CO_2 Fixation and Rubber Deposition in the Bark and Leaves of Guayule during Winter

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Summary

The chloroplasts found in the stem tissue of *Parthenium argentatum* (guayule) which had passed through one winter fixed a considerable amount of CO_2 and a relatively large proportion was incorporated into rubber. In this region of the stem the cells were not completely filled with rubber and new cells were still generated by the vascular and cork cambia. While the cells in the oldest part of the two-year-old plants appeared to be filled to capacity with rubber, ^{14}C was still incorporated into *cis*-polyisoprene. This indicated that despite the presence of copious amounts of rubber some of these cells still had the ability to synthesize rubber. In the parts of the stem produced by the current season's growth most of the ^{14}C was incorporated into the aqueous extract and not into rubber which suggests that it was used for general metabolism. At present it is not known which precursors, if any, are obtained directly from the chloroplasts and are used as substrate for rubber production. This aspect is currently receiving attention.

Key words: Parthenium argentatum, rubber biosynthesis, chloroplasts, $^{14}CO_2$ fixation, ultrastructure.

Introduction

In guayule rubber deposition takes place mainly during winter when the night temperatures are below 7 °C (Bonner, 1943; Goss et al., 1984). The plant is semi-deciduous, and although the majority of the leaves abscise in winter a cluster of small simple leaves remains at the shoot apex. These leaves form the basis of the new seasons growth (Lloyd, 1911). The bulk of the chloroplasts concerned with CO_2 fixation reside in the mesophyll cells of this reduced number of leaves. There is however, also a considerable population of chloroplasts in the three peripheral layers of parenchyma cells of the secondary cortex of the stem, immediately centripetal to the phellogen. Since the reserve carbohydrate, inulin also accumulates during winter (Traub and Slattery, 1946), and does not provide a carbon source for isoprenoid biosynthesis (Bonner and Galston, 1947) photosynthesis can be expected to proceed through the winter to produce materials for these two processes. The extent to which the stem chloroplasts contribute to the reserves of this plant is however, not known. While never addressing stem chloroplasts in particular there are some experiments which indicate that they may play a role in CO_2 fixation. Defoliation of guayule plants in autumn did not completely eliminate rubber-production (Bonner and Galston, 1947),

while field grown plants which were defoliated continuously survived for a period of six months (Benedict, 1949). During this time the rubber content did not decrease.

In this study $^{14}CO_2$ was used to determine the total amount of fixation in the stem attributable to bark chloroplasts and the amount contributed by the leaves. The incorporation of $^{14}CO_2$ derived from both sources into rubber was also estimated.

In order to determine the distribution of the products of photosynthesis through the tissue, experimental material was chosen to represent several stages of maturity with respect to rubber deposition. The radioactive material was extracted in each case and analysed. Representative samples of the tissues were prepared for electron microscopy in order to relate the ultrastructural appearance of the cells involved to the biochemical processes proceeding within them. It is known that rubber deposition in young tissue is cytoplasmic and to a lesser extent vacuolar, while in the mature bark where commercial quantities of rubber are synthesized it occurs in the vacuolar sap only (Backhaus and Walsh, 1983). In these cells the process of deposition can be observed and compared with the observations made by Dickenson (1969) with respect to *Hevea* rubber in the aqueous serum of its latex.

Materials and Methods

Plant material and chemical analysis. Plants of *Parthenium argentatum* (guayule) measuring about 35 cm having winter leaves and numerous subsidiary shoots were cut at the base of the stem from two-year-old plants in June (winter) and placed in a beaker of water. Half of the explants were defoliated and the other half left intact.

The explants were placed on a glass plate and covered with a large bell jar which was sealed with vaseline. A few drops of concentrated sulphuric acid was dropped onto ^{14}C $BaCO_3$ (specific activity 1.91 GBq mmole^{-1}) in a pill vial via a glass tube to release $^{14}CO_2$. The explants were exposed to the radioactive $^{14}CO_2$ for 6 h and were subsequently kept under short days (8 h) and exposed to an irradiance of 30 Wm^{-2}. The day temperature was 24 °C and the night temperature 5 °C. The explants were harvested after 3 days and divided into three parts (Fig. 1). Sample A represented tissue taken at a maximum of 5 cm from the shoot apex which derived from the current season's growth (upper stem). Sample B was taken between 5 and 20 cm from the shoot apex and represented tissue which had been through at least one winter (middle stem). Sample C was taken in the oldest part of the stem 5 cm from ground level and represented tissue which had survived two winters (lower stem). The leaves of the intact plants were also retained for analysis. The material was dried at 60 °C for 24 h and ground to homogenous powders.

