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Enzymology of Phytosterol Transformations

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CONTENTS

I. INTRODUCTION

The purpose of this chapter is to present a summary of several aspects of the enzymology of phytosterol production. Enzymes are known as the agents of metabolic turnover. They also serve as cellular determinants to sequence enzymic steps, and control the rate and type of intermediates and end products formed by cells. Historically, the organic approach (spectroscopic identifications of endogenously formed compounds and feeding/trapping experiments with radioactive compounds fed to whole plant tissues) was used to investigate pathway sequencing of sterols in plants.[1] However, recent developments in preparing cell-free systems and the availability of a variety of sterol and substrate analogs for testing with microsome-bound enzymes have made possible the examination of pathway sequencing directly at the enzyme level. Studies on the enzymology of phytosterol synthesis have lagged behind similar enzymology studies performed with animals. The major reason for studying cholesterol biosynthesis in animals is that impaired cholesterol production results in heart disease. No equivalent disease state from altered sterol production is known to occur in plants.

The pharmaceutical industry has been interested in phytosterols for many years, for their value as starting material in the preparation of animal steroid hormones. The approach used by industrial chemists to locate suitable sterols for chemical modification was to analyze the sterol composition of plants from different geographic regions and plant families. However, there is now agronomic interest in elucidating the enzymology of phytosterol production. In contrast to animals, where the investigator determines *rates* of sterol production, in studies on plants (and fungi) the investigator focuses on distinguishing different sterol pathways. Using this approach the agrochemical industry recently commercialized a series of sterol biosynthesis inhibitors which *modify* the sterol composition of pathogenic fungi, thereby preventing the spread of the fungal disease.[2,3] Alternate approaches are under consideration to enhance the efficiency in crop production from attack by pests and pathogens.[4,5]

Phytosterols are a class of natural products which possess the tetracyclic ring system. They are synthesized by plants (including algae) which operate photosynthetically at some stage of their development or evolutionary history.[1,6] Phytosterols may be distinguished from "zoosterols" — sterols produced by animals, by the presence of alkyl groups at C-24 in the sterol side chain. However, the enzymology to produce cholesterol is present in plants,[7] as it is in fungi.[8] Alternatively, fungi may produce 24-alkyl sterols which are structurally similar to plants.[9] Thus, differences in sterol distribution patterns in plants which have been used in the past to distinguish plants from fungi and marine organisms may not be as different as once thought. In fact, there are cogent reasons to believe, the type and amount of sterols produced in plants are related to developmental regulation[7,10,11] and to phylogenetics.[1,12,13] The

apparent differences in sterol compositions in plants may be the result of different levels and amounts of enzymes (perhaps including families of isozymes in different plant families) that act on the sterol molecule.[13] In this chapter we review the enzymic parameters currently used by investigators to determine pathway sequencing of sterol transformations.

The carbon numbering system and nomenclature of phytosterols, particularly with respect to the sterol side chain carbons, has been discussed elsewhere,[11,13] as shown in Figure 1.

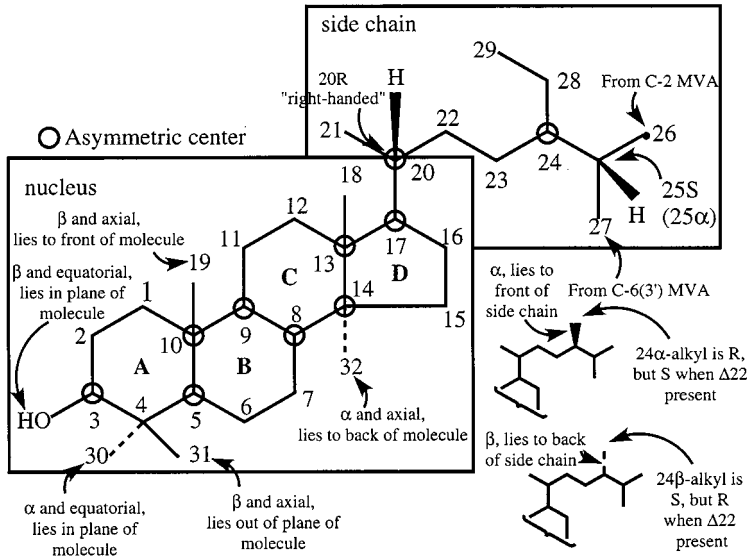

Figure 1 Structure and carbon numbering of phytosterols.

II. ENZYMATIC FORMATION OF STIGMASTEROL FROM CYCLOARTENOL

A. PATHWAYS OF STEROL GENESIS AND TRANSFORMATION

The sterol pathway is distinct from the isopentenoid (isoprenoid; terpenoid) pathway which gives rise to sterols, and the steroid hormone pathway which leads to the production of highly oxygenated compounds, e.g., brassinosteroids[14] and ecdysteroids.[15] In plants the ultimate source of carbon for sterol synthesis is obtained from photosynthesis. Carbon flux proceeds from 6-carbon saccharides through glycolytic sequences to 2-carbon acetyl CoA units. The C-2 units are converted to a 6-carbon intermediate mevalonic acid, which is converted to the basic 5-carbon unit, Δ^3-isopentyl pyrophosphate, of the isopentenoid pathway. Novel lipid shunts, e.g., the Popjak shunt[16] and the Rohmer shunt,[17] have been found to redirect carbon flux from sugar and amino acids into isopentyl pyrophosphate, effectively bypassing the intermediacy of mevalonic acid. We recently discovered, by incubating deuterated water to germinating corn seedlings, that mevalonic acid may not be an intermediate to phytosterols.[11] Hence, the essential role of HMGCoA reductase activity as a rate-determining step generally in phytosterol biosynthesis[18,19] requires reexamination.

