The Biosynthesis of Rubber

INCORPORATION OF MEVALONATE AND ISOPENTENYL PYROPHOSPHATE INTO RUBBER BY *HEVEA BRASILIENSIS*-LATEX FRACTIONS

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The position of mevalonic acid and isopentenyl pyrophosphate (3-methylbut-3-enyl pyrophosphate) as key intermediates in the biosynthesis of isoprenoid compounds is now firmly established, as also is the route by which mevalonic acid is converted into isopentenyl pyrophosphate. This transformation occurs through three ATP-dependent steps with the intermediate formation of mevalonate 5-phosphate and mevalonate 5-pyrophosphate (Tchen, 1957, 1958; de Waard & Popják, 1959; Chaykin, Law, Phillips, Tchen & Bloch, 1958; Bloch, Chaykin, Phillips & de Waard, 1959; Henning, Möslin & Lynen, 1959; de Waard, Phillips & Bloch, 1959). 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 conversion of isopentenyl pyrophosphate into a number of *trans*-isoprenoid compounds. Because of the ease with which the pyrophosphate anion is eliminated from an allylic compound such as dimethylallyl pyrophosphate, the latter behaves as an electrophilic reagent capable of attacking the methylene group of isopentenyl pyrophosphate, with regeneration of an allylic pyrophosphate end group.

Several workers (Park & Bonner, 1958; Kekwick *et al.* 1959; Lynen & Henning, 1960; Archer, Ayrey, Cockbain & McSweeney, 1961) have shown that mevalonate and isopentenyl pyrophosphate are incorporated into high-molecular-weight rubber by freshly tapped *Hevea brasiliensis* latex, and it has been assumed (Lynen & Henning, 1960) that isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate is again a necessary initial step in the formation of the polyisoprene chain. However, experimental evidence for this view with respect to rubber biosynthesis is still required, particularly since it is not known whether rubber possesses a terminal isopropylidene group (as would be expected if the polyisoprene chain were initiated from dimethylallyl pyrophosphate).

No work has been reported, as yet, on the effect of the different cytological components of latex on the incorporation of mevalonate or isopentenyl

pyrophosphate into rubber. In addition to rubber particles, aqueous serum and a small proportion of microsomes and mitochondria, freshly tapped *Hevea* latex contains specialized particles, the most abundant of which are spherical bodies of about 3 μ diameter, bounded by an osmotically sensitive membrane and possessing a liquid interior in which other material may be suspended (Ruinen, 1950; Schoon & Phoa, 1956). These spherical bodies have been named 'lutoids' by Homans & van Gils (1948). A detailed cytological investigation of *Hevea brasiliensis* latex and latex vessels has been carried out by P. B. Dickenson (unpublished work).

In the present paper studies on the incorporation into rubber of mevalonate and isopentenyl pyrophosphate by the three components rubber, aqueous serum and lutoid particles are described.

MATERIALS AND METHODS

Latex. Fresh *Hevea brasiliensis* latex was obtained from two sources: (i) by pricking the branches of 3-year-old budded trees of clone PB 86 growing in a tropical greenhouse attached to these Laboratories; (ii) from a stand of trees (clone PB 186) in regular tapping at the Rubber Research Institute of Ceylon. This latex was collected into ice-cooled flasks, centrifuged at 2000g to remove lutoid particles and transported by air in polythene bottles surrounded by ice. It arrived at these Laboratories within 36 hr. of tapping.

[1-¹⁴C]Isopentenyl pyrophosphate (lithium salt). Barium [¹⁴C]carbonate (1.972 g.) (1.0 mc/m-mole) was used as a source of ¹⁴CO₂ for the carbonation of the Grignard reagent from 1-chloro-2-methylprop-2-ene in apparatus similar to that of Dauben, Reid & Yankwich (1947). This gave [1-¹⁴C]isopentenoid acid (3-methyl[1-¹⁴C]but-3-enoic acid) which was reduced to [1-¹⁴C]isopentenol with lithium aluminium hydride (Yuan & Bloch, 1959). Two trial experiments on the same scale gave a yield of isopentenol of 63% (based on barium carbonate). On the assumption of this yield in the radioactive preparation, the alcohol was diluted fivefold with inactive isopentenol and converted into lithium [¹⁴C]isopentenyl *N*-ethylphosphoramidate. This was phosphorylated with a solution of 'dioxan phosphate' in anhydrous dioxan (Baer, 1944). [1-¹⁴C]Isopentenyl pyrophosphate was isolated as the lithium salt from the mixture of phosphates and pyrophosphates by a modification of the method of Eggerer & Lynen (1960). The acidified barium isopentenyl pyrophosphate, as

obtained by these authors, was treated with the theoretical amount of lithium sulphate, and the clear solution, obtained after removal of barium sulphate, was brought to pH 8 with lithium hydroxide. Lithium [^{14}C]isopentenyl pyrophosphate was precipitated with ethanol. The product (520 mg.), which appeared to be almost pure by paper-chromatographic assay (solvent system 1; see below) had a specific activity of 1.87×10^4 counts/sec./mg.

