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# BIOSYNTHESIS OF LATEX TRITERPENES IN EUPHORBIA; EVIDENCE FOR A DUAL SYNTHESIS

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## SUMMARY

α-Amyrin, esterified germanicol and β-amyrin have been identified as major constituents of *E. pulcherrima* latex. In freshly tapped latex only a part of the latex triterpenes were synthesized from acetate and mevalonate. By feeding $^{14}C$ sugars or acetic acid to intact plants and subsequent analysis of tapped latex is synthesis of all the latex triterpenes could be demonstrated. Three other *Euphorbia* species were studied for comparison and the results obtained are discussed. A dual mechanism of latex triterpene synthesis and a tentative model of latex particle formation is proposed.

## 1. INTRODUCTION

The scattered literature about the ultrastructure of latex cells and laticifers shows the unique composition of these specialized cells. This complex composition results from a drastic change of the cell content immediately after the initiation of these cells. A progressively degenerating cytoplasm in young laticifers of *Hevea brasiliensis* was described by ARCHER et al. (1963) and DICKENSON (1965). In *Achras sapota* the first latex particles appear simultaneously with the lysis of the transverse cell walls which is preceded by a breakdown of cell organelles (SASSEN 1965).

Non-articulated laticifers start their hyphen-like growth throughout the plant immediately after their initiation in the seedlings. Large structural changes take place in the growing tips of these laticifers. MARTY (1970) reported a lysis of cytoplasm in the apices of the non-articulated laticifers of *E. characias* resulting in a large central vacuole. This vacuole, containing the latex particles at maturity, is surrounded by a thin layer of cytoplasm, as is in *Ficus elastica* (HEINRICH 1970), *Euphorbia lathyrus* and *E. marginata* (MORITZ & FROHNE 1967, SCHULZE et al. 1967). Regarding the localisation of latex particles and the type of laticifers, Schulze et al. reported a nearly exclusive vacuolar localisation in non-articulated laticifers *(E. marginata)* and an exclusive presence in the cytoplasm of articulated laticifers, which frequently have a polydisperse vacuolar system (e.g. in *Taraxacum officinale*). In very young non-articulated laticifers of *E. marginata*, however, latex particles are also present in the cytoplasm (SCHULZE et al. 1967) and in *E. characias* particles have been observed both in the vacuole and in the cytoplasm (MARTY 1970, MARTY quoted by

MATILE 1975). *In E. pulcherrima* laticifers more recently their exclusive presence in the central vacuole has been stated (SCHNEPF 1964). The EM pictures of this author justify the suggestions that in mature laticifers of this plant species only the vacuolar phase is expelled at tapping, as was half a century ago already demonstrated by BOBILIOFF (1925) in a number of *Euphorbia* species. As according to the literature data *Euphorbia* latex would be largely a vacuolar fluid, tapped latex of *E. pulcherrima* nevertheless showed a remarkable *in vitro* triterpene synthesis (GROENEVELD 1976).

The present paper gives the results of a detailed study of triterpene and triterpene particle biosynthesis in *E. pulcherrima*. Some other *Euphorbia* species (*E. palustris* L., *E. characias* L. and *E. lathyrus* L.) are included in this study for reasons of comparison.

## 2. PLANTS AND METHODS

### 2.1 Plants

Poinsettia plants (*Euphorbia pulcherrima* Willd. ex Klotsch) were grown in the greenhouse and cultivated on water culture or soil. Specimens of *E. palustris* L., *E. characias* L. and *E. lathyrus* L. were grown in a container with soil placed outdoors.

### 2.2 Sampling

Most of the work reported here was carried out using samples of latex from the leafstalks of expanding, nearly fullgrown leaves of *E. pulcherrima*. After cutting the petiole with a razor blade the rapid expelling drops of latex were collected in a small glass vial. The sample was gently shaken to obtain a homogeneous mixture and after removal of an unavoidable small coagulum this latex was used in the incorporation experiments.

Latex samples from *E. palustris*, *E. characias* and *E. lathyrus* were taken from the young parts of well-growing stems. This latex was collected in 20 or 50 $\mu$l capillary pipettes as described earlier (GROENEVELD 1976). Incorporation experiments with radioactive precursors, extraction of triterpenoids from latex and plant tissue, gel filtration of latex particles, thin layer chromatography and gas liquid chromatography were carried out as described before (GROENEVELD 1976, GROENEVELD & KONING 1976).

Samples of 50–300 $\mu$l latex were centrifuged upon discontinuous sucrose gradients of 0.65 ml tubes, inserted into an adaptor and centrifuged in a spinco SW 50 L rotor. After the run the sucrose gradient was frozen and the particle fractions excised and processed as described before (GROENEVELD & KONING 1976).

### 2.3 Isolation of major triterpenes

Triterpene esters were separated on preparative thin layer plates (silicagel G) and scrapings were extracted with light petroleum/acetone (1/1). Water was added to obtain two layers and the light petroleum fraction was evaporated.

