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Characterisation of Zone 1 and Zone 2 Rubber Particles in *Hevea brasiliensis* Latex

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Abstract

Rubber particles of *Hevea brasiliensis* latex separate into two main zones (Zone 1 and Zone 2) upon high speed centrifugation. Zone 2 rubber particles are small (0.35 µm diameter and below) but numerous and they constitute about 94% of the total rubber particles, with Zone 1 particles making up the remainder. Nevertheless, the small numbers of Zone 1 rubber particles account for about 93% of the rubber volume and 70% of the total area of all rubber particles because of their greater size. The frequency distribution of rubber particle sizes indicates that Zone 1 rubber particles and Zone 2 rubber particles might be two separate populations. Zone 2 rubber particles are more sensitive than Zone 1 particles to destabilisation and electron microscope examination reveals that destabilised lutoids in the latex have small rubber particles of the Zone 2 variety adhering to their surfaces. Molecular characterisation of rubber reveals that both Zone 1 and Zone 2 particles have molecular weights ranging from 10^4 to 10^7. Zone 2 particles contain rubber with higher average molecular weight. Examination of the molecular weight distribution curves show that the lower molecular weight of Zone 1 rubber is mainly due to the presence of a low molecular weight peak (approx. 10^5) in its bimodal distribution. This peak is frequently indistinct in the largely unimodal Zone 2 rubber. A high molecular weight peak (approx. 1.1×10^6) is present in both Zone 1 and Zone 2 rubber. There is about twice as much total gel rubber in Zone 2 rubber (29%) than in Zone 1 rubber (15%). As a large proportion of the latex obtained from the rubber tree is derived from a very small number of Zone 1 rubber particles of exceptionally large size, their prevalence in latex has an important bearing on the economic crop.

Full paper follows

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Rubber particles of Hevea brasiliensis *latex separate into two main zones (Zone 1 and Zone 2) upon high speed centrifugation. Zone 2 rubber particles are small (0.35 µm diameter and below) but numerous and they constitute about 94% of the total rubber particles, with Zone 1 particles making up the remainder. Nevertheless, the small numbers of Zone 1 rubber particles account for about 93% of the rubber volume and 70% of the total area of all rubber particles because of their greater size. The frequency distribution of rubber particle sizes indicate that Zone 1 rubber particles and Zone 2 rubber particles might be two separate populations. Zone 2 rubber particles are more sensitive than Zone 1 particles to destabilisation and electron microscope examination reveals that destabilised lutoids in the latex have small rubber particles of the Zone 2 variety adhering to their surfaces. Molecular characterisation of rubber reveals that both Zone 1 and Zone 2 particles have molecular weights ranging from* 10^4 *to* 10^7*. Zone 2 particles contain rubber with higher average molecular weight. Examination of the molecular weight distribution curves show that the lower molecular weight of Zone 1 rubber is mainly due to the presence of a low molecular weight peak (approx.* 10^5*) in its bimodal distribution. This peak is frequently indistinct in the largely unimodal Zone 2 rubber. A high molecular weight peak (approx.* 1.1×10^6*) is present in both Zone 1 and Zone 2 rubber. There is about twice as much total gel rubber in Zone 2 rubber (29%) than in Zone 1 rubber (15%). As a large proportion of the latex obtained from the rubber tree is derived from a very small number of Zone 1 rubber particles of exceptionally large size, their prevalence in latex has an important bearing on the economic crop.*

Commercial natural rubber is derived from rubber particles in *Hevea brasiliensis* latex which is a polydispersed system. These rubber particles occur mainly in spherical form consisting of *cis*-polyisoprene molecules enclosed and strongly protected by a negatively charged thin film of adsorbed lipids and protein components[1,2]. *Hevea* rubber particles have been reported to occur in a range of sizes, with diameters from 0.005 to 3 µm. The mean particle diameter is about 0.1 µm, with the size distribution of the particles being described as unimodal, with a long 'tail' of larger rubber particles[3-7]. Moir[8] reported that rubber particles that were centripetally separated out from the serum by ultracentrifugation formed two distinct zones of rubber cream: a large Zone 1 fraction which was in the form of a white rubber cream with a consistency of paste and a minor Zone 2 fraction of denser rubber particles that gave rise to a gelatinous white cream with a light blue tint *(Figure 1)*.

This paper presents results of a study on the Zone 1 and Zone 2 rubber particles with respect to various physiological characteristics, including their size, surface proteins and rubber molecular weights and their distribution. The significance of Zone 1 and Zone 2 rubber particles in natural rubber as an economic product is discussed.

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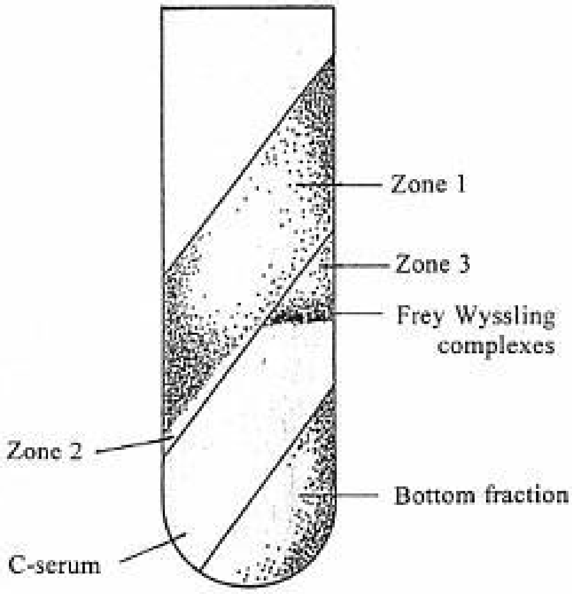


Figure 1. Centrifuged fresh Hevea brasiliensis *latex.*

MATERIALS AND METHODS

Sampling of Zone 1 and Zone 2 Rubber Particles

Latex from *Hevea brasiliensis* trees (clone RRIM 600) tapped half spiral alternate daily (1/2S d/2) was collected in chilled containers and centrifuged at 43 000 g (max) on a Sorvall RC5B centrifuge for 1 h to obtain Zone 1 rubber particles and for 2 h for Zone 2 rubber particles. Zone 1 rubber cream was sampled from the centre portion of the zone after latex centrifugation. To obtain Zone 2 rubber cream, the latex serum (C-serum) was drained away. The entire rubber phase (Zones 1 and 2 together) was then scooped out and the Zone 2 cream at the bottom of the rubber phase was skimmed off and collected with a spatula.

