Rubber Production in Guayule: Determination of Rubber Producing Potential

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ABSTRACT

Optimum conditions for the rapid, efficient, nondestructive determination of rubber producing potential in guayule (Parthenium argentatum) were established. The rubber producing potential may be defined as the ability of the plant material to synthesize rubber from a precursor under specified conditions. To achieve this, stem slices taken from the first 5 centimeters of branches were incubated with $^{14}$Cacetate as precursor in 0.1 molar phosphate buffer (pH 6.5) at 26°C for 16 hours in the light. The $^{14}$C from labeled acetate and acetyl coenzymeA were efficiently incorporated into rubber whereas the $^{14}$C from both mevalonic acid (MVA) and isopentenylypyrophosphate (IPP) were poorly incorporated. Incorporation of 68.6% of the $^{14}$C from labeled IPP into the acetone extractable material suggests that most of the IPP was channeled down the lower terpenoid branch of the polyisoprene biosynthetic pathway. The incorporation of $^{14}$C from labeled acetate into rubber was most efficient at temperatures between 20 and 25°C. The rubber producing potential was also found to be dependent on light intensity. The roots which represent about one-third of the plant biomass not only had the highest rubber producing potential but also contained the highest amount of rubber (7.6%), indicating that the root system could be a major source of rubber. The mature stem bark also had a high rubber content and rubber producing potential, whereas the young stem had a low rubber content and a lower potential for producing rubber. The leaves showed little potential to incorporate labeled acetate into rubber and no more than 0.5% rubber was found in guayule leaves.

As a result of increasing demands for natural rubber there has been a great renewal of interest in the Mexican desert shrub guayule (Parthenium argentatum Gray). This research is aimed primarily at increasing the rubber yields so that the crop may be commercially more viable. The principal repository for rubber is the bark and in earlier programs attention was directed toward improving the bark/wood ratio (1). Emphasis is still placed on increasing the rubber bearing parenchyma tissue (30). The effect of environmental conditions on this and growth generally are receiving attention (17), together with selection and breeding programs (18). A more recent approach however, is the use of bioregulators for chemical stimulation of rubber biosynthesis (11, 24, 29).

To facilitate this research a means of quantitating the rubber producing potential of the test material under different environmental conditions is essential. Several methods have been developed for laboratory analysis and identification of rubber, including chemical extraction (11, 19), gravimetric analysis (12), a low-resolution proton magnetic resonance technique (28), 13C NMR analysis (23), IR spectroscopy of stem cross sections (8) and examination with light and EM (7, 20). It is however often necessary to obtain figures which reflect only the increase in rubber after a particular treatment and not the total rubber content. To obtain this information, using assays for the total rubber content in tissues, the rubber content of the control material must be deducted from the content of the treated material. The rubber content of guayule plants is by no means uniform, so that a large number of plants must be analyzed to give statistical validity to this approach, and plants must be selected to ensure that the rubber content of the control and test material is identical at the initiation of the experiment. To avoid these difficulties the concept of measuring the rubber producing potential by means of a radiochemical assay is suggested.

Acetyl-CoA, the active form of acetate is well established as the principal precursor for isoprenoid biosynthesis. It is well known that [14C]acetate (6, 23) and [14CO2] (22) are incorporated into guayule rubber. This rubber can be extracted efficiently and has been shown to be 90% pure by NMR analysis (22, 23). Since the original observation that acetate is incorporated into rubber (6) many researchers have used this fact to elucidate rubber production in guayule plants (2, 10, 23). It is, however, surprising that no one has as yet attempted to use this information to develop a technique to establish the rubber producing potential of guayule plants. The rubber producing potential is the ability of the plant material to incorporate excess labeled precursor into rubber under specified conditions. A rapid efficient nondestructive radiochemical method to determine rubber producing potential of the plants is developed here. This method will not only facilitate current breeding programs in that the plants potential for producing rubber could be assessed without destroying the plants, but could also lead to a better understanding of rubber biosynthesis, in that the effect of environmental conditions and chemical stimuli on rubber producing potential and thus rubber biosynthesis could be rapidly and efficiently studied on a weekly or even daily basis if necessary using the same plants. It is well established that the rate of rubber production varies throughout the year (13) so that the total rubber content and the rubber producing potential are not synonymous. The main advantage of the radiochemical assay over the other analytical methods is that the same plant can be tested prior to treatment and then repeatedly under specified conditions. This would to a certain extent alleviate the difficulty of selecting plants with the same initial rubber content for an experiment.

