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STRUCTURE AND PROPERTIES OF A 2-CHLOROETHYLPHOSPHONIC ACID (ETHEPHON) METABOLITE FROM *HEVEA BRASILIENSIS* BARK

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; rubber; metabolism; 2-chloroethylphosphonic acid; Ethephon; ethylene; yield-stimulation.

Abstract—The main metabolite formed on incubating 2-chloroethylphosphonic acid (2-CEPA) with excised bark from *Hevea brasiliensis* is a conjugate which has been identified as β-D-glucopyranose-1-(2-chloroethyl)phosphonate. The compound decomposes readily in acid to 2-CEPA and glucose, and is converted partially to ethylene in hot alkali. On incubation with *Hevea* leaf or bark, extensive conversion to ethylene occurs, probably via the enzymic formation of 2-CEPA. The enzymes emulsin and acid phosphatase also catalyse the decomposition of the conjugate to 2-CEPA and hence ethylene.

INTRODUCTION

2-Chloroethylphosphonic acid (2-CEPA, Ethephon) is now widely used to regulate many physiological processes in plants [1]: in *Hevea brasiliensis* it is applied to the bark or tapping cut to stimulate latex flow and thus increase rubber yield [2]. The acid itself is stable, but the di-anion readily breaks down to ethylene [3–6], and it is this substance which is the actual regulatory compound [7–10]. The metabolism of 2-CEPA has been examined in several species [11–23], but metabolites have been found in only a few [11, 12, 14, 19, 21, 23]. In almost all cases, the extent of metabolism has been small, only single metabolites have been detected, and none has been identified. However, in experiments with *H. brasiliensis*, the results have been quite different. Young stem and leaves from this plant transform 2-CEPA extensively to a number of compounds, one of which has been identified as 2-hydroxyethylphosphonic acid (2-HEPA), and another as, probably, a conjugate of 2-CEPA with an unidentified plant substance [14, 21]. In this paper, experiments on the metabolism of 2-CEPA in bark excised from mature *Hevea* are described, and the isolation, identification and properties of the major metabolite, reported. The relevance of the results to the findings with other species and to the action of 2-CEPA as a yield stimulant, is discussed.

RESULTS

Metabolism of 2-CEPA-[^{14}C] *in* Hevea *bark* in vitro

In the initial experiment on the metabolism of 2-CEPA in mature bark a single metabolite was detected. After a short lag period, this accumulated in a linear fashion for up to 72 hr incubation and was not formed in bark which had been steamed. The compound eluted earlier than 2-CEPA from an anion-exchanger and was acid-labile [21]. In later experiments, small additional early peaks

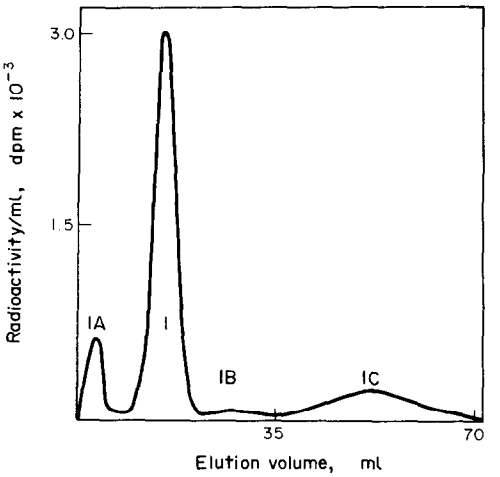

Fig. 1. Ion-exchange fractionation of metabolites of 2-CEPA formed in *Hevea* bark. The sample (10^5 dpm), of an extract made from mature bark which had been incubated for 72 hr with 2-CEPA-[^{14}C], was chromatographed on a QAE-Sephadex A-25 formate column (20 × 0.5 cm) using 50 mM ammonium formate buffer, pH 6.6 for elution. Peak 1 is the conjugate; peaks 1A, B and C are usually absent.

sporadically appeared in the elution diagrams (e.g. 1A in Fig. 1). The substances responsible were not identified, but peak 1C (Fig. 1) was in the position expected of 2-HEPA [21]. Analysis of bark extracts on ion-exchange columns in the chloride form, showed that more than 90% of their radioactivity could be accounted for by the ^{14}C in the acid-labile metabolite (conjugate—see below), in the compounds which showed up intermittently, and in residual 2-CEPA. Since *ca* 60% of the 2-CEPA disappeared (mainly as ethylene) during the 72 hr of the incubation, then a maximum of 4% of the 2-CEPA applied

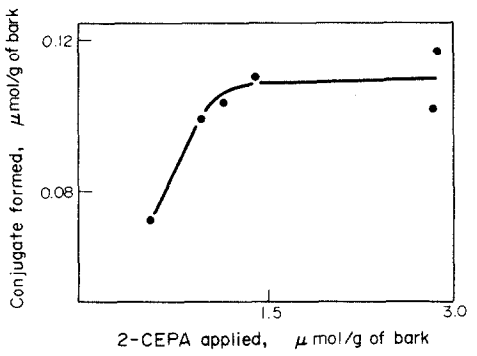

Fig. 2. Effect of amount of 2-CEPA applied on amount of conjugate formed in mature *Hevea* bark. Squares of bark, treated with varying quantities of 2-CEPA-[^{14}C], were incubated for 72 hr at 25° and the conjugate formed then extracted and determined (see Experimental).

to the bark could have been converted to water-extractable compounds which remained undetected. Fig. 2 shows that the extent of formation of the conjugate is dependent on the amount of 2-CEPA applied to the bark, and that the system virtually becomes saturated in this case at *ca* 1 μmol of CEPA per g of bark. In 4 experiments, at loadings of 0.8–1.3 μmol per g of bark, the extent of conjugate formation after 72 hr was 9–22% of the 2-CEPA applied, or 16–34% of the total 2-CEPA decomposed, and the mean rate of synthesis was 1.4–2.4 nmol per g of bark per hr. These values may be minimal for the reasons given below.