A third zone *(Figure 1)* in centrifuged latex (Zone 3) also contains a small number of rubber particles that are believed to be similar[8] to those of Zone 2. (Zone 3 decreases in size with prolonged centrifugation as Zone 2 increases). In this paper, references to Zone 2 rubber particles also cover particles from Zone 3.

Electron Microscopy and Rubber Particle Size Measurement

To determine rubber particle size distribution, latex from mature RRIM 600 trees was collected under chilled conditions. A drop of latex was fixed for 24 h in 2 ml of a fixative consisting of 1% osmium tetroxide in 0.1 *M* phosphate buffer (pH 7.9). Using a wire loop, the fixed material was transferred on to collodion-coated grids and air dried. The grids were then washed in water, face downwards and were examined with a Philips EM300 electron microscope. To assess whole latex rubber particle size distribution, measurements were made of all the rubber particles appearing on enlarged photographic prints (20 000× magnification) of electron micrographs. Size distribution of Zone 1 rubber particles were based on measurements of rubber particles 0.15 µm diameter and above. In each case, measurements were made by three operators to minimise error. Each operator measured more than 1000 rubber particles per sample. The size distribution data for Zone 2 rubber particles was produced from further enlarged photographic prints (180 000× magnification). A total of 934 particles of 0.2 µm diameter and below were measured.

Ultrathin sections of lutoids and rubber particles in latex vessels of the bark of the rubber tree just below the tapping cut were examined under the electron microscope. Small segments (1 mm cube) of bark samples were fixed overnight in 2% osmium tetroxide in 0.1*M* phosphate buffer (pH 7.0). Sucrose (0.35*M*) was added to control tonicity. The tissues were then dehydrated in a graded alcohol series and embedded in styrene methacrylate. Ultrathin sections (60 nm – 80 nm) were mounted on collodian-coated copper grids and double-stained with saturated uranyl acetate for 1 h and lead citrate for 15 min[9] before examination in a Philips EM300 transmission electron microscope. Many of the small rubber particles had diameters below the thickness of the ultrathin sections. Such rubber particles were therefore frequently unsectioned. In calculating the volume of rubber contained in rubber particles of a particular size, the thickness of the rubber particle surface layer (10 nm approx.[4]) was disregarded.

Determination of Molecular Weights and Gel Content of Rubber

Zone 1 and Zone 2 rubber fractions from centrifuged latex were film-dried at room temperature. A 0.3% solution of each rubber sample in tetrahydrofuran was prepared and filtered through a pad of 0.1 µm porosity before gel permeation chromatographic analysis. Gel permeation chromatography was carried out in a Waters GPC Model 200 using Styragel columns of porosities 10^3, 10^4, 10^5 and 10^6 Å. Measurements were made in tetrahydrofuran at 35°C at a flow rate of 1.0 ml min^{-1} as described previously[10].

To determine the gel content of rubber, small pieces of rubber samples (0.20 g) were solubilised in 100 ml of toulene in a reagent bottle kept in the dark for 24 h at room temperature. The contents were then centrifuged at 24 300 g (max) in a Sigma High Speed Centrifuge (Model 3SMK) for 20 min. The sedimented gel layer was separated and its dry weight measured to give the macrogel content. Microgel content was estimated by the difference in concentration of aliquots (20 ml each) of the supernatant before and after treatment with anhydrous calcium sulphate[11]. Total gel content was the sum of the macrogel and microgel contents.

RESULTS

Rubber Particle Size and Surface Area

Rubber particle size distribution in whole latex. The particle size frequency distribution of a fresh sample of RRIM 600 latex as given in *Figure 2a* was very similar to results published previously[6,7]. The median particle diameter was 0.1 µm, but a more accurate mean value was

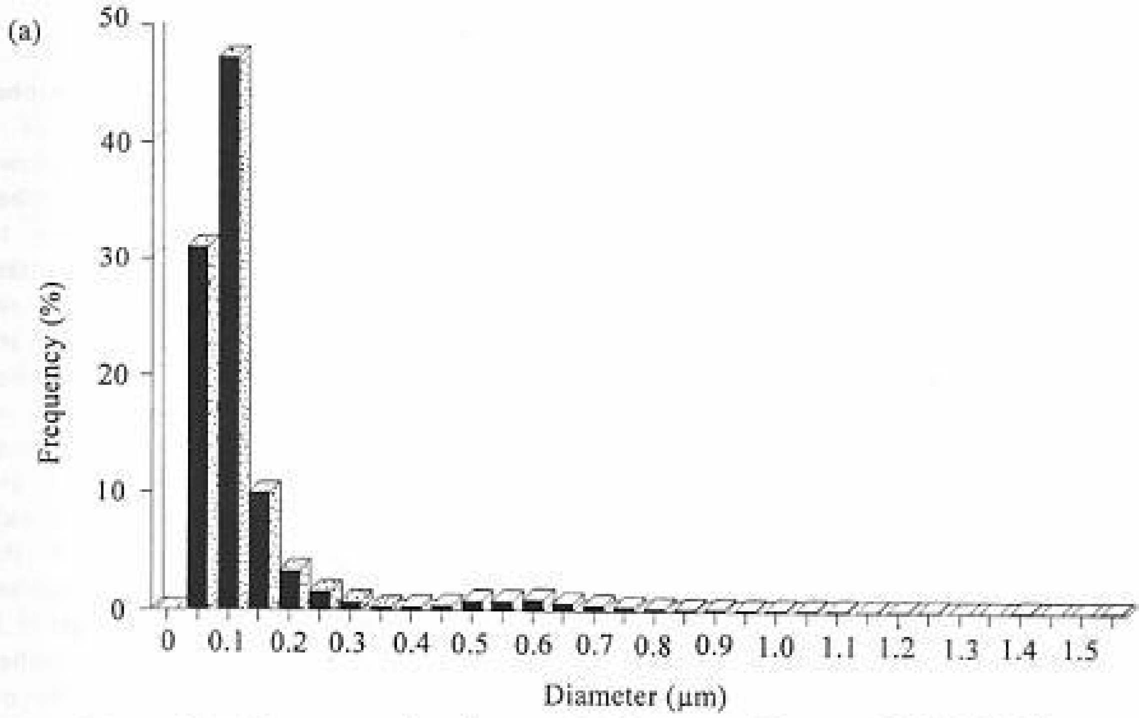


Figure 2(a). Frequency distribution of rubber particle sizes of RRIM 600 latex. (Zone 2 rubber particles are stippled.)