The remaining esters were saponified and subsequently acetylated, as were the triterpenols. The prepared triterpenyl acetates were separated on $AgNO_3$ impregnated thin layer plates and major constituents of each fraction extracted as described. These acetates were recristallized three times from diethylether-methanol solutions. Melting points were determined in open capillaries. The major compound of the triterpenyl acetates revealed a m.p. of 173–175°C, after acetylation 270–274°C (litt. germanicol 176–177°C, germanicyl acetate 275–276°C); triterpenyl poinsettinates 193–196°C, after acetylation 239–243°C (litt. β-amyrin 195–196, β-amyrin acetate 240–242°C); free triterpenols 185–188°C, after acetylation 226°C (litt. α-amyrin 186, α-amyrin acetate 228°C).

## 3. RESULTS

### 3.1 Composition of latex triterpenes

A brief qualitative composition of latex triterpenes is given in *table 1*. In agreement with the reports of PONSINET & OURISSON (1968a) the majority of the triterpenes in *Euphorbia pulcherrima* latex is esterified and 79% of the free triterpenols was cochromatographed with α-amyrin (TLC, GLC). The esterified triterpenes (74% of total triterpene mass) separated into two groups on silica gel G: 43% of these esters appeared to be triterpenyl acetates, 57% was esterified with fatty acids.

According to WARNAAR (1976) over 60% of the esterified fatty acids appeared to be unsaturated C-10 fatty acids, hitherto unknown in plant latices. In concert with WARNAAR in this work all triterpenes esterified with fatty acids are mentioned as triterpenyl poincettiates. The unsaponifiable moieties of both acetates and poincettiates revealed nearly identical gaschromatograms after saponification (and acetylation). Five identical retention times were found for the various constituents (in gas liquid chromatography on 3% SE-30) and only slight deviations in their mutual composition. About 82% of the triterpene moiety of the acetates and about 84% of the triterpene moiety of the poincettiates revealed the retention time measured for β-amyrin. A mass fragmentation pattern closely related to that of β-amyrin (ABRAMSON et al. 1968) was found in the poincettiates. Mass fragments of germanicol were dominant

Table 1. Amount and composition of triterpenes in latex of four *Euphorbia* species. Figures were obtained by gas liquid chromatography of acetylated saponified triterpenes on 3% SE-30 at 260°C; 5α-cholestane was used as internal standard.

| | plant part | concentration | free triterpenols | esterified triterpenols |
|---|---|---|---|---|
| E. pulcherrima | petiole | 30 mg/ml | 26% | 74% |
| E. palustris | stem | 31 mg/ml | 68% | 32% |
| E. characias | stem | 80 mg/ml | 99% | 1% |
| E. lathyrus | stem | 47 mg/ml | 73% | 27% |

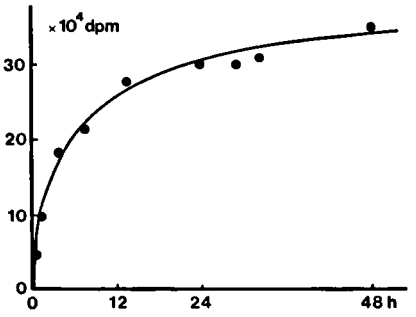



Fig. 1. *In vitro* incorporation of 3 μCi 2-$^{14}$C acetate into triterpenes versus time. 400 μl latex of *E. pulcherrima*.

in the major component of the triterpenyl acetates. (Germanicol has also been reported as a constituent of the wood of this plant species by GIBBS et al. 1967). Melting points support these findings. Therefore the majority of the triterpenes in *E. pulcherrima* latex is pentacyclic.

The minor triterpenes of both esters revealed different mass fragmentation patterns and have not been identified; neither were the triterpenes of *E. palustris E. characias* and *E. lathyrus*. It could be shown, however, that in contrast to *E. pulcherrima* the triterpene esters are a minority in these species. The free triterpenols are main constituents in these latices *(table 1)*.

### 3.2 In vitro experiments

#### 3.2.1 Incorporation of 2–$^{14}$C acetic acid into triterpenes of the latex of *E. pulcherrima* versus time

2μCi2–$^{14}$C acetate (40 μl) was administered to 400 μl latex at room temperature. At different times aliquots of 40 μl latex were extracted with petroleum ether/acetone. The soluble compounds were separated by chromatography on alumina columns and the obtained mixtures of triterpenols and triterpene esters were saponified and acetylated. The radioactivity in the obtained triterpenyl acetates was measured. The results presented in *fig. 1* show that most triterpene synthesis occurs within 12 hours of incubation. After 20 hrs. about 3% of the administered $^{14}$C is converted into triterpenes.

The accumulation of radioactive squalene as reported by PONSINET & OURISSON (1967) in *E. helioscopia* latex was not observed. As maximal synthesis was found in latex tapped from the leafstalks of expanding immature leaves *(table 2)*, most experiments have been carried out with such latex. The remarkable difference in the esterified $^{14}$C-triterpene-to-$^{14}$C-triterpenol ratio also shown in table 3 could be characteristic for the site of tapping, but also depends on the incubation time *(table 3)*.