Dimethylallyl pyrophosphate (ammonium salt). Dimethylallyl alcohol was prepared by reduction of dimethylacrylic acid with lithium aluminium hydride. The alcohol (22.5 m-moles) was phosphorylated by using the method of Cramer & Böhm (1959) as described by Childs & Bloch (1962) for the preparation of farnesyl pyrophosphate, with the following modification. The phosphorylation was carried out at room temperature and the dark solution so produced was treated with a mixture of 0.1 N-ammonia and ether. The ammoniacal layer was then washed with ether and freeze-dried. The phosphates were precipitated from an ammoniacal solution of the residue by the addition of barium acetate solution. The barium salts were suspended in water, treated with the theoretical amount of sodium sulphate solution (calculated from the barium content), and stirred for 48 hr. The clear supernatant solution was freeze-dried and dimethylallyl pyrophosphate (R_F approx. 0.4) was separated from the mixture by preparative paper chromatography in solvent 4 (EDTA solution being replaced by water), the excised strip being eluted with 0.3% ammonia.

Adenosine triphosphate. This was obtained from Nutritional Biochemicals Corp., and was neutralized with sodium hydrogen carbonate before use. Tween 80 was used as received from Honeywell and Stein Ltd.

DL-[^{14}C]Mevalonic acid lactone. This was obtained as a solution in benzene from The Radiochemical Centre, Amersham, Bucks. The lactone had a specific activity of $4.6 \mu\text{C}/\text{mg}$.

Buffers. 0.11 M-Tris-HCl buffer, pH 7.2, 0.05 M-tris-HCl buffer, pH 7.9, and the phosphate and imidazole buffers of Clark & Lubs (1916) and Mertz & Owen (1940), respectively, were used. Systems containing lutoid particles were usually buffered with 0.11 M-tris-HCl, pH 7.2, containing mannitol (0.4 M) (referred to below as 'tris-mannitol' buffer); at this pH and osmolarity the lutoid particles were relatively stable.

Centrifuging. Low-speed separations were carried out on a refrigerated MSE centrifuge. Ultracentrifuging of latex for the production of serum was performed on a Spinco model L centrifuge with the SW 39 head at 39000 rev./min. (175000 g max.) for 1–3 hr. at 0° .

Paper chromatography. The following solvent systems were used with Whatman 3MM paper: (1) *tert.*-butanol-formic acid-water (20:5:8, by vol.) (descending) (Bloch *et al.* 1959); (2) *tert.*-pentanol-acetic acid-water (4:1:2, by vol.) (ascending) (Lynen, Eggerer, Henning & Kessel, 1958); (3) *n*-butanol-formic acid-water (73:13:10, by vol.) (ascending) (Tchen, 1957); (4) propan-1-ol-aq. ammonia (sp.gr. 0.880)–2 mM-EDTA (free acid) (6:3:1, by vol.) (descending, with Whatman no. 1 paper) (Eggleston & Hems, 1952).

Phosphate spots on the chromatograms were detected with the molybdate-acetone spray of Burrows, Grylls & Harrison (1952), by using the ultraviolet development method described by Bandurski & Axelrod (1951). ATP

and its decomposition products were detected by the photographic method of Markham & Smith (1949). Radioautograms were prepared with Kodak X-ray film with an exposure time of 14 days.

Rubber content of latex samples. A sample of latex containing 5–50 mg. of rubber was weighed into a tared microbeaker of capacity 0.4 ml. and evaporated to dryness. One drop of benzene was then added to swell the rubber and cause the particles to cohere strongly. After being dried, the film of rubber was washed successively with 5% (v/v) acetic acid in ethanol and with water and then digested for 40 min. with boiling *N*-KOH. After further washing with water the purified rubber samples were allowed to stand overnight in ethanol and finally dried to constant weight at 80° under vacuum. The reproducibility was better than 1%.

Isolation of lutoid particles. Fresh latex from the greenhouse trees was mixed immediately with an equal volume of ice-cold tris-mannitol buffer, pH 7.2. The latex mixture was then diluted with another 5 vol. of the tris-mannitol buffer and centrifuged at 1750 g for 10 min. at 0° , when the lutoids separated as the main component of the bottom fraction; the supernatant suspension of rubber particles was discarded. The bottom fraction was resuspended in about 10 vol. of the tris-mannitol buffer at 0° , with slow stirring, and recentrifuged as described above. After the supernatant had been discarded this operation was repeated once more to give finally a suspension of lutoid particles in buffer substantially free from rubber.

Lysis of lutoid particles. Rupture of the membrane surrounding the lutoid particles was effected by adding buffer of sufficiently low osmolarity (0.05 M-tris-HCl buffer, pH 7.2) and freezing the mixture at -15° for 10 min.

