Concerning the Role of Lanosterol and Cycloartenol in Steroid Biosynthesis*

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SUMMARY

An homogenate of rat liver which was capable of converting 24,28,24-dien-3β-ol to 24-hydroxycholesterol 5-ene-3β-ol failed to convert 24,28,24-dien-3β-ol to 24-hydroxycholesterol 5-ene-3β-ol. However, both substrates underwent reduction at C-24, 25. 24, 28, 24-dien-3β-ol was not converted to 24,28,24-dien-3β-ol. Both the presence and biosynthesis (from 24,28,24-dien-3β-ol) of 24,28,24-dien-3β-ol were demonstrated in vivo in corn seedlings. The latter were also shown to contain 24,28,24-dien-3β-ol as their dominant sterol with lesser amounts of 24,28,24-dien-3β-ol and 24,28,24-dien-3β-ol. A very small amount of cholesterol 5-ene-3β-ol and traces of 24,28,24-dien-3β-ol were detected. The three major sterols as well as stigmast-5, 24(28)-diene-3β-ol and 24-methylene-24(28)-diene-3β-ol were shown to be labeled after incubation with 24,28,24-dien-3β-ol. The yields were nearly the same from either substrate. The latter data, which were obtained in vivo with an alga, confirm earlier observations made in vivo with an alga and with tissue cultures of an angiosperm. They indicate that lanosterol, 24,28,24-dien-3β-ol (lanosterol) and its 9,19-cyclo derivative (cycloartenol) are both acceptable precursors in the biosynthesis of the major sterols of photosynthetic organisms. However, the data obtained with the liver homogenate demonstrate that a typical (non-photosynthetic) tissue of the animal kingdom is more restricted in its enzymatic capability. The liver preparation did not contain an isomerase for the opening of the 9,19-cyclo grouping, and, except for the 24-reductase, the enzymes in the pathway would not accept the 9,19-cyclosteroid. Consequently, discrimination between lanosterol, 24,28,24-dien-3β-ol and its 9,19-cyclo derivative in liver is made at two points in the sterol pathway (cyclization and utilization), whereas in photosynthetic systems only one point (cyclization) governs the choice.

The discovery that cycloartenol (I), the 9,19-cyclo derivative of lanosterol (II), is the most widespread 4,4,14-trimethyl sterol in photosynthetic organisms has prompted the suggestions (1, 2, 5) that cycloartenol is the intermediate in the biosynthesis of the steroids which these organisms contain. The hypothesis has recently received considerable verification through the experimental conversion of cycloartenol to 24-alkyl sterols in the alga Ochromonas malhamensis (6) and in the angiosperm Nicotiana tabacum (7), as well as by the isolation of the squelene 2,3-oxide cyclase required for the formation of cycloartenol from O. malhamensis (8) and Phaseolus vulgaris (9).

1 Although lanosterol and its derivatives have been reported to be present in both fungi and some members of the Euphorbiae, the accumulated evidence currently indicates that cycloartenol is the most widespread 4,4,14-trimethyl sterol in photosynthetic organisms. This conclusion stems from observations in the laboratories of Schreiber (1), Benveniste, Hirth, and Olivecrona (2). The literature has recently been reviewed by Good (3).

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Since this information strongly suggests a molecular difference in the pathways operating in the photosynthetic versus the non-photosynthetic branches of biology, it affords an opportunity to examine their evolutionary origins and the influence of evolution on regulatory phenomena. In particular, the utilization of cycloartenol for steroid biosynthesis requires that an isomerase be present to rearrange the molecule with opening of the 9,10-ring and formation of a double bond, e.g., the conversion of cycloartenol to lanosterol. If the photosynthetic and nonphotosynthetic organisms both had a common origin in an organism utilizing cycloartenol, the isomerase might still be present as a vestige in the animal kingdom. On the other hand, if both had a common ancestor which utilized lanosterol, the photosynthetic organisms might accept lanosterol as well as cycloartenol. The common ancestor which utilized lanosterol, the photosynthetic vestige in the animal kingdom. On the other hand, if both had a common ancestor which utilized lanosterol, the photosynthetic organisms might accept lanosterol as well as cycloartenol. The latter has already been found to be the case with O. malhamensis (6) and N. tabacum (7). However, similar studies of the two substrates in animal tissue have not been previously made.

To shed further light on this problem we have undertaken a comparison of the utilizations of cycloartenol and lanosterol in rat liver homogenates and in vivo in corn seedlings. Previous plant experimentation had employed lower plants (6), or else substrates were used (18) which precluded the latter has already been found to be the case with O. malhamensis (6) and N. tabacum (7). However, similar studies of the two substrates in animal tissue have not been previously made.

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Experimental Procedure

Radiochemicals p, J-2-14C-Mevalonic acid dibenzylethylene-diamine salt (14.08 μCi per mg) was supplied by New England Nuclear. Tritiated water (specific activity 1 Ci per g) and 4-14C-cholesterol were also obtained from this firm. Cycloartenol and lanosterol, labeled with either 14C or 3H, were prepared as described in subsequent paragraphs.

Mass Spectroscopy—Spectra were determined by the Morgan-Schaffer Corporation, Montreal, Canada, at 90° with a direct inlet. The instrument was an Hitachi RMU-GD distributed by the Perkin-Elmer Corporation, Norwalk, Connecticut, unless otherwise noted.

