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Biosynthesis of natural rubber (NR) in different rubber-producing species

K. CORNISH, The Ohio State University, USA

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Abstract: Rubber biosynthesis in plants underpins the production of this strategically vital polymer. A fundamental understanding of the regulation of rate and polymer quality is essential to the development of alternate rubber-producing crops and new rubber materials with novel properties. Alternate rubber crops are needed to meet the projected shortfalls in global rubber production caused by the burgeoning economies of China and India.

Key words: biochemistry, initiator, monomer, natural rubber, polyisoprene, polymer.

1.1 Introduction

Natural rubber is the fourth most important natural resource of the modern earth, after air, water, and petroleum (History Channel, June 9, 2004). However, due to its pervasive utility, it is also one of our most underrated, taken-for-granted natural products. There are at least 40,000 different products made with natural rubber and over 400 medical devices (Mooibroek and Cornish, 2000). The large amount of irreplaceable natural rubber needed in the military, industrial, transportation, medical and consumer sectors have led to natural rubber being repeatedly defined as a strategic raw material over the last 70 years. Although many synthetic rubber (derived from petroleum) applications can be met with natural rubber, the converse is not true. For example, although all tires contain a significant proportion of natural rubber, the higher the performance required, the greater the amount of the natural rubber component: truck tires are 90–100%, airplane tires are 100%, and navy jet tires on aircraft carriers are single-use 100% natural rubber tires. Almost all commercial natural rubber is tapped from a single species, *Hevea brasiliensis*, the *para* rubber tree. Production predominately occurs in plantations and small holdings in South-east Asia, a region which produces about 90% of global natural rubber. Africa produces around 10% and South America less than 1% because of the endemic South American leaf blight, a fatal fungal disease caused by *Microcyclus ulei* infection (Furtado *et al.*, 2008; Lieberei, 2007; Rocha *et al.*, 2011). The genetic diversity of cultivated *H. brasiliensis* is extremely low, advanced lines are grown as clonal scions on

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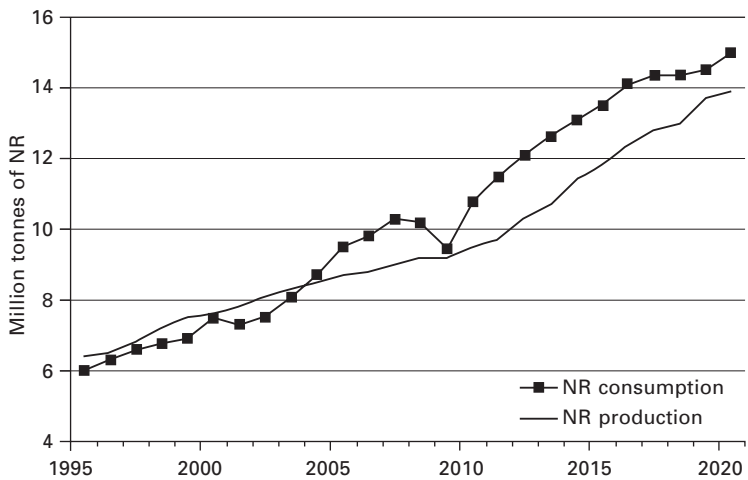
seedling root stocks, and most, if not all, modern lines are *M. ulei* sensitive. Thus, *H. brasiliensis* is at constant risk of crop failure.

The expanding economies of China and India have already eroded the small gains achieved in the rubber supply during the economic downturn of 2008–11, and shortages are burgeoning (Fig. 1.1). The independent International Rubber Study Group (IRSG, Singapore) has predicted a 1.5–3 million metric ton global shortfall between supply and demand by 2020 – the United States imports 1.2 million MT/yr. Like many countries, the United States is currently totally dependent upon the import of natural rubber (NR); in its case, for more than 1.2 million metric tons per year from tropically-grown sources. The economic importance of maintaining a steady supply of NR is highlighted by the fact that the United States rubber products trade in 2011 was worth over $18 billion.

The use of a single species to generate the global supply of a strategy commodity is not necessary, and has happened for rubber more as a matter of chance than anything else. In contrast, many crops are used to supply starch to humans even though the composition and quality of the different crops is dissimilar (e.g., potatoes, wheat, and rice). Similarly, many different plants (Mooibroek and Cornish, 2000), and some *Lactarius sp.* fungi (Mekkriengkrai *et al.*, 2004; Ohya *et al.*, 1997, 1998) make rubber and a few of these, as wild plants, have been used over past centuries and still

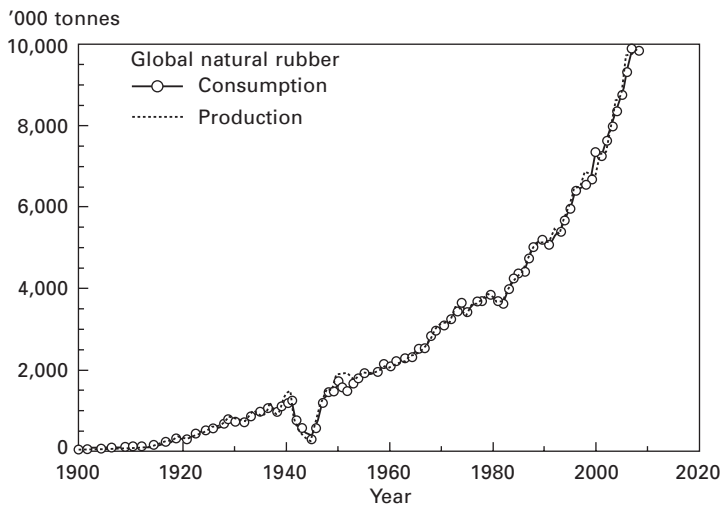


1.1 Annual global production and consumption of natural rubber from 1995 to 2012 with projections to 2020. The economic downturn reduced the rate of consumption from 2007 to 2009, allowing earlier projected shortfalls to be halved (data from the International Rubber Study Group). It should be noted that some of the shortfall could be made up by increased tapping of existing trees (Dock No, IRSG, personal communication), labor permitting.

could be developed as crops. *Hevea brasiliensis* is actually quite a recent crop because, until 120 years ago, we did not know how to compound rubber and generate desired product performance (Finlay, 2013). Wild rubber was used but had few applications. Since then, the enormous investment in all aspects of commercial production has led to the remarkable expansion of the crop that we have seen, particularly in South-east Asia, and until 2005, consumption of rubber has closely matched production (Fig. 1.2).

Of the many plants capable of natural rubber production, two temperate species stand out as commercial candidates, *Parthenium argentatum* (guayule) and *Taraxacum kok-saghyz* (Kazak dandelion, also known as Russian dandelion and Buckeye Gold). These alternate rubber species are under development at a number of universities and companies on several continents. At the present time, *P. argentatum* is ahead of *T. kok-saghyz*, commercially. However, there is a large pilot plant for *T. kok-saghyz* processing in Wooster, Ohio, USA. Also, rubber and latex from *T. kok-saghyz* is similar to that from *H. brasiliensis* in composition and performance (Cornish *et al.*, 2012). This similarity includes latex and rubber-particle bound proteins that cross react with Type I latex allergy, and so this rubber is a supplement to *H. brasiliensis* rubber, but not a circumallergenic rubber or latex, like that from *P. argentatum* (Cornish, 2012).

