Purification of a Prenyltransferase That Elongates cis-Polyisoprene Rubber from the Latex of *Hevea brasiliensis* 

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We have purified “rubber transferase” from latex of the commercial rubber tree *Hevea brasiliensis* and find that it is a dimer with a monomeric molecular mass of 38,000 Da, requires Mg²⁺, and is stabilized by thiol in agreement with studies of a partially purified preparation previously described (Archer, B. L., and Cockbain, E. G. (1969) Methods Enzymol. 15, 476–480). Greater than 90% of the [1-14C]isopentenyl pyrophosphate which is incorporated into deproteinized rubber particles by the purified prenyltransferase is added to high molecular mass polysoprene (>20,000 Da). Purified prenyltransferase and deproteinized rubber particles reconstitute 40–60% of the biosynthetic activity of whole latex in samples matched for rubber content. Incorporation is linear with added rubber particles up to at least 10 mg/ml rubber or 20 μm rubber molecules (based on a number average molecular mass of 500,000 Da).

Prenyltransferase concentrations estimated in whole latex (0.37% or 160 nM) are sufficient to saturate all elongation sites in whole latex, and addition of purified prenyltransferase does not increase [1-14C]isopentenyl pyrophosphate incorporation. Deproteinized rubber particles can be titrated with the pure enzyme (Kₑ = 9 nM) demonstrating that the fraction of rubber molecules available for addition is low (approximately 0.01%). An estimated 7,000 isoprene units are added per complex at a rate of 1/s in a typical assay.

*Hevea* prenyltransferase catalyzes the formation of cis-isoprene in the presence of rubber particles. However, in the absence of rubber particles and in the presence of dimethylallyl pyrophosphate, the purified prenyltransferase catalyzes the formation of geranyl pyrophosphate and all *trans*-farnesyl pyrophosphate as demonstrated by thin layer chromatography, gas chromatography, and molecular exclusion chromatography.

Rubber transferase (1–5) is a prenyltransferase that catalyzes the polymerization of isopentenyl pyrophosphate (IPP) into rubber. Like other prenyltransferases this enzyme requires a divalent cation (Mg²⁺) to catalyze thousands of 1–4 cis condensations (Z-oligomerization) of IPP, the prenyl acceptor, to rubber, the prenyl donor, before random termination occurs. Other prenyltransferases terminate after a characteristic and comparatively low number of additions of IPP to the allylic pyrophosphate substrate. FPP synthase (from porcine and avian liver, *Bacillus subtilis*, plants and yeast) catalyzes two *trans* additions to DMAPP (6–10); geranylgeranyl pyrophosphate synthase catalyzes three *trans* additions to DMAPP (11, 12); hexaprenyl pyrophosphate synthase catalyzes three *trans* additions to *t,t*-FPP (13); heptaprenyl pyrophosphate synthase catalyzes four *trans* additions to *t,t*-FPP (14); solanesyl (nonaprenyl) pyrophosphate synthase catalyzes six to seven *trans* additions to GPP (15); undecaprenyl pyrophosphate synthase catalyzes seven to eight cis additions to *t,t*-FPP (12, 16–18); and dolichol pyrophosphate synthase catalyzes 13–18 cis additions to *t,t*-FPP (19).

Production shows loss of termination precision with increasing chain length and even FPP synthase is observed to make a small amount of all-*trans* geranylgeranyl pyrophosphate (*C₀*) at less than 1% of the rate of FPP formation (6, 20). The nature of the allylic prenyl donor (21) or the Mg²⁺ concentration (22) affects the chain length made by other prenyltransferases by a few isoprene units. These fluctuations in termination precision are modest compared with the prenyltransferase from the rubber tree *Hevea brasiliensis*. Its product varies between 100,000 and 4,000,000 Da (3), representing between 1,500 and 60,000 isoprene additions per molecule.

Whole latex obtained by tapping the laticifers of *H. brasiliensis* contains rubber transferase and one of its substrates, rubber particles. Centrifugation of whole latex produces well defined fractions for use in studies of rubber biosynthesis (3, 23, 24). Rubber particles between 50 and 1500 nm diameter (5, 23, 25–28), microsomes, microfibrils (29), mitochondria, and organelles of incompletely understood function called lutoids and Frey-Wyssling particles are suspended in C-serum. Studies of the biogenesis of laticifers reveal that these vesicles result from the resorption of cell walls between longitudinally contiguous rows of cells (2). Thus, C-serum, a complex mixture of soluble proteins, enzymes, metabolites, and salts is derived from the soluble component of the cytosol from these specialized cells. Guayule rubber biosynthesis occurs in the cytoplasm of leaf mesophyll and stem parenchyma cells (30–32); however, these cells do not fuse to form laticifers found in *H. brasiliensis*.

Archer et al. (1) and Madhavan and Benedict (30) partially purified rubber transferase from *H. brasiliensis* and the guayule *Parthenium argentatum*. There is no report of a homogeneous preparation of rubber transferase. The partially pure enzymes from both species show farnesyl pyrophosphate synthase (FPP synthase) activity. We present a method for purifying rubber transferase from the latex of *H. brasiliensis*.
to greater than 95% homogeneity and describe some of its properties.

