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Biotransformations of putative phytoecdysteroid biosynthetic precursors in tissue cultures of *Polypodium vulgare*

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Incubation of calli and prothalli of *Polypodium vulgare* with different tritium-labelled ecdysteroids has led to modification of some previous assumptions about the biosynthesis of ecdysteroids in plants. Thus, 25-deoxy-20-hydroxyecdysone was transformed efficiently in both tissues into 20-hydroxyecdysone (20E), but no 25-deoxyecdysteroids such as pterosterone and inokosterone were formed. Likewise, incubation of 2-deoxy-ecdysone (2dE) produced exclusively ecdysone (E) and 20E, indicating a high 2-hydroxylase activity in both tissues, despite calli not producing phytoecdysteroids. This 2-hydroxylation was also evident in the transformation of 2,22-dideoxyecdysone (2,22dE) into 22-deoxyecdysone (22dE). Different ecdysteroids that do not occur in *P. vulgare* were formed in the incubation of 3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE) by 3α-reduction and 3β-reduction and 25-hydroxylation processes. The fact that 22,25-dideoxyecdysone and 22dE were the only 2-hydroxylated products formed in this case suggests that only compounds bearing a 3β-hydroxyl group are substrates for the 2-hydroxylase. Surprisingly, 22-hydroxylation was never observed with either 2,22dE or 3D2,22,25dE, raising the possibility that it could occur at an early step in the biosynthetic pathway. In this respect, labelled 22*R*-hydroxycholesterol was efficiently converted into E and 20E, whereas 22*S*-hydroxycholesterol was not transformed into ecdysteroids, because of its unsuitable configuration at C22. Finally, the conversion of 25-hydroxycholesterol into E and 20E was greatly enhanced after thermal treatment of prothalli which induces the release of previously stored ecdysteroids. Thus, *P. vulgare* prothalli and calli appear to be particularly suitable models for the study of ecdysteroid biosynthesis and its regulation in plants.

Keywords: 20-hydroxyecdysone; fern; hydroxycholesterol derivatives; *in vitro* cultures; induction.

Many plant species produce C_{27}–C_{29} ecdysteroids of a wide structural diversity [1–5]. These analogues of insect molting hormone (20-hydroxyecdysone, 20E) are secondary metabolites which are expected to provide some protection to plants against non-adapted phytophagous insect species [6]. Although the presence of such compounds in plants was demonstrated more than 30 years ago, little has been learnt since about their biosynthetic pathway(s), and whether it operates in a sequence that is similar to or different from that in Arthropods is not known.

In vivo and *in vitro* labelling experiments have established that these compounds are biosynthesized from mevalonate via C_{27}, C_{28} and C_{29} phytosterols. Cholesterol has been proved to be the intermediate in the biosynthesis of C_{27} phytoecdysteroids in *Taxus baccata* [7]. In biosynthetic studies with *Ajuga reptans* roots transformed with *Agrobacterium rhizhogenes*, 24-methyl-cholesterol and 24-ethylcholesterol have been proposed as intermediates for the biosynthesis of C_{28} and C_{29} ecdysteroids, respectively [8]. Adler & Grebenok [9] proposed lathosterol (5α-cholest-7-en-3β-ol) as the logical precursor of ecdysteroid biosynthesis in *Spinacia oleracea*. In *Polypodium vulgare* wild plant, label incorporation from radiolabelled mevalonate into 20E and 7-dehydrocholesterol was detected [10]. Later on, Davies *et al*. [11] observed that, whereas the 4α proton of cholesterol was retained, the corresponding 3α and 4β protons were rearranged to the C4 and C5 positions, respectively, in the 20E produced from this precursor. From these results, these authors suggested a mechanism for the formation of the *cis* A/B ring junction and proposed 7-dehydrocholesterol and 7-dehydro-5,6-epoxycholesterol as required intermediates in the biosynthesis of ecdysteroids. These results have, however, been recently questioned, as studies using deuterium-labelled precursors in *A. reptans* and NMR analysis of the 20E formed thereafter showed that 3α, 4α and 4β protons were retained at their respective positions, and the authors proposed a more direct mechanism for the 7-ene-6-one formation [12]. The formation of the same intermediate, 7-dehydro-5,6-epoxy-cholesterol, was also postulated [12], but this proposal has not yet been directly tested because of its great instability.

Data on the sequence of reactions are scarce. It was previously shown that ecdysone (E) can be converted into 20E in both *Sesuvium portulacastrum* [13] and *S. oleracea* [14,15], that *Achyranthes fauriei* can convert 22,25-dideoxyecdysone

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Abbreviations: E, ecdysone, 2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one; 20E, 20-hydroxyecdysone; 25d20E, ponasterone A, 25-deoxy-20-hydroxyecdysone; 2dE, 2-deoxyecdysone; 22dE, 22-deoxyecdysone; 2,22dE, 2,22-dideoxyecdysone; 22,25dE, 22,25-dideoxyecdysone; 2,22,25dE, 2,22,25-trideoxyecdysone; 3α-22dE, 3α-22deoxyecdysone; 3α-2,22dE, 3α-2,22-dideoxyecdysone; 3α-2,22,25dE, 3α-2,22,25-trideoxyecdysone; 3D22dE, 3-dehydro-22-deoxyecdysone; 3D2,22dE, 3-dehydro-2,22-dideoxyecdysone; 3D2,22,25dE, 3-dehydro-2,22,25-trideoxyecdysone.

