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Review

Similarities and differences in rubber biochemistry among plant species

Katrina Cornish*

USDA-ARS, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710, USA

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Abstract

This report reviews aspects of the biochemical regulation of rubber yield and rubber quality in three contrasting rubber-producing species, *Hevea brasiliensis*, *Parthenium argentatum* and *Ficus elastica*. Although many similarities are revealed, considerable differences also exist in enzymatic mechanisms regulating biosynthetic rate and the molecular weight of the rubber biopolymers produced. In all three species, rubber molecule initiation, biosynthetic rate and molecular weight, in vitro, are dependent upon substrate concentration and the ratio of isopentenyl pyrophosphate (IPP, the elongation substrate, or monomer) and farnesyl pyrophosphate (FPP, an initiator), but these parameters are affected by intrinsic properties of the rubber transferases as well. All three rubber transferases are capable of producing a wide range of rubber molecular weight, depending upon substrate concentration, clearly demonstrating that the transferases are not the prime determinants of product size in vivo. However, despite these commonalities, considerable differences exist between the species with respect to cosubstrate effects, binding constants, effective concentration ranges, and the role of negative cooperativity in vitro. The *P. argentatum* rubber transferase appears to exert more control over the molecular weight it produces than the other two species and may, therefore, provide the best prospect for the source of genes for transformation of annual crop species.The kinetic data, from the three contrasting rubber-producing species, also were used to develop a model of the rubber transferase active site in which, in addition to separate IPP and allylic-PP binding sites, there exists a hydrophobic region that interacts with the linear portion of allylic-PP initiator proximal to the pyrophosphate. Substrate affinity increases until the active site is traversed and the rubber interior of the rubber particle is reached. The kinetic data suggest that the hydrophobic region in *H. brasiliensis* and *F. elastica* is about 1.8 nm long but only 1.3 nm in *P. argentatum*. The estimates are supported by measurements of the rubber particle monolayer membrane using electron paramagnetic resonance spectroscopy. Published by Elsevier Science Ltd.

Keywords: *Ficus elastica*; Moraceae; *Hevea brasiliensis*; Euphorbiaceae; *Parthenium argentatum*; Compositae; Natural rubber; Rubber transferase; Electron paramagnetic resonance spectroscopy

Contents

* Tel.: +1-510-559-5950; fax: +1-510-559-5663.
E-mail address: kcornish@pw.usda.gov

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1. Introduction

All natural rubber currently in commercial use comes from a single plant species, the Brazilian rubber tree (*Hevea brasiliensis*), and the United States is completely dependent on imports from developing countries (Davis, 1997), for the manufacture of more than 40,000 products, including more than 400 medical devices. Asia now produces about 90% of the world demand for *H. brasiliensis* natural rubber, which, in 1998, was 6.61 million tonnes [40% of the total rubber demand; the remaining 60% is synthetic rubber produced from petroleum (data from the International Rubber Study Group)]. The United States, with no natural rubber production, is the largest single consumer of this material. In 1998, the United States imported nearly 1.2 million tonnes of natural rubber for product manufacture, at a cost approaching $2,000,000,000. In addition, the United States imports a considerable quantity of finished goods: in 1998, valued at $8,060,000,000 containing 349,000 tonnes of natural rubber (data from US Department of Commerce, Office of Trade and Economic Analysis). Natural rubber consumption closely tracks production, preventing the accumulation of large stockpiles (Fig. 1) (Mooibroek and Cornish, 2000).

Primarily due to its molecular structure and high molecular weight (>1 million Da), natural rubber has high performance properties that cannot be matched by synthetic rubber produced from petroleum. These properties include resilience, elasticity, abrasion and impact resistance, efficient heat dispersion, and malleability at cold temperatures. Despite the best efforts of the chemical industry, these properties have not been achieved by synthetic materials, or non-rubber natural polymers or blends, in many high performance applications, including tires, and surgical gloves and latex balloon devices. As a result, the amount of synthetic rubber produced has reached a plateau, whilst annual natural rubber production is steadily increasing to meet demand (Fig. 2), and probably will soon exceed the synthetic rubber market share. The *H. brasiliensis* crop consists almost entirely of plantation-grown clonal trees, and is one of the most genetically-restricted crops under cultivation. This lack of genetic diversity leaves the crop highly susceptible to pathogenic attack and failure, as demonstrated by the devastation caused by leaf blight in South American plantations (Davis, 1997). A dependable supply is endangered by many other factors, including diminishing acreage as growers in developing countries move away from rubber farming toward higher value agriculture, increasing global demand, and changing political positions (Reisch, 1995; Davis, 1997). Higher-yielding germplasm cannot indefinitely meet increased natural rubber demand and many new plantations will be required. *H. brasiliensis* also has strict climatic requirements, which limit its cultivation to specific tropical regions.

In addition to strategic and economic incentives to develop a renewable US-based natural rubber supply, the recent widespread occurrence of life-threatening "latex allergy" to *H. brasiliensis* rubber products, which

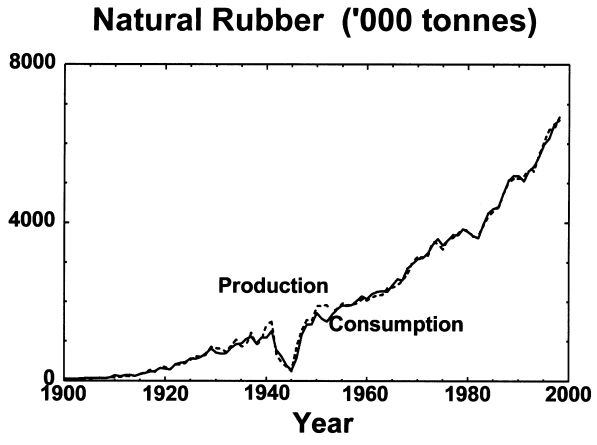

Fig. 1. Global production (dashed line) and consumption (solid line) of natural rubber during the twentieth century.