Authentic Materials—Commercial cholesterol (IV-a) (Fisher) purified by recrystallization from methanol-chloroform formed needle-like crystals, m.p. 147–149° Similar purification of sitosterol (IV-d) (Calbiochem) yielded a mixture, m.p. 138–139°, consisting of 67% sitosterol, 20% campesterol (Structure IV-b), and 13% stigmasterol (Structure III-d) as judged by gas-liquid chromatography. Pure lanosterol (Structure II) was prepared from the commercial material (Mann) by recrystallization from methanol-chloroform followed by acetylation (acetic anhydride-pyridine) and subsequent chromatography on thin layers of silver nitrate-impregnated silica gel. The purified lanosterol acetate was saponified, and the free alcohol (I) was recrystallized from methanol-chloroform to give needles, m.p. 136–138° (literature m.p. 138–139°). Cycloartenol (I) was extracted with acetone in a Soxhlet apparatus from dried, ground seeds of Strychnos nux vomica (21) and purified as the acetate by chromatography on thin layers of silver nitrate-impregnated silica gel. From methanol-chloroform the acetate formed plates, m.p. 120–122° (literature, m.p. 122–124° (21)). Gas-liquid chromatographic analysis showed only one peak which had the same retention time as authentic cycloartenyl acetate. The mass spectrum showed peaks at m/e 468 (molecular ion), 453, 408, 393, 366, 357, 339, 297, 286, and 175, values which are consistent with the published data for cycloartenol (23–25). Saponification yielded the free alcohol (I) as needle-like crystals, m.p. 107–110° (literature m.p. 99–115°, solvated (21)) after recrystallization from methanol-chloroform followed by decocation in a vacuum at 60°. Gas-liquid chromatographic analysis showed the presence of only one component. Cycloartenyl acetate (3β-acetate of Structure VI-a) was prepared by the catalytic reduction of cycloartenyl acetate according to the method of Barton (26). The product was recrystallized from methanol and exhibited a melting point of 130–131° (literature (26), m.p. 132–133° (26)). 24-Methylene cycloartenol (VI-b) was the kind gift of Dr. M. Shimizu and was also isolated from coffee oil. α- and β-Amyrin were commercial material supplied by Pierce Chemical Company, Rockford, Illinois. Pollinastanol was the kind gift of Dr. M. Barbier and E. Lederer, Institut de Chimie des Substances Naturelles, Gif-Sur-Yvette, France. The plant sources of fucosterol and isofucosterol and synthesis of ostreasterol have been previously described (27).

Bromination and Debrornination—Sterols were purified via bromination and debrornination according to the method of Schwenk and Werthessen (28).

Plants Used for Metabolic Studies—Seeds of the corn Zea mays (var. Silver Cross Bantam) were obtained from W. Atlee Burpee Company, Philadelphia, Pennsylvania. They were grown for 10 to 14 days in a greenhouse after which the stems were cut just above the roots. The seedlings (about 12 cm) devoid of roots (0.32 g per seedling) were used for metabolic studies. Similar seedlings of Z. mays (var. White Horsetooth) were used for nonmetabolic studies.

Chromographic Procedures—Unless otherwise noted chromatography on a column of alumina was performed with adsorbent (neutral, activity grade I, manufactured by M. Wochm-Excheweg of Germany and distributed by Aluphar Chemicals, New Orleans, Louisiana) which was deactivated with 3% (w/v) of water. Lipids were eluted with increasing amounts of ether in ligroin. 4, 4-Dimethyl compounds were removed first, followed by 4-methyl compounds, followed by compounds lacking substitution at C-4. An unequivocal determination of the nature...
of each fraction was possible from the \( R_F \) obtained by chromatography on a thin layer of silica gel. Several types of thin layer chromatography were used. The silica gel was manufactured by E. Merck A. G. of Darmstadt, Germany, and supplied by Brinkmann Instruments, Inc., Westbury, New York. Unless otherwise noted, plates of Silica Gel G (0.25-mm thickness) were used which had been impregnated or sprayed with Rhodamine 6G (29). With this technique, the lipid-containing regions could be visualized as fluorescent bands or spots under ultraviolet light. Chloroform was used as the developing solvent unless otherwise noted in nonargentation chromatography. Regions of the plate containing the required materials were scraped off and the adsorbed material eluted with ether. Although \( R_F \) values varied somewhat from day to day, a typical separation gave \( R_F \) values for squalene, cycloartenol (I), lanosterol (II), cholesterol (IV-a), and sitosterol (IV-d) of 0.80, 0.25, 0.25, 0.14 and 0.14 respectively. Mixtures of steryl acetates differing in the number or position of double bonds (e.g. lanosterol acetate (acetate of II) and 24,25-dihydrolanosterol acetate (acetate of VII-a)) were separated by chromatography on thin layers (0.25 mm) of Silica Gel G impregnated with 10% (w/w) of silver nitrate (30). The 4,4-dimethyl steryl acetates (lanosterol acetate, etc.) were separated using benzene-hexane (50:50, v/v) as the developing solvent. Gas-liquid chromatography was carried out on a device equipped with on-column injector port, flame ionization detector, and an FID (28). Preparative chromatography was carried out on the F and M instrument by utilizing a stream splitter which allowed 10% of the effluent to pass through the detector. The remaining 90% passed into a glass capillary tube connected to one arm of the splitter by means of a Swagelok attachment and a rubber septum. The capillary was situated outside the instrument at ambient temperature and this effected the efficient condensation of the separated materials (31). With this technique, it was possible to change fractions at intervals of 1 min with less than a 5-s delay during changeover. The condensed material was transferred to scintillation vials by flushing the capillaries with ether delivered by a 100-μl syringe. The same instrumental system was used in conjunction with a Nuclear-Chicago proportional counter for continuous measurement of radioactivity in the effluent.

Radioassay—Radioactivity was assayed on a Nuclear-Chicago Scintillation System 724 at an efficiency of 75% for \(^{14}C\) and 30% for \(^{3}H\). Radioscanning of developed chromatoplates was accomplished on an Atomic Accessories Model RSC-363 radiochromatogram scanner fitted with a model 1-004 integrator.

