Eur. J. Biochem. *218*, 267–271 (1993)

The separate roles of plant *cis* and *trans* prenyl transferases in *cis*-1,4-polyisoprene biosynthesis

Katrina CORNISH

USDA-ARS, Western Regional Research Center, Albany, USA

(Received July 15, 1993) – EJB 931059/3

In plants, the elongation of *cis*-1,4-polyisoprene (natural rubber, $M_r > 10^6$) requires a small *trans*-allylic diphosphate ($\leqslant C_{20}$) initiator. The *trans*-allylic diphosphates are hydrophilic cytosolic compounds, whereas *cis*-1,4-polyisoprene is hydrophobic and compartmentalised in subcellular rubber particles.

In this paper, it is demonstrated that soluble *trans*-prenyl transferase from latex of *Hevea brasiliensis* functions solely as farnesyl diphosphate synthase, and plays no direct role in *cis*-1,4-polyisoprene elongation. The *cis*-1,4-prenyl transferase is firmly associated with the *H. brasiliensis* rubber particle, as is also the case in other rubber-producing species [Archer, B. L., Audley, B. G., Cockbain, E. G. & McSweeney, G. P. (1963) *Biochem. J. 89*, 565–574; Madhavan, S., Greenblatt, G. A., Foster, M. A. & Benedict, C. R. (1989) *Plant Physiol. 89*, 506–511; Siler, D. J. & Cornish, K. (1993) *Phytochemistry 32*, 1097–1102]. The experimental data explain and refute previous reports in which soluble *trans*-prenyl transferase isolated from *H. brasiliensis* latex was attributed both *trans*-prenyl transferase and *cis*-prenyl transferase activities [Light, D. R. & Dennis, M. S. (1989) *J. Biol. Chem. 264*, 18589–18597; Light, D. R., Lazarus, R. A. & Dennis, M. S. (1989) *J. Biol. Chem. 264*, 18598–18607].

Thus, it appears that plant prenyl transferases are comparable to animal enzyme systems in which *trans*-prenyl transferases are soluble enzymes whilst *cis*-prenyl transferases are membrane-bound [Ericcson, J., Runquist, M., Thelin, A., Andersson, M., Chojnacki, T. & Dallner, G. (1992) *J. Biol Chem. 268*, 832–838].

Hevea brasiliensis is currently the world's sole commercial source of natural rubber (*cis*-1,4-polyisoprene). In this species, the rubber is produced in the form of cytoplasmic rubber particles contained within a living latex produced in laticifers (d'Auzac et al., 1989). Natural rubber is made almost entirely of isoprene units derived from isopentenyl diphosphate (IPP). The polymerization of the natural rubber polymer is catalyzed by the enzyme rubber transferase. Rubber transferase, a *cis*-prenyl transferase, requires divalent cations (such as Mg^{2+} or Mn^{2+}) for activity, but rubber transferase, IPP and Mg^{2+} together will not result in rubber biosynthesis; a second substrate, an allylic diphosphate, is needed to initiate the polymerization process (Archer and Audley, 1987; Cornish and Backhaus, 1990; Madhavan et al., 1989). Hence, rubber molecule formation requires three distinct biochemical processes: (a) initiation, which requires an allylic diphosphate molecule (synthesis of allylic diphosphates is catalysed by *trans*-prenyl transferase enzymes); (b) elongation, the rubber transferase-catalyzed *cis*-1,4-polymerization of isoprene units from IPP; (c) termination, the release of the polymer from the rubber transferase. The first two steps are detailed in Fig. 1.