The biosynthetic steps from acetyl CoA to squalene production are anaerobic.[1] Oxidation of squalene to 2,3-oxido squalene (squalene oxide: SO) is the first aerobic step that precedes phytosterol formation. Cyclization of SO is anaerobic and stereoselective to give six possible isomeric tetracyclic products: cycloartenol, lanosterol, parkeol, euphol, tirucallol, 10α-cucurbitacin, and dammarenol.[13] Of the six tetracycles, only cycloartenol, lanosterol, and parkeol are steroidal. The other three tetracycles are triterpenoids. The transformation of phytosterols begins with the formation of 4,4,14-trimethyl tetracycles and proceeds to completion with the synthesis of 4,4,14-trisdesmethyl tetracycles. As shown in Figure 2, squalene oxide may cyclize to cycloartenol or lanosterol. Both structural isomers may be converted to Δ^5-24-alkyl sterol end products. Because Δ^5-sterols accumulate and function in cell physiology as membrane inserts, we suggested formation of cycloartenol and stigmasterol (and related Δ^5-sterols, e.g., sitosterol) establish the start and finish of the phytosterol transformation pathway.[13,20] Plants normally produce several Δ^5-24-alkyl sterol end products and a variety of 9β,19-cyclopropyl and Δ^8- and Δ^7-sterol intermediates. However, not all Δ^5-sterols are end products; some are intermediates. Criteria that may be used to distinguish an intermediate from an end product were discussed.[20]

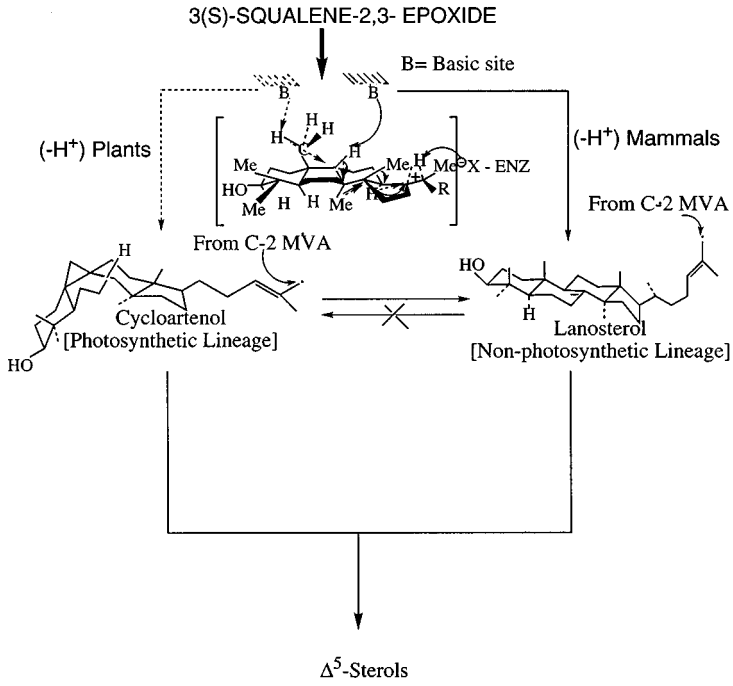

Figure 2 Classic two-pathway model showing the cycloartenol-lanosterol bifurcation.

There are now over 250 naturally occurring sterols which have been characterized from plant isolation studies,[21] and as many as 60 sterols were identified from the same source, *Zea mays* (corn).[11] The diversity of phytosterols is often associated with the variation in the sterol side chain features. Multiple C-24 alkylation pathways have been identified in plants, as shown in Figure 3. The steps controlling C-14 demethylation and opening of the 9β,19-cyclopropane ring also give rise to alternate products and hence to the evolution of alternate pathways to cycloartenol transformation (Figures 4 and 5).

Plants are thought to transform sterols by a series of criss-crossing pathways. The major pathway in advanced vascular plants leads to a sterol profile characterized by a mixture of stigmasterol (24α-ethyl cholesta-5,22-dienol), sitosterol (24α-ethyl cholest-5-enol) and campesterol (24α-methyl cholest-5-enol). Various pathways have been considered leading from cycloartenol to 24-methyl and 24-ethyl sterol end products. Since all the sterolic enzymes may lack a high degree of sterol specificity, and enzymes may lack a high degree of mechanistic specificity e.g., isomerases which promote reversible double bond rearrangements from 8(9)- to 7(8)- or 8(14)-positions, multiple pathways have been envisaged converging into the pathway that leads to sitosterol.[22]

The transformation of cycloartenol to stigmasterol has been mapped by the natural product approach[1,3] and more recently by isolating microsome-bound enzymes that catalyze sterol transformations. Investigations on corn, sunflower, and bean sterol enzymology show the expected mechanistic outcome for each step, but that differences exist in K_m values obtained on a range of substrates tested. The results show there exists a strict dependence for select molecular features of sterols in binding to enzymes.[23–31] The enzyme studies and the similarity in natural product distribution of sterols in several plants[7,11] indicate the route taken by cycloartenol to stigmasterol may be rather uniform, involving the order of steps shown in Figure 6: (a) anaerobic methylation of cycloartenol to 24(28)-methylene cycloartanol; (b) aerobic C-4 demethylation to yield a 3-keto sterol; (c) anaerobic reduction of the 3-keto sterol to 3β-OH to produce cycloeucalenol; (d) anaerobic opening of the 9β,19-cyclopropane ring to produce a $\Delta^{8(9)}$-sterol, obtusifoliol; (e) aerobic demethylation at C-14 to yield $\Delta^{8,14}$-sterol; (f) anaerobic saturation of the 14(15)-bond to yield a $\Delta^{8(9)}$-sterol; (g) anaerobic isomerization of the 8(9)-bond to the 7(8)-position; (h) methylation of the 24(28)-exocyclic bond to produce a 24(28)-ethylidene; (i) aerobic C-4 demethylation followed by reduction of the 3-keto sterol intermediate to produce a 4-desmethyl Δ^7-sterol; (j) aerobic desaturation to produce a Δ^5-sterol; (k) anaerobic reduction of the Δ^7-bond; (l) anaerobic isomerization of the 24(28)-bond to the 24(25)-position; (m) anaerobic saturation of the 24(25)-bond to yield a 24α-ethyl sterol (sitosterol); (n) introduction of the *trans*-22,23-double bond aerobically to produce stigmasterol.

