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A COMPARATIVE INVESTIGATION OF THE PARTICLE FRACTIONS FROM HOYA, DISCHIDIA AND EUPHORBIA LATICES

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SUMMARY

Particle size, morphology and lipid composition of the latex particle fractions of 18 *Hoya*, 3 *Dischidia* and 5 *Euphorbia* species were investigated. Of these latices only two showed in vitro triterpene biosynthesis.

Some aspects of particle biosynthesis are discussed.

1. INTRODUCTION

The particles of a number of rubber and terpenoid containing plant latices have diverse sizes ranging from less than 0.01 μm in *Taraxacum kok-saghyz* (HENDRICKS et al. 1944) to 50 μm and more in *Musa* (TRECUL 1867, LLOYD 1928). Early reports about particle diameters in several plant latices are only dealing with minimum and maximum sizes determined with a light microscope (JUMELLE 1903, HENRI 1908, FICKENDEY 1909, HAUSER 1927) and range in general from 0.5 to 3–4 μm. TRUMBULL (1942) and BLONDEAU & CURTIS (1946) reported the approximate size of *Cryptostegia grandiflora* latex particles being 0.2–1.5 μm resp. 0.6–1.4 μm in diameter. LUCAS (1938) determined a particle diameter distribution curve of *Hevea brasiliensis* by means of ultra-violet microscopy resulting in the broad range from 0.09–2.0 μm. With the aid of an electron microscope the existance of smaller rubber particles was demonstrated in *Hevea* (HENDRICKS et al. 1944, SCHMIDT & KELSEY 1951, VAN DEN TEMPEL 1952, SCHOON & VAN DER BIE 1955).

Indirectly particle diameter distribution curves were determined by centrifuging as was done e.g. on *Hevea* by HESSELS 1943, SCHMIDT & KELSEY 1951 and on *Funtumia elastica* by VAN DIE 1955. In addition many latices have been investigated chemically for their particle content (VAN DIE 1955, BOITEAU & RATSIMANGA 1964, PONSINET & OURISSON 1968a). Some latices appeared to be able to synthetize their particle compounds in vitro (BANDURSKY & TEAS 1957, KLEINSCHMIDT & MOTHES 1959, PONSINET & OURISSON 1967). In the present paper studies on the particle diameter frequency distribution, particle morphology and lipid composition and incorporation of acetate, mevalonate, and isopentenyl pyrophosphate into triterpenols by tapped latex of several plants are described.

2. MATERIALS AND METHODS

Species of *Hoya*, *Dischidia*, and *Euphorbia* were cultivated in the green house. After incision with a razor blade latex was collected with 20 or 50 µl capillaries. Aliquots of 20 µl latex were diluted with 0.5 ml of a phosphate buffer pH 8.1 of a suitable strength (*table 1*) and chromatographed on a Sephadex G-100 column (30 × 0.9 cm). Parts of the eluted particle suspension were fixed with OsO_4 and investigated electron optically as described before (GROENEVELD 1976).

Total terpenoid extracts of Sephadex filtrated particle suspensions were chromatographed on thin layer as described before (GROENEVELD 1976).

The dry weight of the particle fraction was determined after adding 0.3–0.5 ml 1.5 M sucrose containing 1% acetic acid to aliquots of 150–200 µl latex and subsequent freezing; the coagulated suspension was centrifuged at 60,000 g, the top layer squeezed, washed with water, and dried to a constant weight at 60 °C.

Incorporation experiments. 2.5 µCi Na-acetate 2-C^{14} in 50 µl (specific activity 55.8 m Ci/mmol) was added to 0.2 ml freshly tapped latex and incubated at 27 °C during 16 hours. Light petroleum extracts were made as described before (GROENEVELD 1976) and saponified with 5% KOH in ethanol/benzene (9/1, v/v) during 1½ hours at 90 °C. Unsaponifiable compounds were extracted with light petroleum (b.r. 40–60 °C) and chromatographed on columns of aluminium oxide (Merck, Aluminium Oxid standardisiert, Aktivitätsstufe II–III Brockmann) by a stepwise increase of peroxide free anhydrous diethyl ether in light petroleum (E/P) (modified after GOAD & GOODWIN 1966). The fraction eluted with 10% E/P contained squalene and farnesol (partly), 30% E/P the triterpenols (4,4 dimethylsterols) and farnesol (partly), 50% E/P the sterolfraction (4-desmehylsterols).