MATERIALS AND METHODS

Reagents—[1-14C]Isopentenyl pyrophosphate (~57 Ci/mol) was purchased from Amersham Corp. [4-14C]Isopentenyl pyrophosphate (50.4 Ci/mol) was purchased from Du Pont-New England Nuclear and used in a limited number of initial studies. Unlabeled isopentenyl pyrophosphate (IPP) was synthesized by published procedures of Davison et al. The ammonium salt was a gift from Dr. C. Dale Poulter, University of Utah, and Brian Froehler, Genentech, Inc. The ammonium salt of dimethylallyl pyrophosphate (DMAPP) was synthesized by the published procedures of Davison et al. (47) and was a gift from Dr. D. A. Widdowson and Dr. C. Pemberton, Imperial College. Affi-Gel 501, a sulfhydryl-specific organomercouric affinity column and Triton X-100 were purchased form Bio-Rad. Hydrophobic chromatography kits were purchased from Pharmacia LKB Biotechnology Inc.; Hexyl-agarose and Sephacryl S-200 were purchased from Sigma; DE52 molecular exclusion column was purchased from LKB-Producer AB; and a 7.5 x 600-mm Ultro Pac TSK-G3000 SW high performance molecular exclusion column was purchased from LKB-Producer AB; Dolichol (C50), dolichol mixture (C90-C160), solanesol (C46), and geraniol (C10 cis) were purchased from Sigma. Nerol (C10 trans) was purchased from Peak Scientific Products. Thiokol/Ventron Division. Mixtures of varying ratios of isomers of farnesol (C15) were a gift from Givaudan or purchased from Fluka AG, Buchs SG, or Aldrich. Isopentenol (3-methyl-2-buten-1-ol), N-chlorosuccinamide, methyl sulfide, di-methylphosphonate, and n-butyl lithium were purchased from Aldrich. All other chemicals were of analytical grade.

Shipping Latex—Freshly tapped latex from uncultivated H. brasiliensis in Costa Rica was immediately diluted with 300 ml of 300 mM Tris-HCl, pH 7.5, 0.03% sodium azide/700 ml latex, quickly frozen in 1-liter bottles, and shipped to this laboratory on dry ice. Dilution with buffered azide attenuates the loss of latex due to coagulation during the tapping, freezing, shipping, and thawing procedures. In general, latex was collected from trees which had never been tapped or had not been tapped for several months. However, in some cases latex samples were collected from trees tapped every 3 days for at least 1 month. Reliable methods of latex collection and shipment were the result of the efforts of Charles Hunter, Grajfas Tropicales S.A., San Jose, Costa Rica. In preliminary studies small volumes of latex (5–10 ml) were shipped overnight on wet ice by Dr. Paul K. Soderholm, Subtropical Horticultural Research Station, Miami, FL. Quantitative Estimation of Rubber Content—For routine comparisons of samples containing rubber particles, whole latex and deproteinated rubber particles in order to convert the A280 to mg/ml of rubber. Samples were filtered and washed as in the standard assay (see below) and dried in a vacuum oven. The weight was determined by weighing the filter before and after filtration of the rubber. Control filters were carried through to correct for changes in the weight of the filter (+5% loss). Two of the above filters and two control filters prepared without prenyltransferase were dissolved in 10% (v/v) farnesol in evacuated sealed tubes at 110°C. The dried hydrolysates were run by standard methods and stained with Coomassie Blue (Bio-Rad Laboratories) or silver stained by published methods (49).

Isolation of Particles—Deproteinized rubber particles with low intrinsic prenyltransferase activity were prepared by a modification

Gel Permeation Chromatography—Separation of soluble rubber from its polymer in chain lengths > 4,000 Da (4400 Daltons) was achieved on a 500 Å-Styragel (Waters Associates, Inc.) with an exclusion limit of 20,000 Da (tetrahydrofuran or toluene, 1 ml/min, 25°C, refractive index detector, Waters Associates, Inc.). In some cases, at higher sample loads, precipitation of rubber on the column was encountered, and this could be avoided by running the column at 100°C. Samples for injection were prepared from rubber trapped on 0.22-μm filters. A sample containing 5.5 mg/ml deproteinated rubber particles, 2.2 mg/ml purified prenyltransferase (57 nM), 0.3 mM [1-14C]IPP (0.21 Ci/mol), 1 mM MgSO4, 10 mM DTT, and 50 mM Tris, pH 7.5, in a final volume of 10 ml was incubated 180 min and filtered in 0.5-ml aliquots. The filters were washed with 1 ml of whole latex and ethanol, and air-dried as in the standard assay described above. Two of the above filters and two control filters prepared without prenyltransferase were dissolved in scintillant. Four of the remaining filters were extracted overnight and rinsed twice with benzene. After the pooled benzene extract (2.5 ml) was extracted with 0.1 ml of 6 N NaOH, the rubber was precipitated from the benzene by the addition of 2.5 ml of ethanol. The precipitated rubber was collected by centrifugation. Two of the samples of extracted rubber were redissolved in benzene for scintillation counting along with the benzene/ethanol supernatant and the NaOH extract. The remaining rubber samples were dissolved in 10% (v/v) farnesol in ethanol and injected on the a-Styragel column.