Future improvements in rubber yield per area of *H. brasiliensis* and the development of alternate natural rubber crops require an understanding of

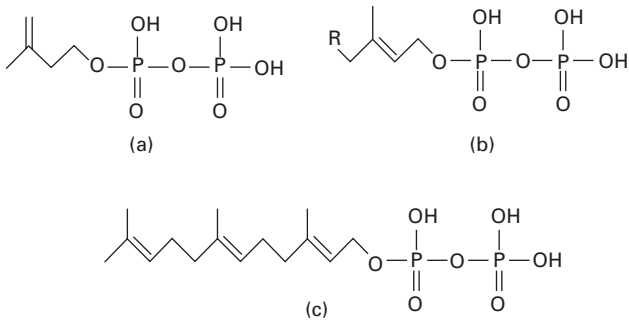


1.2 Annual global production and consumption of natural rubber from 1900 to 2008. The impact of World War II is clear and led to the Emergency Rubber Project. However, the world needs ten times more rubber than it needed in the 1940s, and demand will at least double by 2030 (data from the International Rubber Study Group).

how the rubber is made, and how these mechanisms relate to both yield and quality. This understanding can direct both genetic engineering approaches, and plant breeding. Without such knowledge, such efforts rely heavily on serendipitous discoveries, and research over many years is required. In this chapter, I attempt to describe some of the commonalities and complexities of rubber biosynthesis and what we know, at this time, about the regulation of rubber biosynthetic rate, chain transfer and final molecular weight in evolutionarily-divergent rubber-producing species.

1.2 Rubber biosynthesis

Rubber biosynthesis requires two distinct pyrophosphate substrates and a divalent cation activator, usually magnesium ions in the living plant system (Archer and Audley, 1967, 1987; Cornish, 2001a, 2001b; Cornish and Backhaus, 1990). The Mg^{2+} is essential for binding the pyrophosphate (PP) substrates into the active site. The first substrate, which initiates the polymerization reaction, is an allylic pyrophosphate (APP), which appears to be the C_{15} farensyl-PP (FPP) *in vivo* (Fig. 1.3c). However, apart from a single initiator, the rest of the rubber polymer is made from the non-allylic-PP, isopentenyl pyrophosphate (IPP) (Fig. 1.3a). IPP is isomerized to DMAPP (Fig. 1.3b) and then two condensation reactions, catalyzed by the *trans* prenyl transferase farnesyl pyrophosphate synthase, sequentially add two IPPs to the DMAPP to make FPP. Thus, the final rubber polymer is actually *trans trans*-$[cis]_n$-polyisoprene, when n is an indeterminate number that is around 30,000 for most high performance rubbers. The head group of the elongating rubber molecule functions as the APP during polymerization. The reaction is an alkylation by prenyl transfer from the non-allylic pyrophosphate monomer IPP (nucleophile) to the initiator APP (electrophile) (Walsh, 1979).



1.3 (a) Structure of IPP; (b) structure of APP, where APP is dimethyl allylic pyrophosphate (DMAPP) if R = H, APP is geranyl pyrophosphate (GPP) if R = C_5H_9, APP is farnesyl pyrophosphate (FPP) if R = $C_{10}H_{17}$; (c) structure of FPP. In solution, the pyrophosphate groups will be ionized to varying degrees.

Each rubber polymerase can produce many rubber molecules sequentially, and multiple enzymes are present on each enzymatically-active rubber particle (Castillón and Cornish, 1999). However, the mechanism by which chain termination occurs is poorly understood and may not be the same in different species. *In vitro*, chain length, and therefore termination, is closely tied to the rate of the chain transfer reaction (the displacement of the elongating rubber molecule by a new APP initiator) which, in turn, is governed by the concentration, APP identity, and APP:IPP ratio, the magnesium cofactor concentration and A_{max}, and the species-specific rubber transferase K_m's of these compounds (Castillón and Cornish, 1999; Cornish, 1993, Cornish and Backhaus, 1990; Cornish *et al*., 2000; Cornish and Scott, 2005; Cornish and Siler, 1995, 1996; da Costa *et al*., 2005, 2006; Espy *et al*., 2006; Scott *et al*., 2003; Siler and Cornish, 1995). However, if this were the only mechanism, the concentration of substrates and activators in rubber-producing tissues must be very tightly regulated, at least in species making rubber in laticifers, because the rubber polydispersity is quite narrow (Cornish *et al*., 1993). Low polymer polydispersity means that the same molecular weight is made all the time in a specific species, even though different species make rubber of different chain lengths. We have only found broad-based rubber transferase-regulated polymer chain length in *Parthenium argentatum* (guayule), a species that makes high molecular weight rubber in generalized bark parenchyma cells. Tight control of cytoplasmic APP and IPP concentrations may be possible in a latificer, because this organ is partially separated from the tissues and functions essential to life. However, these isoprenoid substrates are used by many other enzymatic processes in plants, and their abundance varies with season, stage of plant growth and development. This suggests that other endogenous factors may be involved in the maintenance of low polydispersity.

The substrates for rubber biosynthesis, IPP (the monomer), and its APP catabolites (the initiators), are synthesized from carbohydrates via acetyl-coenzyme A, 3-hydroxy-3-methylglutaryl-coenzyme A reductase and mevalonate. The plastid-localized deoxy-xylulose/methyl-erythritol phosphate pathway also produces IPP (Lichtenthaler *et al*., 1997; Rohmer *et al*., 1993). Plastidic IPP can move from the chloroplast to the cytosol, where it would be available for rubber biosynthesis, but it is not known how much cross-talk between the two compartments actually occurs (Kumar *et al*., 2012).

Rubber transferase is an unusual enzyme in that the specific binding of the APP initiator seems to be confined to the C_5 APP end of the initiating molecule and does not directly involve the entire substrate (Cornish, 2001a, 2001b). The K_m^{APP} is dependent upon the size and stereochemistry of the APP initiator, with binding affinity increasing with initiator length until the interior of the rubber particle is reached. Length, in this case, refers to the minimized chemical structure (much shorter in an all *cis*-APP than an all

trans one) and not to the number of carbons in the polymer chain (Table 1.1). Many different APPs have been shown, in *in vitro* assays, to effectively initiate rubber biosynthesis ((Archer and Audley, 1987; Castillón and Cornish, 1999; Cornish, 2001a, 2001b; Cornish and Scott, 2005) (Tables 1.2–1.4). The use of labeled and derivatized APPs to study rubber biosynthesis is limited

Table 1.1 Intramolecular lengths of different substrates in rubber biosynthesis. Molecules measured are minimized structures

Substrate	Entire molecule (terminal P to terminal C)	Entire hydrocarbon region (first to last C)	Linear region (from terminal P)	Linear region (from C adjacent to OPP)
DMAPP/IPP	0.885	0.387	0.885	0.387
GPP	1.345	0.842	1.345	0.842
NPP	0.908	0.712	0.870	0.387
tt-FPP	1.822	1.311	1.822	1.311
tc-FPP	1.367	1.179	0.880	0.387
cc-FPP	1.470	1.004	1.470	1.004
ttt-GGPP	2.189	1.738	2.189	1.738
ttc-GGPP	1.767	1.594	0.870	0.387
ttccc-HPP	2.648	2.375	1.825	1.433
ttccccc-OPP	3.163	1.992	2.385	1.992

Note: All measurements are based on straight lines between the centers of the two atoms designated and are expressed in nm.