The triterpenol and sterol fractions were acetylated in a mixture of acetic anhydride/pyridine 1/2 (90 °C during 2 hours) extracted with light petroleum and chromatographed on aluminium oxide. The acetates were eluted with 10% E/P and chromatographed on thin layer plates (silicagel G impregnated with 15% $AgNO_3$, w/w) developed in benzene/petroleum ether, (2/3, v/v in order to separate the triterpene acetates from farneso acetate. Rf values: β-amyrine acetate 0.5, lupeol acetate 0.2, stigmasterol acetate 0.45, farnesol acetate 0.02, geraniol acetate 0.05. Radioactive triterpene- and sterol acetates were scraped off and counted in a liquid scintillation counter.

3. RESULTS

The buffer used in gel filtration of the latex of *Hoya australis* also appeared to be useful for the isolation of the particle fraction of a few other latices. Of some *Hoya* species, however, the latices flocculated during or after gel filtration, or during their fixation in OsO_4. This could be prevented by an increase of buffer strength.

As far as the *Hoya* species are concerned, the buffer concentrations given in

Table 1. Qualitative lipid composition and average diameter of latex particles in *Hoya*, *Dischidia* and *Euphorbia* latices. Buffer concentration near the minimal buffer strength necessary to prevent flocculation during gel filtration.

	Triterpenol	Triterpene acetates	Triterpene cinnamates	Triterpenes esterified with fatty acids	Average particle diameter (μm)	Standard deviation	Number of measured particles	Mol. buffer
H. bella Hook.	+	+	+	+	0.41	0.04	(554)	0.10
H. coronaria Blume	+	+	+	−	0.36	0.05	(279)	0.10
H. shepherdi Short	+	+	+	−	0.35	0.04	(444)	0.10
H. australis R.Br. ex Traill	+	+	+	tr.	0.34	0.05	(320)	0.12
H. ovalifolia Wight & Arn.	+	+	+	−	0.34	0.04	(332)	0.12
H. cinnamomifolia Hook.	+	+	+	−	0.33	0.05	(559)	0.12
H. spec.	+	−	+	−	0.32	0.06	(232)	0.12
H. bandaensis Schlechter	+	+	+	−	0.28	0.04	(326)	0.15
H. diversifolia Blume	tr.	+	+	−	0.28	0.04	(241)	0.15
H. macrophylla Blume	+	+	+	−	0.27	0.04	(567)	0.15
H. obovata Decne	−	+	+	−	0.26	0.04	(170)	0.20
H. lacunosa Blume	+	+	−	−	0.26	0.05	(235)	0.20
H. fraterna Blume	tr.	+	+	−	0.25	0.03	(276)	0.20
H. latifolia Don.	+	+	+	−	0.25	0.04	(191)	0.20
H. crassipes Turcz.	tr.	+	+	−	0.24	0.04	(227)	0.20
H. longuifolia Wallich	+	+	+	−	0.23	0.04	(359)	0.25
H. pseudolanceolata Cost.	+	tr.	+	+	0.22	0.04	(205)	0.22
H. imperialis Lindl.	+	+	−	+	0.20	0.03	(240)	0.25
H. multiflora Bl.	+	+	+	+	0.18	0.04	(235)	0.30
D. membranifolia (?)	+	+	−	−	0.35	0.06	(601)	0.10
D. spec.	+	+	−	−	0.47	0.05	(201)	0.10
D. bengalensis Colebr.	+	+	−	−	0.53	0.04	(345)	0.10
E. balsamifera Ait.	+	−	−	−	0.22	0.05	(429)	0.20
E. lactea Haw.	+	−	−	−	0.24	0.08	(502)	0.20
E. tirucalli L.	+	−	−	−	0.24	0.07	(493)	0.20
E. milii Desm.	+	+	−	+	0.31	0.09	(321)	0.12
E. pulcherrima Willd. ex Klotsch	+	+	−	+	0.28	0.06	(418)	0.2 M KCl + 0.01% Tween

