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# EVIDENCE FOR CONVERSION OF ARACHIDONIC ACID TO HYDROXYICOSATETRAENOIC ACIDS BY A CELL-FREE HOMOGENATE FROM MAIZE SEEDLINGS

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**Key Word Index**—*Zea mays*; Gramineae; cell-free homogenate; arachidonic acid; hydroxyicosatetraenoic acids.

**Abstract**—The conversion of arachidonic acid to the hydroxyicosatetraenoic acids 5-HETE, [5(*S*)-hydroxy-6,8,11,14,-icosatetraenoic acid], 12-HETE, [12(*S*)-hydroxy-5,8,10,14-icosatetraenoic acid] and 15-HETE, [15(*S*)-hydroxy-5,8,11,13-icosatetraenoic acid] by a 10 000 *g* cell-free supernatant of a *Zea mays* seedlings homogenate is described. For metabolic pathway detection [1-$^{14}$C]arachidonic acid was added. The hydroxyicosatetraenoic acids were isolated by TLC and HPLC and purified to constant specific radioactivity against authentic [$^{3}$H]-labelled 5-HETE, 12-HETE and 15-HETE by co-chromatography.

## INTRODUCTION

The most important fatty acid constituent of membrane phospholipids in mammalian cells is arachidonic acid (*all-Z*)-5,8,11,14-icosatetraenoic acid. It has long been known that release of arachidonic acid and subsequent oxidation leads to a wide variety of biologically active compounds, such as prostaglandins, prostacyclin, thromboxanes, leukotrienes, lipoxins and mono-, polyhydroxy- and hydroperoxy fatty acids [1, 2].

The occurrence of arachidonic acid in higher plant materials is now well established [3]. Furthermore, several prostaglandins have also been identified in higher plants [4, 5]. Hitherto studies on the capacity of homogenates of higher plant materials, or enzymes isolated from such sources, metabolizing arachidonic acid are limited. 2-Lipoxygenase isolated from soybean can transform arachidonic acid *in vitro* into prostaglandin $F_{2\alpha}$[6]. Recent work has shown, that when arachidonic acid was incubated with homogenates of potato tubers, two isomers of 6-*trans*-leukotriene $B_4$, epimeric at C-12, were formed in addition to the major product 5(*S*)-hydroperoxy-6-*trans*-8,11,14,-*cis*-icosatetraenoic acid (5-HPETE) [7].

When maize seedlings homogenates were used for the investigation of *c*AMP dependent protein kinases, a number of compounds where found to interfere with common *c*AMP dependent protein kinase assays [8, 9]. Some of these compounds showed more lipophilic than hydrophilic characteristics and could be extracted with ethyl acetate. Subsequently, the occurrence of lipophilic peroxides could be detected by a non-enzymatic method [10]. Because such peroxides are known to be intermediates in the biosynthetic pathway to the above highly active biological compounds in mammals [2], [1-$^{14}$C]-arachidonic acid was added to maize seedling homogenates. As described in this paper, a number of labelled products could be observed after radio-TLC analysis. Subsequent purification of some of these compounds yielded for the first time from a higher plant tissue homogenate the hydroxyicosatetraenoic acids, 5(*S*)-hydroxy-6,8,11,14,-icosatetraenoic acid (5-HETE), 12(*S*)-hydroxy-5,8,10,14-icosatetraenoic acid (12-HETE) and 15(*S*)-hydroxy-5,8,11,13-icosatetraenoic acid (15-HETE).

## RESULTS AND DISCUSSION

After 20 min of incubation at 37°, 10 000 *g* supernatants of maize seedling homogenates converted [1-$^{14}$C]-labelled arachidonic acid into a number of labelled products as evident from radio-TLC. The major products were unmetabolized [1-$^{14}$C]arachidonic acid ($R_f$ 0.87), [1-$^{14}$C]5-HETE ($R_f$ 0.75), [1-$^{14}$C]-12-HETE and [1-$^{14}$C]-15-HETE. [1-$^{14}$C]-12-HETE and [1-$^{14}$C]-15-HETE were not well resolved by the solvent systems used. Further purification of 5-HETE could be achieved by TLC, whereas additional HPLC purification of [1-$^{14}$C]-12-HETE and [1-$^{14}$C]-15-HETE was necessary [13] (Table 1).

After pre-purification in all cases, known amounts of authentic [$^{3}$H]-labelled 5-HETE and 12-HETE and 15-HETE were added and co-chromatographied during the further purification steps. As shown in Table 2, after only three TLC purifications a constant specific radioactivity could be obtained for [1-$^{14}$C]-5-HETE against authentic [$^{3}$H]-5-HETE. When [$^{3}$H]-labelled 12-HETE and 15-HETE, had been added to the separated eluates of [1-$^{14}$C]-12 and -15 HETE first HPLC, HPLC in solvent 1 was repeated under the same conditions. Thereafter conditions were changed and a second HPLC purification was performed [14]. As shown in Table 1, constant specific radioactivity of [1-$^{14}$C]-12-HETE/[$^{3}$H]-12-HETE and of [1-$^{14}$C]-15-HETE/[$^{3}$H]-15-HETE was obtained after the last two HPLC purification steps. The values found for the retention times correspond to those given in ref. [14].

