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DECOMPOSITION OF 2-CHLOROETHYLPHOSPHONIC ACID IN STEMS AND LEAVES OF *HEVEA BRASILIENSIS*

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; 2-chloroethylphosphonic acid; 2-hydroxyethylphosphonic acid; ethylene; plant growth regulator.

Abstract—The plant growth regulator 2-chloroethylphosphonic acid is converted to at least 13 compounds other than ethylene in leaf and stem tissue of *Hevea brasiliensis*. A minor component of the products is probably 2-hydroxyethylphosphonic acid.

INTRODUCTION

2-CHLOROETHYLPHOSPHONIC ACID (2-CEPA) is now well known as a plant growth regulator.[1] Its effects on plants include induction of flowering,[2] maturation and abscission of fruit and leaves,[3,4] seed germination[5] and rubber latex yield-stimulation.[6] In all cases its biological activity has been ascribed to the ethylene formed when it decomposes in aqueous solution above pH 3·5.[2,7] The few studies which have been published on the decomposition of 2-CEPA in plant tissue indicate that the compound is poorly metabolized. Weaver *et al.*[8] could not detect any metabolites when 2-CEPA-^{14}C was applied to berries of grape (*Vitis vinivinifera*) and Martin *et al.*[9] obtained similar results in experiments with walnut (*Juglans regia*). Yamaguchi *et al.*[10] treated tomato (*Lycopersicon esculentum*) and summer squash plants (*Cucurbita pepo*) with 2-CEPA-^{14}C. With tomato, all the radioactivity extracted from the tissues after incubation for 7 days had the same R_f as 2-CEPA on PCs. However, in the case of squash, an unidentified radioactive compound was detected in leaves and fruit; $^{14}CO_2$ production was less than 0·2% of the applied radioactivity. Edgerton and Hatch[11] studied the metabolism of 2-CEPA-^{14}C in apples and cherries (*Prunus avium* and *P. cerasus*). Only 2-CEPA was detected in the fruits, but about 5% of the applied radioactivity appeared in an unknown metabolite in cherry leaves. This paper reports experiments on the decomposition of 2-CEPA-^{14}C in vegetative tissue of *Hevea brasiliensis*, which show that up to 39% of the 2-CEPA applied may be converted into a range of non-volatile products.

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RESULTS AND DISCUSSION

Preliminary experiments showed that satisfactory TLCs could not be obtained using extracts unless an initial separation on an anion-exchange chromatography column was carried out. The elution diagrams obtained with this procedure are given in Fig. 1. Virtually all the water-soluble radioactive compounds present were anionic at pH 5·5, since less than 2% of the radioactivity applied to the columns was washed off with water. It was shown by TLC that fractions 4 and 5 from the stem extract and 6 and 7 from the leaf extract all contained 2-CEPA-^{14}C and only traces of other radioactive materials. This splitting of 2-CEPA into two peaks was not unexpected since it had been observed previously that radiochemi-

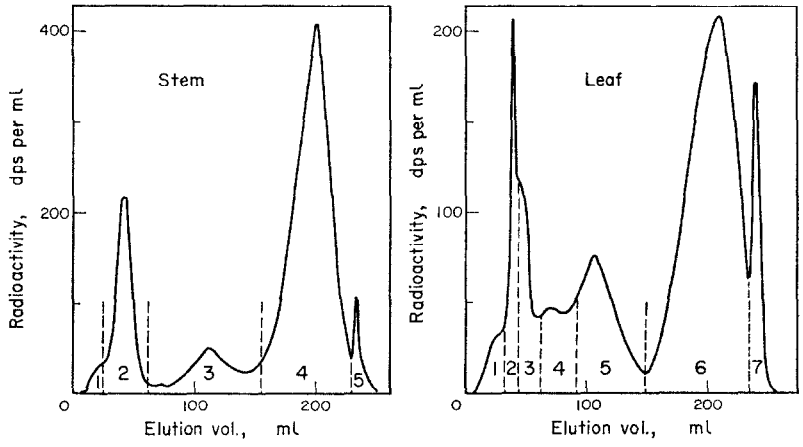

FIG. 1. CHROMATOGRAPHY OF EXTRACTS OF *Hevea brasiliensis* STEM AND LEAF TISSUE AFTER TREATMENT WITH 2-CEPA-^{14}C FOR 72 HR.