When the radioactive triterpene esters were submitted to thin layer chromatography most radioactivity was found in the triterpenyl poincettiates. After saponification and subsequent acetylation the prepared triterpenyl acetates were separated on $AgNO_3$ impregnated thin layer plates. As the majority of the prepared triterpenyl acetates has an Rf between 0.4 and 0.7 and no separate single spots could be scraped from the thin layer plates, scannings are pre-

Table 2. Incorporation of 2-$^{14}$C acetate into free triterpenols and the triterpene moiety of triterpene esters by latex obtained from several plant parts of *E. pulcherrima*. 150 $\mu$l latex, 20 $\mu$l 2-$^{14}$C acetate solution (1 $\mu$Ci), 20 h of incorporation at room temperature.

| | | dpm | triterpene ester-to-triterpenol ratio |
|---|---|---|---|
| young leaves | triterpene ester | 89,800 | |
| | free triterpenol | 21,600 | 4.2 |
| mature leaves | triterpene ester | 136,000 | |
| | free triterpenol | 19,990 | 7.0 |
| petiole of expanding leaves | triterpene ester | 146,400 | |
| | free triterpenol | 21,400 | 6.8 |
| stem | triterpene ester | 50,800 | |
| | free triterpenol | 12,000 | 4.2 |

Table 3. Incorporation of 2-$^{14}$C acetate into free triterpenols and the triterpene moiety of the triterpene esters versus time. 200 $\mu$l latex of *E. pulcherrima*, 1 $\mu$Ci 2-$^{14}$C acetate (20 $\mu$l solution in water)

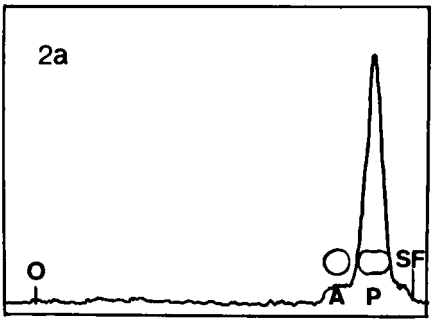
| incorporation time | | radio-activity in dpm | triterpene ester-to-triterpenol ratio |
|---|---|---|---|
| 3 hours | triterpenols | 13,650 | 7.2 |
| | triterpene esters | 98,200 | |
| 8 hours | triterpenols | 14,100 | 8.6 |
| | triterpene esters | 121,600 | |
| 24 hours | triterpenols | 16,700 | 9.8 |
| | triterpene esters | 163,250 | |

sented in *fig. 2*. The triterpenols of the esters showed a similar $^{14}$C distribution pattern as was found for the free triterpenols represented in *fig. 2c*. The $^{14}$C distribution corresponded with the distribution of mass, as was measured in radiogaschromatography.

The mass of the acetylated free triterpenols did not correspond to the $^{14}$C distribution: the major compound $\alpha$-amyrin did not cochromatograph with the main peak of activity *(fig. 2c)*. Apparently not all free triterpenols were synthesized in equal proportions.

### 3.2.2 Incorporation at different conditions

Several investigators showed the yield of acetate incorporation into rubber *(Hevea)* or triterpenes *(Euphorbia)* to be sensitive to physical modifications. With *E. pulcherrima* experiments were carried out on the influence of the degree of dilution on *in vitro* triterpene synthesis. Immediately after 2-$^{14}$C acetate



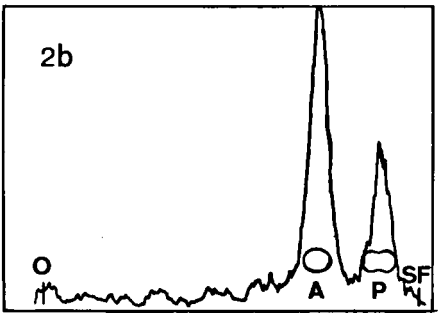
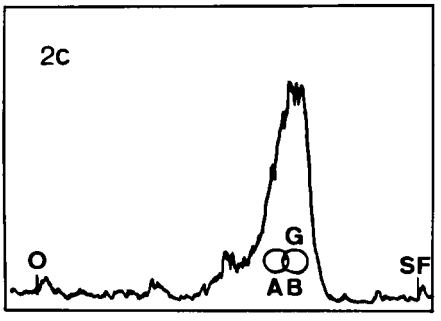




Fig. 2a and 2b. Radiochromatograms of triterpene esters of *E. pulcherrima* latex labelled *in vitro* with 2-$^{14}$C acetate (2a) and labelled *in vivo* with u-$^{14}$C sucrose (2b). Letters indicate positions of zones corresponding to compounds: A, triterpenyl acetates, P, triterpenyl poincettiates. The origin is at 0 and the solvent front at SF. Silica gel G thin layer chromatography, cyclohexane-ethyl acetate 9–1 (v/v).