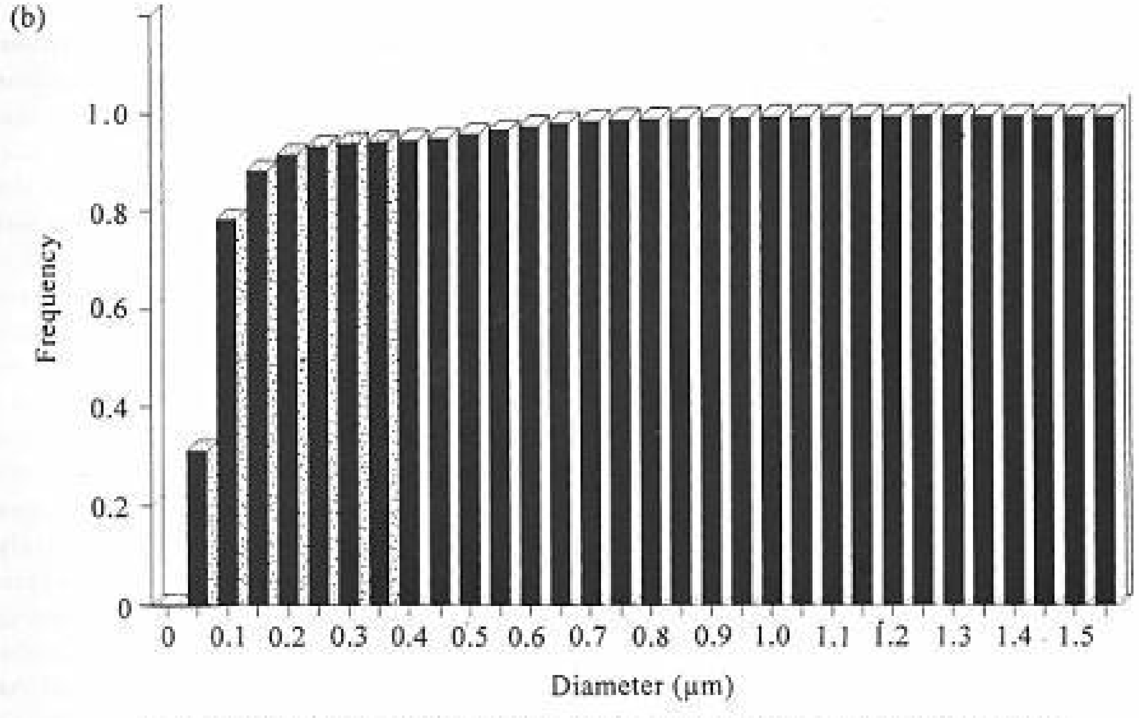


Figure 2(b). Cumulative frequency distribution of rubber particle sizes. (Zone 2 rubber particles are stippled.)

obtained by dividing the particles into smaller class sizes (see below). Rubber particles of 0.2 µm and smaller accounted for 92% of the total number of particles *(Figure 2b)*. Although rubber particles of diameters greater than about 0.3 µm were relatively rare, small numbers of particles of up to 1.55 µm diameter were recorded.

The volume of rubber in the latex derived from the various sizes of the rubber particles depended on both the volume of the particles and their frequency of occurrence in the latex. As seen in *Figure 3a*, the particles that contributed most to the total volume of rubber in the latex were those of diameter of 0.6 µm. It is noteworthy that the very large particles (*e.g.* those of 1.4 µm and 1.55 µm diameter) contributed very significantly to the volume of rubber despite their being extremely rare. On the other hand, the small rubber particles (*e.g.* those of 0.1 µm diameter) accounted for little of the total mass of rubber in latex despite their abundance.

Rubber particle size distribution in Zone 1 and Zone 2. The separation of rubber particles latex centrifugation according to size could be expected to follow Stoke's Law governing the passage of very small spheres in a fluid medium. Small particles reach their terminal velocity sooner than large particles and this augments size separation of the rubber particles. In a tube of centrifuged latex, the small rubber particles are deposited in Zones 2 and 3 below the larger particles in Zone 1 *(Figure 1)*. Still smaller rubber particles would remain suspended in the serum.

Dry weight measurements of the rubber found in Zone 1 and in Zones 2 and 3 of centrifuged latex showed that the rubber particles in Zones 2 and 3 made up 7.3% of the total rubber. The cumulative volume of rubber contributed by rubber particles of a progressively increasing diameter is shown in *Figure 3b*. The sum total of rubber contributed by rubber particles up to 0.35 µm made up 7.46% of the total mass of rubber. Large rubber particles that are normally associated with Zone 1 are generally absent in the Zone 2 rubber cream[12]. In the Zone 1 rubber cream, on the other hand, small rubber particles that are normally associated with Zone 2 are also encountered[13]. However, these small particles would contribute negligibly to the total volume of the mainly large rubber particles in the Zone 1 rubber cream. If it were assumed that most of the rubber particles were separated exclusively on the basis of size, Zone 1 rubber particles would be those of 0.4 µm diameter and larger, while Zone 2 rubber particles would be those 0.35 µm and below.

Surface area of Zone 1 and Zone 2 rubber particles. While it is obvious that a large rubber particle would have a larger surface area than one that is smaller, the total surface area contributed by particles of any particular size category would depend both on the size as well as the abundance of the particles. Because of their large numbers, Zone 2 rubber particles of 0.1 µm diameter accounted for the greatest total surface area of any size category despite the small size of the particles *(Figure 4a)*. Zone 1 particles of 0.6 µm were next in importance in terms of their contribution to the total surface area of rubber particles in latex. From *Figure 4b*, it can be seen that cumulatively, Zone 2 particles made up 30% of the total area of all rubber particles despite their making up only 7% – 8% of the volume of all the rubber particles in latex. This is owing to the fact that smaller rubber particles have proportionately larger surface areas as per unit mass.