Preparation of \(^1\text{C}\)-Lanosterol from \(^2\text{H}\)-Mevalonate in Liver Homogenate—To 16 ml of a rat liver homogenate prepared according to the method of Bucher (32) was added water (3 ml) containing NADH (32 mg), NADPH (8 mg), ATP (16 mg), and glutathione (20 mg). \(^2\text{H}\)-Mevalonate (22.0 μg) was added as a solution in 1.0 ml of water. A solution of unlabeled lanosterol (5.0 mg) in 0.5 ml of acetone was also added as a trapping agent. The homogenate was aerobically incubated for 3 hours at 37°C after which a solution of KOH (5 g) in ethanol (100 ml) was added and the mixture saponified under reflux for 1½ hours. The neutral lipids (75.14 mg, 12.97 × 10^6 dpm) were isolated by extraction with ether. \(^1\text{C}\)-Lanosterol, isolated by thin layer chromatography (both with and without silver nitrate), contained 3.71 × 10^6 dpm (1.52 × 10^9 dpm per mg). A portion of this material was subjected to gas-liquid chromatography. The peak of radioactivity coincided with the peak of mass for lanosterol. In addition, after admixture with unlabeled lanosterol and three recrystallizations from methanol, one sample of the \(^1\text{C}\)-lanosterol maintained a constant specific activity (1640 dpm per mg, 1800 dpm per mg, and 1830 dpm per mg, respectively). The specific absence of any contaminating 4-desmethyl sterol was demonstrated by thin layer chromatography. The maximum level of contamination by cholesterol in particular was not more than 0.1%.

Preparation of \(2\text{H}\)-Lanosterol—Pure lanosterol was oxidized to the ketone (lanosta-8,24-dien-3-one) by the method of Bentley et al. (21). It (8.0 mg, m.p. 80–82°C, \( R_F \) 5.93 μl; literature, m.p. 81–82°C (20)) was dissolved in 1.0 ml of n-hexane and passed through a column of 3.0 ml of alumina (basic) which contained 0.12 ml of H_2O (120 mCi) (33). After elution with 5% ether-hexane a portion of the isolated ketone (5.12 mg, 3.93 × 10^6 dpm per mg) was reduced with excess lithium aluminium hydride in 2.0 ml of ether at reflux for 2.0 hours. After destruction of the excess hydride with moist ether, \(2\text{H}\)-lanosterol (4.64 mg, 3.81 × 10^6 dpm per mg) was isolated. It was purified by thin
layer chromatography and finally crystallized from methanol-
chloroform yielding colorless needles, m.p. 135-136° (literature
m.p. 138-139° (20)). 3.37 x 10^5 dpm per mg. When the product
was submitted to gas-liquid chromatography as the acetate with
continuous measurement of radioactivity in the effluent, a single
radioactivity peak (relative retention time 1.67) was obtained.
When the free alcohol was crystallized from methanol-chloro-
form four times together with authentic material no loss of label
occurred.

Preparation of 2-H-Cycloartenol—The labeled cycloartenol was
prepared as described for the correspondingly labeled lanosterol.
The final product (2-H-cycloartenol, 4.15 mg) was crystallized from
methanol-chloroform and dried under reduced pressure at
room temperature for 48 hours (m.p. 108-113°, 1.78 x 10^5 dpm
per mg). The literature (21, 26) records difficulties in obtaining
a sharp melting point for cycloartenol, the highest recorded
value being 115° (21) and the lowest 85-92° (26). This appears
to be a problem of solvation (21). We observed the same prob-