The lipophilic peroxides which had been observed through the course of reaction-time of the homogenate

Table 1. Purification of TLC and HPLC pre-purified [1-$^{14}$C]-arachidonic acid metabolite [1-$^{14}$C]-12-HETE and [1-$^{14}$C]-15-HETE to constant specific radioactivity by cochromatography with 400 000 dpm of authentic [$^{3}$H]-12-HETE and 800 000 dpm of authentic [$^{3}$H]-15-HETE (see Experimental)

| Compounds | Chromatography | Radioactivity. (dpm) of: [1-$^{14}$C]-12-HETE [1-$^{14}$C]-15-HETE | Radioactivity. (dpm) of: [$^{3}$H]-12-HETE [$^{3}$H]-15-HETE | Specific radioactivity (dpm) [1-$^{14}$C]-12-HETE/[$^{3}$H]-12-HETE [1-$^{14}$C]-15-HETE/[$^{3}$H]-15-HETE |
|---|---|---|---|---|
| [1-$^{14}$C]-12-HETE + [$^{3}$H]-12-HETE | HPLC/solvent 1 | 250 000 | 400 000 | 0.62 |
| [1-$^{14}$C]-15-HETE + [$^{3}$H]-15-HETE | HPLC/solvent 1 | 580 000 | 800 000 | 0.72 |
| [1-$^{14}$C]-12-HETE + [$^{3}$H]-12-HETE | HPLC/solvent 2 | 180 000 | 288 000 | 0.62 |
| [1-$^{14}$C]-15-HETE + [$^{3}$H]-15-HETE | HPLC/solvent 2 | 450 000 | 620 000 | 0.72 |

Table 2. Purification of TLC pre-purified [1-$^{14}$C]-arachidonic acid metabolite [1-$^{14}$C]-5-HETE to constant specific radioactivity by cochromatography with 200 000 dpm of authentic [$^{3}$H]-5-HETE (see Experimental)

| Compounds | Chromatography | Radioactivity (dpm) of: [1-$^{14}$C]-5-HETE | Radioactivity (dpm) of: [$^{3}$H]-5-HETE | Specific radioactivity (dpm) $\frac{[1\text{-}^{14}C]\text{-5-HETE}}{[^{3}H]\text{-5-HETE}}$ |
|---|---|---|---|---|
| [1-$^{14}$C]-5-HETE + [$^{3}$H]-5-HETE | TLC/solvent A | 180 000 | 200 000 | 0.90 |
| [1-$^{14}$C]-5-HETE + [$^{3}$H]-5-HETE | TLC/solvent B | 166 000 | 181 000 | 0.91 |
| [1-$^{14}$C]-5-HETE + [$^{3}$H]-5-HETE | TLC/solvent A | 151 000 | 167 700 | 0.90 |

with the added arachidonic acid (20 min) were isolated in only small quantities. Semiquantitative measurements (see Experimental [10]), demonstrated the complex character of the oxidation reactions. However, there is good evidence that the corresponding hydroperoxides are the precursors of 5-HETE, 12-HETE and 15-HETE.

Whereas [1-$^{14}$C]-5-HETE and [1-$^{14}$C]-12-HETE are found at the same order of magnitude of radioactivity (Tables 1 and 2), [1-$^{14}$C]-15-HETE was found to contain more than twice as much radioactivity (Table 1). The latter compound is the main precursor of lipoxin A [15].

5-HETE, 12-HETE and 15-HETE, known as intermediates of thromboxane, leukotriene and lipoxin formation in mammals [1, 2, 16, 17] are now provisionally identified for the first time from higher plant material. In mammals two main pathways are involved in the regulation of cell function, proliferation and differentiation: the calciumphosphatidyl inositol and the cyclic nucleotide (cAMP, cGMP) cascades. The herein reported metabolic pathway of arachidonic acid, an important constituent of membrane phospholipids in mammals and some plants [18] and investigations in the field of cAMP-dependent regulation [8, 9, 19], suggest that similar regulation mechanisms may occur in the plant kingdom.

## EXPERIMENTAL

Conditions for aseptic growth and the harvesting of maize seedlings are described elsewhere [11]. [1-$^{14}$C]-Arachidonic acid ((*all-Z*-)-5,8,11,14-eicosatetraenoic acid; 58 mCi mM$^{-1}$, 1.85 MBq), [$^{3}$H]-5-HETE, 5(*S*)-hydroxy[5,6,8,9,11,12,14,15-$^{3}$H] eicosatetraenoic acid; [$^{3}$H]-12-HETE, 12(*s*)-hydroxy-[5,6,8,9,11,12,14,15-$^{3}$H]eicosatetraenoic acid; [$^{3}$H]-15-HETE, 15(*S*)-hydroxy [5,6,8,9,11,12,14,15-$^{3}$H]eicosatetraenoic acid; each 100 Ci mM$^{-1}$, 3.7 TBq, were obtained from Amersham.