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(22,25dE; ecdysteroid abbreviations used are based on those of Lafont *et al*. [16]) into 20E and 25-deoxy-20,26-dihydroxyecdysone [17] and more recently that *A. reptans* hairy root cultures can convert ketol (2,14,22,25-tetradeoxyecdysone) into 20E [12]. These data are therefore for a few steps only, which are thought to be among the last ones in the case of Arthropods; in animals, it is generally accepted that hydroxylations at positions 25, 22, 2 and 20 take place after nucleus functionalization and in this preferential sequence [18]. Major limitations in the case of plants derive from the lack of suitable *in vitro* systems.

We have previously shown that *in vitro* cultures of *P. vulgare* prothalli produce high amounts of ecdysteroids (0.7% dry weight), with a variety of chemical structures, 20E being the most abundant [19,20]. Using radiolabelled compounds, we found that the seven ecdysteroids present in these tissues, i.e. E, 20E, 24-hydroxyecdysone, abutasterone (20,24-dihydroxyecdysone), polypodine B (5,20-dihydroxyecdysone), inokosterone (25-deoxy-20,26-dihydroxyecdysone) and pterosterone (25-deoxy-20,24-dihydroxyecdysone), could be produced from biosynthetic precursors such as mevalonate and cholesterol, whereas all 25-hydroxylated ecdysteroids may be produced, in part, but not exclusively, from E [21]. We recently demonstrated that ecdysteroid biosynthesis in prothalli can be greatly stimulated by immersing these tissues in hot water (45 °C) [22].

We report here on the results of investigations on the biosynthetic steps of phytoecdysteroids using incorporation of labelled ecdysteroid and cholesterol derivatives by the above tissue cultures. Likewise, although *in vitro* calli cultures of *P. vulgare* do not produce ecdysteroids [23], they have the enzymatic capacity to carry out different reactions implied in the biosynthetic routes of these compounds. Taking advantage of the morphological characteristics of this plant material that facilitates the incorporation of labelled compounds, we also studied biotransformations of selected substrates related to ecdysteroid biosynthesis and previously shown to be efficiently converted by arthropod steroidogenic organs [24,25].

MATERIALS AND METHODS

Chemicals

The tritium-labelled compounds were obtained from different sources: [23,23,24,24-$^3H_4$]E of specific activity 88 Ci·mmol^{-1} was purchased from NEN (DuPont, Germany); [23,23,24,24-$^3H_4$]2dE, [22,23-$^3H_2$]2,22dE and [1α,2α-$^3H_2$]3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE) of specific activity 40–100 Ci·mmol^{-1} were kindly provided by C. Hétru and J. Hoffmann (CNRS, Strasbourg, France); [^{3}H]25-deoxy-20-hydroxyecdysone (25d20E; specific activity 180 Ci·mmol^{-1}) was a gift from H. Kayser (Novartis, Basle, Switzerland); [22,23-$^3H_2$]3D2,22dE was prepared from [22,23-$^3H_2$]2,22dE as described by Blais *et al*. [26]; [1α,2α-$^3H_2$]3α-2,22,25dE and [22,23-$^3H_2$]3α-2,22dE were prepared by $NaBH_4$ reduction of [1α,2α-$^3H_2$] 3D2,22,25dE and [22,23-$^3H_2$]3D2,22dE, respectively, by the procedure of Dinan & Rees [27]; [22,23-$^3H_2$]3α-22dE was prepared from 22dE by the procedure of Milner & Rees [28]; [22-^{3}H]22*R*-hydroxycholesterol, [22-^{3}H]22*S*-hydroxycholesterol (specific activity 15.5 Ci·mmol^{-1}) and [26,27-^{3}H]25-hydroxycholesterol (specific activity 80 Ci·mmol^{-1}) were provided by J. Lacy (NEN).

Most reference unlabelled ecdysteroids were isolated and identified in the laboratory. Reference 22dE was a gift from F. Baker, Novartis, and 2,22,25dE from C. Hétru.

In vitro cultures

P. vulgare prothalli was produced as described by Camps *et al*. [19] in 1/2 MS culture medium supplemented with sucrose (30 g·L^{-1}) and agar (8 g·L^{-1}), adjusted to pH 5.7. Callus formation was induced in prothallus clusters cultured in MS basal medium supplemented with 3 mg·L^{-1} 2,4-dichlorophenoxyacetic acid. After a few days in culture, an increase in prothallus thickness was observed. The formation of undifferentiated cell clusters in the upper border of the prothalli was clearly distinguishable after 4 weeks. Therefore, a green friable callus could be isolated from individual prothalli and subcultured in petri dishes, in the same culture medium. All cultures were incubated at 22 ± 2 °C under 16 h light (Sylvania cool white, 5 μM·s^{-1}·m^2). Selected callus lines could be maintained in *in vitro* conditions by subculturing every 6 weeks [23].

Incorporation of radioactive precursors

Small prothalli (≈ 25 mg fresh weight) or calli (≈ 150 mg fresh weight) were cultured in small petri dishes (6 cm internal diameter) in the corresponding medium mentioned above. After 2 weeks, 1 μCi labelled 25d20E, 2dE, 2,22dE, 3D2,22,25dE, 3α-2,22,25dE, cholesterol or cholesterol derivatives were topically applied. After treatment for different time periods, samples were lyophilized, and radiolabelled ecdysteroids were extracted with methanol (4 × 1 mL) and quantified by HPLC with an on-line radioactivity monitor for detection of labelled compounds. Experiments were carried out at least in triplicate.