Structure and general properties of the conjugate

The retention on anion-exchangers showed that the molecule contained at least one acidic group and electrophoresis at pH 2, alongside acids of known pK, suggested a phosphonic hydroxyl (pK, <2 [24]); 2-CEPA was formed on acid hydrolysis. The compound therefore appeared to be a monoester, or similar derivative, of 2-CEPA; i.e. a conjugate of the acid with some substance in the bark. The only functional group detected in the conjugating residue was OH, and hydrolysis (0.1 M HCl, 60°, 1 hr) gave a sugar, identified as glucose by TLC in solvents A, E and G, and as D-glucose by incubation with D-glucose oxidase (EC 1.1.3.4) followed by separation of the resulting D-gluconic-δ-lactone electrophoretically at pH 9.2 [25]. The glucose content of the conjugate was 0.8 mol per mol. From these results the likely structure was D-glucose-1-(2-chloroethyl)phosphonate and this was confirmed by the PMR spectrum (below). The PMR data also establish that the compound is the 1-β-ester since the glucose residue is in the D-form, as shown above. R_f values of the conjugate in solvents A–D (cellulose), E, F (Si gel G), and G (kieselguhr), were (2-CEPA values in brackets): A, 0.25, (0.5); B, 0.55, (0.2); C, 0.35 (streaks), (0.5); D, 0.35, (0.6); E, 0.7; F, 0.7; G, 0.1, (0.0). On polyethyleneimine-cellulose (PEI-cellulose), R_f values were, in buffer A, 0.7 (0.5); B, 0.5 (0.2); C, 0.85 (0.5). On cellulose layers, 5 nmol of the conjugate could just be detected with acidic molybdate and 20 nmol with $AgNO_3$–ethanolic KOH (slow reaction). On Si gel G, 20 nmol was just detectable with acidic $KMnO_4$ (slow reaction) and 40 nmol with anisaldehyde–H_2SO_4 (olive-green spot). The compound was not detectable with the $NaIO_4$–*p*-anisidine reagent, though this reagent worked well with sugars on Si gel G, but not on cellulose. However, the anticipated reaction with periodate took place readily in solution and could be useful for identification purposes. Conjugate-[^{14}C] (1.4 mM) was incubated with 0.05 M $NaIO_4$ in H_2SO_4 (pH 3.5) at 20° for 3 hr followed by TLC (solvent A), autoradiography, and staining with $AgNO_3$–ethanolic KOH. Four immediately-reacting spots were observed; only one of these was radioactive (R_f 0.55) and it corresponded exactly in position and shape with the image on the autoradiograph. Control experiments established that the new labelled compound was not 2-CEPA, (which has almost the same R_f, but only reacts very slowly with the $AgNO_3$ reagent), and it was probably the expected dialdehyde [26]. Acetylation of the conjugate with Ac_2O in Py overnight at room temperature apparently gave a single compound (R_f 0.85, solvent E).

PMR spectrum of conjugate

(270 MHz, D_2O, *t*-BuOH standard): δ 4.91 (1H, *dd*, $J_{1,2}$ = 8 Hz, $J_{1,p}$ = 8 Hz, C-1), 3.32 (1H, *dd*, $J_{1,2}$ = 8 Hz, $J_{2,3}$ = 9 Hz, C-2), 3.52 (1H, *dd*, $J_{2,3}$ = 9 Hz, $J_{3,4}$ = 9 Hz, C-3), 3.40 (1H, *dd*, $J_{3,4}$ = 9 Hz, $J_{4,5}$ = 9.2 Hz, J_{gem} = 12.5 Hz, C-4), 3.50 (1H, *m*, $J_{4,5}$ = 9 Hz, $J_{5,6a}$ = 2 Hz, C-5), 3.90 (1H, *dd*, $J_{5,6a}$ = 2 Hz, J_{gem} = 12.5 Hz, C-6), 3.71 (1H, *dd*, $J_{5,6b}$ = 5.5 Hz, J_{gem} = 12.5 Hz, C-6), 3.77 (2H, *dt*, $J_{1',2'}$ = 8.2 Hz, $J_{2',p}$ = 9.2 Hz, C-2'), 2.24 (2H, *dt*, $J_{1',2'}$ = 8.5 Hz, $J_{1',p}$ = 18 Hz, C-1'). N.B. The double doublets arising from the protons on C-1, C-2, C-3 and C-4 coincidentally appear as triplets due to the close similarity of the coupling constants.

Stability in acid and alkali

The conjugate was only slightly decomposed by 0.01 M HCl on heating at 60° for 1.5 hr, but was completely hydrolysed in 0.1 M HCl at 60° for 1 hr. Passage down a column of a strong cation resin in the H^+ form did not cause breakdown, but evaporation of solutions of the conjugate in HCl, either in a N_2 stream at room temperature, or by freeze-drying, led to substantial or complete decomposition. The first order rate constant for hydrolysis in M HCl at 33° was 0.025/min and the compound is therefore more acid-labile than β-D-glucose-1-phosphate under the same conditions (*k*, 0.015/min) [27]. It is possible that during acid hydrolysis of the phosphonate ester, rapid migration of the esterifying group via a cyclic intermediate occurs, as is the case with phosphate esters of sugars [28]. The stability of the conjugate, in the kinds of acidic extraction media used by many workers in investigations of 2-CEPA metabolism in various plants, was also examined. This could only be done in a rough way, since a number of key details of extraction procedures (e.g. time of contact with extractant) had been omitted in the published accounts. It was assumed that extraction at room temperature had been used and that compounds extracted from the tissue would have been in contact with the acidic media for at least 1 hr. The results showed that <1% of the conjugate was hydrolysed in 1 hr at 20° in extraction media (2)–(5) and >90% in the more strongly acidic medium (1) (see Experimental for details of media).