The ground material was placed in cellulose thimbles with a wad of glasswool inserted above the samples. Each of the three stem explant portions harvested as well as the leaves was extracted for 8 h using a Soxhlet apparatus with water, acetone and petroleum ether (40–60 °C) in succession. The extracts were evaporated to dryness and made to constant volume (50 ml) using the same respective solvents as before. Three 1 ml sub-samples of these extracts were placed in glass counting vials and bleached with 0.6 ml H_2O_2 at 60 °C for 2 h; 10 ml of Ready Solve EP cocktail was added to the samples which were well shaken and kept in the dark for 12 h. The radioactivity was then recorded using a Beckman LS 3800 scintillation counter.

The remaining petroleum ether samples were taken to dryness for a NMR spectral analysis for rubber. During this process the rubber was separated from the waxes by acetone and CCl_4 partitioning. The rubber and wax samples were subsequently dissolved in petroleum ether and the radioactivity in each solution again recorded.

Microscopy. Transverse sections of leaves and stem sections to represent regions A, B and C on the experimental material were dissected out. Cubes (2 mm^2) were placed in 6 % glutaraldehyde in 0.5 M cacodylate buffer; post fixed in 2 % osmium tetroxide in the same buffer; dehydrated in a graded acetone series and propylene oxide; embeddad in Epon-Araldite resin and polymerized for 48 h at 70 °C. Semi-thin sections (2 μm) were cut with a glass knife on a LKB ultra microtome III and stained with toluidine blue for light microscopy. Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate (Reynolds, 1963) and photographed with a CX 100 Jeol electron microscope.

Results

In this experiment the ^{14}C incorporated into the water soluble, acetone soluble and petroleum ether soluble fractions of intact and defoliated explants was determined. The major objective being to establish the contribution of stem chloroplasts to rubber production. All results and calculations are based on the radioactivity detected in these three extracts. To facilitate comparison between explants and plant components, radioactivity was calculated to represent dpm g^{-1} dry material. Both explants fixed $^{14}CO_2$ during the course of the experiment. The non-defoliated explants contained 86.5 % of the total radioactivity recovered, indicating that a considerable proportion of $^{14}CO_2$ was fixed by the small tufts of leaves present at the shoot apices. The chloroplasts found in the stems did fix $^{14}CO_2$ as defoliated explants contained 14.5 % of the total recovered radioactivity. Despite the fact that less ^{14}C was incorporated by the defoliated explants it can be seen that these explants as a whole were apparently more efficient in incorporating the ^{14}C into the petroleum ether fraction

Table 1: Percentage radioactivity associated with the water, acetone and petroleum ether extracts of the complete intact and defoliated guaylue explants 3 days after exposure to $^{14}CO_2$.

Extract	Intact	Defoliated
Water	76.3 (67.0)*)	71.0
Acetone	19.8 (27.0)*)	19.6
Petroleum ether	3.9 (6.0)*)	9.4

*) Results in brackets indicate percentage radioactivity when the leaves were not included.

Table 2: Percentage radioactivity in the water, acetone and petroleum ether extracts obtained from different parts of intact and defoliated guayle explants which had been exposed to $^{14}CO_2$. The radioactivity for each extract is expressed as a percentage for the plant component analysed.

Plant Material extracted	Intact			Defoliated		
	Water	Acetone	Petroleum *ether*	Water	Acetone	Petroleum *ether*
Leaves	93.3	6.6	0.1	–	–	–
Upper Stem	56.4	41.6	2.0	76.2	22.4	1.4
Middle Stem	78.4	13.6	8.0	61.3	18.5	20.2
Lower Stem	52.3	42.1	5.6	78.6	18.0	3.4