Isothermal Denaturation of Dominant Sterols of Corn Seed-
ings—The neutral fraction after saponification of 44 seedlings
was chromatographed on a column of alumina with a gradient
elution system of n-hexane into ether. One of several peaks of
material contained 5.91 mg which, when subjected to thin layer
chromatography, gave a single spot which had moved the same
distance as authentic sitosterol (R f 0.16). Gas-liquid chro-
matography revealed three major components and a trace of sub-
stance with the retention time of cholesterol. The relative retention
times of the major compounds were 1.31, 1.39, and 1.60. These retention times corresponded, respectively, to those
of authentic campesterol (1.31), stigmasterol (1.36), and sitos-
erol (1.59). They constituted 17%, 21%, and 60% respectively,
of the mixture. The remaining 2% was a component with the relative retention time of cholesterol (1.00). After acetylation of the mixed sterols and argentation chromatography,
a major spot coincident with that of sitosterol acetate was ob-
tained which indicated that nearly all of the sterol possessed
only one double bond. The mixture of free alcohols was crystal-
ized twice from methanol-chloroform to give needles, m.p. 133-
136°. The infrared spectrum of the sterol mixture exhibited a
weak peak at 907 cm⁻¹ corresponding to the presence of some
Δ²-sterol. The absorption at λ max 11.91, 12.12, and 12.40 μ
was precisely the same as that found generally for Δ²-sterols.
These data are in agreement with the assignments based on
chromatographic data. The structures were further confirmed
by the mass spectrum of the mixed acetates. Weak molecular
ion peaks occurred at m/e 456 and 442, the former being the
stronger. The m/e values correspond to composition of C₃₃H₆₀₂
and C₃₂H₅₈O, respectively. These are equivalent to compositions
of C₃₃H₆₀O and C₃₂H₅₈O for the free sterols (24-ethyl-
and 24-methylcholesterol, respectively). A molecular ion peak at
m/e 454 of the same intensity as the one at m/e 442 also was
apparent and indicated the presence of a diene (C₁₃H₂₆O or
C₁₃H₂₄O as the free sterol). The essential absence of a corre-
sponding peak at m/e 394 for loss of CH₂COOH from the latter
molecular ion indicated the presence of a Δ²-bond, since strong
allylic cleavage with loss of the side chain effectively competes
with loss of CH₂COOH. The empirical formula and presence of
a Δ²-bond are consistent with stigmastanol being the third
component. A very strong peak for the principal molecular
ion (m/e 456) less CH₂COOH appeared, however, at m/e 396.
A weaker peak at m/e 382 was also derived by loss of CH₂COOH
from the molecular ion of m/e 442. Strong peaks at m/e 255,
derived from loss of CH₂COOH and side chain from both molec-
ular ions (m/e 456 and 442), and at m/e 213, derived from loss
of CH₂COOH and side chain and 42 mass units (24, 34) from
the same two molecular ions, were in agreement with the two
components possessing one double bond in the ring system
and no methyl groups at C-4 and C-14. The weakness of the mole-
cular ion peaks at m/e 456 and 442 compared to the peaks for
M₊ less CH₂COOH at m/e 380 and 362 was in agreement with the
chromatographic assignment of this nuclear unsaturation as Δ¹
rather than Δ², for example, since the former much more readily lose CH₂COOH yielding the Δ¹,4-diene (35). Fur-
themore, the extinction coefficient at 215 nm was 323 which is charac-
teristic (36) of Δ₂-sterols (ε₉₀ 700) rather than Δ₁- (ε₉₀ 3000)
Δ₂- (ε₉₀ 4000) or Δ₄(14)-sterols (ε₉₀ 8100) and the Liebermu-
Buroward color was identical with that for Δ₂ sterols rather than
with the color produced from Δ₁- (37) or Δ₂-sterols (5, 38). It
was also observed that no prominent peaks appeared at m/e 143
and 158 which are characteristic (35) of Δ₁,4-unsaturation. The
absence of a Δ₁,4-system was corroborated by the failure of the
mixture to exhibit absorption at 272, 282, and 294 nm in the
ultraviolet region of the spectrum (39). In summary, the accu-
mulated information is consistent with an assignment of structure
to the dominant sterols as follows. The major component was
24₆-ethylcholesterol-5-en-3β-ol (sitosterol, Structure IV-d, or cli-
stonasterol, Structure IV-e). The two minor components, each
present in approximately the same quantities, were 24₆-methyl-
cholesterol-5-en-3β-ol (campesterol, IV-b), or 5,6-dehydroergostanol,
IV-e) and 24₆-ethylcholesta-5,22-dien-3β-ol (poriferasterol,
III-e, or stigmastanol, III-d). In addition, a trace of cholesterol-
5-en-3β-ol (cholesterol, IV-a) was present. The sterol mixture
amounted to 44 mg/100 g of wet tissue.

Isolation and Identification of Cycloartenol, Cyclopentanol, and
3-Methylenecyclopentanol from Corn Seedlings—The extract of 9.0
kg of corn (obtained from extraction with ethanol in a Soxhlet
apparatus) was partitioned between water and ether. The ether-
soluble material (61.9 g) was separated into two fractions by elution
from a column of 600 g of anionotropic alumina, Brockman grade III, with (a) 2% ether in ligroin (0 liters) yield-
ing 7.0 g and (b) 0 liters each of 50% ether in ligroin and 100% ether
yielding 18.5 g. Fraction b after saponification yielded 4.1 g of lipid.
From this by chromatography on a column of alumina (200 g) were obtained 8.1 g of solid material by elution
with 40% ether in ligroin and 100% ether (2 liters each). 3β-
Hydroxyxysteroids were obtained by the digitonin procedure (40,
41). They were separated into three structural groups (4-
decamethyl-, 4a-methyl-, and 4,4-dimethylsterols) by chro-
matography on alumina (100 g of Brockman Grade III) according
to the method of Good and Goodwin (5). The fraction contain-
ing 4,4-dimethyl sterol was chromatographed on preparative
thin layer plates of silica gel. The material which moved with
The movement of sterols was observed as the acetate of sterol I in argentation chromatography. Since it has the same rate of movement as lanosterol, it was observed in some cases that the major peaks obtained after argentation chromatography were not present in Fraction B. Although the data for Component 1 are similar to those obtained prior to argentation chromatography, it should have been present in Fraction B. However, Fraction B exhibited no peak in gas-liquid chromatography for the acetate of lanosterol. Eighty-eight percent of the eluted material was in the peak corresponding to the acetate of cycloartenol and 12% in a slower moving peak (relative retention times 2.69, 3.16, and 2.04 on SE-30, QF-1, and Hi-EFF-SB, respectively). The acetate of lanosterol moved at a faster rate in all three systems. The 12% component had the same retention time values as the acetate of 24-methylene-cycloartanol and apparently represented some overlap of Fractions A and B.

The cycloartenyl acetate (acetate of I) isolated from Fraction B in the argentation chromatography gave the following m/e peaks in mass spectroscopy: 468, 453, 408, 393, 365, 339, 297, and 216, when examined by Mrs. A. Ball of the University of Liverpool with an AEI MS-12 spectrometer equipped with a direct inlet. The data agree with the literature (23-25). The 24-methylene-cycloartanol acetate (acetate of VI-b) isolated from Fraction A and the cycloartenyl acetate (acetate of VI-a) isolated from Fraction C showed the following m/e peaks on the AEI instrument: 452, 422, 407, 379, 300, and 297, and 470, 455, 410, 395, 367, 357, 341, 207, and 288, respectively.