Prothalli clusters (≈ 25 mg fresh weight) were immersed in 5 mL water at 45 °C for 40 min [22] and returned to small petri dishes (6 cm internal diameter) with the appropriate culture medium. Then, 1 μCi labelled 25-hydroxycholesterol was topically applied to the prothalli; they were then incubated for 20 h and the ecdysteroids extracted and quantified by HPLC.

Enzymatic hydrolysis

Aliquots of tissue culture extracts (100 000 c.p.m.) were evaporated and dissolved in 2 mL 50 mM sodium acetate buffer, pH 5.4, and incubated overnight with ≈ 1 mg β-glucuronidase (from *Helix pomatia*-H1 type, Sigma G 0751) at 35 °C. Ecdysteroids were then adsorbed on a C_{18} Sep-Pak cartridge (Millipore) and eluted with 3 mL ethanol.

HPLC analysis

Analyses were monitored at 242 nm with a diode array detector or at 254 nm with a UV detector and with on-line radioactivity monitor (Flo-One A250; Packard) for the detection of labelled compounds and quantified with a chromatographic software. Different reversed-phase (RP) HPLC columns of Spherisorb ODS2 [5 μm, 15 (or 25) cm × 4.6 mm internal diameter; Tracer, Waters or Shandon] and different conditions based on multistep gradients of the ternary solvent mixture acetonitrile/propan-2-ol/water were used as follows: acetonitrile/propan-2-ol/water (0 : 7 : 93, by vol.) for 10 min followed by a gradient to 8 : 7 : 85 over 10 min, these conditions being maintained for 20 min, and a second gradient applied to 58 : 7 : 35 for 10 min in a Tracer column eluted at 1.2 mL·min^{-1} and 55 °C (RP-1); a gradient of acetonitrile/propan-2-ol/water starting from 8 : 7 : 85 to 78 : 7 : 15 over 40 min and maintained under these conditions for 5 min in a

Waters column eluted at 1 mL·min^{-1} and 30 °C (RP-2); a multistep gradient of solvent B (acetonitrile/propan-2-ol, 5 : 2, v/v) and 0.1% trifluoroacetic acid in water: 15–25% B for 2 min, 25% B for 6 min, 25–75% B for 20 min, 75% B for 27 min, 75–90% B for 5 min and 90% B for 5 min in a Shandon column at 1 mL·min^{-1} and room temperature or in a Waters column at 1 mL·min^{-1} and 30 °C (RP-3); a multistep gradient of solvent B and 0.1% trifluoroacetic acid in water: 15–25% B for 2 min, 25% B for 6 min, 25–75% B for 20 min, 75% B for 22 min, 75–90% B for 5 min, 90–100% B for 5 min and 100% B for 15 min in a Shandon column eluted at 1 mL·min^{-1} and room temperature (RP-4).

Alternatively, a normal-phase (NP) HPLC column (Zorbax-SIL, 25 cm × 4.6 mm internal diameter) eluted at 1 mL·min^{-1} and room temperature was used with different mixtures of cyclohexane/propan-2-ol/water: 100 : 40 : 3 (NP-1); 100 : 30 : 1.5 (NP-2); 200 : 25 : 0.5 (NP-3).

RESULTS

Metabolism of 25d20E

Ponasterone A (25d20E) is one of the minor components of *P. vulgare* [21] and could be the precursor of the 25-deoxy-ecdysteroids in this plant. When this precursor was incubated with prothalli and calli, it was converted into 20E at different rates. After 22 h, 83% of the applied product was transformed in prothalli and 92% in calli. The transformation was complete after 5 days, and 20E was the only compound detected (RP-1, RP-2, RP-3 and NP-2 HPLC systems) even after treatment of the incubation extracts with β-glucuronidase. These results show that the 25-hydroxylation is a fast and efficient process in both tissues and can work with a polyhydroxylated substrate. On the other hand, 25-deoxyecdysteroids (pterosterone and inokosterone) must be produced from another precursor different from 25d20E, which means that 24-hydroxylations and 26-hydroxylations must take place before hydroxylation at C20.

Metabolism of 2dE

The 2-hydroxylation step is known to be (one of) the last step(s) of the ecdysone biosynthesis in arthropods [29]. Although it can be assumed that this is also the case in plants, so far experimental evidence is lacking. Therefore, we incubated [23,24-$^3H_4$]2dE with prothalli and calli of *P. vulgare*. As shown in Fig. 1, this labelled precursor was transformed into E and 20E, this metabolism being more efficient in calli than in prothalli. Thus, whereas in prothalli 40% of transformation of precursor was observed after 24 h, the transformation was complete by this time in calli, with 20E being the predominant product (RP-1, RP-2 and NP-1 HPLC systems). These results establish that in plants also the C2 position is easily hydroxylated and that 2dE is a substrate for the 2-hydroxylase. However, it cannot be definitely concluded from these results that 2dE is an intermediate in the biosynthesis of phytoecdysteroids in prothalli of *P. vulgare* and that 2-hydroxylation effectively takes place at a late step in the biosynthetic pathway.

