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SOME MORPHOLOGICAL AND CHEMICAL CHARACTERISTICS OF THE PURIFIED TERPENOID PARTICLES OF THE LATEX OF HOYA AUSTRALIS R.BR. EX TRAILL.

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SUMMARY

A method is described for the isolation, purification and extraction of the triterpene particles of *Hoya australis* R. Br. ex Traill. (Asclepiadaceae) and *Euphorbia milii* Desm. (Euphorbiaceae).

Chemical data and morphological properties of the triterpene particles of *H. australis* are presented and discussed.

1. INTRODUCTION

Latices of many plant species can be regarded as colloid systems. Small particles in general ranging from 0.01–5 μm in diameter and containing rubber or other terpenoids are dispersed in an aqueous serum phase. Most investigations in this field have been done with latex of *Hevea brasiliensis* in which the dominant particulate phase is rubber hydrocarbon. These particles make up 30–45% of the latex volume and range in size from about 0,03 to 3 μm with a majority smaller than 0.1 μm (LUCAS 1938, VAN DEN TEMPEL 1952).

Moreover, so-called lutoids occur in *Hevea* latex (e.g. HOMANS & VAN GILS 1948, RUINEN 1950), which take up 10–20% of the latex volume. This organelle, which has a vacuolar or a lysosomal character (WIERSUM 1957, PUJARNISCLE 1968), has a widespread occurrence in latices throughout the plant kingdom (SOUTHORN 1964). Several other types of particles and organelles are present in *Hevea* latex but only in small amounts.

Separation of the various structures mentioned may be accomplished by centrifugation of freshly tapped whole latex (e.g. MOIR 1959), or especially for lutoid purification by isopycnic centrifugation of diluted latex (PUJARNISCLE 1968). In both cases the rubber particles move centripetally to the top. Redispersion of this top layer and repeated centrifugation leads to a more or less pure rubber-particle fraction.

In most other latex plant species the particles contain triterpene alcohols and their esters as main constituents (VAN DIE 1955). In addition, the latices of plants like *Papaver somniferum* are characterized by a multitude of small vesicles, which probably contain the alkaloids (THURESON-KLEIN 1970). Whole latex of this plant species centrifuged at 100,000 g forms six zones resembling

those reported by MOIR (1959) for *Hevea* latex (FAIRBAIRN et al. 1968). The alkaloid containing vacuoles of *Chelidonium majus* could be separated from serum compounds by centrifugation in a suitable osmoticum (MATILE et al. 1970).

The present paper reports investigations of the latex particles of *Hoya australis* (Asclepiadaceae). It describes attempts to purify them without prior coagulation. The purified particles are morphologically studied with the (scanning) electron microscope. In addition the paper gives a number of physico-chemical characteristics of the purified particles. Morphological and chemical evidence for the existence of a particle envelope of a proteinaceous nature is presented.

2. MATERIALS AND METHODS

Hoya australis R.Br. ex Traill. and *Euphorbia milii* Desm. were cultivated in the greenhouse. *Hoya* latex was collected from plants growing on a gravel culture, with 50 μl capillaries after incision with a razor blade. *Euphorbia milii* was usually tapped at the stem, *Hoya australis* at the leaf stalks. Freshly tapped latex samples were diluted at least five times with elution buffer before applying to a sephadex G-100 column. After the gel filtration the sephadex was resuspended in elution buffer in order to check whether the particle recovery was complete, as indicated by a clear supernatant.

Liquid electrofocussing was performed according to the instructions from the manufacturer (LKB, Sweden) in a 110 ml column. Ampholine concentration was 1 %, final voltage 800 V, the time 20 hours.

The triterpenoid fraction was obtained by repeated extraction of the coagulum or particle precipitate with aceton followed by benzene. Both extracts were mixed, centrifuged and the supernatant evaporated and redissolved in light petroleum. Diluted aqueous particle suspensions were extracted with light petroleum after an equal volume of aceton was added. After vigorous shaking in a stoppered tube ethanol was added till the light petroleum phase, containing the triterpenoids, separated from the aqueous aceton-alcohol layer.