**Incubation of Labeled Substrates with Corn Seedlings and Isolation of Neutral Lipids**—Fourteen-day-old seedlings were placed in glass scintillation-counting vials and an aqueous solution (2 ml) of 2-14C-mevalonate or an aqueous emulsion (2 ml) of labeled cycloartenol (I) or lanosterol (II) was added. The latter was prepared by the addition of 4 ml of a 0.5% solution of Tween 20 in acetone to the substrate followed by the addition of 8 ml of a 0.1% solution of Tween 20 in water. Removal of the acetone in a stream of nitrogen and vigorous agitation of the resultant mixture in a mechanical shaker produced the final emulsion. Incubation was carried out for 48 hours (unless otherwise noted) at room temperature. Transpiration was aided by a stream of air, and water was added to the vials as needed. About 90% of the labeled substrates entered the plants. The seedlings were cut into small pieces and macerated with ethanol in a Waring Blender for 20 s. An aqueous solution of potassium hydroxide was used to prepare the sterols.
radioactivity when submitted to gas-liquid chromatography. The free alcohol (I) showed an RF (0.23) and a relative retention labeled lanosterol. During seven successive crystallizations P-sitosterol showed that there was less than 0.35% of this sterol peaks and an inflection. The most strongly labeled material Seedlings-One hundred seedlings were incubated with racemic in the ether-soluble material (132.6 mg) after saponification contained from 2-14C-mevalonate as described for 14C-cycloartenol. After acetylation it was chromatographed preparatively in the...terol acetate. The fraction corresponding to the acetates of cycloartanol (VI-a) was cocrystallized (chloroform-methanol) with the acetate of 24-methylenecycloartanol (VI-b) was associated with the acetate of 24-methylenesterols but also cr- and fl-amyrin when cocrystallized with authentic VI-a acetate.

Preparation of 14C-Cycloartenol from 2-14C-Mevalonate in Corn Seedlings—One hundred seedlings were incubated with racemic 2-14C-mevalonate (0.3 x 10^6 dpm) for 24 hours. Radioactivity in the ether-soluble material (132.6 mg) after saponification corresponded to a 61% conversion of one enantiomer of mevalonate to lipids. The material was subjected to thin layer chromatography which revealed radioactivity in four major peaks and an inflection. The most strongly labeled material had an RF of 0.27 which was identical with that of an authentic cycloartenol. It was acetylated and purified by argentation chromatography which separates not only 24,25-dihydro- and 24-methylenosterols but also alpha- and beta-amyrin from Delta4,4-dimethylsterols as previously described (22). The cycloartenol acetate so obtained (1.6 x 10^6 dpm) yielded a single peak of radioactivity when submitted to gas-liquid chromatography. The free alcohol (I) showed an RF (0.23) and a relative retention time (1.80) identical with that of authentic cycloartenol. The alcohol, when mixed with authentic cycloartenol and submitted to gas-liquid chromatography, yielded a peak of radioactivity corresponding to the mass peak. When a portion was admixed with authentic unlabeled cycloartenol, the specific activity did not change during three successive crystallizations from methanoll-chloroform. That the labeled substance was not lanosterol was unequivocally demonstrated by cocrystallization with unlabeled lanosterol. During seven successive crystallizations from methanol-chloroform, the specific activity decreased steadily and approached zero. The last value indicated that less than 1.3% of the label could have been associated with lanosterol. Similar studies of the loss of label after admixture with beta-sitosterol showed that there was less than 0.35% of this sterol present.

Biosynthesis of Labeled 24-Methylenecycloartenol and Alpha- and Beta-Amyrin from 2-14C-Mevalonate in Corn Seedlings—In another experiment the fraction from argentation chromatography associated with the acetate of 24-methylenecycloartenol (VI-b) was cocrystallized (chloroform methanol) with the acetate of authentic VI-b three times without significant loss of label. The fraction corresponding to the acetates of cycloartenol (VI-a) or alpha- and beta-amyrin when cocrystallized with authentic VI-a acetate led to removal of the label but to its retention when cocrystallized three times with alpha-amyrin acetate or beta-amyrin acetate. When submitted to gas-liquid chromatography two radioactive peaks were observed with retention times identical with those of alpha- and beta-amyrin acetates.

Biosynthesis of 4-Desmethyl Sterols from 2-14C-Mevalonate in Corn Seedlings—Labeled material with an RF of 0.17 was obtained from 2-14C-mevalonate as described for 14C-cycloartenol. After acetylation it was chromatographed preparatively in the argentation system. Two radioactive bands were observed, one which had moved at the same rate as sitosteryl acetate and one much less intense band which had moved at the same rate as 24-ethyldenecholesterol (V-b and V-c). Unlabeled sitosteryl acetate was added to give a specific activity of 6,860 dpm per mg. After re-re cristallization (methanol-chloroform) the specific activity decreased to 5,490 dpm per mg but was not decreased with two further recrystallizations (6,040 and 5,270 dpm per mg, respectively).