Table 1.2 Binding constants (K_m) and maximum reaction velocity (V_{max}) of the *Parthenium argentatum* rubber transferase

Substrate	Size (No. of carbons)	Stereoisomer	Apparent K_m (μM)	V_{max} (μmol IPP/g dry rubber/4h@16°C)
IPP	5	–	350*	–
DMAPP	5	–	2.56*	1.40
GPP	10	*trans*	0.49*	1.74
NPP	10	*cis*	0.08	2.01
FPP	15	*trans,trans*	0.019*	1.18
FPP	15	*trans,cis*	0.69	1.74
FPP	15	*cis,cis*	1.89	1.74
GGPP	20	*trans,trans,trans*	0.016*	1.78

Kinetic values for the allylic pyrophosphates were determined at 16°C in the presence of 5 mM [^{14}C] isopentenyl pyrophosphate (IPP) and 1.25 mM $MgSO_4$. DMAPP, dimethyl allyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; NPP, neryl pyrophosphate. The maximum reaction velocities tabulated are the means of two experiments, while the K_ms* are the mean of two. V_{max} varies with the activity of the rubber particle preparation used.

Table 1.3 Binding constants (K_m) and maximum reaction velocity (V_{max}) of the *Hevea brasiliensis* rubber transferase

Substrate	Size (No. of carbons)	Stereoisomer	Apparent K_m (μM)	V_{max} (μmol IPP/g dry rubber/4h@25°C)
IPP	5	–	300	–
DMAPP	5	–	10	0.7
GPP	10	*trans*	3	1.1
NPP	10	*cis*	3	0.8
FPP	15	*trans,trans*	1	1.5
FPP	15	*trans,cis*	1	0.5
FPP	15	*cis,cis*	2	1.9
GGPP	20	*trans,trans,trans*	1.5	2.0
GGPP	20	*trans,trans,cis*	–	2.7
HPP	30	*trans,trans,cis,cis,cis*	1	0.95
OPP	40	*trans,trans,cis,cis,cis,cis,cis*	1	1.0
SPP	45	*all trans*	5	0.65

Kinetic values for the allylic pyrophosphates were determined at 25°C in the presence of 5 mM [^{14}C] isopentenyl pyrophosphate (IPP) and 1.25 mM $MgSO_4$. DMAPP, dimethyl allyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HPP, hexaprenyl pyrophosphate; NPP, neryl pyrophosphate; OPP octaprenyl pyrophosphate; SPP, solanesyl pyrophosphate.

Table 1.4 Binding constants (K_m) and maximum reaction velocity (V_{max}) of the *Ficus elastica* rubber transferase

Substrate	Size (No. of carbons)	Stereoisomer	Apparent K_m (μM)	V_{max} (μmol IPP/g dry rubber/4h@25°C)
IPP	5	–	160	–
DMAPP	5	–	8	0.30
GPP	10	*trans*	1	0.49
NPP	10	*cis*	–	0.59
FPP	15	*trans,trans*	0.2	0.85
FPP	15	*trans,cis*	–	0.69
FPP	15	*cis,cis*	–	0.60
GGPP	20	*trans,trans,trans*	0.38	1.17
GGPP	20	*trans,trans,cis*	–	1.52
HPP	30	*trans,trans,cis,cis,cis*	–	0.27
OPP	40	*trans,trans,cis,cis,cis,cis,cis*	–	0.17
SPP	45	*all trans*	–	0.19

Kinetic values for the allylic pyrophosphates were determined at 25°C in the presence of 5 mM [^{14}C] isopentenyl pyrophosphate (IPP) and 1.25 mM $MgSO_4$. DMAPP, dimethyl allyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HPP, hexaprenyl pyrophospahe; NPP, neryl pyrophosphate; OPP octaprenyl pyrophosphate; SPP, solanesyl pyrophosphate.

only by the solubility (Lichtenthaler *et al*., 1997; Rohmer *et al*., 1993) of the APP, and the solubility of large or complex APP molecules can be enhanced by the use of noninhibitory concentrations of detergents. This ability has proven a powerful tool in biochemical studies and has led to biochemically-based models of the active site (Cornish, 2001a, 2001b). Derivatized APPs that no longer contain a terminal C_5 APP may act as substrate competitors of various types (Xie *et al*., 2008; DeGraw *et al*., 2007; Henry *et al*., 2009).

In contrast, the IPP binding site is highly specific and no IPP analogs, to date, have been incorporated into the rubber polymer in *in vitro* assays. There is some very interesting work, from the Puskas group, however, indicating isoprene can be incorporated under specific circumstances, but the mechanisms for this are not fully understood, and it may not be relevant *in planta* (Chiang *et al*., 2009, 2011; Kostjuk *et al*., 2011; Forestier *et al*., 2009; Lindsay *et al*., 2008; Puskas *et al*., 2006; Puskas, 1994; Puskas and Wilds, 1994).

It has also been shown that APP competitively inhibits IPP binding at the IPP binding site, but that IPP enhances APP binding at the APP binding site (Castillón and Cornish, 1999; Scott *et al*., 2003). Thus, it appears that both nonallylic and allylic pyrophosphates are behaving as substrate analogs capable of allylic pyrophosphate substrate activation.

1.3 Rubber particles and rubber biosynthesis

Rubber particles from different species have highly species-specific complements of lipids and proteins, and these can change with rubber particle age. One of the proteins, or protein complexes, is responsible for rubber biosynthesis. This biological catalyst, rubber transferase or rubber polymerase (EC 2.5.1.20), is embedded, probably as a complex, in the monolayer membrane of cytosolic rubber particles (Cornish *et al*., 1999; Wood and Cornish, 2000; Siler *et al*., 1997; Backhaus and Walsh, 1983, Cornish and Backhaus, 1990). A combination of structural and kinetic studies indicates that the substrates for rubber biosynthesis enter the rubber particle at the surface and the rubber polymer is elongated to the interior of the rubber particle on the far side of the monolayer biomembrane (Cornish, 2001a, 2001b; Cornish *et al*., 1999; Wood and Cornish, 2000). Extension of the elongating rubber polymer into the hydrophobic rubber interior of the particle is probably essential to the continued polymerization reaction. The aqueous-organic interface provided by the rubber particle monolayer biomembrane is probably required and accounts for the general lack of success in identification of solubilized rubber transferase activity. Only one report has been published, for the *P. argentatum* rubber transferase (Benedict *et al*., 2009), but this has not yet been reproduced.

The organic aqueous interface between the aqueous cytosol and the organic rubber interior is essential to the synthesis of high molecular weight hydrocarbon rubber chains. This is because the rubber is synthesized from hydrophilic pyrophosphates in the cytoplasm, and then the hydrocarbon rubber polymer must be elongated into the hydrophobic interior of the rubber particles.

A combination of structural and kinetic studies indicates that the polymer passes through a hydrophobic column as it traverses the membrane (Cornish, 2001a, 2001b; Cornish *et al*., 1999). The physical length of this column was determined by kinetic characterization, using different APP initiators, and electroparamagnetic spin probe analysis. These contrasting methods yielded the same conclusions, with the distance from the APP specific binding site to the rubber polymer particle interior being equivalent to the size of an all *trans* GGPP for *H. brasiliensis* and *F.elastica* and an all *trans* FPP for *P. argentatum* (Table 1.4).