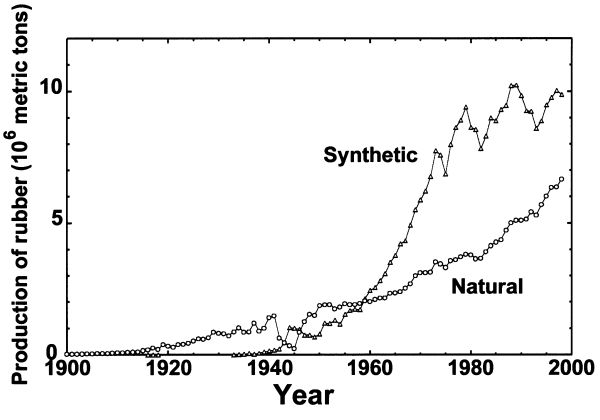

Fig. 2. Global production of natural (○) and synthetic (△) rubber during the twentieth century. Synthetic rubber production was established largely because of the drop in natural rubber production during World War II. Rubber consumption figures have closely tracked production.

affects an estimated 20,000,000 Americans, strengthens the need for development of alternative sources of natural rubber (Morales et al., 1989; Slater, 1989; Tomazic et al., 1992; Kekwick 1993; Pailhories, 1993; Ownby et al., 1994; Nakayama et al., 1996; Siler and Cornish, 1994a; Carey et al., 1995; Siler et al., 1996). Also, although synthetic rubber cannot replace natural rubber in many applications, the converse is not true. Large-scale, domestic natural rubber production will be needed in the future, even without a natural rubber supply crisis, to first supplement, then replace the 60% market share currently enjoyed by synthetic rubbers because these are derived from petroleum, a non-renewable resource. A decline in global oil production may begin within the next 10 (Campbell and Laherrere, 1998) or 20 (Monastersky, 1998) years. Thus, commercialization and natural rubber production from domestic crops is in the best short- and long-term interests of developed countries such as the United States.

Some 2500 plant species, many of which are tropical, produce natural rubber (Bowers, 1990; Ray, 1993). Most, however, do not produce the high molecular weight polymers required for high performance commercial products [molecular weight is strongly correlated with rubber quality (Swanson et al., 1979)]. Of the possible rubber-producing plant species for the US, *Parthenium argentatum* (guayule) has received the most sustained research and development effort over the years (Bowers, 1990; Whitworth and Whitehead, 1991) and is closest to domestication and commercial production. Currently, guayule is being introduced as a biennial crop in the southwestern United States to supply high performance, nonallergenic latex (Siler and Cornish, 1994a; Siler et al., 1996; Cornish and Lytle, 1999) to the medical products market (Cornish, 1996, 1998, patents exclusively licensed to Yulex Corporation). This crop may be able to fully supply the medical market but is unlikely to generate sufficient rubber for other large markets, such as tires, because it cannot tolerate the snowy winters and severe temperatures experienced by much of the United States and many other temperate countries. Also, although it may prove practical to generate new higher yielding lines, it cannot be grown as an annual crop because it produces its rubber in bark parenchyma cells and most of the rubber is synthesized during winter months. Thus, one season's growth gives a plant containing little bark parenchyma and little rubber. Additional alternative rubber-producing annual crops are, therefore, required to supply rubber to meet non-medical market requirements, and to engender a much-needed biodiversity.

Ideally, rubber-producing crop plants should be rapidly-growing, have large biomass, and be annual crops, which can be more readily included in on-farm crop rotations and farming systems, and could be readily planted and plowed-out in response to market needs and farmer production considerations. Cropping systems, such as trees, that must remain in place for many years are not well suited to changing market demand and fluctuations. Most of the 2500 known rubber-producing plant species produce small amounts of poor quality, low molecular weight rubber. Successful conversion of candidate crop species, whether or not they already produce rubber naturally, into domestic rubber-producing crops requires the generation of lines capable of producing commercially-viable yields of high quality rubber. This can only be accomplished by basing genetic engineering strategies upon a thorough understanding of biochemical factors affecting rubber yield (biosynthetic rate, substrate availability and rubber particle size, number and ontogeny) and rubber quality (primarily rubber molecular weight).

2. Rubber particles and their components

Many different dicotyledonous plants make natural rubber. Most of this review concentrates on the similarities and differences in rubber production among three plant species, each from a different superorder of the Dicotyledonae, i.e. *Hevea brasiliensi* Müll. Arg. (Rosidae), *Ficus elastica* Roxb. (Dilleniidae), and *Parthenium argentatum* Gray (Asteridae). In all three species, natural rubber is compartmentalized within subcellular rubber particles, located in the cytosol of cells, whether these be specialized laticifers (pipe-like anastomosized cell systems which produce latex), as in *H. brasiliensis* (d'Auzac et al., 1989) and *F. elastica* (Heinrich, 1970) or generalised cells, such as the bark parenchyma of *P. argentatum* (Whitworth and Whitehead, 1991). The average size of the particles varies among species [e.g. *F. elastica* 3.8 μm, *Euphorbia lactiflua* 0.42 μm (Siler et al., 1997; Wood and Cornish, 2000)], and differently-sized subsets of particles can even be found in the same plant. *H. brasiliensis*, for example, has two distinct subsets of particles with mean diameters of 1.0 and 0.2 μm (Cornish et al., 1993; Wood and Cornish, 2000).

Very little is known about rubber particle ontogeny, largely because small, electron-dense rubber particles cannot be readily distinguished from other small electron-dense particles in cytoplasm (Backhaus, 1985). However, studies of rubber particle structure and biochemical components suggest a likely origin in the rough endoplasmic reticulum. The overall structure of rubber particles is similar in all species examined so far, in that they contain a homogeneous rubber core surrounded by an intact monolayer membrane (Cornish et al., 2000; Wood and Cornish, 2000). The glycosylated moieties of some of the particle-bound proteins and the hydrophilic head groups of the phospholipids enable the particles to interface with the aqueous cytosol. However, within the shared architecture, the rubber particle components are quite different, the compartmentalized

