Plant Sterol Metabolism*

ENZYMATIC CLEAVAGE OF THE 9β,19β-CYCLOPROPANE RING OF CYCLOPROPYL STEROLS IN BRAMBLE TISSUE CULTURES

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SUMMARY

A cell-free enzyme preparation obtained from bramble tissues (Rubus fruticosus) grown in vitro was found to be capable of opening the cyclopropane ring of cycloeucalenol, producing obtusifoliol. The identification of this substance was based on the use of radiochromatography, formation of functional derivatives, and crystallization to constant specific radioactivity. A boiled preparation failed to mediate the reaction, proving that the opening of the 9β,19β-cyclopropane ring is enzymatic. Enzymatic activity was bound to the microsomal fraction and was not dependent upon the addition of exogenous ATP or NADH.

Microsomes from other higher plant species (germinating peas and tobacco tissue cultures) were found to be active in transforming cycloeucalenol into obtusifoliol, but microsomes from rabbit liver were unable to perform this reaction.

24-Methylene cycloartenol, cycloartenol, and cycloeucalenol, which are ubiquitous constituents of higher plants, were tested as substrates for the enzymatic reaction. The 4,4-dimethyl sterols are very poor substrates, whereas cycloeucalenol is efficiently converted. In a second group of experiments, three substrates differing only by the number of methyl substituents in position 4 were incubated with the microsomal fraction. Cycloeucalenol (a 4α-methyl sterol) and 24-methylene pollinastanol (a 4-desmethyl sterol) were converted to obtusifoliol and 4-desmethyl obtusifoliol, respectively. But 24-methylene cycloartenol (a 4,4-dimethyl sterol) was not transformed, which suggests that the 4β-methyl group hinders this reaction.

On the basis of the results obtained, a tentative mechanism for the opening of the cyclopropane ring is proposed.

The analysis and identification of plant sterols (1–4) raise new problems in the understanding of phytosterol biosynthesis: one such problem concerns the nature of the product of 2(3) oxidosqualene cyclization. Following the establishment of lanosterol1 as the precursor of sterols in animals and fungi (5), the identification of lanosterol as the precursor of higher plant sterols. However, lanosterol has never been identified in any higher plant tissue. Large quantities of lanosterol have been found in the latex of euphorbia species but sterols are not synthesized in the latex (6–8).

By contrast, cycloartenol, 24-methylene cycloartenol, and cycloeucalenol are ubiquitously present in photosynthetic plant tissues and this has led various authors to suggest that cycloartenol may replace lanosterol in phytosterol biosynthesis and to propose the intermediacy of 9β,19β-cyclopropyl sterols in the biosynthetic pathway leading to phytosterols (9–11) (Scheme 1).

2(3)-oxidosqualene \(\rightarrow\) cycloartenol \(\rightarrow\) 24-methylene cycloartenol \(\rightarrow\) cycloeucalenol \(\rightarrow\) obtusifoliol \(\rightarrow\) 24-methylene lophenol \(\rightarrow\) phytosterols

Scheme 1

This hypothesis was based on the following experimental results. (a) Cyclopropyl sterols, especially cycloartenol, became rapidly labeled following incubation of plant tissues with [1,4-14C]-acetate or [2,3-14C]mevalonate (12–14) and their specific radioactivities, when compared to those of phytosterols, were in agreement with those measured for sterols in plants.

1 The trivial names used are: cycloartenol, 4,4,14α-trimethyl 9β,19β-cyclo-5α-cholest-24-en-3β-ol; lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; parkeol, 4,4,14α-trimethyl-5α-cholesta-9(11),24-dien-3β-ol; 24-methylene cycloartenol, 4,4,14α-trimethyl-9β,19β-cyclo-5α-ergosta-24(28)-en-3β-ol; 24-methylene lanostenol, 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β-ol; 24-keto cycloartenol, 4,4,14α-trimethyl-24-oxo-9β,19β-cyclo-5α-cholestan-3β-ol; 24-keto lanosterol, 4,4,14α-trimethyl-24-oxo-5α-cholest-8-en-3β-ol; cycloeucalenol, 4α,14α-dimethyl-9β,19β-cyclo-5α-ergosta-24(28)-en-3β-ol; obtusifoliol, 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol; 31-nor-cycloartenol, 4α,14α-dimethyl-9β,19β-cyclo-5α-cholesta-8,24-dien-3β-ol; 31-nor-lanosterol, 4α,14α-dimethyl-5α-cholesta-8,24-dien-3β-ol; 24-methylene lophenol, 4α,14α-dimethyl-5α-ergosta-7,24(28)-dien-3β-ol; 24-ethylidene lophenol, 4α methyl 5α-stigmasta-7,24(28)-dien-3β-ol; 24-methylene pol linastanol, 14α-methyl 9β,19β-cyclo-5α-ergosta-24(28)-en-3β-ol; 4-desmethyl obtusifoliol, 14α-methyl 5α-ergosta-8,24(28)-dien-3β-ol; poriferasterol, 24(5)-24-ethyl-cholesta-5,8-dien-3β-ol; campesterol, 24(8)-24-ethyl-cholesta-5,8-dien-3β-ol.