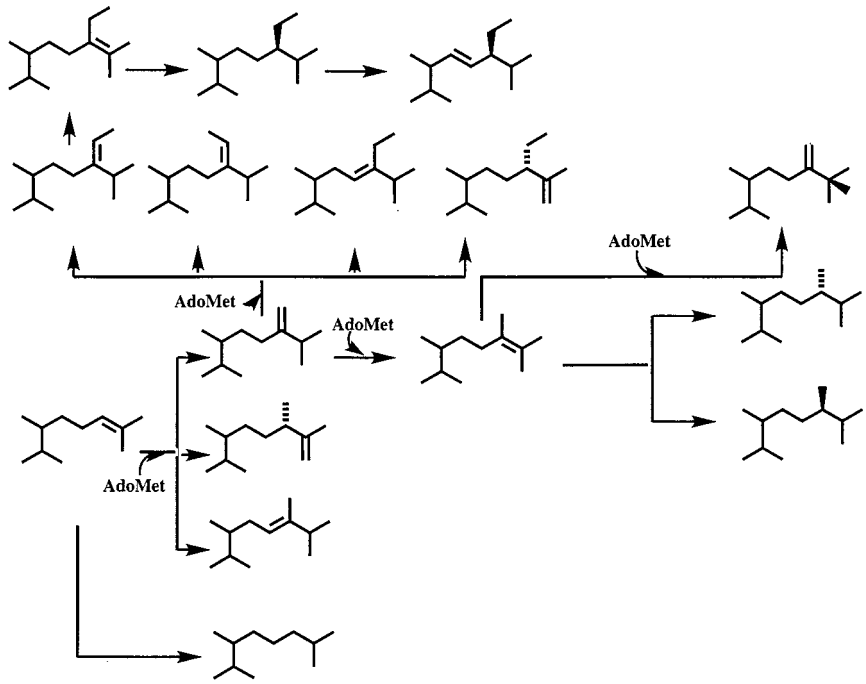

Figure 3 Pathways for biomethylation of the phytosterol side chain.

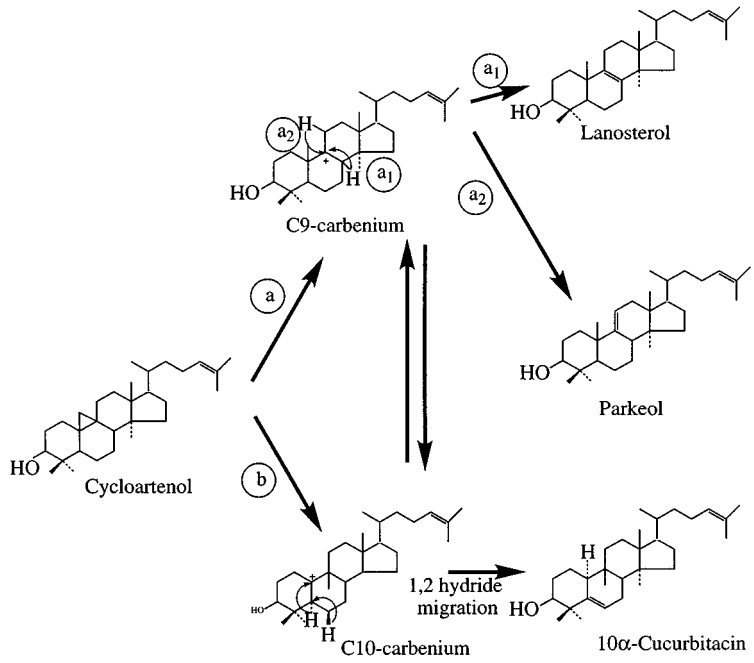

Figure 4 Pathways for cycloartenol transformation.

Conversion of cycloartenol to sitosterol and stigmasterol involves two biomethylation steps and the resulting end products are stereochemically pure 24α-ethyl. The production of a mixture of 24α- and β-methyl cholesterols appears to be developmentally delayed relative to 24-ethyl sterol production in actively growing tissue.[11] There has been much speculation regarding the precursor-product relationship

