Plant Physiol. (1993) 103: 535–542

Seasonal Variations in Rubber Biosynthesis, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, and Rubber Transferase Activities in *Parthenium argentatum* in the Chihuahuan Desert[1]

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The rubber content and the activities of enzymes in the polyisoprenoid pathway in *Parthenium argentatum* (guayule) were examined throughout the growing season in field plots in the Chihuahuan Desert. The rubber content of the plants was low in July and August and slowly increased until October. From October to December there was a rapid increase in rubber formation (per plant) from 589.0 mg to 4438.0 mg. The percentage of rubber in the plants increased from 0.7% (mg/g dry weight) in August and 1.27% in October to 5.5% in December. The rapid increase in rubber formation may result from exposing the plants to low temperatures of 5 to 7°C. The activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) was 21.1 nmol mevalonic acid (MVA) h^{-1} g^{-1} fresh weight in the bark of the lower stems in June during seedling growth and decreased to 5.1 nmol MVA h^{-1} g^{-1} fresh weight in July and 2.9 nmol MVA h^{-1} g^{-1} fresh weight in September. From October to December, the activity increased from 5.0 to 29.9 nmol MVA h^{-1} g^{-1} fresh weight. The activity of rubber transferase was 65.5 nmol isopentenyl pyrophosphate (IPP) h^{-1} g^{-1} fresh weight in the bark in September and increased to 357.5 nmol IPP h^{-1} g^{-1} fresh weight in December. The rapid increase in the activities of HMGR and rubber transferase coincided with the rapid increase in rubber formation. The activities of MVA kinase and IPP isomerase did not significantly increase in the fall and winter. A tomato HMGR-1 cDNA probe containing a highly conserved C-terminal region of HMGR genes hybridized at low stringency with several bands on blots of *Hin*dIII-digested genomic DNA from guayule. In northern blots with the HMGR-1 cDNA probe at low stringency, HMGR mRNA was high in June and November, corresponding to periods of high HMGR activity during seedling growth and rapid increase in rubber formation. The seasonal variations in rubber formation and HMGR mRNA, HMGR activity, and rubber transferase activity may be due to low temperature stimulation in the fall and winter months.

Investigations on the biochemistry and physiology of rubber biosynthesis have centered on *Hevea brasiliensis*, the commercial rubber tree of the tropics, and *Parthenium argentatum* (guayule), a Chihuahuan Desert plant of interest as a source of industrial rubber for the United States. The biochemistry of the polymerization of IPP to form new rubber chains catalyzed by a rubber transferase bound to rubber particles is the same in both species (Archer and Audley, 1987; Madhavan et al., 1988), but an outstanding feature of rubber formation in guayule is the stimulation of rubber biosynthesis by the exposure of the plants to low temperatures, similar to those existing in the fall and winter in the Chihuahuan Desert (Bonner, 1943; Downes and Tonnet, 1985; van Staden et al., 1986).

Bonner (1943) demonstrated that the exposure of guayule plants to 27°C during the day and 7°C at night with an 8-h photoperiod for 4 months in a controlled-temperature greenhouse induced a 4-fold increase in rubber formation compared with plants grown at 27°C during the day and 27°C at night with an 8-h photoperiod. van Staden et al. (1986) have demonstrated that rubber biosynthesis in guayule plants in South Africa is strictly a winter phenomenon. Downes and Tonnet (1985) studied the effect of low temperature on rubber accumulation in field-grown and phytotron-grown guayule plants. In fields near Narrabri and Canberra, Australia, the rubber percentage in the guayule plants was higher after the winter than after the summer, indicating that cooler temperatures were favorable for rubber formation. In the phytotron experiments, the rubber percentage was much higher in guayule plants maintained at 15°C-day and 10°C-night temperatures with a 16-h photoperiod than plants maintained at 24°C-day and 19°C-night temperatures with a 16-h photoperiod.

The manner in which guayule interacts with the cold temperatures to produce rubber has not been elucidated. An EM study of rubber formation in guayule plants by Goss et al. (1984) showed that in plants grown at 27 to 32°C days and 7°C nights with an 8-h photoperiod, there was a substantial increase in rubber particles in the cortical parenchyma cells compared with plants grown at 27 to 32°C days and 21 to 24°C nights with an 8-h photoperiod. There also was a severalfold increase in the incorporation of [^{14}C]acetate and [^{14}C]mevalonate into rubber in the stems of the cold-treated plants, which suggests that a faster rate of reactions in the

[1] This work was supported in part by a grant from Texas Advanced Technology Program and The Texas Agricultural Experiment Station, College Station, TX.

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl pyrophosphate; MVA, mevalonic acid; MVA-DBED salt, mevalonic acid-dibenzylethylenediammonium salt; MVA-5-P, mevalonic-5-phosphate.

polyisoprenoid pathway accounted for the increase in rubber biosynthesis. Rubber transferase activity in homogenates of the bark from lower stems of guayule plants exposed to the low fall and winter temperatures in the Chihuahuan Desert showed a 12-fold increase over the activity in plants during the summer months (Madhavan et al., 1988). The low temperatures may induce the expression of genes coding for the synthesis of enzymes involved in rubber biosynthesis in guayule plants (Bonner, 1975).