Metabolism of 2,22dE

As mentioned above for the 2-hydroxylation, data on 22-hydroxylation in plants are also scarce. When [^{3}H]2,22dE was incubated with prothalli of *P. vulgare*, the corresponding

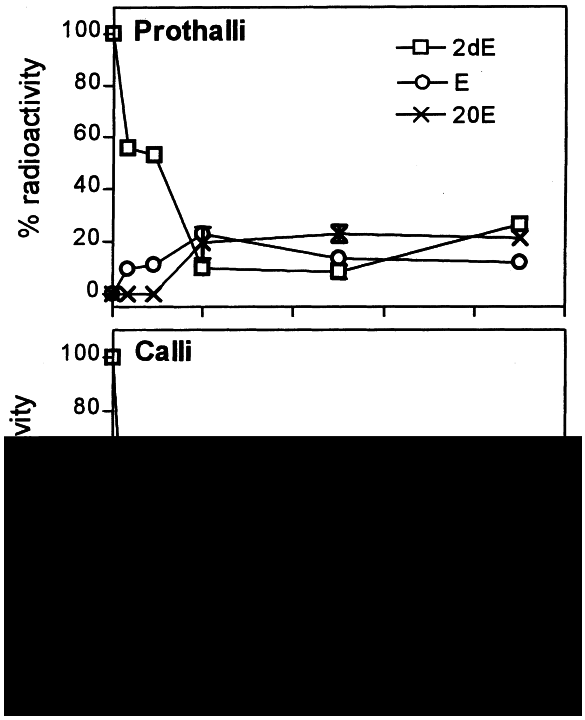

Fig. 1. Biotransformation of 2dE in *P. vulgare*. Radiolabelled 2dE was topically applied to prothalli and calli. After treatment for different time periods, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and quantified by HPLC. Values are mean ± SD (n = 3).

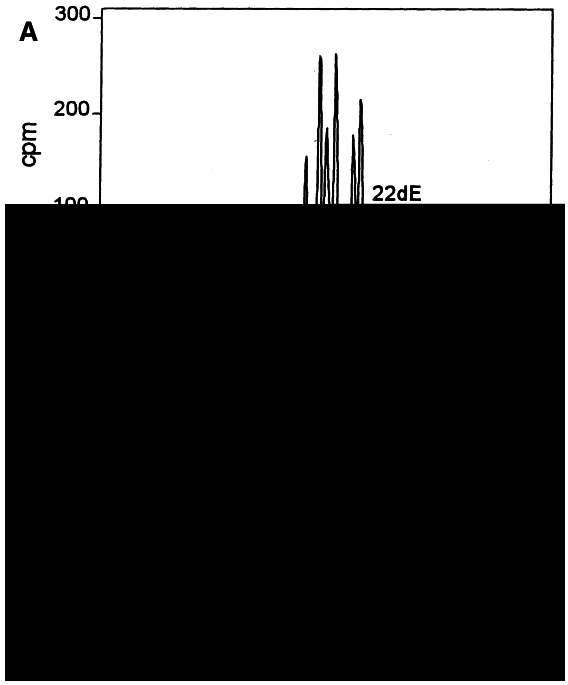

Fig. 2. Biotransformation of 2,22dE in prothalli of *P. vulgare*. Radiolabelled 2,22dE was topically applied to prothalli. After 2 days, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and analyzed by HPLC before (A) or after (B) treatment with β-glucuronidase. Chromatographic HPLC system RP-3.

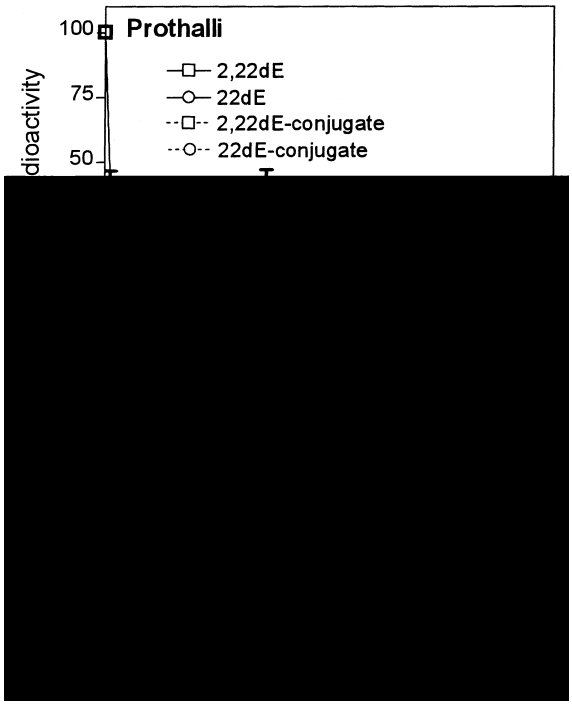

Fig. 3. Biotransformation of 2,22dE in *P. vulgare*. Radiolabelled 2,22dE was topically applied to prothalli and calli. After treatment for different time periods, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and quantified by HPLC. Values are mean ± SD (n = 3).

radiochromatograms revealed a complex pattern of metabolites that was greatly simplified by treatment of the extracts with β-glucuronidase (Fig. 2), 22dE and the starting compound being the main compounds identified (RP-3 and NP-2 HPLC systems). The evolution of this biotransformation depicted in Fig. 3 is remarkable. At short incubation times (12–24 h), a high percentage of total radioactivity can be attributed to 22dE, which disappears with time, suggesting that the biotransformations take place on the free ecdysteroids and then a conjugation occurs that prevents further metabolism of the ecdysteroids formed. From the polarity of the conjugates and their chromatographic behaviour, they could be glucosides, but direct evidence has still to be obtained.