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ment with precursor to product relationships (15). (b) Labeled cycloartenol has been isolated after anaerobic incubations of different cell-free extracts with labeled 2(3)-oxidosqualene (18-18); in no case was lanosterol detected. (c) When the incubations contained also S-adenosylmethionine, labeled 24-methylenecycloartenol was isolated (19). Furthermore, it has been demonstrated with a cell-free extract of peas that cycloartenol could be a substrate for a C-24 transmethylation reaction leading to 24-methylene cycloartenol (20). (d) Finally, labeled cycloartenol (21), 24-methylene cycloartenol, and cycloartenol (22) are converted in vivo to phytosterols by tissue cultures of Nicotiana tabacum (21) and to poriferasterol by the alga Ochrosumnas malhamensis (22).

If 98,193-cycloproplyl sterols such as cycloartenol, 24-methylene cycloartenol, or cycloartenol are intermediates in the biosynthesis of plant sterols, then an enzyme capable of opening the cyclopropane ring should be present in plants which would distinguish plants from animals and fungi. Furthermore, the absence of lanosterol and the ubiquitous presence of obtusifoliol in higher plants would suggest that this enzyme probably acts on cycloeucalenol (23).

We therefore investigated the ability of a cell-free extract from bramble tissues grown in vitro to open the cyclopropane ring of cycloeucalenol and other related substrates. Short preliminary reports on part of this work have been published (24, 25).

EXPERIMENTAL PROCEDURE

Materials

Tissue

Bramble (Rubus fruticosus) tissue cultures were obtained in 1948 by Dr. J. Morel (Versailles, France) from stems and have been described elsewhere (26). These tissues were grown under continuous white light at 25°C on a synthetic medium having the following composition: mineral solution of Heller (26) (100 ml), thiamine (1 mg), glucose (50 g), Bacto-agar (Difco, 12.5 g), and twice distilled water (900 ml). They consist mainly of undifferentiated protodifferentiated and heterotrophic cells, capable of growing in the absence of an exogenous source of indoleacetic acid.

Analytical Materials

Conventional techniques for thin layer chromatography were used throughout this study for separation and identification of the substances. Precoated thin layer plates (Merek F 254, 0.25 mm) were generally used. For argentation chromatography, thin layer plates were immersed for 1 min in a solution of 20% AgNO3 in ethanol-water (3:1, v/v); after which they were allowed to dry at room temperature for 1 hour; finally they were activated at 110°C for 30 min. Thin layer plates were sprayed with ethanolic berberin hydrochloride (0.1%), and the compounds were visualized under ultraviolet light. Radiochromatograms were scanned on a Berthold radiochromatogram scanner LB 2722. Radioactivity was determined by liquid scintillation counting with an Intertechnique ABAC, SL-40 counter. Basic alumina (Woehl, grade III) was used for column chromatography.

Gas-liquid chromatography was carried out on a Varian Aerograph model 1100 gas chromatograph equipped with a flame ionization detector. Coiled columns (1.50 m x 3 mm) were packed with either 1% OV-17, or 1% SE-30 on 80 to 100 mesh hexamethyldisilasane-treated Chromosorb W. Mass spectrometry was carried out on a Thomson-Houston THN-208 mass spectrometer at 170°C with a direct inlet.