table 1 may be regarded as near the minimum values needed to prevent flocculation. In these solutions particle suspensions remained stable during at least 6 hours, and also during fixation. Similar results were obtained with *Euphorbia* latices. Latex from *E. pulcherrima* flocculated immediately after dilution with phosphate buffer regardless its ionic strength and pH, but good gel filtration results could be obtained by using a 0.2 M KCl solution-containing 0.01% Tween – 80. This solution appeared to be useful in all other investigated latices too.

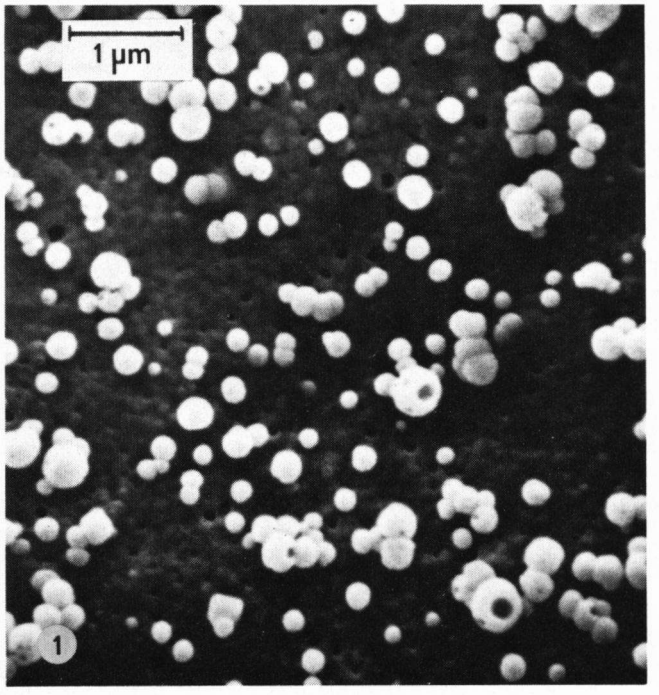
Latex was obtained from stem, leaves, leafstalks, young and older parts of specimen of *Hoya australis*. The triterpene particles of these samples were examined on diameter frequency distribution by scanning electron microscopy (SEM), as well as on general appearance in ultra thin sections. The particle populations of these various latex samples appeared to be very uniform throughout the plant (*table 2*). Like *Hoya* species, the investigated species of *Dischidia* and *Euphorbia* also have non-articulated laticifers. Here too no differences in particle morphology could be observed in latex samples of various plant parts.

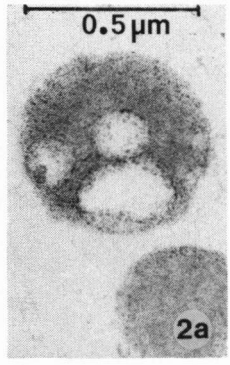
All investigated *Hoya*, *Dischidia*, and *Euphorbia* species showed globular particles with a smooth surface in scanning electron microscopy. Of *Euphorbia milii* and *E. pulcherrima* some particles, especially the larger ones, were provided with a "hole" (*fig. 1*). This structure was observed after gel filtration in either phosphate buffer or in a 0.2 M KCl solution containing 0.01% Tween – 80. In thin sections particles with an identical structure did occur (*fig. 2a, b*). Here, the particle mass locally has a concave surface to which a membrane-like vesicle is attached.