Since the hydrophobic rubber molecule is packaged inside a rubber particle, but is formed from hydrophilic diphosphate substrates obtained from the cytoplasm, the polymerization reaction must take place at the particle surface. Particle-bound rubber transferases have been demonstrated in the rubber-producing species *Parthenium argentatum* (Cornish and Backhaus, 1990; Madhavan et al., 1989) and *Ficus elastica* (Siler and Cornish, 1993). It has been reported that rubber transferase may be bound to the rubber particle in *H. brasiliensis* (Archer et al., 1963; Audley and Archer, 1988; Berndt, 1963). However, it also has been postulated that rubber biosynthesis, in *H. brasiliensis*, is mediated instead by the association of a soluble *trans*-prenyl transferase (Light and Dennis, 1989) with a small, particle-bound protein, rubber-elongation factor (Dennis and Light, 1989; Light et al., 1989).

In this paper, *H. brasiliensis* rubber biosynthesis is re-examined and the conflicting models described above are finally resolved.

MATERIALS AND METHODS

Plant material

H. brasiliensis buffered, living latex, from clone PB260, was generously provided by J. R. Bugansky (Senior Botanist,

Correspondence to K. Cornish, USDA-ARS-WRRC-PBT, 800 Buchanan Street, Albany, CA 94710, USA

Fax: +1 510 5595777.

Abbreviations. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; OMBPP, 3,4-oxido-3-methyl-1-butyl diphosphate.

Enzymes. Rubber transferase (EC 2.5.1.20); isopentenyl-diphosphate isomerase (EC 5.3.3.2).

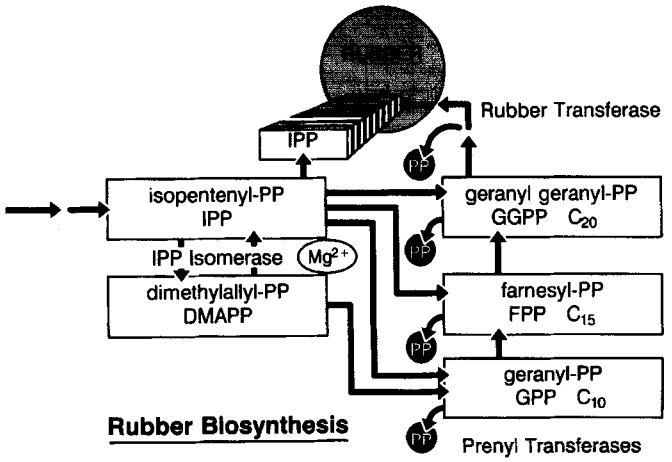

Fig. 1. Natural rubber biosynthesis from isopentenyl diphosphate. The synthesis of the allylic diphosphates occurs in the cytosol. All the allylic diphosphates shown can initiate rubber-molecule formation in isolated rubber particles, not only geranyl geranyl-*PP* (GGPP) as simply shown here. The *cis*-1,4-polymerization of isoprene into rubber is catalyzed by a particle-bound rubber transferase.

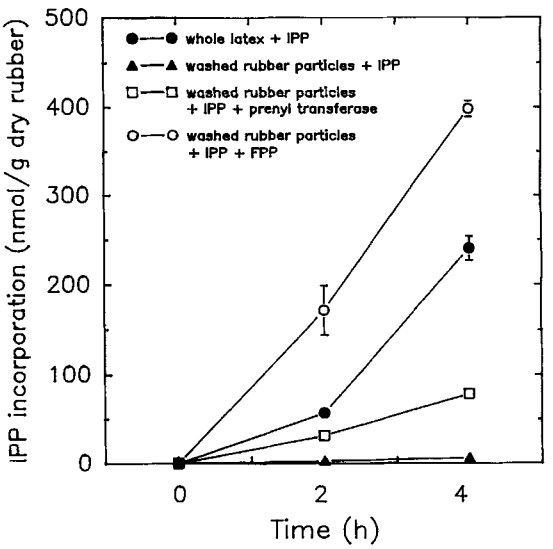

Fig. 2. Rubber transferase activity in whole latex and purified rubber particles. 80 μM IPP, 20 μM FPP and 12 μg · cm^{-3} prenyl transferase were added as indicated. Reactions were carried out at 26°C. Each value is the mean of three experiments ± SE. Experimental details are described in Materials and Methods. (●) Whole latex + IPP; (▲) washed rubber particles + IPP; (□) washed rubber particles + IPP + prenyl transferase; (○) washed rubber particles + IPP + FPP.