When a solution of labelled conjugate was heated in NaOH at pH 12.5, some radioactivity was lost from the solution and ethylene (identified by GLC [10]) formed. The reaction was investigated quantitatively under conditions similar to those previously used to study the stability of 2-CEPA [29] and the results are given in

Table 1. Formation of ethylene from the conjugate on heating at 80° in alkali

Heating time (hr)	pH	$^{14}C_2H_4$ formed	^{14}C remaining in soln	^{14}C recovered
0.5	9.3	0.4	107	107
3.0	9.3	0.5	105	106
0.5	12.5	10.7	88.5	99
3.0	12.5	31.8	74.2	106

Conjugate-[^{14}C] (*ca* 50 nmol) was heated in 2 ml of alkali as described under Experimental. Results are expressed as % initial conjugate.

Table 1, where it can be seen that a considerable amount of ethylene is produced in hot alkali. The extent of formation of the gas is, however, less than that found with 2-CEPA (96% decomposition to ethylene in 0.5 hr at 80° and pH 9.3 [29]).

Decomposition in bark and leaves

Fig. 3 shows that on incubation of conjugate with leaf or bark from a young seedling, or bark from a mature tree, a considerable proportion of the conjugate is converted to ethylene in 72 hr. The decomposition appears to be at least partially enzymic, since heat treatment of the tissue greatly reduces the rate of formation of the gas. The apparently smaller effect of prior steaming on the decomposition taking place in leaf, is probably a reflection of the shorter steaming time used with the leaf (see Experimental). The shorter period was chosen so as to minimise possible breakdown of the conjugate during heating. In the experiments, the amount of conjugate applied was

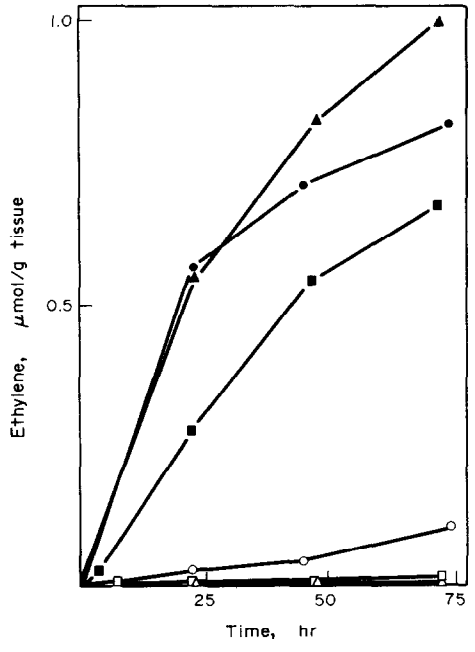

Fig. 3. Formation of ethylene from the glucose conjugate of 2-CEPA on incubation with *Hevea* tissue. Inner mature bark (▲), young bark (■) and leaf (●), were incubated at 30° with 1.9, 1.0 and 1.6 μmol of conjugate-[^{14}C] per g of tissue, respectively, and steamed controls (inner mature bark (△), young bark (□) and leaf (○)) treated in the same way. The method of ethylene determination and other details are given under Experimental.

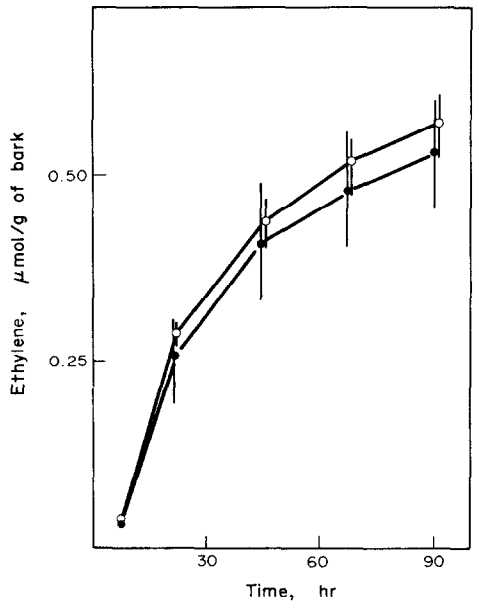

Fig. 4. Formation of ethylene from 2-CEPA and its glucose conjugate on incubation with youg *Hevea* bark. Samples (100 mg) of bark were treated in triplicate with aqueous solns (pH 5.5) containing 0.1 μmol of either 2-CEPA-[^{14}C] (●) or conjugate-[^{14}C] (○) and incubated at 30°. The points show the mean amounts of ethylene formed from each compound and the vertical bars indicate max. and min. values.

1–1.9 μmol per g of tissue and the initial rates (nmol/g/hr) of ethylene evolution corrected for the rates in heated tissue, were, at 30° (duplicate determinations): leaf, 22, 18; inner mature bark, 24, 15; young bark, 12, 11. Further experiments using the same amount of conjugate were run at 25° with mature bark. Over 72 hr, the time course of ethylene formation was virtually linear and the rates of ethylene evolution (nmol/g/hr) were: inner bark, 9, 8; outer bark, 8. If, as seems probable, the mechanism of ethylene production involves 2-CEPA as intermediate, then the enzymic formation of the acid is likely to be faster than the rate of ethylene appearance indicates, for several reasons: e.g. accumulation of 2-CEPA in the tissue; incomplete trapping of ethylene; removal of 2-CEPA by metabolism.

The curves obtained for ethylene formation on incubating young bark with either conjugate or 2-CEPA are given in Fig. 4. The rates of ethylene release from both compounds were almost the same throughout the experiment. Initial rates (nmol/g/hr) were: from conjugate, 13, 12, 14; i.e. about the same as above; and from 2-CEPA, 9, 13 and 15.