One leaf and the stem segment were extracted with 30 ml and 60 ml respectively of 0·02 M 2-CEPA (see text). Samples of the extracts (leaf 19·5 ml, 20 400 dps; stem 27·5 ml, 27 800 dps) were adjusted to pH 5·5 with NaOH and applied to columns (30 × 1·1 cm) of Bio-Rad AG1-X10 chloride. The columns were washed with water (30 ml) and the compounds eluted with 225 ml of 0·05 M LiCl followed by 45 ml of 0·5 M LiCl; 3 ml fractions were collected. The recoveries of radioactivity from the columns were 93% (leaf) and 90% (stem). The numbers under the curves refer to the fractions used for 2-D TLC. The amount of 2-CEPA-^{14}C applied to the leaf was 114 000 dps and to the stem segment, 277 500 dps.

cally pure 2-CEPA-^{14}C can be resolved into a major and a minor peak when chromatographed on the anion-exchange resin, and that the proportion of the 2-CEPA eluted with 0·5 M LiCl in the second (minor) peak decreased as the load of 2-CEPA applied to the column was increased. The appearance of two peaks on the column chromatogram of 2-CEPA-^{14}C was not due to the presence of a labelled impurity, since the eluted materials co-chromatographed with authentic 2-CEPA in the four solvent systems given below, and on heating in alkali, 96% of the radioactivity of each peak was converted to ethylene. 2-D-TLC of fractions 1–3 (stem) and 1–5 (leaf) showed that each fraction contained many labelled compounds. In all, at least 13 compounds were detected from the leaves and 20 from the stem. Many of the spots visible on autoradiographs of the fractions from the leaf did not correspond with those from the stem. Fraction 2 from both leaf and stem contained the largest number of labelled compounds. One of the minor components of this fraction was tentatively identified as 2-hydroxyethylphosphonic acid. This compound is formed in small amounts (3–4%) over a wide range of pH values when 2-CEPA is incubated for several days

at room temperature in buffer solutions (Audley and Archer, to be published). The total amounts of radioactivity in non-volatile, water-soluble compounds separable from 2-CEPA by anion-exchange chromatography, expressed as percentages of the radioactivity applied to the tissues, were 10% (leaf) and 5% (stem). No $^{14}CO_2$ was detected in the experiment with stem segments. Smaller samples of extracts of six more leaves which had been incubated with 2-CEPA-^{14}C were chromatographed on 20 × 0·5 cm columns of anion-exchange resin. The elution diagrams were very similar to those shown in Fig. 1. Table 1 gives the amount of applied 2-CEPA-^{14}C converted by the leaves to products resolvable from 2-CEPA by anion-exchange chromatography. In one leaf as much as 39% of the 2-CEPA applied was converted to such products. This corresponds to 47% of the 2-CEPA decomposed. Non-enzymic formation of the labelled compounds is most unlikely, since very little 2-CEPA-^{14}C was converted to labelled, involatile, water-soluble compounds when incubated with a steamed leaf, boiled leaf extract, or a suspension of disrupted leaf tissue (Table 1). The small amount of radioactive material produced in incubations *b*–*e* (Table 1) can be largely accounted for by the formation of 2-hydroxyethylphosphonic acid as described above.

TABLE 1. FORMATION OF WATER-SOLUBLE RADIOACTIVE COMPOUNDS IN LEAVES AND LEAF EXTRACTS OF *Hevea brasiliensis* INCUBATED WITH 2-CEPA-^{14}C FOR 72 HR

Material incubated	Total radioactivity extracted after incubation (dps)	Total radioactivity in fractions separated from 2-CEPA by anion-exchange chromatography as % of 2-CEPA-^{14}C incubated
a Leaf	42 100 ± 16 700*	19 ± 10*
b Steamed leaf	61 800	2·2
c Leaf homogenate	46 500	3·1
d Boiled leaf homogenate	34 400	1·6
e Boiled concentrated leaf extract	50 200	1·6
f Unincubated 2-CEPA-^{14}C	—	0·25†

* Mean value (± s.d.) of six incubations.

† As % of 2-CEPA-^{14}C chromatographed.

Each leaf was extracted with 30 ml of 0·02 M 2-CEPA (see text). Mixtures *c*, *d* and *e* were adjusted to pH 3 with HCl after incubation, centrifuged, and each supernatant made up to 25 ml with 0·02 M 2-CEPA. After counting, samples of the extracts (2–5 ml) were adjusted to pH 5·5 with NaOH and applied to columns (20 × 0·5 cm) of Bio-Rad AG1–X10 chloride* the columns washed with water (10 ml) and the compounds eluted with 50 ml of 0·05 M LiCl followed by 10 ml of 0·5 M LiCl; 1 ml fractions were collected and used wholly for determination of radioactivity. Recovery of radioactivity from the columns was 88–108 %. The amount of 2-CEPA-^{14}C used in each incubation was 114 000 dps.