Goodyear Tire & Rubber Co.) from plantation trees grown in Sumatra, as described (Siler and Cornish, 1993).

Enzymes and isomerase inhibitor

Purified *H. brasiliensis* prenyl transferase, greater than 95% purity (Light and Dennis, 1989), was the generous gift of Mark S. Dennis (Genentech). IPP isomerase (Anderson et al., 1989) and 3,4-oxido-3-methyl-1-butyl diphosphate (OMBPP), a site-directed specific inhibitor of isomerase activity (Muehlbacher and Poulter, 1988) were generously supplied by C. Dale Poulter, University of Utah, USA.

Purification of washed rubber particles

Washed rubber particles were prepared from whole latex of *H. brasiliensis* by a centrifugation/flotation procedure described for *P. argentatum* (Cornish and Backhaus, 1990) with some modification of centrifugation speed and duration (Siler and Cornish, 1993).

Assay of prenyl transferase activity

Reactions were performed in a volume of 500 μl, containing 80 μM [^{14}C]IPP (1.16 μCi · $mmol^{-1}$), 50 mM Tris/HCl, pH 7.5, 1 mM $MgSO_4$ and 10 mM dithiothrietol. Treatments included combinations of 4 μg prenyl transferase, 1 μg IPP isomerase (19 μmol · min^{-1} · mg^{-1}), 20 μM OMBPP and 20 μM dimethylallyl diphosphate (DMAPP). Reactions were incubated for 40 min at 25°C. All reactions were stopped by the addition of 25 μl 1 M EDTA. Enough solid NaCl was added to each tube to saturate the samples with some NaCl left undissolved. The samples were then partitioned three times against 750 μl aliquots of n-butanol. The radioactivity in the combined n-butanol fractions was determined by liquid scintillation spectroscopy. Background values were determined in the presence of 25 μl 1 M EDTA and were subtracted.

Assay of rubber transferase activity

Rubber transferase activity of rubber particle preparations from *H. brasiliensis* was determined by a method previously described for *P. argentatum* (Cornish and Backhaus, 1990). Whole latex or washed rubber particles were incubated in 5 mM dithiothreitol, 1 mM $MgSO_4$, 100 mM Tris/HCl, pH 7.5, with appropriate concentrations of [^{14}C]IPP, farnesyl diphosphate (FPP), DMAPP, prenyl transferase and OMBPP. Except where stated otherwise, 80 μM [^{14}C]IPP (1.16 μCi · $mmol^{-1}$) and 20 μM FPP were used in the rubber-transferase-activity assays. The rubber particles (4–12 mg · $reaction^{-1}$) were harvested by filtration, the mass determined, the filters washed to remove unincorporated radiolabelled IPP, and the radioactivity on the filters (the newly synthesized polyisoprene) determined by liquid scintillation spectroscopy.

RESULTS

Rubber transferase activity in whole latex of *H. brasiliensis*, assayed in the presence of [^{14}C]IPP, is shown in Fig. 2. Washing the rubber particles using the centrifugation/flotation procedure (see Materials and Methods) almost completely eliminates IPP incorporation. The addition of soluble *trans*-prenyl transferase to washed rubber particles and IPP restored some rubber transferase activity, although not to the level observed in whole latex. These results are in agreement with the findings of Light and Dennis (1989), in which the addition of the prenyl transferase to rubber particles, partly purified by gel filtration, slightly increased rubber transferase activity. One possible explanation for the partial reconstitution of activity is that prenyl transferase acts as rubber transferase, as has been proposed (Light and Dennis, 1989). However, when FPP (at a concentration 25 times the apparent rubber transferase FPP K_m) was added to washed rubber particles and IPP, a rate of IPP incorporation far above that in whole latex or in washed rubber particles containing IPP and prenyl transferase was observed (Fig. 2). FPP is known to be an effective initiator of rubber molecule formation (Archer and Audley, 1987; Cornish and Backhaus, 1990).