Curve Fitting—The fit of data to computer to equations in the text was achieved by an iterative approach in which parameters are adjusted by the Marquardt-Levenberg method until a least squares solution is reached. All fits were calculated through the use of RS/1 (BBN Research Systems, Bolt Beranek and Newman Inc.) run on a VAX 11780 with a UNIX operating system (4.2 BSD version, Bell Labs).

Amino Acid Analysis—Following carboxymethylation in urea, prenyltransferase was hydrolyzed for 24 h with constant boiling HCl in evacuated sealed tubes at 100°C. The dried hydrolysates were analyzed on a Beckman 6300 amino acid analyzer equipped with a ninhydrin detector using an automated program. The concentration of tryptophan was not determined.

Isolation of Particles—Deproteinized rubber particles with low intrinsic prenyltransferase activity were prepared by a modification

3 R. Marcinko, personal communication.
of the procedure described by Archer et al. (1, 33, 34). A 1-liter bottle of diluted whole latex was thawed and centrifuged at 2000 × g for 10 min to remove coagulated rubber (bottoming pellet), dimers and intact (bottom: bottom) latex. The remaining whole latex was decanted and frozen in 10-ml portions. Freshly depolymerized rubber particles were prepared from 10 ml of whole latex by size exclusion chromatography on a Sepharose S-300 column (5 × 50 cm) equilibrated in 50 mM Tris-HCl, pH 7.5, and run at 0.64 ml/min. The latex particles were rinsed with this volume while the serum proteins were retained in the column. Particles from a typical column had an absorbance at 280 nm of 300. These particles were used in the rubber transferase assay and could be stored frozen at -80 °C for several weeks without appreciable coagulation or loss of function. The Sepharose S-300 column was rinsed after each use with fresh rubber thawed and centrifuged at 27,500 × g (27,500) for 2 h causing most of the rubber to coagulate at the top of the centrifuge bottle. The supernatant fluid was then centrifuged at 9000 rpm for 2 h causing most of the rubber to coagulate at the top of the centrifuge bottle. The supernatant fluid was then centrifuged at 9000 rpm for 2 h causing most of the rubber to coagulate at the top of

Rubber Elution Assay—Rubber transferase was assayed by measuring its ability to add monomeric isopentenyl pyrophosphate to existing depolymerized latex particles. A 0.5-ml incubation mixture contained 0.41-0.45 ml of depolymerized rubber particles or whole latex, 50 mM Tris-HCl, 1 mM MgSO4, 10 mM DTT, and 0.5 mM [1-3H]IPP (specific activity = 0.4 Ci/mol) and added prenyltransferase if appropriate. The reaction was incubated for the appropriate time, and rubber was removed by treatment with 0.25 mM EDTA, pH 8.0. Each sample was then filtered with an Amicon vacuum filtration manifold and 0.22-μm cellulose acetate/cellulose nitrate filters (Millipore GSWP02500) which provided sufficient flow but still retained the latex particles. The most coarse of latex particles on the filter was removed by centrifugation at 2-15 × g for 1 min and washed in HCl followed by two 5-15 min washes with ethanol. Radioactivity on the filters was determined in a toluene-based scintillation mixture (Quanti- titer, Mallinkrodt). If the filters remained in scintillant overnight the rubber dissolved, the filters became transparent, and the radioactivity could not be determined.

The second method was rapid and was based on a method used to extract differentially long chain allylic phosphates from [14C]IPP (18). At neutral pH, IPP was not partitioned into the butanol phase (15), whereas FPP was partitioned nearly equally in the aqueous and the organic phases. Thus, the reaction was quenched by adding 0.05 ml of 0.25 mM EDTA, H2O was added to 1 ml, the mixture was extracted with 2 ml of 1-butanol (the butanol phase expands to 2.5 ml owing to H2O uptake), 1.5 ml of the butanol phase was back-extracted with 0.5 ml of H2O, and 1.0 ml of the butanol phase was counted. The overall extraction yield was 25% with this method. It was easier to use with samples that had a high concentration of protein since these samples form an intractable emulsion when extracted with hexane. The efficiency of 14C counting was 0.7 cpm/dpm. The original intent of the acid and ethanol washes was to protonate and remove, respectively, adventitious [14C]IPP. However, we note that Archer and Audley (2) recently reported that in contrast to our method these treatments do not cause turbidity. Archer and Audley (2) achieved an efficiency of 1.5 cpm/dpm. Furthermore, the rubber dissolved, the filters themselves became transparent, and the radioactivity could not be determined.

The broadness of this band resulted from the nonlinearity of the assay with respect to enzyme concentration and from aggregation of a small percentage of the enzyme that could be detected in the void volume by SDS-PAGE (data not shown).