rubber being of much lower molecular weight in *F. elastica* than in the other two species, and all three possessing a species-specific complement of lipids and proteins (Cornish et al., 1993; Siler et al., 1997). The lipid fraction of *P. argentatum* rubber particles is typical of many plant cell membranes, and appears to reflect the very ordinary cells (bark parenchyma) in which the rubber particles are formed. It seems likely that the rubber particle membrane in different species is made up of readily available lipid components, and that highly conserved structure and chemical composition are not required. Also, the amount of protein associated with rubber particles varies tremendously between species, ranging from 4.8% in *E. lactiflua* to less than 0.1% in *P. argentatum* (Siler et al., 1997), and the number of different proteins is remarkably different. The most complicated rubber particles currently known are those of *H. brasiliensis*. These contain upwards of 80 different proteins across a size range of 5 to over 200 kDa. However, other rubber particles contain far fewer proteins, perhaps less than 10 in *F. elastica* and *P. argentatum*, for example (Cornish et al., 1993). The architecturally-analogous cytoplasmic oil bodies (Huang, 1992) are much more highly conserved from one species to another in their protein and lipid components, but these organelles must export their contents, not merely retain them, and so require a sophisticated secretion system. Rubber is a secondary product that is not catabolized during the life of the plant, and rubber particles only need to effectively compartmentalize the rubber. The non-conserved nature of the particular rubber particle biomembrane components suggests that few, if any, specific genes may be required to generate a rubber particle once a rubber transferase enzyme begins rubber synthesis. Thus, normally non-rubber-producing species transformed with genes encoding rubber transferase may be able to effectively compartmentalize rubber using cytosolic membrane components. Information on factors regulating the number of rubber particles made in a tissue will probably not be obtained until transformed plants with enhanced rubber biosynthetic activity have been generated.

Very large proteins or protein complexes appear to be present in all species (Cornish et al., 1993; Siler and Cornish, 1993, 1994a,b), and they all contain an integral, membrane-bound, rubber-synthesizing *cis*-prenyl transferase enzyme or enzyme complex (rubber transferase, EC 2.5.1.20). This enzyme synthesizes rubber from hydrophilic substrates obtained from the cytoplasm and the hydrophobic polymer is compartmentalized into the rubber particle interior. Thus, the polymerization reaction must take place at the rubber particle surface, and it has been proven that rubber transferase is an integral membrane protein or protein complex (Madhavan et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Cornish and Siler, 1996).

The identity of the rubber particle-bound rubber transferase remains equivocal. Two different proteins on *H. brasiliensis* rubber particles have been implicated in activity, namely the 14.6 kD "rubber elongation factor" (Dennis and Light, 1989) and the 24 kD "small rubber particle protein" (Oh et al., 1999). Although the case for both has yet to be substantiated, the data supporting the role of the 24 kD protein seems more convincing than for the 14.6 kD protein (Cornish, 1993), which relied solely on immunoinhibition of activity in latex, not in washed rubber particles. Oh et al. (1999), used a combination of immunoinhibition and direct addition of expressed protein to affect activity. However, it seems worthwhile to point out that meaningful immunoinhibition experiments must be performed using purified antibody. Crude sera can lead to reproducible and substantial inhibition of rubber transferase activity in washed rubber particles that is not antibody specific (Fig. 3). Also, immunoblots of sera against *H. brasiliensis* latex proteins have shown that experimental animals frequently contain antibodies to *H. brasiliensis* latex proteins before being challenged with purified protein(s) (Siler and Cornish, unpublished). The animals have presumably reacted to contact with the latex gloves worn by their handlers and/or the rubber stoppers commonly used to support the water supply tubes.

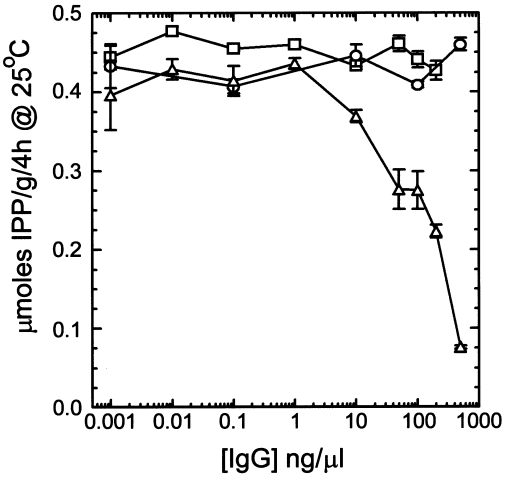

Fig. 3. Rubber transferase activity in rubber particles purified from *Hevea brasiliensis* latex. Ascites fluid (crude sera) from mice challenged with a low-density lipoprotein was the generous gift of Dr. Alex Karu, UC Berkeley Hybridoma Center. IgG was purified from the ascites fluid by chromatography on a 1 ml bed volume Protein G column (Hi-Trap, Pharmacia) according to the manufacturer's instructions. A non-specific purified mouse ascites IgG (Pierce) was used as a negative control. The serum and purified IgG samples were incubated with the rubber particles for 15 h on ice before the remaining rubber transferase activity was assayed as described (Cornish, 1993). 465 µg dw of rubber particles in 500 µl were assayed for 4 h at 25°C in imM IPP, 20 µM FPP and 1 mM Mg^{2+}. Each value is the mean of 3±S.E. In the absence of IgG or ascites, 0.412±0.034 µmol IPP were incorporated. The experiment was repeated with similar results (not shown).Key: (○) control IgG; (Δ) serum; (□) purified lipoprotein IgG.

Thus, pre-immune bleed evaluations are essential and, in this laboratory, we use animals born and reared in latex-free environments.

The situation has been complicated again recently by the identification and cloning of a bacterial gene encoding undecaprenyl diphosphate synthase (Shimizu et al., 1998), another member of the *cis*-prenyl transferase family, followed by the isolation of various related enzymes through sequence homology. Although it seemed possible that rubber transferase also might be identified as a homologue, it seems increasingly unlikely that the homology between the two enzymes is high enough for successful homogue-based cloning. This is perhaps not surprising, because the undecaprenyl diphosphate synthase is a soluble enzyme and makes relatively short polymer (55 carbons), whereas the rubber transferase is membrane-bound, possibly a complex, and can make product many thousands of carbons long (Cornish et al., 1993, 2000; Castillón and Cornish, 1999).