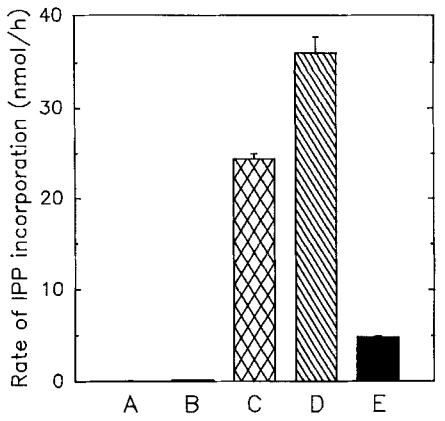

Fig. 3. Soluble prenyl transferase activity in the presence of (A) IPP, (B) IPP + prenyl transferase, (C) IPP + prenyl transferase + DMAPP, (D) IPP + prenyl transferase + isomerase, and (E) IPP + prenyl transferase + isomerase + OMBPP (isomerase inhibitor). Concentrations were as follows: IPP, 80 μM; prenyl transferase, 4 μg · cm^{-3}; DMAPP, 20 μM; isomerase, 2 μg · cm^{-3}; OMBPP, 20 μM. The reactions were incubated for 40 min at 26°C. Each value is the mean of three experiments ± SE. Experimental details were as described in Materials and Methods.

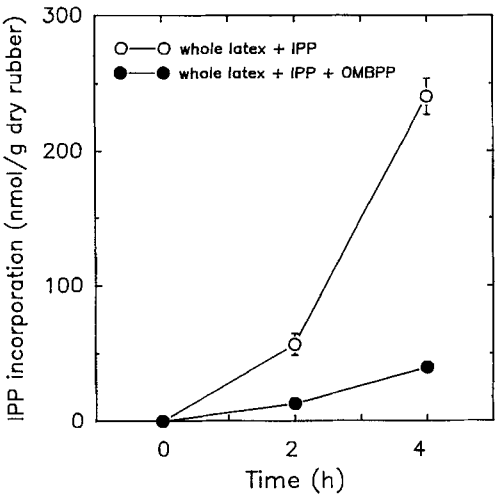

Fig. 4. Rubber transferase activity in the whole latex in the presence and absence of 20 μM OMBPP (isomerase inhibitor). OMBPP was incubated on ice with whole latex for 1 h before the activity assay was begun. Each value is the mean of three experiments ± SE. Experimental details were as described in Materials and Methods. (○) Whole latex + IPP; (●) whole latex + IPP + OMBPP.

Therefore, the observed stimulatory effect of the soluble *trans*-prenyl transferase on rubber biosynthesis can be explained without requiring a role as rubber transferase. The prenyl transferase functions as an FPP synthase in the absence of rubber particles, given a suitable substrate mixture (Light and Dennis, 1989; Fig. 3). If the prenyl transferase supplies FPP to the system, a stimulation of the IPP incorporation rate would still occur. Light and Dennis (1989) did not examine the effect of FPP or other allylic diphosphates on rubber biosynthesis. Nevertheless, in solution, prenyl transferases require both IPP and DMAPP (see Fig. 1) as substrates in order to synthesize allylic diphosphates of size C_{10} and above. If the *H. brasiliensis* prenyl transferase is stimulating rubber formation by synthesizing FPP initiator molecules, this presupposes the presence of either DMAPP or of IPP isomerase, which makes DMAPP from IPP, in the *in vitro* assay (see Fig. 1). Prenyl transferase cannot make FPP in the absence of DMAPP.