In this paper we report the activity of HMGR and other enzymes of the polyisoprenoid pathway in guayule plants growing in field plots in the Chihuahuan Desert. Seasonal variations in rubber formation and the activities of HMGR and rubber transferase were observed that may be due to low-temperature stimulation in the fall and winter months. Examination of guayule RNA with a HMGR-1 cDNA from tomatoes suggests that the increase in HMGR activity may be associated with an increase in the level of HMGR mRNA.

MATERIALS AND METHODS

Chemicals

DL-3-Hydroxy-3-methylglutaryl-[3-^{14}C]CoA, [2-^{14}C]sodium acetate, and *RS*-mevalonic-[2-^{14}C]DBED salt were purchased from New England Nuclear. [1-^{14}C]IPP ammonium salt was purchased from Amersham. Mevinolin was purchased from Sigma. HMGR-1 cDNA was a gift from Drs. J.O. Narita and W. Gruissem (University of California, Berkeley).

Plant Material

Guayule (*Parthenium argentatum*) seed N-576 was germinated in the greenhouse in small pots containing vermiculite. After 2 months the seedlings were transplanted to field plots in the Chihuahuan Desert at the Texas Agricultural Experiment Station (Fort Stockton, TX) in May, 1992. The seedlings were planted in 30-foot rows that were replicated three times randomly throughout the field plots.

Temperature

The exposure of guayule plants to several months of daily temperatures of about 27°C (days) and 7°C (nights) resulted in a stimulation of rubber formation (Bonner, 1943; Goss et al., 1984). In the Chihuahuan Desert field plot experiments, the accumulative number of hours of 5 to 7°C from May to December were calculated from continuous daily air temperature recordings in the field plots.

Enzyme Extraction and Assay

HMGR

HMGR activity in guayule stems was assayed by the procedure of Ji et al. (1992). Bark was removed from the lower stems of the harvested plants, rinsed with distilled H_2O, and blotted dry with paper towels. One-half gram of the bark was ground to a powder in a mortar in liquid N_2. The powder was homogenized in 6 mL of 10 mM Tris-HCl buffer, pH 7.0, containing 0.35 M Suc, 30 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% BSA, and 3.5% (w/v) PVP. PVP absorbs phenolics and was used to prevent polyphenol oxidase activity in the tissue. The homogenate was centrifuged at 5,000*g* for 10 min and the supernatant fraction was collected and centrifuged at 105,000*g* for 60 min in a Beckman L8-M ultracentrifuge. The pelleted membranes were suspended in 75 μL of 0.2 M potassium phosphate buffer, pH 6.9, containing 25 mM DTT.

The enzyme assay mixture contained 0.3 μmol of DTT, 0.24 μmol of NADPH, 1.4 μmol of Tris-HCl buffer, pH 7.6, 5 of μL pelleted membrane suspension, and 14 nmol of DL-[3-^{14}C]HMG-CoA containing 0.12 μCi of radioactivity to a final volume of 21 μL. The reaction mixture was incubated at 30°C for 60 min and the reaction was stopped by adding 2 μL of 6 M HCl and 2 μL of 1 M MVA lactone. The mixture was incubated at room temperature for 2 h for MVA lactonization followed by centrifugation at 12,000*g* for 3 min to pellet the membrane fragments. Ten microliters of the supernatant fraction was applied to Whatman LK 50F silica gel TLC plates. The plates were developed in 50 mL of diethylether:acetone (3:1, v/v) for 20 min and analyzed by autoradiography. The R_F values for HMG-CoA and MVA lactone were 0.0 and 0.9, respectively. The radioactive HMG-CoA and MVA lactone were scraped from the plates. Fisher ScintiVerse BD scintillation cocktail was added to the powder for analysis in a Beckman LS-200 liquid scintillation spectrometer. HMGR activity was expressed as nmol MVA h^{-1} mg^{-1} protein or nmol MVA h^{-1} g^{-1} fresh weight. To establish the developmental curve for HMGR activity, the mean of the activity of three individual plants was plotted against the time of year. Lines representing the range of activity in the three plants were also drawn on the developmental curve.

In these analyses, the HMGR activity in the 105,000*g* membrane pellet represents the total HMGR activity in the guayule bark. Grinding the woody bark of guayule in liquid N_2 was necessary to disrupt the cells and obtain total HMGR activity. As a result of grinding in liquid N_2, the chloroplasts and mitochondria present in the bark tissue were also disrupted. The 5,000*g* pellet did not contain any detectable HMGR activity, but the HMGR activity in the 105,000*g* pellet represents microsomal, chloroplast, and mitochondrial activity. The exact proportion of HMGR activity in the organelles and microsomal membranes in guayule bark was not determined.