Biosynthesis of 4-Desmethyl Sterols from 4-14C-Cycloartenol (Structure I) and 4-14C-Lanosterol (Structure II)—4-14C-Cycloartenol diluted with carrier material (6.32 x 10^6 dpm; 2.43 x 10^6 dpm per mg) was administered to 44 corn seedlings (13.0 g). Similarly, 5.85 x 10^6 dpm of 14C-lanosterol (1.52 x 10^6 dpm per mg) was added to another 44 seedlings. Each batch was macerated in ethanol and saponified, and the neutral lipids (90.0 mg, 3.38 x 10^6 dpm, labeled from 14C-cycloartenol and 64.7 mg, 5.17 x 10^6 dpm, labeled from 4-14C-lanosterol) were extracted with ether. The neutral lipids obtained from each batch of corn were chromatographed on a thin layer of silica gel. In both cases two radioactive peaks were observed. The major one corresponding to unchanged substrate and the minor one corresponding to 4-desmethyl sterols. The 4-desmethyl sterol fraction labeled from 4-14C-lanosterol and 4-14C-cycloartenol contained 0.8 x 10^4 dpm and 1.4 x 10^4 dpm, respectively. After acetylation the sterol fractions were submitted to argentation chromatography. In each case two radioactive peaks were observed. They corresponded to the acetates of the Delta-sterols (IV-a to e) and of the Delta4,24(28)-sterols (V-b and V-c). From the 4-14C-cycloartenol incubation, after preparative argentation chromatography, 1.7 mg (1.78 x 10^6 dpm) of Delta-steryl acetate and a negligible weight (3.35 x 10^5 dpm) of Delta4,24(28)-steryl acetate were obtained. From the 4-14C-lanosterol incubation in the same manner was obtained 1.6 mg (1.46 x 10^6 dpm) of Delta-steryl acetate and a negligible weight (2.13 x 10^6 dpm) of Delta4,24(28)-steryl-steryl acetates. After addition of unlabeled sitosteryl acetate, a portion of each sample of Delta-steryl acetate was hydrolyzed and a portion of the alcohols formed was converted to the 5,6-dibromide and debrominated with regeneration of the Delta-sterols. The acetates, the alcohols, and the alcohols purified via the dibromides were cocrystallized with sitosterol or its acetate as shown in Table III. Most of the label was retained in the crystals in all cases indicating that the dominant Delta-sterols (IV-b or IV-c and IV-d or IV-e) of the plant had been labeled. No attempt was made to identify the Delta4,24(28)-sterol fraction further, but it was probably 28-isofucosterol (V-c) for reasons discussed in the section on "Results and Discussion."

Metabolism of 2-14H-Cycloartenol and 2-14H-Lanosterol in Seedlings of Z. mays—2-14H-Lanosterol (3.89 x 10^6 dpm, 3.37 x 10^6 dpm per mg) was administered to 36 seedlings (15 g of fresh weight). 2-14H-Cycloartenol (3.41 x 10^6 dpm, 1.78 x 10^6 dpm per mg) was administered to a similar batch. After an incubation period of 45 hours the neutral lipids were obtained. The radioactive 4-desmethyl sterols (2.01 mg, 6.79 x 10^6 dpm, labeled from 2-14H-lanosterol; 3.72 mg, 7.47 x 10^4 dpm, labeled from 2-14H-cycloartenol) were isolated by successive column and thin layer chromatography. Portions of the radioactive 4-desmethyl sterols were submitted to gas-liquid chromatography. Two radioactive peaks were observed in both cases. The retention times corresponded to the endogenous 24-methyl- and 24-ethylcholesterol of the plant. However, argentation chromatography of other portions (after acetylation) revealed, in both cases, the presence of steroidal monoenes and dienes in an approximate ratio of 1:1. The former moved at the same rate as 24-methyl- and 24-ethylcholesterol, while the rate of movement of most of the diene coincided with that of 24-ethyldenecholesterol (V-b...
or V-c). In these chromatograms standard samples of the cis- and trans-∆(23)-isomers (isofucosterol and fucosterol) did not separate, and the configuration of the metabolite could not be established. In addition to the 24-ethylidenecholesterol, small amounts of radioactivity moved at the same rate as did the diene, stigmasterol. The exact amounts of the metabolites were determined from preparative argentation chromatography. Three distinct bands were observed. The results are summarized in Table IV. To the monoene fraction was added an authentic label (Table V). The metabolites mixed with the carrier were also oxidized with chromic acid in acetone (42, 43). The previously unreported stigmast-5-en-3-one (mixed with ergost-5 en-3-one), m.p. 120-126° and 123-128°, respectively, from cycloartenol and lanosterol, was isolated. Both samples exhibited the expected band at 5.83 μ for a 3-keto group and moved at the same rate in thin layer chromatography as did authentic cholest-5-en-3-one. Both samples retained radioactivity through two recrystallizations (Table V). In another experiment with a single peak with the same retention time as that of authentic cholest-5-en-3-one, m.p. 78-81° and 80-83°, respectively, from cycloartenol and lanosterol, was isolated. Both samples exhibited the expected band at 5.83 μ for a 3-keto group and moved at the same rate in thin layer chromatography as did authentic cholest-5-en-3-one. Both samples retained radioactivity through two recrystallizations (Table V).

In Table IV the specific radioactivities of steryl acetates derived from the Wiley-Chromatography samples were measured. The values are calculated from the amount of radioactivity in total sterols after alumina chromatography and from the ratio of radioactivities in appropriate bands in argentation chromatography. Further identification is given under "Experimental Procedure."

### Table III

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<td>1.3</td>
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### Table IV

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<td>D1-Stigmasten-3-one</td>
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of 2-\(^{3}\)H-cycloartenol (2.38 \(\times\) 10\(^6\) dpm, 1.78 \(\times\) 10\(^6\) dpm per mg, Experiment B) and 2-\(^{3}\)H-lanosterol (3.08 \(\times\) 10\(^6\) dpm, 3.37 \(\times\) 10\(^6\) dpm per mg, Experiment C), respectively. After incubation for 3 hours at 37\(^\circ\)C, the neutral lipids were isolated from each preparation. They contained 2.14 \(\times\) 10\(^6\) dpm (13.8 mg, \(A\)), 2.00 \(\times\) 10\(^6\) dpm (18.6 mg, \(B\)), and 2.60 \(\times\) 10\(^6\) dpm (18.1 mg, \(C\)), respectively.