The presence of IPP isomerase was demonstrated in a series of experiments. When isomerase was added to a mixture of IPP and prenyl transferase, FPP was synthesized from IPP and DMAPP, which was made from the IPP by IPP isomerase (Fig. 3). OMBPP is an effective inhibitor of yeast IPP isomerase activity (Muehlbacher and Poulter, 1988) and the addition of OMBPP inhibited the formation of FPP (Fig. 3). This inhibition must be mediated by inhibition of DMAPP production by the isomerase because assays in the presence of DMAPP and OMBPP demonstrated that OMBPP neither inhibits nor stimulates prenyl transferase activity itself (data not shown).

OMBPP also inhibited incorporation of IPP into rubber by whole latex (Fig. 4) although, as was the case with prenyl transferase, OMBPP neither inhibited nor stimulated rubber transferase activity (data not shown). Thus, whole latex contains a substantial amount of active IPP isomerase.

In similar experiments, a low level of isomerase activity was also detected in highly purified washed rubber particles (data not shown). Unlike the washed rubber particles used here, the rubber particles used by Light and Dennis (1989) were purified by gel filtration on S-300. This type of rubber particle preparation, even when made using longer columns and higher bed volumes than those used by Light and Dennis (1989), is not as free of soluble latex components as are the washed rubber particle preparations (data not shown). Therefore, it is extremely likely that substantial levels of IPP isomerase were present in the gel-filtered particle preparations (Light and Dennis, 1989).

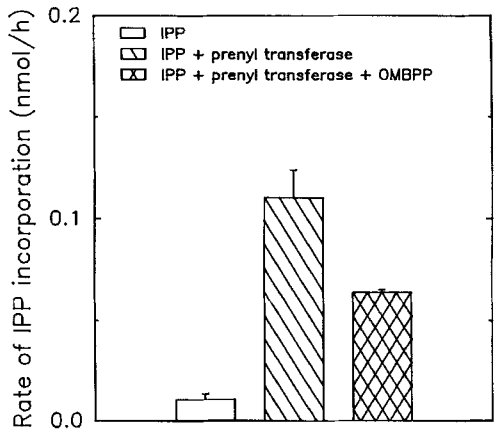

Fig. 5. Soluble prenyl transferase activity assayed in the presence of IPP and OMBPP (isomerase inhibitor). Concentrations were as follows: IPP, 80 μM; prenyl transferase, 4 μg · cm^{-3}; OMBPP, 20 μM. The reactions were incubated for 40 min at 26°C. Each value is the mean of three experiments ± SE. Experimental details were as described in Materials and Methods. (□) IPP; (▨) IPP + prenyl transferase; (▩) IPP + prenyl transferase + OMBPP.

Furthermore, the biosynthesis of FPP by prenyl transferase in the presence of IPP alone, although very low (Fig. 3), was significantly above the assay background (Fig. 5, see IPP with IPP and prenyl transferase). Addition of OMBPP resulted in a decrease in the rate of FPP formation in IPP containing prenyl transferase, indicating that the prenyl transferase preparation itself also had a low level of isomerase contamination (Fig. 5). The prenyl transferase preparation is the

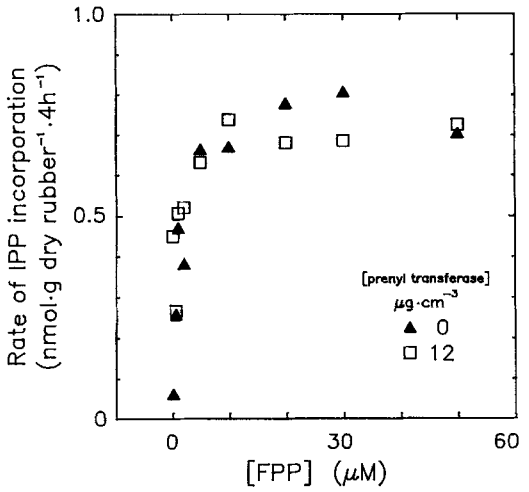

Fig. 6. FPP concentration dependence of rubber transferase activity in whole latex in the presence and absence of soluble prenyl transferase. Reactions were carried out in 1 mM IPP (2.5 times the rubber transferase apparent IPP K_m) and incubated for 4 h at 26°C. Experimental details were as described in Materials and Methods. (▲) 0 μg · cm^{-3} prenyl transferase; (□) 12 μg · cm^{-3} prenyl transferase.