MVA Kinase

Bark was removed from the lower stems of the harvested plants, rinsed with distilled H_2O, and blotted dry with paper towels. One-half gram of bark tissue was ground to a powder in a mortar with liquid N_2. The powder was homogenized in 6 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM GSH, pH 8.0, 5 mM NaCN, and 0.125% BSA. The NaCN and BSA were used to inhibit the polyphenol oxidase activity in the bark tissue. An aliquot of the homogenate was used to assay the total activity of MVA kinase, IPP isomerase, and rubber transferase. The assay mixtures for MVA kinase contained 5.0 μmol of potassium phosphate buffer, pH 7.0, 0.5 μmol of DTT, 0.25 μmol of $MgCl_2$, 0.2 μmol of ATP, and 36.5 μL of crude homogenate to a final volume of 50.0 μL. The reaction mixtures were incubated at 30°C for 60 min and the reactions

were stopped by placing the reaction tubes in boiling H_2O for 2 min. The mixtures were centrifuged at 12,000*g* for 3 min to remove the denatured protein. Ten microliters of the supernatant was applied to Whatman No. 1 paper. The paper was developed with propanol:NH_4OH:H_2O (6:3:1, v/v/v) for 12 h using descending chromatography. The dried chromatograms were analyzed by autoradiography. The R_F values for MVA and MVA-5-P on the chromatograms were 0.75 and 0.20, respectively. Radioactive MVA-5-P was located on the chromatograms, cut out, and analyzed by liquid scintillation counting in the presence of Fisher ScintiVerse BD scintillation cocktail. MVA kinase activity was expressed in nmol MVA-5-P h^{-1} g^{-1} fresh weight. The MVA kinase activity in the bark of the lower stems of guayule plants was determined by assaying two plants at each sampling throughout the growing season.

IPP Isomerase

IPP isomerase activity in the homogenates of the bark from the lower stem was determined by the procedures of Scatterwhite (1985) and Madhavan et al. (1988). The reaction mixtures contained 1.2 μmol of $MgCl_2$, 1.8 μmol of potassium fluoride, 0.75 μmol of GSH, pH 8.0, 27 nmol of [1-^{14}C]IPP containing 30.5 × 10^6 cpm of radioactivity, and 90 μL of the crude homogenate to a final volume of 150 μL. The reaction mixtures were incubated at 30°C for 15 min and the reactions were stopped by adding 0.2 mL of 25% HCl in methanol and 0.5 mL of H_2O. The reaction mixtures were reincubated at 30°C for 30 min and the radioactive alcohols were extracted with hexane. An aliquot of the hexane was assayed for radioactivity in the Beckman liquid scintillation spectrometer. IPP isomerase activity was expressed in nmol DMAPP h^{-1} g^{-1} fresh weight. The IPP isomerase activity in the bark was determined by assaying two plants at each sampling throughout the growing season. The addition of potassium fluoride to the reaction mixtures inhibited 80% of the phosphatase activity in the stem homogenates.

Rubber Transferase

The activity of the rubber transferase bound to the rubber particles in homogenates of the bark from the lower stems was determined by the procedure of Madhavan et al. (1988). The reaction mixtures contained 0.75 μmol of $MgCl_2$, 0.75 μmol of GSH, pH 8.0, 27 nmol of [1-^{14}C]IPP containing 30.5 × 10^6 cpm of radioactivity, and 90 μL of the crude stem homogenate to a final volume of 150 μL. The reaction mixtures were incubated at 30°C for 60 min and the reactions were stopped with the addition of 0.5 mL of ethanol. The reaction mixtures were extracted with hexane until no further radioactivity was extracted. The hexane extract was evaporated to 200 μL, and 2 mg of carrier rubber dissolved in hexane was added to the extract. The rubber was precipitated with 3 volumes of acetone. The rubber was collected by centrifugation at 10,000*g* for 15 min and washed with acetone until no radioactivity could be detected. The rubber was dissolved in toluene and assayed for radioactivity in a Beckman liquid scintillation spectrometer. The rubber transferase activity was expressed as nmol IPP h^{-1} g^{-1} fresh weight. To establish the developmental curve for rubber transferase activity, the mean of the activity of three individual plants was plotted against the time of year. Lines representing the range of activity between the three plants were also drawn on the developmental curve.

Analysis of Rubber Content

The rubber content of the guayule plants was determined by the procedure of Black et al. (1983). The defoliated guayule plants were air dried and cut into small segments with pruning shears and then ground in a Retsch ZM-1 centrifugal mill. The ground sample was dried in an oven at 50°C for 3 h. Two grams of the oven-dried material was homogenized in 67 mL of acetone in a Polytron homogenizer for 30 s at a setting of 7. The homogenized sample was centrifuged at 6,000*g* for 5 min and the acetone supernatant fraction was removed. The pellet was extracted once more with acetone, which extracts the terpenoid resin. The rubber was extracted from the pellet by homogenizing it with 67 mL of cyclohexane in a Polytron homogenizer for 30 s at a setting of 7. The homogenate was centrifuged and the pellet was reextracted with cyclohexane in the Polytron homogenizer. The cyclohexane extracts were evaporated to dryness in a forced-air oven at 105°C. The rubber was weighed and the rubber content was calculated. The rubber content was expressed as mg rubber per plant and as percent dry weight. To obtain the developmental curve for the rubber content of the guayule plants, the mean of the rubber content for three individual plants was plotted against the time of year.