Triterpenoid extracts were chromatographed on 0.25 mm layers of Kiezelgel G (Merk) and developed in cyclohexaneethylacetate (9 : 1, v/v). The triterpene-esters were separated on thin layers impregnated with 15% $AgNO_3$ (w/w) and developed with benzene-hexane (2 : 3, v/v) (KEMP & MERCER (1968). β-Amyrine, β-amyrine-acetate, benzoate, cinnamate, and palmitate were used as references as was in gas liquid chromatography. Triterpene benzoates and cinnamates, which co-chromatographed on thin layer, were detected and identified by their u.v. absorption spectrum. Polar lipids were extracted and chromatographed on thin layer as described by LEPAGE (1964). A chloroplast extract was used as a reference sample.

Total terpenoid-lipid extracts were chromatographed on Varaport 3% SE-30, packed in a glass column, length 1.80 m, int. diam. 4 mm. The starting

temperature was 200 °C, rising 2 °/min. to a final temperature of 300 °C (KEMP & MERCER 1968).

Electron microscopy: Immediately after tapping an amount of latex was fixed in 6% glutaraldehyde in a 0.1 M phosphate buffer of pH 7.2 at 2 °C during 5 hours. The glutaraldehyde was subsequently eliminated by repeated washings with phosphate buffer. Post-fixation was carried out with 1% OsO_4 in buffer. Particle suspensions on the other hand were fixed by dilution with an equal volume of a 2% OsO_4 solution in buffer. If necessary, the suspension was centrifuged for a few minutes at 600 g and the pellet resuspended in a small volume of supernatant.

The fixed suspension was thoroughly mixed in an equal volume of 2% agar of 45 °C and afterwards immediately chilled (DICKENSON 1969). Parts of this particles containing agar were dehydratated with ethanol-propylene oxide and embedded in epon. Sections were contrasted with uranylacetate and lead citrate.

Scanning electron microscopy: A few microliters OsO_4 fixed particle suspension in a suitable dilution were put on a millipore filter (MF type VS) and after drying at room temperature a small strip of it was mounted with adhesive on a microscope support. Pictures were made with an angle of 5° and an enlargement of 5700 ×.

Diameters were measured from 17,100 × enlargements with an interval of 0.5 mm, equal to 30 nm. To what extent the triterpenoid particles increase in volume by OsO_4 hardening is unknown hitherto. For the rubber particles of *Hevea brasiliensis* hardened with bromine (Brown, quoted by SCHOON & VAN DER BIE 1955) a diameter increase of about 10% is measured.

3. EXPERIMENTS

Preliminary investigations showed that freshly tapped latex of *Hoya australis* (pH 4.5) contains microscopically visible aggregates of particles besides the individual ones. A dilution of this latex with water causes a rapid flocculation, that is an enlargement of the particle aggregates to macroscopically visible dimensions. In vivo the latex of *H. australis* contains at least 0.14 M Mg^{2+} (GROENEVELD 1975). If diluted with a Mg^{2+} containing solution $\geqslant$0.04 M a stable suspension is obtained independent of its particle concentration. In this suspension, however, the microscopically visible particle aggregates originally present in the latex remain unaltered.

With KCl solution $\geqslant$0.1 M stable suspensions are also obtained and in addition a gradual break-up of the apparently loosely bound particle aggregates takes place, which is enhanced by increasing the pH of the solution. Attempts were subsequently made to separate the latex particles from serum compounds by means of gel chromatography. Acid buffer solutions, containing either a sufficient amount of Mg^{2+}, or K^+ gave always rise to considerable particle losses, apparently by their staying behind in the gel column, and much tailing was obtained during the particle elution. In the eluted particle fractions clus-

ters of tens of particles were observed.

In an alkaline Mg^{2+} containing buffer these particle aggregations appeared still to be present, but substitution of Mg^{2+} by K^{+} led to a decrease of the aggregation grade. A complete recovery without appreciable tailing and without aggregates of any significance was obtained if a 0.1 M phosphate buffer of pH 8.1 containing 0.05 M KCl was used. These optimal conditions of gel chromatography of *Hoya australis* latex appeared to be useful for *Euphorbia milii* latex too. Typical elution patterns of both latices are shown in *fig. 1*. *Centrifugation:* After sephadex chromatography the particle fraction of *H. australis* obtained in a 0.10 M phosphate buffer was diluted with water (1 : 1) and centrifuged at 58,000 g. The precipitated particles could be resuspended in a small amount of water and thus be concentrated. The suspensions obtained were stable for about 24 hours and were used for density determinations, for isoelectric focussing, and for chemical analysis.