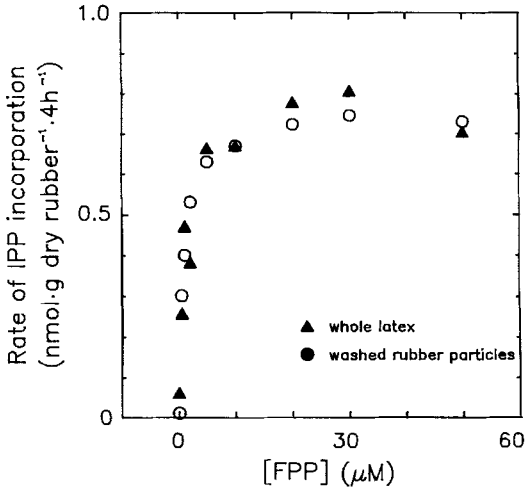

Fig. 7. FPP concentration dependence of rubber transferase activity in whole latex and washed rubber particles. Reactions were carried out in 1 mM IPP (2.5 times the rubber transferase apparent IPP K_m) and incubated for 4 h at 26°C. Experimental details were as described in Materials and Methods. (▲) Whole latex; (●) washed rubber particles.

same as that used by Light and Dennis (1989), confirming the presence of IPP isomerase in their experiments.

Even though an FPP-mediated effect of the prenyl transferase on rubber biosynthesis now seems clear, experiments were also carried out to reveal any effect that the prenyl transferase might have independent of its ability to synthesize FPP. Therefore, the effect of prenyl transferase on rubber biosynthesis in whole latex and by washed rubber particles was examined over a concentration range of FPP, the product of the *trans*-prenyl transferase in solution. Prenyl transferase had no effect on rubber transferase catalyzed IPP incorporation in whole latex, even at FPP levels substantially above the apparent FPP rubber transferase K_m of 0.8 μM (Fig. 6). The possibility that this was caused by a non-limiting concentration of prenyl transferase already in the whole latex was discounted as the FPP concentration dependence of IPP incorporation into whole latex was identical to that of washed rubber particles, from which the non-rubber particle components of whole latex, including prenyl transferase, had been removed (Fig. 7).

DISCUSSION

The results presented here demonstrate that soluble *trans*-prenyl transferase has no discernable effect on the *cis*-1,4 polymerization of natural rubber. The role of prenyl transferase as an FPP synthase (Light and Dennis, 1989) can account for the stimulation of rubber biosynthesis observed in the presence of rubber particles and IPP (Light and Dennis, 1989; Fig. 2). The synthesis of allylic diphosphate, such as FPP, is an essential step in rubber biosynthesis; no elongation can take place without rubber molecule initiation by an allylic diphosphate (see Fig. 1). Since only one initiator molecule is required for each rubber molecule, made from many thousands of isoprene monmers, a very small amount of DMAPP or IPP isomerase in a rubber-particle *in vitro* assay of IPP incorporation, would permit the synthesis, by prenyl transferase, of enough FPP to allow a considerable production of rubber. Washed rubber particles contained isomerase activity and the prenyl transferase preparations also had detectable isomerase activity (Fig. 5). Thus, the required source of DMAPP to support FPP synthesis by the soluble *trans*-prenyl transferase was present in the assays.