Authentic Materials

Cycloartenol was extracted with light petroleum in a Soxhlet apparatus from a piece of eucalyptus wood (Eucalyptus robusta) kindly supplied by Dr. E. B. Huddleston (Sydney, Australia), and was purified by column chromatography. The alcohol was recrystallized from methanol giving needles, m.p. 138-140°C (literature m.p. (27) 140°C). Gas-liquid chromatography analysis showed a single peak which had the same retention time as authentic cycloartenol. The mass spectrum of the acetate showed a fragmentation pattern consistent with published data (28): m/e (relative intensity) 468(10) M+*, 408(100) [M - CH3COOH]+, 333(67) [M - CH3COOH - CH3]+, 300(13) [M - CH3COOH - C5H4]+, 283(18) [M - CH3COOH - side chain]+, and 175(3) [M - CH3COOCH - C5H4 - side chain]+.

Obutusifoliol was extracted with light petroleum in a Soxhlet apparatus from the latex of Euphorbia obtusifolia which was kindly supplied by Prof. Gonzales Gonzales (Las Palmas, Las Canarias, Spain). The extracted product was purified by column chromatography and was then recrystallized from methanol, m.p. 129-130°C (literature m.p. (29) 144°C). Gas-liquid chromatography analysis showed a single peak which had the same retention time as an authentic sample of obtusifoliol. The structure was established by nuclear magnetic resonance spectroscopy.

Cycloartenol and 24-methylene cycloartenol were kindly supplied by Prof. G. Ourisson, Strasbourg, France. Lanosterol was purified from a commercial source by recrystallization of the acetate from methanol.

24-Methylene lanosterol was synthesized from the 24-epoxide of lanosterol acetate. This latter was transformed to 24-ketone by treatment with SnCl2 in benzene (30). The 24-keto lanosteryl acetate was then converted into 24-methylene lanosterol acetate by the method of Bertini et al. (31).

24-Methylene pollinastanol was obtained as described by Knapp et al. (32). Banana peels (Musa sapientum) (10 kg fresh weight) were lyophilized for 1 week. The dried peels were then extracted 3 times with chloroform-methanol (1:1, v/v). After evaporation of the organic solvent, water was added, and the aqueous phase was extracted 3 times with light petroleum. The crude extract was chromatographed first on an alumina column. Elution with 5% diethyl ether in light petroleum gave a sterol ether fraction. After saponification, the unsaponifiable matter was extracted and then chromatographed on an alumina column. The order of elution was: 4,4-dimethyl sterols, 4acetyl sterols, and 4-desmethyl sterols. The 4-desmethyl sterol fraction was acetylated and the acetates were separated by argentation chromatography as described before. One of the two products obtained had the same RF as a 24-methylene derivative. The mass spectrum showed a fragmentation pattern consistent with published data (28).

Radiochemicals

Sodium [3H]borylborate (5 Ci per mmole) and tritiated water (6 Ci per ml) were supplied by the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France).

[5-3H]Cycloartenol—Cycloartenol (175 mg) was dissolved in 20 ml of acetone-benzene (2:1, v/v) and 0.6 ml of Jones' reagent was added dropwise while stirring at 0-5°C under N2. After 10 min, the reaction mixture was diluted with water and extracted with diethyl ether; the organic phase was diluted over Na2SO4 and the solvent was evaporated. The ketone obtained was chromatographed on an alumina column, pure cycloartenol was eluted with benzene-hexane (1:1, v/v), and finally recrystallized from acetonitrile giving needles, m.p. 84-86°C. Cycloartenol (40 mg) was reduced with NaBH4 (20 μmol) in anhydrous diethyl ether (previously distilled over CaH2) under N2 for 1 hour. The resulting cycloeucalenone was extracted, purified by thin layer chromatography, and recrystallized from methanol. The specific radioactivity of the product was adjusted to about 100 μCi per mmole and the labeled product was recrystallized again twice from methanol; the specific radioactivities of crystals from both first and second recrystallization and from the corresponding mother liquors were shown to be 100 μCi per mmole. The purity of the labeled cycloeucalenone was greater than 99% on the basis of gas-liquid chromatography.

[8-3H]Cycloartenol—The same procedure as for cycloeucalenone was used. The specific radioactivity thus obtained was about 65 μCi per mmole.