However, even though the degree of relationship between the UDP synthase and rubber transferase is unknown, immuno-inhibition experiments indicate that rubber transferase activity does appear to be associated with a 376 kDa rubber particle-bound protein in *F. elastica* (Cornish et al., 1994). Cross-specific immunoinhibition and immunoprecipitation studies, with purified IgG antibodies and purified rubber particles, also have indicated that some structural similarities probably exist between the rubber transferases of *F. elastica*, *H. brasiliensis* and *P. argentatum* (Siler and Cornish, 1993, 1994b; Cornish et al., 1994).

3. Rubber biosynthesis

3.1. Regulation of initiation and biosynthetic rate

Natural rubber is produced by a simple side-branch of the ubiquitous isoprenoid pathway (Chappell, 1995) from an allylic pyrophosphate (allylic-PP) and isopentenyl pyrophosphate (IPP) (Fig. 4). Several allylic-PP's, namely dimethylallyl-PP (DMAPP), geranyl-PP (GPP) and *trans,trans*-farnesyl-PP (FPP) are synthesized, by soluble enzymes, in the same cytosolic compartment as rubber transferase and so could be used by the rubber transferase. The IPP polymerized by the rubber transferase is probably derived from the mevalonate pathway, also located in the cytosol (Chappel, 1995). However, IPP derived from the deoxy-xylulose pathway (reviewed by Lichtenthaler, 1999) may diffuse from the plastids and join the cytosolic pool. The importance of the soluble *trans*-prenyl transferases in rubber biosynthesis lies in their synthesis of allylic-PP's, essential initiators of rubber biosynthesis.

The rubber particle-bound rubber transferases of *F. elastica*, *H. brasiliensis* and *P. argentatum* have considerable similarities in their substrate requirements,

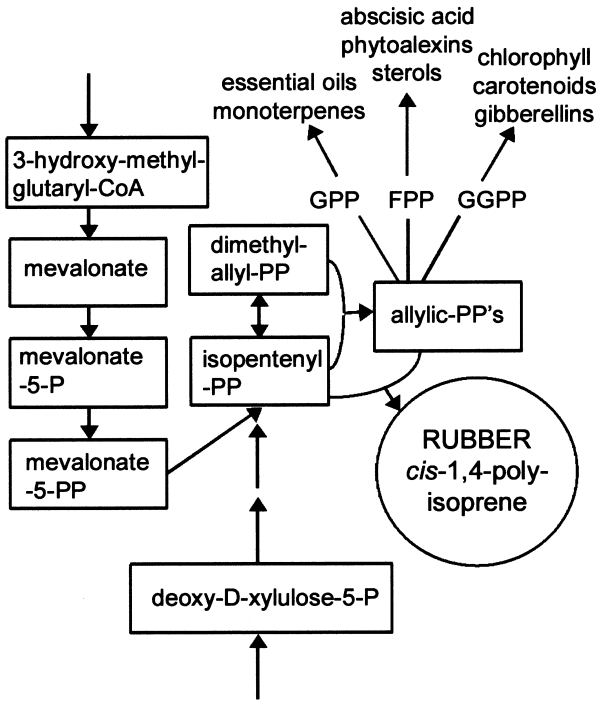

Fig. 4. The relationship of rubber biosynthesis to the isoprenoid pathway. The rubber particle membrane-bound rubber transferase (a *cis*-prenyl transferase) binds an allylic pyrophosphate (allylic-PP) to initiate a new rubber molecule and then elongates the polymer by *cis*-1,4-polymerization of isopentenyl monomers derived from isopentenyl pyrophosphate (IPP). Cytosolic IPP is produced by the mevalonate pathway, but the endogenous pool size may be supplemented by IPP produced by the deoxy-*D*-xylulose pathway in the plastids. Dimethyl allyl pyrophosphate (DMAPP) is produced by IPP-isomerase, and allylic-PP's larger than DMAPP are synthesized by soluble *trans*-prenyl transferases. All the allylic-PP's shown can be used as initiators by rubber transferase. All reactions shown require a divalent cation cofactor such as magnesium. Given the multidisciplinary nature of the intended audience of this paper, a brief summary of essential terms is given. IPP is the "elongation" or "polymerization" substrate, or the "monomer" in the rubber biosynthetic reaction. Polymer "elongation" is equivalent to "polymerization" and to "propagation". Allylic-PP is the initiation substrate, one being needed for each new polymer. Allylic-PP also can displace the elongating rubber molecule from the enzyme's active site and so acts as a "chain transfer agent" in addition to its role as an initiator. PP, pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP = geranylgeranyl pyrophosphate.

kinetic constants, and pH dependencies (Lynen, 1969; Archer and Audley, 1987; Madhavan et al., 1989; Cornish and Siler, 1996; Cornish et al., 1998). Rubber molecules are synthesized from one allylic-PP molecule, which initiates the reaction; the rubber polymer (*cis*-1,4-polyisoprene) is then made by sequential condensations of the non-allylic IPP (divalent cations, such as magnesium or manganese, are a required cofactor) with release of a diphosphate at each condensation (Archer et al., 1963). After initiation and elongation, a termination event occurs in which the rubber molecule is released from the enzyme. However, despite these similarities, remarkable differences exist between these species with

respect to enzymatic reaction mechanisms and product molecular weight.

Many different allylic-PP's are effective initiators of rubber biosynthesis, including DMAPP (C_5), GPP (*trans*, C_{10}), FPP (all-*trans*, C_{15}) and geranylgeranyl-PP (GGPP) (*trans*, C_{20}) (Lynen, 1969; Archer and Audley, 1987; Madhavan et al., 1989; Cornish et al., 1998). Only the first three of these are likely initiators of rubber biosynthesis in vivo because only the corresponding short-chain prenyl transferases share the cytosolic compartment with rubber transferase. Geranylgeranyl-PP synthase is located in the plastids (e.g. Dogbo and Camara, 1987). Also, GPP synthase is not a common cytosolic enzyme and is usually only found in abundance in specialized secretory cells such as in mint oil glands (Burke et al., 1999). However, the gene encoding a GPP synthase is present in *P. argentatum* (from EST homologues, Mau and Cornish, unpublished). Direct NMR-based structural evidence supports FPP as the principal initiator in vivo, at least in *H. brasiliensis* (Tanaka, 1989; Tanaka et al., 1996) and the mushroom *Lactarius volemus* (Ohya and Tanaka, 1998). Also, biochemical studies have shown that the apparent K_m value for FPP is lower than those for GPP or DMAPP in all three species (Table 1) (Cornish et al., 1998) and so the affinity of rubber transferase is greater for FPP than for the other two initiators.