Prenyl transferase is not the only putative, soluble rubber transferase to have been reported in protein extracts of *H. brasiliensis* (Archer et al., 1963; McMullen and McSweeney, 1966). However, in common with the purified prenyl transferase (Light and Dennis, 1989), these other extracts could not synthesize rubber in solution; all had an absolute requirement for rubber particles, *F. elastica* washed rubber particles often being the particles of choice. The rubber particles were thought to provide a source of rubber diphosphate necessary for the reaction. However, it is now clear that rubber particles are not merely inert balls of rubber, but contain a variety of species-specific proteins (Backhaus et al., 1991; Cornish and Backhaus, 1990; Siler and Cornish, 1992; Siler and Cornish, 1993). That these proteins include particle-bound rubber transferases has been reported in three different rubber-producing species, *H. brasiliensis* (Archer et al., 1963; Berndt, 1963), *P. argentatum* (Cornish and Backhaus, 1990; Madhavan et al., 1989) and *F. elastica* (Siler and Cornish, 1993).

The isolation of the *H. brasiliensis* particle-bound rubber transferase has not been achieved as yet. As mentioned earlier, a model was proposed in which *trans*-prenyl transferase interacts with rubber-elongation factor to form rubber transferase, a *cis*-prenyl transferase (Light et al., 1989). Although the appeal of this model is undeniable, it is not supported by the available data. The elimination of *trans*-prenyl transferase as a rubber transferase also casts doubt on the role postulated for rubber-elongation factor in rubber-molecule elongation (Dennis and Light, 1989; Light et al., 1989). Although rubber-elongation factor is indeed an abundant particle-bound protein in *H. brasiliensis*, silver-stained SDS/PAGE analysis revealed that numerous other proteins are also present on purified rubber particles (Siler and Cornish, 1992). Any one of these proteins may be rubber transferase.

If soluble rubber transferases exist in addition to the rubber-particle-bound form, in *H. brasiliensis* latex, the IPP-incorporation rate by whole latex in the presence of excess

FPP (25 times apparent rubber transferase FPP K_m) would have been higher than by the washed rubber particles, and this was not observed (Fig. 7). Rubber biosynthesis can occur in the absence of soluble proteins if these are substituted for by a number of different allylic diphosphate initiator molecules (Archer and Audley, 1987; Audley and Archer, 1988; Berndt, 1963; Cornish and Backhaus, 1990; Madhavan et al., 1989). Thus, it is probable that all soluble extracts reported to have rubber transferase activity (Archer et al., 1963; Light and Dennis, 1989; McMullen and McSweeney, 1966) were actually part of the rubber molecule initiation system, synthesizing essential allylic diphosphates but not *cis*-1,4-polyisoprene.

None of the above reports investigated rubber biosynthesis in the presence of the products of the soluble enzymes. This is the first report in which the effect on rubber biosynthesis of a soluble enzyme, purified *trans*-prenyl transferase, has been examined in the presence of both its allylic diphosphate product and purified rubber particles. If Light and Dennis (1989) had included appropriate allylic diphosphate controls in their experiments, they may not have been reduced to their postulate of two separate roles for a single soluble enzyme, entailing an improbable stereochemical shift from a *trans* to a *cis* reaction product.

In conclusion, the role of the *H. brasiliensis* soluble prenyl transferase in rubber biosynthesis seems to lie solely in its ability to catalyze the synthesis of FPP (Light and Dennis, 1989; Fig. 2), a potent initiator of new rubber molecules. Natural rubber biosynthesis is catalysed by a rubber-particle-bound *cis*-1,4-prenyl transferase that has yet to be isolated. No soluble rubber transferase activity, of any kind, could be detected. Thus, plant prenyl transferases seem to be analogous to those in animals in which *trans*-prenyl transferases are soluble enzymes but *cis*-prenyl transferases are membrane-bound (Ericsson et al., 1992).

I thank D. R. Light and C. D. Poulter for their invaluable advice and assistance and D. J. Siler for her critical review of this research.