24-Methylene[2,4,4-3H]pollinastanol—24-Methylene pollinastanol was oxidized to 24-methylene pollinastanone by action of the Jones reagent as described before. The ketone was first purified by thin

1 The discrepancy between the meeting point reported in the literature and ours for obtusifoliol could be explained by the presence of a slight amount (less than 5%) in our case, as shown by gas-liquid chromatography of euphorbol (27).
layer chromatography (cyclohexane-ethyl acetate (90:10, v/v)) and then recrystallized twice from methanol. The purified ketone was triturated with the method of Klein and Knight (33). The ketone (2 mg) was dissolved in a small volume of benzene-hexane (1:1, v/v), applied to a column of basic alumina previously deactivated with tritiated water (5 Ci per ml), and left on the alumina for 90 min; the triturated ketone was then eluted with benzene-hexane (1:1, v/v) and reduced by LiAlH4 in anhydrous diethyl ether giving a mixture of 3β-OH and 3α-OH isomers. The 3β-OH isomer (Rf 0.40) and the 3α-OH isomer (Rf 0.48) were easily separated by thin layer chromatography (cyclohexane-ethyl acetate (90:10, v/v), two migrations). The 24-methylene-2α,4α-H2pollinastanol (3β-OH) thus obtained was more than 98% pure by gas-liquid chromatography analysis. The specific radioactivity thus obtained was 29 μCi per μmol.

[3α,4α]-24-Methylene cycloartenol was kindly prepared by Dr. R. Labriola, Buenos Aires, Argentina. The 24-epoxide of cycloartenyl acetate was treated with SnCl4 in dry benzene (30). The 24-ketocycloartanyl acetate obtained was then treated with [3α,4α]-24-methylene cycloartenyl acetate, as described elsewhere (34), giving the expected 24-methylene cycloartenol with a specific radioactivity of 25 μCi per μmol.

Methods

Preparation of Subcellular Fractions

Bramble tissues (50 g fresh weight) were homogenized in a loosely fitting Elvehjem-Potte Teflon glass homogenizer in the presence of twice their weight of a buffer containing: 0.25 M saccharose, 0.004 M MgCl2, 0.000 M cysteine, and 0.1 M Tris-HCl (pH 7.0). The extract was filtered through four layers of muslin, and the filtrate was passed through nylon (Blutex, Triplette et Renuard, 50 μm). An initial centrifugation at 150,000 X g for 10 min gave a pellet of crude nuclei and plastids (C1) and a supernatant. This centrifuge at 6,000 X g for 10 min gave a crude mitochondrial pellet (C2) and a second supernatant which was further centrifuged at 120,000 X g for 90 min to give the microsomal pellet (C3) and a supernatant (S120). This was used for the preparation of a soluble supernatant fraction. The pellets (C1, C2, and C3) were resuspended in the following medium: 2 mM Mg2+, 2 mM mercaptoethanol, 20 mM Tris-HCl (pH 7.7) (Medium A).

Preparation of Soluble Supernatant Fraction

Protein of the supernatant S120 was fractionated with solid ammonium sulfate. The fraction which precipitates between 45 and 80% of saturation was dissolved in a minimal volume of Medium A and dialyzed against Medium A for 12 hours. Glycerol (30%, v/v) was finally added to the diazylated enzymes. The mixture was stored at -20°C.

Activity Assays

Assay mixtures contained 20 mM Tris-HCl (pH 7.6), 6 mM Mg2+, 6 mM K+, particles, supernatant fraction, and finally the substrate was dissolved in 50 μl of a solution of 0.5% Tween-80 in acetone. Incubations were carried out for 5 hours at 31°C in an atmosphere of the labeled substrates and terminated by addition of 10% KOH in water. In each experiment, a duplicate consisting of particles and 24-ethylidene lophenyl acetate3 (fastest migrating fraction), cycloartenyl + obtusifoliyl acetate, and 24-methylene cycloartenyl + 24-methylene lanostanyl acetate (slowest migrating fraction). These fractions were analyzed by gas-liquid chromatography and by mass spectrometry as previously described (12). The partially purified acetates were epoxidized with perbenzoic acid and analyzed for 24-methylene obtusifoliyl acetate as described earlier (12). The reaction was carried out at room temperature overnight and was stopped by addition of an aqueous solution of Na2CO3 (5% w/v). Epoxides of 4,4-dimethyl sterol acetates were extracted 3 times with diethyl ether and were chromatographed on thin layer plates (ethyl acetate, cyclohexane (10:90, v/v), two migrations). In these conditions, the 24(28)-epoxide of 24-methylene cycloartenyl acetate could be easily separated from the 8,24(28)-diepoxide of 24-methylene lanostanyl acetate, and the 24-epoxide of cycloartenyl acetate could be separated from the 8,24-diepoxide of lanosteryl acetate.