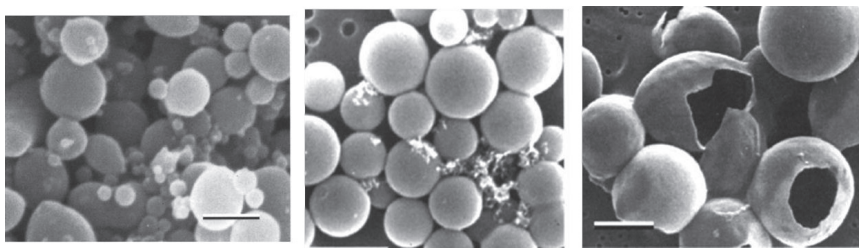
Extension of the elongating rubber polymer into the hydrophobic rubber interior of the particle is probably essential to the continued polymerization reaction. Without the hydrophobic compartment drawing the polymer from the enzyme, the polymer would rapidly block the active site. This type of blockage has been shown in GGPP synthase when site-directed mutagenesis opened the floor of the enzyme's binding pocket. In this case, a significantly longer *trans*-polyisoprene molecule was synthesized, but once this polymer obstructed the channel through hydrophobic interactions coiling up the polymer against the protein, synthesis halted (Tarshis *et al*., 1996).

The physical interaction between the hydrophobic rubber particle interior and the elongating rubber molecule may increase the physical 'tug' on the chain and eventually encourage the chain transfer reaction, by increasing the vibration or strain on the head group and pulling the APP terminus away from the binding site at the moment of prenyl transfer and transient substrate release. It is also possible that the fluidity of the membrane enhances the chain release from the active site. Electron *para* magenetic spin probe analysis demonstrated that the *H. brasiliensis* and *P. argentatum* rubber particle membranes are fluid, whereas those of *Euphorbia lactiflua* (high in protein) and *F. elastica* are stiff (Cornish *et al*., 1999). Furthermore, the fatty acid analysis (Table 1.5) suggests that particle size may be related to fatty acid size (Siler *et al*., 1997). The smallest rubber particles (*E. lactiflua*, mean particle diameter 200 nm) also have the shortest fatty acids in the particle membrane, whereas the largest particles (*F. elastica*, mean particle diameter 4.5 μm) have the longest fatty acids in its rubber particle membrane (Table 1.5). Scanning electron microscopy also clearly shows the stiff membrane of the *F. elastica* particle, and proves that the inner rubber core is fluid (Fig. 1.4) (Wood and Cornish, 2000).

Table 1.5 Sum of neutral, phospho and glycol lipids in rubber particle membranes from four species

Species	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0	24:0	26:0	28:0
H	4		10	4	11						
P	9		2	8	53	28					
F	10			16			4	21	18	13	18
E	64		9	18	9						

Source: H, *Hevea brasiliensis*; P, *Parthenium argentatum*; F, *Ficus elastica*; E, *Euphorbia lactiflua*. Data summarized from Siler *et al.*, 1997.



(a) (b) (c)

1.4 Scanning electron micrograph of rubber particles purified from (a) *Hevea brasiliensis* (bar is 1 μm), (b) *Parthenium argentatum* (same scale as (a)), and (c) *Ficus elastica* (bar is 2 μm).

1.4 Kinetic analyses of rubber transferase

Rubber biosynthesis is dependent upon the concentrations of APP (initiator), IPP (monomer), and magnesium ions (activator). Kinetic constants are best determined for each by varying the concentration of one at a time, while the other two are present at non-limiting, but non-inhibitory, concentrations. These constants can vary over several orders of magnitude in different rubber-producing species for the initiator, and over at least one order of magnitude for the magnesium ion activator, and other activators can be different again. Thus, several species-specific experiments may be required to find the appropriate concentration ranges for good kinetic data. Depending upon which aspect of rubber biosynthesis is under investigation, the initiation reaction, the polymerization reaction, or both simultaneously (as is most common), different kinetic analyses are appropriate (Segel, 1993). We have found that the Michaelis–Menton plot of $1/v$ versus $1/[S]$ generally results in a curved plot leading to a subjective linear regression and doubtful K_m's. The Eadie–Hosftee plot of $v/[S]$ versus $[S]$ generates a linear plot over most concentrations for the IPP polymerization reaction, in the presence of non-limiting initiator concentrations, but very low IPP concentrations and non-limiting IPP concentrations should be deleted. The gradient of the plot is $-K_m^{IPP}$, and the y axis intercept is V_{max}^{IPP} for IPP in the particular

initiator used. However, due to the non-specific hydrophobic binding region in the rubber transferase active site, short initiators also generate curved $v/[S]$ versus $[S]$ plots. In these circumstances, we suggest using the Hill plot of $\log(v\text{-}V_{max})/v$ plotted against $\log [S]$. In this plot, the K_m^{APP} is the x-axis value where $y = 1$.

Similarly, cofactor investigations can be problematical. It is difficult to wash out sufficient Mg^{2+} to bring activity low enough to determine reaction kinetics, because of its tight affinity with the active site, without taking so much time that appreciable enzyme activity is lost during the purification. This problem can be solved by the addition of EDTA to chelate the essential magnesium cation activator and bring the enzyme activity to baseline. Titrating back magnesium will indicate the true origin as activity rises above the baseline value. That particular magnesium concentration becomes the true origin and is subtracted to adjust the x-axis values to the origin. Informative kinetic plots can then be constructed and rate constants determined. These analyses underpin the next sections of the chapter. A recent publication can be consulted to obtain details of rubber transferase assays and related topics (Cornish and Xie, 2012).

1.5 Regulation of biosynthetic rate

1.5.1 Allylic and non-allylic pyrophosphates

Enzymological investigations ideally are performed using a soluble enzyme system where

$$\text{Enzyme} + \text{Substrate} \rightleftharpoons \text{EnzymeSubstrate complex} \rightarrow \text{Enzyme} + \text{Product}$$

Such reactions follow Michaelis–Menton kinetics. However, this equation does not adequately describe rubber biosynthesis, which involves a membrane-bound enzyme, two substrates, a cofactor and a polymeric product that is not fully released from the active site at each substrate addition. This system can be described as:

$$\text{Enzyme} + \text{Substrate}_1 \rightleftharpoons \text{EnzymeSubstrate}_1 + \text{Substrate}_2 \rightarrow \text{EnzymeSubstrate}_2\text{Substrate}_1 + (\text{Substrate}_2)_n \rightarrow \text{Enzyme}\ (\text{Substrate}_2)_{n+1}\text{Substrate}_1 \rightarrow \text{Enzyme} + \text{Product}$$

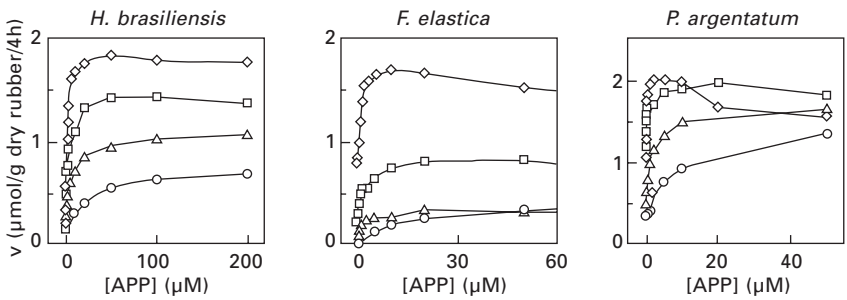
where Substrate_1 is the APP initiator, Substrate_2 is the isopentenyl monomer, and Product ($(\text{Substrate}_2)_n\text{Substrate}_1$) is the *trans*,*trans (cis)n*-1-4 polyisoprene (rubber). Membrane-bound enzyme reactions can be investigated intact

provided that only one enzyme is present that uses the substrates for the reaction under investigation. This, fortunately, has proved to be the case for rubber biosynthesis (Cornish and Backhaus, 1990; Cornish, 1993; Cornish and Siler, 1996).