Incubation with rubber latex. For incubations of latex with isopentenyl pyrophosphate about 200 mg. of the latex sample was weighed into a 7 ml. tube containing a platinum spiral fitted closely round the inner wall of the tube. Any cofactors etc., were then added, followed by a solution of isopentenyl pyrophosphate in water. Incubations with mevalonic acid or its lactone as precursor were started, unless stated otherwise, by adding the required amount of latex to the dried precursor and cofactors. Sodium mevalonate was obtained by heating mevalonic acid lactone with a small excess of sodium hydrogen carbonate solution at 70° for 30 min. Incubations were terminated by the addition of 0.5 ml. of 5% (v/v) acetic acid in ethanol while the tube was rotated rapidly about a vertical axis. The resulting film of coagulated rubber, which was held in place by the platinum spiral, was washed with the above acid coagulant and then with water. It was next covered with *N*-KOH and heated for 40 min. at 100° , after which the rubber was washed, extracted overnight with boiling ethanol, superficially dried and then dissolved in 3 ml. of a 1% (w/v) solution of trichloroacetic acid in toluene. With this treatment, dissolution of the rubber was complete, and the solution was quantitatively transferred to the vessel of an Ekco scintillation counter and the radioactivity measured by standard procedures, with an internal standard for the determination of counter efficiency (approx. 58%). The background varied between 1.2 and 1.5 counts/sec. The ozonolysis method of Kekwick *et al.* (1959) was used for confirming that the observed radioactivity was due to the incorporation of ^{14}C into rubber only.

In systems containing dimethylallyl pyrophosphate in which it was required to demonstrate that the radioactive

rubber was not of very low molecular weight, the latex mixture after incubation was heated with 0.1 ml. of 6N-KOH for 4 hr. at 100° and then acidified and extracted with toluene. Rubber was precipitated from the toluene solution (dried with calcium chloride) by the addition of 4 vol. of ethanol. After repetition of the precipitation process, the final coagulum of rubber was dissolved in toluene and its radioactivity determined as above. This procedure would be expected to free the rubber from polyisoprene hydrocarbons of molecular weight less than about 1000. Squalene (mol.wt. 410), for example, is readily soluble in ethanol-toluene (4:1, v/v) mixture.

In all systems in which the rubber content was too low for it to be coagulated (or precipitated from solution) without appreciable losses, purified commercial latex of negligible gel content was added after the incubation. All incubations were at 25°.

Incubation of lutoid particles or latex serum. Unless stated otherwise, 0.02 ml. of an aqueous solution of isopentenyl pyrophosphate (2.5 mg./ml.) was incubated with 0.2 ml. of serum or lysed lutoid particles in a 15 ml. centrifuge tube with stopper. Incubations with serum were terminated by the addition of 1.0 ml. of ethanol, and those containing lysed lutoids by the addition of 0.01 ml. of 30% (w/v) trichloroacetic acid, unless otherwise stated.

Specific surface area of rubber particles. Osmic acid was used for hardening and staining the rubber particles in latex samples (P. B. Dickenson, unpublished work) and particle-size distribution curves were determined from electron micrographs. The surface area/g. of rubber is given by:

$$S = \frac{6.0 \sum n_i d_i^2}{D \sum n_i d_i^3} \text{ cm.}^2,$$

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

RESULTS

Incorporation of mevalonate into rubber. Table 1 shows the uptake of [2-¹⁴C]mevalonate into rubber when incubated with freshly tapped whole latex or with the same latex diluted (1:1) with tris-mannitol buffer. The addition of ATP increases the rate of incorporation, as might be expected from the fact that the conversion of mevalonate into isopentenyl pyrophosphate is ATP-dependent (Tehen, 1957, 1958).

To obtain further information on the relatively low rate of incorporation in system (b) of Table 1, a sample of the diluted latex was separated by centrifuging at 1750 *g* for 10 min. at 0° into a top fraction containing most of the rubber, and a bottom fraction which contained all the lutoid particles. The uncentrifuged latex, the top fraction, and a mixture (1:1, w/w) of the top and bottom fractions were then incubated separately with [2-¹⁴C]mevalonate (Table 2). Also examined was a mixture of top and bottom fractions in which the latter had been frozen at -15° for 10 min. to lyse the lutoid particles.

The results with latex B (Table 2) show that lutoids are not required for the incorporation of mevalonate into rubber. Indeed, the low count obtained with latex D indicates that the presence of lysed lutoid particles causes a retardation in the rate of uptake of mevalonate, which can be more than restored, however, by the addition of sufficient ATP.

To determine whether lysed lutoids decompose ATP, lutoid particles (from 2 ml. of latex A) essentially free from rubber and the original serum constituents of the latex were lysed by freezing with 0.4 ml. of 0.05M-tris-hydrochloric acid buffer, pH 7.2. A sample (0.1 ml.) of the mixture was then incubated with 1.2 μ moles of ATP at 25° for 3 hr. Analysis of the reaction products by paper chromatography, with the propan-1-ol-aq. ammonia-EDTA solvent of Eggleston & Hems (1952), showed that most of the ATP had been decomposed into ADP, AMP and other (unidentified) compounds of higher R_f values. No decomposition of the ATP was observed when the lysed lutoids were boiled before incubation or omitted from the incubation mixture.

Table 1. *Incorporation of mevalonate into rubber in undiluted or diluted whole latex*

Each incubation mixture contained 0.378 μ mole of DL-[2-¹⁴C]mevalonate (9380 counts/sec.) and either 250 mg. of undiluted latex or 125 mg. of undiluted latex plus 125 mg. of tris-mannitol buffer, pH 7.2. Incubation was at 25° for 1 hr.

Latex	Added ATP (μ moles)	Incorporation of mevalonate into rubber	
		(counts/sec.)	(μ m-moles)
(a) Undiluted	—	271	10.9
(b) Diluted	—	32	1.3
(c) Diluted	2.5	822	33.1

Table 2. *Incorporation of mevalonate into rubber in whole or fractionated latex*

Each incubation mixture contained 0.378 μ mole of DL-[2-¹⁴C]mevalonate (9380 counts/sec.) and 250 mg. of latex or fractionated latex. Incubation was at 25° for 1 hr.

