (1): 1–124.

DJERASSI, C., H. BUDZIKIEWICZ & J. M. WILSON (1962): Mass spectrometry in structural and stereochemical problems of unsaturated pentacyclic triterpenoids. *Tetrahedron Lett.* **7**: 263–270.

FAIRBAIRN, J. W., M. DJOTÉ & A. PATERSON (1968): The alkaloids of Papaver somniferum VII. Biosynthetic activity of the isolated latex. *Phytochemistry* **7**: 2111–2116.

GOAD, L. J., & T. W. GOODWIN (1966): The biosynthesis of sterols in higher plants. *Biochem. J.* **99**: 735–746.

GOODWIN, T. W. (1971): Biosynthesis of carotenoids and plant triterpenes. *Biochem. J.* **123**: 293–329.

GROENEVELD, H. W. (1976a): Some morphological and chemical characteristics of the purified terpenoid particles of Hoya australis R. Br. ex Traill. *Acta Bot. Neerl.* **25**: 1–13.

— (1976b): A comparative investigation of the particle fractions from Hoya, Dischidia and Euphorbia latices. *Acta Bot. Neerl.* **25** (2): 167–177.

GRUNWALD, C. (1970): Sterol distribution in ultracellular organelles isolated from tobacco leaves. *Plant Physiol.* **45**: 663–666.

HEINRICH, G. (1970): Electronenmikroskopische Untersuchung der Milchröhren von Ficus elastica. *Protoplasma* **60**: 317–323.

KASPRZIJK, Z. (1969): Incorporation of 1-^{14}C acetate into triterpenoids of Calendula officinalis. *Phytochemistry* **8**: 1921–26.

— (1971): Rate of incorporation of $^{14}CO_2$, 1-C^{14}-acetate and 2-C^{14}-DL mevalonate into fatty acids and triterpenoids in the shoots of Calendula officinalis at different light intensities. *Phytochemistry* **10**: 797–805.

KERN, W. & W. HASELBECK (1942): Über die Inhaltsstoffe der Asclepiadacea Hoya carnosa I. Mitteilung. *Arch. Pharm. Ber. Dtsch. Pharmaz. Ges.* **280**: 340–348.

LISS, I (1961): Vorkommen und Bildung von 3,4-Dihydroxyphenyl-alanine in den Geweben und im Latex von Euphorbia lathyris. *Flora* **151**: 351–367.

LOOMIS, W. D. & R. CROTEAU (1973): Biochemistry and physiology of lower terpenoids in: *Terpenoids: structure, biogenesis and distribution.* V.C. RUNECKLES & T. J. MABRY eds. Acad. Press New York and London: 147–185.

MAHLBERG, P. G. (1959): Karyokinesis in the non-articulated laticifers of Nerium oleander L. *Phytomorphology* **9** (2): 110–118.

MARTY, F. (1968): Infrastructure des laticifères differenciés d'Euphorbia characias. *C.R. Acad. Sci. Paris* **267** série D: 299–302.

— (1970): Les peroxysomes (microbodies) des laticifères d'Euphorbia characias L. *J. Microsc.* **9**: 923–948.

— (1971): Vesicules autophagiques des laticifères différenciés d'Euphorbia characias L. *C. R. Acad. Sci. Paris* **272**, série D: 399–402.

MEISSNER, L. (1966): Über den RNS- und Proteingehalt isolierter Milchsäfte und den Einbau radioaktiv markierter Aminosäuren in die Latex Proteinen. *Flora, Abt. A* **156**: 634–654.

MORITZ, O. (1967): *Einführung in die Pharmazeutische Biologie.* Gustav Fischer Verlag-Stuttgart 4. Aufl.: p. 205.

MÜLLER, P. & H. R. SCHÜTTE (1971): Studien zu Bildung und Stoffwechsel phenolischer Latex-Aminosäuren. *Biochem. Physiol. Pflanzen* **162**: 234–244.

PONSINET, G. & G. OURISSON (1967): Biosynthèse in vitro des triterpènes dans le latex d'Euphorbia. *Phytochemistry* **6**: 1235–43.

— & — (1968): Aspects particuliers de la biosynthèse des triterpènes dans le latex d'Euphorbia. *Phytochemistry* **7**: 757–764.

POPOVICI, H. (1926): Contribution à l'étude cytologique des laticifères. *C. R. Acad. Sci. Paris* **183**: 143–145.

PROKOFIEV, A. A. (1939): Rubber formation in plants. *Bull. Acad. Sci. URSS. Biol.* **6**: 908–923.

