

The Biosynthesis of Rubber

INCORPORATION OF ISOPENTENYL PYROPHOSPHATE INTO PURIFIED RUBBER PARTICLES BY A SOLUBLE LATEX-SERUM ENZYME

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1. The rubber particles in *Hevea brasiliensis* latex have been partially purified by 'washing' with buffer solution, and separated into active fractions of different particle size. 2. The enzyme responsible for incorporating isopentenyl pyrophosphate into rubber is distributed between the surface of the rubber particles and the aqueous serum phase of the latex. The enzyme at the surface can be removed or inactivated if the rubber particles are washed sufficiently with buffer solution. Enzyme in the serum phase can be concentrated by fractional precipitation with ammonium sulphate. 3. To incorporate isopentenyl pyrophosphate into rubber *in vitro*, active rubber particles are required as well as enzyme and soluble cofactors. The activity of the rubber particles per unit surface area increases with diminishing particle size.

It is now firmly established that, in the biosynthesis of isoprenoid compounds, including rubber (Lynen & Henning, 1960; Archer, Ayrey, Cockbain & McSweeney, 1961), isopentenyl pyrophosphate (3-methylbut-3-enyl pyrophosphate) is an essential intermediate. It has also been demonstrated by Agranoff, Eggerer, Henning & Lynen (1960) that the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate by isopentenyl pyrophosphate isomerase is an essential initial step in the biosynthesis of a number of *trans*-isoprenoid compounds. The electrophilic dimethylallyl pyrophosphate is believed to attack the methylene group of isopentenyl pyrophosphate with regeneration of an allylic pyrophosphate end group, thus allowing further reaction with isopentenyl pyrophosphate to occur. In *Hevea brasiliensis* latex, Archer, Audley, Cockbain & McSweeney (1963) have shown that under suitable conditions the rate of incorporation of isopentenyl pyrophosphate into rubber can be accelerated by the addition of dimethylallyl pyrophosphate, indicating that this compound can also initiate the formation of long *cis*-polyisoprene chains. The above authors concluded that isopentenyl pyrophosphate was incorporated into rubber *in vitro* at the surface of the rubber particles in the latex, mainly by reaction with rubber molecules already present and possessing a terminal allylic pyrophosphate group, i.e. the reaction measured was predominantly chain extension.

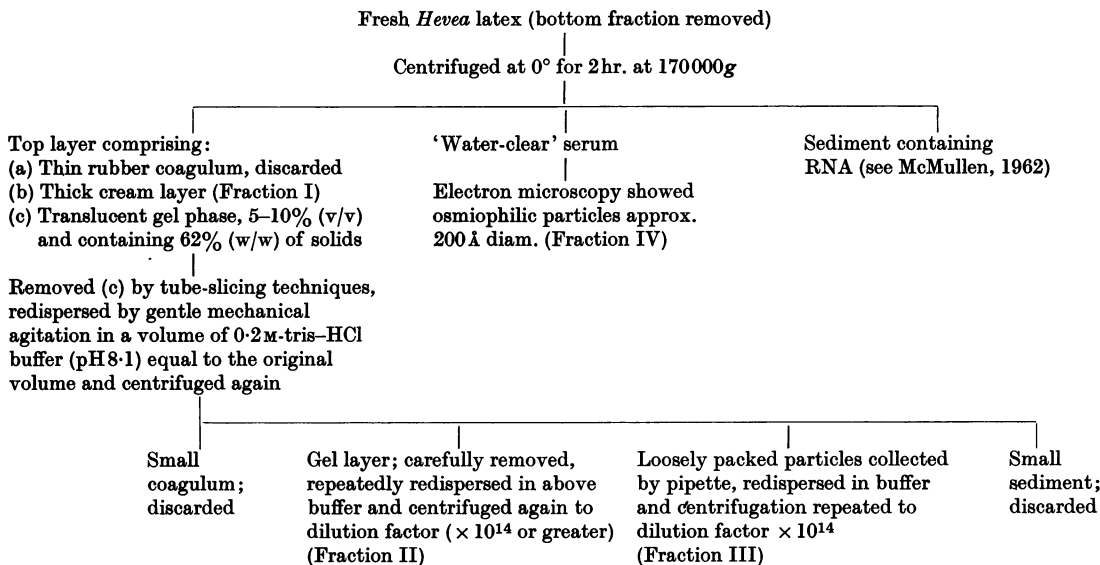
Hevea brasiliensis latex is a complex system cytologically (Dickenson, 1964) but it is readily separated by centrifuging into three main fractions consisting respectively of rubber particles, aqueous serum and a relatively heavy fraction containing non-rubber particles. In the present paper, a study has been made of the incorporation of isopentenyl pyrophosphate into the purified rubber particles (fractionated according to particle size) and of the effect of different latex-serum protein fractions on the incorporation.