In *H. brasiliensis*, *F. elastica* and *P. argentatum*, many other allylic-PP's can initiate rubber polymerization in vitro (Archer and Audley, 1987; Cornish et al., 1998). Thus, rubber transferases in general are very tolerant of both size and stereochemistry of the allylic-PP. This is in contrast to most known prenyl transferases which have strict substrate structural requirements for the elongation reactions they catalyze. Nonetheless, in vitro experiments have shown that the particular initiator supplied to the rubber transferase has a considerable impact on rubber biosynthetic rate. This appears to result from different initiation rates being caused by different $K_m^{\text{allylic-PP}}$'s as well as by direct effects on the IPP incorporation rate (Cornish and Siler, 1995; Castillón and Cornish, 1999; Cornish et al., 2000). In general, biosynthetic rate increases with the size of the *trans*-allylic-PP from DMAPP until either FPP (in *P. argentatum*) or GGPP (in *H. brasiliensis* and *F. elastica*) (Cornish and Siler, 1995; Cornish et al., 1998). In line with the increase in biosynthetic rate, the apparent K_m for the different allylic-PP's declines (Table 1).

Table 1
K_m's of three rubber transferases[a]

Initiator	*Hevea brasiliensis*	*Ficus elastica* (μM)	*Parthenium argentatum*
DMAPP	13.2	33.9	3.4
GPP	3.9	1.6	0.7
FPP	1.5	0.6	0.01
GGPP	1.4	0.3	0.01
IPP in FPP	363	115	385

[a] K_m's were determined from Hill plots of log $\upsilon/(V_{max}-\upsilon)$ against log substrate concentration. Assays in varying allylic-PP were performed at 25°C for *H. brasiliensis* and *F. elastica* and 16°C for *P. argentatum* in the presence of 1 mM [^{14}C]IPP. Assays in varying IPP were performed similarly except that 20 μM FPP was used.

Kinetic data were used to develop a model of the rubber transferase active site which contains a catalytic site and separate IPP and allylic-PP binding sites. The allylic-PP binding site is composed of a specific binding region which recognizes the allylic-PP moiety and a non-specific region which interacts with the hydrophobic portion of allylic-PP initiator. Increasing the length of the initiator increases the hydrophobic interaction and thus the affinity of the enzyme for the substrate. Substrate affinity increases until the active site is traversed and the interior of the rubber particle is reached. At this point, no additional change in affinity occurs with an increase in initiator length. The kinetic data indicate that FPP is of sufficient size to traverse the rubber transferase active site in *P. argentatum*, because V_{max} and K_m are similar for FPP and GGPP in this species, whereas GGPP is large enough to do the same in the rubber transferase active sites of *H. brasiliensis* and *F. elastica*. The model was successfully tested using a variety of stereoisomers of the allylic-PP's (unpublished data). The data suggest that the hydrophobic region in *H. brasiliensis* and *F. elastica* is about 1.8 nm long but is only 1.3 nm in *P. argentatum*. These estimates are supported by measurements of the *P. argentatum* rubber particle monolayer membrane using electron paramagnetic resonance spectroscopy (Cornish et al., 1999). When the hydrophilic pyrophosphate region of FPP is aligned with the hydrophilic phospholipid region of the monolayer membrane, the rubber interior of the particle is reached at almost exactly the same place (Fig. 5). This seems noteworthy because the tertiary structure of the enzyme or enzyme complex would not make such a coincidence actually necessary, even though a minimization of active site structure has obvious advantages.

3.2. Regulation of molecular weight

Many different plant species produce natural rubber, but only a few produce the high molecular weights required for high performance commercial products. As mentioned earlier, the biochemical regulation of molecular weight must be understood so that genetic-engineering strategies may be designed that increase yield while maintaining or conferring high molecular weight. In vitro investigations have now shown that, in the three different species discussed in this review, rubber