Isopycnic centrifugation of *H. australis* particles on discontinuous sucrose gradients in K^{+} or Mg^{2+} containing buffers resulted in a density varying from 1.008 to 1.045. Untreated freshly tapped latex appeared to have a similar particle fraction density range.

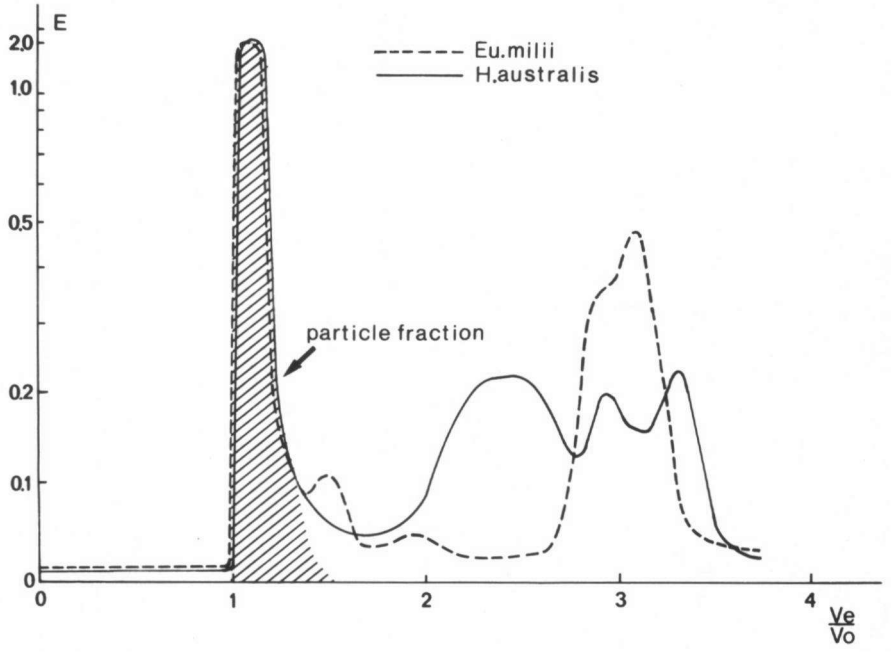

Fig. 1. Elution pattern of freshly tapped latex after filtration through a Sephadex G 100 column (90 × 0.9 cm). Absorbance 280 nm. 0.1 M phosphate buffer pH 8.1 containing 0.05 M KCl. 0.25 ml latex in both cases.

Isoelectric focussing: In contrast to the particles of freshly tapped latex, the purified stable particle suspension with low ionic strength could be used in isoelectric focussing experiments. An isoelectric point was found of 3.2, which was independent of the sucrose gradient applied.

Chemical analyses: A purified particle fraction was resuspended in water and centrifuged. The precipitate was freeze dried and nitrogen determinations were made by using standard micro Kjeldahl techniques. The N-content appeared to be 0.84% of the particle dry weight, which corresponds with approximately 5.2% of protein.

A part of the freeze dried particle fraction was hydrolyzed for 16 hours at 110°C in 6 N HCl. Gas chromatography as used by BORSTLAP (1972) showed the following amino acids to be present: alanine, valine, glycine, isoleucine, leucine, threonine, serine, phenylalanine, asparagine, proline, glutamine, lysine.

A freeze dried particle fraction dissolves for its greater part in ethanol and the residue dissolves completely in benzene. As the latter gave a precipitate after ethanol addition, it probably contains rubber hydrocarbon. Besides traces of sterol and sterol esters, the light petroleum extract contains triterpenols, triterpene acetates and triterpenecinnamates, which could be demonstrated by thin layer chromatography. In light petroleum extracts of particle suspensions and in particle fractions obtained by isopycnic centrifugation the same compounds are present. Gaschromatographically all particle fractions obtained are very uniform in triterpenoid composition, as shown in *table 1.* Phospholipids or glycolipids could not be detected in freeze dried particle preparations, not even in a freshly tapped latex sample as large as 0.75 ml.