Rubber biosynthesis is an unusual reaction in that the rubber transferase can accept any one of a number of APPs as the initiating molecule (Archer and Audley, 1967, 1987; Cornish, 1993). However, the structure and size of the initiating molecule affect the IPP condensation reaction rate as the rubber molecule polymerizes. In non-limiting APP concentrations, the longer the carbon chain of the initiator, up to the C_{15} farnesyl pyrophosphate (FPP), in *P. argentatum*, the higher the rate (v) of IPP incorporation into rubber by the rubber transferase (Fig. 1.5) (Cornish, 2001a, 2001b; Cornish and Scott, 2005). In this species, the C_{20} initiator, geranylgeranyl pyrophosphate (GGPP) leads to a lower IPP polymerization rate with only the C_5 dimethyl allyl pyrophosphate (DMAPP) giving a slower rate. However, in *H. brasiliensis* and *F. elastica*, rate increases with APP size up to the C_{20} all *trans*-GGPP (Fig. 1.5). These effects are caused by the hydrophobic non-specific binding region in the active site described above. It is also clear that the higher the concentration of IPP, the more rubber is made up to a saturating [IPP] (Fig. 1.5).

1.5.2 Allylic pyrophosphate isomers

Isomers and analogs of APPs have been extensively used to investigate rubber biosynthesis and, especially, the functioning of the active site. In one unpublished study on *P. argentatum* only, pairs of APPs were used simultaneously. The pyrophosphates were mixed in pairs such that a total allylic pyrophosphate concentration of 20 μM was maintained, and the



1.5 [^{14}C] IPP incorporation by purified rubber particles, as a function of the concentration of different initiators. DMAPP, dimethyl allyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate. All assays were performed in 1 mM IPP.

concentration of one APP pyrophosphate ranged from 0 to 20 μM while the concentration of the other ranged from 20 to 0 μM. Interactions between the initiating substrates become apparent and these also presumably could occur *in vivo*. Thus, if the APP binding site has no preference for either APP in the pair, the plot would show a horizontal linear relationship from the V_{max} of one by itself, moving through the concentration range to the V_{max} of the other by itself. If, however, the rubber transferase did bind one of the pair preferentially, as one would expect from the different K_m's, a non-linear plot would result. In the case of a concave plot, the enzyme preferentially binds the APP present at the right-hand *y*-axis, and if convex, the APP present at the left-hand *y*-axis. In this manner, a horizontal linear plot indicated that the *P. argentatum* rubber transferase does not distinguish between a *trans*,*trans*-FPP and *trans*,*tran*,*trans*-GGPP, but has a higher affinity for both of these substrates than for GPP or DMAPP (concave plots with the smaller substrate to the left of the plot (unpublished data, not shown).

The results of this investigation also demonstrate kinetically that the *P. argentatum* rubber transferase only performs condensations of additional IPP into the elongating rubber chain in the *cis* configuration. It had seemed possible that short allylic pyrophosphates, such as DMAPP or GPP, may first be converted into all *trans*-FPP initiators (Light and Dennis, 1989). These longer *trans*-allylic pyrophosphates would then be bound by the rubber transferase and initiate formation of the *cis*-polyisoprene as occurs from all-*trans*-FPP *in vivo*. The two possible models would generate different stereo-intermediates as the new rubber molecule begins to form, depending upon the particular initiator, and lead to different shapes of the pair's plots.

If *trans*:

DMAPP → GPP → *trans*,*trans*-FPP → *trans*,*trans*,$(cis)_n$-OPP

and

NPP → *cis*,*trans*-FPP → *cis*,*trans*,*trans*,$(\text{cis})_n$-OPP

and

cis,*cis*-FPP → *cis*,*cis*,*trans*-APP → *cis*,*cis*,*trans*$(cis)_n$-OPP

If *cis*:

DMAPP → NPP → *cis*,*cis*-FPP → all(*cis*)-OPP

The data (not shown) are consistent with *cis*-polymerization.

1.5.3 Time courses of substrate incorporation in *H. brasiliensis* and *P. argentatum*

Time courses of IPP incorporation (unpublished) were taken out to 8 hours in [FPP] of 1/10 K_m^{FPP}, (0.15 μM) K_m^{FPP} (1.5 μM) and 10 × K_m^{FPP} (15 μM)

for *H. brasisliensis* and in K_m^{FPP} (0.015 μM), $10 \times K_m^{FPP}$ (0.15 μM) and $100 \times K_m^{FPP}$ (15 μM FPP) for *P. argentatum*. The time courses were performed in 8 mM Mg^{2+} and IPP concentrations approximating K_m^{IPP} and $10 \times K_m^{IPP}$. In *P. argentatum*, at K_m [IPP] and [FPP], polymer size did not increase beyond the normal *in vivo* size after 4 h, while in *H. brasiliensis* the polymer size grew to twice its normal *in vivo* size in 8 h. This suggests that the laticifer controls *in vivo* molecular weight in *H. brasiliensis*. *P. argentatum* has no laticifer control, of course. Also, it takes approximately 2 h for the rubber molecules to be released from the active size and for new initiation to occur. This can be interpreted as it taking two hours for a rubber molecule to become mature. In the very limited [FPP] in *P. argentatum*, no reinitiation takes place, because of the rubber tranferase FPP negative cooperativity at this concentration, and the rubber molecular weight increased throughout the 8 h of the experiment.

1.5.4 Role of cofactor/activator

Rubber transferases from *H. brasiliensis*, *P. argentatum* and *F. elastica* can use magnesium and manganese interchangably to achieve maximum velocity. The concentration of activator required for maximum velocity is defined as $[A]_{max}$. The $[A]_{max}^{Mg2+}$ in *F. elastica* is 10 times the $[A]_{max}^{Mg2+}$ for either *H. brasiliensis* or *P. argentatum* (Table 1.6) (Scott *et al.*, 2003). For each species, the $[A]_{max}^{Mg2+}$ is essentially the same as the $[A]_{max}^{Mg2+}$. The differences in $[A]_{max}^{Mg2+}$ correlate with the actual endogenous Mg^{2+} concentrations in the latex of living plants. Extremely low Mn^{2+} levels *in vivo* indicate that Mg^{2+} is the RuT cofactor in living *H. brasiliensis* and *F. elastica* trees (Table 1.6).