METHODS AND MATERIALS

Latex. Fresh *Hevea brasiliensis* latex was obtained from a group of trees in regular tapping at the Rubber Research Institute of Ceylon. This latex was collected in ice-cooled flasks, centrifuged at 2000g for 10 min. to remove a bottom fraction consisting mainly of non-rubber particles, and transported to England by air in polythene bottles surrounded by ice. The latex was used within 2 weeks of tapping since a gradual decrease in the biological activity of the latex occurs with time. The total solids from the aqueous serum phase of fresh latex were kindly provided, as freeze-dried material, by the Rubber Research Institute of Malaya as described previously (McMullen, 1959). The solids were stored at -25° and were reconstituted to a serum by dissolving in distilled water immediately before use.

Cofactors from latex serum. An aqueous solution containing cofactors required for the incorporation of isopentenyl pyrophosphate into rubber was obtained by passing fresh or reconstituted latex serum through a tightened dialysis membrane under pressure (Adair, 1956). The porosity of the membranes used was approx. 0.013 ml./sec./cm.²/dyne and the pressure applied was 440 mm. Hg. Blockage of the membrane occurred unless the serum was centrifuged

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Scheme 1. Centrifugal fractionation and purification of rubber particles.

beforehand for at least 3 hr. at 170000g to remove some of the small amount of rubber still present in the serum. The membrane, holding about 0.5–1.0 ml. of the serum, was immersed in a narrow tube containing 0.25–0.5 ml. of 0.2M-tris-HCl buffer (pH 8.1) such that the final dilution of cofactors was not greater than 50%. The u.v.-absorption data of a typical solution after such treatment were: $E_{280m\mu}^{1cm.} = 14$, $E_{265m\mu}^{1cm.} = 28$ (max. value), $E_{233m\mu}^{1cm.} = 9$ (min. value) and $E_{280}/E_{260} = 0.50$, indicating the presence of nucleotide components. The composition of latex serum with respect to nucleotide and thiol components has been previously described (McMullen, 1960).

Centrifugal fractionation of latex. Latex received from the Rubber Research Institute of Ceylon, which had already been centrifuged at 2000g to remove the bottom fraction of non-rubber particles, was subjected to a further centrifugal fractionation procedure, summarized in the flow diagram (Scheme 1). The rubber particles were thus separated into four Fractions (I–IV) of differing size–frequency distribution. Fractions II and III were 'washed' by diluting several times with a large proportion of buffer solution and centrifuging again, as indicated in Scheme 1. The rubber particles in these fractions were much more stable, colloidal, to such washing than were the larger particles in Fraction I, which tended to flocculate. The very small particles in Fraction IV (diam. approx. 200 Å) could not be concentrated appreciably by centrifuging at 170000g. A more concentrated suspension of rubber particles of approximately the same size was obtained from the freeze-dried aqueous serum solids supplied by the Rubber Research Institute of Malaya. These solids contained small rubber particles that had not been removed when the original latex was centrifuged. By dissolving 10g. of the serum solids in 12 ml. of 0.2M-tris-HCl buffer (pH 8.1), centrifuging the solution at 170000g for 3 hr., discarding the upper third of the solution, which contained relatively large particles, and repeating the

centrifugation, a solution was left containing very small rubber particles with a similar size–frequency distribution to that of Fraction IV, but more concentrated.