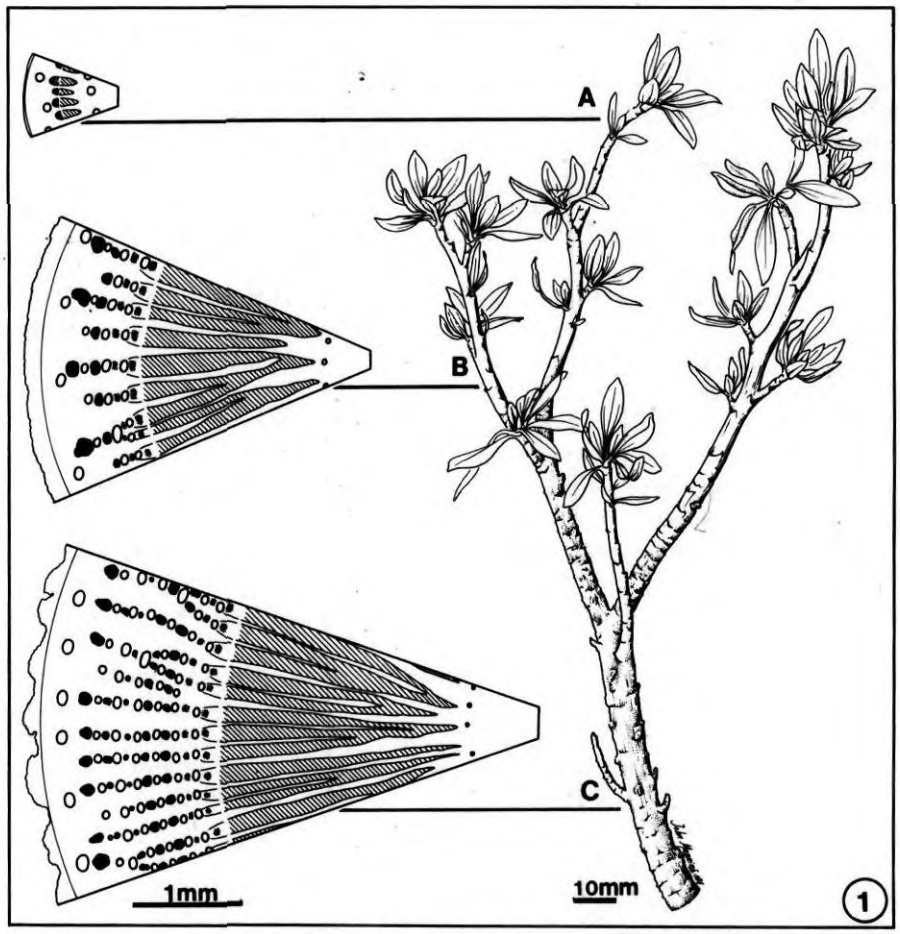


Fig. 1: Line drawing of a winter shoot of guayule showing regions A, B C used for chemical and ultrastructural investigations. The regions are accompanied by plan diagrams representing their anatomy. In the plan drawings hatching represents xylem; stippling = phloem; and cross hatching = schlerenchyma and lignified non-functional phloem. Open circular areas represent resin canals. A (upper stem) indicates tissue of the current year's growth, B (middle stem) tissue which has passed through one winter, C (lower stem) old tissue at the base of the stem which has passed through two winters.

(Table 1). When the leaves of the non-defoliated explants were omitted from the calculations it is clear that percentage wise more ^{14}C was incorporated into the rubber extracts. The increase being due to the elimination of the diluting effect of the leaves which contained a considerable proportion (93.3 %) of radioactivity in the water extract. The results in Table 2 indicate that only 0.1 % of the fixed $^{14}CO_2$ ended up in

the petroleum ether component of the leaves. Irrespective of the plant component analysed most radioactivity always occurred in the water extracts. In both the intact and defoliated explants low levels of the fixed ^{14}C was recovered from the petroleum ether extracts obtained from the upper stem material. The highest degree of ^{14}C incorporation into rubber occurred in the middle portions of the stem of the explants. The presence of ^{14}C labelled rubber in the petroleum ether extracts was confirmed by NMR analysis. After the removal of waxes and other impurities from the petroleum ether extracts by means of CCl_4 partitioning 91.8 % of the original radioactivity present in this extract was recovered in the rubber component. This component gave a NMR spectrum which indicated that it contained over 90 % *cis*-polyisoprene.