[^{14}C]Acetate Incorporation into Rubber in Guayule Stems

Plants were harvested in October and the bark was cut from the base of the stems, rinsed in distilled H_2O, and blotted with paper towels. One gram of bark tissue was cut into slices 0.5 mm thick × 2 cm in length with a razor blade. The bark slices were incubated for 4 h at 30°C in 5.0 mL of Linsmaier and Skoog medium (1965) with 178 nmol of sodium acetate containing 10 μCi of [2-^{14}C]sodium acetate and 100 μM mevinolin. The control flasks did not contain the mevinolin inhibitor. At the end of the incubation period, the radioactive medium was removed and the slices were washed five times with 10 mL of distilled H_2O. The slices were then extracted several times with acetone followed by the extraction of rubber with hexane. An aliquot of the hexane extract was assayed for radioactivity.

DNA Extraction

DNA was extracted from leaves of guayule and tomato by the procedure of Dellaporta et al. (1984) and purified by CsCl gradient centrifugation by the procedure of Sambrook et al. (1989). Three grams of the leaves were frozen in liquid N_2 before they were ground to a powder with a mortar and pestle in liquid N_2. The powder was extracted with 15.0 mL of 100 mM Tris-HCl buffer, pH 8.0, containing 50 mM EDTA, 500 mM NaCl, and 10 mM mercaptoethanol. One milliliter of a 20% SDS solution was added to the extract and the mixture was vortexed and incubated for 10 min at 65°C. Following

the incubation, 5.0 mL of 5 M potassium acetate was added and the extract was incubated for 20 min at 0°C to precipitate the protein and polysaccharide. The mixture was centrifuged at 25,000*g* for 20 min and the supernatant fraction was extracted with 10 mL of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged at 20,000*g* for 20 min. The extraction was repeated once more. The combined top aqueous phases were transferred to a test tube containing 10 mL of ice-cold isopropanol and placed at −20°C for 30 min. The precipitated DNA was wound on a glass rod and allowed to dry at room temperature. The DNA was dissolved in 1.0 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM EDTA. CsCl powder and ethidium bromide were added to the DNA solution to a final volume of 14.0 mL. The density of the CsCl solution was 1.55 g mL^{-1} and the concentration of the ethidium bromide was 740 μg mL^{-1}. The solution was centrifuged for 24 h at 53,000 rpm with a Ti 70–1 rotor at 20°C. The DNA appeared as a clear red band in the center of the gradient and was collected with a disposable syringe. The ethidium bromide was removed by extracting the DNA solution with 10 mL of isopropanol saturated with H_2O and NaCl. The DNA solution was diluted with 3 volumes of H_2O and the DNA precipitated with 2 volumes of ethanol. The DNA was spun out with a glass rod, washed with 70% ethanol, and dried at room temperature. The DNA was dissolved in 100 μL of 0.1 M Tris-HCl, EDTA buffer, pH 8.0, and stored at 4°C.

Southern Blots

The procedure for the DNA blots was that described by Southern (1975). Two micrograms of DNA was digested overnight with 20 units of *Hin*dIII. The digested DNA was electrophoretically separated on 1% agarose and transferred to nylon membrane obtained from Sigma. Prehybridization was done in 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 100 μg mL^{-1} denatured salmon testes DNA at 65°C for 3 h. The HMGR-1 cDNA probe was labeled with a Random Primed DNA Labeling Kit obtained from United States Biochemical. Hybridization was done in 6× SSC, 0.5% SDS, and 100 μg mL^{-1} of denatured salmon testes DNA at 65°C for 24 h. Washing was done at room temperature in 2× SSC and 0.5% SDS for 5 min, 2× SSC and 0.1% SDS for 15 min, and 0.1× SSC and 0.5% SDS for 1 h. The exposure time for autoradiography was 3 to 5 d.

RNA Isolation

RNA was isolated from guayule bark by the procedure of Sambrook et al. (1989). The bark was removed from the lower stems and immediately frozen in liquid N_2. Three grams of frozen tissue was ground to a powder with a mortar and pestle in liquid N_2. The powder was homogenized in 15 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 4 M guanidine thiocyanate and 1% mercaptoethanol. Sodium lauryl sarcosinate was added to the homogenate to a concentration of 0.5% and the mixture was centrifuged at 5,000*g* for 20 min. The supernatant fraction was extracted with phenol: chloroform:isoamyl alcohol (25:24:1, v/v/v) and centrifuged at 12,000*g* for 20 min. The top aqueous phase was extracted once more with chloroform:isoamyl alcohol (24:1, v/v). The top aqueous phases were combined and layered onto a cushion of 5.7 M CsCl, 0.01 M EDTA, pH 7.5, in a Beckman Quick-seal tube. The tube was sealed and centrifuged at 53,000 rpm in a Beckman Ti 70.1 rotor at 20°C for 18 h. The tube was cut and the supernatant fraction was removed with a syringe. The RNA deposited at the bottom of the tube was washed three times with 70% ethanol and dried. The RNA was further purified by dissolving it in 300 μL of Tris-HCl, EDTA buffer, pH 7.5, adding 30 μL of 3 M sodium acetate, and precipitating the RNA with 3 volumes of ethanol. The yield by this procedure is 180 μg RNA g^{-1} of guayule bark.