Electron microscopy and size–frequency distribution. Suspensions of rubber particles were treated with 1% osmium tetroxide solution, sprayed on carbon film and photographed in a Siemens Elmiskop I electron microscope at an instrumental magnification $\times 20000$. The preparations were shadowed with gold–palladium at 25° to confirm the reliability of particle diameter measurements. Approximately 500 particles in each preparation were measured to obtain the size–distribution curves and hence the specific surface areas (cm.²) of the rubber particles, given by:

$$S = \frac{6.0 \sum n_i d_i^2}{D \sum n_i d_i^3}$$

where n_i is the number of particles of diameter d_i cm. and D is the density of rubber (0.93).

Determination of rubber content. A portion (500 mg.) of the rubber-containing suspension (containing about 50 mg. of rubber) was heated at 90–100° for $\frac{1}{2}$ hr. with an equal volume of 20% (w/w) NaCl soln. The coagulated rubber was washed and purified as described below (incubations with isopentenyl pyrophosphate).

Release of adsorbed components by hot 10% NaCl solution. The NaCl supernatant obtained by the above procedure was made up to 3.5 ml. with 0.2M-tris-HCl buffer, pH 8.1, and the u.v. absorption determined at 260 m μ and 280 m μ . The amount of pentose in the NaCl supernatant was estimated by the orcinol method of Mejbaum as modified by Markham (1955). Mixtures of crystalline bovine serum albumin and pure yeast nucleic acid were used for calibration purposes.

Fractionation of latex-serum proteins. A portion (2g.) of freeze-dried latex-serum solids was dissolved in 10 ml. of 0.2M-tris-HCl buffer, pH 8.1, followed by the stepwise addition of neutral saturated (NH₄)₂SO₄ solution. The

protein fractions were then desalted by dialysis under N_2 . The temperature of all solutions was maintained at 0–5° throughout. The protein fractions so obtained were passed through Millipore filters of grade GS (0.22 μ) and checked for contamination by small rubber particles under the electron microscope. Different fractions are denoted by the symbols $S_{x/y}$, where x and y refer to the limits of percentage saturation with $(NH_4)_2SO_4$ between which the protein was precipitated.

Incubations with isopentenyl pyrophosphate. About 150 mg. of rubber suspension, containing 5–20 mg. of rubber, was weighed into a small stoppered test tube. Latex serum (or a serum protein fraction) and any required cofactors were then added, followed by a solution of [^{14}C]isopentenyl pyrophosphate in water, to give a total volume of approx. 200 μ l. Incubations were carried out at 30° with continuous gentle mechanical agitation, and the reaction was terminated by adding an aqueous solution of 0.5 mg. of *p*-chloromercuribenzoate, for incubations of short duration. To recover the rubber, the tube and contents were spun axially while 0.5 ml. of 1% acetic acid in ethanol was gradually added to create a film of rubber coagulum around the lower half of the tube. Inert 'carrier' rubber latex of 70% rubber content (which gave no incorporation of ^{14}C on prolonged incubation with serum and labelled isopentenyl pyrophosphate) was added just before coagulation, where necessary, to raise the total rubber content to at least 15%. At this concentration, coagulation was complete and the acidic ethanol supernatant contained no detectable particles of rubber. The coagulum was allowed to stand for $\frac{1}{2}$ –1 hr. to consolidate, washed in several changes of water and boiled in *N*-KOH soln. for 1 hr. After further washing, the samples were continuously extracted overnight with hot ethanol, dried under a stream of N_2 at 30° and dissolved in 1 ml. of warm, dry toluene containing 0.5% of trichloroacetic acid. The rubber solution was quantitatively transferred to 10 ml. phials and diluted to a volume of 4 ml. with dry toluene; 1 ml. of scintillation solution [containing 1.5% 2,5-diphenyl-oxazole and 0.15% 1,4-bis-(5-phenyloxazol-2-yl)benzene] was added and the radioactivity determined at 20° in an Ekco model 610 counter. The counting efficiency as measured by internal standard ([^{14}C]toluene) was approx. 30%, with a counting error of 3% and a background of 12.0 counts/min.