The microscopic and ultrastructural data obtained for the different regions of the explants analysed correlate well with the biochemical data obtained. The stem tissue represented in region A (Fig. 1) has been described several times (Lloyd, 1911; Artschwager, 1943; Gilliland and Van Staden, 1983). In the winter tissue analysed in this experiment the resin content was high (± 10 %) causing the resin canals to be distended. Rubber particles occurred in all the ground parenchyma cells both in the vacuole and the cytoplasm. These cells were however, not filled to capacity. In this part of the stem about three layers of suberized phellem had developed. Immediately centripetal to the phellogen the parenchyma cells contained chloroplasts which were usually slightly larger than leaf chloroplasts (Figs. 2 and 3). Occasional chloroplasts occurred throughout the ground parenchyma.

In the intermediate region labelled B (Fig. 1), about 12 cm from the shoot apex, considerable secondary development had taken place as the tissue was about 4 months older. There were 3 or 4 concentric rings of resin canals alternating with strands of phloem and sclerenchyma respectively. All but the most recently formed sieve tubes were lignified and apparently non-functional since the companion cells had collapsed (Figs. 4 and 5). The epithelial cells of the resin canals contained large irregular masses of rubber in the cytoplasm (Fig. 6). Most of the ground parenchyma cells had spherical rubber particles ranging in size from 0.1 μm to 1.5 μm floating in the vacuolar sap and no cytoplasmic rubber. They were always surrounded by an osmophilic film which was sometimes diffuse (Backhaus and Walsh, 1983) (Fig. 7). Some cells contained many tiny rubber particles (Fig. 8). There were approximately 30 chloroplasts per cell in the outer layers of the cortex and occasional chloroplasts throughout the ground parenchyma. These cells all had a thin peripheral layer of cytoplasm with mitochondria and E. R. (Fig. 9). A few chloroplasts had prolamellar bodies indicating a lack of light (Fig. 10). The stem at this stage had a deep layer of phellem (Fig. 1 B). The width of the bark was ± 1 mm and the diameter of the stem ± 6 mm. All early workers emphasised the fact that the amount of rubber produced is a function of the wood : bark ratio (Addicott and Pankhurst, 1944; Bonner and Galston, 1947). In region C (Fig. 1), about 30 cm from the shoot apex, the wood : bark ratio was the same as that in region B, the diameter being 9 mm and the bark width 1.5 mm. This tissue was by far the most mature with respect to rubber storage, particularly the cells in

close proximity with the lignified phloem. In these cells although they appeared viable the rubber particles in the vacuole were so tightly packed together that they had lost their spherical shape and some had coalesced (Fig. 11). Sometimes they were so compressed that the cell wall appeared to have ruptured (Fig. 12). Rubber does not pass through plasmodesmata. Rubber deposition, however, still seemed to be proceeding in the peripheral layers of the cortex where there were a large number of chloroplasts and where ^{14}C was incorporated into the petroleum ether fraction which contains rubber (Table 2). Plastoglobuli were common in chloroplasts associated with rubber accumulation and starch grains rare (Fig. 13).

Discussion

The radiochemical experiments demonstrated that bark chloroplasts are capable of making a contribution to CO_2 fixation, and that some of the products of this fixation are incorporated into *cis*-polyisoprene and isoprenoid compounds. In an experiment lasting three days the amount of rubber produced can be expected to be very small. Goss et al. (1984) recorded amounts of rubber produced naturally by plants subjected to low temperatures over a period of 182 days. The increase was about 1 % in 30 days, so that amounts in the order of 0.1 % could be expected in three days. For this reason it was considered more practical to express rubber production as a percentage of the total radioactivity recovered in the water, acetone and petroleum ether soluble fractions, particularly as most of the radioactivity (over 90 %) associated with the petroleum ether fraction was *cis*-polyisoprene.

In spite of the contribution made by the bark chloroplasts, however, the leaves remain the principal source of photosynthates, which are translocated to the stems and roots as water soluble compounds. In this experiment the contribution of the leaves was estimated to be in the order of 86.5 %. The high value is attributable to the superior numbers of chloroplasts in the leaves. The mesophyll cells which are all palisade mesophyll cells and very compactly arranged contain about 60 chloroplasts per cell whereas the peripheral bark cells contain only about 30 chloroplasts per cell.

Fig. 2: Chloroplast (CL) from a peripheral parenchyma cell in the stem cortex in the middle region (B).