Portions of each fraction (1.5 \(\times\) 10\(^6\) dpm, \(A\); 1.47 \(\times\) 10\(^6\) dpm, \(B\); 1.90 \(\times\) 10\(^6\) dpm, \(C\)) were chromatographed on an alumina column and the respective cholesterol-containing fractions isolated. Those contained 8.05 \(\times\) 10\(^4\) dpm (2.02 mg), 4.46 \(\times\) 10\(^4\) dpm (2.25 mg), and 1.70 \(\times\) 10\(^4\) dpm (2.58 mg), respectively. The latter amount of radioactivity, which subsequently was not significantly separable from cholesterol, corresponded to a 7.5% yield from the 2-\(^{3}\)H-lanosterol incubated. The 4,4-dimethylsterol fractions contained 1.07 \(\times\) 10\(^6\) dpm, 1.24 \(\times\) 10\(^6\) dpm, and 1.12 \(\times\) 10\(^6\) dpm, respectively.

The cholesterol-containing fraction from Experiments A and B and a portion of that from C (0.46 mg, 3.16 \(\times\) 10\(^4\) dpm) were further purified by thin layer chromatography. Most of the radioactivity, (2.31 \(\times\) 10\(^4\) dpm, 1.47 mg; and 1.80 \(\times\) 10\(^4\) dpm, 0.37 mg) from Experiments B and C, respectively, remained associated with the cholesterol fraction. However, only 1.89 \(\times\) 10\(^4\) dpm was found in the cholesterol fraction from Experiment A. Acetylation of these fractions followed by further thin layer chromatography led to the retention of radioactivity (1.38 \(\times\) 10\(^4\) dpm) in the cholesterol acetate from Experiment C, but only 0.5 \(\times\) 10\(^4\) dpm (1.04 mg) and 4.2 \(\times\) 10\(^3\) dpm (1.05 mg) were recovered in the cholesterol acetate fraction (\(R_s\) = 0.65) from Experiments A and B, respectively. The value in Experiment B corresponds to not more than a 0.02% incorporation of label into cholesterol from 2-\(^{3}\)H-cycloartenol. In another incubation of 2-\(^{3}\)H-cycloartenol the cholesterol-containing fraction was mixed with unlabeled cholesterol. After two crystallizations from methanol-chloroform no label at all could be detected, and the sensitivity of the measurement indicated that less than 0.01% conversion to cholesterol could have occurred. The possibility that pollinastanol (VIII), which could theoretically be derived from cycloartenol by demethylation at C-4 and reduction of the \(\Delta^2\)-bond, was formed in Experiment B was eliminated. The acetate of authentic pollinastanol moved in argentation chromatography with \(R_s\) 2.0, but no label with this \(R_s\) was found in Experiment B. The radiochemical identity of the cholesterol labeled from 2-\(^{3}\)H-lanosterol was confirmed by admixture with unlabeled cholesterol and several recrystallizations from methanol-chloroform. The label was retained. Another portion of the cholesterol labeled from 2-\(^{3}\)H-lanosterol was diluted with carrier and oxidized by chromic acid in acetone (42, 43) to give cholest-5-en-3-one, \(m_p\) 125-127\(^\circ\) (literature, \(m_p\) 127\(^\circ\) (45)), \(\lambda_{max}\) 5.86 \(\mu\). Label was retained through four recrystallizations (from acetone).

The presence of tritium at C-2 was proven by refluxing the 2-\(^{3}\)H-cholest-5-en-3-one in 10% ethanolic KOH solution for 10 min. The cholest-4-en-3-one formed crystals from methanol—m.p. 80-81\(^\circ\), literature, \(m_p\) 76-79\(^\circ\) (46), \(\lambda_{max}\) 245 nm (\(\epsilon_7,400\)) 6.01 \(\mu\) (literature, 242 nm, (\(\epsilon_7,400\)), 6.01 \(\mu\) (46))—and was completely devoid of radioactivity.

A portion (63%) of the 4,4-dimethyl sterols (isolated by aluminum chromatography) from the incubation of 2-\(^{3}\)H-cycloartenol was acetylated and submitted to argentation thin layer chromatography together with an authentic mixture of lanosterol and 24,25-dihydrolanosterol acetate as carriers. The lanosterol and cycloartenol type molecules are not distinguishable in this chromatographic system. Two radioactive bands were observed with \(R_s\) values corresponding to the two materials added as carrier. The region associated with the 24,25-dihydro compound was rechromatographed several times. Since in the last chromatogram the 24,25-dihydro compound lost a negligible amount of its radioactivity, we assume the final amount of label represented pure cycloartanyl acetate. After correction for losses in the chromatograms, the amount of label corresponding to cycloartanyl acetate in the original sample was 29,000 dpm. This corresponds to a 1.9% yield of cycloartenol in the incubation. Similar sequential chromatography of the acetylated products from the incubation with 2-\(^{3}\)H-lanosterol showed that 0.9% of 24,25-dihydrolanosterol was formed. By contrast not more than 0.06% of cycloartenol was observed after sequential chromatography of the acetylated products from the incubation of 2-\(^{3}\)H-cycloartenol with inactivated enzyme. The discrimination between the identities of cycloartanyl acetate and 24,25-dihydrolanosteryl acetate rests primarily on the fact that cholesterol was formed only from lanosterol. Consequently, no isomerase for the opening of the cycloprenyl ring was present, and the 4,4-dimethyl product from cycloartenol must have retained the nuclear structure of the substrate. The absence of the isomerase was also indicated by data from cocrystallization. After admixture of unlabeled lanosteryl acetate with the acetylated 4,4-dimethylsteryl fraction from the 2-\(^{3}\)H-cycloartenol incubation and seven recrystallizations from methanol-chloroform, 96% of the label was lost. Consequently, the formation of lanosterol was insignificant.