[^{14}C]Isopentenyl pyrophosphate (lithium salt). This was prepared as described by Archer *et al.* (1963), and possessed specific activity 11.22×10^5 counts/min./mg.

Centrifuging. Low-speed separations were carried out with a refrigerated MSE centrifuge. High-speed centrifuging was performed with a Spinco model L centrifuge, usually with the SW 39 rotor at 39 000 rev./min. (175 000 g max.) at 0°.

Reagents and chemicals. These were A.R. grade when available. Tris was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) and crystalline bovine serum albumin from Armour Laboratories (Eastbourne, Sussex).

RESULTS AND DISCUSSION

Fig. 1 shows electron micrographs of the rubber particles in Fractions II, III and IV obtained by the centrifugal fractionation procedure described in Scheme 1. Typical particle size–frequency curves

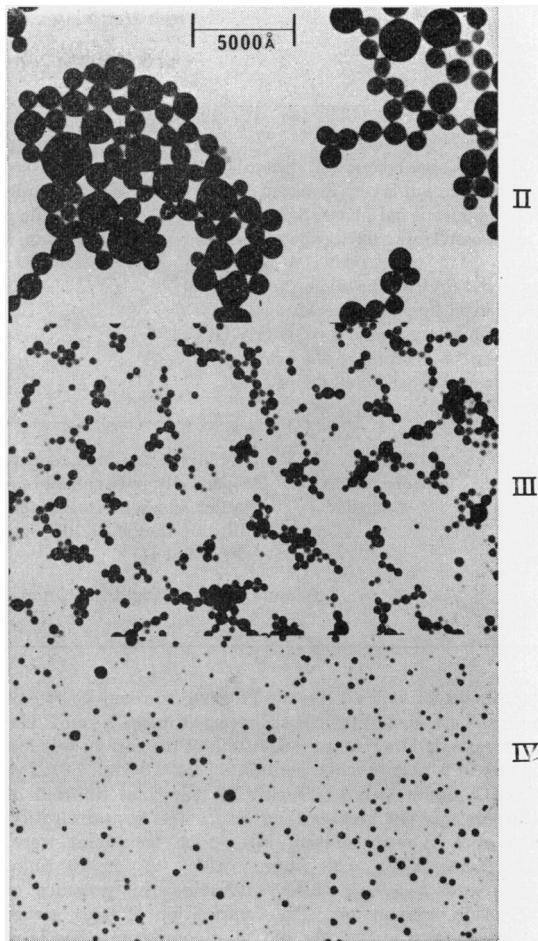


Fig. 1. Electron micrograph of rubber latex fractions II, III and IV: osmium stained. Instrumental magnification $\times 20000$.

for the fractions are shown in Fig. 2. The rubber particles of Fraction I were not examined for size distribution because of their tendency to coagulate on washing and centrifuging again. Previous studies on the nature of the adsorbed film surrounding the rubber particles in *Hevea brasiliensis* latex have established that the film is a complex one, containing as major components proteins and phospholipids (Baker, 1940; Tunnicliffe, 1954). To ascertain qualitatively whether nucleotidic materials were present on the surface of the washed rubber suspensions studied in the present work, 500 mg. samples of Fractions II and III each containing approx. 50 mg. of rubber were heated at 100° for $\frac{1}{2}$ hr. with an equal volume of 20% sodium chloride solution. After removal of the coagulated rubber, the supernatant

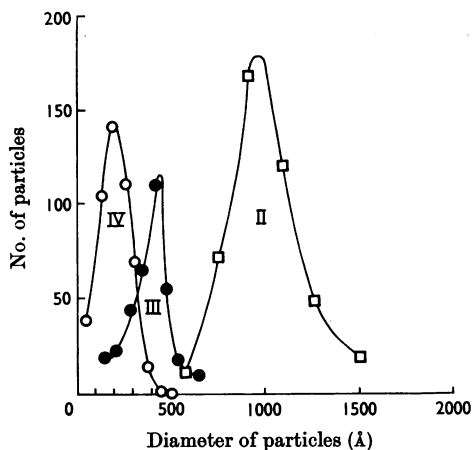


Fig. 2. Size-frequency distribution curves of rubber-latex Fractions II, III and IV. Specific surface areas of the rubber particles ($m^2/g.$) for Fractions II, III and IV were 49, 143 and 244 respectively.