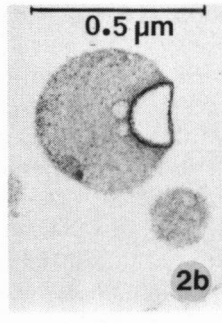
In sectioned particles of *Hoya australis* sometimes a similar structure can be observed (*fig. 3a, 3b*), which differs from that found in the *Euphorbia* species. The concave part of the particle surface is apparently not covered with a membrane-like envelope, but this is extruded from the deformed particle, as was shown earlier (GROENEVELD 1976). Similar phenomena could be regularly observed in flocculated particle suspensions of *H. australis* (*fig. 4*), obtained by decreasing the buffer concentration from 0.1 M to 0.02 M phosphate. The induced flocculation had led to coalescence, accompanied by a deformation

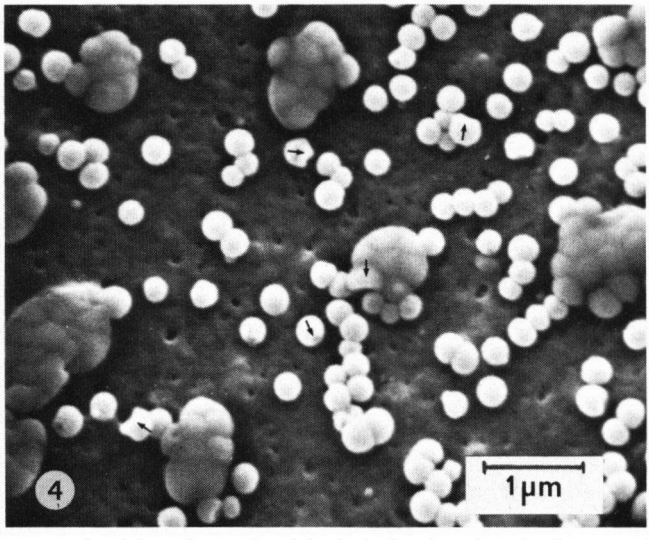
Table 2. Average particle diameter of several latex samples of *Hoya australis*.

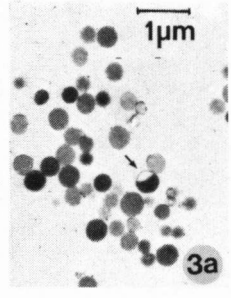
Site of tapping	Average diameter
All parts of the plant	0.33 μm
All parts of the plant	0.34 μm
All parts of the plant	0.33 μm
All parts of the plant	0.34 μm
Top of the stem	0.35 μm
Young leaf	0.33 μm
Young stem	0.34 μm
Old leaf	0.35 μm
Old stem	0.34 μm
Average diameter	0.34 μm











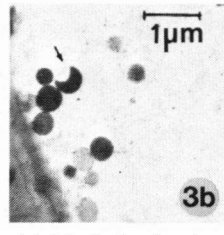


Fig. 1. Particles of *E. milii* with "holes". Phosphate buffer pH 8.0; 0.1 M, OsO_4 fixation. SEM.

Fig. 2a, b. Ultra thin sections of paticles of *E. milii* with membranous vesicles. Phosphate buffer pH 8.0; 0.1 M, OsO_4 fixation.

Fig. 3a, b. Ultra thin sections of particles of *H. australis*. Two deformed particles between globular ones. Phosphate buffer pH 8.0; 0.1 M, OsO_4 fixation.

Fig. 4. Partly coalesced particle fraction of *H. australis*, de formed particles indicated by arrows. Phosphate buffer pH 8.0; 0.1 M, OsO_4 fixation. SEM.

of the globular particle shape. Such particle deformations, however, were not observed in the investigated latices of which stable suspensions were put on the millipore filter.

The results of the particle diameter frequency distribution measurements of a number of latices are given in histograms in *fig. 5*; average diameter, standard deviation, number of particles measured, and the buffer concentrations em-- ployed in gel filtration are listed in *table 1*.

Some qualitative data on the chemical composition of the various latices obtained by thin layer chromatography are also shown in *table 1*. They are in agreement with more detailed data on *Hoya* latex obtained by WARNAAR & KNOLLEMA (1976). Sterols were present as traces only, the main components being triterpene acetates in *Dischidia* and triterpene cinnamates in *Hoya* in addition to free triterpenols. Some quantitative data on several particle populations are presented in *table 3*.