4α-Methyl Sterols—These were treated as the 4,4-dimethyl sterols. After argemotin chromatography, three fractions were obtained corresponding to cycloartenyl + lanostanyl acetates (fastest migrating fraction), parkeyl acetate, and 24-methylene cycloartenyl + 24-methylene lanostanyl acetates (slowest migrating fraction). After epoxidation and chromatography of the epoxides of 4α-methyl sterol acetates, the 24(28)-epoxide of cycloartenyl acetate could be separated from the 8,24(28)-diepoxide of obtusifoliyl acetate.

4-Desmethyl Sterols—After argemotin chromatography, three fractions were obtained, corresponding to sioasteryl + campesterol acetates (fastest migrating fraction), isofucosterol acetate and 24-methylene pollinastanyl + 4-desmethyl obtusifoliyl acetate (slowest migrating fraction). Using the same techniques as described before, the 24(28)-epoxide of 24-methylene pollinastanyl acetate could be easily separated from the 8,24(28)-diepoxide of 4α-methyl obtusifoliyl acetate.

In most cases, the analytical procedure was completed by crystallization of the purified epoxides of sterol acetates to a constant specific radioactivity.

Results

Evidence for Enzymatic Opening of Cyclopropane Ring of Cycloartenol

A supernatant (S1) and a control consisting of an identical enzyme preparation inactivated by heating at 100°C for 30 min were incubated with [3H]cycloeucalenol (Table I). After thin layer chromatography of the nonsaponifiable fraction, which separates 4,4-dimethyl sterols (Rf 0.45) from 4α-methyl sterols (Rf 0.40) and from 4-desmethyl sterols (Rf 0.25), radioscanning showed the presence of only one peak, with an Rf value corresponding to that of 4α-methyl sterols. After acetylations, the 4α-methylsterol acetates were separated by argemotin chromatography into 24-methylene lophenyl acetate (fastest migrating fraction), parkeyl acetate, and 24-methylene cycloartenyl + 24-methylene lanostanyl acetate (slowest migrating fraction). These fractions were analyzed by gas-liquid chromatography and by mass spectrometry as previously described (12).
Enzymatic opening of 9β,19β-cyclopropane ring of cycloeucalenol
A crude supernatant $S_1$ (10 ml) of bramble homogenate was used in the experiment; the assay mixture contained 0.25 mM sucrose, 4 mM Mg$^{2+}$, 5 mM cysteine, 0.1 M Tris-HCl (pH 7.6), and 5 μM [3H]-cycloeucalenol.

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Radioactivity of substrate</th>
<th>Radioactivity recovered in 24,28-methylene sterols</th>
<th>24(28)-Epoxide of cycloeucalenyl acetate</th>
<th>8,24(28)-Diepoxide of obtusifoliyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment ($S_1$)</td>
<td>$10 \times 10^4$</td>
<td>$9.4 \times 10^4$</td>
<td>$2.3 \times 10^4$</td>
<td>$1.5 \times 10^4$</td>
</tr>
<tr>
<td>Control (boiled)</td>
<td>$11 \times 10^4$</td>
<td>$10.2 \times 10^4$</td>
<td>$2.0 \times 10^4$</td>
<td>$0.002 \times 10^4$</td>
</tr>
</tbody>
</table>

TABLE II
Variation of specific radioactivity of diepoxide of obtusifoliyl acetate during recrystallization
Carrier diepoxide of obtusifoliyl acetate (11.75 mg) was added to the labeled diepoxide of obtusifoliyl acetate (8.5 × 10$^4$ dpm). The specific radioactivity of the crude mixture before recrystallization was 72,000 ± 4,000 dpm per mg.

Intracellular localization of enzymatic activity
Assays were performed under standard conditions; particles (2 ml ± 3.4 mg of proteins) were incubated in the presence of 10 μM [3H]-cycloeucalenol and, where indicated, soluble supernatant fraction (2 ml = 2 mg of proteins).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Diepoxide of obtusifoliyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/mg of membrane proteins</td>
</tr>
<tr>
<td>C1</td>
<td>0.22</td>
</tr>
<tr>
<td>C2</td>
<td>0.30</td>
</tr>
<tr>
<td>C10</td>
<td>2.75</td>
</tr>
<tr>
<td>C120 + soluble supernatant fraction</td>
<td>2.85</td>
</tr>
<tr>
<td>Soluble supernatant fraction alone</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Conclusions
The results (Table III) show that the highest enzymatic activity was associated with microsomes and that the soluble supernatant fraction showed only a very low activity and was unable to stimulate significantly the activity of the microsomal fraction.