Latex A: whole latex diluted with an equal weight of tris-mannitol buffer, pH 7.2. Latex B: top fraction (free of lutoids) from latex A. Latex C: mixture (1:1) of latex B with the bottom fraction from latex A. Latex D: as latex C, but with the lutoids of the bottom fraction disrupted by freezing at -15° for 10 min.

Latex	Added ATP (μ moles)	Incorporation of mevalonate into rubber	
		(counts/sec.)	(μ m-moles)
A	—	124	5.0
B	—	180	7.3
C	—	151	6.1
D	—	39	1.6
C	2.5	700	28.2
D	2.5	746	30.1

Formation of isopentenyl pyrophosphate and prior intermediates from mevalonate. It is shown below that isopentenyl pyrophosphate is incorporated into rubber only very slowly if the great majority of the rubber particles in latex are removed by high-speed centrifuging. To obtain a build-up of isopentenyl pyrophosphate or prior intermediates, therefore, latex serum free of lutoids and containing very little rubber was incubated with [2-¹⁴C]mevalonic lactone and ATP, and the products were chromatographed with the *n*-butanol-formic acid-water solvent system of Tehen (1957). Four reaction products were detected, three of them having R_F values identical with the values reported by Witting & Porter (1959) for mevalonate 5-phosphate, mevalonate 5-pyrophosphate and isopentenyl pyrophosphate (Table 3). The fourth component has not been identified.

Incorporation of isopentenyl pyrophosphate into rubber. To determine which fractions of latex are required for the uptake of isopentenyl pyrophosphate into rubber, whole latex diluted with approx. 4 vol. of tris-mannitol buffer, pH 7.2, was separated into a top fraction and a bottom fraction (the latter containing all the lutoid particles) by centrifuging at 1750 *g* for 10 min. A sample of the top fraction was then centrifuged at 175000 *g* for 60 min. to obtain a serum of very low rubber content. Incubations with isopentenyl pyrophosphate were carried out with the systems listed in Table 4, from which it is clear that lutoid particles or other components of the bottom fraction are not required for the uptake of isopentenyl pyrophosphate into rubber, and that the activity of the serum phase is very low. Electron-microscopical examination of serum III showed that some of the smallest rubber particles (less than 0.07 μ diameter) had not been removed and the very small uptake of isopentenyl pyrophosphate in this serum fraction was attributed to the presence of residual rubber.

In view of the results of Table 4, the effect of different variables on the rate of incorporation of isopentenyl pyrophosphate into rubber was studied with latex from which the bottom fraction had been removed by slow-speed centrifuging to give 'top-fraction latex'. The main advantage of using this fraction, rather than whole latex, is that the presence of disrupted lutoid particles which decompose isopentenyl pyrophosphate as a result of phosphatase activity (see below) is avoided.

Effect of isopentenyl pyrophosphate concentration, rubber concentration and pH. The effect of isopentenyl pyrophosphate concentration on its rate of incorporation into rubber is illustrated in Fig. 1. The plots are linear and indicate an order of reaction, with respect to isopentenyl pyrophosphate, appreciably less than unity. In fact the difference in rates

for concentrations of 0.19 and 0.94 μ mole of isopentenyl pyrophosphate/100 mg. of latex was negligible, and the former concentration was therefore normally used in subsequent incubations. The activities of most latex samples decreased to about one-half of their original values after 3-4 days of storage at 0° in closed vessels.

In studying the effect of rubber content, samples of top-fraction latex were diluted with essentially rubber-free serum obtained by high-speed centrifuging of a mixture (1:1, w/w) of top-fraction latex and tris-hydrochloric acid buffer, pH 7.9, so as to give a series of samples with rubber contents ranging from about zero to 23% by weight. The rate of incorporation of isopentenyl pyrophosphate in each sample was then determined from the linear uptake-time plots. The rates varied directly with rubber content up to a concentration of about 15% of rubber (Fig. 2).

Table 3. R_F values of reaction products from the incubation of [2-¹⁴C]mevalonic lactone and adenosine triphosphate with latex serum

Compound	Probable identity	R_F	
		Observed	Witting & Porter (1959)
1	Mevalonate 5-pyrophosphate	0.03	0.03
2	Unidentified	0.13	—
3	Mevalonate 5-phosphate	0.18	0.18
4	Isopentenyl pyrophosphate	0.30	0.32

Table 4. Incorporation of isopentenyl pyrophosphate into rubber by whole latex or latex fractions

Each incubation mixture contained 350 mg. of diluted latex or serum, at pH 7.2, and 0.0186 μ mole of isopentenyl pyrophosphate (577 counts/sec.). Sample II was prepared by centrifuging sample I at 1750 *g* for 10 min. and discarding the bottom fraction containing all the lutoid particles. The serum (sample III) was obtained by centrifuging sample II at 17500 *g* for 60 min. Incubation was at 25° for 20 min. For the assay of sample III carrier rubber was added after the incubation.