Enzymes that utilize metals may be either metalloenzymes or metal-activated enzymes. If an enzyme is a metalloenzyme, the metal is tightly bound with high affinity and is an integral component of the enzyme. If the metal is not always associated with the enzyme, but effects activity, the enzyme is classified as using metals as activators (King, 2003). Metals are essential activators for the catalytic activity of prenyl transferases but no *cis* or *trans* prenyl transferases characterized so far have been true metalloenzymes, i.e. the metal activator in the prenyl transferase is not a structural component

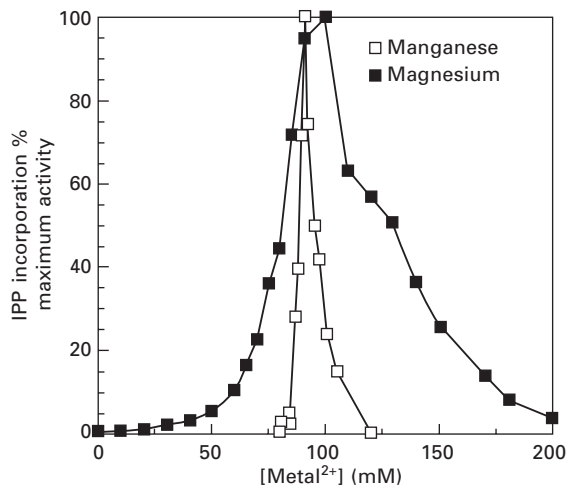
Table 1.6 Latex cation concentration by inductively coupled plasma, and kinetic determination of A_{max}

Species	$[Mg^{2+}]$ mM		$[Mn^{2+}]$ mM	
	ICP	A_{max}	ICP	A_{max}
H. brasiliensis	12	10	0.014	4
F. elastica	53	70	0.012	11
P. argentatum	?	8	?	5

of the enzyme. It is well established that rubber transferase activity can be readily eliminated by EDTA and the 20 mM EDTA was sufficient to eliminate activity for all three species of purified rubber particles and agreed with an earlier report for *Ficus carica* (Kang *et al*., 2000). When the activity was restored by the addition of either Mg^{2+} or Mn^{2+} (Fig. 1.6), the response indicated that rubber transferase is acting as a metal-activated enzyme (Scott *et al*., 2003).

Kinetic analyses demonstrate that FPP-Mg^{2+} and FPP-Mn^{2+} are active substrates for rubber molecule initiation, although free FPP and metal cations, Mg^{2+} and Mn^{2+}, can interact independently at the active site with the following relative dissociation constants $K_d^{FPP} < K_d^{FPP\text{-}Metal} < K_d^{E\text{-}Metal}$. Similarly, IPP-$Mg^{2+}$ and IPP-Mn^{2+} are active substrates for rubber molecule polymerization. Although metal cations can interact independently at the active site with the relative dissociation constant $K_d^{IPP\text{-}Metal} < K_d^{E\text{-}Metal}$, unlike FPP, IPP alone does not interact independently. The results suggest that *H. brasiliensis* could use [Mg^{2+}] as a regulatory mechanism for rubber biosynthesis and molecular weight *in vivo*. A similar response to [Mg^{2+}] was discovered in *P. argentatum*, at the same concentrations as *H. brasiliensis*, although somewhat smaller in degree.

This regulatory mechanism would work as follows. At a low level of Mg^{2+}, only a small amount of rubber is synthesized, as the [FPP.Mg] and [IPP.Mg] are low. Increasing the [Mg^{2+}] leads to a higher amount of FPP. Mg and IPP.Mg in solution, resulting in higher FPP and IPP incorporation



1.6 Concentration dependencies of magnesium and manganese cations on [^{14}C] IPP incorporation by rubber transferase in enzymatically active purified rubber particles from *F. elastica* pretreated with 20 mM EDTA. IPP incorporation was measured in the presence of 20 μM FPP, 200 μM IPP, and for both metals.

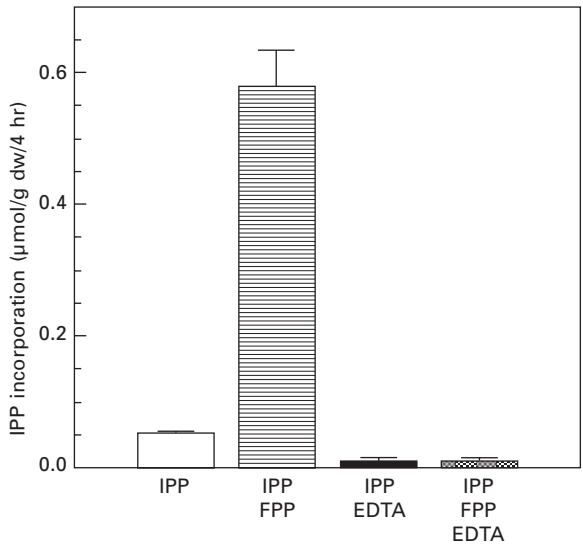
rates (Scott *et al*., 2003). However, at yet higher [Mg], the metal ion can interact directly with the rubber transferase inhibiting enzyme activity causing a decrease in IPP incorporation rate (Fig. 1.6). [Mg^{2+}] has a stronger effect on IPP incorporation rate than on FPP incorporation rate and, as the [Mg^{2+}] affects both the initiation rate and the elongation rate, it also affects the rubber molecular weight.

The affinity of the enzyme for IPP.Mg is greatly affected by the [Mg^{2+}]. The change in affinity is so great (160-fold in *H. brasiliensis*) that it is likely due to a conformational change of the rubber transferase, similar to that which occurs in porphorbilinogen synthase (PBGS). The affinity of PBGS for aminolevulinic acid, its substrate, increases by two orders of magnitude in the presence of magnesium, where PBGS then exists as an octamer (Breinig *et al*., 2003). Rubber transferase undergoes a similar change in affinity when the exogenous magnesium concentration was increased from 4 mM to 8 mM. The huge change in K_m^{IPP} with Mg^{2+} strongly suggests that the enzyme, after the conformational restructuring, may then be a true metalloezyme. Neither FPS (*trans*-prenyl transferase) nor UPPS (*cis*-prenyl transferase) are metalloenzymes, which may or may not be true for other prenyl transferases in general (Laskovics and Poulter, 1981).

1.5.5 Initiator-dependent and independent polymerization

Virtually all investigations of rubber biosynthesis have relied on purified enzymatically-active rubber particles as the source of enzyme (Cornish and Xie, 2012). However, in rubber particles with flexible or fluid membranes, the process of purification releases all polymers that were incomplete from the active site of the rubber transferase (Fig. 1.7; cf. IPP FPP bar with IPP, FPP, EDTA bar). Thus, the polymerization process cannot be studied independently of the initiation process in these species using standard assays. However, *F. elastica* rubber particles have rigid, waxy membranes and some elongating rubber allylic pyrophosphate chains remain in their active sites during purification (Fig. 1.7; cf. IPP bar with IPP EDTA bar) (Espy *et al*., 2006). This allows the polymerization process itself to be studied.