Likewise, when the transformation of 2,22dE was carried out in calli, HPLC analysis of the extracts revealed the formation of two conjugated products, which, after treatment with β-glucuronidase, afforded 2,22dE and 22dE. As shown in Fig. 3, after the first day only conjugates of 2,22dE and 22dE are present in the extracts, the transformation into the conjugate of 22dE being complete after 3 weeks. No other radiolabelled phytoecdysteroid was observed during these incubations, suggesting that 2,22dE is not an intermediate in ecdysteroid biosynthesis in *P. vulgare*. It is important to note here that hydroxylation at position C22 was never obtained.

Metabolism of 3D2,22,25dE

Incubation mixtures of this substrate with prothalli of *P. vulgare* gave complex chromatographic patterns and revealed that it was not a precursor of the ecdysteroids occurring in this plant. Other compounds were formed that could be identified by HPLC (RP-3, NP-2 and NP-3 HPLC systems) after treatment of the extracts with β-glucuronidase and comparison with authentic references, such as 3α-2,22,25-trideoxyecdysone (3α-2,22,25dE), 3-dehydro-2,22-dideoxyecdysone (3D,2,22dE), and 3α-2,22-dideoxyecdysone (3α-2,22dE).

Another abundant metabolite (compound X) was formed which exhibited no change in its HPLC retention time after incubation with β-glucuronidase, indicating that it is not a conjugated metabolite or that more drastic hydrolytic conditions are required to release the free ecydysteroid. To identify its structure, incubations of prothalli with labelled 3α-2,22, 25dE were carried out. After 3 days, 3α-2,22dE and this metabolite (compound X) were formed, suggesting that it should have a 3α structure. However, its HPLC retention time was not coincident with that of different synthetic samples such as 3α-22-deoxyecdysone (3α-22dE) and 3-dehydro-22-deoxyecdysone (3D22dE). The kinetics of the biotransformation of 3D2,22,25dE depicted in Fig. 4 show that this compound is very rapidly transformed into 3α-2,22,25dE during the first 2 days and then this new compound is further metabolized into 3α-2,22dE and perhaps into other unidentified 3α derivatives (Fig. 4). Long incubation times result in increased amounts of more polar products, probably resulting from further different hydroxylations.

Similarly, diverse incubations of 3D2,22,25dE with calli of *P. vulgare* were carrried out. With the same techniques as described above 3α-2,22,25dE, 2,22,25-trideoxyecdysone (2,22,25dE), 3D2,22dE, 22,25dE, 2,22dE, and 22dE were identified. The kinetics of this biotransformation are depicted in Fig. 4. It is noteworthy that after 24 h, 50% of the original substrate had been metabolized to 3α-2,22,25dE and 2,22, 25dE by 3α-reductase and 3β-reductase (Fig. 4), whereas after 5 days metabolites generated by hydroxylase activities

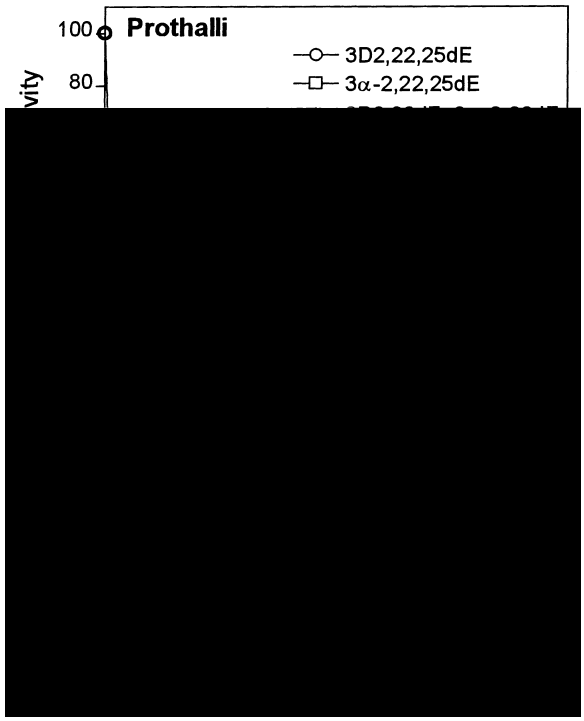

Fig. 4. Biotransformation of 3D2,22,25dE in *P. vulgare*. Radiolabelled 3D2,22,25dE was topically applied to prothalli and calli. After treatment for different time periods, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and quantified by HPLC. Values are mean ± SD (n = 3).