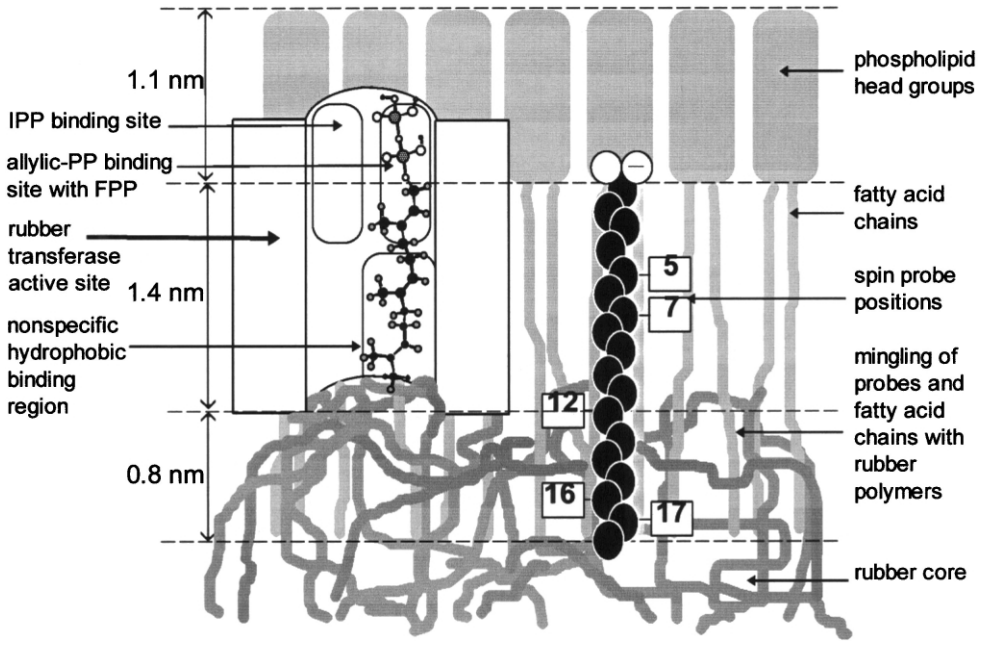

Fig. 5. A schema depicting, to scale, a section of the surface monolayer biomembrane surrounding a *Parthenium argentatum* rubber particle and the intermingling of the phospholipid fatty acid chains with the pure rubber core (from Cornish et al., 1999). The major phospholipids are phosphatidyl choline and the major fatty acids are 16:0, 18:0, 18:1, 18:2 and 18:3 comprising 95% of the phospholipids (Siler et al.,1997). The predominate protein, allene oxide synthese, a 52 kDa glycoprotein (Pan et al., 1995), is a partially embedded amphiphilic protein (not shown) that penetrates the monolayer only as far as the 5- and possibly the 7-doxyl spin probe positions. The orientation of the stearic acid spin probes in the monolayer is depicted with the stearic acid chain in the all *trans* configuration and the doxyl spin probe shown as a rectangular box attached to a specific methylene carbon (black ball) indicated by the number in the box. The positions of all the probes used are depicted on a single stearic acid instead of on individual ones for the sake of space, and indicate the region of the monolayer probed. The stearic acid chain is aligned parallel to the phospholipid fatty acid chains with its polar head group localized in the aqueous media between the phospholipid head groups. The intermingling of the fatty acid chains of the phospholipids with the pure rubber of the particle core begins at the C-12 doxyl position and ends somewhere in the vicinity of the terminal methyl of the stearic acid chain. The rubber transferase active site with FPP bound into the allylic-PP binding site is positioned so that the hydrophilic pyrophosphate of FPP is aligned to the polar head group region of the membrane. The kinetic studies suggest that FPP traverses the active site as shown. Both models show the same physical position for the membrane-rubber interface.

molecular weight is greatly affected by substrate concentration and type, and especially by the concentration of allylic-PP initiator. In general, the higher the allylic-PP concentration, the lower the rubber molecular weight that results, while the converse is true for IPP, i.e. the higher the concentration of IPP the greater the molecular weight (Castillón and Cornish, 1999; Cornish et al., 2000). It appears that competition for the allylic-PP binding (initiation) site occurs between free allylic-PP (although it should be noted that only FPP has been tested in all three species) and the allylic-PP moiety of the growing polymer. This site binds the original FPP initiator and all the allylic-PP intermediates formed as the rubber polymer grows (the terminal five-carbon unit always includes the pyrophosphate and allylic carbon). The higher the FPP concentration, the more likely that free FPP, acting as a reinitiation (or chain transfer) reagent, could displace the polymer and initiate a new rubber molecule, which in turn accounts for the shortening of the rubber polymer observed with increasing FPP concentration. The affinity of FPP would probably not be very much lower than the affinity of the growing rubber polymer because only a relatively small piece of the rubber molecule appears to be positioned within the active site at any time (see Fig. 5 and related discription). The same effect of increasing concentration causing shorter polymers is caused by GPP in *P. argentatum* and would probably occur in the other two species as well.

Presumably, the rubber transferase enzymes would be similarly affected in vivo. The polymer molecular weights produced by rubber transferase vary without exhaustion of free substrate, and in many cases without large changes in substrate concentration. Competition between FPP and the IPP donor site which could signal a non-productive terminal extensions and hence chain extension seems unlikely because such competition has only been observed in the *P. argentatum* rubber transferase (Castillón and Cornish, 1999) but not in the *H. brasiliensis* or *F. elastica* rubber transferases (Cornish et al., 2000). The rubber transferase system in vitro does appear to be able to terminate the polymers and initiate new molecules. For example, the incorporation rate of allylic-PP, under saturating concentrations of IPP and either FPP or GPP, was linear over 6 h in *P. argentatum* (Cornish and Siler, 1995) and so the rate of termination and reinitiation (the chain transfer reaction rate),

remained constant under these conditions. Very little is known about the termination event of rubber polymer biosynthesis. Different termination functional groups have been characterized by NMR spectroscopic analyses and indicate that rubber dephosphorylation and release may involve esterification, cyclization or hydrolysis in different species (Tanaka, 1989; Tanaka et al., 1996).

The studies of molecular weight regulation in vitro, summarized above, clearly indicate that additional mechanisms must be involved in regulating chain length in vivo. Also, although the three rubber transferases appear to be similar in many ways, a closer look at their regulation of rubber molecular weight reveals quite different mechanisms. For example, the *P. argentatum* and *H. brasiliensis* rubber transferase enzymes, despite their different allylic-PP kinetic constants and kinetic behavior in vitro (Table 1), produce similar molecular weights in any particular substrate mixture (Castillón and Cornish, 1999; Cornish et al, 2000). However, the *F. elastica* enzyme consistently produces rubber twice as large as in the other two species, even though this is primarily a low molecular weight producer in vivo (Cornish et al., 1993). This higher molecular weight may be caused by the lower K_m^{IPP} (Table 1), which is a third that of the other two species in the presence of FPP. The greater affinity of the *F. elastica* rubber transferase for IPP may lead to a more rapid polymerization (elongation or propagation) rate which, in turn, could produce the higher overall molecular weights observed over the four hour reaction period used. Also, K_m's reflect the substrate concentration around which the particular enzyme operates most effectively and are believed to reflect the endogenous, or physiological, substrate concentration in vivo (Segel, 1993). Thus, the rubber transferase K_m^{IPP} and the allylic-PP K_m's can be considered to predict the ideal conditions for the enzyme (high IPP levels, low allylic-PP levels). The high rubber transferase K_m^{IPP} ensures that rubber is made at times when IPP is non-limiting for other prenyl transferases, all of which have lower K_m^{IPP}'s much closer to the allylic-PP K_m's (reviewed by Ogura and Koyama, 1998). Under optimal conditions, and if no other factors are involved, the rubber molecular weight synthesized would presumably reflect the substrate equilibrium, and can be calculated from the K_m^{IPP} to $K_m^{allylic\text{-}PP}$ ratio. In *P. argentatum*, the ratio of K_m^{IPP} to K_m^{FPP} does translate to match the high molecular weight of over 1,000,000 Da produced in vivo (Table 2) in this species. Similarly, in *F. elastica* the same ratio is consistent with the low molecular weight it produces in vivo (Table 2). However, in *H. brasiliensis*, the K_m^{IPP} to $K_m^{allylic\text{-}PP}$ ratio predicts that *H. brasiliensis* would produce low molecular weight rubber in vivo (Table 2), which is, of course, untrue.