For this reason only differences greater than 30% have been considered as significant in the work described below.

Cofactors Required for Enzymatic Cleavage of Cyclopropane Ring of Cycloeucalenol
Microsomes (2 ml) corresponding to 3.5 mg of proteins were incubated under standard conditions and in the presence of ATP (4 mM) and NADH (0.5 mM). No significant difference (+25%) in obtusifoliol formation could be observed whether ATP and NADH were present or not. Thus ATP and NADH do not seem to play any role in the cyclopropane ring-opening reaction.

Substrate Specificity of Enzyme
Behavior of Cyclopropyl Sterols Present Ubiquitously in Higher Plants Toward Enzyme—In most plant tissues, cycloartenol and 24-methylene cycloartanol are present together with cycloeucalenol. A biogenetic scheme leading to phytosterols (15) postulated that these three compounds could be substrates of a cyclopropane ring-opening enzyme. This ring-opening reaction is important in the transformation of cycloeucalenol into obtusifoliol has taken place...
possible since authentic 4-desmethyl obtusifoliol was not availa-
respectively. Complete identification of the latter was not
converted at about the same rate to obtusifoliol and a product
lanostenol, cycloeucalenol and 24-methylene pollinastanol were
24-methylene cycloartanol was not converted to 24-methylene
carbon 4). Results presented in Table V show clearly that while
methyl groups on carbon 4; i.e. 24-methylene cycloartanol (two
hand, and 24-methylene cycloartanol and cycloeucalenol, on the
other, is the absence in cycloeucalenol of the 4cy-methyl group.
With this in mind, a second set of experiments was performed
in higher plants, only cycloeucalenol was efficiently attacked.

Influence of Number of Methyl groups in four on enzymatic opening of
9,19β-cyclopropane ring; comparative incubations of
24-methylene cycloartanol, cycloeucalenol, and
24-methylene pollinastanol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Radioactivity in 8,24(28)-diepoxide (dpm)</th>
<th>Amount of substrate converted (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-Methylene cycloartanol</td>
<td>2.4 x 10⁶</td>
<td>75</td>
</tr>
<tr>
<td>Cycloeucalenol</td>
<td>4.0 x 10⁶</td>
<td>30</td>
</tr>
<tr>
<td>24-Methylene pollinastanol</td>
<td>2.1 x 10⁶</td>
<td>40</td>
</tr>
</tbody>
</table>

* Radioactivity in 8,24(28)-diepoxide of 24-methylene lanosteryl acetate.
  b Radioactivity in 8,24(28)-diepoxide of 24-methylene lanostenyl acetate.
  c Radioactivity in 8,24(28)-diepoxide of 24-methylene cycloartanol.
  d Diepoxide of cycloartenyl acetate.
  e Diepoxide of 24-methylene lanostenyl acetate.
  f Diepoxide of obtusifoliyl acetate.
the enzyme, giving Compound III as proposed previously by Rees et al. (19). Specific attack and elimination of a proton at C-19 by a basic radical of the enzyme leads to the formation of the cyclopropene ring and to the subsequent trans-elimination of radical B, giving cycloartenol IV (in that case, trans-elimination of proton 8 and radical B which give lanosterol would be forbidden). After two more enzymatic transformations (24-methylation and 4-demethylation), cycloartenol gives cycloeucalenol VI which is a 4α-methyl compound. By a "push-pull" mechanism involving perhaps the same radicals A and B of the enzymatic system, cycloeucalenol would be transformed into intermediate VII which could be identical with III but without a 4β-methyl. In this last intermediate, trans-elimination of proton 8 and radical B would be possible and would be performed by another radical C, leading to obtusifoliol VIII (23).

The opening of the cyclopropane ring of cycloeucalenol involves unfavorable 4β-methyl, 10-methyl interactions, i.e. high transition state energy, in intermediate III which do not exist in the case of intermediate VII derived from cycloeucalenol. Therefore, from a thermodynamic and kinetic point of view, one would expect the opening of the cyclopropane ring to be more favorable in the case of cycloeucalenol compared to cycloartenol. Another critical step in this hypothetical scheme would be the proton elimination from the intermediates III and VII. This operation could be induced by a specific active group of the enzymatic protein and would also be controlled by specific structural characteristics of the substrate. Possibilities are the number of methyl groups at C-4 and their stereochemistry. Our laboratory is investigating the influence of the stereochemistry at the C-4 position by using the 4β-methyl isomer of cycloeucalenol.