To make purification by bexyl agarose chromatography most efficient, isocratic conditions were chosen in which rubber transferase did not bind to the column material, but was slightly retarded. The active pool from the Sepharose S-200 column was concentrated via Amicon filtration (YM30) to 10 ml and dialyzed against 2 liters of 50 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM MgSO4, 0.1% dithiothreitol, and 0.1% sodium azide (0.7 ml/min). A single broad band of rubber transferase activity was eluted with a linear salt gradient from 0 to 125 mM NaCl (Fig. 2B). Dithiothreitol (10 mM) which interfered with the UV detection was added to each fraction immediately after elution. Rubber transferase could be stored for at least 6 months at -80 °C in 25 mM Tris-HCl, pH 8, 10 mM dithiothreitol, 1 mM MgSO4, 0.3% glycerol, and 0.1% sodium azide.

RESULTS

Characterization of Whole Latex—Much of the published data on rubber transferase was obtained using freshly tapped latex in a laboratory with an adjoining greenhouse (5), so it was useful to compare the properties of our frozen and shipped material with those reported for the freshly tapped material. Archer et al. (35) characterized the composition of freshly tapped H. brasiliensis latex and found that it is 33% (w/v) rubber and that serum is 0.5% (w/v) protein. We monitored these two values over the course of many different shipments and prenyltransferase purifications.

Rubber content was calculated from the measured absorbance at 280 nm and converted to milligrams of rubber per ml with a factor of 0.06 mg A280 per mg of rubber. Some latex samples used contain as little as 4% rubber owing to extensive coagulation, but many others contain as much as 30% rubber. A freeze sample of uncoagulated latex rushed overnight on wet ice from Florida had an A280 of 5500 or 33% rubber in agreement with Archer et al. (35). Two frozen and thawed samples from Costa Rica had A280 values as high as 5100 (31%) and 4100
latex as shown in Table I. This discrepancy could be attributed with boiling ethanol, was performed with 10-300 mg centrifuge volume (0.5 ml reaction volume). In contrast, the essay used by other laboratories (1, 2, 5, 30, 37) in which the sample was dried, treated with acetic acid/ethanol, digested with hot KOH, and exhaustively extracted, was performed with 10-300 mg of rubber/assay. The smaller amount of total rubber is recommended in this assay owing to the capacity of the filter. Under our assay conditions the radioactivity incorporated was linear with respect to added rubber particle concentration at least up to 10 mg/ml rubber (Fig. 1) consistent with the observations of Archer et al.(5).

In the absence of purified prenyltransferase (see Table II), deproteinated latex particles in the standard assay mixture incorporated 300-700 dpm [14C]IPP into deproteinated rubber particles in contrast to 70-350 dpm observed due to nonspecific trapping of [14C]IPP in the rubber during filtration of the same particles in the presence of the inhibitor EDTA. EDTA inhibits rubber biosynthesis by complexing the requisite Mg2+. When a sample of purified prenyltransferase was added to the latex particles, 1,500-15,000 dpm [14C]IPP were incorporated. This incorporation was linear with respect to time for up to at least 4 h. The background activity in deproteinated particles could represent between 0 and 30% of the reconstituted system (for example see Table III) and was dependent upon the flow rate of the S-300 column as well as the age of the particles.

Prenyltransferase Purification—The purification of prenyltransferase shown in Table II and Fig. 2 and described under “Materials and Methods” consisted of a combination of gel exclusion, ion exchange, and hydrophobic chromatography. Based on the observed yields the prenyltransferase represented 0.37% of the soluble protein in serum. It could not be distinguished in SDS-PAGE of C-serum (Fig. 2A, lane A).

The final anion exchange chromatography step is shown in Fig. 2B. This chromatogram illustrates one of the major difficulties encountered when the *Hevea* prenyltransferase was purified using the rubber elongation assay as the sole assay to analyze columns. The activity peak in Fig. 2B is three to four times broader than the relatively sharp A360 peak. Because the rubber elongation assay is nonlinear with respect to prenyltransferase (Fig. 3), this assay greatly underestimated the amount of enzyme in the peak tubes relative to the side fractions. After the *Hevea* rubber transferase prenyltransferase was purified to homogeneity by a procedure monitored with the rubber elongation assay (Fig. 2B, insert), we discovered that it is an FPP synthase. The concentration dependence of the FPP synthase assay with respect to prenyltransferase (Fig. 3) is linear at higher enzyme concentrations, and thus it was much easier to pool fractions with maximal activity from any given chromatographic step. By using the FPP synthase assay the original purification scheme could be abbreviated considerably to that shown in Table II. All steps in the purification yielded only one component of FPP synthase activity which also coincided with rubber elongation activity (although the activity peak was broader with the latter assay). We were unable to detect any FPP synthase activity in *Hevea* latex other than the activity associated with the rubber transferase prenyltransferase.