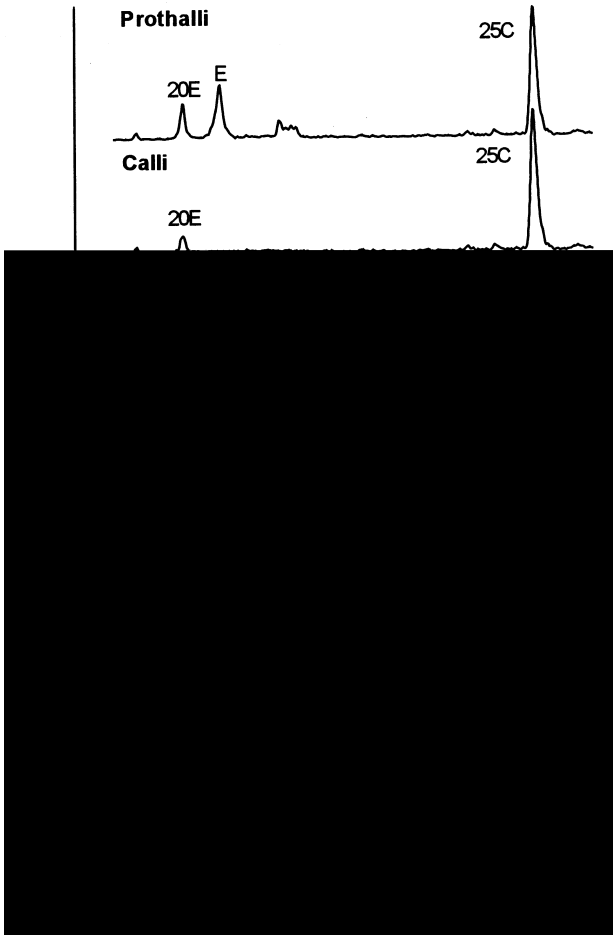

Fig. 5. Biotransformation of cholesterol analogues in *P. vulgare*. Radiolabelled 25-hydroxycholesterol (25-C), 22*R*-hydroxycholesterol (22*R*-C) and 22*S*-hydroxycholesterol (22*S*-C) were topically applied to prothalli and calli. After 3 days, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and analysed by HPLC. Chromatographic HPLC system RP-4.

(2-hydroxylase and 25-hydroxylase) were mainly detected. Again, it is remarkable that no 22-hydroxylation was observed.

Metabolism of cholesterol and some analogues

Previous experiments had shown that [4-^{14}C]cholesterol is converted into ecdysteroids after a one-week incubation with prothalli [21]. It was of interest to compare the efficiency of conversion of cholesterol and various available hydroxylated derivatives (22-hydroxycholesterol and 25-hydroxycholesterol). Of these, 25-hydroxycholesterol was shown to be very efficiently metabolized *in vitro* by arthropod molting glands [25]. Indeed, incubation of prothalli and calli from *P. vulgare* with 25-[^{3}H]hydroxycholesterol afforded significant conversion into E and 20E after 3 days (Fig. 5). The conversion was very efficient with prothalli (28.5% E + 20E) and lower, but still significant (8%) with calli, where only labelled 20E was found. The chromatograms were very simple, with almost no accumulation of metabolites of intermediate polarity between E and 25-hydroxycholesterol. By comparison, incubation with [^{3}H]cholesterol resulted in an overall 2% conversion into E + 20E with prothalli, whereas it was not detectable with calli (results not shown).

In a previous paper we reported that the metabolism of labelled E to 20E in *P. vulgare* prothalli was stimulated when this tissue was immersed in water at 45 °C for 1 h [22]. Based on these results, *P. vulgare* prothalli were submitted to this treatment and the metabolism of 25-hydroxycholesterol was studied after a short (20 h) labelling period. As shown in Fig. 6, a 10-fold increase in labelled 20E formation was observed as compared with controls. In these experiments, the chromatograms were analysed over a longer period which allowed detection of a lot of metabolites less polar than 25-hydroxycholesterol, which presumably represent cholesterol esters (results not shown). These compounds were not further investigated.

The availability of labelled 22*R*-hydroxycholesterol and 22*S*-hydroxycholesterol allowed us to perform experiments with these molecules. It is important to notice that 22*R*-hydroxycholesterol has the same stereochemistry at C22 as ecdysteroids, whereas 22*S*-hydroxycholesterol has the same sterochemistry as brassinosteroids. HPLC analysis of the extracts of the biotransformation of 22*R*-hydroxycholesterol in prothalli (Fig. 5) revealed that this product was transformed into E and 20E to a large extent after 3 days (RP-3 and RP-4 HPLC systems). The conversion rate was very similar to that obtained with 25-hydroxycholesterol. Once again, transformation in calli (Fig. 5) occurred to a smaller extent than in prothalli. It is noteworthy that in calli, after 3 days, E was not detected, as expected because of its transformation into 20E in this culture. In contrast, no polar metabolites eluted in the 20E-E area were obtained during labelling experiments using 22*S*-hydroxycholesterol (Fig. 5). Whether the metabolites formed had brassinosteroid-related structures could not be

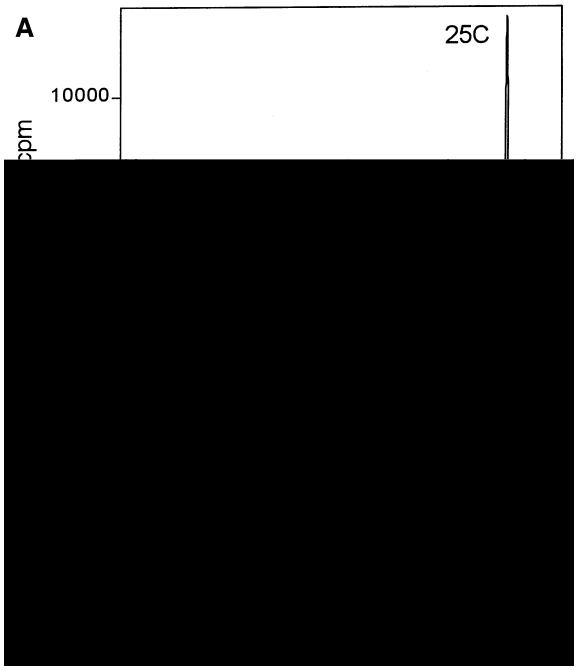

Fig. 6. Effect of a thermal treatment on 25-hydroxycholesterol (25-C) conversion by *P. vulgare* prothalli. (A) Radiolabelled 25-hydroxycholesterol was topically applied to prothalli and, after 20 h, samples were lyophilized, and radiolabelled ecdysteroids extracted with methanol and analysed by HPLC. (B) Prothalli were immersed in water at 45 °C for 40 min before the addition of 25-hydroxycholesterol. Chromatographic HPLC system RP-4.

further investigated because of the lack of suitable reference molecules.