gave in both cases a positive ribose reaction with orcinol and a reproducible ratio of u.v. absorption at $280m\mu$ and $260m\mu$, approx. 0.86, suggesting the presence of a significant amount of nucleotidic material as well as protein on the surface of the washed rubber particles. If all the absorption were due to RNA and protein, the above ratio (0.86) would correspond to a protein containing about 5% of RNA (Layne, 1957).

The total nitrogen content of the washed Fraction II was approx. 0.3% of the weight of rubber. After the rubber had been coagulated, dissolved in toluene containing 0.5% (w/v) trichloroacetic acid, centrifuged and reprecipitated by the addition of an equal volume of methanol, the nitrogen content of the purified rubber was still 0.07% (w/w). This was close to the nitrogen content of *Hevea* rubber after treatment of the latex with proteolytic enzymes (Baker, 1940), indicating that some of the nitrogen at the surface of the rubber particles is particularly difficult to remove and may even be chemically bound to the rubber.

Incorporation of isopentenyl pyrophosphate into rubber. If, in the preparation of latex Fractions II and III, washing of the rubber particles was stopped when the serum had been diluted about 1000-fold with buffer solution (0.2M-tris-hydrochloric acid, pH 8.1), the suspensions would no longer incorporate significant amounts of isopentenyl pyrophosphate into rubber. The original activity could be largely restored, however, by adding a solution of soluble cofactors obtained by dialysing fresh latex serum. It was concluded from this that sufficient enzyme remained on the surface of the rubber particles,

Table 1. Effect of undialysed latex serum on the incorporation of $[1-^{14}C]$ isopentenyl pyrophosphate into the washed rubber particles of latex Fraction II

The rubber particles in samples of latex (Fraction II) were washed by repeated dilution and recentrifugation with 0.2M-tris-HCl buffer, pH 8.1, until the original aqueous serum had been diluted 2.7×10^{11} or 6.6×10^{14} times (serum dilution factor). Samples of the washed suspensions were incubated for 18 hr. at 30° with $50\mu g.$ of isopentenyl pyrophosphate (56100 counts/min.) and 40 mg. of undialysed latex serum (or 40 mg. of cofactor solution only). Total volume of incubation mixture, 0.2 ml.

Serum dilution factor	Wt. of rubber (mg.)	Isoprene residue incorporated ($\mu g./mg.$ of rubber)	
		With added cofactors only	With added latex serum
0	11.0	1.38	—
2.7×10^{11}	8.4	0.05	0.87
6.6×10^{14}	8.3	0.05	0.91

after dilution of the serum phase 1000-fold, for most of the original activity to be restored, provided that the necessary soluble cofactors were added. When further washing of the rubber particles was carried out, as in the normal preparation of Fractions II and III, the ability of the rubber suspension to incorporate isopentenyl pyrophosphate after addition of soluble cofactors gradually decreased. The rate of incorporation approached zero when the aqueous phase of the suspension had been diluted 10^{10} times or more. If at this stage, however, fresh latex serum (essentially rubber-free) was added to the suspension instead of the dialysed cofactor solution, the original activity of the rubber was largely restored, as illustrated in Table 1. Almost identical results were obtained when a serum protein fraction $S_{50/55}$ (see below) and soluble cofactor solution were substituted for the whole latex serum. These experiments demonstrate that the enzyme system responsible for the incorporation of isopentenyl pyrophosphate into rubber is present in latex serum as well as at the surface of unwashed rubber particles. When whole latex serum, or the $S_{50/55}$ protein fraction plus soluble cofactors, was incubated with labelled isopentenyl pyrophosphate in the absence of active rubber particles (inactive carrier rubber being added at the end of the incubation), no significant amount of radioactivity was incorporated into the rubber. These results support the view that, *in vitro*, reaction of the isopentenyl pyrophosphate occurs with existing rubber molecules possessing a terminal allylic pyrophosphate group.