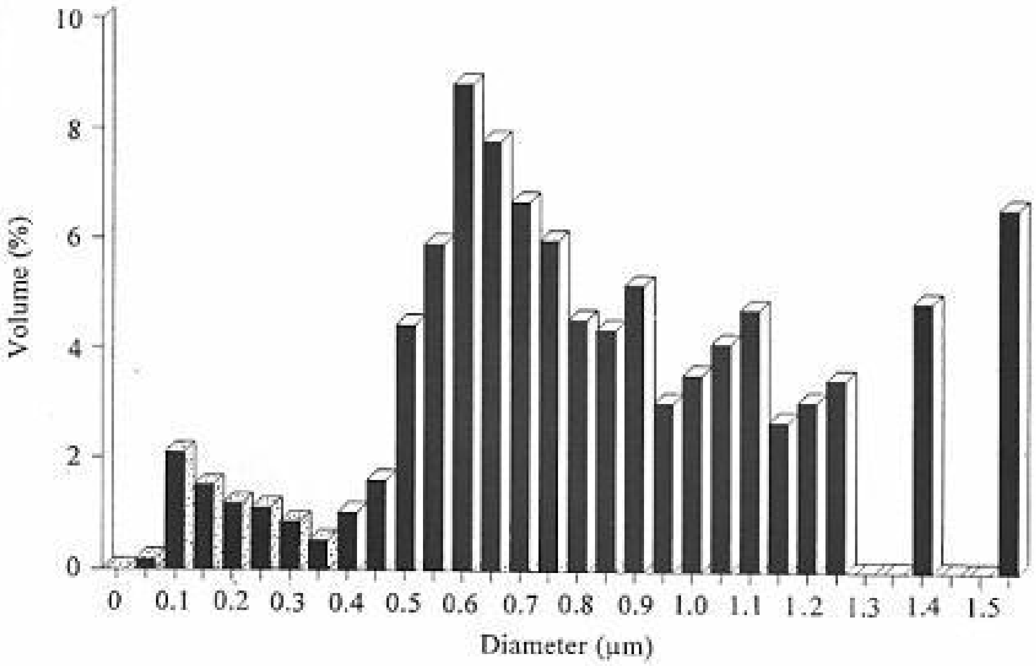


Figure 3(a). Combined volume of rubber contributed by rubber particles of various sizes in RRIM 600 latex (% of total). (Zone 2 rubber particles are stippled.)

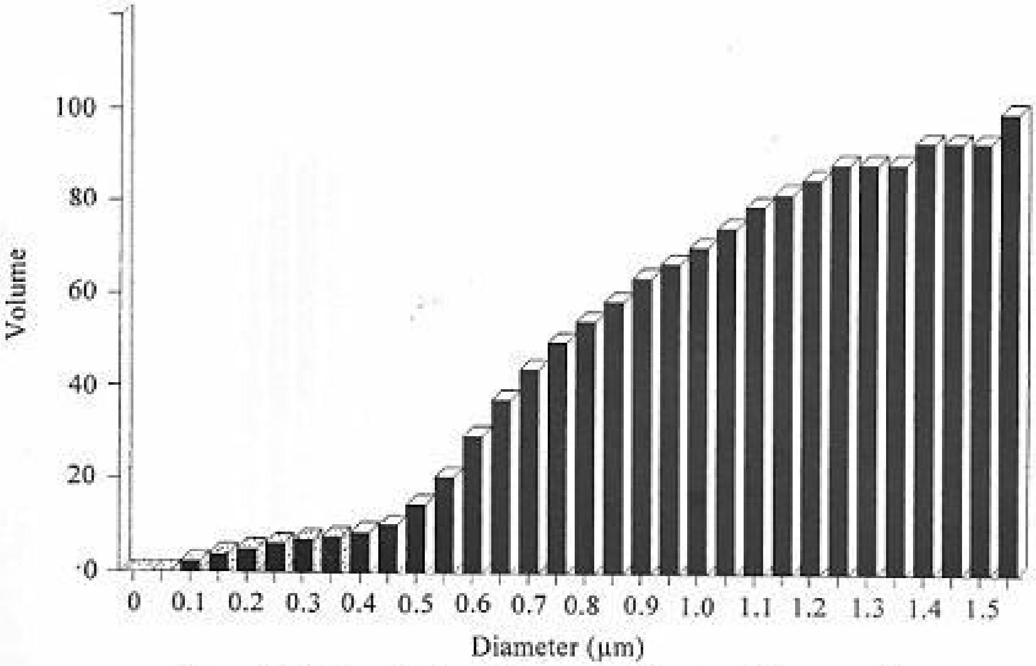


Figure 3(b). Cumulative volume of rubber in rubber particles of various sizes. (Zone 2 rubber particles are stippled.)

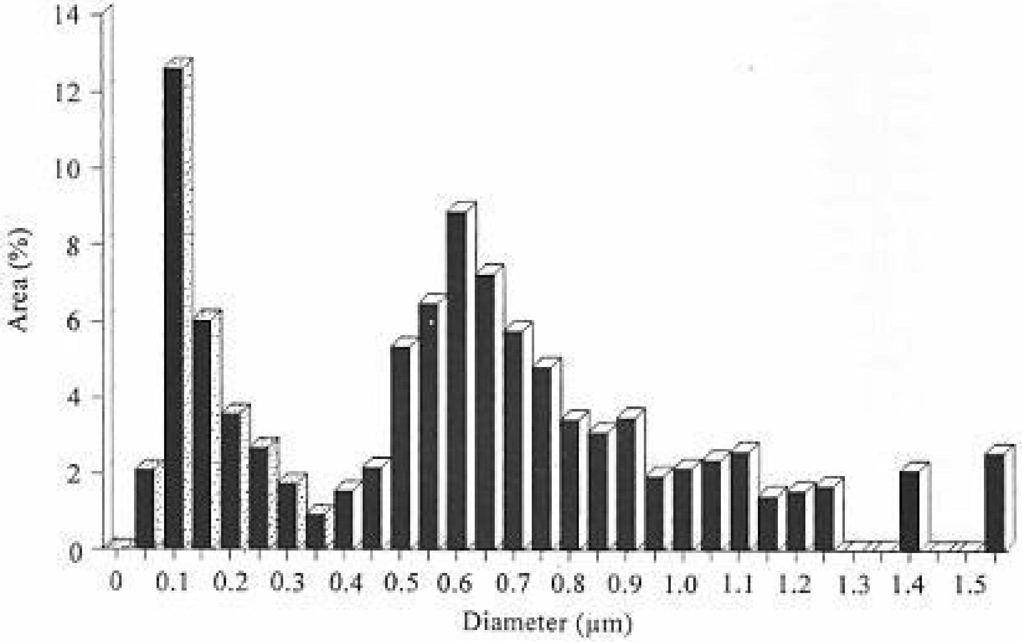


Figure 4(a). Combined surface area contributed by rubber particles of various sizes in RRIM 600 latex (% of total). (Zone 2 rubber particles are stippled.)