MATERIALS AND METHODS

Plant Materials and Chemicals. Thirteen- to 16-month-old guayule plants (Parthenium argentatum Gray) grown in 35 cm pots in the open during the autumn and winter of 1985 were...
used as a source of plant material. [U-\(^{14}\)C]Acetic acid sodium salt (2.15 GBq mmol\(^{-1}\)), [\(^{14}\)C]acetate-CoA (2.14 GBq mmol\(^{-1}\)), [\(^{2-}\)\(^{14}\)C]malonic acid (2.15 GBq mmol\(^{-1}\)) and [\(^{1}\)\(^{14}\)C]isopentenylpyrophosphate (2.1 GBq mmol\(^{-1}\)) were purchased from Amer- 

**Sample Preparation.** All experiments were conducted using the first 5 cm of the young branches collected randomly from the 100 experimental plants used for this study. After removal of the leaves the stem material was cut transversely into 0.5 to 1.0 mm slices. These slices were mixed to increase the randomization of the material and between 1 and 2 g (equal amounts within an experiment) of material were used per replicate. A minimum of 3 replicates were used for each treatment. The sliced plant material was added to 10 ml distilled H\(_2\)O containing the respective radioactive precursor (equimolar amounts of radioactivity within an experiment). The material was incubated at different temperature and light regimes for various times. At the completion of the experiment the reaction was terminated with boiling 80% ethanol and the radioactivity incorporated into the water soluble, acetone soluble and petroleum ether fractions determined.

**Determination of Radioactive Incorporation.** After terminating the reaction with 80% ethanol the tissue slices were subsequently washed three times in boiling 80% ethanol and then dried to constant weight at 50°C (48 h). The dried material was ground in a mill through a 20 mesh screen, placed in a cellulose thimble and the weight of the material to be extracted was recorded. This material was then extracted in a Soxhlet apparatus with distilled H\(_2\)O, acetone and petroleum ether (40-60°C) in succession. The three fractions obtained in this manner were brought to constant volume (10 ml) by flash evaporation. Aliquots (1 ml) of the respective fractions were placed in 8 ml scintillation vials and 4 ml of Beckman Ready-Solve EP added to each. The vials were left in the dark for 12 h whereafter the radioactivity (dpm) was recorded using a Beckman 3800 instrument. The radioactivity recovered in each fraction was subsequently expressed as a percentage of the total radioactivity recovered in the aqueous, acetone and petroleum ether fractions. The Duncan multiple range test procedure, (P = 0.05) which gives a 95% confidence level and F ratios, was used to determine whether the results obtained were statistically significant. A minimum of three replications were used for each treatment.

**Optimization of Assay Parameters.** The purpose of this study was to develop an efficient means of estimating the rubber producing potential of guayule tissue. As the optimum conditions for this assay were unknown, a large number of parameters had to be investigated in succession. A suitable rubber precursor was selected after initial experiments where \([^{1}\text{C}]\text{acetate (21.8 KBq, 2.15 GBq mmol}^{-1}\), \([^{1}\text{C}]\text{acetate-CoA (23.7 KBq, 2.14 GBq mmol}^{-1}\), \([2-^{1}\text{C}]\text{MVA}^{2} (22.0 KBq, 2.15 GBq mmol}^{-1}\), and \([1-\text{C}]\text{IPP (23.3 KBq, 2.1 GBq mmol}^{-1}\) were incubated with 1.5 g plant material (samples in May 1985 from 13-month-old plants) in distilled H\(_2\)O at pH 7.0 for 3 h at 30°C in the dark. This experiment resulted in the selection of \([^{1}\text{C}]\text{acetate as the precursor for subsequent experimentation. To optimize extraction of the incorporated radioactivity 1.6 g plant material sampled in May 1985 from 13-month-old plants was treated with \([^{1}\text{C}]\text{acetate (19.8 KBq, 2.15 GBq mmol}^{-1}\). The treated material was extracted for 8, 16, and 24 h in each solvent, respectively. This indicated that 8 h was sufficient in each case. The optimum pH for the reaction was established by incubating the 1.6 g material sampled in June 1985 from 14-month-old plants with \([^{1}\text{C}]\text{acetate (29.4 KBq, 2.15 GBq mmol}^{-1}\) in distilled H\(_2\)O ranging from 5.5 to 7.8. Subsequent to this a pH of 6.5 was used for further experimentation. The optimum temperature for the reaction was selected after experimenting with a temperature range 5 to 40°C, on 1.3 g material sampled in July 1985 from 15-month-old plants incubated with \([^{1}\text{C}]\text{acetate (20.4 KBq, 2.15 GBq mmol}^{-1}\). In August 1985 1.4 g samples of material from 16-month-old plants treated with \([^{1}\text{C}]\text{acetate (18.8 KBq, 2.15 GBq mmol}^{-1}\) were used to investigate incubation time. The incubation time was selected from a range from 3 to 24 h. A comparison was made between the use of distilled H\(_2\)O (pH 6.5) and 0.1 m phosphate buffer (pH 6.5) as the reaction medium with 1 g material from 16-month-old plants treated in August 1985 with \([^{1}\text{C}]\text{acetate (9.1 KBq, 2.15 GBq mmol}^{-1}\). Finally the effect of light was determined by incubating 1.1 g material sampled in August 1985 from 16-month-old plants with \([^{1}\text{C}]\text{acetate (10.4 KBq, 2.15 GBq mmol}^{-1}\) in bright light (80 W m\(^{-2}\)), dull light (8.5 W m\(^{-2}\)) and in the dark.