Northern Blots

The procedure for the northern blots was described by Sambrook et al. (1989). Thirty micrograms of guayule stem RNA was denatured with formamide and formaldehyde at 65°C for 15 min and subjected to electrophoresis on a 2.2 M formaldehyde/1.0% agarose gel. The remaining procedure was similar to that described previously for the Southern blots. The blot membrane was Amersham Hybond-ECL.

RESULTS

Growth and Rubber Formation in Guayule in Desert Field Plots

The growth and rubber content of guayule plants were determined throughout the growing season in field plots in

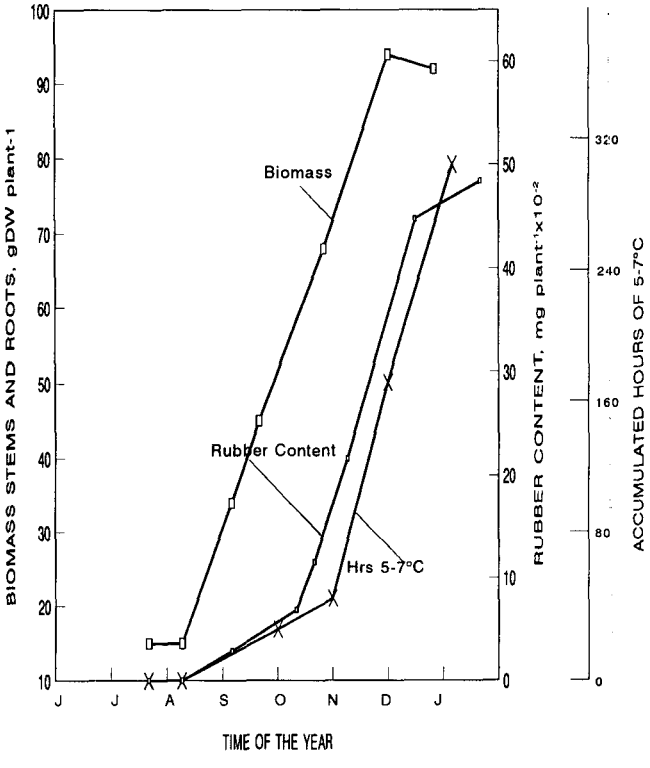

Figure 1. The development of biomass and rubber content of guayule plants in Chihuahuan Desert field plots.

Table I. *HMGR activity from guayule bark*

The reaction mixture contained the following: 0.3 μmol of DTT, 0.24 μmol of NADPH, 14 nmol [3-^{14}C]HMG-CoA, 5 μL microsomal preparation; the final volume was 21 μL. The reaction mixture was incubated at 30°C for 60 min.

Reaction Mixture	HMGR Activity
	nmol MVA h^{-1} mg^{-1} protein
Complete	25.6
−DTT	20.5
−NADPH	0.1
+Mevinolin (24 μM)	0.1
Boiled enzyme	0

the Chihuahuan Desert in Fort Stockton, TX (Fig. 1). The plants were transplanted to the field plots in May. On July 19 and August 11, the dry weight of the stems and roots of the guayule plants was about 15.0 g dry weight plant^{-1}. The growth of the plants increased from 33.3 g dry weight plant^{-1} on September 6 to 96.3 g dry weight plant^{-1} on November 11 to 90.7 g dry weight plant^{-1} on December 19.

Rubber is deposited primarily in the cortical parenchyma cells of the stems and roots of the guayule plants (Goss et al., 1984). The rubber content in the stems and roots of the guayule plants in the field plots on August 6 was 30.0 mg plant^{-1} and slowly increased to 589.0 mg plant^{-1} on October 14. From October 14 to December 19, there was a rapid increase in rubber content from 589.0 mg plant^{-1} to 4438.0 mg plant^{-1}. This linear rate of rubber biosynthesis was correlated to the cumulative number of hours of temperature between 5 and 7°C. The marked increase in rubber biosynthesis occurred on October 14, when the growth of the stems and roots was about 65% complete. Some of the increase in rubber per plant was due to an increase in the size of the plant, but the major amount of increase was due to an increased percentage of rubber in the stems and roots. The percentage of rubber in the guayule plants increased from 0.7% (mg g^{-1} dry weight) in August and 1.27% in October to 5.5% by December 19. The rubber content was 6.5% in the January samples. The increase in the percentage of rubber in the cortical parenchyma cells of the stem and roots again occurred after the biomass accumulation was 65% complete. The results demonstrated that the fall and winter temperatures of 5 to 7°C in the Chihuahuan Desert may stimulate the polyisoprene pathway in the guayule plants.