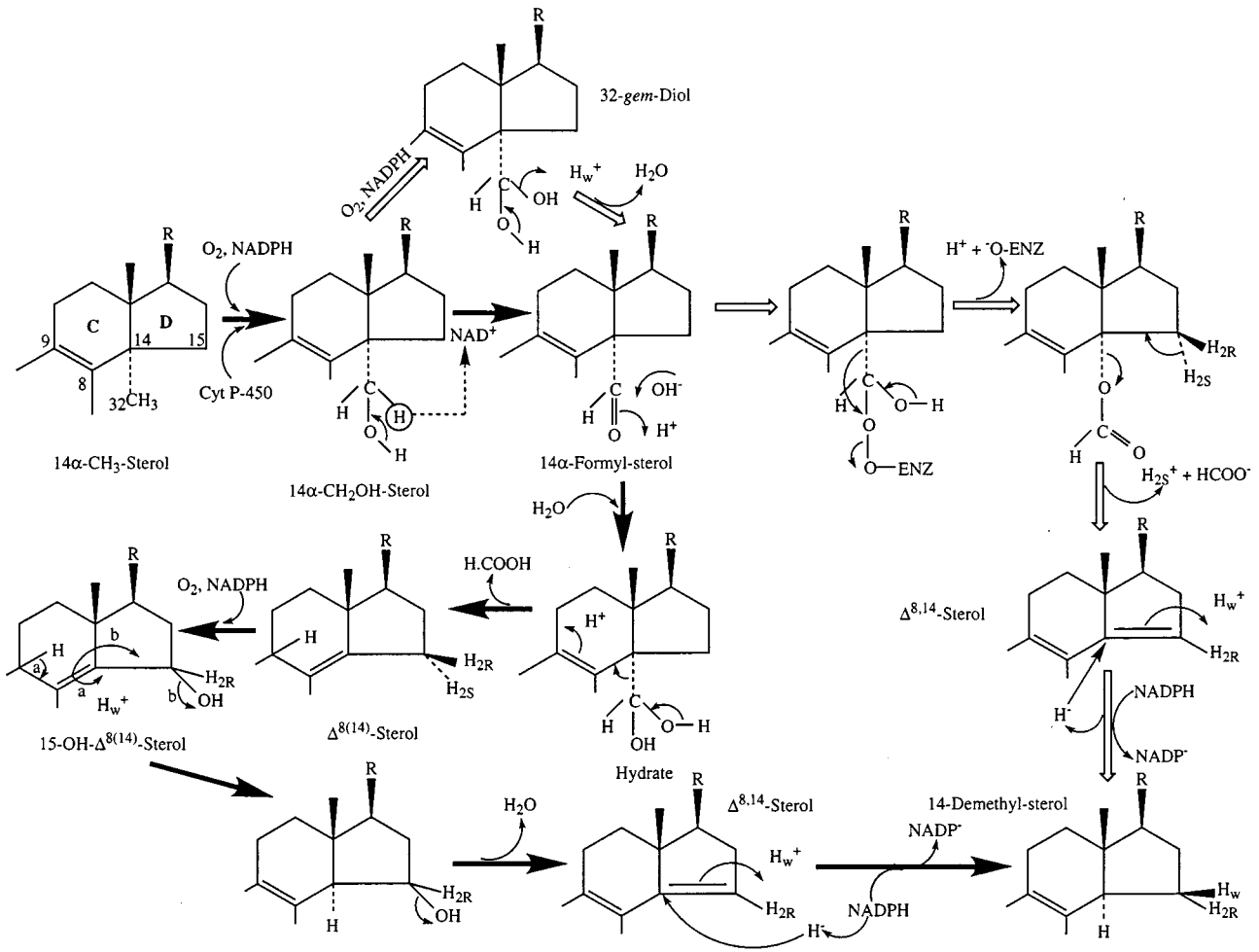

Figure 5 Pathways for 14-demethylation of phytosterols.

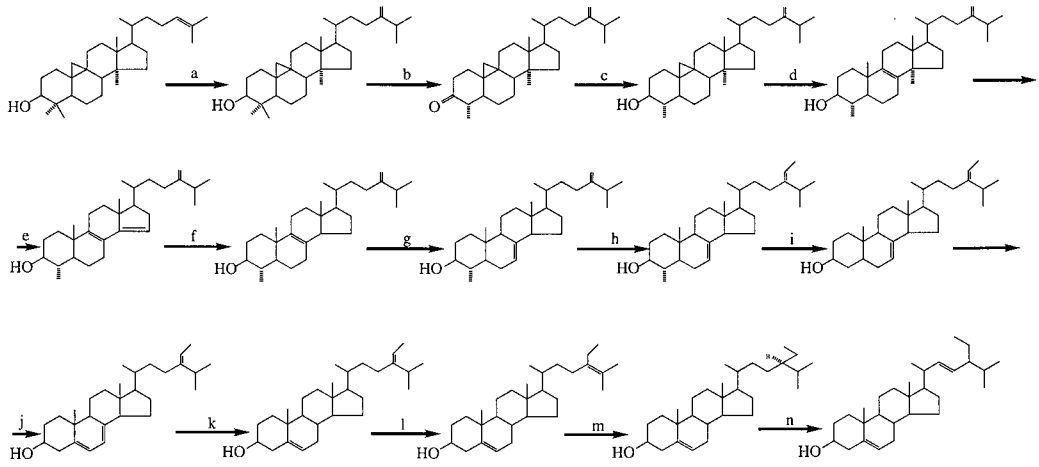

Figure 6 Hypothetical kinetically favored pathway to 24-ethyl sterols in sorghum, corn, and sunflower.

of $\Delta^{25(27)}$-24-methyl and $\Delta^{23(24)}$-24-methyl sterols with formation of specifically 24β-methyl sterols in grass plants (Figure 3 reviewed in References 7 and 11). Recently, we prepared a series of [3-^{3}H]24-alkene sterol isomers (specific activity 5×10^8 dpm/mg), ergosta-5,24(28)-dienol, ergosta-5,24(25)-dienol, ergosta-5,25(27)-dienol, and ergosta-5,23(24)-dienol and incubated the sterol samples with 8-day sheath samples as described.[11] Under conditions that ergosta-5,24(28)-dienol and ergosta-5,24(25)-dienol were incorporated (0.1%) into HPLC pure 24α- and β-methyl cholesterol, ergosta-5,25(27)-dienol and ergosta-5,23(24)-dienol were recovered from the tissues in unchanged form. Ergosta-5,24(28)-dienol was also converted to sitosterol by the sheath tissues in low yield (0.01%) (Guo and Nes, unpublished). The results show that corn seedlings operate multiple sterol pathways that give rise to specific end products and the pathways do not necessarily cross-over as a result of enzymes that lack substrate specificity.