System	Isopentenyl pyrophosphate incorporated into rubber	
	(counts/sec.)	(μ m-moles)
Sample I (diluted whole latex)	282	9.1
Sample II (top fraction from sample I)	335	10.8
Sample III (serum from sample II)	3	0.1

The effect of pH over the range 6.1–7.9 is shown in Fig. 3. Because of the low buffering capacity of tris-hydrochloric acid below pH 7.2, this system was examined only between pH 7.2 and 7.9. Over this range, it gave the same results as the imidazole-hydrochloric acid buffers. Phosphate appears to have a retarding effect on the rate of incorporation, as compared with the other two buffers.

Effect of surface area of rubber particles. A sample of top-fraction latex was diluted with 2 vol. of 0.11M-tris-hydrochloric acid buffer, pH 7.9, containing 0.1% of Tween 80 to increase the colloidal

stability of the latex. Preliminary experiments had shown that this concentration of Tween 80 did not affect appreciably the rate of incorporation of isopentenyl pyrophosphate into rubber. The latex was then separated into four fractions of different average particle size by centrifuging. The particle-size range in each fraction was narrowed further by diluting with the serum from a high-speed-centrifuged sample of the buffered latex, and recentrifuging. The rubber content of each was then determined and the particle-size distribution and specific surface area of the particles were obtained from electron micrographs. The rate of incorporation of isopentenyl pyrophosphate in each fraction is given in Table 5, after correction for differences in rubber content (Fig. 2).

In addition to providing evidence that incorporation of isopentenyl pyrophosphate occurs at the surface of the rubber particles, Table 5 indicates that the smaller particles may be somewhat more effective, per unit surface area, than the larger ones.

Role of dimethylallyl pyrophosphate. The uptake of isopentenyl pyrophosphate into rubber in the experiments described above could be due to the formation of new rubber from a 'starter' molecule such as dimethylallyl pyrophosphate, or to the incorporation of isopentenyl pyrophosphate into existing rubber chains (most probably by reaction with terminal allylic pyrophosphate groups on the rubber). In an attempt to distinguish between these possibilities, top-fraction latex was incubated with [1-¹⁴C]isopentenyl pyrophosphate in the

Fig. 1. Effect of isopentenyl pyrophosphate concentration on its rate of incorporation into rubber in top-fraction latex. Each incubation mixture contained 100 mg. of latex, 100 mg. of tris-HCl buffer, pH 7.9, and the following amounts (dissolved in 0.1 ml. of water) of [1-¹⁴C]isopentenyl pyrophosphate: ▽, 0.048 μmole; ●, 0.095 μmole; ×, 0.19 μmole; ○, 0.94 μmole.

Fig. 2. Effect of rubber content on the rate of incorporation of isopentenyl pyrophosphate into rubber in diluted top-fraction latex. Each incubation mixture contained 230 mg. of diluted latex (with serum plus tris-HCl buffer, pH 7.9, as diluent) and 0.19 μmole of [1-¹⁴C]isopentenyl pyrophosphate (944 counts/sec.) in 0.02 ml. of water.

Fig. 3. Effect of pH on the rate of incorporation of isopentenyl pyrophosphate into rubber in top-fraction latex. Each incubation mixture contained 84 mg. of latex, 146 mg. of buffer solution [imidazole-HCl (●) or sodium pyrophosphate (○)] and 0.19 μmole of [1-¹⁴C]isopentenyl pyrophosphate (944 counts/sec.) in 0.02 ml. of water.

Table 5. *Influence of size of rubber particles (from top-fraction latex) on the rate of incorporation of [1-¹⁴C]isopentenyl pyrophosphate into rubber*

Experimental details are given in the text.

	Latex A	Latex B	Latex C	Latex D
Relative centrifugal-force range for separation (g)	< 4000	4000-7000	7000-10000	10000-50000
Rubber content (% w/w)	13.1	14.0	7.84	7.33
Mean particle diameter $\pm$ s.d. (μ)	1.11 ± 0.15	0.37 ± 0.13	0.13 ± 0.06	0.08 ± 0.02
Specific surface area of rubber (m. ² /g.)	5.2	13.5	37	71
Rate of incorporation of isopentenyl pyrophosphate per g. of rubber (counts/sec./min. of incubation)	8.5	18.5	162	513
Rate of incorporation of isopentenyl pyrophosphate per cm. ² of rubber surface (counts/sec./min. of incubation)	1.6	1.4	4.4	7.2

Fig. 4. Effect of dimethylallyl pyrophosphate on the rate of incorporation of isopentenyl pyrophosphate into rubber in top-fraction latex. Each incubation mixture contained 100 mg. of latex, 100 mg. of tris-HCl buffer, pH 7.9, 1.25 μ moles of $MgSO_4$, 0.19 μ mole of [1-¹⁴C]isopentenyl pyrophosphate (944 counts/sec.) in 0.02 ml. of water and either 20 mg. of water (○) or 1.134 μ moles of dimethylallyl pyrophosphate in 0.02 ml. of water (●).

presence of unlabelled dimethylallyl pyrophosphate. Unexpectedly, the latter retarded the rate of incorporation of the isopentenyl pyrophosphate into rubber; indeed the rate became effectively zero (Fig. 4) while the percentage incorporation was still low (the retardation occurs whether magnesium is added as a cofactor or not).