The Development of the Activities of Enzymes in the Polyisoprene Pathway

HMGR

The activity of HMGR in guayule stems, which is the primary tissue of rubber biosynthesis, has not been previously described. The data in Table I show the requirements for HMGR activity in the membrane pellet isolated from the homogenates of the bark of guayule stems. In the complete reaction mixture, 25.1 nmol MVA h^{-1} mg^{-1} protein was produced by the enzymic reduction of HMG-CoA. In the absence of NADPH or in the presence of boiled enzyme, there was no formation of [^{14}C]MVA. Mevinolin at a concentration of 24 μM completely inhibited the enzymic reaction. Mevinolin (100 μM) added to guayule bark tissue inhibited the incorporation of [^{14}C]acetate into rubber by 98.2%. This indicates that HMGR is functional during rubber biosynthesis. In separate experiments, we have shown that the HMGR activity in the membrane pellet is linear for 90 min at 30°C, is linear with protein concentration from 0 to 40 μg, and has a pH optimum between pH 6.5 to 7.5.

The effect of HMG-CoA concentration on HMGR activity is shown in Figure 2. The membrane HMGR activity is saturated at 125 μM HMG-CoA. The double reciprocal plot of substrate versus velocity shows that the K_m for the membrane HMGR is 24 μM. The K_m values of HMGR in membrane pellets from pea, radish, and maize for HMG-CoA are 160, 1.5, and 10 μM, respectively (Russell, 1985; Bach et al., 1990). The optimum temperature for the HMGR activity in stem homogenates is 35 to 45°C. The development of HMGR activity in guayule stem homogenates throughout the growing season is shown in Figure 3. The HMGR activity was 21.2 nmol MVA h^{-1} g^{-1} fresh weight on June 29 and decreased to low values throughout the summer months. HMGR activity increased to 5.0 nmol MVA h^{-1} g^{-1} fresh weight in the October 1 samples and reached a value of 29.9 nmol MVA h^{-1} g^{-1} fresh weight in the plants harvested on December 20. From October to December, there was a 6-fold increase in HMGR activity in the bark tissue from the lower stems of the guayule plants.

Rubber Transferase

The rubber transferase activity in homogenates of guayule stems is tightly bound to the rubber particles (Madhavan et

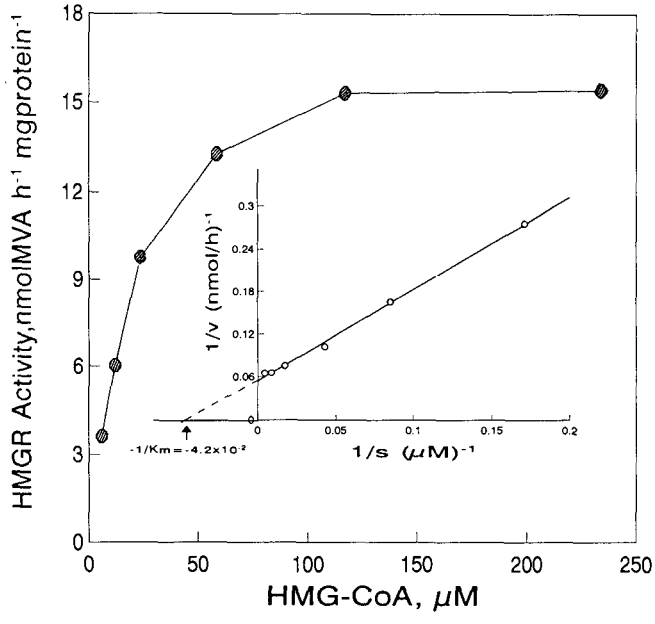

Figure 2. The effect of HMG-CoA concentrations on HMGR activity in stem homogenates from guayule. The inset shows a double reciprocal plot of the velocity of HMGR against HMG-CoA concentration.

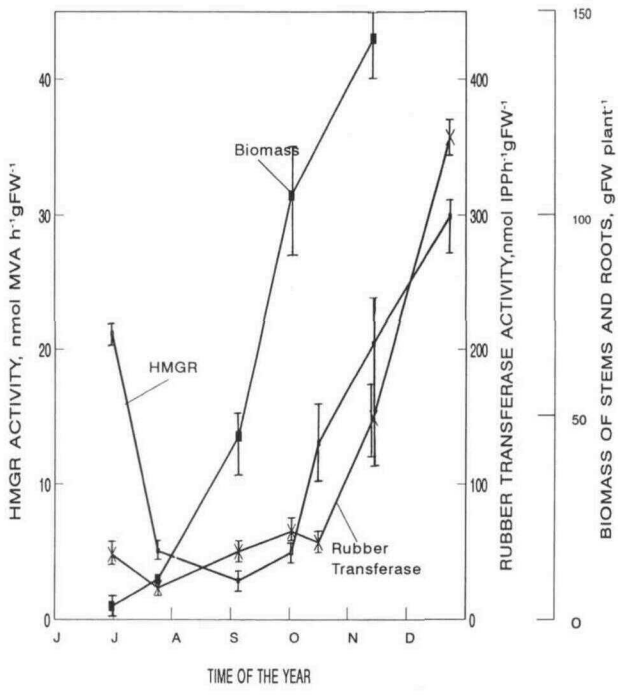

Figure 3. The development of biomass, HMGR, and rubber transferase activities in guayule plants throughout the growing season in Chihuahuan Desert field plots.