Freshly tapped latex of all investigated *Hoya*'s, *Dischidia membranifolia*, and *Euphorbia balsamifera*, *E. lactea* and *E. tirucalli* were found to be unable to synthesize triterpenols from acetate in vitro. Even after a 16 hours' incubation period no incorporation of acetate into triterpenes or triterpene esters could be detected. Also attempts with labelled mevalonate and isopentenyl pyrophosphate were negative. Latices of *E. milii* and *E. pulcherrima*, however, showed a distinct incorporation of acetate in the triterpenol fraction as summarized in *table 4*.

4. DISCUSSION

The particle populations of the investigated *Hoya* and *Dischidia* species are characterised by a narrow diameter distribution range, those of *Euphorbia*

Table 3. Dry particle content (dry weight of the particle fraction as a percentage of latex fresh weight), number of particles per ul latex and interficial area (total particle surface in 1 ul latex) of some *Euphorbia*, *Dischidia* and *Hoya* latices.

	dry particle content (%)	number of particles per µl latex (× 10^9)	interfacial area (cm^2)
Euphorbia balsamifera	7.7	11.9	19
E. tirucalli	18.0	12.1	23
E. lactea	18.0	18	36.9
Dischidia spec.	24.6	3.9	29.2
Hoya australis	7.6	3.4	11.9
H. cinnamomifolia	8.0	4.1	14.1
H. obovata	8.8	9.2	19.6
H. lacunosa	9.3	10	20.6
H. shepherdii	9.5	3.7	15.3
H. coronaria	12.7	4.9	20.5
H. multiflora	24.0	78	80.1
H. imperialis	28.0	68	83.4

Table 4. Incorporation of acetic acid 2-C^{14} (sodium salt) into the triterpenol and sterol fraction of *Euphorbia milii* and *E. pulcherrima* latex. 0.2 ml latex incubated with 50 µl isotope solution (= 2.5 µCi) during 16 hours at 27°C.

	Triterpenol	Sterol
E. milii	72,000 dpm	1,100 dpm
E. pulcherrima	122,000 dpm	700 dpm

latices have a somewhat wider one and resemble those of *Funtumia elastica* (VAN DIE 1955). For *Hoya* species a uniform particle population is probably always present throughout the whole plant. The possible existence of two distinct size classes e.g. as reported for whip latex in *Cryptostegia* (0.2 and 1.1 µm, BLONDEAU & CURTIS 1946) has been carefully investigated but no evidence for it could be found.