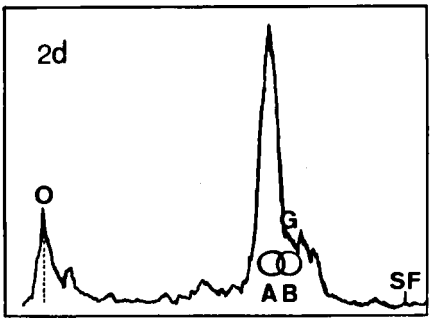




Fig. 2c and 2d. Radiochromatograms of acetylated triterpenols of *E. pulcherrima* latex labelled *in vitro* with 2-$^{14}$C acetate (2c), and labelled *in vivo* with u-$^{14}$C sucrose (2d). Letters indicate positions of zones corresponding to standards: A, α-amyrin acetate; B, β-amyrin acetate, and G, germanicyl acetate. The origin is at 0 and the solvent front at SF. $AgNO_3$ impregnated silica gel G thin layer chromatography (15%, w/w) developed twice in benzene/petroleum ether 40–60, 3/2.

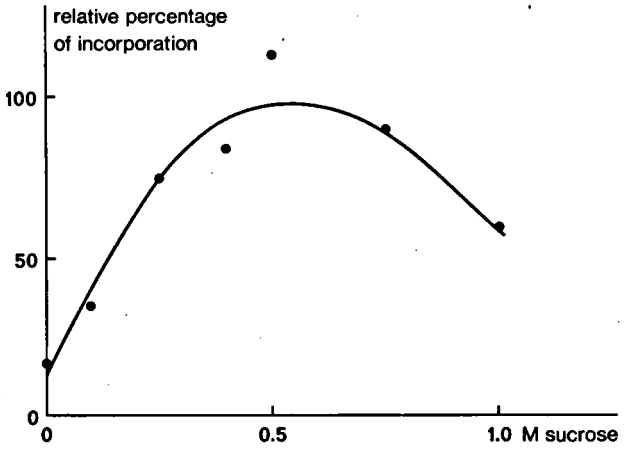



Fig. 3. Effect of dilution and sucrose concentration on triterpene synthesis in latex of *E. pulcherrima*. 24 h of incubation, 50 µl latex, 450 µl sucrose solution. Rate of synthesis of undiluted latex is 100%.

administration; aliquots of latex were diluted 10 times with several sucrose concentrations. Results presented in *fig. 3* show that a final concentration of 0.5 M sucrose is optimal in triterpene biosynthesis. At this concentration the rate of triterpene synthesis approximated that of undiluted latex. Dilution with water or salt solutions gave a drastic decrease of the $^{14}C$ triterpene yield.

3.2.3 $^{14}C$ incorporation into different particle fractions

When latex of *E. pulcherrima* was centrifuged at 35,000 rpm during 90 minutes three distinct phases were obtained: a top layer of triterpene particles, a clear serum phase and a bottom fraction in which starch grains were also present. Almost all triterpene particles (99.3–99.7% of triterpene mass) migrated to the top. The top layer could be partly resuspended in a few milliliters of 0.6 M KCl; about 2/3 of it was coagulated (sticky mass). The bottom fraction contained 0.7–0.3% of the total triterpene mass present in the latex sample.

Globular and complex triterpene particles (described in detail by GROENEVELD 1976) appeared to be present in both obtained particle fractions. When latex was centrifuged upon a discontinuous sucrose gradient (1.3–1.8–2.0–2.5 M sucrose), the bottom fraction was separated into several subfractions. About 0.01% of the triterpenes migrated to the bottom (density >2.5 M sucrose) while at the serum/1.3 M sucrose interface and at the 1.3/1.8 M sucrose interface the remaining 0.3–0.7% of the triterpenes was found as triterpene particles. All obtained particle fractions were identical in triterpene composition (GLC).

Latex incubated during 22 hrs. with 2–$^{14}C$ acetic acid or 2–$^{14}C$ mevalonic acid and centrifuged as described above yielded a $^{14}C$ triterpene distribution pattern as presented in *table 4*.

Table 4. Yield of $^{14}C$ triterpenes of different particle fractions obtained by isopycnic centrifugation after *in vitro* 2-$^{14}C$ acetate incorporation (1 $\mu$Ci) by 250 $\mu$l latex, versus time. After 22 hours the latex was centrifuged on a discontinuous sucrose gradient at 35,000 rpm (Spinco, SW-50L) during 90 minutes. The centripetally moved particles were suspended in 0.6 M KCl solution yielding a coagulated sticky mass ($I^A$) and a stable particle suspension ($I^B$). A small pellet was present at the bottom (III), the remaining particle fractions between serum phase and 2.0 M sucrose layer (II). Total triterpene mass was measured by gas liquid chromatography of the unsaponifiable part of the petroleum ether extract of each particle fraction.