Activity of latex-serum protein fractions. Partial separation of the latex-serum proteins was effected,

Table 2. *Effect of rubber particle size on the incorporation of isopentenyl pyrophosphate*

Samples of latex Fractions II-IV in 0.2M-tris-HCl buffer, pH 8.1, and containing 5-10mg. of rubber, were incubated for 18 hr. at 30° with 250 µg. of [^{14}C]isopentenyl pyrophosphate (280500 counts/min.). For Fractions II and III, 40mg. of latex serum, centrifuged at 170000g for 3 hr., was added.

Latex fraction	Diameter of particles at peak of size-frequency curve (Å)	Specific surface area of rubber (m. ² /mg.)	Isoprene residues incorporated (µg./mg. of rubber)	Area of rubber surface per incorporated isoprene residue (Å ²)
II	900	50	2.0	280
III	400	140	10.0	160
IV	200	240	40.0	70

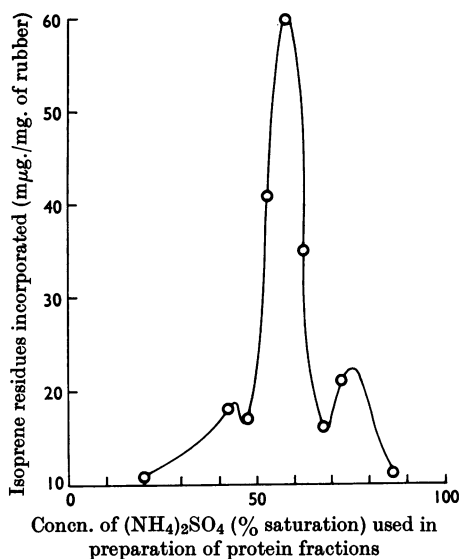


Fig. 3. Relative activities of latex-serum protein fractions as shown by isopentenyl pyrophosphate incorporation into the rubber of latex Fraction II. Samples of latex Fraction II in 0.2M-tris-HCl buffer, pH 8.1, and containing 10mg. of rubber, were incubated for 30 min. at 30° with 50 µg. of [^{14}C]isopentenyl pyrophosphate (56100 counts/min.), 30mg. of cofactor solution and 30mg. of different protein fractions prepared from latex serum by precipitation with ammonium sulphate at the percentage saturation indicated. Total volume of incubation mixture, 0.2 ml.

as described in a previous section, by fractional precipitation with ammonium sulphate. Proteins precipitated by up to 30% saturation with ammonium sulphate were contaminated by very small rubber particles which had been present in the freeze-dried latex-serum solids. If, however, these proteins were first removed, subsequent fractions could be shown by electron microscopy to be essentially free from rubber or any other particulate material.

The relative activities of the different protein fractions (at the same concentration) with respect

to incorporation of isopentenyl pyrophosphate into rubber are shown in Fig. 3. The greatest activity resides in the fraction precipitated in the range 50-65% saturation with ammonium sulphate, i.e. the $S_{50/65}$ fraction. Much smaller activities, possibly not significant, were shown by the $S_{35/45}$ and $S_{70/80}$ fractions.

Influence of rubber particle size. Previous studies, with unwashed fractions of fresh latex, by Archer *et al.* (1963) suggested a higher specific rate of incorporation of isopentenyl pyrophosphate into rubber by the smaller rubber particles. In confirmation of these results, Table 2 shows the amount of isopentenyl pyrophosphate incorporated per milligram of rubber by the Fractions II, III and IV. These fractions were obtained from the same original latex sample, Fraction IV being unwashed for reasons explained above. From electron-microscope measurements of the specific surface area of the rubber particles, the area of surface per incorporated isoprene residue can be calculated. Table 2 shows that this area is large even for the smallest-particle-size fraction (higher than would be required, for example, for a monomolecular layer of close-packed isoprene residues).

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