Microsomes prepared from rabbit liver were unable to open the cyclopropane ring of cycloeucalenol and to transform it into obtusifoliol. As cycloeucalenol was shown to be a suitable substrate for testing the presence of a cyclopropane ring-opening enzyme, our results suggest that this enzyme may be restricted to higher plants or perhaps more generally to photosynthetic eukaryotes. Gibbons et al. (39) reached the same conclusion in showing that a cell-free extract from rat liver did not transform cycloartenol to lanosterol.

Finally, as neither cycloartenol nor 24-methylene cycloartenol is a substrate for the enzyme, it is possible to rule out lanosterol and 24-methylene lanosterol from the major phytosterol pathway. The results of the present study support a modification of a previous scheme (15) in which it was proposed that lanosterol and 24-methylene lanosterol were intermediates. It is suggested (Scheme 3) that a branch point exists at the level of cycloartenol, which may be methylated to give 24-methylene cycloartenol following the pathway postulated by other workers (11), or demethylated to give 31-nor-cycloartenol. This latter compound may be methylated to give cycloeucalenol or may also be a substrate for the enzyme discussed here leading to 31-nor-lanosterol. The passage from the pathway represented by broken lines to the pathway represented by full lines involves the reaction of C-24 methylation which has been demonstrated in vitro with a microsomal system from bramble tissue cultures (19) and with a cell-free extract of pea seedlings (20). Finally the two parallel routes converge at obtusifoliol. This scheme is in agreement with the kinetic results (19), with the absence of lanosterol and the occurring

### Table VI

**Comparison of cycloeucalenol-obtusifoliol isomerase activity in microsomes obtained from different organisms**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Radioactivity of substrate</th>
<th>Substrate concentration</th>
<th>Radioactivity in dephosphorylated obtusifoliol acetate</th>
<th>Amount of substrate converted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm</td>
<td>μM</td>
<td>dpm</td>
<td>mmole/mg of proteins</td>
</tr>
<tr>
<td>Bramble tissue cultures</td>
<td>$9.4 \times 10^4$</td>
<td>4.7</td>
<td>$1,120 \times 10^4$</td>
<td>1.5</td>
</tr>
<tr>
<td>Germinating pea seeds</td>
<td>$13 \times 10^4$</td>
<td>13</td>
<td>$180 \times 10^4$</td>
<td>0.023</td>
</tr>
<tr>
<td>Tobacco tissue cultures</td>
<td>$6.4 \times 10^4$</td>
<td>8.2</td>
<td>$160 \times 10^4$</td>
<td>0.12</td>
</tr>
<tr>
<td>Rabbit liver</td>
<td>$4.9 \times 10^4$</td>
<td>11.6</td>
<td>$&lt;10^4$</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Discussion**

Evidence has been given for the presence of an enzyme capable of opening the cyclopropane ring of cycloeucalenol in a cell-free extract of bramble tissue cultures. This enzyme transforms cycloeucalenol into obtusifoliol. Demonstration of this enzymatic activity in cell-free extracts prepared from plant tissues belonging to three different families (rosaceae, solanaceae, and legumes), support the generality of this reaction in plants.

The enzymatic activity is tightly bound to microsomal membranes, and soluble supernatant enzymes are unable to stimulate significantly the activity of microsomes. Thus it could be concluded that a soluble protein factor such as that involved in rat liver squalene epoxidase (37), or such as the sterol carrier protein described in many other steps of cholesterol biosynthesis (38), does not seem to be involved here.

Studies of the enzyme specificity toward natural cyclopropyl sterols possessing a different number of methyl groups on carbon 4 have surprisingly shown that 4,4-dimethyl cyclopropyl sterols (24-methylene cycloartenol and cycloartenol) are very poor substrates, whereas cycloeucalenol which is a 4α-methyl sterol is a good substrate as is 24-methylene pollinastanol, a 4-desmethyl cyclopropyl sterol. This suggests that the presence in 4,4-dimethyl cyclopropyl sterols of a 4β-methyl group hinders the action of the enzyme in some way, either by interfering with the binding of enzyme and substrate or by hindering the approach of a chemical group essential for the opening of the cyclopropane ring.