The dominant sterols synthesized by vascular plants possess a chiral 24-alkyl group. For chemotaxonomic purposes, the configuration at C-24 has been used to disassociate primitive from advanced plants. As plants become more advanced, there is an apparent shift in the type of 24-alkyl sterols produced. Algae produce predominantly 24β-alkyl sterols, whereas highly advanced vascular plants produce mainly 24α-alkyl sterols.[1] The different 24-alkyl stereochemistries, particularly with respect to the second biomethylation product, are formed as a result of mechanistically different enzyme systems. For instance, the chirality of 24α-ethyl sterols is produced by the action of a 24-ethyl reductase, whereas the β-ethyl configuration is determined by the action of a C-24 sterol methyl transferase (SMT). In the first biomethylation reaction the configuration of the C-24 methyl group may be determined by the face of methyl cation attack, producing 24β-methyl sterols. However, the C-24β-methyl configuration may also be produced by reduction of the 24,25-double bond in 24-methyl desmosterol (ergosta-5,24(25)-dienol), a natural intermediate in the formation of campesterol and 24-epicampesterol (Figure 3). Thus, there are two routes which lead to the same stereochemical outcome. Tracheophytes as primitive as the Verbenaceae synthesize 24β-ethyl sterols from seed to flowering plant.[1] Similar primitive plants from the Crassulaceae and Cucurbitaceae synthesize 24β-ethyl sterols and 24α-ethyl sterols. The relative distribution of the 24-alkyl epimers in these plants has been found to depend on ontogeny.[21] Cucurbits possess 24β-ethyl sterols in seeds which disappear during germination and the 24β-ethyl sterols are completely replaced with 24α-ethyl sterols in seedlings.[31] In the crassulacean *Kalanchoe diagremontiana*, roots produced exclusively 24α-ethyl cholest-5-enol and 24α-ethyl cholest-5,22-dienol whereas the leaves produced exclusively 24β-ethyl cholesta-5,25(27)-dienol and 24β-ethyl cholesta-5,22,25(27)-trienol.[10]

The anatomical differences in sterol composition may be the result of developmental regulation of sterol sequencing, which has been referred to as an "evolutionary recapitulation" of the phytosterol biosynthetic pathways. The ability for some plants to produce a mixture of 24-ethyl sterol epimers while other plants produce one or the other type of 24-ethyl stereochemistry indicates that sterol biomethylation patterns in plants may be controlled phylogenetically by the evolution of structurally different SMT enzymes which are expressed uniquely in some, but not in other, plants. A second possibility is that a

family of SMTs (isozymes) are synthesized by plants, and they are expressed at different levels during plant development. A third possibility is that there are relatively few catalytically different sterol enzymes in a given pathway and the product distribution is the result of kinetic (transition state catalysis), thermodynamic (rate of product utilization), and allosteric (competing sterol, cofactor or some other modulator) control mechanisms acting on the development of the enzyme-substrate complex and subsequent catalysis (rate and product distribution).

B. REACTION VERSUS KINETIC MECHANISMS

The first step in determining the mechanism of action of an enzyme is to establish the course of the reaction and the attendant sterol and cofactor/coenzyme affinity for the enzyme in terms of its catalytic competence (viz., by measuring apparent K_m and V_{max} values). The second is to establish the order of addition of substrates and release of products and to determine whether the maximal velocity is a measure of the rate of the step in which covalent change takes place, i.e., the step identified as the "rate-determining step" in the reaction coordinate.

There have been few reports on the enzymology of sterol transformations, and most of them are of C-24 biomethylation reactions. In no study has a pure enzyme been studied. Therefore, caution must be exercised when drawing conclusions from data on apparent V_{max} values. The K_m values are more secure and may provide information on the relative differences of sterol affinity to enzymes. When enzymes are studied from anatomically similar plant tissues the different K_m values may reveal the uniformity and differences in pathway sequencing.

The reaction mechanisms of phytosterol transformation have been examined in cell-free systems in a variety of plants, as reviewed in detail elsewhere.[1] Structural requirements of sterols to bind and undergo catalysis by several microsome-bound/solubilized enzymes from corn and sunflower have also been studied. It is unclear from the paucity of enzymic data, which kinetic pattern operates for a given sterolic enzyme, e.g., a single-displacement (sequential) bisubstrate mechanism or that of a ping-pong mechanism. Unfortunately, product inhibition experiments may not detect the desired outcome using microsomes and kinetic patterns from transition state analogs assayed with microsome preparations may not give a true representation of the kinetic behavior of the pure enzyme. Thus, until the plant enzymes are purified we are left with important gaps in our understanding of their structure and function.

C. CHARACTERIZATION OF MICROSOME-BOUND ENZYMES

As noted earlier in the article, no sterolic enzyme from plants has been purified. Therefore, there is no information on the subunit architecture, native molecular weight, or turnover number for any of the sterolic enzymes from plants. The microsome-bound enzyme properties of several mechanistically different enzymes from corn and sunflower have been studied. Generally, the properties of the enzyme which are reported are on: catalytic competence for different sterol substrates (K_m and V_{max}), time of incubation, pH dependence, temperature profile, level of microsomal protein which gives maximal enzyme activity, cofactor requirements, and product distribution(s). The enzymes for which there is much information are the (*S*)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase, cycloeucalenol to obtusifoliol isomerase, 24(28)-methylene cycloartanol 4-demethylase, sterone reductase, obtusifoliol 14-demethylase, $\Delta^{5,7}$-Δ^{7}-reductase, $\Delta^{14(15)}$-reductase, and $\Delta^{8(9)}$- to 7(8)-isomerase.[24–31] The combination of mechanistic data on reaction course and on substrate binding studies show the stereoselectivity operating in phytosterol transformations in corn and sunflower. For instance, metabolism of the 24,25-bond proceeds to the 24(28)-position, 9β,19-cyclopropyl ring opening proceeds to the 8(9)-position, and C-14 demethylation proceeds to the 14(15)-position.[26,27,29] If alternate outcomes are possible and alternate products are found in the plant, the plant may produce an isoform of that enzyme and its expression is developmentally regulated.