DISCUSSION

The results of the different metabolic studies with prothalli and calli of *P. vulgare* are summarized in Fig. 7.

Conversion of distal putative precursors by prothalli and calli

In our previous work with *P. vulgare* prothalli, we showed that this material produces significant amounts of labelled 25-deoxy-20,24-dihydroxyecdysone and 25-deoxy-20,26-dihydroxyecdysone after 1 week of incubation with labelled mevalonate [21]. We suggested that 25d20E could be a putative precursor of these ecdysteroids, although it was detected in minor amounts in this tissue. However, the present results disprove this assumption, indicating that 25d20E is transformed into 20E efficiently in both tissues by fast 25-hydroxylation and no hydroxyl group is introduced at C24 or C26. The above ecdysteroids must be produced from another precursor, i.e. hydroxylation at C24 or C26 must take place at an earlier step. This was confirmed when using labelled 2dE; incubations of 2dE in both tissues exclusively produced E and 20E, and again no metabolites hydroxylated at positions C24 or C26 were observed. Hydroxylation at C2 requires a 3β-OH group, and does not take place with compounds bearing a 3α-OH (Fig. 7). On the other hand, 25-hydroxylation does not have such requirements, as it can occur whatever the functionalization at C3 (3α-OH, 3β-OH or 3-oxo). It is noteworthy that, although calli are unable to produce ecdysteroids from endogenous substrates, they nevertheless possess 2-hydroxylase, 20-hydroxylase and 25-hydroxylase activities.

Conversion of less polar molecules

The metabolism of 2,22dE in both tissues afforded mainly 22dE, also being more efficient in calli. Remarkably, only 2-hydroxylation occurred in this case and, apparently, hydroxylation at C22 or any other sites was not observed. Similar results were obtained in the case of labelling experiments with 3D2,22,25dE. This opens up the possibility that hydroxylation at C22 may take place at a very early stage of ecdysteroid biosynthesis in *P. vulgare*. Interestingly, 22-hydroxylation is thought to represent an early step in the biosynthesis of brassinosteroids [30], which are structurally related molecules. It is therefore possible that the stereochemistry of this 22-OH would then engage hydroxysterols in the ecdysteroid or the brassinosteroid pathway. Labelling experiments with 22*R*-hydroxycholesterol and 22*S*-hydroxycholesterol seem to support this hypothesis. This may, however, not apply to all plants, as labelled 22,25dE was converted into 20E in *A. fauriei* [17], and labelled 2,14,22,25-tetradeoxyecdysone was converted into 20E in *A. reptans* [12].

In contrast with the previous precursors, 2,22dE was converted to a large extent into polar conjugates (of 2,22dE and 22dE), which, from their behaviour, are probably glucosides. Whether these molecules represent a more soluble transport form or a process involved in the storage in cell vacuoles is at present not known. Anyway, this reaction seems to prevent any further hydroxylation reaction, although with calli upon long-term labelling (21 days) 2,22dE conjugates are progressively replaced by 22dE conjugates. Conjugation with sugars is a common process in plants and is known to occur for cardenolides [31] brassinosteroids [32] and ecdysteroids [33],

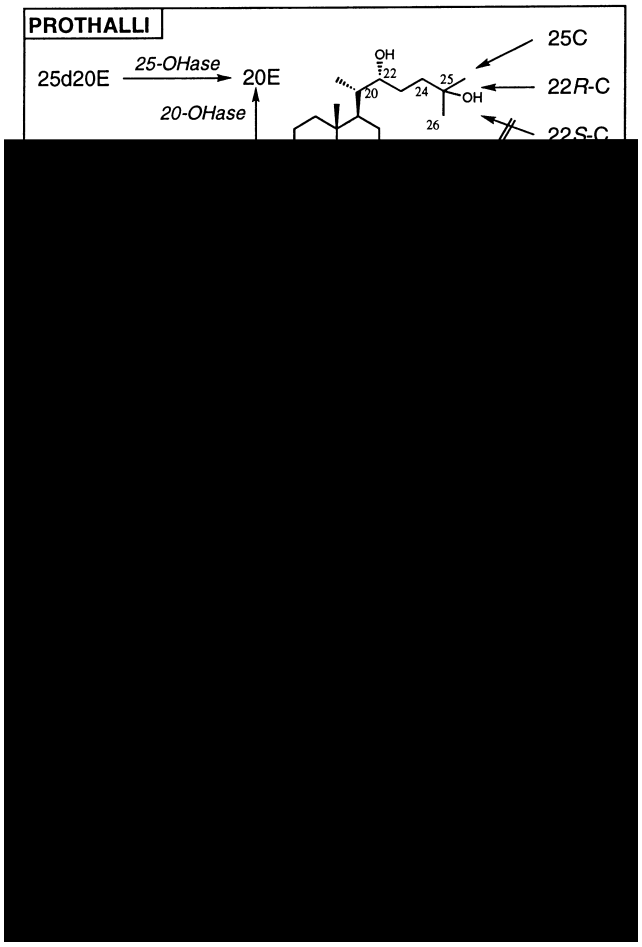

Fig. 7. Summary of the biotransformation of different substrates in *P. vulgare*. Putative enzymes involved are indicated. Abbreviations: 25-OHase, ecdysteroid 25-hydroxylase; 20-OHase, ecdysone 20-hydroxylase; 2-OHase, ecdysteroid 2-hydroxylase; 3β-reductase, 3β-hydroxysteroid-5β-oxidoreductase; 3α-reductase, 3α-hydroxysteroid-5β-oxidoreductase.