al., 1988). The polymerization reaction catalyzed by the bound rubber transferase consists of the repetitive additions of IPP to an allylic-pyrophosphate initiator to form a high mol wt rubber polymer. In crude stem homogenates, an allylic-pyrophosphate such as DMAPP is not required for the polymerization reaction because of the presence of an active IPP isomerase furnishing DMAPP from the IPP in the reaction mixtures. The optimum temperature for rubber transferase activity in guayule stem homogenates is 27°C.

The development of rubber transferase activity in guayule stems throughout the growing season is shown in Figure 3. Rubber transferase activity was 48.8 nmol IPP h^{-1} g^{-1} fresh weight on June 29, decreasing to 23.7 nmol h^{-1} g^{-1} fresh weight in July and increasing to 65.5 and 57.3 nmol IPP h^{-1} g^{-1} fresh weight on September 29 and October 14, respectively. Rubber transferase activity increased linearly from October 14 to December 20, at which point the rubber transferase activity was 357.5 nmol IPP h^{-1} g^{-1} fresh weight. The rapid increase in both HMGR and rubber transferase activities from October 14 to December 20 nearly coincided with the rapid increase in rubber formation (Fig. 1). The high HMGR activity was associated with growth of the seedlings. The higher activity of rubber transferase in the June plants compared with the activity in the July plants may have been due to the exposure of the seedlings to colder temperatures in May and June. From October to December there was about a 6-fold increase in rubber transferase activity in the bark tissue.

We have shown in separate experiments that IPP isomerase activity in guayule stem homogenates fluctuated from 632 nmol DMAPP h^{-1} g^{-1} fresh weight in plants harvested in June to 670 nmol DMAPP h^{-1} g^{-1} fresh weight in plants harvested on October 14 to 400 nmol DMAPP h^{-1} g^{-1} fresh weight in plants harvested on December 20. MVA kinase activity fluctuates between 2376 nmol MVA-5-P h^{-1} g^{-1} fresh weight in plants harvested in June to 3156 nmol MVA-5-P h^{-1} g^{-1} fresh weight in plants harvested on December 20. The activities of both of the enzymes are high from June to December and are probably not regulated by low temperature.

Southern Blots

Figure 4 shows Southern blots of tomato (lane 1) and guayule (lane 2) genomic DNA digested with *Hin*dIII using an HMGR-1 cDNA probe from tomato. Multiple copies of HMGR genes exist in tomato and the homology between the HMGR-1 cDNA and guayule DNA is sufficient to demonstrate the presence of multiple copies of HMGR genes in guayule. Multiple copies of HMGR genes exist in other plants (Chin et al., 1982; Caelles et al., 1989; Yang et al., 1991; Choi et al., 1992) in contrast to a single copy of the HMGR gene in mammals (Osborne et al., 1985).

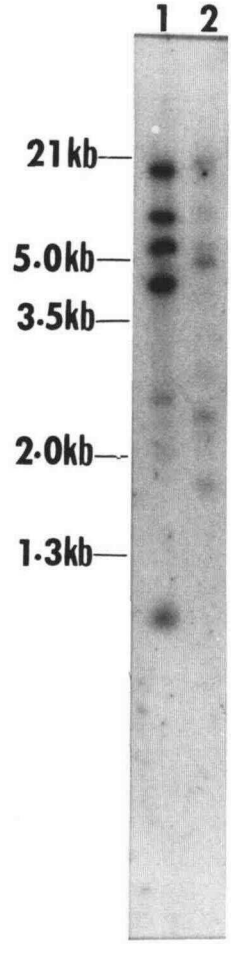

Figure 4. Southern blots of tomato (lane 1) and guayule (lane 2) genomic DNA digested with *Hin*dIII using an HMGR-1 cDNA probe from tomatoes.

Northern Blots

Northern blots (Fig. 5) show that using tomato HMGR-1 cDNA probe at low stringency, HMGR mRNA was detectable throughout the growing season in guayule stems. The development of HMGR mRNA in guayule stems was high in June, decreased to lower levels in July and August, and increased in September, October, and November. The high levels of HMGR mRNA in June and November corresponded to the high level of HMGR in June and to the high level of HMGR during the linear rate of rubber synthesis (Fig. 3).