III. THE SEARCH FOR THE RATE-LIMITING ENZYME

Much of the research on regulation of phytosterol biosynthesis has been associated with studies on measuring activity of HMGCoA reductase (HMGR), an enzyme that converts HMGCoA to mevalonic acid. The regulatory importance of HMGR may be a consequence of the fact that reduction of HMGCoA to mevalonic acid is the first committed step in isopentenoid metabolism. There is often a strong correlation between the level of HMGR activity in plants and the rate of sterol production.[18,19] However, most investigators measure total Δ^5-sterol levels, rather than measuring all of the sterol mass in the plant tissue. Interestingly, in contrast to animals where one gene encodes HMGR, there are multiple genes encoding HMGR in plants. Different numbers of genes are produced in plants.[32]

We suggest that proof of a critical slow step in phytosterol genesis requires measuring the activity of HMGR with that of pathway enzymes directly involved in sterol transformation, while concurrently measuring the temporal flux of carbon through the pathway and measuring change in the actual concentrations of intermediates and end products *in vivo*. The change in HMGR activity may mediate the level of cycloartenol, but it does not follow that HMGR should affect the genesis of all of the Δ^5-sterol end products, particularly if one or more enzymes that transform cycloartenol to sitosterol are controlled at the level of differential gene expression. We assume a fundamental control of phytosterol transformation comes from a simple mechanism where the enzymes that act on sterols respond to changes in the concentration of their substrates. When the substrate concentration rises, the reaction goes faster in most cases. The increased rate of removal of the substrate (viz., into ester droplets or into membrane sites) prevents further rise in its concentration. When the level rises to a point where there is no available sink, then the end product may bind to sterol enzymes and downregulate their activity. In support of our hypothesis, the level of cycloartenol was increased severalfold by amplifying the activity of HMGR through molecular biology techniques.[4] The level of Δ^5-sterol end products was not increased beyond the level of free sterol which could be inserted into the membrane rather, cycloartenol and related intermediates accumulated in the plant. In a study of plant sterol mutants which overproduced sterol, the extra sterol was esterified, suggesting a requirement for sterol homeostasis that is associated with sterol controlled membrane fluidity.[33] We found that sitosterol downregulated SMT activity in sunflower,[28] which is consistent with end-product control on the first step in the pathway.

We recently measured HMGR and SMT activities in corn seedlings following seed germination and correlated this information with the amount and types of sterols produced by the seedlings during maturation and with kinetic studies involving pulse-chase experiments with 8-day seedlings incubated with radiolabeled acetate and mevalonate. As the data in Figure 7 show, HMGR activity was not correlated with anatomical development and Δ^5-sterol production, whereas SMT activity was associated with Δ^5-sterol production. The sterol composition of the sheath was different from blade from which sheath tissue originates. Blades produce mainly Δ^5-24-ethyl sterols, whereas sheaths produce mainly $\Delta^{5,23(24)}$-24-methyl sterols. The difference in sterol production is related to a shift in the C-24 biomethylation enzyme activities (Figure 7). Hence, the SMT may be a key regulatory enzyme in phytosterol synthesis. Additional evidence that favors the SMT as the critical slow step in phytosterol transformation is based on the observations from sunflower and corn, which show that, of the enzymes that may act on cycloartenol, the SMT has the highest degree of sterol specificity and is a relatively slow-acting enzyme.

We observed product inhibition from 24(28)-methylene cycloartanol using a soluble corn SMT preparation (K_i 100 μM) and with a microsome-bound sunflower preparation.[28] In corn, HMGR activity was not sensitive to incubation with phytosterols, sitosterol, or 24(28)-methylene cycloartanol (up to a concentration of 100 μM), which is essential for an enzyme to be a rate-limiting enzyme of sterol production. Alternatively, the observation that sitosterol downregulates SMT activity in sunflower indicates the ultimate product of the sequence inhibits the first reaction peculiar to phytosterol transformations.

IV. TRANSITION STATE ANALOGS (TSA)

The first transition state analog tested in a cell-free preparation from plants was triparanol,[34] which was found to inhibit the biomethylation reaction in a similar manner to its effect on reduction of the 24,25-double bond from rats. Subsequently, a series of rationally designed sterol biosynthesis inhibitors were prepared synthetically to block the biomethylation reaction and other steps in cycloartenol conversion to sitosterol.[3,28] There are also several examples of TSA of phytosterol transformation that occur as natural products.[2,3] So far all the transition state inhibitors which have been tested in cell-free systems are reversible inhibitors.*

V. ALTERNATE C-24 METHYLATION PATHWAYS: EVIDENCE FOR TWO DISTINCT (*S*)-ADENOSYL-L-METHIONINE: $\Delta^{24(25)}$-STEROL METHYL TRANSFERASE ENZYMES FROM THE SAME PLANT

The mechanism of biomethylation of phytosterol posits a series of stereochemically related events — electrophilic alkylations, rearrangements, and deprotonations that may arise from a common set of

* After this chapter was complete we prepared and tested a 26,27-cyclopropylidene cycloartenol that inhibited the SMT from core. This new substrate analog was found to be a suicide substrate (Nes and Guo, submitted).