Action of hydrolases

The action on the conjugate of some widely occurring hydrolases was examined using ethylene production as a criterion of 2-CEPA formation. The enzymes used, which were as supplied by the manufacturers, and which presumably differed in their purities, were arbitrarily compared on a weight basis; results are shown in Table 2. Alkaline phosphatase and carboxylesterase had virtually no activity, phosphodiesterase I, slight activity at high concentration and acid phosphatase, considerable activity. Emulsin was the most effective and was studied in more detail. Using this enzyme at pH 5, it was demonstrated

Table 2. Effect of hydrolases on the conjugate

Enzyme	Enzyme concn (% wt/vol)	Conjugate concn (mM)	pH	t_1 (hr)	C_2H_4 formed	t_2 (hr)	Total C_2H_4 formed
Carboxylesterase	1 in 100*	5.9	7.5	23.5	< 0.5	22.5	<0.5
	1 in 10	5.9		23.5	< 0.5	22.5	<0.5
Alkaline phosphatase	0.0005	1.0	9.5	18	< 0.5	30	<0.5
	0.1	6.0		24	0.5	24	0.7
Phosphodiesterase I	0.005	1.0	8.8	19	< 0.5	24	<0.5
	0.1	6.0		24	1.5	24	3.6
Acid phosphatase	0.005	1.0	4.6	21	0.5	24	<1
	0.1	6.0		24	0.5	24	19
	0.1	6.0		24	0.5	24	20
Emulsin	0.01	5.9	5.0	23.5	0.5	23.5	3
	0.1	5.9		23.5	5.0	23.5	43
	1.0	0.59	7.0	22	35	24	67

Conjugate-[^{14}C] and enzyme were incubated for t_1 hr at the pH indicated and any $^{14}C_2H_4$ formed counted. The pH was then raised to 9.5 for t_2 hr and $^{14}C_2H_4$ again measured; further details are given under Experimental. $^{14}C_2H_4$ formed is given as % initial conjugate-[^{14}C]. Boiled and no-enzyme controls gave conversions of <0.5%.

* Carboxylesterase concentrations are expressed as dilutions of the $(NH_4)_2SO_4$ preparation supplied by the manufacturer.

that as conjugate disappears, 2-CEPA accumulates, and the curves for substrate decomposition and product formation showed a pronounced lag phase (*ca* 1 hr) (Fig. 5); the maximum rate of 2-CEPA formation was *ca* 19 nmol per hr. In a repeat of the experiment, the lag phase was *ca* 2 hr and the maximum rate of 2-CEPA formation *ca* 55 nmol/hr. The results establish that the conjugate is a substrate for emulsin, though a poor one, since measurements under identical conditions, but using *p*-nitrophenyl-*β*-D-glucoside [30] gave a decomposition rate of *ca* 2 × 10^5 nmol/hr. The reason for the lag phase (which was also observed in an experiment at pH 7) is not known, but as the conjugate was not completely pure, the presence of an inhibitor, of the type described in [31] might explain the effect; different amounts of impurities in the conjugate samples might also be responsible for the poor agreement in the rates of 2-CEPA formation.

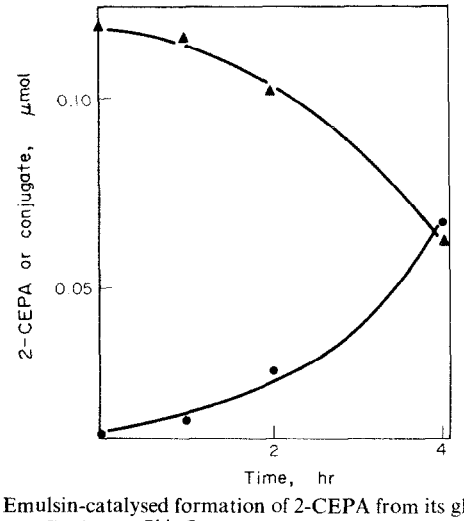

Fig. 5. Emulsin-catalysed formation of 2-CEPA from its glucose conjugate. Conjugate-[^{14}C] (4.9 mM) was incubated at 30° with 0.2% emulsin in 50 mM NaOAc buffer, pH 5 and the amount of conjugate decomposed (▲) and 2-CEPA formed (●) determined using the TLC method; the figures plotted have been corrected for a recovery of ^{14}C of 88 ± 1% (see Experimental).

Emulsin and other glycosidases will catalyse the synthesis as well as the decomposition of glycosides [32], so the possibility of using emulsin to synthesize the conjugate was examined. 2-CEPA (1.4 M) and D-glucose (1.1 M) were incubated at pH 5 with 1% emulsin at 30°. Samples taken after 1, 3, 4 and 10 days incubation were analysed by TLC on cellulose in solvent A and on PEI-cellulose in 50 mM NaCl. No conjugate was seen, but a compound which was probably a glucose-*β*-glucoside was found. This substance, which appeared after one day, was not formed when heated enzyme was used; i.e. under conditions in which emulsin appeared to be active synthetically, no conjugate synthesis was effected.

Conjugate as a possible constituent of the metabolites of 2-CEPA in leaves and tissue culture

At least one of the products formed by the metabolism of 2-CEPA in *Hevea* leaves is probably an acid-labile conjugate [21] and the chromatographic properties of this substance suggest that it is the same as that formed in bark. Thus on the 2-D TLC map of leaf metabolites of 2-CEPA exposed to acid during isolation (Fig. 6 in ref. [21]), spot 4 may represent 'bark conjugate' which escaped decomposition; leaf metabolites isolated in the absence of acid and run in the 2D-system also gave a strong spot in the conjugate position (unpublished results). Similar evidence suggests that the compound is a component of the metabolite mixture produced from 2-CEPA by cultures of *Hevea* callus tissue [23].