DISCUSSION

Rubber formation in guayule plants was examined throughout the growing season from May to December in Chihuahuan Desert field plots. Rubber biosynthesis was not significant during the summer months but was induced in developing plants exposed to the low fall and winter temperatures. During the months of September, October, November, and December, rubber biosynthesis in guayule plants was closely correlated to the cumulative number of hours of temperature of 5 to 7°C. The biomass of the plants also increased as the plants experienced the cooler temperatures of the fall, but growth occurred at temperatures higher than those inducing rubber biosynthesis. At the time of the initiation of the linear phase of rubber biosynthesis, the growth of the plants was 65% complete. During this rapid period of rubber biosynthesis, there was about an 8-fold increase in the rubber content of the plants. It appears that the induction of the polyisoprene pathway in guayule plants occurs as a result of an interaction of developing plants with the low temperatures of the fall and winter in the Chihuahuan Desert.

These observations agree with earlier results obtained under controlled growth conditions. Rubber biosynthesis was increased 3- to 4-fold in guayule plants grown at 27°C during the day and 7°C at night compared with control plants grown at a day and night temperature of 27°C (Bonner, 1943). Exposure of guayule plants to 6 months of 27 to 32°C for days and 7°C for nights resulted in a 2-fold stimulation of rubber formation over control plants grown at 27 to 32°C for days and 21 to 24°C for nights (Goss et al., 1984). The control plants contained 2.18% rubber and the cold-treated plants contained 5.69% rubber. Electron photomicrographs of the cortical parenchyma cells showed a high concentration of rubber particles in the cold-treated plants and an increase in [^{14}C]acetate incorporation into rubber. In this study, the increase in [^{14}C]acetate incorporation into rubber suggested that a faster rate of the reactions of the polyisoprene pathway is responsible for the increase in rubber formation.

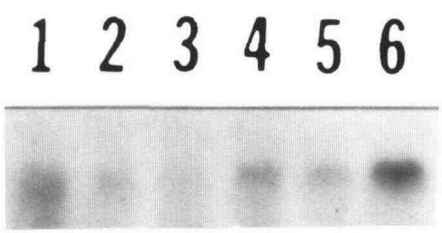

Figure 5. The lanes 1, 2, 3, 4, 5, and 6 show RNA extracted from the bark tissue used for the analysis of HMGR and rubber transferase activities from plants harvested in June, July, August, September, October, and November.

Rubber transferase activity has been characterized in crude homogenates of guayule stems and is stimulated 12-fold by the low temperatures of the fall and winter in the Chihuahuan Desert (Madhavan et al., 1988). HMGR activity has been studied in the membrane fraction of the latex of *H. brasiliensis* (Sipat, 1985) but has not been characterized in stem tissues from guayule plants. HMGR activity in guayule stem homogenates is totally inhibited by 24 μM mevinolin, and the incorporation of [^{14}C]acetate into rubber in guayule bark slices is inhibited by 100 μM mevinolin. This demonstrates that rubber synthesis in guayule is dependent on carbon flow through HMG-CoA. This is different from other studies. Mevinolin inhibits phytosterol in radish seedlings without affecting the biosynthesis of ubiquinone, Chl, or carotene synthesis (Bach et al., 1990). Tomato fruit at the 0.3- to 0.4-cm stage of development injected with mevinolin inhibits further fruit development and HMGR activity, which is presumably required for phytosterol biosynthesis during cell division. Mevinolin injected into fruit at the 1.5- to 2.0-cm stage of development did not inhibit carotene synthesis (Narita and Gruissem, 1989). Four different tomato genes for HMGR have been identified and cloned (Gruissem et al., 1991). HMGR-1 expression and HMGR activity are high in the early stages of fruit development during rapid cell division and probable phytosterol biosynthesis. The expression of HMGR-2 occurs during lycopene synthesis and fruit ripening, but no HMGR activity was measurable. The final stage of fruit ripening was not dependent on HMGR activity but may use preexisting pools of MVA for lycopene biosynthesis (Narita and Gruissem, 1989).

The development of HMGR and rubber transferase activities in guayule plants throughout the growing season shows that the activities of the two enzymes develop together as the plants experience cold temperatures and develop almost simultaneously with the increase in polyisoprene biosynthesis. It would appear that the low activities of HMGR and rubber transferase during the summer months limits the polyisoprene pathway. The high activity of HMGR during June occurs during an active period of seedling growth. The activities of MVA kinase and IPP isomerase are high throughout the growing season and are not stimulated in plants exposed to cold temperatures. The development of the activities of these enzymes is not similar to the developmental pattern of rubber biosynthesis.

The development of HMGR activity in guayule plants from October to December appears to support polyisoprene biosynthesis. In preliminary nucleic acid investigations using HMGR-1 cDNA probe from tomatoes, multiple copies of HMGR genes were demonstrated in guayule DNA. The examination of RNA from guayule stems with the tomato HMGR-1 cDNA probe demonstrated higher levels of HMGR mRNA in plants harvested in June and November. The levels of HMGR mRNA correspond to the high rates of HMGR activity in guayule plants in June during seedling growth and in November during the period of rapid rubber biosynthesis.

In conclusion, rubber biosynthesis in guayule plants occurs during the low temperature of the fall and winter in the

Chihuahuan Desert. The increase in carbon flow through the polyisoprene pathway may in part be accounted for by a stimulation of HMGR mRNA and HMGR and rubber transferase activities.

Received March 12, 1993; accepted June 13, 1993.

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