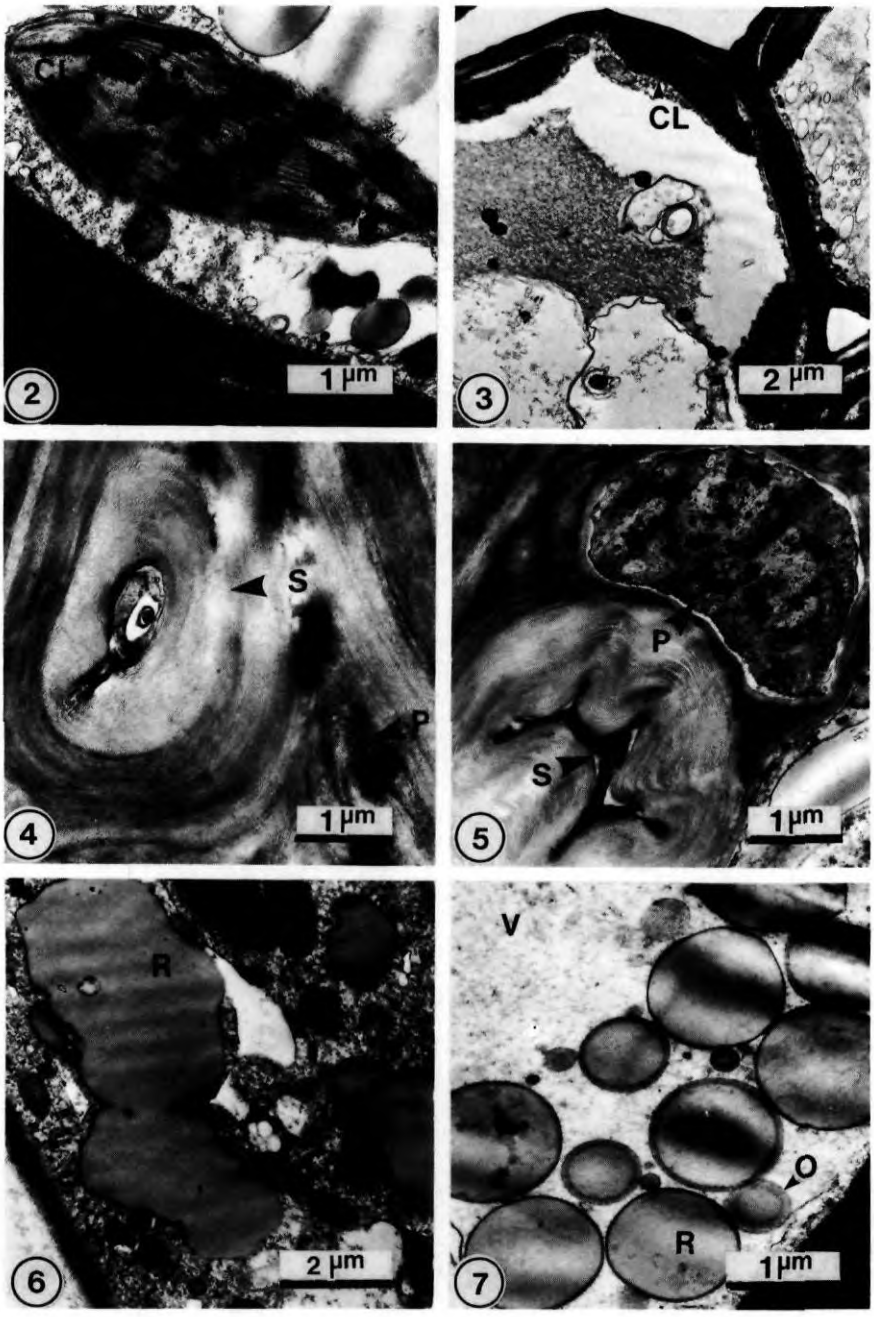
Fig. 3: Part of a mesophyll cell from a winter leaf showing chloroplasts (CL).

Fig. 4: Lignified non-functional sieve tube (S) with a collapsed companion cell (P) from the lower region (C) of the stem.

Fig. 5: Apparently functional sieve tube (S) with nacreous thickening and companion cell (P) with viable cytoplasm from the middle region (B) of the stem.

Fig. 6: Irregularly shaped rubber particles (R) in the cytoplasm of an epithelial cell from a resin canal in the upper region (A) of the stem.