RESULTS AND DISCUSSION

The 4,4,14-tridesmethyl sterols (44 mg/100 g of wet tissue lacking roots) of corn seedlings were shown to be composed of cholesterol, and its 24-methyl-, 24-ethyl-, and 24-ethyl-\(\Delta^2\)-derivatives in a ratio of 2:17:60:21. No other desmethylsterols were observed, but very small amounts (about 0.1 mg/100 g of wet tissue lacking roots) of cycloartenol, cycloartenol, and 24-methylenecycloartenol were isolated in ratios of 4:1:1. Incubation (by stem feeding) of the seedlings with 2-\(^{14}\)C-mevalonate led principally to labeled cycloartenol. 24-Methylenecycloartenol and 24-methyl- and 24-ethylcholesterol were among the compounds which were also labeled. No lanosterol was formed. This was demonstrated by the essentially complete loss of label when the \(^{14}\)C-cycloartenol (which had not been submitted to a procedure which separates Structures I and II) was submitted to cocrystallization with lanosterol. The label was retained when the biosynthetic product was cocrystallized with cycloartenol. We believe this is the first time the biosynthesis of cycloartenol and the lack of biosynthesis of lanosterol have been demonstrated in a higher plant under conditions in which practically the whole organism is used. The results are in agreement with the hypothesis (1-3, 5) and previous evidence for the role of cycloartenol rather than lanosterol as the first polycyclic compound in the pathway to sterols in plants. However, previous work (6, 7, 19, 47) has shown that in the cases examined (\(O.\) malhamensis, \(N.\) tabacum tissue cultures, germinating \(Pineus\) pinea seeds, and homogenates of pea seedlings) plants readily convert lanosterol to various other sterols including the dominant sterol of \(O.\) malhamensis. In \(O.\) malhamensis, \(N.\) tabacum tissue cultures, and homogenates of pea seedlings label from cycloartenol was not incorporated at a very much larger rate than...
was label from lanosterol (6, 7, 19). This suggests that plants
do not discriminate between the two intermediates (I and II).
This was confirmed in the present work.

2-Tritiocycloartenol and 2-tritiolanosterol (of approximately
the same specific activity) led, by stem feeding of the corn seed-
lings, to the plants' dominant sterols, 24-methyl- and 24-ethyl-
cholesterol. In addition, evidence was obtained with both
substrates for incorporation of label into 24-ethylcholesta-5-
7,22-dien-3β-ol and into 24-ethylidenecholesterol. The latter
was probably isofucosterol (27, 48, 49). Considerably
more of the latter was observed from I and II than from meval-
olone, presumably as a reflection of the larger amounts of I and
II used and the regulatory nature (27, 48) of the metabolism of
the 24 ethyldiene group. In a similar fashion, the same
metabolites were obtained from 14C-cycloartenol and 14C-lanos-
sterol (derived from 2-14C-mevalonate in the corn seedlings and
liver homogenates, respectively). Quantitatively both the H-
and 14C-cycloartenol gave slightly higher yields of metabolites
than did the correspondingly labeled lanosterol (Table IV), but
in all cases the yield of the seedlings' dominant sterols (24-
methyl- and 24-ethylcholesterol) was about 1%. This means
that in an angiosperm, under conditions which approximate the
normal living situation, the plant cannot significantly distinguish
lanosterol from cycloartenol, and, consequently, qualitative and
quantitative regulation of which intermediate (I or II) is in-
volved must reside exclusively at the cyclization of 2,3-oxido-
squalene. However, in the animal kingdom, discrimination
between I and II was found to be much more extensive. 2-
Tritiolanosterol was incubated with a rat liver homogenate, and
2-tritiocholesterol was obtained in a 0.7% yield. 24,25-Dihy-
drolanosterol was obtained in a 0.9% yield. By contrast, under the
same conditions, 2-tritiohcyloartenol gave no labeled cholesterol.
We could have detected a conversion of 0.01%. A compound
which is presumed to be 2-tritio-24,25-dihydrcycloartenol
(cycloartenol VI-a), since it moved in argentation chromatog-
raphy with the expected rate, was obtained in a 1.9% yield.
Also, a labeled metabolite (in a 0.6% yield) was obtained which
has not been identified beyond the fact that it did not have the
properties of cholesterol, cholesterol, or pollinastanol. Thus,
cycloartenol is not only not apparently formed in mammals
(10, 12, 13), but one or more crucial enzymes in the pathway
between lanosterol and cholesterol will not accept cycloartenol.
This was further demonstrated by the absence of pollinastanol
(VIII) as a metabolite of cycloartenol. Had cycloartenol been
acceptable to the enzymes, even in the absence of an isomerase
for opening of the 3-membered ring, pollinastanol should have
been obtained. The data also show that no isomerase was
present, since neither lanosterol nor cholesterol was formed from
cycloartenol. The intermediacy of lanosterol in the mammalian
tissue is therefore insured in three ways, viz. by the nature of
the 2,3-oxidosqualene cyclase, the specificity of the enzymes in
the pathway after cyclization, and by the absence of an isomerase.

The evolutionary question posed in the introductory section is,
of course, left unanswered. Had there been an isomerase pres-
ent, it would be reasonable to interpret its presence as indicative
of a common origin of animals and photosynthetic plants in
organisms utilizing cycloartenol. Unfortunately, one can not
draw an unequivocal conclusion in the reverse sense from the
negative data, since the isomerase, being unused, might have been
lost in some genetic way during evolutional development.

Nevertheless, the absence of an identifiable isomerase in mam-

malian tissue is in accord, as is the intermediacy of lanosterol
rather than cycloartenol in fungi (14-17), with the tentative
supposition that photosynthetic and nonphotosynthetic systems
had partly or wholly different origins. Similarly, if higher plants
arose by symbiosis of photosynthetic and nonphotosynthetic
organisms, the chloroplastic and nonchloroplastic pathways of
sterol biosynthesis may proceed in the former case via cyclo-
artenol and in the latter via lanosterol. Experiments to test
this hypothesis are in progress.

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Geoffrey F. Gibbons, L. John Goad, T. W. Goodwin and William R. Nes


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