The presence of initiator-independent incorporation of IPP by enzymatically-active rubber particles purified from *F. elastica* led to the conclusion that pre-existing, partially-formed rubber chains are present in *F. elastica* purified rubber particles, which are further elongated *in vitro* upon the addition of IPP (Fig. 1.8). In agreement with the earlier time courses for *H. brasiliensis* and *P. argentatum*, it takes about 2 h to complete a rubber molecule (Fig. 1.8(b)). FPP appears to serve as both the chain transfer agent and the reinitiation agent. Even though IPP has been shown to enhance APP binding at the APP binding site, it cannot reinitiate the elongating rubber chain. The fact that the rubber chain reaches a finite size after two hours (Espy *et al*., 2006),



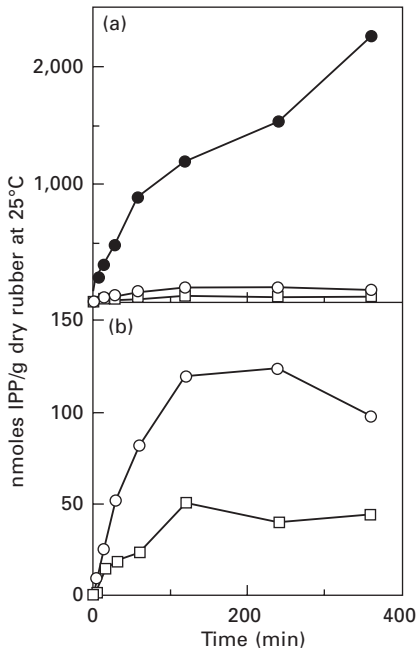
1.7 [^{14}C] IPP incorporation by purified *F. elastica* buoyant rubber particles in the presence or absence of 20 mM EDTA, and in the presence or absence of 20 µM FPP.

suggests that the IPP may have displaced the mature molecules which then have left the active site but cannot reinitiaite. This also lends weight to the hypothesis that physical interaction plays a role in rubber molecular weight as this occurred in the absence of an APP chain transfer agent; if the rubber was still in place, the chain should have kept elongating because it is not limiting. Separate kinetic characterization of the IPP incorporation reactions showed that K_m^{IPP} is lower for the initiator-independent reaction than for initiator-dependent IPP polymerization, probably because of competition between the substrates for the opposite binding site (Table 1.7).

At the optimal [Mg^{2+}] for maximum rubber synthesis in *F. elastica* (A_{max}^{Mg2+} = 100 mM), the K_m^{IPP} is at a minimum for both the initiator-dependent and initiation-independent IPP condensation reactions, and the K_m^{FPP} is at a minimum for the initiator-dependent IPP condensation reaction, indicating that this [Mg^{2+}] results in the highest affinity of both substrates to bind to RuT. For all Mg^{2+} concentrations studied, the $K_m^{FPP} < K_m^{IPP}$initiator-independent < K_m^{IPP} initiator-dependent (Scott *et al.*, 2003).

1.6 Regulation of molecular weight

The molecular weight of the rubber made during *in vitro* assays is primarily governed by the rate of the chain transfer reaction, i.e. the termination and release of an existing rubber polymer molecule, coupled with the initiation of



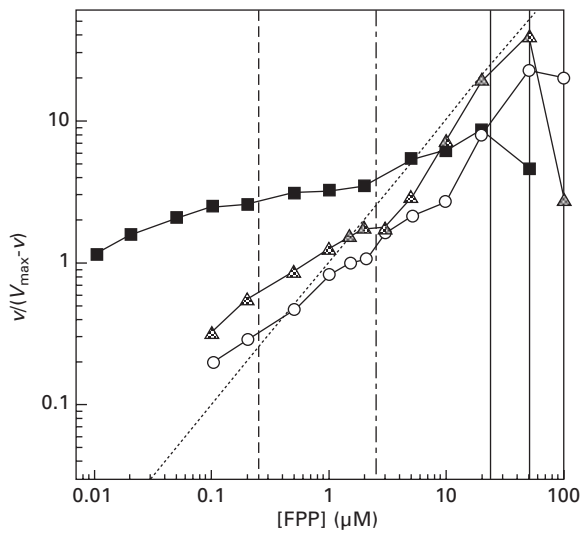
1.8 Time course of IPP incorporation by purified *F. elastica* rubber particles. (a) Effect in the presence of non-limiting levels of DMAPP (20 μM) initiator and 200 μM ^{14}C-IPP (20 μM) (•), and in the presence of 1 mM (□) or 5 mM (o) IPP, without an allylic pyrophosphate initiator. (b) The same data plotted in (a) but on a different scale.

Table 1.7 Kinetic constants at various [Mg^{2+}] for the initiator-dependent reaction, initiator-independent reaction, and initiator dependence reaction for rubber transferase in *F. elastica* purified rubber particles

Polymerization reaction	Substrates [ ] denotes varying substrate	Mg^{2+} (mM)	$K_m^{IPP, FPP}$ (mM, μM)	V_{max} (μmol/gdw/4h)
Initiator-dependent	[IPP], FPP	75	17.5	2.20
		100	15.8	1.50
		130	23.0	1.25
Initiator-independent	[IPP]	75	1.90	0.11
		100	0.60	0.049
		130	1.52	0.055
Initiator dependence	[FPP], IPP	50	0.257	0.225
		75	0.075	0.097
		100	0.070	0.056
		130	0.158	0.107

a new molecule in the same active site. Its regulation is strongly dependent upon substrate and activator identity, concentration and ratios, and especially the relationship of substrate concentration to K_m. Fortunately, it is possible to determine the mean molecular weight of the rubber made under any set of conditions by dual label experiments, in which the initiator is tritiated and ^{14}C-IPP is used as the source of monomer. The mean molecular weight is calculated based on the ratio of the ^{3}H:^{14}C because each rubber polymer contains a single initiator. The molecular weight distribution of new rubber requires a gel permeation chromatograph coupled to both a mass detector of some type and a radioisotope detector (Benedict *et al*., 1990; Espy *et al*., 2006). However, very few laboratories have such equipment, whereas the mean molecular weight method is readily accessible by any basic biochemistry laboratory with a radiation license.

It has previously been shown that rubber molecular weight *in vitro* is highly dependent upon the concentration and ratio of IPP and FPP (Cornish and Siler, 1995; Castillón and Cornish, 1999; Cornish *et al*., 2000). As substrate concentrations increase, while maintaining a constant substrate ratio (Fig. 1.9), the rubber molecular weight produced by the *P. argentatum* rubber transferase decreases. Thus, FPP appears to exert the predominant



1.9 Hill plot of the incorporation of ^{14}C-IPP (1 mM) into rubber across a wide concentration range of FPP initiator. In this plot the intercept of the plotted line with $y = 1$, is the K_m^{FPP}. The diagonal thin dashed line (·····) is the line Michaelis–Menton kinetics would produce. The vertical bold dashed line (– – –) and the bold dotted and dashed lines (– · –) are the conditions referred to in the text for the molecular weight time courses.

regulatory effect and the higher the concentration of FPP, the lower the molecular weight. GGPP behaves similarly to FPP in *in vitro* assays.