Fig. 7: Spherical rubber particles (R) in vacuolar sap (V) of a parenchyma cell in the middle stem (B). Rubber particles are surrounded by an osmophilic layer (O). The protoplast is very thin but appears partially functional, occasionally it is disorganised.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The large amount of $^{14}CO_2$ incorporated into water soluble substances must include materials for general growth and metabolism as well as precursors for carbohydrate reserves, notably inulin (Traub and Slattery, 1946). This fraction must also include the early precursors for all the isoprenoid compounds synthesised in the plant. The resin which is a mixture of terpenes and terpenoids (Benedict, 1984) has the same precursors as rubber up to the stage of isopentenylpyrophosphate (IPP) production. The best documented precursor of IPP is acetate which occurs in the cells as acetyl-CoA (Arreguin et al., 1951; Rabinowitz and Teas, 1961; Benedict et al., 1983).

The most obvious source of acetyl-CoA would be glycolysis but all attempts to incorporate fructose, glucose and sucrose into rubber have proved unsatisfactory (Teas and Bandurski, 1956; Goss et al., 1984). This directs attention to other possible sources of early precursors. Recent work has shown that the products of photosynthesis include a number of low molecular weight compounds notably glycollic acid (Goodwin and Mercer, 1972) whose main role is in the formation of glycine and serine in association with peroxisomes. Serine is a source of acetyl-CoA (Lehninger, 1975). In this way it would be possible for the chloroplasts to contribute by a very direct route to producing rubber precursors. Peroxisomes are frequent in the tissue. In any event precursors must be present in the aqueous fraction.

The acetone extracts can be expected to contain a variety of compounds which make up the resinous component of the tissue. These include α-pinene, dipentene, cadiene, carotenoids, partheniols, linolenic acid and *trans*-cinnamic acid (Belmares and Jimenez, 1978) most of which are derived from isoprenoid precursors. In our plants 10% resin is generally recorded in winter. Since this is a fluid and the resin canal system is interconnected, movement of resin can be expected equalising its distribution throughout the plant. Relatively high amounts of ^{14}C were detected in the acetone fraction in all regions of the stem.

The quantities of ^{14}C associated with the rubber (petroleum ether) fractions varied with respect to the plant region analysed. This variation could however, be most readily accounted for by the anatomy and ultrastructure of the stem component

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Fig. 8: Part of a parenchyma cell from the stem cortex in the middle region (B) showing numerous minute rubber particles (R).

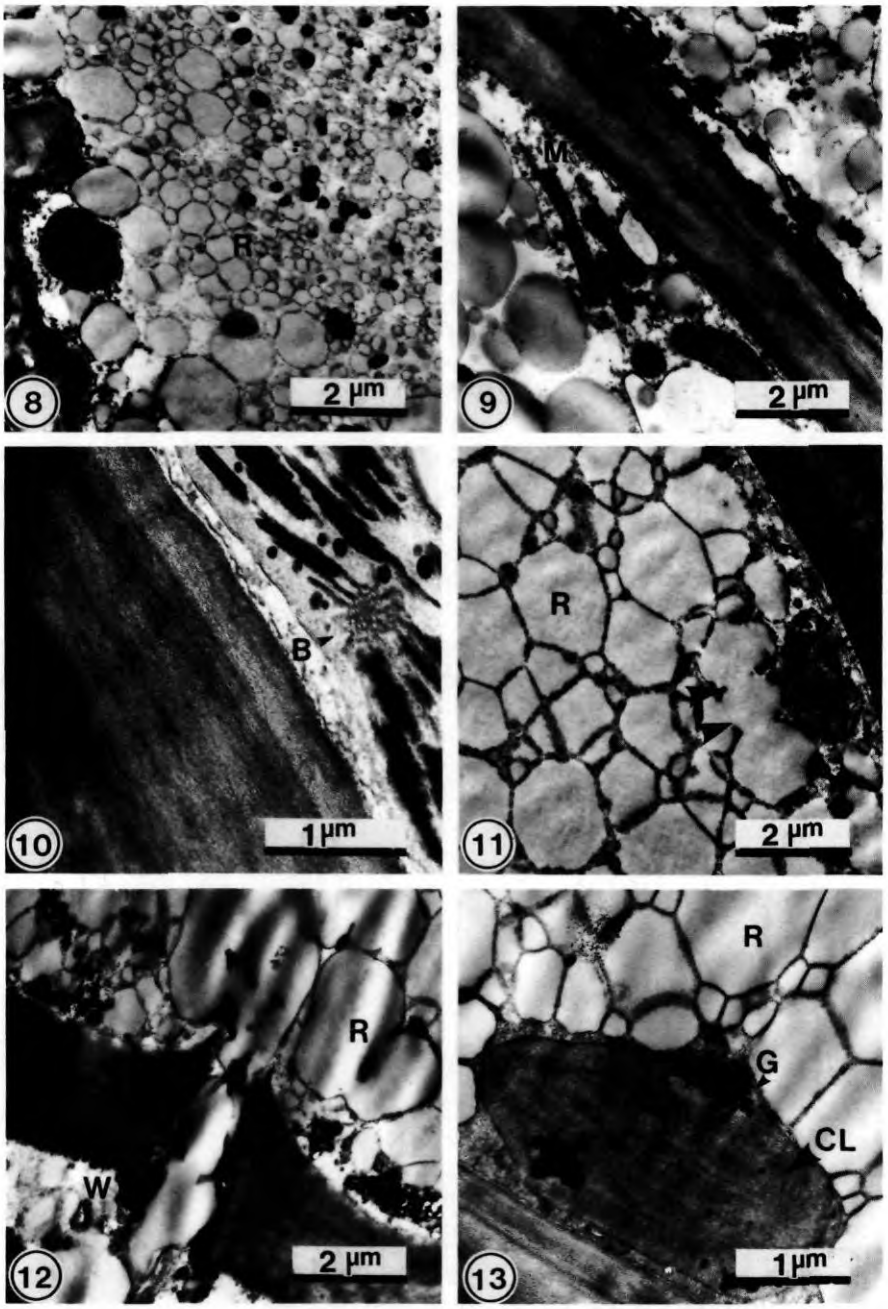
Fig. 9: Mitochondria (M) in the peripheral layer of cytoplasm from a parenchyma cell in the stem cortex in the middle region (B).