for example. In *P. vulgare,* no glucosides of the polar ecdysteroids have been isolated so far, but glucosides of ecdysteroid-related molecules are known (see osladin [34] and polypodosaponin [35]). Therefore, the present finding is not unexpected, although its significance remains to be determined.

Incubations with 3D2,22,25dE in both tissues led to the formation of a complex array of metabolites such as 22dE, 22,25dE, 2,22dE, 3D2,22dE, 3α-2,22dE, 2,22,25dE, and 3α-2,22,25dE (Fig. 7). They provide evidence for the enzymatic potentialities of these tissues, which contain a 3α-reductase (prothalli and calli) and a 3β-reductase (calli). Whether these enzymes use ecdysteroids as endogenous substrates cannot, however, be concluded. In contrast, the lack of 3β-reductase detection in prothalli, which produce large amounts of ecdysteroids, led us to assume that, in contrast with Arthropods [36], the biosynthesis of ecdysteroids does not involve 3-oxo intermediates. Such a conclusion agrees with the retention of the 3α-proton observed in *A. reptans* during the conversion of cholesterol into 20E [12].

Conversion of cholesterol analogues

The metabolic studies of cholesterol analogues with prothalli and calli of *P. vulgare* showed that both 22*R*-hydroxycholes-

terol and 25-hydroxycholesterol were transformed into E and 20E, indicating that early hydroxylation of cholesterol at C22 or C25 does not prevent its conversion into ecdysteroids. The fact that the corresponding incubations with 22*S*-hydroxycholesterol did not lead to the formation of ecdysteroids is in agreement with its inappropriate configuration at C22. The conversion rates of 22*R*-hydroxycholesterol and 25-hydroxycholesterol were much higher than those of cholesterol, and this may merit two explanations which are not exclusive of each other: (a) these molecules are more water-soluble and can thus diffuse more rapidly; (2) they are not diluted by endogenous pools.

Of particular interest was the finding that calli converted, although at a low rate, 22*R*-hydroxycholesterol and 25-hydroxycholesterol into 20E. Labelling studies with putative precursors had already shown that, although calli do not produce ecdysteroids, they contain the hydroxylases at C2, C20 and C25. Considering that calli do not convert cholesterol itself into ecdysteroids but they do convert its two hydroxylated derivatives, it is tempting to suggest, by analogy with what is known for vertebrate steroidogenic organs, that calli lack a functional carrier protein involved in the transport of cholesterol to the first enzyme of the biosynthetic pathway, whereas a carrier protein is not required with the more hydrophilic sterols.

The efficient conversion of 25-hydroxycholesterol (and 22*R*-hydroxycholesterol) into ecdysteroids obtained in *P. vulgare* cannot be generalized to other plant systems. Similar experiments performed with two other ecdysteroid-producing systems, i.e. *Silene otites* seedlings (M. Garcia and R. Lafont, unpublished data) and *Serratula tinctoria* hairy root (J. P. Delbecque, M. F. Corio-Costet and R. Lafont, unpublished data), produced completely negative results. These results are reminiscent of those obtained with several insect systems [29], and clearly demonstrate that the substrate-specificity of biosynthetic enzymes at least differs among species. Whether this means that the biosynthetic pathway also differs between plants and animals and also between plant species remains an open question.

Future trends

Elucidating the biosynthetic pathway(s) of ecdysteroids in plants is a very stimulating challenge; how plants produce the same molecules as insect molting hormones, whether the pathway is similar or different and whether it was 'invented' only once or several times during evolution represent fascinating questions, as ecdysteroids are produced by species belonging to both Thallophytes and Trachaeophytes (ferns, gymnosperms and angiosperms). Characterization of the enzymes should accompany the elucidation of the different steps. Until now, almost nothing has been published in this area, and more is known for brassinosteroids as the result of the identification of dwarf mutants of *Arabidopsis* [30,37]. Such an approach is, however, not feasible in the present case, as no special phenotype can be expected from the presence/absence of ecdysteroids in a plant. We must therefore rely innitially on biochemical approaches, as recently adopted for the ecdysone 20-mono-oxygenase of spinach [38]. This approach will at first be used to characterize the type of enzyme involved in a given step (e.g. cytochrome P450 mono-oxygenase, 3-oxosteroid reductase), and then enzyme purification will allow its molecular characterization.

A second question of interest concerns the regulation of ecdysteroid production. It has been repeatedly observed that cell suspensions or callus cultures from ecdysteroid-producing species produce at best very small amounts of ecdysteroids, while tissue and hairy-root cultures produce much larger amounts [23,39–41]. This means that tissue organization is required for adequate productivity. In this respect, the prothalli and calli of *P. vulgare* appear to be adequate systems for analysing this problem. In addition, it is possible to induce a high rate of synthesis by thermal treatment [22], and it should be possible to test the effect of other factors in this system.

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