In contrast, molecular weight increases with IPP concentration (Cornish and Siler, 1995, Castillón and Cornish, 1999; Cornish *et al*., 2000), but the degree to which this occurs depends upon the presence or absence of negative cooperativity in the particular APP identity and concentration, and on whether or not the concentration of APP or IPP is limiting (da Costa *et al*., 2005, 2006). A comparison was made among the *P. argentatum*, *F. elastica* and *H. brasiliensis* rubber transferases using FPP as the initiator (da Costa *et al*., 2005, 2006). When FPP was limiting, molecular weight increased in all three species in an IPP-dependent manner, as non-limiting IPP concentrations continued to increase. Limiting the chain transfer reaction (replacement of the elongation rubber polymer with a new initiator) allows polymer elongation to continue. However, 0.25 μM FPP is not a limiting initiator concentration for the *P. argentatum* rubber transferase (Table 1.2), which has a much lower K_m^{FPP} than the other two species (Tables 1.3 and 1.4). Nonetheless, in this case, the IPP concentration dependent molecular weight increase is still mediated by inhibition of the chain transfer reaction because of the negativity cooperativity exerted by this enzyme. At this FPP concentration, the first FPP bound to the enzyme, which initiates the polymerization reaction, also impedes access of additional FPP molecules to the active site. This 'negative cooperativity' occurs between 0.1 and 2.0 μM FPP in the *P. argentatum* rubber transferase (Fig. 1.9) (da Costa *et al*., 2005, 2006).

This experiment was repeated at the higher FPP concentration of 2.5 μM, above the K_m^{FPP} for all three rubber transferases (Tables 1.1–1.3), and outside the negative cooperativity concentration range for the *P. argentatum* rubber transferase. As expected, in the absence of substrate limitations or negative cooperativity, we found no IPP concentration-dependent molecular weight increases above 500 μM IPP for either the *H. brasiliensis* or the *P. argentatum* rubber transferase. However, this particular concentration matches a relatively short concentration span of FPP negative cooperativity in *F. elastica* (2–3 μM FPP) (da Costa *et al*., 2005, 2006).

Thus, in general, under non-limiting IPP and FPP concentrations, rubber molecular weights were independent of IPP concentration, except where the chain transfer reaction was inhibited because of negative cooperativity. However, molecular weight regulation differs among the three rubber transferases because of their differences in intrinsic substrate affinities (K_m's) and the concentration range of initiating substrate at which the chain transfer reaction is inhibited.

The *P. argentatum* rubber transferase has additional unusual features not shared by the *H. brasiliensis*, *T. kok-saghyz* or *F. elastica*, which appear to allow rubber production of consistent molecular weight without adversely

affecting plant growth and development. *P. argentatum* makes rubber in parenchyma cells which are not separated from the main living plant as are laticifers. This means that it is subject to movement of assimilate from essential processes to rubber, and the rubber is likewise affected. For example, rubber is largely synthesized during the winter, when the plant is essentially dormant and can afford to use large quantities of photo-assimilate on a compartmentalized secondary product, which it cannot catabolize (Ji *et al.*, 1993; Cornish and Backhaus, 2003). During the summer, when *P. argentatum* is rapidly growing and is dependent upon the isoprenoid pathway to sustain the developmental processes, the high rubber transferase K_m^{IPP}, which is at least 10-fold greater than known competing enzymes in the cytosol, ensures that rubber can only be made when IPP is not required for vital reactions. However, the K_m^{FPP} is smaller than the K_m^{FPP} reported for the other FPP-utilizing enzymes, indicating that rubber transferase can compete successfully for FPP in the presence of cytosolic FPP-requiring enzymes. Substrate deficits are avoided, in this case, because the FPP negative cooperativity of the *P. argentatum* rubber transferase ensures that rubber biosynthesis does not deplete the FPP pool to the detriment of plant growth and development. Also, the inhibition of the chain transfer reaction ensures that the rubber is made to a mature high molecular weight throughout the year, instead of short rubber in the summer and long rubber during the winter. It is noteworthy that the *F. elastica* and *H. brasiliensis* rubber transferases exhibit much less FPP negative cooperativity than the *P. argentatum* transferase (Fig. 1.8). This may reflect the disparate location of rubber production in the three species. *F. elastica* and *H. brasiliensis* both synthesize rubber in laticifers which are anatomically fairly autonomous of the rest of the plant. Thus, production in laticifers depends on a flow of photo-assimilate to the laticifer which then regulates the rate of rubber biosynthesis. When something goes amiss with regulation, rubber biosynthesis may cease, but the tree continues to live (e.g. tapping panel dryness (Krishnakumar *et al.*, 2001)). In contrast, *P. argentatum* synthesizes its rubber in generalized bark parenchyma cells, which may make the process of rubber biosynthesis more dependent on developmental processes.

1.7 Identification and purification of rubber transferase

Rubber transferase is not the only *cis*-prenyl transferase in plants capable of producing *cis*-polyisoprene. However, the substrate binding constants are quite different among the different *cis*-prenyl transferases. For example, the soluble UPPSs have a K_m^{IPP} much lower than that for the rubber transferases (2–8 μM compared to 150–400 μM) (Schmidt *et al.*, 2010b, Lu *et al.*, 2009; Chen *et al.*, 2005; Apfel *et al.*, 1999; Pan *et al.*, 2000) as do the DDPPS of

the endoplasmic reticulum (5–6.7 μM) (Rebl *et al.*, 2009; Cunillera, 2000). In addition, the reaction A_{max}^{Mg2+} is very different, with a maximum reported for the UPPS of 2 mM, for the DDPPS of 1 mM, and for rubber transferase up to 100 mM. Also, rubber transferase is the only member of this class capable of indeterminate polymer lengths of at least 1 million g/mol under appropriate substrate conditions. *Cis*-prenyl transferase contamination is common in rubber particle preparations especially from latex-producing species which have copious amounts of soluble *cis*-prenyl transferase (Post *et al.*, 2012; Schmidt *et al.*, 2010a, 2010b). Given the same substrates as rubber transferase, the *cis*-prenyl transferases also can make product, but this is always of low molecular weight. As mentioned above, well-washed rubber particles have been shown to possess only one enzyme with IPP binding ability: if an enzyme other than rubber transferase was also bound to the rubber particles, then it would be expected that a curved plot of v against $v/[S]$ would be obtained when the IPP concentration dependence of IPP incorporation rate is assayed in non-limiting initiator and activator. Instead, linear plots are generated indicating a single binding site (Cornish, 2001a, 2001b; Cornish and Scott, 2005). However, the product(s) of *cis*-prenyl transferases could still be involved in rubber biosynthesis by serving as initiators (Cornish, 1993). Many different allyic-PPs, whether *cis* or *trans* or a combination of both stereochemistries, are effective initiators *in vitro* (Tables 1.1–1.3).

In vitro assays should employ limiting APP concentrations and excess IPP to permit the enzyme in question to produce its maximum product length. This is one approach to distinguish the two classes of *cis*-prenyl transferases. Checking the K_m and linearity of the IPP incorporation reaction in IPP plots of v against $v/[S]$ is another approach. A third method entails determining the IPP V_{max} under constant substrate concentrations in rubber particle preparations purified to different extents while the *cis*-prenyl transferase amount in question is correlated using SDS-PAGE. A combination of these methods is likely required in the absence of an expression system proving high molecular weight rubber synthesis with binding constants matching those of the rubber particle-bound system.

1.8 Conclusions

Current understanding of rubber biosynthesis is incomplete, and there are many opportunities for progress. As alternate rubber species are introduced into the crop portfolio, species-specific differences in rubber biosynthesis and subsequent macromolecular structure and performance of the natural rubber polymers give researchers enormous scope for fascinating and pertinent research.

1.9 Acknowledgments

I acknowledge the many who have contributed to our current knowledge of rubber biosynthesis and look forward to the innovative advances I expect to see in the next few years. I also thank the United States Department of Agriculture for their support of my earlier research and the Ohio Department of Development, Third Frontier for their support of my current efforts.

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