Fig. 10: Chloroplast with prolamellar body (B) in the cytoplasm of a parenchyma cell in an interfascicular ray in the lower region (C) of the stem.

Fig. 11: Tightly packed rubber particles (R) from a parenchyma cell in the stem cortex in the lower region (C) of the stem. Note coalescence of rubber particles (arrows).

Fig. 12: Cell in the stem cortex in the lower region (C) where rubber (R) is passing through the cell wall (W).

Fig. 13: Chloroplast (CL) with plastoglobuli (G) in a cell completely filled with rubber (R) in a cortical cell in the lower region (C) of the stem.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11
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W
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12
R
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1 µm
13

concerned. In region A (Fig. 1) the amount of ^{14}C incorporated was small which is consistent with the small assay figure for rubber recorded in young tissue, usually less than 2 %. This rubber is mainly cytoplasmic. The leaves contain only 0.5 % rubber although they incorporated a large amount of ^{14}C into components extracted with water.

In region B (Fig. 1), there are large parenchyma cells in the cortex and the interfascicular rays with a peripheral layer of cytoplasm. Archer and Audley (1973) showed that the extension of rubber molecules suspended in an aqueous medium in *Hevea* latex takes place at the surface of existing rubber particles. The same process appears to be taking place in these cells. In some cases the existence of a starter molecule dimethylallylpyrophosphate as suggested by Lynen (1969) is indicated by the occurrence of many tiny rubber particles.

Region C (Fig. 1) of the stem showed lower incorporation of ^{14}C into rubber than region B. The cells in this part of the stem are the oldest and the majority of them are already filled with rubber having been through two winters. This is known to be the major region for rubber accumulation with time. Analysis of the bark of 18 month old plants removed near the base showed that it contained 8 % rubber. In three-year-old plants this value had risen to 14 %. New rubber deposition can only be expected in the new cells produced by the cork and vascular cambia. These cells are very similar in appearance to the stem parenchyma cells found in region B. The old cells in the interfascicular rays, however, still appeared viable in spite of their numerous highly compressed rubber particles. Mitochondria, nuclei and chloroplasts were still intact and numerous in the thin layer of cytoplasm lining the cell walls. There were many ribosomes and the cytoplasm was not disorganised. The presence of mitochondria as sources of ATP required in several steps in the biosynthetic pathway to rubber is very important.

Whether the acetyl-CoA required for isoprenoid biosynthesis is obtained by a direct route from chloroplasts or not, these organelles do appear to be very closely associated with its biosynthesis. The chloroplasts in the peripheral layers of cells in the bark are particularly well placed for this purpose.