| incorporation time | particle fraction | radioactivity in dpm | triterpene mass in $\mu$g | dpm/$\mu$g |
|---|---|---|---|---|
| | $I^A$ | 4,000 | 11,150 | 0.4 |
| | $I^B$ | 14,500 | 4,160 | 3.4 |
| 3 hours | II | 4,700 | 109 | 41 |
| | III | 16,500 | 2 | 8168 |
| | $I^A$ | 12,920 | 10,310 | 1.2 |
| | $I^B$ | 24,800 | 4,750 | 5.2 |
| 8 hours | II | 5,400 | 102 | 53 |
| | III | 27,800 | 2 | 12600 |
| | $I^A$ | 30,000 | 10,000 | 3.0 |
| | $I^B$ | 19,900 | 6,000 | 3.3 |
| 24 hours | II | 10,200 | 140 | 74 |
| | III | 37,700 | 2 | 18800 |

Most $^{14}C$ incorporated into the triterpenes was found in the top layer, but about 20% occurred in the bottom fraction resulting in a very high specific activity of the latter. In the serum phase labelled triterpenes were never observed. Thin layer chromatography showed a uniform $^{14}C$-distribution over the triterpenes of all obtained fractions. In addition all obtained particle fractions demonstrated a steady increase of the $^{14}C$ content of the triterpenes with increasing incubation time *(table 4)*. In agreement with the experiments of PONSINET & OURISSON (1968b) no indication about migrations of $^{14}C$ triterpenes from one fraction to another was observed. In general all fractions had their own $^{14}C$-esterified triterpene/triterpenol ratio.

3.2.4 Incorporation experiments with isolated fractions

Particle fractions isolated prior to $^{14}C$-acetate administration were characterized by a drastic reduction in ability to incorporate $^{14}C$ into triterpenes compared with untreated latex. The results presented in *table 5* demonstrate that two of the latex particle fractions obtained by isopycnic centrifugation can synthesize triterpenes. The distribution of $^{14}C$ triterpenes over the two prepared fractions, however, was not identical to those obtained by fractionation after

Table 5. Incorporation of 2-$^{14}$C acetate into triterpenes by isolated particle fractions after 24 h at room temperature. The top and bottom fractions were obtained after the centrifugation of 400 $\mu$l latex.

| | | radio-activity | yield |
|---|---|---|---|
| top fraction | + 3.5 $\mu$Ci | 1,200 dpm | 0.015% |
| bottom fraction | + 2.5 $\mu$Ci | 20,400 dpm | 0.4% |
| 400 $\mu$l latex | + 1.0 $\mu$Ci | 152,500 dpm | 6.9% |

$^{14}$C incorporation. In addition an improved separation of the bottom fraction showed that the heaviest fraction which gave the highest specific activity in previous experiments had now lost all incorporation ability.

The very low incorporation rate of the isolated latex fractions did not allow incorporation experiments with more purified fractions. They show both top and bottom fractions to be able to synthesize triterpenes. Moreover, the obtained $^{14}$C triterpene fractions all had the same $^{14}$C distribution on thin layer chromatograms as untreated latex after $^{14}$C acetate incorporation.

### 3.2.5 Incorporation experiments with different precursors

Several types of labelled precursors were employed to investigate triterpene biosynthesis *(table 6)*. Mevalonic acid gave the highest recovery in $^{14}$C triterpenes; of acetic acid and pyruvic acid smaller amounts were incorporated, while even malonic acid appeared to be a potential triterpene precursor. The incorporation of glucose label was negligible just as remarkably that of isopentenylpyrophosphate. The label of all used precursors was incorporated into $^{14}$C triterpenols and mainly into $^{14}$C-triterpenyl poincettiates. Compared with the triterpenyl poincettiates the triterpenyl acetates were not or slightly labelled (0–9% of total $^{14}$C triterpenes).

Table 6. Yield of $^{14}$C-triterpenes after *in vitro* incorporation of several isotopes (350 $\mu$l latex, 50 $\mu$l isotope solution, 24 h incorporation at room temperature).

| | | yield | $\mu$mol incorp. |
|---|---|---|---|
| u-$^{14}$C sucrose | 2.9 mCi/mmol | not detectable | |
| u-$^{14}$C glucose | 3 mCi/mmol | 0.001% | |
| 2-$^{14}$C Na-pyruvate | 10 mCi/mmol | 0.85% | $31.8 \times 10^{-4}$ |
| 1-$^{14}$C malonic acid | 14.5 mCi/mmol | 0.16% | $7.6 \times 10^{-4}$ |
| 2-$^{14}$C acetic acid | 56 mCi/mmol | 4.25% | $18.9 \times 10^{-4}$ |
| 2-$^{14}$C mevalonic acid | 10 mCi/mmol | 5.14% | $85.3 \times 10^{-4}$ |
| 1-$^{14}$C isopentenyl-pyrophosphate | 61 mCi/mmol | 0.08% | $0.34 \times 10^{-4}$ |

### 3.3 *In vivo* incorporation experiments

#### 3.3.1 Incorporation in Euphorbia pulcherrima

Apart from the described *in vitro* incorporation experiments, latex triterpene biosynthesis was also studied by means of *in vivo* experiments. As even long isolated stem parts did not expell latex after 24 h at repeated incision, small potted plants were employed. Aliquots of 50–150 $\mu$l isotope solution were injected with 20 or 50 $\mu$l capillaries into the hollow stem which is normally present from the third internode onward counting from apex to base. Plants were incubated in daylight at room temperature. After 24–48 h all the leaves were checked on radioactivity with a GM counter and latex was tapped as described before from the most radioactive leaf onward. Afterwards, stem, leafstalks and midribs, and laminae were extracted with acetone and petroleum ether, and processed as the latex.