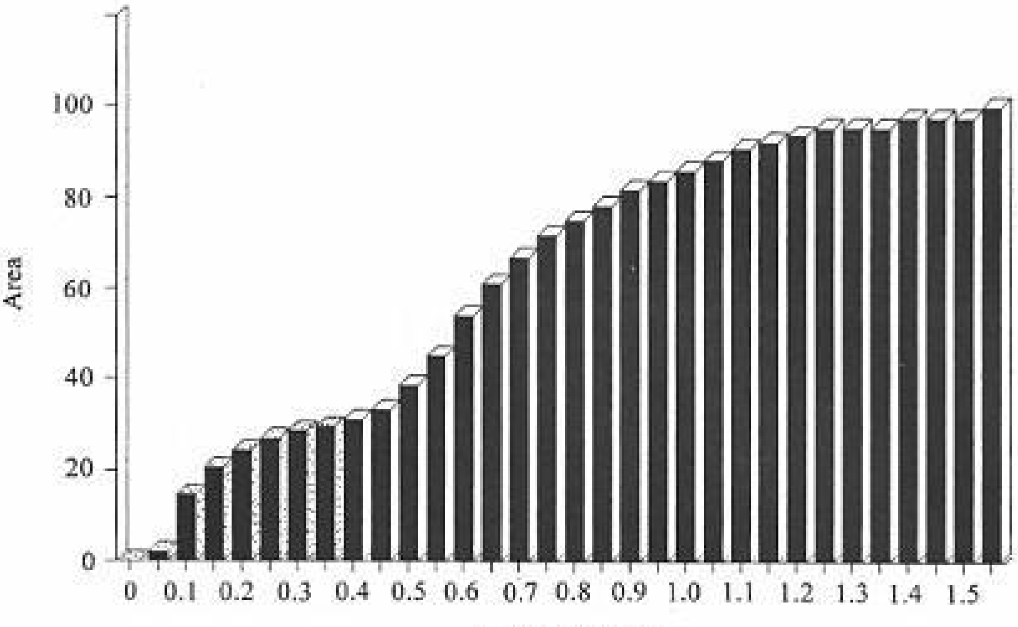


Figure 4(b). Cumulative surface area of rubber particles of various sizes. (Zone 2 rubber particles are stippled.)

Zone 1 and Zone 2 Rubber Particles as Separate Populations

To investigate whether Zone 1 and Zone 2 rubber particles were in fact two separate populations, about 1000 each of the small (0.01 µm to 0.19 µm) and large rubber particles (0.15 µm to 1 µm) were measured. In previous analyses (such as that presented in *Figure 2a*), frequency distributions were based on the entire rubber particle population. As Zone 1 particles constituted only about 6% of the total number of rubber particles in latex, a sampling of, say, 1000 particles in latex would yield only about 60 Zone 1 particles. This number, when spread over a number of size classes (24 classes in the present study) was insufficient for a proper assessment of the particle size frequency distribution specifically of Zone 1 rubber particles. In the present study, therefore, the sizes of about 1300 rubber particles between 0.15 µm and 1 µm were measured and categorised into 18 size classes.

The size frequency distribution of the small rubber particles (0.01 µm – 0.19 µm) was a Gaussian curve with a mean diameter of about 0.07 µm *(Figure 5a)*, which is very similar to the mean reported by van den Tempel[4]. The size frequency distribution of the large particles (0.15 µm – 1 µm) included the tail end of Zone 1 rubber particles (particles up to about 0.35 µm diameter) followed by a good approximation of a Gaussian distribution of the larger Zone 1 particles with a mean diameter of about 0.55 µm – 0.6 µm *(Figure 5b)*. Hence, two distinct rubber particle species were discernible in the distribution of rubber particle sizes in whole latex. *Figure 5b* showed that the two species overlapped where the rubber particles were about 0.35 µm to 0.4 µm diameter. It is significant that the size transition between Zone 1 and Zone 2 particles is also at this point (as noted above), in agreement with the proposition that Zone 1 and Zone 2 particles are separate populations.

Rubber Particles and the Destabilisation of Lutoids

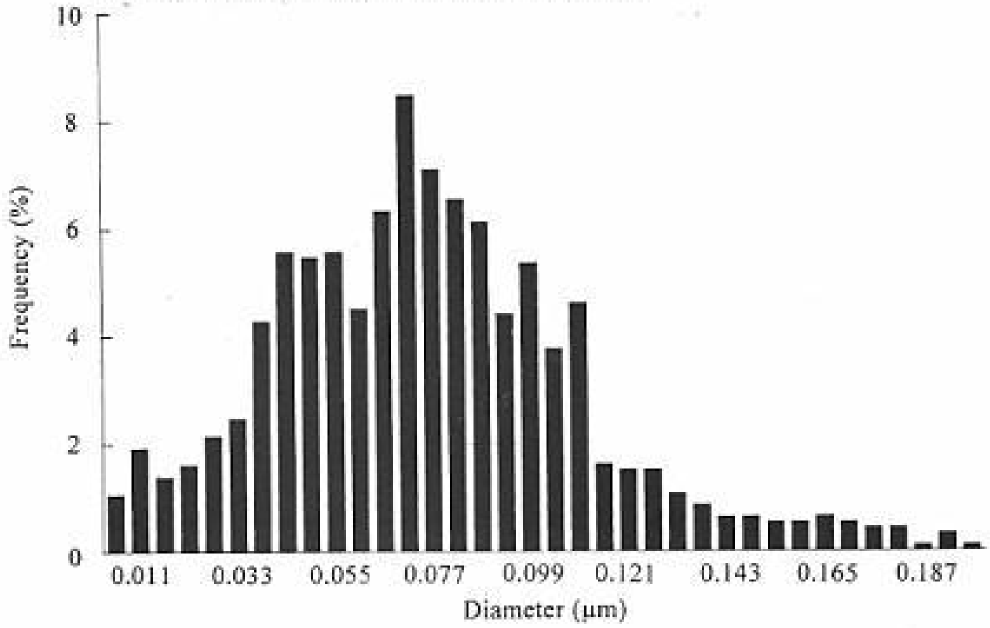
Destabilisation of latex particles can generally occur as a flocculation of rubber particles or a coagulation in which individual rubber particle surfaces fuse into a larger mass of rubber. Such destabilised rubber particles are seen in the plugs at the cut ends of latex vessels after latex flow has stopped[14]. Lutoids contain a latex destabilising agent and damaged lutoids are often associated with destabilised rubber particles[14]. While both Zone 1 and Zone 2 rubber particles could be *flocculated* by these destabilising agents under specified conditions, only Zone 2 rubber particles – but not Zone 1 rubber particles – could be induced to *coagulate*[15].