Inspection of Fig. 3 reveals why the FPP synthase reaction

### Table I

**Comparison of FPP synthase activity in whole latex and in C-serum**

| Product formed | Activity in whole latex relative to C-serum |
|---------------|-----------------------------------------|
| Whole latex   | C-serum                                  |
| dpm/0.01 ml   | %                                       |
| 10,000 ± 320  | 11,100 ± 390                            |

*Average of four determinations ± S.D.*
was the more useful of the two for rapidly monitoring columns during the purification of *Hevea* prenyltransferase. The FPP synthase assay, when diluted to 10 nM prenyltransferase or less, resulted in the underestimation of the activity in the side fractions from a column, but the peak fractions coincided exactly with the peak of enzyme. However, if the rubber elongation assay was used, all fractions greater than 15–20 nM prenyltransferase showed nearly the same response in the assay. In practice this was the most common problem; peaks with the latter assay tended to be broad and flat making it difficult to pool the collected fractions.

This prenyltransferase did not bind to a neryl methylphosphophosphophosphonate affinity column, the *cis* analog of the geranyl methylphosphosphophosphonate affinity column successfully used to purify FPP synthase from avian and porcine liver (38). It was even more surprising that the prenyltransferase also did not bind to the latter column since the purified protein was found to have FPP synthase activity. In both cases over 90% of the activity flowed through and did not bind to either the *cis or trans* analog containing column. No additional activity was eluted at up to 50 mM pyrophosphate. Thus affinity chromatography of this type has not proven to be a viable approach to purification.

**Characterization of Prenyltransferase—**Prenyltransferase from the Mono Q column was at least 95% pure by SDS gel electrophoresis after silver staining which revealed a single band at 38,000 daltons (Fig. 2B, insert). Characterization of the purified native material on a standardized TSK-G3000 SW column in 0.2 M sodium phosphate, pH 6.5, gave a native molecular mass of 70,000 daltons. Thus, rubber transferase is a dimer of identical monomeric subunits. No sequence greater than 0.5% (5 pmol) was detected when 40 μg (1 nmol) of purified rubber transferase was subjected to NH$_4$-terminal sequence determination. The protein was washed from the analytical filter, digested with CNBr, and subjected to sequence analysis again. A mixture of four to five sequences at the 30–120 pmol level were detected. Thus, the NH$_2$ terminus of the enzyme is blocked, and contaminating proteins are either present in low amounts or are blocked. The amino acid composition of the purified prenyltransferase is compared to other purified prenyltransferases in Table IV.

A high level of DTT (10 mM) was required for maximal incorporation of [*4C*]IPP into rubber; 2-mercaptoethanol was not as effective at equal concentrations. Free small molecular weight thiols were not required for catalysis in the rubber elongation assay, however, since the same stimulation could be achieved by reducing the prenyltransferase with DTT followed by dialysis to remove the mercaptan before the prenyltransferase was added to assay (data not shown). In addition rubber transferase activity of the purified prenyltransferase was completely inhibited by thiol alkylating agents such as iodoacetate confirming the observation of Archer and Cockbain (1) on the partially pure protein. The divalent cations Zn$^{2+}$ and Ca$^{2+}$ (0.1–1 mM) completely inhibited rubber elongation activity, while both Mg$^{2+}$ and Mn$^{2+}$ were stimulatory and were maximal at 1 mM; Ca$^{2+}$ and Pb$^{2+}$ were nearly as effective at 5 mM. Sodium pyrophosphate and sodium phosphate were also found to be inhibitors of rubber transferase activity with apparent $K_i$ values of 5 and 200 mM, respectively (0.3 mM IPP).

**Percent Reconstitution of Whole Latex Activity—**When samples of whole latex and deproteinated rubber particles plus prenyltransferase were matched for rubber content it was possible to reconstitute 40–60% of the activity of whole latex by using the purified system. The addition of purified prenyltransferase to diluted whole latex did not increase its rubber biosynthetic activity even when the final concentration of prenyltransferase was two to four times higher than the endogenous level of the enzyme (Table III). Thus, the prenyltransferase concentration in whole latex was saturating even though the molar ratio of rubber molecules to prenyltransferase was 140 in the experiment in Table III. Saturation was observed in samples in which this ratio was as high as 1500. The lack of 100% reconstitution and the lack of stimulation of whole latex by added prenyltransferase could be due to a slow rate of formation of an active prenyltransferase complex with its rubber substrate. However, preincubation of the prenyltransferase with rubber particles for up to 5 h did not result in an increase (or decrease) in the measured rate indicating that complex formation is rapid.