Ion-exchange chromatography: A gel filtrated particle suspension could be retained by DEAE ion-exchanger at pH $\geqslant$ 4.

Despite the precarious recovery, which makes this technique less suitable

Table 1. The relative triterpenol/triterpene ester composition of latex and particle fractions of *Hoya australis*. The percentages are calculated from peak areas in the gas chromatograms.

Hoya australis latex	Triterpenol	Triterpene acetate	Triterpene cinnamate
Total latex	6.5%	25.5%	67.9%
Coagulum	7.4%	28.6%	64.2%
Isopycnic centrifugation:			
density 1.008–1.017	7.2%	26.1%	66.1%
1.017–1.026	5.5%	28.6%	65.9%
1.026–1.035	6.1%	24.3%	69.9%
1.035–1.045	7.8%	26.0%	66.1%
Sephadex washed. Particle fraction	6.0%	27.9%	67.0%

in particle separation, no indication is obtained for the presence of different types of particles according to their proteinaceous properties.

Electron microscopy of latex particles: Ultra-thin sections of isolated particles gave well-prepared electron microscopic pictures, as shown in *plates 1–4*. The terpenoid particles of *H. australis* appeared to be quite uniform spheres with a variable electron density. A membrane-like envelope could regularly be observed *(plate 2, 3)*. As the latex particles of these species, besides a small amount of rubber hydrocarbon, contain mainly triterpene alcohols and triterpene esters which are easily soluble in ethanol and acetone, it leaves no doubt that special care has to be taken in preparing the sections of isolated particles. Embedding a particles containing agar block in a mixture of plastic/acetone led to a picture as shown in *plate 1*. The oblate form of these particles is very probably caused by the centrifugal force applied during the preparation of the particle pellet.

Dehydration with an alcohol/propylene oxide sequence improved the quality of the pictures. They show a homogenous more or less electron-dense particle again, occasionally surrounded by a thin, probably proteinaceous membrane-like structure. The inner layer of this envelope is often more electron-dense than the outer one *(plate 2)*. Sometimes this envelope is more or less extruded. The particles prepared in this way also show differences in electron density.

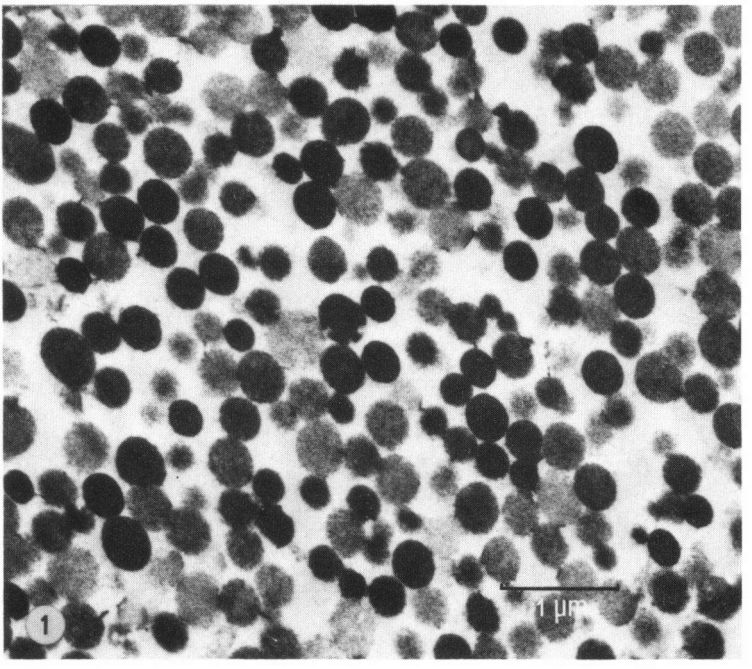

Plate 1. Gel filtrated particle fraction from *Hoya australis*. Dehydration with acetone Membrane-like filaments are attached to some particles. 18,800 ×.