DISCUSSION

β-D-Glucose-1-(2-chloroethyl)phosphonate is the main water-extractable substance produced when *Hevea* bark from mature trees is incubated with 2-CEPA and it seems most likely, for several reasons, that it is the bark itself which is enzymically effecting the transformation of the acid, rather than microorganisms inevitably present. Penetration of 2-CEPA into the scraped bark is relatively swift (Archer, B. L., unpublished result) and contact between 2-CEPA and microorganisms would be minimised; no microbial (e.g. mould) growth was detected by

eye at any time on the treated surface, which tended to dry, though a little mould could be seen sometimes on the lower, damper surface; *Hevea* cells grown under sterile conditions with 2-CEPA in the medium also synthesize the conjugate, or a very similar substance [23].

The amount of 2-CEPA converted to the conjugate *in vitro* is considerable, and may be greater than appears at first sight. In the mature bark, the observed rate of synthesis of the conjugate is several times less than the apparent rate of degradation, measured under similar conditions. If it is assumed that the rate of penetration of 2-CEPA is not rate-limiting, then the results imply either that the true rate of synthesis is considerably greater than the measured rate, or that the conjugate is protected from attack by spacial separation of the synthetic and degradative enzymes. Possibly the true situation lies between these extremes. Leaves and young bark also have a substantial capacity to degrade the conjugate (Fig. 3) and the above arguments would apply to the synthesis of the conjugate, or similar substances, in these parts of the plant [14].

The conjugate appears to be a substrate, albeit a poor one, of β-glucosidase (emulsin) and acid phosphatase, though as these enzymes were, very probably, not pure, it is not certain that they are truly the catalytic species. In [24] it has been shown that nitrophenyl esters of phosphonic acids are readily decomposed by highly purified acid phosphatase from wheat germ, but no data are given on the effect of the enzyme on aliphatic phosphonate esters. Aryl monoesters of phosphonic acids are good substrates of phosphodiesterase I, but the alkyl esters are not [33, 34], and in agreement with this, it has been shown that the conjugate is scarcely attacked by this enzyme. Intestinal alkaline phosphatase hydrolyses neither alkyl nor aryl esters of phosphonic acids [34] and its inactivity with the conjugate is in line with these results. That the conjugate may be a substrate for β-glucosidase is of some interest, since the structure of the enzymically-labile portion of the molecule is much more akin to that of a phosphodiester than to that of a glycoside as defined in [35]. Although many enzymes will only attack phosphonates if they are structurally similar to their normal substrates, this is not always true; some phosphonates which have no isosteric phosphate analogue in living organisms are substrates for widely distributed enzymes [33, 36]. The conjugate appears to be an example of this second type of substance and it may be a substrate of enzymes other than the two quoted above. The decomposition of the conjugate to ethylene in *Hevea* tissue needs an enzyme (or enzymes) which has not been identified. The likely mechanism is that the conjugate is converted to 2-CEPA, hydrolytically or otherwise, and ethylene arises spontaneously from this substance. In young bark, ethylene is formed from the conjugate and from 2-CEPA at about the same rate (Fig. 4); i.e. the enzymic step is not rate-limiting. This finding can be compared with the results of work done with *Cucumis sativus* L. (cucumber) using the 2-chloroethylmonoester of 2-CEPA [37]. Plants treated with 2-CEPA evolved ethylene at an initial rate (nmol/g/hr) of *ca* 8 falling after three days to *ca* 1.8. The rate for the ester was much lower: 0.9 falling to 0.45. In this experiment, therefore, the rate of decomposition and/or penetration of the ester was quite different from that of the parent acid. In cucumber, the physiological effects of the ester persist longer than those of the acid and this may be due to the relative rates of ethylene formation. The mechanism of conversion of the 2-chloroethyl ester to ethylene has not been elucidated.

The isolation of the conjugate as a definite metabolic product of 2-CEPA, and the observation that the metabolite is itself, apparently, a substrate for two common enzymes, adds weight to the view that the growth regulator can be metabolized readily in *H. brasiliensis*. The question then arises as to the reason for the largely negative findings in other species (see Introduction). It is unlikely that a facile ability to metabolize 2-CEPA would be restricted to *Hevea* and the answer probably lies in differences in experimental approach. Virtually all the plants used by others have been horticultural species and the tracer 2-CEPA has been applied usually near the levels required in practice to produce the desired physiological effects. These levels (per unit wt of tissue) are lower than used here, and in the previous work with leaves and young stem [14, 21], and the difference may be important. Fig. 2 illustrates that as expected, the amount of 2-CEPA metabolized in mature bark is positively dependent on the level of the chemical applied and a similar result is found in tissue culture experiments [23]. In the experiments with mature bark and young stem of *Hevea*, the water-repellent surface was removed to promote absorption, and petiolar feeding was employed with the leaves; both these procedures would increase the chance of the 2-CEPA rapidly reaching sites of metabolism. In the work with the other plants, the tracer was usually applied to the untreated surfaces of leaves or of fruit, sometimes over a small area: data in some of the papers show that penetration was poor [13, 16, 20]. Acidic extraction mixtures have been used widely in studies of 2-CEPA metabolism [11, 13–18, 20, 22] and in most of these media the conjugate is reasonably stable. However, in experiments in which methanolic HCl was used [13, 15–18, 22], and especially when the extracts were concentrated, or dried, failure to detect the conjugate, or other similarly acid-labile metabolites, could be attributable to the use of this extractant.

Ethylene formation on heating in alkali has been used to estimate residual 2-CEPA in plant tissue [11, 20], though the validity of the method has been questioned [29]. The present findings raise further doubts, because it has been shown that a 2-CEPA metabolite also can form appreciable quantities of ethylene in hot alkali.