In vitro biochemical studies, showing that all three species can make high and low molecular weight rubber depending upon substrate concentrations, demonstrate that the rubber transferases, themselves, are not the cause of the low molecular weight rubber normally produced by *F. elastica*, nor of the high molecular weight produced by *P. argentatum* and *H. brasiliensis*. Other *cis*-prenyl transferases investigated make fixed product sizes as do most *trans*-prenyl transferases (Ogura and Koyama, 1998; Wang and Ohnuma, 1999). The rubber transferases clearly represent a distinct class of *cis*-prenyl transferases, despite similar substrate and cofactor requirements and similar pH dependencies.

Table 2
Some kinetic parameters and projected and in vivo rubber molecular weights produced by three different rubber transferases[a]

Parameter	*Hevea brasiliensis*	*Ficus elastica*	*Parthenium argentatum*
K_m^{IPP}/K_m^{FPP}	242	192	38,500
Projected M_r from K_m ratio (Da)	16,632	13,232	2,618,176
Actual rubber M_r in vivo (Da)	$>10^6$	10^3–10^4	$>10^6$

[a] The ratios of apparent K_m's were calculated from Table 1. The projected rubber molecular weight (M_r) from in vitro experiments (Refs. 41 and 42) was calculated, assuming that the ratio of K_m^{IPP}/K_m^{FPP} reflects the number of isoprene units incorporated, as (K_m^{IPP}/K_m^{FPP}) 68 + 176. (Molecular weight of isoprene subunit = 68, pyrophosphate with one negative charge = 176).

3.3. The role of negative cooperativity

It appears for rubber transferases, in general, that once an allylic-PP binds to the active site, the polymer that begins to form restricts access to the allylic-PP binding site by free allylic-PP. This probably occurs because the enzyme has a high affinity for the end of the rubber polymer. The enzyme product is not soluble and is certainly not fully released from the active site during the polymerization reaction. Thus, IPP polymerization can proceed even though the apparent K_m^{IPP} is much higher than K_m^{FPP} (Table 1). However, in addition to the affinity of the rubber transferases for the allylic-PP terminal C_{15} or C_{20} portion of the growing polymer, Hill plots of IPP incorporation rate as a function of FPP concentration demonstrate, by gradients $\ll 1$, that all three rubber transferases exhibit regions of strong negative cooperativity (Fig. 6) (Cornish et al., 2000). At these FPP concentrations, an additional hinderance to the binding of FPP to the allylic-PP binding site occurs, in that after one FPP has bound to the allylic-PP site it becomes much more difficult for a second molecule to bind. This change in binding affinity probably results from a conformational change in the enzyme caused by the first FPP binding event. Thus, free FPP cannot easily displace the elongating rubber polymer, i.e. the chain transfer reaction is inhibited over the FPP negative cooperativity concentration range. The *P. argentatum*

rubber transferase shows a remarkably wide concentration range of negative cooperativity, operating from its K_m^{FPP} to more than two orders of magnitude above it (Fig. 6). In contrast, *H. brasiliensis* and *F. elastica* have much shorter concentration ranges of strong negative cooperativity. In *H. brasiliensis*, the negative cooperativity region falls at the high end of the range for *P. argentatum*, whereas that of *F. elastica* falls just beyond the *P. argentatum* range (Fig. 6). Another measure of the negative cooperativity operating in these rubber transferases is reflected by the ratio of the FPP concentration at 0.75 V_{max} ($y=3$ in Fig. 6) to the FPP concentration at 0.5 V_{max} ($y=1$ in Fig. 6). Values of 3 reflect Michaelis-Menton kinetics, less than 3 indicate positive cooperativity, and greater than 3 indicate negative cooperativity (Segel, 1993). Using this measure, it is clear that negative cooperativity is much stronger in *P. argentatum* than in the other two species ($[S]_{0.75}/[S]_{0.5}$ for *H. brasiliensis* is 6.91, for *F. elastica* is 8.04, and for *P. argentatum* is 53.8).

The FPP negative cooperativity also appears directly to affect IPP incorporation rate, although IPP incorporation is dependent upon IPP concentration in *P. argentatum* (Castillón and Cornish, 1999). In *H. brasiliensis* and *F. elastica*, IPP only enhanced IPP incorporation at FPP concentrations where negative cooperative operated, when IPP and FPP concentrations were rate-limiting (Cornish et al., 2000).

When the regulation of molecular weight is considered, it is clear that the negative cooperativity properties of the different rubber transferases directly affect the molecular weight they produce under different substrate conditions, beyond the effects mediated by their different K_m's. For example, as described earlier, for *P. argentatum* rubber transferase, Hill plots of $\upsilon/(V_{max}-\upsilon)$ against [S] indicate negative cooperativity (gradient $\ll 1$, Fig. 6) over a much wider concentration range than in the other two species. Over this range, the *P. argentatum* rubber transferase active site is insensitive to the presence of unbound FPP, and the rubber polymers produced are considerably larger than would be the case if free FPP had the same ability to compete with the elongating polymers, and displace the rubber molecules from the active site, that it has at concentrations outside this negative cooperativity range (as discussed in the previous section). Also, in all three species, it appears that increasing IPP concentrations only can increase the molecular weight of the rubber polymers either when the initiator concentration is limiting (below the $K_m^{allylic\text{-}PP}$) or, under nonlimiting concentrations, when the particular enzyme exhibits negative cooperativity (Fig. 7; Cornish et al., 2000).