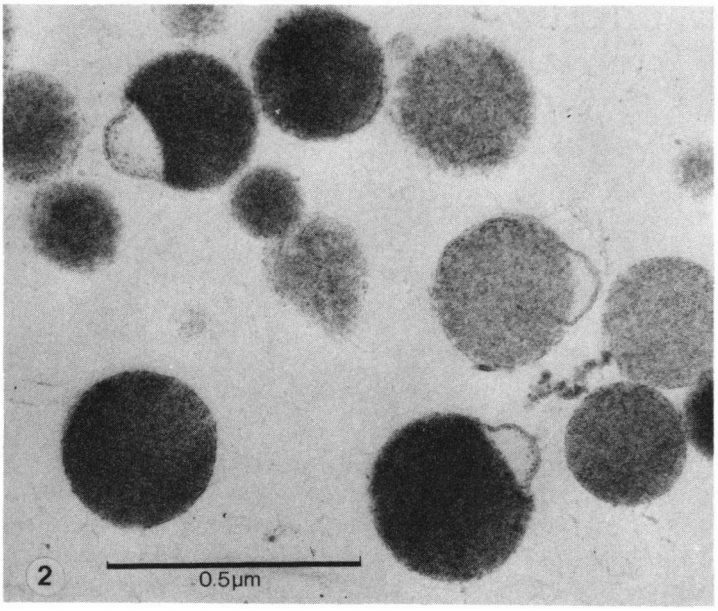

Plate 2. Gel filtrated particles from *H. australis* OsO_4 fixation. 0.1 M phospate buffer. pH 8.1 0.05 M KCl. 81,000 ×.

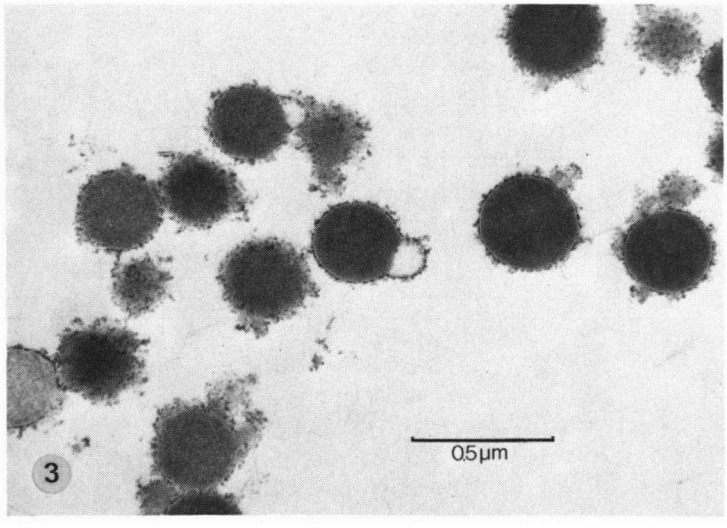

Plate 3. Particles from *H. australis*, OsO_4 fixed, immediately after tapping. Serum compounds are precipitated at the particle surface. 46,500 ×.

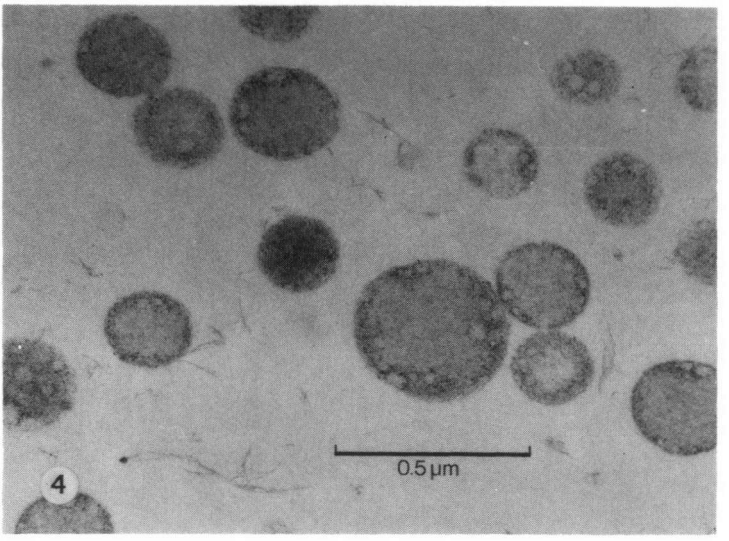

Plate 4. OsO_4 fixed particles of *Euphorbia milii* after gel filtration. 62,000 ×.