Results of a typical experiment are presented in *table 7:* 0.5% of the supplied u-$^{14}$C sucrose activity was incorporated into all the triterpenes either present as free triterpenols or esterified. About 35% of these $^{14}$C triterpenes was expelled with the latex at tapping. Of the remaining part, the stem had most $^{14}$C triterpenes. The distribution of the $^{14}$C over the triterpene esters and triterpenols differed from that found after *in vitro* incorporation *(fig. 2a)*. Most $^{14}$C of the esters was found in the triterpenyl acetates *(fig. 2b)*. The triterpenols of the acetates and poincettiates, separated as acetates on $AgNO_3$ impregnated thin layer sheets, showed identical $^{14}$C distributions. A labelling of the free triterpenols proportional to their mass was established too, as most activity chromatographed with the major component $\alpha$-amyrin *(fig. 2d)*. Apart of the $^{14}$C distribution over the different triterpenes in these *in vivo* incorporation experiments, a specific distribution of $^{14}$C triterpenes over the different particle fractions (obtained by isopycnic centrifugation) was obtained. This is shown in a double label experiment presented in *table 8:* u-$^{14}$C sucrose was administered to an intact plant as described before and latex was tapped after 24 h. 200 $\mu$Ci $^{3}$H-acetate was added to this $^{14}$C labelled latex, and after 24 h subsequently centrifuged on a discontinuous sucrose gradient (35,000 rpm,

Table 7. $^{14}$C distribution over the triterpenols and the triterpene moieties of the triterpene esters of the latex and the several plant parts of *E. pulcherrima* from which the latex was tapped, after *in vivo* incorporation of 25 $\mu$Ci u-$^{14}$C sucrose during 24 h.

| | LATEX dpm $\times 10^3$ | STEM dpm $\times 10^3$ | PETIOLE dpm $\times 10^3$ | LEAF dpm $\times 10^3$ |
|---|---|---|---|---|
| triterpene esters | 74.6 | 80.9 | 42.72 | 12.9 |
| triterpenyl acetates | 52.2 | 49.35 | 27.97 | 9.3 |
| triterpenyl poincettiates | 22.3 | 31.55 | 14.74 | 3.7 |
| triterpenols | 61.1 | 63.99 | 34.18 | 8.9 |
| fatty acids | 8.5 | 14.75 | 3.75 | 1.7 |
| free triterpenols | 16.99 | 19.2 | 7.7 | 5.05 |
| sterols | – | 4.95 | .75 | .45 |

Table 8. 300 μl $^{14}$C-labelled latex (obtained after *in vivo* feeding of u-$^{14}$C sucrose) was incubated with 200 μCi $^{3}$H-acetate during 24 h. This latex was centrifuged during 90 minutes on a discontinuous sucrose gradient and the particle fractions of the top (I), at the serum/1,3M sucrose interface (II), at the 1.3 M sucrose/1.5 M sucrose interface (III) and of the bottom pellet (below 2.5 M sucrose) (IV) were excised after freezing and processed.

| | ESTERIFIED TRITERPENES | | TRITERPENOLS | |
|---|---|---|---|---|
| | dpm $^{14}$C | dpm $^{3}$H | dpm $^{14}$C | dpm $^{3}$H |
| I | 81,550 (99%) | 2,424,300 (79%) | 15,500 (100%) | 195,300 (63%) |
| II | 700 (1%) | 282,850 (9%) | – | 39,850 (13%) |
| III | – | 90,450 (3%) | – | 27,350 (9%) |
| IV | – | 272,250 (9%) | – | 47,700 (15%) |

90 min.). The obtained particle fractions were excised and radioactivity in the triterpenes of these particle fractions measured. Nearly all $^{14}$C triterpenes were found in the top layer, while only 77.5% of the $^{3}$H triterpenes were present in this particle fraction. The remaining $^{3}$H-triterpenes was distributed over the other particle fractions, comparable to the distribution presented in *table 4*.

In contrast to the laticiferous system of several *Hoya* species in which sugars were the only effective precursors in *in vivo* latex triterpene synthesis, *Euphorbia pulcherrima* also transferred $^{14}$C-acetate into triterpenes *in vivo*. In experiments with this precursor nearly all $^{14}$C triterpenes were also found in the centripetally moving particle fraction. The distribution of $^{14}$C over the triterpene esters and triterpenols resembled that obtained with sucrose as precursor *(table 9)*.