The factors already known to control the conversion of 2-CEPA to ethylene in *Hevea*, and which presumably influence the form of the latex-yield vs. time curve [38], are complex. They include rates of translocation of the acid into and out of the bark [21], local pH values within and without the bark cells, and temperature. To these must now be added enzymic formation of the conjugate. In the usual yield-stimulation procedure, the amount of 2-CEPA applied per unit area of bark [38], would be considerably larger than in the experiments with mature bark described here, and therefore conjugate synthesis would be expected to proceed at the maximum rate (c.f. Fig. 2), at least initially. Since the true rate of conjugate synthesis may be higher than the observed rate (see above), it is evident that a large fraction of the 2-CEPA might be conjugated under field conditions. Whether the conjugate is then stored for relatively long periods or whether the ethylene is released rapidly, cannot be decided at present. It is however clear that the yield response could be influenced by conjugate formation. The apparent rate of conjugate synthesis is high enough for the reaction to

play a role in the control of the early events in the yield response. For example, an increase in latex flow (yield) is observable about 24 hr after stimulant application and rises to a maximum at about 4 days; increased polysome synthesis in the latex is detectable after 12 hr and is also maximal at 4 days [39].

The main metabolic fate of 2-CEPA in *Hevea brasiliensis* appears to be conjugate formation and conversion to 2-HEPA; 2-HEPA is also metabolized, partly by conjugation ([21], and unpublished results). 'Conjugate' is used in its usual sense, and implies covalent linkage of the administered substance or a product of it, with some endogenous compound. The word has been used by others to mean the non-covalent complexes of sugars and 2-CEPA, supposedly formed in fruit [16–18]. It is not known whether young stem from *Hevea* produces conjugates, but the elution patterns, on ion-exchangers, of metabolites from such tissue (see Fig. 1 of ref. [14]), suggests this is so. In mature bark, conjugation of 2-CEPA with glucose seems to be the major metabolic route. It is unlikely that significant amounts of 2-CEPA are converted to CO_2, or water-insoluble material, since these processes are minor in young stem, leaves, tissue cultures of *Hevea* [14, 21, 23], and in other plants [11, 20, 22]. The reason why mature bark produces essentially a single compound, whereas the green organs produce many, is unknown. Photosynthetic activity *per se* is not implicated, since tissue cultures of *Hevea*, grown in the dark, synthesize many metabolites [23]. It is not unreasonable to suppose that the observations reflect the greater proportion of older cells in the mature bark. So far no effects which might be ascribed to possible toxic properties of 2-CEPA or its metabolites have been seen in *Hevea* treated with the growth regulator. On the contrary, evidence which does indicate that 2-CEPA and/or its metabolites might inhibit certain enzymes, has been published recently [40, 41].

The mechanism of biosynthesis of the conjugate is of some interest. It is likely that uridine-diphosphate-glucose or some other donor molecule is required. Studies of the reaction in cell-free extracts and identification of the enzymes taking part would be a useful contribution to the currently active subject of phosphonate metabolism [36].

EXPERIMENTAL

Radiochemical materials and methods. 2-CEPA-[^{14}C] was prepared and its radiochemical purity (99%) assessed as described previously [14]. For most expts it was diluted to a sp. act. of 1.17×10^5 dpm/μmol with 2-CEPA purified by crystallization from C_6H_6. Radioactivity was determined by scintillation counting using a mixture of 0.9% butyl-PBD in toluene and Triton X-100 (2:1, by vol.) or for non-aqueous samples, 0.6% butyl-PBD in toluene. Severe chemiluminescence was encountered on counting samples containing aq. bark extract, but this was eliminated by diluting and acidifying (HCl, *ca* pH 1) before mixing with the phosphor.

Separation methods. TLC was on cellulose in A, *n*-BuOH–HOAc–H_2O (12:3:5); B, EtOH–NH_4OH–H_2O (38:5:8); C, aq. PhOH (72% w/w); D, *n*-BuOH–$EtCO_2H$–H_2O (26:12:17); on Si gel G in E, EtOH–$CHCl_3$–H_2O (3:1:1) and F, EtOH–H_2O (4:1); on kieselguhr G plates buffered with 20 mM NaOAc in G, EtOAc–*iso*-PrOH–H_2O (65:23:12). PEI-cellulose plates [42] were run in the following buffers: A, 0.5 M NaOAc, pH 4.4; B, 50 mM [Cl^-] imidazole–HCl, pH 6.6; C, 0.2 M [Cl^-] imidazole–HCl, pH 7. Sugars and other compounds were detected with $AgNO_3$–ethanolic KOH, acidic $KMnO_4$, $NaIO_4$–*p*-anisidine, *p*-aminophenol [43] and anisaldehyde–H_2SO_4 [44]. Phosphorus-containing compounds were detected by spraying with acidic-molybdate [45] followed by exposure to daylight, or in the case of PEI-cellulose, followed by spraying with $SnCl_2$ and exposure to NH_3 [29]. Radioactive compounds were located by scanning or autoradiography [14]. Generally, elution was with MeOH. For quantitation of labelled bands, the relevant areas were transferred to counting phials, soaked in MeOH (2 ml for 1 hr) scintillator added, and after clearing, radioactivity determined. Thin-layer electrophoresis was done on acid-washed cellulose [46] in either HCO_2H, pH 2, or in 0.25 M $NaHCO_3$ buffer, pH 9.2 at 200 V (applied) for 1.5–2 hr. Anion-exchange chromatography was on columns of QAE-Sephadex A-25 formate at pH 6.6 (sorption in 10 mM HCO_2NH_4 buffer, elution with 50 mM HCO_2NH_4 buffer), or QAE-Sephadex A-25 chloride at the same pH (sorption in 10 mM [Cl^-] imidazole-HCl buffer, elution with 50 mM [Cl^-] imidazole-HCl buffer) [21].