Electron microscopy was carried out on the contents of latex vessels in *Hevea* bark. It was frequently observed that lutoids that were in their early stages of destabilisation (when their membranes were partially depolarised[16]) had small rubber particles of the Zone 2 variety adhering to their surfaces *(Figure 6)*. Zone 1 rubber particles, on the other hand, were not commonly seen adhering to such lutoids although these larger rubber particles would also constitute a part of the coagulum mass when latex destabilisation was advanced as when latex vessel plugs were formed[14].

Rubber of Zone 1 and Zone 2 Particles: Molecular Characterisation

Molecular weight averages. In the latex system, each rubber particle is expected to contain hundreds to thousands of rubber molecules within its enclosing interface of lipids and proteins. There is, however, no apparent correlation between the molecular weight averages of these rubber molecules and the diameter of the particles containing them[17].

(a) Small particles of diameters 0.01 to 0.19 µm.



(b) Large particles of diameters 0.15 to 1 µm. (Results of 3 operators.)

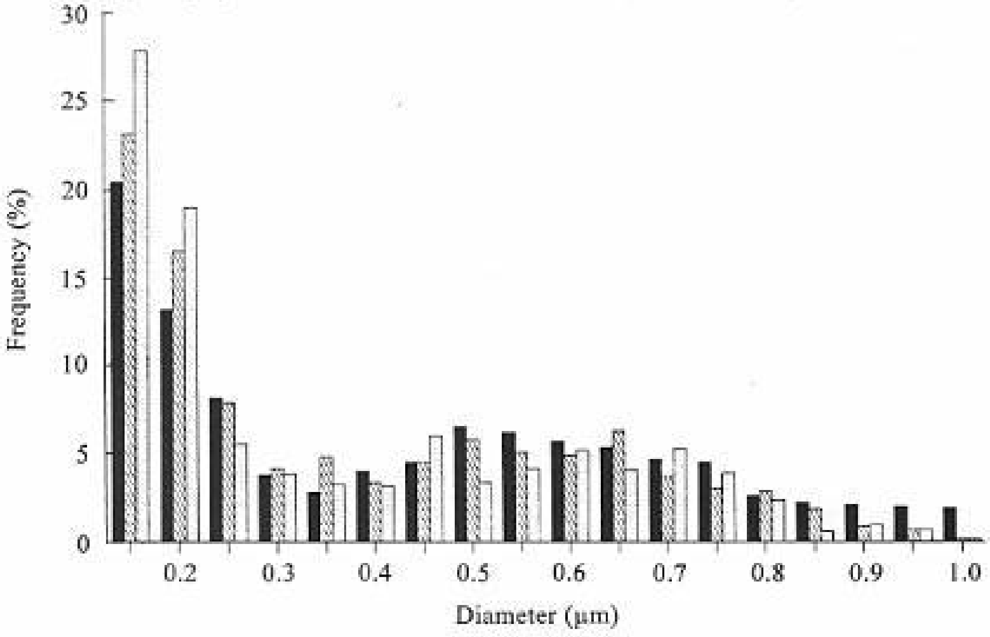


Figure 5. Size frequency distribution of small and large rubber particles in fresh latex.

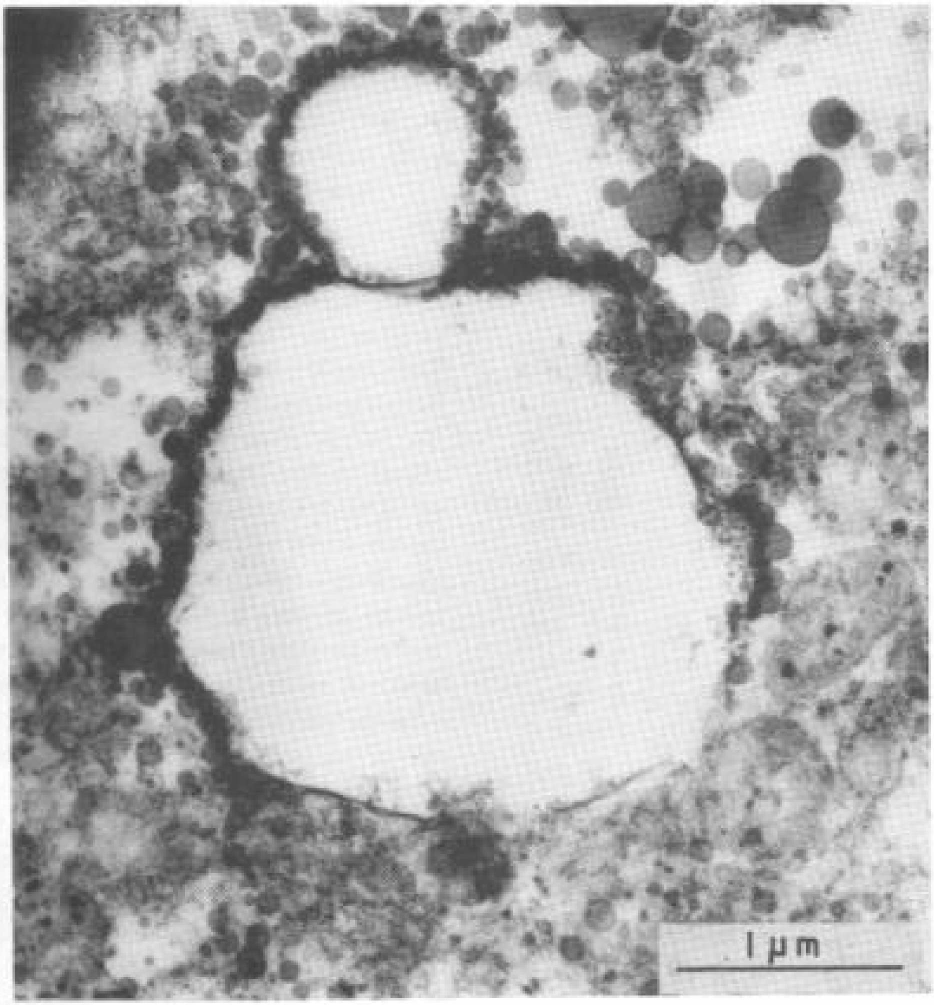


Figure 6. Destabilised lutoids in Hevea *latex with small (Zone 2) rubber particles adhering to the lutoid membranes.*