Despite a relatively low yield sugars remain the most specific precursors for latex triterpene synthesis in isolated stem parts *(table 10)*. In *in vivo* experiments with *E. pulcherrima*, acetate and mevalonate are apparently mainly incorporated into non-latex triterpenes and phytosterols as is shown in an experiment with isolated stem parts. As in incorporation experiments with *Hoya* (GROENEVELD & KONING), large amounts of the administered acetate or mevalonate – $^{14}$C were incorporated into triterpenes and phytosterols not localized in latex of the stem.

Table 9. Distribution of $^{14}$C over triterpenols and triterpene moiety of triterpene esters in latex after 24 h *in vivo* incorporation of u-$^{14}$C sucrose (20 μCi, 2.9 mCi/mmol) and 2-$^{14}$C acetate (7 μCi, 56 mCi/mmol) by *E. pulcherrima* plants.

| | u-$^{14}$C sucrose dpm | 2-$^{14}$C acetate dpm |
|---|---|---|
| triterpenols | 74,000 | 20,000 |
| triterpene esters | 134,250 | 69,000 |
| triterpenyl acetates | 97,990 (71%) | 30,650 (44%) |
| triterpenyl poincettiates | 39,800 (29%) | 38,430 (56%) |

Table 10. Incorporation of 2-$^{14}$C acetate (5 $\mu$Ci), 2-$^{14}$C mevalonate (2.5 $\mu$Ci) and u-$^{14}$C sucrose (12,5 $\mu$Ci) into triterpene and sterol ester fractions, free triterpenols and sterols by isolated apical stem parts of *E. pulcherrima* during 24 h at room temperature.

| | acetate | mevalonate | sucrose |
|---|---|---|---|
| ester fraction | 590,000 dpm | 1,615,700 dpm | 439,000 dpm |
| triterpenols | 105,600 dpm | 550,690 dpm | 159,350 dpm |
| sterols | 126,790 dpm | 442,500 dpm | 23,490 dpm |

3.3.2 Incorporation experiments with *E. palustris*, *E. characias*, *E. lathyrus*
2–$^{14}$C acetate was supplied to latex tapped from young parts of well growing plants. The results *(table 11a)* showed that most radioactivity occurred in the triterpene esters of these latices.

Within these latices, however, most triterpenes occur as free triterpenols *(table 1)*. Thin layer chromatography of the acetylated triterpenols of the free and esterified triterpenes showed that not all components were labelled and that the distribution of $^{14}$C was not proportional to the distribution of the mass. Apparently also in these latices only some of the latex triterpenes are synthesized *in vitro*. *In vivo* incorporation experiments with u-$^{14}$C sucrose gave most $^{14}$C in the free triterpenols (table 11b). Only *E. lathyrus* could be used for these experiments. As *E. characias* and *E. palustris* do not have a hollow stem $^{14}$C sucrose administration in the usual way was impossible. Therefore the technique with stem segments was used (GROENEVELD & KONING 1976), but from these no latex could be obtained after 24 h of incubation. The whole stem part was extracted and processed as described before (GROENEVELD & KONING 1976). The results presented in *table 11b* show most $^{14}$C is found in the free triterpenols. The composition of these triterpenol fractions was found to be

Table 11a. *In vitro* incorporation of 2-$^{14}$C acetate into free triterpenols and the triterpene moiety of the triterpene esters by latices of several *Euphorbia* species. 100 $\mu$l latex, 20 $\mu$l (= 1 $\mu$Ci) 2-$^{14}$C acetate during 20 h at room temperature.

| | *triterpene esters* | *triterpenols* |
|---|---|---|
| *E. palustris* | 39,690 dpm | 11,000 dpm |
| *E. characias* | 110,000 dpm | 18,750 dpm |
| *E. lathyrus* | 409,000 dpm | 48,500 dpm |

Table 11b. *In vivo* incorporation of u-$^{14}$C sucrose into triterpenes (triterpenols and triterpene moiety of triterpene esters) by several *Euphorbia* species. 25 $\mu$Ci, u-$^{14}$C sucrose, (100 $\mu$l during 24 h at room temperature).

| | *triterpene esters* | *triterpenols* | *sterols* |
|---|---|---|---|
| *E. palustris* | 12,750 dpm | 635,000 dpm | 56,500 dpm |
| *E. characias* | 9,350 dpm | 640,000 dpm | 38,200 dpm |
| *E. lathyrus* (latex) | 28,300 dpm | 54,400 dpm | – |

identical to those of the latex triterpenols (GLC). The relatively low yield of $^{14}C$ sterols in these experiments (as was found in *E. pulcherrima (table 10))* indicates the $^{14}C$ triterpenols represent the latex triterpenols. As in *E. pulcherrima*, in these experiments all the triterpenols were labelled (thin layer chromatography).