Plant material. Leaves and young bark were obtained from seedlings (2- to 3-yr-old) growing locally in a greenhouse. Samples were kept in H_2O-satd containers and used as quickly as possible after removal from the plants. Mature bark from near the tapping panel was obtained from trees growing in Malaysia. The bark, with some adhering wood, was sealed in polythene envelopes and air-freighted at 0°; such bark was usually at least 48 hr old before it was used.

Incubation of 2-CEPA-[^{14}C] with mature bark. Squares of bark (5 × 5 cm, 15–20 g) from mature *H. brasiliensis* were freed from adhering wood, scraped to remove the external dead layer, and 0.16 ml of aq. 2-CEPA-[^{14}C], (*ca* 1 μCi, pH 3–4) applied over the whole scraped surface of each piece. Any excess liquid was allowed to dry superficially and each bark sample then incubated, treated surface upwards, at 25° for 72 hr, in an atmosphere saturated with H_2O; the air was replaced every 24 hr.

Determination of conjugate-[^{14}C] and 2-CEPA-[^{14}C], in mature bark. Each square of bark, incubated as above, was finely chopped, transferred to a centrifuge tube containing 50 ml of H_2O and 50 ml of $CHCl_3$ (ice-cold), and finely disintegrated with a Willems Polytron; omission of $CHCl_3$ resulted in jamming of the machine with coagulated rubber. The slurry was centrifuged at 4°, the clear H_2O-layer removed, and the residual suspension extracted with 2 further 50 ml portions of H_2O using the Polytron. The pooled aq. layers were freeze-dried and the $CHCl_3$, which contained virtually no ^{14}C, discarded. This procedure extracted at least 95% of the labelled H_2O-solubles. The solid was suspended in 25 ml of H_2O, centrifuged, and a sample of the supernatant ($6–12 \times 10^4$ dpm) fractionated on an anion-exchange column (20 × 0.5 cm, formate) as above. The conjugate-[^{14}C] eluted early (peak 1, Fig. 1) free of other labelled materials; 2-CEPA-[^{14}C], the main radioactive component of the extract was strongly retained on the column. When it was required to determine 2-CEPA-[^{14}C] in the bark extracts, chromatography was done with the chloride form of the exchanger. With such columns the conjugate was usually (see Results) the first peak to elute and 2-CEPA the last. To show that this peak was due to 2-CEPA only, a sample of the bulked fractions was passed down a cation column (H^+) form to remove imidazole [14] and the effluent freeze-dried. TLC in solvents A and B showed that the only labelled compound present was 2-CEPA, and on heating at pH 9 for 5 hr at 60°, 95% of the ^{14}C was lost as a labelled gas, presumably C_2H_4. It is apparent from the findings in this paper that these tests are not foolproof, since the peak may have contained, besides 2-CEPA, labelled substances, which like the conjugate, readily break down in acid to 2-CEPA. However, such substances probably would have been separated from 2-CEPA on the column in the first place.

Isolation of conjugate. Using the above method, 19.5 × 5 cm squares of bark (465 g) were treated with 2-CEPA-[^{14}C] (*ca* 1 μmol per g bark), incubated for 72 hr and extracted; the amount of conjugate formed was 46 μmol. The extract was freeze-dried, the residue extracted twice with MeOH and the soln rotary evapd to a thick paste. Portions of this, each containing *ca* 6–7 μmol of conjugate, were then treated as follows:— the paste was extracted ×3 with 10 mM HCO_2NH_4 buffer, the soln

(50 ml) chromatographed on a 20 × 2.8 cm formate column as above, and the fractions containing the conjugate (eluting between 430 and 930 ml), freeze-dried. Batches of this solid, each containing *ca* 4 μmol of conjugate, were taken up in H_2O (5 ml) and chromatographed on columns of charcoal (B.D.H.; MFC grade, 1 × 1.2 cm diam.). After application of the sample, each column was washed with H_2O (30 ml) and the conjugate eluted with MeOH (400 ml); additional conjugate could be eluted with 5% Py–MeOH. For reasonable recoveries (>75%), the size of the column relative to the load was critical. Further purification was by TLC (50 nmol conjugate per plate) first on Si gel G in solvent E and then twice on cellulose in solvent A; plates were prewashed with solvent, and MeOH. Conjugate isolated by this procedure was used for most of the expts described in this paper, and though not necessarily pure, contained no extraneous materials reacting with $AgNO_3$–ethanolic KOH, or acidic $KMnO_4$ when rechromatographed as above on Si gel G or cellulose; solvent B (cellulose) was sometimes used to effect further purification. The yield was 22 μmol (sp. act. 1.17 × 10^5 dpm/μmol).

Estimation of $^{14}C_2H_4$. Except in one case (see below), all expts in which $^{14}C_2H_4$ was measured, were done in an apparatus comprising a 7 cm B 10 test tube connected at 45°, by a short wide-bore tube, to a 4 cm B 10 test tube. The joints, lightly greased, were kept tight by means of rubber bands. The longer tube contained the C_2H_4-forming system and the shorter, 0.2 ml of 0.25 M $Hg(ClO_4)_2$ in 2 M $HClO_4$ to trap $^{14}C_2H_4$ [47]. At suitable times, the radioactive trapping solns were replaced with fresh reagent and counted. Tests on this apparatus, run at pH 9.5 and 30° with 2-CEPA-[^{14}C] showed that 78% of the calculated amount [5] of $^{14}C_2H_4$ liberated was absorbed by the perchlorate When determining $^{14}C_2H_4$ formed on incubating conjugate with pieces of leaf, Warburg vessels were used. The pieces, floating in buffer, were in the main compartment and the side-arm contained 0.15 ml of the C_2H_4-trapping mixture.