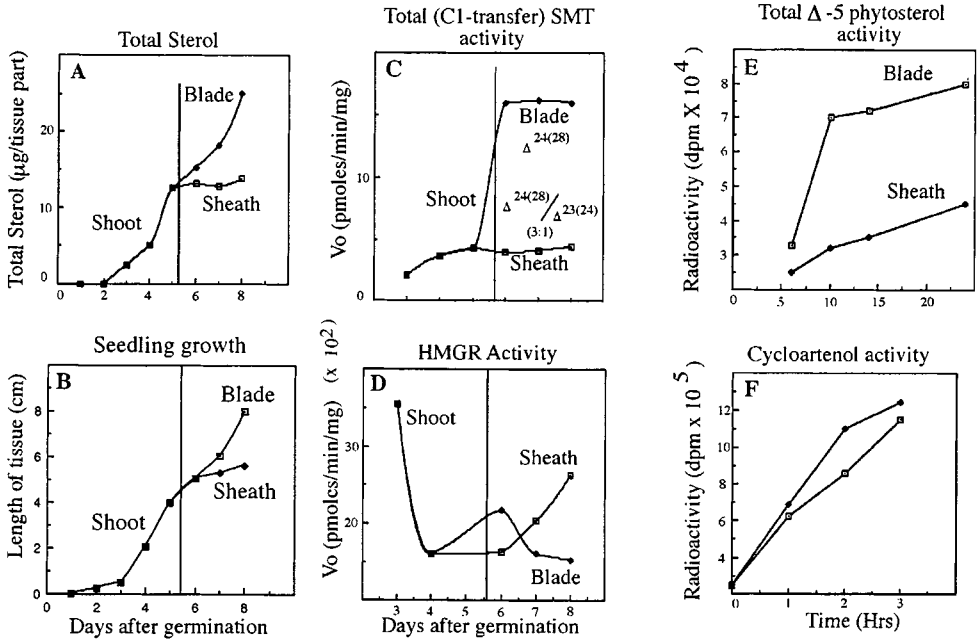

Figure 7 Sterol content and enzyme activities (HMGR and SMT) in developing corn seedling tissues (Venkatramesh, M. and Nes, W.D., unpublished): (A) Total sterol levels were determined by GLC of the non-saponifiable lipid fraction; (B) length of tissue measured as a function of seedling development from seed germination. (C) SMT activity measured in microsome preparations from the corresponding tissues. Assays were performed with 50 μ*M* each of cycloartenol (c1a and c1b) or 24-methylene lophenol (c2) and [$^{3}H_{3}$-*methyl*]AdoMet as substrates (by the methods used in Reference 28); further, C1a represents the biomethylation of cycloartenol to 24(28)-methylene cycloartanol (as in step "a" in Figure 6) and C1b represents the biomethylation of cycloartenol to cyclosadol (as in Figure 8) while C2 represents the biomethylation of 24(28)-methylene lophenol to citrostadienol (as in step "h" in Figure 6). (D) HMG-CoA reductase (HMGR) activity assayed from microsome preparations according to the methods of Bach et al.[40] (E) Pulse chase study using intact corn seedlings incubated with ^{14}C-mevalonic acid to determine carbon flow into total Δ^{5}-sterols (cf. Reference 11). (F) Pulse chase study with ^{14}C-mevalonic acid to determine carbon flow into cycloartenol (cf. Reference 11).

enzymatic intermediates. However, it is unclear whether the alternate products of the biomethylation reaction occur from the action of a single enzyme or multiple enzymes that give rise to distinct C-24 methylated sterols. Assuming three major transition state intermediates exist in the biomethylation reaction progress and the variant sterol side chain conformations contribute to the mechanism (Figure 3), then it should be possible to detect an induced kinetic isotope effect (KIE) on the rate of product formation using deuterium-labeled substrates. Induced KIEs which result from an isotopically sensitive branching pathway involving one enzyme may result from primary or secondary deuterium isotope effects which *accelerate* the rate of formation of one enzymatic product by *suppressing* the rate of formation of a second enzymatic product, such that there is no significant change in the overall rate of the reaction.[35] For two enzymes, induced KIEs may produce singular rate changes that lead to slowed formation of the only 24-alkene isomer derived from deuterium elimination,[36] or they may produce concurrent rate reductions in the formation of both 24-alkene sterol products.

One approach used in this laboratory to distinguish the operation of one or two enzymes consists of measuring the rate changes and product ratios under saturating conditions resulting from incubations with a cell-free system of corn[37] and yeast[38] involving AdoMet/ [$^{2}H_{3}$-methyl] AdoMet and a sterol acceptor molecule which may possess one or another isotopic substitution. As we discovered for the $\Delta^{24(28)}$ SMT from yeast k_H/k_D = 1.3, whereas the $\Delta^{23(24)}$24-methyl SMT and $\Delta^{24(28)}$ SMT from corn gave KIE of 1.8 and 1.3, respectively. Whereas there was only one SMT detected in yeast, two SMTs were detected in corn based on the KIE studies. In further support that corn produced two SMTs was the fact that apparent K_m (a measure of sterol binding to the different SMT enzymes) for cycloartenol and lanosterol conversion to the $\Delta^{24(28)}$-producing SMT was 30 μ*M* and 19 μ*M* whereas the K_m for cycloartenol and lanosterol for the $\Delta^{23(24)}$24-methyl-producing SMT was 10 μ*M* and 7 μ*M*, respectively. Rate changes

that result from incubation with the different sterol substrates assayed with the corn enzymes are shown in Table 1.[37] The summation of the results reported here (cf. Figure 8) indicate that under physiological conditions both 24(28)-methylene cycloartanol and cyclosadol ($\Delta^{23(24)}$-24-methylcycloartanol) arise naturally from different biomethylation enzymes.