Latex particles of *Euphorbia milii* prepared in a similar manner have a different submicroscopic structure *(plate 4)*. Their homogeneous content is not surrounded by a thin envelope but the outer layer is more or less impregnated with more electron-dense material and shows vacuole-like structures, sometimes lined with electron-dense material.

Although the distribution of particle diameters can be calculated from ultra thin sections scanning electron microscopy was chosen for this purpose, which has the advantage that possible coalescence of particles can be detected. A gel filtrated particle suspension in phosphate buffer of pH 8.1 fixed in 1% OsO_4 solution and dried on a support gave unsatisfactory pictures. Large particle aggregations and crystals of buffer-salts did not permit a proper view into the particle population (as was the case with polystyrene particles). Addition of small amounts of ethanol gave no improvement. In order to obtain a good

Table 2. Characteristics of the particle fraction of *Hoya australis* R.Br. ex Traill.

isoelectric point	3.2
density	1.008–1.045
dry weight	62 mg/ml latex
amount of protein	5.2% of particle dry weight
average particle diameter	0,340 µm
smallest particle diameter	0,140 µm
largest particle diameter	0.42 µm
particle concentration	3.02×10^9/µl latex
interfacial area	11.6 cm^2/µl latex

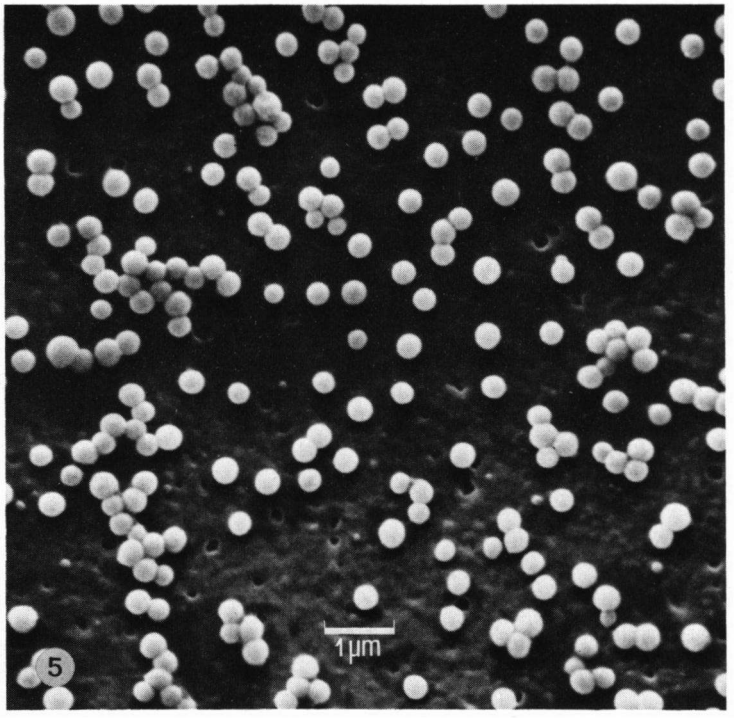

Plate 5. Sephadex washed particle fraction *H. australis* . 11,400 ×. 0.1 M phosphate buffer pH 8.1 containing 0.05 M KCl. OsO_4 fixation, scanning electron microscope.

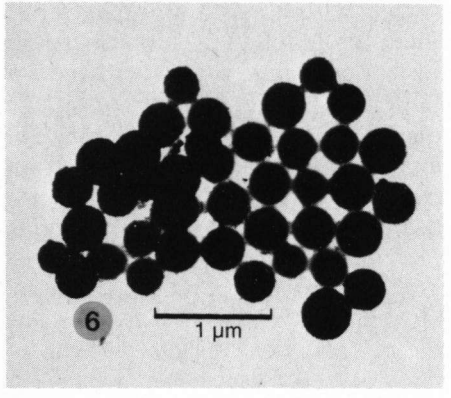

Plate 6. Sephadex washed particle fraction *H. australis*. 19,200 ×. Buffer: 0.05 M TRIS-HCl pH 7.8 containing 0.1 M $MgCl_4$. OsO_4 fixation, transmission electron-microscope.

particle distribution a drop of a fixed particle suspension was put on a millipore filter, which after drying was mounted on the microscope support. A homogenous and uniform particle fraction could be observed *(plate 5)*. Particles eluted in a TRIS-HCl buffer of pH 7.9, and containing $MgCl_2$, are clustered in micro aggregates of several µm as shown in *plate 6*.