*Preparation of cell-free homogenate.* Five-day-old etiolated maize seedlings were frozen in liquid $N_2$ and stored at −20°. Seedlings were homogenized (2 g fr. wt ml$^{-1}$ buffer) in a pre-cooled mortar and buffer (0°). The buffer contained: 30 mM Tris–HCl, 1 mM $MnCl_2$, 1 mM EDTA, 2 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 6.8). After filtration through a nylon cloth, the crude extract was centrifuged for 30 min at 10,000 *g* at 0°. The supernatant was used for expts and stored in 5 ml portions at −20°.

*Conversion of arachidonic acid by homogenate supernatant.* [1-$^{14}$C]Arachidonic acid ($5 \times 10^7$ dpm) and 10 μg unlabelled arachidonic acid (higher amounts were found to be inhibitory) were incubated with vigorous shaking at 37° with 5 ml of supernatant. After low-speed centrifugation (1000 *g* for 5 min) the supernatant was collected and the pellet re-extracted with the same solvent mixt. To the combined exts, $CHCl_3$ and $H_2O$ was added to give a final mixt of $CHCl_3$–MeOH–$H_2O$ (8:4:3). The ensuing layers were sepd by centrifugation.

*Separation and identification of reaction products.* The $CHCl_3$ layers were evapd to dryness and aliquots taken for TLC on silica gel G with A: EtOAc–hexane–HOAc (30:50:1) and B: $CHCl_3$–MeOH–HOAc–$H_2O$ (200:200:5:4) [12]. Plates were air-dried and radioactive components detected using a TLC scanner. Zones corresponding to standards of [$^{3}$H]-5-HETE, [$^{3}$H]-12-HETE and [$^{3}$H]-15-HETE were scraped off the plates, eluted with MeOH and dried under vacuum. $R_f$ values were [1-$^{14}$C]-15-HETE, 0.60; [1-$^{14}$C]-12-HETE, 0.63; [1-$^{14}$C]-5-HETE, 0.75 and for unmetabolized [1-$^{14}$C]arachidonic acid, 0.87. Other zones with $R_f$ values: 0.07, 0.11, 0.36–0.53, 0.67 and 0.94 are part of further investigations in this laboratory.

For the next purification step isolated [1-$^{14}$C]-5-HETE ($R_f$ 0.75) was co-chromatographed with 200 000 dpm of authentic [$^{3}$H]-5-HETE by TLC in solvent systems A and B. The zones, containing [1-$^{14}$C]-5-HETE were scraped off and eluted with MeOH. Aliquots were taken and the relative amounts of [$^{14}$C]- and [$^{3}$H]- determined by liquid scintillation counting (LSC). The MeOH extracts of the TLC zones of [1-$^{14}$C]-12-HETE ($R_f$ 0.63) and [1-$^{14}$C]-15-HETE ($R_f$ 0.60) were analysed by HPLC as described in ref. [13] with slight modifications. The solvent system of 0.1% HOAc contained 55% MeCN and was eluted at 1 ml min$^{-1}$ ($C_{18}$ODS, 5 μm). Aliquots of frs were taken and the radioactivity measured by LSC. To the eluates of the sepd frs of [1-$^{14}$C]-12-HETE and [1-$^{14}$C]-15-HETE authentic [$^{3}$H]-12-HETE (400 000 dpm) and 15-HETE (800 000 dpm) were added. Radioactivity was first determined as described for the purification of 5-HETE. When the first HPLC was repeated, eluates were evapd and a second HPLC performed [14] under the following conditions: column 25 × 0.4 cm, 3μ Spherisorb ODS2, linear gradient from 100% MeOH–MeCN–2 mM $H_3PO_4$ (1:1:3) to 100% MeOH–MeCN–2 mM $H_3PO_4$ (2:2:1) in 45 min, then isocratic flow rate 0.7 ml min$^{-1}$. $R_f$ (min) of [1-$^{14}$C]-12-HETE/[$^{3}$H]-12-HETE (56.2) [1-$^{14}$C]-15-HETE/[$^{3}$H]-15-HETE (54.4) corresponded to those given in ref. [14]. Constant sp. act. was achieved following the two HPLC steps.

*Organic peroxide formation.* When organic peroxide formation was measured, 0.5 ml of the reaction mixt (5 ml supernatant) were taken at 5 min intervals and extracted twice with 1 ml EtOAc. The organic layer was removed *in vacuo*. The residue was dissolved in MeOH and the amount of peroxide present determined in arbitrary units from calibration plots of changes in fluorescence per time against standard plots of *t*-butyl-hydroperoxide reaction with homovanillic acid [10].

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