Table 1 Product Distribution and Rates of Sterol C-24 Biomethylated Alkene Formation by SMT Enzymes from Corn Sheaths with Deuterium-Labeled Sterol and AdoMet Substrates

Substrate	Product Distribution $\Delta^{24(28)}$-sterol/$\Delta^{23(24)}$-sterol	V_{max} (pmol/min/mg) $\Delta^{24(28)}$-sterol	$\Delta^{23(24)}$-sterol
Cycloartenol and [$^3H_3$-*methyl*]AdoMet[a]	2.28/1	2.6	0.9
[24-^{2}H]Cycloartenol and [$^3H_3$-*methyl*]AdoMet[a]	2.40/1	2.5	0.9
[3-^{3}H]Cycloartenol and AdoMet	2.54/1	2.7	0.9
[3-^{3}H]Cycloartenol and [$^2H_3$-*methyl*]AdoMet	4.76/1	2.4	0.5
Lanosterol and [$^3H_3$-*methyl*]AdoMet[a]	1.56/1	1.3	0.6
[24-^{2}H]Lanosterol and [$^3H_3$-*methyl*]AdoMet[a]	1.53/1	1.3	0.6
[3-^{3}H]Lanosterol and AdoMet	1.63/1	1.2	0.6
[3-^{3}H]Lanosterol and [$^2H_3$-*methyl*]AdoMet	3.51/1	1.0	0.3
[23-$^2H_2$]Lanosterol and [$^3H_3$-*methyl*]AdoMet[a]	3.31/1	1.3	0.3

Note: 24-Alkene sterol production was determined by standard assay of aliquot of the same enzyme preparation at the saturating substrate concentration of 75 μ*M*; distribution of 24-alkene sterol products was determined by radio reversed-phase high performance liquid chromatographic analysis and the experiment was run in quadruplicate with no significant variation in product distribution; the variation in V_{max} never exceeded ± 0.05 pmol/min/mg protein. Endogenous level of microsomal cycloartenol, 24(28)-methylenecycloartanol and cyclosadol in the corn tissue was found to be in a ratio of 62:27:11; the products were present at 0.37 μmol. In the soluble SMT preparation the level of endogenous sterol decreased by a factor of 10.

[a] A calculation factor of 1.5 was used to account for the loss of one tritium atom from the [$^3H_3$-*methyl*]AdoMet, which was incorporated into the $\Delta^{23(24)}$-24-methyl sterol.

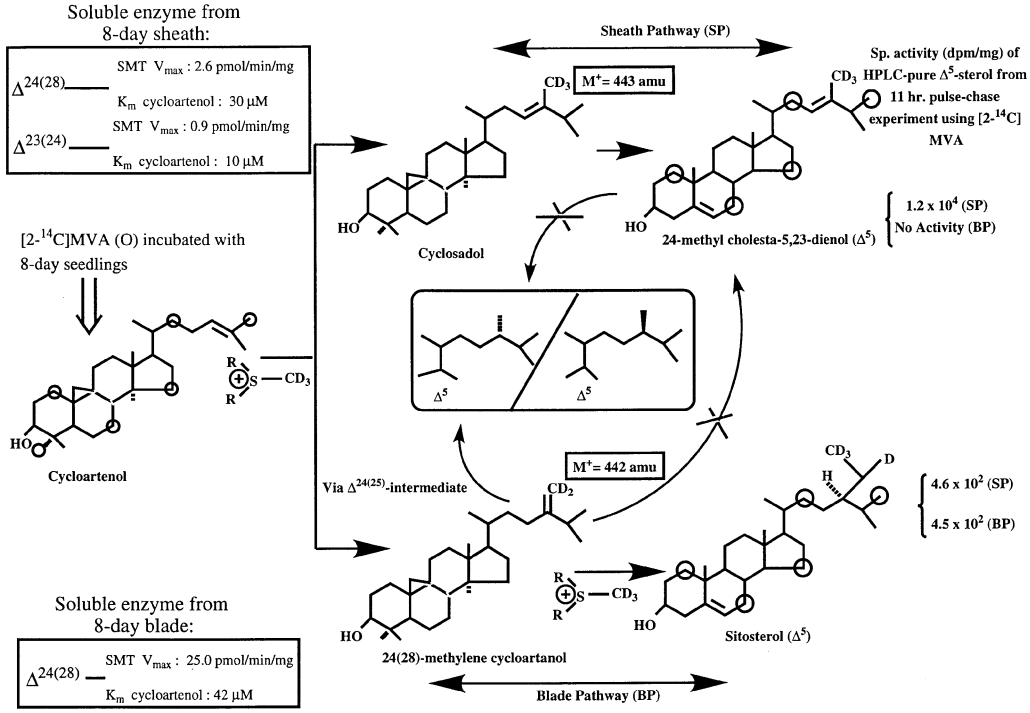

Figure 8 Results from several experiments, intact seedlings fed different sterol precursors and cell-free SMT preparations that show corn produces alternate biomethylation pathways that give rise to different Δ^5-sterol end products.

VI. FUTURE DIRECTIONS

The purpose to elucidate the uniformity and differences in the phytosterol synthetic pathways is to better understand which enzyme or set of enzymes may be worth tinkering with at the molecular biology level or to target for inhibition by a rationally designed sterol biosynthesis inhibitor. The future holds the production of plant mutants with specific changes in sterol composition which may provide a resource for the investigation of sterol-controlled membrane structure and function. It is expected that foreign sterol genes may be introduced into plant cultures, transformed to be herbicide resistant.[39] Specific sterol enzymes will be identified, their genes cloned, sequenced, and expressed in a suitable vector. Isolation and characterization of these sterol genes and their gene products will allow for site-mutagenesis experiments to follow. The mutated genes may be introduced into plants (transgenic plants) to study sterol functions in plant physiology or simply to affect broad-based resistance to insects and nematodes. Key to all these studies is for sterol enzymes from plants to be purified, from which antibodies may be produced and other information may be obtained, such as the primary structure, subunit composition, and native molecular weight.

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