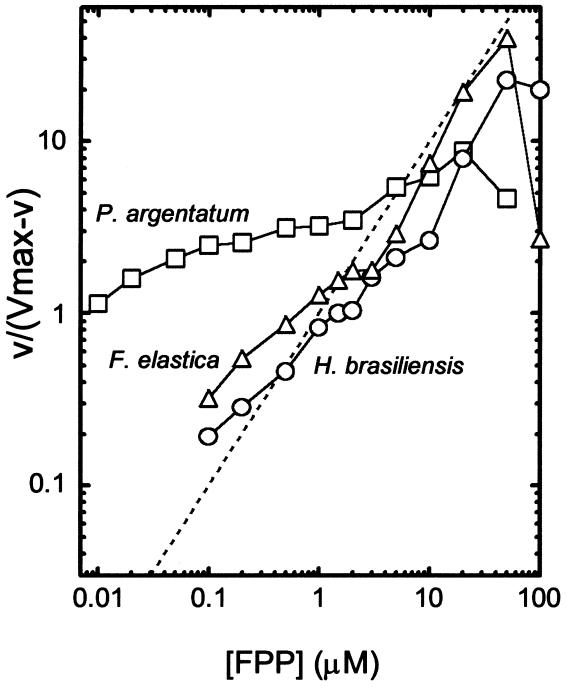

Fig. 6. Hill plot of IPP incorporation rate as a function of FPP concentration for *Hevea brasiliensis*, *Ficus elastica* and *Parthenium argentatum*. The intercept at $y=1$ is the K_m. Regions with a gradient $<<1$ indicate concentration ranges of negative cooperativity.

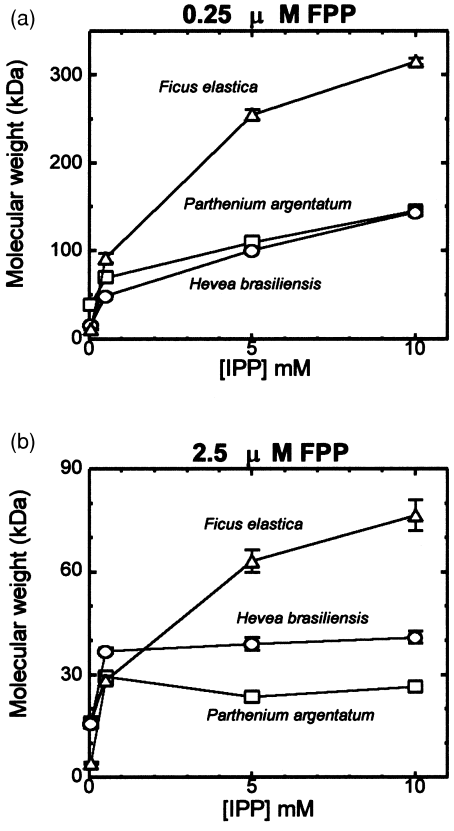

Fig. 7. Mean molecular weight (kDa) of rubber molecules synthesized by *Hevea brasiliensis*, *Ficus elastica* and *Parthenium argentatum* at various IPP concentrations at either (a) 0.25 μM or (b) 2.5 μM FPP. Each value is the mean of three replicates ± S.E.

3.4. Competition and activation

Additional similarities and differences between the three rubber transferases become apparent when co-substrate effects are considered (Castillón and Cornish, 1999; Cornish et al., 2000). For example, substrate activation appears to operate in the *P. argentatum* and *H. brasiliensis* rubber transferases. In these enzymes, IPP enhances the rate of FPP binding, but is significant in *H. brasiliensis* only at rate-limiting FPP concentrations instead of at all FPP concentrations as in *P. argentatum*. By contrast, the *F. elastica* rubber transferase was insensitive to increasing IPP concentrations and is the only species of the three in which IPP may slightly inhibit FPP incorporation at all FPP concentrations tested.

In all three species, the initiator FPP appears to activate IPP incorporation, but the *P. argentatum* rubber transferase differs from the other two in that IPP incorporation rate was enhanced by increasing FPP concentration at all IPP concentrations. By contrast, for the *H. brasiliensis* and *F. elastica* rubber transferases, IPP incorporation rates were only markedly FPP-dependent at rate-limiting IPP concentrations of near or below the K_m^{IPP}'s.

For the *H. brasiliensis* and *F. elastica* rubber transferases, under conditions where the FPP concentration is near or below the K_m^{FPP}, but when the IPP concentration is above K_m^{IPP}, IPP incorporation rate is a function of the IPP concentration and is insensitive to FPP concentration. This is apparent also for the *P. argentatum* rubber transferase when GPP is used is the initiator. It seems likely that the same result would be found with FPP, in this species, but lower FPP concentrations, than as yet explored, would be needed to achieve conditions below its K_m^{FPP}.

Although competition at the allylic-PP site has not been observed (Cornish et al., 2000), IPP and allylic-PP do appear to compete for the IPP binding site in the *P. argentatum* rubber transferase (Castillón and Cornish, 1999), although catalysis only occurs if IPP is bound. Interestingly, this also is a species-specific phenomenon, such competition not being apparent with either *H. brasiliensis* or *F. elastica* rubber transferase.

4. Conclusions

Prospects for the biotechnological development of alternative rubber producing crop plants suitable for temperate zone agriculture will improve as our knowledge of the factors regulating rubber biosynthesis progresses. In vitro investigations have indicated promising strategies for the enhancement of rubber yield, such as the transformation of rubber-producing plants with genes for substrate synthesis. However, the production of high molecular weight rubber seems more uncertain because in vitro biochemical studies have shown that a combination of factors must contribute to the molecular weight of rubber produced in vivo. The *P. argentatum* rubber transferase appears to exert more control over the molecular weight it produces than those from *H. brasiliensis* or *F. elastica* and the corresponding gene(s) may, therefore, provide the best prospect for genetic transformation of candidate annual crop species. However, in vitro experiments have not yet provided a unifying biochemical mechanism that could explain the different rubber molecular weights found in vivo in different plant species, such as the high molecular weight rubber in *H. brasiliensis* and *P. argentatum* and the low molecular weight rubber in *F. elastica*. It is possible that these evolutionarily-divergent species employ different mechanisms to regulate molecular weight but, given the general similarities in rubber biosynthesis enjoyed by the species, similar endogenous regulatory factors probably operate in vivo. Thus, molecular weight probably will be affected by endogenous substrate concentrations, the intrinsic properties of the rubber transferases involved and by other, as yet unknown, endogenous factors. These factors, and their interaction, must be understood before effective metabolic engineering strategies can be designed to maximize biosynthetic rate while simultaneously maintaining or conferring high rubber molecular weight. It may then be possible to achieve the goal of engineering temperate zone annual crops to produce commercially-viable yields of high quality rubber.

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