The inhibitory effect of dimethylallyl pyrophosphate is not due to isotope dilution by any isopentenyl pyrophosphate which might be formed from the dimethylallyl pyrophosphate by isomerase activity. Not only is the shape of the rate curve in Fig. 4 different from that to be expected for a simple dilution effect, but investigations by D. Barnard (unpublished work) have shown that the inhibition is caused by the enzymic formation of non-rubber products in latex serum due to the reaction of isopentenyl pyrophosphate with di-

methylallyl pyrophosphate. Steps were therefore taken to replace the serum by an enzymically inactive solution without preventing incorporation of isopentenyl pyrophosphate into the rubber. This was achieved by the procedure shown in Fig. 5, in which the small and medium-sized rubber particles in top-fraction latex were repeatedly washed with the boiled serum from a sample of top-fraction latex previously diluted with an equal weight of tris-hydrochloric acid buffer, pH 7.9. The purpose of removing the larger rubber particles (see Scheme 1) was to increase the colloidal stability of the latex, since the larger particles have a greater tendency to coalesce during centrifuging.

The washed rubber suspension, and also separate samples of the serum obtained from a portion of the suspension by high-speed centrifugation, were then incubated with [1-¹⁴C]isopentenyl pyrophosphate with and without the addition of unlabelled dimethylallyl pyrophosphate. The conversion of isopentenyl pyrophosphate into non-rubber products in the serum was found to be negligible whether dimethylallyl pyrophosphate was added or not, but the rate of incorporation of isopentenyl pyrophosphate into rubber in the suspension was considerably increased by the addition of dimethylallyl pyrophosphate (Fig. 5), indicating that the latter was acting as a 'starter' for the formation of new rubber chains. No significant decrease in the radioactivity of either rubber sample occurred when it was precipitated from toluene solution by 4 vol. of ethanol, thus showing that none of the rubber was of very low molecular weight.

The effect of pretreating latex with acid solutions that might be expected to remove any allylic pyrophosphate groups from the rubber was also studied. Top-fraction latex was diluted (1:1, w/w) with tris-hydrochloric acid buffer, pH 7.9, and brought to pH 2.0 by adding it to the required amount of N-hydrochloric acid. After standing for 5 min. the mixture was added to sufficient N-sodium hydroxide to restore the pH to 7.9. Microscopical exami-

Scheme 1. Scheme for replacement of serum in top-fraction latex.

Fig. 5. Effect of dimethylallyl pyrophosphate on the rate of incorporation of isopentenyl pyrophosphate into rubber in a suspension of rubber particles in boiled latex serum plus tris-HCl buffer, pH 7.9. Each incubation mixture contained 85 mg. of rubber in 41 mg. of boiled latex serum plus 74 mg. of tris-HCl buffer, pH 7.9, 0.19 μ mole of [1- 14 C]-isopentenyl pyrophosphate (944 counts/sec.) in 0.02 ml. of water, and either 0.02 ml. of water (●) or 1.134 μ moles of dimethylallyl pyrophosphate in 0.02 ml. of water (O).

nation gave no evidence of particle aggregation. The rate of incorporation of isopentenyl pyrophosphate into rubber, in this latex, was extremely small even when rubber-free latex serum (not acid-treated) prepared by the high-speed centrifuging of top-fraction latex was added (Table 6). Experiments in which the latex was adjusted to pH 5.2 and allowed to stand for 1 hr. or 16 hr. before re-adjusting to pH 7.9 gave similar results (Table 6), except that the loss of activity of the latex occurred more slowly than at pH 2.

Action of lysed lutoid particles on isopentenyl pyrophosphate. Attempts to demonstrate the

presence of isopentenyl pyrophosphate isomerase in rubber latex have not yet succeeded, but during the present work it was shown that lysed lutoid particles can rapidly hydrolyse isopentenyl pyrophosphate to isopentenol by phosphatase action.

A rubber-free suspension of lutoid particles, prepared and lysed as described in the Materials and Methods section, was incubated with [1- 14 C]-isopentenyl pyrophosphate for 1 hr. The mixture was then saturated with sodium chloride and extracted several times with benzene to remove all extractable radioactivity. Controls showed that only an insignificant amount of isopentenyl pyrophosphate was extracted from aqueous solution. Before the last extraction, the mixture was heated at 80° for 5 min. to inhibit any further enzyme activity. Samples of the extracted mixture were then acidified to pH 2 to convert any dimethylallyl pyrophosphate or other allylic pyrophosphates into the corresponding alcohols, and after standing at 20° (overnight for convenience) were re-extracted with benzene. Table 7 (Expts. 2 and 3) shows that nearly 60% of the initial isopentenyl pyrophosphate was converted into benzene-soluble material, but that no further radioactivity could be extracted after acidification, thus indicating that no allylic pyrophosphates were formed during the incubation. In parallel experiments with unlabelled isopentenyl pyrophosphate, the benzene-soluble material was identified by gas-liquid chromatography as isopentenol; no other reaction product was detectable.