## 4. DISCUSSION

Compared with *Hevea brasiliensis* latex the investigated latex of *E. pulcherrima* is characterized by several remarkable differences. The magnesium and potassium ions are equally dispersed throughout the latex (GROENEVELD, unpublished) and no Mg-rich organelles as the lutoids of *Hevea* (RIBAILLIER et al. 1971) were detected. The *in vitro* synthesized amounts of $^{14}C$ triterpenes are not proportional to the particle mass as was the case in rubber biosynthesis in Hevea (MCMULLEN & MCSWEENEY, 1966). This means that not all particles or the whole particle surface is involved in the biosynthesis of particle constituents. In view of the $^{14}C$-triterpene distribution after ultracentrifugation and the influence of sugar solutions on synthesis a special organelle might be involved in this biosynthesis. In contrast to the findings with *E. helioscopia* (PONSINET & OURISSON, 1968b) this *in vitro* synthesis takes place in all prepared latex particle fractions and is not restricted to a "bottom fraction". The present work, however, clearly shows that this *in vitro* biosynthesis is incomplete. In all investigated *Euphorbia* latices the $^{14}C$-triterpene ester/$^{14}C$-free triterpenol ratio is not proportional to the corresponding mass ratio.

In *E. pulcherrima* latex almost only the triterpenes of the poincettiates are labelled (predominantly $\beta$-amyrin). Germanicyl acetate and free $\alpha$-amyrin are scarcely synthesized *in vitro*. $^{14}C$-sugar supply to intact plants leads to a more proportional $^{14}C$ distribution over the various triterpenes and as far as this could be measured on $AgNO_3$-thin layer plates, $\alpha$-amyrin, germanicyl acetate and $\beta$-amyrin poincettiate were labelled in a more or less equal specific activity. As irrespective of the applied precursor only the triterpenyl poincettiates and some of the free triterpenols were synthesized *in vitro* the existence of two triterpene synthesizing systems is supposed.

The participation of two systems, viz. the expelled latex and the laticiferous system as a whole, is explained by a hypothetic scheme presented in *fig. 4*. In all investigated *Euphorbia's* the majority of all triterpenols and triterpene esters of the latex particle is supposed to be synthesized in a thin layer of cytoplasm. The presence of this cytoplasm was demonstrated in *E. lathyrus* (MORITZ & FROHNE 1976), in *E. characias* (MARTY 1970), and *E. marginata* (SCHULZE et al. 1967). Marty supplied EM evidence for a particle biosynthesis in this cytoplasm and a final secretion into the central vacuole of the laticifers of *E. characias* (MARTY 1968), as was shown in the laticifers of *Nelumbo nucifera* (ESAU & KOSAKAI 1975). The present work shows that in *E. pulcherrima* sucrose as well as some other compounds e.g. acetic acid, can reach this cy-

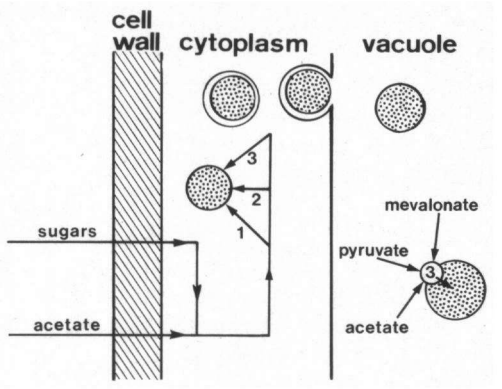



Fig. 4. Tentative model of triterpene particle biosynthesis in *E. pulcherrima* laticifers. Figures represent biosynthesis of free triterpenols (1), triterpenyl acetates (2) and triterpenyl poincettiates (3).

toplasm in intact plants and are involved in the triterpene biosynthesis of all latex triterpenes.

Within the vacuolar part of the laticiferous system another synthesizing system is present, still active after tapping. As triterpenyl acetates are formed scarcely from sugars by expelled tapped latex no parts of the cytoplasmic system are supposed to be expelled.

In several *Euphorbia* latices complex particles or particles with attached vesicles have been reported, e.g. in *E. marginata* (SCHULZE et al. 1967), *E. pulcherrima* (SCHNEPF 1964), and *E. milii* (GROENEVELD 1976). The influence of sugar concentration on the *in vitro* triterpene biosynthesis and the absence of incorporation rates proportional to particle mass (surface) might indicate that this vesicle is the site of *in vitro* triterpene biosynthesis. A new particle population is not supposed to be initiated by this biosynthetic system as all obtained particle fractions showed a uniform triterpene composition. A transfer of newly synthesized triterpenes from vesicle to particle is a more probable explanation.

This *in vitro* system may be active in the intact laticifer, if the required substrates are present in the vacuolar phase. The contribution of each of both systems to the total triterpene mass in the particles cannot easily be determined. The *in vitro* and *in vivo* incorporation experiments yielded $^{14}$C-triterpenyl poincettiates. The distribution of $^{14}$C triterpenes over the particle fractions obtained by isopycnic centrifugation in *in vivo* incorporation experiments *(table 8)* indicates that nearly all triterpenes of the latex particles are synthesized in the cytoplasm of the laticifer.

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