**Titration of Active Ends—**As observed with partially pure preparations from both *Hevea* and guayule (1, 3, 4, 30), [*4C*]IPP transferase and have obtained amino acid sequence data from eight tryptic fragments (1, QWWER; 2, VLYNXLK; 3, LQGVPTEYEBEN-
A. SDS-PAGE analysis (15% acrylamide, Coomassie-stained) of purification steps shown in Table III. Activity during this purification was followed by the FPP synthase assay, and purified fractions were later verified to catalyze the incorporation of [1-14C]IPP into deproteinated rubber particles. Lane A, C-serum (centrifuged twice to remove rubber particles); lane B, 25-65% ammonium sulfate precipitate resuspended and dialyzed; lane C, pool of protein that does not bind to DE52 cellulose (50 mM potassium phosphate, pH 8.0); lane D, pooled active fractions from Sephacryl S-200 molecular exclusion column; lane E, pooled active fractions from chromatography on hexyl-Sepharose; lane F, active fractions from FPLC on Mono Q anion exchanger. Molecular masses (kDa) of marker proteins are shown. B. Chromatography of prenyltransferase on final Mono Q column. The preparation was essentially identical to that shown in Table II except that [1-14C]IPP incorporation into deproteinated rubber particles was the sole assay used throughout the purification. Also, hydrophobic chromatography was performed on an octyl-Sepharose column and an additional anion exchange step (QAE-Sephadex) was used. Insert shows silver-stained SDS-PAGE (15% acrylamide) analysis of 0.01 ml out of 0.55 ml of active fractions from the final Mono Q column. The Abs 280 absorbing material in fractions 25–32 does not contain protein (SDS-PAGE analysis) and may be DTT. Assay conditions were 0.15 mM [1-14C] IPP, 0.21 Ci/mmol, 1 mM MgSO4, 10 mM DTT, 2 mg of rubber particles, and 50 mM Tris, pH 7.5, in a final volume of 1 ml.

IPP incorporation into rubber molecules was nonlinear (Fig. 3, closed triangles) with respect to enzyme concentration as the prenyltransferase saturates the available elongation sites on the surface of the rubber particles. The total concentration of rubber molecules in whole latex was 0.66 mM (33% w/v rubber; rubber M₄ = 500,000) and the concentration of prenyltransferase dimer was 160 nM (based on 0.37% of C-serum protein, Table II). When purified enzyme was used to artificially double the prenyltransferase content of whole latex, no increase in rubber biosynthesis was observed. Thus the prenyltransferase content (50–100 nM) in diluted latex was sufficient to saturate the rubber molecules that were competent for further addition of IPP (Table III).

The availability of pure prenyltransferase allowed the titration and quantitative estimation of the elongation sites on the rubber particles. The activity dependence on prenyltransferase concentration shown in Fig. 3 (closed triangles) could be fit to the following equation (39):

\[ v = \frac{V_{\text{max}}/2S}{(M + S + K) - (4M + S + K - 4MS)^{\frac{1}{2}}} \]  

where \( v \) = rate; \( V_{\text{max}} \) = rate at saturating prenyltransferase (both in disintegrations/min); \( M \) = concentration of prenyltransferase monomer; \( S \) = concentration of elongation sites associated with the rubber particles; \( K \) = binding constant for prenyltransferase to its elongation site. The concentration of prenyltransferase monomer was used since at the concentrations used the prenyltransferase was predominantly monomeric (see below). When the rubber particles shown in Fig. 3 which contain 8.6 mg/ml rubber or 17 µm rubber molecules (using \( M₄ = 5 \times 10^6 \) for rubber) were titrated the best fit to this equation revealed that there were 2 nM elongation sites that bound the prenyltransferase with a relatively high affinity of 9 nM. Thus only ~0.01% of the available rubber molecules were competent for addition.

Attempts to Initiate New Rubber with the Purified Prenyltransferase—Archer et al. (4, 5) reported that DMAPP stim-
The rubber filtration assay described under "Materials and Methods" contained 0.5 mM [14C]IPP (0.4 Ci/mmol), 1 mM MgSO4, 10 mM DTT, and 50 mM Tris, pH 7.5, incubated 70 min in a final volume of 0.4 mL.

Comparison of the rubber biosynthetic activity of whole latex and deproteinated rubber particles with added purified prenyltransferase enzyme

| Components in assay | Rubber in assay | Enzyme in assay | IPP added to rubber | Activity relative to whole latex |
|---------------------|----------------|-----------------|--------------------|------------------|
| Whole latex          | 2.5 (12.5)     | 91              | 5520 ± 210         | 100              |
| Whole latex + 1.24 mg, enzyme | 2.5 (12.5) | 173               | 5520 ± 170         | 100              |
| Deproteinated particles | 1.7 (8.5) | 547 ± 20        |                | 9.9              |
| Deproteinated particles, 1.24 mg enzyme | 1.7 (8.5) | 82               | 2210 ± 45        | 40               |

* Weight of rubber calculated from A280 due to light scattering by rubber particles ("Materials and Methods")

** Total prenyltransferase (added + endogenous). The endogenous prenyltransferase in serum was calculated using 0.003 mg/mg total protein.

† Average of duplicate determinations and deviation from the mean.

‡ Latex was from trees tapped every 1.24 month.