**Assay Application.** To assess the potential of the optimum incubation conditions arrived at, different tissues from 16-month-old guayule plants were subjected to analysis. Two g samples from leaves, young stems (first 5 cm of branches), 1-year-old stems (5-15 cm of the branches), bark and root tissue sampled in August 1985 were incubated with \([^{1}\text{C}]\text{acetate (9.7 KBq, 2.15 GBq mmol}^{-1}\) at pH 6.5 in phosphate buffer at 26°C for 16 h in the light. The incorporation of radioactive into the aqueous, acetone and petroleum ether fractions was subsequently established.

The percentage rubber (petroleum ether extractables) for the above tissue types was determined by Soxhlet extraction and expressed on a dry weight basis. The rubber extracted in petroleum ether using this method has been shown to be 90% pure by NMR analysis (22).

**Microscopy.** Various tissues of 16-month-old guayule plants were prepared for light and EM (21).

**RESULTS**

Preliminary studies indicated that tissue taken from the first 5 cm of the branches (young tissue) could be sliced readily and was the most suitable for use in this study and the development of a rapid assay technique. Tissue used for microscopic analysis was taken from interfascicular rays between phloem bundles. In the cells of the young stem tissue representing the current season’s growth (5 cm from the shoot apex) rubber particles were sparse (Fig. 1). In the tissue from an interfascicular ray which had been subjected to one winter of growth (5-15 cm from apex) the rubber particles were more numerous (Fig. 2). The mature bark

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2 Abbreviations: MVA, mevalonic acid; IPP, isopentenylpyrophosphate.
from 16-month-old plants taken 3 cm from ground level contained cells where the rubber was very compactly arranged (Fig. 3). Cells from mature root bark also contained compactly arranged rubber particles (Fig. 4).

Initial experiments with the different precursors indicated that the $^{14}$C from acetate and acetyl-CoA were the most efficiently incorporated into rubber (Fig. 5). Acetyl-CoA was probably hydrolyzed prior to being absorbed by the tissue. For this reason and in order to reduce the cost factor it was decided to use $[^{14}C]$ acetate in all subsequent experiments. The incorporation of the $^{14}$C from MVA and IPP into rubber was unexpectedly low, 1.65% and 3.67%, respectively. As for Acetyl-CoA, IPP was probably hydrolyzed prior to being absorbed by the tissue. Significant however, is the high incorporation of $^{14}$C from IPP into the acetone extractable material which includes the resin.

The pH studies indicated that 6.5 was optimum for the incorporation of $^{14}$C from acetate into rubber in guayule stem slices (Fig. 6). A decrease in pH to 5.5 greatly reduced the incorporation of $^{14}$C by the stem tissue. From the results it would also seem as if a high pH value, in the region of 7.8 could be optimal for resin production.

The results with respect to temperature indicated that optimum incorporation of $^{14}$C from acetate into rubber occurred over a temperature range of 20 to 35°C (Fig. 7). Both higher and lower temperatures significantly reduced rubber production. Resin production was favored at 40°C.

Figure 8 shows that with respect to rubber production best results were obtained if the incubation time was in the vicinity 12 to 24 h. For convenience 16 h was used in all subsequent experiments.

The use of a 0.1 m phosphate buffer indicated that with respect to rubber production it had no advantage over distilled H$_2$O as an incubation medium (Fig. 9). However, incorporation of radioactivity into the acetone fraction was favored with the buffer.