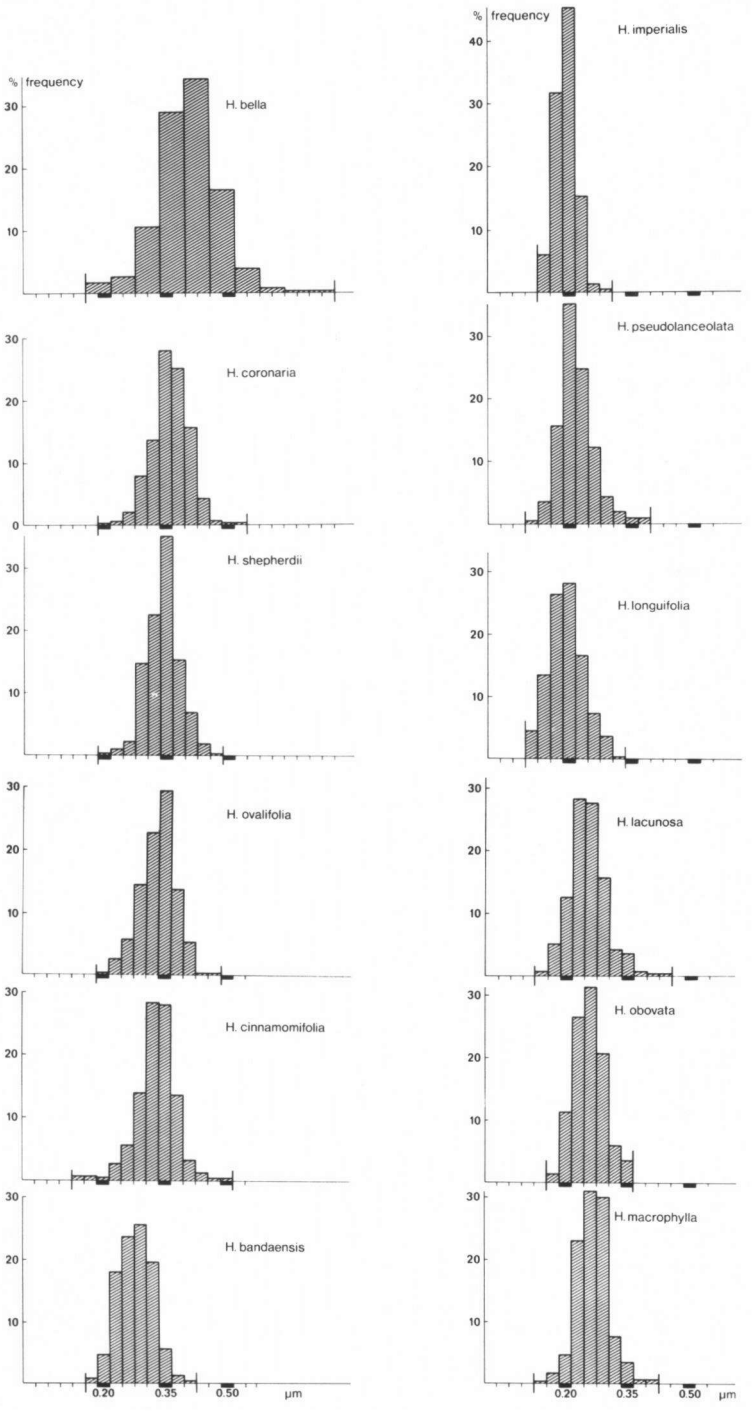
Within the genus *Hoya* an inverse correlation appeared to exist between the average particle diameter and the buffer concentration required for stabilizing the particle suspension. This supports the view that the different particle diameter distributions shown in *fig. 5* and *table 1* are characteristic features of the investigated *Hoya* latices, and not produced by different degrees of swelling caused by OsO_4 treatment.

In all investigated species a more or less sharply defined order of size of the smallest particles can be observed. Minimum particle size exceeds by at least a factor 4 the millipore filter grade (0.025 µm). In addition, the fluffy particle surface (inherent to the method used) gives the smallest particles a foggy image, which means that particles with a 1.5 mm diameter in a 17,000 enlargement (corresponding with 0.09 µm diameter) can be recognised as a real latex particle. Very small particles like the smallest ones (0.03 µm diameter) reported for *Hevea brasiliensis* latex (BATEMAN 1963) can not be detected with this method. The symmetric particle distributions found in the present work, however, do not suggest the existence of such very small particles in the investigated latices.

The largest particle diameter in each species could also be stated. As particle coalescence is visible in the scanning electron microscope (*fig. 4*), the suggestion of SCHOON & PHOA (1956), that the largest particles are built up from smaller ones seems improbable for *Hoya* latices.

The particle diameter frequency distributions of the latices of the investigated Asclepiadaceae are in most cases nearly symmetrical. In some species maximum frequency is found at a diameter smaller than the average one, while in other species the maximum diameter frequency exceeds the average one. All investigated species differ greatly from *Hevea brasiliensis*, which has an asymmetrical distribution curve, ranging from 0.03 µm to 1–2 µm with a maximum frequency at 0.1 µm.

No correlation could be found between particle size and particle composition in general. In most *Hoya* species triterpenyl cinnamates are present and the average particle diameter ranges from 0.18 µm to 0.41 µm. Within this range,


% frequency
H. bella
H. imperialis
H. coronaria
H. pseudolanceolata
H. shepherdii
H. longifolia
H. ovalifolia
H. lacunosa
H. cinnamomifolia
H. obovata
H. bandaensis
H. macrophylla
0.20
0.35
0.50
µm