### Table III

| Amino acid | Expected no. of amino acids in prenyltransferase |
|-----------|-----------------------------------------------|
| Hevea     | Porcineb                                   |
| Axs       | 41 ± 4                                     |
| Thr       | 35 ± 2                                     |
| Ser       | 23 ± 3                                     |
| Gln       | 43 ± 5                                     |
| Pro       | 11 ± 2                                     |
| Gly       | 35 ± 4                                     |
| Ala       | 26 ± 2                                     |
| Cys (CM)  | 6 ± 1                                      |
| Val       | 28 ± 5                                     |
| Met       | 5 ± 1                                      |
| Ile       | 14 ± 2                                     |
| Leu       | 38 ± 4                                     |
| Tyr       | 14 ± 3                                     |
| Phe       | 12 ± 2                                     |
| His       | 8 ± 1                                      |
| Lys       | 28 ± 4                                     |
| Arg       | 12 ± 2                                     |
| Trp       | ND*                                        |
| Avian     | 4.6                                         |

* Based on a monomer molecular mass of 38,000 Da and an average amino acid molecular weight of 105. Average and standard deviation of eight determinations.

† Based on a monomer molecular mass of 38,500 Da (50, 51).

‡ Based on a monomer molecular mass of 38,400 Da (50, 52).

§ Based on a monomer molecular mass of 38,500 Da (51, 53).

ND, not determined.

### Table IV

Amino acid analysis of purified Hevea prenyltransferase compared with other purified prenyltransferases

Analysis of the enzymatically hydrolyzed products derived from DMAPP and IPP by GC and GC/MS revealed that the C10 product was geraniol (trans) and that the C16 product was t-t-farnesol with a trace (less than 3%) of either cis-trans-farnesol or trans,cis-farnesol. Thus, the FPP synthetase activity present in other partially purified rubber transferase preparations (4, 5, 30) is a property of the purified enzyme.

### Incorporation of [14C]IPP into High Molecular Weight Polysoprene

With the observation that the purified prenyltransferase catalyzed the trans addition of IPP to DMAPP and GPP to make low molecular weight t-t-FPP it was imperative to demonstrate that the rubber elongation assay measures the incorporation of IPP into high molecular weight rubber. Two benzene extractions of radiolabeled rubber trapped on assay filters dissolved 76% of the radioactivity. No radioactivity was extracted from the benzene solution by 6 n NaOH, therefore low molecular weight organic pyrophosphates were not present. Addition of an equal volume of ethanol precipitated the rubber (43, 44) which was removed by centrifugation. No radioactivity remained in the benzene-ethanol supernatant fluid. The rubber film was redissolved in benzene, and 70% of the radioactivity of the original benzene extract was in this solution. Losses were due to the fact that rubber solutions left a film on glassware during transfer steps. As shown in Fig. 4, when chromatographed in toluene on a 500 Å -Stryagel column, 95% of the radioactivity injected was eluted at a molecular mass greater than 20,000 Da (exclusion limit of the column). Less than 1.5% of the injected radioactivity was found in the region where farnesol (222 Da) was eluted. In contrast, when incubations of purified prenyltransferase with DMAPP and [14C]IPP were acid-hydrolyzed, dried, extracted with benzene, and injected under the same conditions, the radioactivity was coeluted with farnesol, a major subfraction was eluted with geraniol, and a few percent were eluted in the C20 region. In the latter incubations no counts were observed in the high molecular weight region (>C20).

### Concentration Dependence of FPP Synthase Assay

As discussed above, variation of enzyme concentration in the rubber transferase assay revealed a nonlinear concave down enzyme dependence (Fig. 3, closed triangles). In contrast, variation of the prenyltransferase in the FPP synthase assay revealed a nonlinear concave up enzyme dependence that could be fit to a model by a rate dependence in which dissociated monomers to the major spots at C10 and C16. This material of apparent higher molecular weight is attributed to hydrocarbon producing rearrangements of [14C]IPP and/or [14C]GPP during acid hydrolysis.
DISCUSSION

The prenyltransferase purified from *H. brasiliensis* catalyzed the rubber elongation reaction in the presence of deproteinated rubber particles (cis addition of IPP) but had FPP synthase activity (trans addition of IPP) when assayed with DMAPP or GPP. FPP synthase activity which copurified with rubber transferase (4, 30) is a property of the purified protein. In the absence of rubber particles, the prenyltransferase catalyzed the formation of all trans-FPP from DMAPP and IPP rather than all cis-polyisoprene. All of the FPP synthase activity in whole latex is ascribed to this prenyltransferase. FPP synthase activities in whole latex and rubber particle free serum were identical (Table I) and both activities were copurified from serum. The identical prenyltransferase was purified reproducibly from *Hevea* latex whether the FPP synthase assay or the rubber elongation assay was used to follow the activity during purification.

The concentration of prenyltransferase dimer in freshly tapped whole latex was estimated from the purification yield shown in Table II to be 160 nM, after correcting for dilution. A similar determination of 180 nM was made from the direct comparison of FPP synthase activity of purified prenyltransferase to the activity in whole latex. In contrast, the rubber content of whole latex was measured to be 330 g/liter (38), which translates to 660 mM rubber molecules by using a number average molecular mass of 500,000 Da for rubber. We calculated from this analysis that there was a very large excess of potential substrate to prenyltransferase in the whole latex. Thus, if all molecules were competent for addition, increasing the concentration of prenyltransferase (when IPP was not limiting) would have increased rubber biosynthesis. The addition of purified, concentrated prenyltransferase to diluted whole latex did not increase the incorporation of [14C]IPP into rubber (Table III). Thus the low concentration of prenyltransferase in whole latex was sufficient to saturate the rubber molecules that were competent for addition. Therefore, very few rubber molecules in whole latex could accept additional IPP.