TABLE 1. MOLECULAR WEIGHT AVERAGES OF ZONE 1 AND ZONE 2 RUBBER PARTICLES FROM FOUR CLONES

Rubber sample		$M_w \times 10^{-5}$	$M_n \times 10^{-5}$	M_w/M_n
PR 261	Zone 1	9.94	0.85	11.6
	Zone 2	11.98	2.00	5.8
RRIM 600	Zone 1	16.44	1.54	10.6
	Zone 2	25.48	2.66	9.6
RRIM 901	Zone 1	14.98	1.60	9.4
	Zone 2	15.41	1.70	9.1
RRIM 905	Zone 1	14.18	1.36	10.5
	Zone 2	17.06	1.53	11.2

M_w = Weight average molecular weight
M_n = Number average molecular weight
M_w/M_n = Polydispersity

TABLE 2. GEL CONTENT (%) IN ZONE 1 AND ZONE 2 RUBBER FRACTIONS FROM RRIM 600 LATEX

Centrifugation zone		Macrogel	Microgel	Total gel
Zone 1	Expt. 1	7.9	6.1	14.0
	Expt. 2	13.2	2.0	15.2
	Expt. 3	14.1	2.7	16.8
	Mean	11.7	3.6	15.3
Zone 2	Expt. 1	25.0	3.4	28.4
	Expt. 2	26.3	2.7	28.0
	Expt. 3	24.0	6.1	30.1
	Mean	25.1	4.1	28.8

In fact, there was indication that higher molecular weights were associated with the smaller particles[18]. This was indeed confirmed in the present study using gel permeation chromatography, which revealed that the smaller Zone 2 particles consistently contained rubber of higher molecular weight averages than the larger Zone 1 particles. *Table 1* shows the weight averages (M_w) and number averages (M_n) of rubber from four different clones.

Molecular weight distribution. Examination of the molecular weight distribution of these rubber samples revealed yet another interesting finding. Although both Zone 1 and Zone 2 particles had rubber molecules of similar molecular weights ranging from 10^4 to 10^7 approximately, they exhibited different molecular weight distribution behaviour in the four clones studied *(Figure 7).* While rubber molecules in Zone 1 demonstrated a distinctly bimodal distribution with a low molecular weight peak at about 10^5 and a high molecular weight peak at $1 - 2 \times 10^6$, Zone 2 rubber indicated a skewed unimodal distribution with a shoulder in the low molecular weight region and a prominent high molecular weight peak similar to that in Zone 1 rubber particles. This was the case in three of the four clones studied, the exception being RRIM 905 which showed a more pronounced low molecular weight shoulder. Some variation was detected between clones as shown by their polydispersity M_w/M_n *(Table 1).* Generally, Zone 1 rubber had considerable quantities of both the low molecular weight rubber besides the high molecular weight molecules, whereas Zone 2 rubber had a predominance of the higher molecular weight components. It would appear that in the bimodal distribution of molecular weight characteristic in the majority of *Hevea* clones[10], the low molecular weight peak is derived largely from Zone 1 rubber. Because of the small amount of Zone 2 rubber in latex, its contribution to the overall molecular weight distribution will be very small.

Gel content. An examination of the gel content of Zone 1 and Zone 2 rubber from RRIM 600 showed that there was twice as much gel in Zone 2 rubber (29%) than in Zone 1 rubber (15%)*(Table 2).* Most of the gel in the rubber of both zones was macrogel. This implies that there was more crosslinking between the high molecular weight rubber in the smaller Zone 2 than the larger Zone 1 particles. The trend, if any, in the microgel component was not apparent.

DISCUSSION

Gomez and Samsidar Hamzah[7] reported that latex from untapped potted seedlings (1-2 months old) showed two peaks in rubber particle size distribution: one at 0.08 µm and another at 0.24 µm. In budded mature trees that were tapped, only one peak at about 0.12 µm was observed. That rubber particles in latex come in a range of sizes is not surprising. But are all these particles the same, differing only in size? Do the smaller Zone 2 particles 'grow' into the larger Zone 1 particles, or are Zone 1 and Zone 2 rubber particles essentially two different species?

There have been indications from past observations that rubber particles in Zones 1 and 2 are two separate populations. Zone 1 and Zone 2 rubber particles differ in appearance and consistency, suggesting that differences exist beyond their dimensions. Moir[8] found that rubber particles in the two zones differ in the biochemical make-up of their surfaces by demonstrating dissimilar stain uptake. The separateness of Zone 1 and Zone 2 rubber particles is strongly corroborated by the present study that shows the presence of two apparently separate size frequency distributions for the large and small rubber particles consistent with their delineation into Zone 1 and Zone 2