The particle diameters of *H. australis* latex showed a slight asymmetric

distribution curve with a top at 0.34 μm and minima and maxima of 0.140 and 0.42 μm respectively. Examination of this fraction in a transmission view showed particles with diameters within the same range *(plate 6)*.

4. DISCUSSION

In a highly complex natural colloid system as a plant latex the stability of the particle suspension is influenced by a hydration layer and its electric charge, while serum proteins also interfere with the suspension stability (e.g. SOUTHORN & EDWIN 1968, SOUTHORN & ESAH YIP 1968).

As latex of *Hoya australis* may be diluted with a solution of 0.1 M KCl or 0.04 $MgCl_2$ without any visible flocculation in contrast to lower concentrations of these salts, the necessity of a minimal ionic strength for suspension stability was expected. On the other hand several reports exist on particle aggregating effects of magnesium salts in *Hevea* latex (HENRI 1908, FREUNDLICH & HAUSER 1925). The considerable degree of tailing during gel filtration of *Hoya* latex in a Mg^{2+} containing eluting system may also be explained by the formation of such aggregates. The quantity of nitrogen found in the present work, the presence of protein amino acids, and the visible particle envelope may be regarded as arguments in favour of the existence of a protein layer, which contains approximately 5.3% of the particle dry weight, and apparently has the low isoelectric point of 3.2. This IEP is somewhat lower than that of 4.04–4.52 found for *Hevea* particles (e.g. BOWLER 1953), but still falls within the range of 3.2–5.1 found for latex particles of several other Euphorbiaceae (MOYER 1935). Assuming a particle population with a uniform diameter of 225 nm, each particle can be covered with a protein film of 2 nm thickness. In that case 5% of the particle mass consists of protein. On the other hand 5% of the particle mass is also taken up by a protein layer of 4 nm thickness in a particle population with the uniform diameter of 450 nm. Considering the narrow range of particle diameter distribution in *H. australis* latex *(fig. 2)* all the particles may be assumed to be covered with a protein film of 3 nm thickness. This protein film may be represented by the bright particle-surrounding line (thickness 3.5–4 nm) visualized by a condensation of serum compounds at the particle surface *(plate 3)*. In the case of washed particles occasional parts of these serum compounds possibly remained at the surface giving the protein envelope a membrane-like structure *(plate 4)*. In latex particles of *E. milii* these membrane-like structures have not been detected.

In *Hevea* the interfacial film consists of a phosphoprotein/lipid complex (TUNNICLIFFE 1954) in which at least some phospholipid is present (SMITH 1954). In *H. australis* particles no phospholipids or other polar lipids normally present in plant membranes could be detected, so the existence of a single or double "unit membrane" around its latex particles is unlikely.

In contrast to the uniform electron density of *Hevea* rubber particles in ultra thin sections (DICKENSON 1969) the particles of *H. australis* have a varying electron density. In addition the specific gravity varies too. No search for a