We reached a similar conclusion when we used purified prenyltransferase to titrate serum free deproteinated rubber particles. Titration of 8.6 mg/ml deproteinated rubber particles (17 mM rubber molecules) (Fig. 3, closed triangles) revealed 2 nM competent allylic pyrophosphate donor sites or 0.01% of the total potential active ends. The purified enzyme had a high affinity (Kd = 9 nM) for these sites. Additional evidence that the number of competent end groups was limiting is that the rubber transferase assay was linear with added deproteinated rubber particles. The titration of a fixed level of enzyme by a range of rubber particles between 0.6–11 mg/ml rubber (1.2–23 mM rubber molecules; Mw = 500,000 Da) is shown in Fig. 1. This titration was limited by the concentration of deproteinized rubber particles which could be obtained by gel exclusion chromatography. Archer et al. (5) have titrated fixed enzyme samples (C-serum) with washed rubber particles concentrated by centrifugation up to a rubber content of 23% (230 mg/ml). They see a linear dependence up to nearly 160 mg/ml. A replot of their data allows an estimate of half-saturation (by extrapolation) of ~360 mg/ml rubber or about 700 mM rubber molecules (Mw = 500,000 Da). Since it is likely that their particles also had only 0.01% active ends, the number of elongation sites in a 700 mM rubber molecule is calculated to be about 70 nM. This last number approaches our estimates of the prenyltransferase concentration in C-serum and suggests Archer et al. (5) had begun to titrate out the available enzyme.

It is possible to estimate the number of sites per rubber particle from the total calculated number of elongation sites. The number of sites was proportional to the diameter of the rubber particles. Particles from *H. brasiliensis* range from between 50 to 1500 nm (5, 23, 25–28). The diameter of rubber particles in our preparation was approximately 250–300 nm (36). By using a

\[ v = \frac{\nu_d M/2 - (K_d M + 8 M K_d) / 8 + K_d / 8}{(M + M_d)} \]  

\[ K_d = \frac{M}{M_d} \]

where \( v \) = observed rate (disintegrations/min); \( \nu_d \) = turnover of the prenyltransferase dimer (disintegrations/min/nM); \( M \) = concentration of prenyltransferase monomer; \( K_d \) = the monomer/dimer dissociation constant. The best fit to the data in Fig. 3 yielded an dissociation constant of 80 nM. Thus, in whole latex and in the enzyme dilutions used in the rubber transferase assays, this prenyltransferase may contain a high concentration dependence of the FPP synthase assay shown in Fig. 3, we have no other evidence that this prenyltransferase dissociated to a monomer at low dilution.

![Fig. 4](image)  

**Fig. 4.** Molecular exclusion chromatography of benzene-soluble radiolabeled products of the rubber elongation assay. Deproteinated rubber particles were labeled with [1-14C]IPP using purified FPP synthase. Samples were filtered and the trapped radiolabeled products of the rubber elongation assay were copurified from serum. The identical prenyltransferase was purified reproducibly from *Hevea* latex whether the FPP synthase assay or the rubber elongation assay was used to follow the activity during purification.

In practice it is easier to purify the prenyltransferase using the FPP synthase assay rather than the rubber elongation assay since in the former assay a linear relationship with respect to enzyme addition can be approached at higher enzyme concentrations whereas in the latter assay the enzyme dependence is nonlinear concave down (Fig. 3).
density of 0.9 g/ml for natural rubber (45) and a rubber content of 8.6 mg/ml, rubber particle diameters of 100, 300, and 500 nm correspond to 30, 1, and 0.24 nm rubber particles. Thus, 1 nM active ends leads to a calculation of from 4 to as low as 0.03 growing ends per rubber particle. Active ends may not be evenly distributed among all particles. Larger particles may have no growing ends whereas smaller particles may have multiple growing ends. Archer et al. (5) have shown that small diameter washed rubber particles have a disproportionately high rate of [14C]IPP incorporation even when corrections were made for their greater surface to volume ratio.

A low percentage of rubber molecules in our preparation was competent to add to IPP. We expect the loss of elongation sites in latex occurs as a consequence of the head growth mechanism (46) of rubber polymerization in which the polymer rather than the monomer is activated. Three mechanisms for activated allylic pyrophosphate loss from rubber molecules can be used to calculate the maximum turnover per active site for each of the assays. In a typical assay an elongation complex added approximately 7000 isoprene units at a rate of 60 min⁻¹ in the rubber elongation assay (Fig. 3, closed triangles). The most efficient mechanism for the polymerization is for the elongation complex, which includes the Hevea prenyltransferase, to remain associated with the individual rubber molecule during these thousands of additions. One active site in the fully associated prenyltransferase dimer incorporated IPP into GPP and t,t-FPP at a rate of 600 min⁻¹. A 10-fold difference in the rates of prenylation in each reaction indicates they may not have the same rate determining step.

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