The results of these experiments suggest that 2-CEPA is metabolized by *Hevea brasiliensis* tissue to a significant extent, but it is also possible that the compounds formed may have

**Note added in proof.* Later experiments have shown that new batches of Bio-Rad AG1 anion-exchange resin (X2, X8 or X10) give inferior separations to those reported above. However, good resolution of 2-CEPA and its metabolites has recently been obtained using columns of QAE-Sephadex A-25 (Cl) with 0·07 M imidazole–0·05 M HCl buffer, pH 6·6 as eluant.

arisen in part from the metabolism of ethylene released by the non-enzymic decomposition of 2-CEPA. However, it is unlikely that most of the compounds observed were formed from ethylene. since work carried out with a variety of plant tissues indicates that ethylene is poorly metabolized.[12]

EXPERIMENTAL

2-*CEPA*-1-2-^{14}C was prepared by a modification of the method of Maynard and Swan (1963) and purified by the ion-exchange chromatographic method used to analyse the tissue extracts (see below). The product was further purified by TLC on cellulose in solvent (*a*). The 2-CEPA-^{14}C band (R_F, 0·7) was eluted with MeOH. The radiochemical purity as determined by TLC in solvents (*a*) to (*d*) was greater than 99% (sp. act. 2·2 mCi/mmol). The aqueous solutions of 2-CEPA-^{14}C were adjusted to pH 6–7 with NaOH immediately before use. Unlabelled 2-CEPA was obtained from Amchem Products Inc., Ambler, Penna., U.S.A.

Measurement of radioactivity. Scintillation counting was used with a mixture of 0·9% 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)—1,3,4-oxadiazole (butyl-PBD) in toluene, and Triton X100 (2:1, v/v) as the phosphor. ^{14}C-Ethylene was estimated by absorbing samples mixed with 1 ml of carrier ethylene in vials containing 2 ml of 0·2 M mercuric acetate in 4% HOAc in MeOH, followed by scintillation counting in 0·6% butyl-PBD in toluene. Counting efficiencies varied from 65 to 75%.

Incubation of tissues and extracts with 2-*CEPA*-^{14}*C*. Mature leaves, (about 3 g each), and pieces of green stem were obtained from 2-year-old seedlings of *Hevea brasiliensis* grown in a greenhouse. To avoid blockage of xylem vessels by latex exuded from the cut petiole, each leaf was placed under H_2O immediately after its removal from the plant; a band of bark about 1 cm wide was scraped from the petiole at a distance of about 3 cm from the cut end and the exposed wood was then cut through diagonally. The leaves were placed upright in a current of air to increase transpiration and a solution of 2-CEPA-^{14}C (3·1 μCi) in 0·25 ml H_2O fed to each leaf via the petiole. At no time were the ends of the petioles allowed to become dry. Quantitative uptake of the radioactive solutions was obtained in about 3 hr. One leaf was then steamed for 3 min and incubated in a H_2O-saturated atmosphere. The other leaves were incubated with the cut-ends of their petioles under H_2O. A homogenate was prepared by disintegrating a single untreated leaf in 20 ml of 0·05 M sodium phosphate buffer, pH 7·0, for 40 sec at full speed in a Whillems Polytron Type 20 homogenizer (Northern Media Supply Ltd.), and a portion of the suspension was boiled for 3 min. A more concentrated solution was obtained by similarly homogenizing three leaves in 60 ml H_2O, centrifuging, and freeze-drying the supernatant. The residue was dissolved in 12 ml of 0·05 M sodium phosphate buffer, pH 7·0, and a portion boiled for 3 min. 3-ml portions of the boiled and unboiled homogenates and of the concentrated extract were each incubated with 2-CEPA-^{14}C (3·1 μCi). For the experiments with stem, the bark of a piece 20 cm long and 7 mm dia. was lightly scraped with a scalpel to remove the cuticle and 2-CEPA-^{14}C (7·5 μCi) applied uniformly over the scraped surface. The stem segment was incubated in a stoppered tube over 0·02 M NaOH to trap $^{14}CO_2$ and the air in the tube was replaced every 24 hr. All incubations were carried out at 25° for 72 hr under continuous illumination (4500 lx).

Chromatography. After incubation, the tissues were homogenized for 40 sec in 0·02 M unlabelled 2-CEPA in the Whillems homogenizer, the suspensions centrifuged, and samples of the supernatants counted. Portions of the extracts were then chromatographed on columns of Bio-Rad AG1-X10 anion-exchange resin (Cl^- form) using LiCl as eluant (see Fig. 1 and Table 1). Fractions from the columns required for TLC (Fig. 1) were passed down columns (4 × 1 cm) of Bio-Rad AG 50 W-X12 cation-exchange resin (H^+ form) and the eluates freeze-dried. The residues were dissolved in 80% EtOH and one spot (10 μl, 200 dps) applied to each TLC plate.

TLC was carried out on cellulose MN 300 with solvent (a), *n*-BuOH–HOAc–H_2O (12:3:5) or (b) *iso*-butyric acid –NH_3–H_2O (22:1:10). Two-dimensional chromatograms (20 × 20 cm) were run first in 72% (w/w) phenol in H_2O (solvent c) and then in HOPr–*n*–BuOH–H_2O (12:26:17) (solvent d). For autoradiography, the plates were wrapped in Melanex film and kept in contact with Kodirex X-ray film for 2 weeks.

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