To take account of the possibility that dimethylallyl pyrophosphate might have been formed during the incubations, but hydrolysed almost completely to the alcohol by phosphatase action and extracted as such before acidification, a large pool of unlabelled dimethylallyl pyrophosphate was included

Table 6. *Incorporation of isopentenyl pyrophosphate into rubber by latex pretreated with hydrochloric acid*

Each incubation mixture contained 0.19 μ mole of [1^{14}C]isopentenyl pyrophosphate (917 counts/sec.). The pH of the incubation mixtures was 7.9. Incubation was at 25° for 30 min.

Expt. no.	Wt. of latex (mg.)	Pretreatment of latex	Additional* tris-HCl, pH 7.9 (mg.)	Additional* latex serum (mg.)	Incorporation of isopentenyl pyrophosphate into rubber (counts/sec.)
1	150	5 min. at pH 2	80	—	2
2	150	5 min. at pH 2	—	80	3
3	150†	None	80	—	105
4	200	16 hr. at pH 5.2	—	—	3
5	200	1 hr. at pH 5.2	—	—	28
6	200†	None	—	—	150

* These additions were made immediately before incubation.

† In these controls sodium chloride was added in amount equal to that formed in the corresponding pretreatment process.

Table 7. *Hydrolysis of isopentenyl pyrophosphate by lysed lutoid particles*

Each incubation mixture contained 0.19 μ mole of [1^{14}C]isopentenyl pyrophosphate (917 counts/sec.) in 0.02 ml. of water. Incubation was at 25° for 1 hr.

(a) Expts. 2, 3, 4 and 5 each contained one-sixth of the lutoids (lysed) obtained from 1.0 ml. of greenhouse latex; the suspension medium was 0.05M-tris-HCl buffer, pH 7.2; (b) 0.05M-tris-HCl buffer, pH 7.2; (c) solution of 1.9 μ moles of dimethylallyl pyrophosphate in 0.08 ml. of water.

Expt. no.	Additions (ml.)				Radioactive material extractable by benzene (counts/sec.)	
	Lysed lutoid suspension (a)	Buffer solution (b)	Dimethylallyl pyrophosphate solution (c)	Water	Before acidification	After acidification (additional counts/sec.)
1	—	0.10	—	0.08	3	2
2	0.10	—	—	0.08	515	4
3	0.10	—	—	0.08	546	2
4	0.10	—	0.08	—	42	2
5	0.10	—	0.08	—	79	2

in some of the incubations. Under these conditions (Table 7: Expts. 4 and 5) much less of the isopentenyl pyrophosphate was hydrolysed, owing to the competitive substrate action of dimethylallyl pyrophosphate, but acidification of the incubation mixture again indicated that the lysed lutoids possessed no isopentenyl pyrophosphate-isomerase activity.

DISCUSSION

Evidence that mevalonate is converted into isopentenyl pyrophosphate in the aqueous phase of latex, through the intermediate formation of mevalonate 5-phosphate and mevalonate 5-pyrophosphate, is provided by the radioautograph results summarized in Table 3. There is no reason to assume that the unidentified compound was another intermediate in this pathway.

Table 5 (with Fig. 2) demonstrates that the incorporation of isopentenyl pyrophosphate into rubber occurs at the surface of existing rubber particles. Electron microscopy showed that the latex fraction of largest particle size in Table 5

contained no particles less than 0.3 μ in diameter; further, all the particles were spherical and of uniform electron density after staining with osmic acid. These observations confirm that the uptake of isopentenyl pyrophosphate could not have been due to particles other than rubber, and suggest that the mechanism by which rubber particles increase in size, *in vivo*, is by the formation of rubber molecules at the surface of the particles rather than by the colloidal aggregation of the particles themselves (though this may occur occasionally).

The results of Fig. 5, showing that incorporation of isopentenyl pyrophosphate into a suspension of washed rubber particles is significantly increased by the addition of dimethylallyl pyrophosphate, provide strong evidence that new rubber chains are formed from dimethylallyl pyrophosphate as the 'starter' molecule. The insolubility of this new rubber in ethanol-toluene (4:1, v/v) shows that it could not be material of very low molecular weight. It is reasonable to assume that dimethylallyl pyrophosphate is produced in latex *in vivo* by the isomerization of isopentenyl pyrophosphate, since

this is an obligatory step in the biosynthesis of squalene and sterols in yeast (Agranoff *et al.* 1960), and *Hevea* latex contains about 1% of sterol (mainly β -sitosterol) on a dry-weight basis. Some of this sterol is distributed throughout the rubber particles (Tunnicliffe, 1954). Natural rubber has a wide distribution of molecular weight (from less than 100 000 to 1 000 000 or more) with an average of about 500 000, corresponding to about 7 000 isoprene units/molecule. Thus considerably less dimethylallyl pyrophosphate should be required to form the rubber in latex than to initiate the 1% of sterol. The build-up of the rubber chain from dimethylallyl pyrophosphate presumably occurs by the repeated addition of isopentenyl pyrophosphate, with elimination of pyrophosphate, as proposed by Lynen *et al.* (1958), i.e.