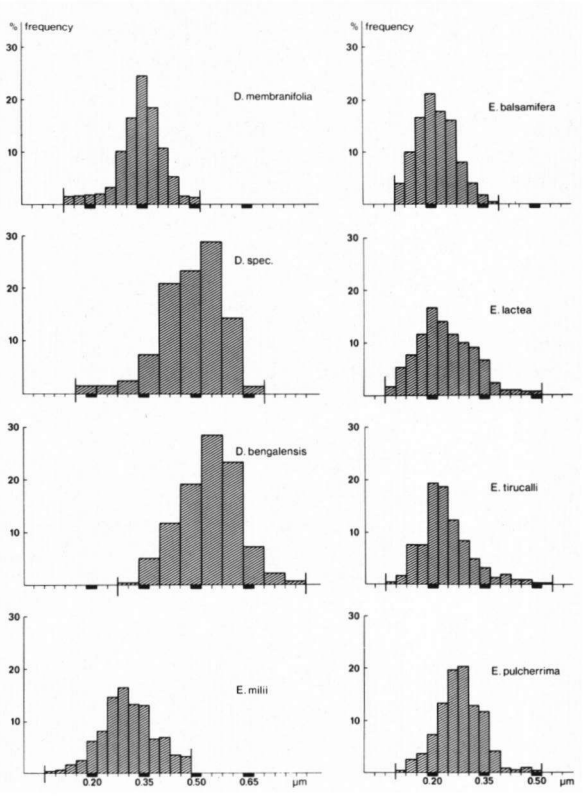


Fig. 5. Particle diameter frequency distribution in several latices; statisticle interval 0.03 or 0.06 µm. The minimum and maximum diameters are marked by vertical dashes.

however, the *Euphorbia* particles are also found, which contain either triterpenols only, or the more complex triterpene esters of *E. milii* and *E. pulcherrima*. Within the genus *Hoya*, *H. obovata* and *H. shepherdii* both only contain triterpene esters, but differ in average particle diameter. *H. bella* and *H. imperialis* both have esterified fatty acids in their particles but have nevertheless a complete different particle population.

A correlation could neither be found between Mg^{2+} or K^+ content of the whole latex and average particle size: *H. multiflora* and *H. bella* only have small amounts of these ions but they differ greatly in particle size. A correlation between particle size and particle concentration does not exist. Latices with relatively small particles may have a high particle concentration (*H. imperialis*, *H. multiflora*) compared with those having a relatively large average particle diameter (*H. australis*). This results in the broad range of interfacial area listed in *table 3*.

Only in the latices of two species an in vitro triterpene biosynthesis could be demonstrated. As predominantly tetracyclic triterpenes are found in *E. milii*

latex while *E. pulcherrima* contains mainly pentacyclic triterpenes (PONSINET & OURISSON 1968a) the ability of an in vitro biosynthesis is apparently not restricted to a special type of triterpene skeleton.

Remarkably, however, these latices are characterised by a relatively high amount of potassium, and a potassium/magnesium ratio > 1, in contrast to all other investigated latices (GROENEVELD 1975). In addition both latices possess particles of which the surface has concave sites. Similar structures can be observed in the pictures of *E. pulcherrima* latex given by SCHNEPF (1964).

In *Hevea brasiliensis* the final stages of the synthesis of the main particle compound occurs at the surface of all particles (MCMULLEN & MCSWEENY 1966). In the latex of *Euphorbia cyparissias*, however, species specific triterpenoids are synthezed in a hitherto unidentified "bottom fraction" obtained by centrifugation (PONSINET & OURISSON 1968b), Within 24 hours these newly synthetized compounds did not disperse into the supernatant in which the majority of the triterpenes were present. In tapped latex of *E. tirucalli*, *E. lactea* and in that of all mentioned *Hoya* species in vitro biosynthesis apparently does not occur. Very probably in these species the triterpene synthesizing system is not expelled with tapping. As far as the particle synthesis is concerned, the model described by POPOVICI (1926) still is an attractive one. In fixed, acid fuchsine stained sections of *Ficus carica* this author observed small rubber particles in a parietal latex vessel cytoplasm and larger ones in a central vacuole. She suggested a particle initiation in the cytoplasm and finally excretion into the vacuole, as was aready suggested by TRECUL (1867) to occur in *Musa* latex. Both the narrow particle diameter frequency distribution in particle populations of *Hoya* species and the inability of in vitro triterpene biosynthesis of many latices can be explained by this model.

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