Formation of $^{14}C_2H_4$ *in bark.* A piece of brown bark was removed from a seedling and rapidly sliced into strips (*ca* 1 × 0.5 cm). Groups of 3 strips were weighed (80–100 mg), 20 μl of an aq. soln, pH 5, of conjugate-[^{14}C] applied to the whole, originally wood-facing, surface of each set, and any excess liquid allowed to just dry. Control strips were steamed 30 min before application of the tracer. Immediately after treatment, each set was put in a C_2H_4-trapping apparatus. Glass wool was used to keep the individual strips apart and each incubation tube contained moistened filter paper. Similar expts were carried out with strips of like wt cut from soft inner mature bark and also with a single piece (*ca* 90 mg) cut from the much harder outer mature bark, after removal of surface flaky material. These expts were run at 25° and 30°.

Formation of $^{14}C_2H_4$ *in leaf.* A small *Hevea* leaf was fed with conjugate-[^{14}C] [14]. The blades were then cut into squares (*ca* 0.5 × 0.5 cm) which were thoroughly mixed, divided into 6 roughly equal lots, and each lot rapidly weighed (30–50 mg). Two of the groups were incubated at 30° as above each in 1 ml of 50 mM K–Pi buffer, pH 6.8 containing 0.4 M sucrose; 2 other groups were similarly treated, after suspension in steam for 3 min. The remaining 2 lots of pieces were each extracted with 10 ml of M HCl (Polytron) and the ^{14}C in the extracts measured. The ^{14}C-contents per unit wt of the two groups were within 10% of each other, showing that the distribution of ^{14}C in the samples was reasonably uniform. Comparison of the calculated total uptake with that measured by counting the feeding soln, established that little ^{14}C had been lost as ethylene, or other volatiles, during tracer application.

Comparative rates of formation of $^{14}C_2H_4$ *from conjugate and 2-CEPA.* Strips of brown bark were peeled from 2 regions of each of 3 seedlings and 6 sets of 3 strips cut. Each set contained bark from different plants and the wt per set was 100 ± 4 mg. Treatment of the strips, 3 lots with conjugate and 3 with 2-CEPA (aq. solns pH 5.5), and incubation at 30° were as above.

Stability of conjugate in acid and alkali. Conjugate-[^{14}C] was heated at 80° in 2 ml of either 50 mM glycine buffer, pH 9.3, or NaOH, pH 12.5. These solns were then rapidly cooled in ice-H_2O and after 2 hr, $^{14}C_2H_4$ determined. Radioactivity remaining in the reaction mixtures was measured after their acidification with M HCl. To study the kinetics of the decomposition of conjugate-[^{14}C] in acid, samples (120 nmol) were heated at 33° in M HCl (25 μl) for 20, 40 and 60 min and the reaction stopped by the addition of M $KHCO_3$ (25 μl). The whole of each mixture was streaked on a 5 cm-wide cellulose TLC plate which was run in solvent A. Conjugate and 2-CEPA were eluted and counted as above; recovery of ^{14}C was 84 ± 1%. A plot of log (dpm in conjugate eluted) against time gave the straight line expected for a first order reaction. Stabilities in acidic extraction media were investigated similarly, except that the conjugate-[^{14}C] was incubated for 1 hr at 20° and the solns were not neutralized before TLC. The extraction media tested were (proportions by vol.,) (1) MeOH 99–conc. HCl 1 [16–18]; (2) 1% HCO_2H in 80% aq. EtOH [20]; (3) MeOH adjusted to pH 1 with HCl [13]; (4) 1% HCO_2H in 95% aq. EtOH [11]; (5) 20 mM 2-CEPA aq. [14].

Action of hydrolases on conjugate-[^{14}C]. Labelled conjugate and the hydrolases were incubated at 30° at an appropriate pH for 24 hr and $^{14}C_2H_4$ formation measured. The pH of the incubation mixture (0.2 ml) was then raised to 9.5 by addition of 0.4 M Na_2CO_3 (20 μl) and 0.1 M ethanolamine–HCl buffer, pH 9.5 (0.5 ml) to fully ionize any 2-CEPA (pK_2 7.0) [5, 6] which had accumulated, the mixture reincubated (*ca* 24 hr, i.e. about 4 half-lives of 2-CEPA [5, 6]) and $^{14}C_2H_4$ again measured. Boiled and no-enzyme controls were done in all cases. The media described in [48, 24, 49] were used in incubations with alkaline phosphatase (EC 3.1.3.1.) from intestinal mucosa, acid phosphatase (EC 3.1.3.2.) from wheat germ, and phosphodiesterase I (EC 3.1.4.1.) from *Crotalus adamanteus* venom, respectively. Carboxylesterase (EC 3.1.1.1.) from hog liver, was tested in 50 mM Tris–HCl buffer, pH 7.5 and β-glucosidase (EC 3.2.1.21.) emulsin) from almonds, in 50 mM NaOAc buffer, pH 5 and in 50 mM imidazole–HCl buffer, pH 7. Checks that the enzymes remained active during the long incubations were made in parallel expts using the appropriate derivatives [50] of *p*-nitrophenol. To measure the rate of conjugate decomposition catalysed by emulsin, conjugate-[^{14}C] and the enzyme were incubated in the above NaOAc buffer (25 μl) and the reaction stopped by adding 12 μl of M HCO_2H in EtOH. The amount of conjugate and 2-CEPA in the mixtures was determined by the method used for determining acid-stability; recovery of ^{14}C was 86–89%.

Glucose determination. Glucose in the conjugate was estimated colorimetrically by the Molisch reaction using internal and external standards [51]. The amount of carbohydrate eluted from the cellulose thin-layers used for purification, was small, and was corrected for by appropriate blanks.

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