— (1944): On the synthesis of rubber by plants. Photosynthesis and rubber formation in plants. *C. R. (Doklady) Acad. Sci. URSS* **45**(7): 307–309.

RAO, A. R. & M. MALAVIYA (1966): The non-articulated laticifers and latex of Tabernaemontana coronaria Willd. *Proc. Nat. Inst. Sci. India* **32**B: 233–242.

— & — (1966): A study of the non-articulated laticifers and the nature of latex in two members of Asclepiadaceae. *Proc. Indian Acad. Sci.* **64**: 45–52.

READ, M. (1972): *Growth and tipburn of lettuce: carbon dioxide enrichment at different light intensity and humidity levels, and rate of incorporation of carbon-14 assimilates into the latex.* Thesis. Wisconsin.

REES, H. H., E. I. MERCER & T. W. GOODWIN (1966): The stereospecific biosynthesis of plant sterols and α- and β-amyrine *Biochem. J.* **99**: 726–734.

RUINEN, J. (1950): Microscopy of the lutoids in Hevea latex. *Ann. Bogor.* **1** (1): 27–48.

RUHLAND, W. & K. RAMSHORN (1938): Anaeroben Gärung in aktiven pflanzlichen Meristemen. *Planta* **28**: 471–514.

SCHAFFSTEIN, G. (1932): Untersuchungen an ungegliederten Milchröhren. *Beih. Bot. Zentralbl.* **49**: 197–220.

SCHENK, W., H. R. SCHÜTTE & R. MOTHES (1962): Über den Stoffwechsel von Pipecolinsäure, 4-Hydroxypipecolinsäure, 4-Aminopipecolinsäure und 4-Oxo-Pipecolinsäure, in Strophanthus scandens. *Flora* **152**: 590–609.

SCHMALHAUSEN, J. (1877): Beiträge zur Kenntnis der Milchsaftbehälter der Pflanzen. *Mém. Acad. Imp. Sci. St. Petersbourg*, Sér. 7, **24**: 1–27.

SCHMIDT, B. & H. VON GUTTENBERG (1953): Untersuchungen über den Gehalt an ätherischen Olen in Abhängigkeit von der Trockenheit. *Pharmazie* **8**: 845–850.

SCHULZE, CH., E. SCHNEPF, & K. MOTHES (1967): Über die Lokalisation der Kautschukpartikel in verschiedenen Typen von Milchröhren. *Flora Abt. A* **158**: 458–460.

SOUTHORN, W. A. (1964): A complex subcellular compound of wide spread occurrence in plant latices. *J. Exp. Bot.* **15**: 616–621.

STEWART, W. S. (1948): Yield, composition and other latex characteristics of Cryptostegia grandiflora. *J. Agric. Res.* **76**: 105–127.

TOLBERT, N. E., A. OESER, R. K. YAMAZAKI, R. H. HAGEMAN & T. KISAKI (1969): A survey of plants for leaf peroxisomes. *Plant Physiol.* **44**. 135–147.

TRÉCUL, M. A. (1867): Des vaisseaux propres et du tannin dans les musacées. *Ann. Sci. Nat.* 5ième série **8**: 283–300.

TREIBER, K. (1891): Über den anatomischen Bau des Stammes der Asclepiadaceae. *Bot. Zentralbl.* **48**: 273–281.

TREUB, M. (1879): Sur la pluralité des noyaux dans certain cellules végétales. *C. R. Acad. Sci. Paris* **89**: 494–496.

VASILEV, A. E. (1970): O lokalisatii syntesa terpenoidov rastitelnoi kletke. *Rastitelni Resursi* **6**: 29–45. (in Russian).

VREEDE, M. C. (1949): Topography of the laticiferous system in the genus Ficus. *Ann. Bot. Gardens, Buitenzorg.* **51** (2): 125–149.

WARNAAR, F. (1976): Cinnamoyl derivatives produced as saponification artifacts during gas chromatographic analysis of esterified cinnamic acid from Hoya latices. *Anal. Biochem.* **71**: 533–539.

— & G. KNOLLEMA (1976): Aromatic and fatty acid components of triterpene esters from Hoya latices. In preparation.

WILDMAN, S. G., F. A. ABEGG, J. A. HELDER & S. B. HENDRICKS (1946): Observations on the inheritance of latex quality in Cryptostegia. *Arch. Biochem.* **10**: 141–155.

WOODWORTH, R. H. (1932): Diaxylary laticiferous cells of Beaumontia grandiflora. *J. Arnold Arboretum* **13**: 35–36.