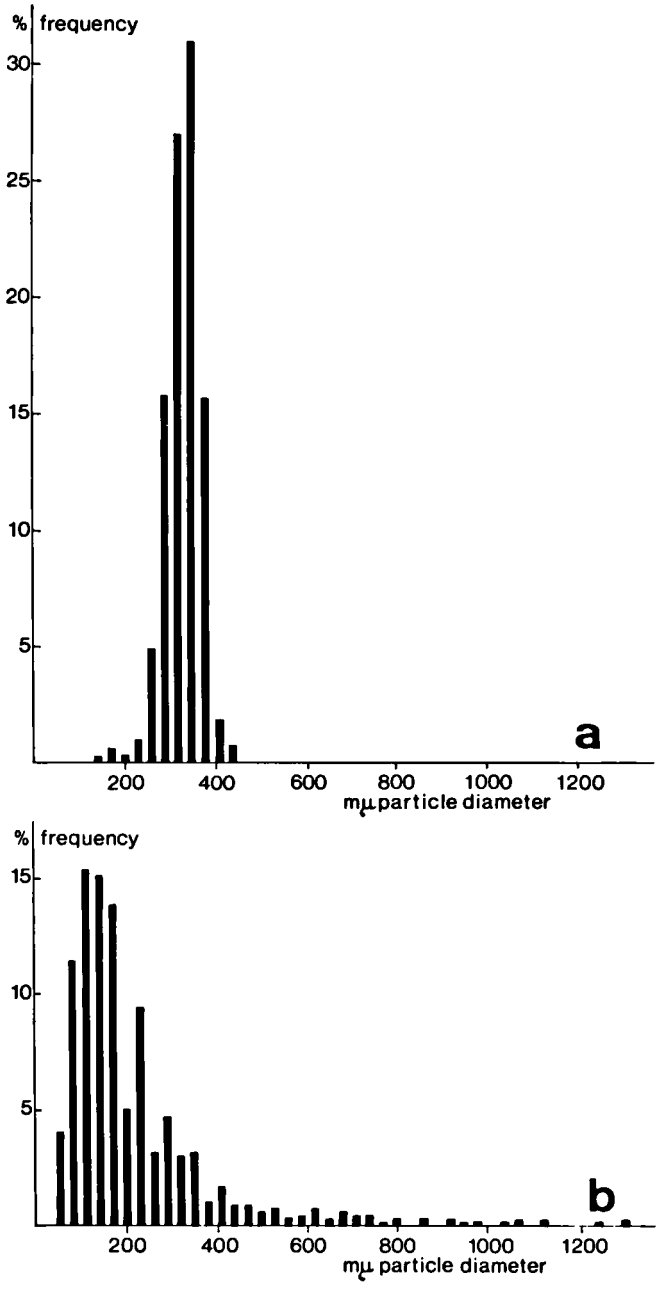

Fig. 2. Particle diameter distribution in *H. australis* (a) (469 particles) compared with *Hevea brasiliensis* (b) (mixture of clonal latices, 1013 particles) given by Schoon & v. d. Bie. Statistical interval 30 nm in both cases.

correlation between specific gravity and electron density has been attempted but obviously these differences in electron density cannot be explained by a different triterpene composition. All particle fractions, whatever their way of preparation had been, possess the same triterpene/triterpene ester composition *(table 1)*. HEINRICH (1970) has reported similar density differences in thin sections of *Ficus elastica* latex particles, which he attributed to different grades of polymerization of the rubber. Unlike *Ficus* latex particles, however, those of *H. australis* only contain small amounts of rubber hydrocarbon, and therefore a more general explanation for the varying electron density seems wanted. Possibly it is caused by different grades of cristallinity in each particle or by the presence of an other unsaturated compound in the particle fraction.

In *Hevea* extensive research on the distribution of particle diameters has been carried out (LUCAS 1938, HESSELS 1947, SCHMIDT & KELSEY 1951, VAN DEN TEMPEL 1951, COCKBAIN 1952, SCHOON & VAN DER BIE 1955). The particle diameter distribution curve is assymetric with a maximum frequency at $\pm$ 100 nm (HESSELS 1947, SCHOON & VAN DER BIE 1955, VAN DEN TEMPEL 1951). A similar particle diameter distribution curve, obtained by centrifugation, is reported by Hessels for ammoniated *Hevea* latex. In *Hevea* the largest particle diameters (1.5–4 μm) measure 50–100 times the smallest ones (30 nm). The particle diameter distribution curve of *Hoya australis* shows the largest particle diameter (420 nm) to be about 3 times the smallest one (140 nm). The curve is slightly assymmetric with a maximum frequency at 340 nm.

The amount of particles calculated to be present in *Hoya australis* latex *(table 1)* is not far from the 1.1×10^9 particles found for *Hevea* latex with a dry rubber content of 40% (van Gils 1941, quoted by VAN DEN TEMPEL 1951). The interfacial area of *H. australis* particles (11.2 $cm^2/\mu l$ latex) is also of the same order as the interfacial area of 7.7 $cm^2/\mu l$ latex calculated for *Hevea* by VAN DEN TEMPEL (1952), and falls within the range of the numbers given by COCKBAIN (1951) for ammoniated latex of several *Hevea* clones.

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