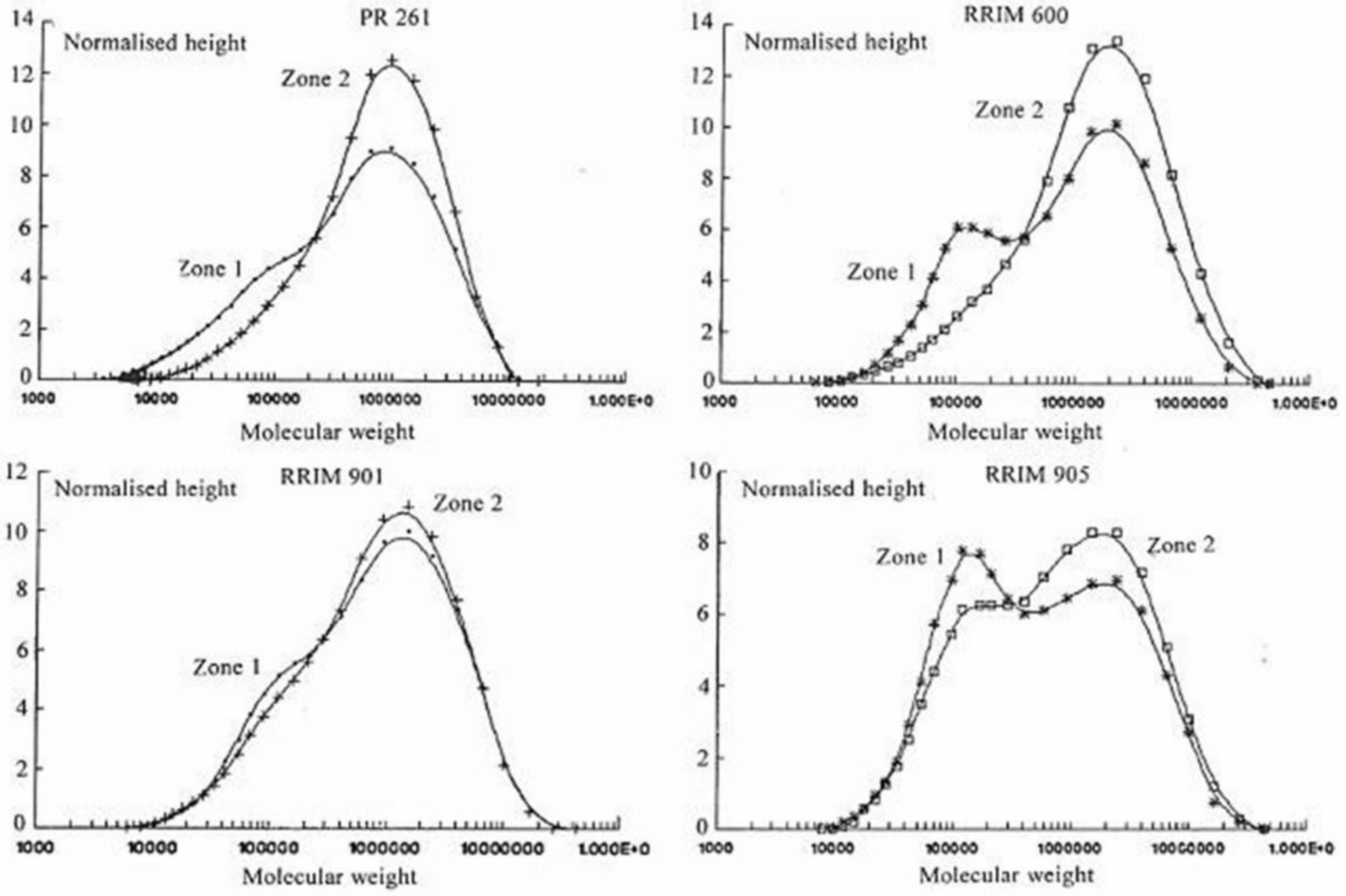


Figure 7. Molecular weight distribution of Zone 1 and Zone 2 rubber from four Hevea brasiliensis *clones.*

particles. The differences observed in the present study with respect to the molecular weight and gel composition of the rubber and the propensity to destabilisation also point to the likelihood of Zone 1 and Zone 2 rubber particles being dissimilar species.

It may not be unreasonable to suppose that the observed molecular differences in Zone 1 and Zone 2 particles are the result of a biosynthetic process of rubber at the particle interface involving more than one enzyme system. While one enzyme system synthesises high molecular weight rubber in all the particles, a second enzymic system responsible for producing lower molecular weight components come into operation when the particles reach a certain optimum size. Although obscure in many of its details, this supposition seems to give a plausible explanation for the predominance of the high molecular weight fraction in Zone 2 particles and the bimodal distribution in Zone 1 particles.

The association between destabilised lutoids and Zone 2 rubber particles *in situ* is consistent with the proposition that, compared with Zone 1 particles, Zone 2 rubber particles are more readily sensitised to destabilisation by B-serum released from damaged lutoids (in association with other factors such as bark sap)[15]. It would seem that during the early stages of lutoid destabilisation when the lutoid membrane surfaces are partially depolarised[16], only the smaller rubber particles are entrapped by the aggregates, while larger rubber particles also participate in the formation of the final latex vessel plugs leading to the cessation of latex flow. Although the Zone 2 rubber particles made up only a small proportion (7% - 8%) of the total rubber particle volume, their total surface area is proportionately much larger (30%) owing to the fact that smaller rubber particles had larger surface area per unit mass. Hence, Zone 2 particles could have an important bearing on rubber biosynthesis and aspects of latex physiology that involves latex destabilisation such as latex vessel plugging and tree dryness.

In terms of contribution to the economic crop, Zone 2 rubber particles play only a very small role. As mentioned, the numerically superior Zone 2 rubber particles constitute only a very small proportion (about 7%) of the rubber volume. Indeed, from the particle size measurements in the present study, the largest single rubber particle measured had a volume equivalent to 88% of the total volume of all the 2357 Zone 2 rubber particles combined. It should be noted that the particular Zone 1 rubber particle had a diameter of 1.55 µm, whereas rubber particles even as large as 3 µm to 6 µm have been reported[19]. The importance of large rubber particles in the rubber crop can be seen from the fact that, in the study sample, the combined volume of the largest 1% of the rubber particles accounted for about 50% of the total rubber in the latex. The combined volume of the largest 6% of the rubber particles (practically all of the Zone 1 rubber particles) accounted for 93% of the total rubber. This is in general agreement with van den Tempel[4] who reported that 85% of the total dispersed rubber phase of latex was accounted for by less than 4% of the largest rubber particles. Hence, a large proportion of the economic rubber crop is derived primarily from a relatively small number of Zone 1 rubber particles of unusually large dimensions.

As most of the rubber crop is derived from the large Zone 1 rubber particles, it is obvious that an increment in the number of these particles would make a significant difference in the latex dry rubber content. The theoretical possibilities are intriguing. Even in the situation where it is assumed that the number of rubber particles in a given volume of latex is unchanged, a very small shift in the proportion of small (Zone 2) rubber particles to large (Zone 1) rubber particles would make a very sizeable change in the rubber content. From the frequency and volume data in the present study, it is calculated that a shift of every 1% median sized (0.1 µm) Zone 2 rubber particles to median sized (0.6 µm) Zone 1 rubber particles would result in an increment of almost 10% dry rubber with the number of rubber particles unchanged.

In most commercial rubber, the Zone 2 rubber would be blended in with the Zone 1 rubber and the presence of the former would not generally be significant. The exception to this would be in the case of skim rubber that is recovered after the rubber cream is extracted by centrifugation for the production of latex concentrate. A large proportion of the rubber particles that fail to be removed as cream would be the smaller Zone 2 rubber particles that remain in the skim which contains 4% – 6% dry rubber content. Skim rubber has many technological properties comparable to higher grade rubbers despite being inferior in a small number of aspects such as high nitrogen and increased scorchiness[20,21].

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