REFERENCES

1. Anderson, M. S., Muehlbacher, M., Street, J., Proffitt, J. & Poulter, C. D. (1989) Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*, *J. Biol. Chem. 246*, 19169–19175.
2. Archer, B. L. & Audley, B. G. (1987) New aspects of rubber biosynthesis, *Bot. J. Linn. Soc. 94*, 181–196.
3. Archer, B. L., Audley, B. G., Cockbain, E. G. & McSweeney, G. P. (1963) The biosynthesis of rubber, *Biochem. J. 89*, 565–574.
4. Audley, B. G. & Archer, B. L. (1988) Biosynthesis of rubber, in *Natural rubber science and technology* (Roberts, A. D., ed.) p. 35, Oxford University Press, London.
5. Backhaus, R. A., Cornish, K., Chen, S.-F., Huang, D.-S. & Bess, V. H. (1991) Purification and characterization of an abundant rubber particle protein from guayule, *Phytochemistry 30*, 2493–2497.
6. Berndt, J. (1963) The biosynthesis of rubber, *US Government Res. Rep. AD-601*, 729.
7. Cornish, K. & Backhaus, R. A. (1990) Rubber transferase activity in rubber particles of guayule, *Phytochemistry 29*, 3809-3813.
8. d'Auzac J., Jacob, J.-L. & Chrestin, H. (1989) Physiology of Rubber Tree Latex, *Physiology of rubber tree latex*, CRC Press, Boca Raton, Florida.
9. Dennis, M. S. & Light, D. R. (1989) Rubber elongation factor from *Hevea brasiliensis*. Identification, characterization and role in rubber biosynthesis, *J. Biol. Chem. 264*, 18608–18617.
10. Ericcson, J., Runquist, M., Thelin, A., Andersson, M., Chojnacki, T. & Dallner, G. (1992) Distribution of prenyltransferases in rat tissues: evidence for a cytosolic all-*trans*-geranylgeranyl diphosphate synthase, *J. Biol Chem. 268*, 832–838.
11. Light, D. R. & Dennis, M. S. (1989) Purification of a prenyl transferase that elongates *cis*-polyisoprene rubber from the latex of *Hevea brasiliensis*, *J. Biol. Chem. 264*, 18589–18597.
12. Light, D. R., Lazarus, R. A. & Dennis, M. S. (1989) Rubber elongation by farnesyl pyrophosphate synthases involves a novel switch in enzyme stereospecificity, *J. Biol. Chem. 264*, 18598–18607.
13. Madhavan, S., Greenblatt, G. A., Foster, M. A. & Benedict, C. R. (1989) Stimulation of isopentenyl pyrophosphate incorporation into polyisoprene in extracts from guayule plants (*Parthenium argentatum* Gray) by low temperature and 2-(3,4-dichloro-phenoxy)triethylamine, *Plant Physiol. 89*, 506–511.
14. McMullen, A. J. & McSweeney, G. P. (1966) Incorporation of isopentenyl pyrophosphate into purified rubber particles by a soluble latex-serum enzyme, *Biochem. J. 101*, 42–47.
15. Muehlbacher, M. & Poulter, C. D. (1988) Isopentenyl-diphosphate isomerase: inactivation of the enzyme with active-site-directed irreversible inhibitors and transit-state analogues, *Biochemistry 27*, 7315–7328.
16. Siler, D. J. & Cornish, K. (1992) Potential for allergy amelioration by rubber particle purification from *Hevea brasiliensis* latex, and for allergy circumvention by use of alternate rubber-producing plants, *Proceedings of the International Latex Conference: Sensitivity to Latex in Medical Devices, 34*, p. 46 FDA, Washington, D. C.
17. Siler, D. J. & Cornish, K. (1993) A protein from *Ficus elastica* rubber particles is related to proteins from *Hevea brasiliensis* and *Parthenium argentatum*, *Phytochemistry 32*, 1097–1102.