In the incubation experiments in which no dimethylallyl pyrophosphate was added, it is unlikely that the formation of new rubber chains accounts for more than a very small proportion of the uptake of isopentenyl pyrophosphate, nearly all of which is probably incorporated into existing rubber chains possessing an allylic pyrophosphate end group. Some support for this view is provided by the large decrease in isopentenyl pyrophosphate uptake (Table 6) caused by acid pretreatment of the latex. In addition, D. Barnard (unpublished work) has shown that treatment of latex with phosphatase causes a large decrease in the subsequent rate of incorporation of isopentenyl pyrophosphate into rubber, which cannot be accounted for by a diminution in isopentenyl pyrophosphate concentration due to phosphatase action. This evidence that rubber with a terminal pyrophosphate group can act as a primer for the further incorporation of isopentenyl pyrophosphate suggests that the build-up of the rubber chain from dimethylallyl pyrophosphate occurs by a series of steps rather than by a single concerted reaction.

The function of the luteoid particles in latex remains unknown. They possess no detectable isopentenyl pyrophosphate-isomerase activity (Table 7), and the incorporation of isopentenyl pyrophosphate or mevalonate into rubber proceeds at least as rapidly after their removal from the latex (Tables 4 and 2). Indeed, the presence of lysed luteoids greatly retarded the incorporation of mevalonate, a result that can be attributed to their activity in hydrolysing ATP. Lysed luteoids also hydrolyse isopentenyl pyrophosphate (Table 7),

and difficulties in obtaining reproducible results for the incorporation of mevalonate or isopentenyl pyrophosphate into rubber may be expected if the luteoids are not removed from the latex, since the membrane surrounding the luteoid particles is very sensitive to disruption by osmotic and other forces.

The mechanism by which new rubber particles are initiated in latex remains obscure. The range in diameter of the rubber particles in typical latex samples examined by van den Tempel (1952) extended from less than 0.05μ to just over 1.0μ , with a peak in the size-frequency curve at about 0.07μ . From van den Tempel's results it can be estimated that the number of particles/g. of rubber was approx. 10^{14} . If each of these particles were initiated by the formation in the serum, or other non-rubber phase, of a rubber molecule of mol.wt. 500 000 (the diameter of which, as a sphere, would be 0.012μ), it can be calculated that only about 1 part in 10 000 of the total isopentenyl pyrophosphate converted into rubber *in vivo* would be required for the production of these molecular 'nuclei'. Such small proportions of isopentenyl pyrophosphate, if converted into rubber nuclei in the serum or other non-rubber phase of our incubation mixtures, would not have been detected. Thus, provided that coalescence between the rubber particles in latex as a result of colloidal factors is not appreciable, the observed particle-size-frequency curve implies that the formation of new rubber molecules at the surface of the particles is a much more rapid process than the initiation of the particle nuclei.

SUMMARY

1. Mevalonate is converted into isopentenyl pyrophosphate in the aqueous phase of *Hevea brasiliensis* latex, through the intermediate formation of mevalonate 5-phosphate and mevalonate 5-pyrophosphate.

2. Isopentenyl pyrophosphate is incorporated into rubber at the surface of the rubber particles in latex. This incorporation is attributed mainly to the reaction of isopentenyl pyrophosphate with rubber molecules originally present and possessing a terminal (allylic) pyrophosphate group.

3. The addition of dimethylallyl pyrophosphate to latex causes a decrease in the uptake of isopentenyl pyrophosphate into rubber owing to the enzymic formation of inhibitors in the aqueous phase of the latex. If the enzymes in the aqueous phase are removed, the incorporation of isopentenyl pyrophosphate into rubber is increased by the addition of dimethylallyl pyrophosphate, indicating that the latter acts as a 'starter' for the formation of new rubber chains.

4. Particle-size distribution data for *Hevea* latex suggest that *in vivo* the rate of initiation of new rubber particles in the aqueous or other non-rubber phase of latex may be very slow, compared with the rate of incorporation of isopentenyl pyrophosphate into rubber at the surface of existing particles. Experimentally, the conversion of isopentenyl pyrophosphate into rubber in the aqueous phase of latex *in vitro* is so small that it could be accounted for by incomplete removal of rubber during centrifugal separation of the aqueous phase.

5. *Hevea brasiliensis* latex contains a proportion of non-rubber particles bounded by an osmotically sensitive membrane (lutoid particles) which, on disruption, hydrolyse ATP and thereby retard the incorporation of mevalonate into rubber. Disrupted lutoids also hydrolyse isopentenyl pyrophosphate to isopentenol, but possess no detectable isopentenyl pyrophosphate-isomerase activity. Lutoid particles are not necessary for the incorporation of isopentenyl pyrophosphate into rubber *in vitro*.

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The Isolation, Characterization and Acid-Base Properties of Ribonucleic Acid from Rabbit-Reticulocyte Ribosomes

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The importance of ribonucleic acids in protein biosynthesis is now well established (Chantrenne, 1961). The incorporation of amino acid residues into peptide linkage in specific sequence appears to be under the control of RNA, ranging from intermediate to high molecular weight (Otaka, Mitsui & Osawa, 1962; Ishihama, Mizuno, Takai, Otaka & Osawa, 1962; Monier, Naono, Hayes, Hayes &

Gros, 1962). The bulk of the ribosomal RNA differs from 'messenger' RNA in nucleotide composition (Volkin & Astrachan, 1956*a, b*; Volkin, Astrachan & Countryman, 1956), rate of metabolic turnover (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961) and metabolic activity in cell-free systems (Nirenberg & Matthaei, 1961; Wood & Berg, 1962). Further, DNA and 'messenger' RNA form hybrid