Light intensity had a marked effect on the incorporation of $^{14}$C from acetate into rubber (Fig. 10) but no effect on its incorporation into acetone extractable material. Bright light (80 W m$^{-2}$) gave better results then incubation in the dark.
Analysis of 16-month-old guayule plants indicated that on a dry weight basis the bark and roots had the highest rubber content (Fig. 11). The lowest amount was found in the leaves while the young stems (first 5 cm) and older stems (5–15 cm of the branches) contained intermediary amounts. Using similar materials as was analyzed for total rubber distribution and subjecting it to the assay technique developed it was found that the leaves had no detectable potential to incorporate the $^{14}$C from acetate into rubber (Fig. 12). No significant difference was found between the rubber producing potential of young and older stems. The bark had a high potential. The roots however, incorporated most $^{14}$C into rubber. On a dry weight basis the bark contributed 16.5 ± 0.6% of the total dry mass of the 16-month-old plants, and it contained 6.25 ± 1.24% rubber. The roots contributed 34.7 ± 3.5% of the total dry mass and they contained 7.57 ± 1.4% rubber.

**DISCUSSION**

The results obtained with this study showed that the optimum conditions for the radiochemical assay for rubber producing potential in guayule stem slices are: $[^{14}]$C acetate as precursor in 0.1 M phosphate buffer (pH 6.5) with incubation conditions of 26°C for 16 h in the light. This method is rapid, efficient and the same plant may be tested prior to and repeatedly during experimentation. Previous experiments have indicated that acetate promotes rubber synthesis in guayule (15). The present results confirm that acetate acts as a precursor for rubber biosynthesis.
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Of interest however, is that it contrasts with results obtained using Hevea latex. Despite the presence of all the necessary enzymes and cofactors (27) the conversion of acetate to rubber in this species was very low (3). This was considered to be due to a lack of mitochondria in the tapped latex and a possible metabolic block in the conversion of acetate to acetyl-CoA (3, 9). Within the guayule stem tissue both these requirements were apparently satisfied as the stem slices used contained large numbers of intact cells with the necessary organelles.

The 14C from both MVA and IPP were poorly incorporated into rubber under the specified experimental conditions. These results again differ from those obtained with Hevea latex where substantial amounts of MVA and IPP were incorporated into high mol wt polysoprene (4, 5, 26). A possible explanation for the poor incorporation of IPP is that this molecule does not easily penetrate membranes and is probably hydrolyzed prior to absorption into the tissue. Poor hydrolysis would thus result in poor incorporation. In the case of IPP the guayule stem slices converted 68.8% of the incorporated radioactivity into acetone extractable material which contains the resin and numerous other components such as fats, terpenes and phytosterols (16). Since resin and rubber are synthesized via the same polysoprene biosynthetic pathway, branching at IPP, it appears that most of the 14C from IPP was channeled down the lower terpenoid branch to produce resin. This aspect warrants further attention as our investigations have repeatedly shown that during summer guayule plants produce more resin than rubber. During winter the converse is true, suggesting a switch in the biosynthetic pathway as a result of changing environmental conditions. It is well established that low temperatures favor rubber production (10, 13). It has been suggested that low night temperatures below 7°C increase the expression of the genes coding for the enzymes involved in rubber synthesis (14). Our results obtained with the temperature experiment supports this suggestion. In this study material obtained from plants grown in winter (with night temperatures in the vicinity of 7°C) incorporated most acetate into rubber when incubated in temperatures between 20 and 35°C. These results are in agreement with the report that best rubber production in guayule occurred with 27°C d followed by 7°C nights (14). Similar results were also reported with respect to acetate incorporation into rubber (23). Light was found to be beneficial with respect to the incorporation of 14C from acetate into rubber. Confirming earlier reports that rubber accumulation was dependent on light intensity (14, 18). This aspect needs further investigation but could be related to the fact that guayule stem tissue has the ability to fix 14CO2 and incorporate a portion of it into rubber (22).

Rubber accumulation in different parts of guayule plants...
depends on the age of the tissue, light intensity, moisture availability, temperature, plant morphology and season (18). The results from this study clearly showed that the bark and root tissue had the highest potential for rubber production. This in spite of the fact that a large number of the cells in these plant parts were filled with rubber particles. Provided the necessary substrate requirements, enzymes, cofactors and organelles are present, these plant components could still synthesize a large amount of rubber. The young stems, where the cells are not yet completely filled with rubber particles, had a lower potential for rubber production. Although rubber transferase has been isolated for guayule leaves (25) they showed little potential to incorporate 14C from acetate into rubber. We have not found more than 0.5% rubber in guayule leaves. The roots not only had the highest rubber producing potential but also yielded the highest amount of rubber. This coupled with the fact that the root system represents about one third of the plants biomass indicates that the root system could be a major source of rubber. This will have to be considered in cultivation practices.

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Correction

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Page 1030, in the legends for Figures 6, 7, and 8, water extractables should be represented by ●, and not ○; and petroleum ether extractables represented by ○ and not ●.