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References

Addicott, F. T. and J. B. Pankhurst: Some anatomical effects of moisture stress in nursery seedlings of guayule. Bot. Gaz. *106*, 208–214 (1944).

Archer, B. L. and B. G. Audley: Rubber, gutta percha and chicle. In: Miller, L. P. (ed.): Phytochemistry, Vol. 2, pp. 310–343. Van Nostrand-Reinhold, New York (1973).

Arreguin, B., J. Bonner, and B. J. Wood: Studies on the mechanism of rubber formation in guayule. III. Experiments with isotopic carbon. Arch. Biochem. Biophys. *31*, 234–247 (1951).

ARTSCHWAGER, E.: Contribution to the morphology and anatomy of guayule (*Parthenium argentatum*). USDA Tech. Bull. no. 842: 1–33 (1943).

BACKHAUS, R. A. and S. WALSH: The ontogeny of rubber formation in guayule, *Parthenium argentatum* Gray. Bot. Gaz. *144*, 391–400 (1983).

BELMARES, H. and L. L. JIMÉNEZ: Resina de Guayule: Characterizacion y Possible Usas Industriales. In: CAMPOS-LÒPEZ, E. and W. MCGINNIS (Eds.): Guayule. Reencuentro en el Desierto, pp. 315. Consejo Nacional de Cienca y Technologia, Mexico City (1978).

BENEDICT, H. M.: A further study on the non-utilization of rubber as a food reserve by guayule. Bot. Gaz. *111*, 36–43 (1949).

BENEDICT, C. R.: Biosynthesis of rubber. In: PORTER, J. W. and S. L. SPURGEON (Eds.): Biosynthesis of isoprenoid compounds. pp. 357–369. John Wiley, New York (1984).

BENEDICT, C. R., P. H. REIBACH, S. MADHAVAN, R. V. STIPANOVIC, J. H. KEITHLY, and H. YOKOYAMA: Effect of 2-(3,4-dichlorophenoxy)-triethylamine on the synthesis of cis-polyisoprene in guayule plants. (*Parthenium argentatum* Gray). Plant Physiol. *72*, 897–899 (1983).

BONNER, J.: Effects of temperature on rubber accumulation by the guayule plant. Bot. Gaz. *105*, 223–243 (1943).

BONNER, J. and A. W. GALSTON: The physiology and biochemistry of rubber formation in plants. Bot. Rev. *13*, 543–596 (1947).

DICKENSON, P. B.: Electron microscopical studies of latex vessel system of *Hevea brasiliensis*. J. Rubber Res. Inst. Malaya. *21*, 543–559 (1969).

GILLILAND, M. G. and J. VAN STADEN: Detection of rubber in guayule (*Parthenium argentatum* Gray) at the ultrastructural level. Z. Pflanzenphysiol. *110*, 285–291 (1983).

GOODWIN, T. W. and E. I. MERCER: Introduction to Plant Biochemistry. Permagon Press, Oxford (1972).

GOSS, R. A., C. R. BENEDICT, J. H. KEITHLY, C. L. NESSLER, and R. D. STIPANOVIC: *cis*-Polyisoprene synthesis in guayule plants (*Parthenium argentatum* Gray) exposed to low, nonfreezing temperature. Plant Physiol. *74*, 534–537 (1984).

LEHNINGER, A. L.: Biochemistry, 2nd Ed. Worth Publishers, New York (1975).

LLOYD, F. E.: Guayule (*Parthenium argentatum* (Gray): A rubber plant of the Chihuahuan desert. Carnegie Institute Washington, D.C. Publ. No. 139 (1911).

LYNEN, F.: Biochemical problems of rubber synthesis. J. Rubber Res. Inst. Malaya. *21*, 389–406 (1969).

RABINOWITZ, J. L. and H. J. TEAS: Proceedings of the National Rubber Research Conference, Kuala Lampur, 1960, Rubber Research Institute of Malaya, pp. 77. Kuala Lampur (1961).

REYNOLDS, E. S.: Use of lead citrate at high pH as an electron opaque stain. J. Cell Biol. *17*, 208–212 (1963).

TEAS, H. J. and R. S. BANDURSKI: The enzymatic synthesis of rubber. J. Amer. Chem. Soc. *78*, 3549–3550 (1956).

TRAUB, H. P. and M. C. SLATTERY: Analysis of levulins, inulin and monosaccharides in guayule. Bot. Gaz. *108*, 295–299 (1946).