This enzyme operates without addition of exogenous ATP or NADH, in agreement with a hypothetical reaction mechanism equivalent to an acid-catalyzed isomerization like that involved in the cyclisation of 2(3)-oxidosqualene. Biosynthesis of cycloartenol involves formation of the 9β,19β-cyclopropane ring; cleavage of this same ring occurs during obtusifoliol formation. The similarities existing between cyclopropane ring cleavage and 4-demethylation, cycloartenol gives cycloeucalenol VI which is a 4α-methyl compound. By a "push-pull" mechanism involving perhaps the same radicals A and B of the enzymatic system, cycloeucalenol would be transformed into intermediate VII which could be identical with III but without a 4β-methyl. In this last intermediate, trans-elimination of proton 8 and radical B would be possible and would be performed by another radical C, leading to obtusifoliol VIII (23).

The opening of the cyclopropane ring of cycloeucalenol involves unfavorable 4β-methyl, 10-methyl interactions, i.e. high transition state energy, in intermediate III which do not exist in the case of intermediate VII derived from cycloeucalenol. Therefore, from a thermodynamic and kinetic point of view, one would expect the opening of the cyclopropane ring to be more favorable in the case of cycloeucalenol compared to cycloartenol. Another critical step in this hypothetical scheme would be the proton elimination from the intermediates III and VII. This operation could be induced by a specific active group of the enzymatic protein and would also be controlled by specific structural characteristics of the substrate. Possibilities are the number of methyl groups at C-4 and their stereochemistry. Our laboratory is investigating the influence of the stereochemistry at the C-4 position by using the 4β-methyl isomer of cycloeucalenol.

Microsomes prepared from rabbit liver were unable to open the cyclopropane ring of cycloeucalenol and to transform it into obtusifoliol. As cycloeucalenol was shown to be a suitable substrate for testing the presence of a cyclopropane ring-opening enzyme, our results suggest that this enzyme may be restricted to higher plants or perhaps more generally to photosynthetic eukaryotes. Gibbons et al. (39) reached the same conclusion in showing that a cell-free extract from rat liver did not transform cycloartenol to lanosterol.

Finally, as neither cycloartenol nor 24-methylene cycloartenol is a substrate for the enzyme, it is possible to rule out lanosterol and 24-methylene lanosterol from the major phytosterol pathway. The results of the present study support a modification of a previous scheme (15) in which it was proposed that lanosterol and 24-methylene lanosterol were intermediates. It is suggested (Scheme 3) that a branch point exists at the level of cycloartenol, which may be methylated to give 24-methylene cycloartenol following the pathway postulated by other workers (11), or demethylated to give 31-nor-cycloartenol. This latter compound may be methylated to give cycloeucalenol or may also be a substrate for the enzyme discussed here leading to 31-nor-lanosterol. The passage from the pathway represented by broken lines to the pathway represented by full lines involves the reaction of C-24 methylation which has been demonstrated in vitro with a microsomal system from bramble tissue cultures (19) and with a cell-free extract of pea seedlings (20). Finally the two parallel routes converge at obtusifoliol. This scheme is in agreement with the kinetic results (19), with the absence of lanosterol and the occurring.

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1. It has been shown (30) that a latex from *Euphorbia lathyris* can transform cycloartenol into lanosterol. This result is consistent with the presence of both cycloartenol and lanosterol in this system; it would imply the involvement of a different reaction mechanism from that proposed in this article to explain the opening of the cyclopropane ring.

2. It has been recently shown in our laboratory that a yeast cell-free extract cannot open the cyclopropane ring of any 4,4-di- methyl, 4α-methyl, and 4-desmethyl cyclopropyl sterol (C. Anding, L. Parks, and G. Ourisson, unpublished results).

3. R. Heintz, unpublished results.
Scheme 2

Scheme 3
rence of cycloeucalenol and obtusifoliol together in higher plants and with the identification in some species of 31-nor-cycloartenol (40) and 31-nor-lanosterol (41); relevant to this last point, the identification of cholesterol in all plant tissues studied up to now supports the idea that 31-nor-cycloartenol and 31-nor-lanosterol are ubiquitously distributed in higher plant kingdom.

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Plant Sterol Metabolism: ENZYMATIC CLEAVAGE OF THE 9β,19β-CYCLOPROPANE RING OF CYCLOPROPYL STEROLS IN BRAMBLE TISSUE CULTURES
Roland Heintz and Pierre Benveniste


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