EVIDENCE FOR DIFFERENCES IN STEROL BIOSYNTHESIS AND DERIVATIZATION IN SORGHUM[1]

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ABSTRACT.—The 24-desalkyl and 24-alkylsterols synthesized by leaves of flowering sorghum plants were identified by a combination of glc, hplc, ms, and nmr. Leaves from plants in the same developmental stage were treated with [2-^{14}C]-acetate, and the radioactivity was associated with individual components of the 4,4-desmethyl sterol fraction determined by a combination of hplc-radioactivity counting and glc/ms. The specific activities of sorghum leaf sterols are in the following decreasing order: cholesterol (1036 dpm/μg)>24α-ethylcholesterol (416 dpm/μg)>24ξ-methylcholesterol (389 dpm/μg)ξ24α-ethylcholesta-5,22-dienol (39 dpm/μg). While all four of these sterols were detected by glc as components of steryl glycosides and steryl esters, only the 24-alkysterols were present as [^{14}C]-labeled steryl glycosides. The differential regulation of sterol biosynthesis and derivatization is discussed in terms of differences in their functions.

The biosynthesis of sterols is believed to occur essentially by the same pathway in "main line" tracheophytes (1), of which sorghum, a C-4 plant, is a member. In the present study, we observed that the biosynthetic bifurcation (2) in which the $\Delta^{24(25)}$-bond alternatively is reduced to produce cholesterol or alkylated to produce campesterol (24α-methylcholesterol),[2] stigmasterol (24α-ethylcholesta-5,22-dienol) or sitosterol (24α-ethylcholesterol) may be significant to the ecological interaction of sorghum with insect (3) and fungal (4,5) pathogens known to depend on the availability of sterols in this host to grow, develop, and reproduce (6,7). Before we continued structure-activity tests involving lipid components of the host with aphids and oomycetous fungi, we had to gain detailed knowledge of the identities, amounts, turnover rates, sites of synthesis, and translocation capabilities of sterols in sorghum at different stages of its maturity. Information on the first three parameters of flowering sorghum will be presented here, while data on the latter two points will be presented elsewhere. Palmer and Bowden have presented some information on sterols in grains (8,9) and maturing grains (10) of *Sorghum vulgare*.

MATERIALS AND METHODS

Sorghum plants [*Sorghum bicolor* (L.) Moench cv IS-809], also referred to as *S. vulgare* (8), were grown from seed for approximately three months, as previously described (6). The shoot used was 36 cm tall with seven visible leaves. The immature inflorescence of this shoot was just emerging and was green. The radioactive material {250 μCi of [2-^{14}C]-acetate, as the sodium salt (58.9 mCi/mMol), purchased from Amersham International Limited} was dissolved in an ethanolic solution of 0.1% silicone oil and 0.1% *DL*-α-tocopherol. Half the solution was painted by the "brush technique" along the midrib (upper surface) of the blade and onto part of the ligule of four leaves. The other half was applied to the plant in the same way 72 h later. After seven days of incubation (seven days from the first treatment) at room temperature (24±2°), the [^{14}C]-treated blade and ligule parts—which we will refer to as leaf tissue—were sectioned from the plant and weighed. Information regarding details of extraction, chromatography, radioactive counting, ms, and nmr have been presented in earlier communications (11-13); also, see Table 1.

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[1]A preliminary report of this study was presented at the American Society of Biological Chemists' Meeting, San Francisco, CA, June 6, 1983.

[2]Replacement of the hydrogen atom with another group can alter the configurational designation in the *R, S* notation; thus, we prefer to use the α,β-designation of Plattner *et al.* as modified by Nes (2). The α-oriented substituent (hydrogen, etc.) is in front, when C-22 is to the right (*trans*-oriented with respect to C-13) in the usual view of the molecule (*cf.* Figure 3). The configuration at C-24 for the ethyl but not methyl cholesterol was proven by nmr (see Results).

TABLE 1.   Chromatographic and Physical Constants of Sterols Isolated from Leaves Treated with [14C]-Acetate

Trivial Name	GLC (RRT_c)[a]			HPLC α_c[b] System		MS (m/z)[e]	nmr
	SE-30	OV-17	SP-1000	1	2		
Cholesterol	1.0	1.0	1.0	1.0	1.0	M^+(386, 100%), M^+-CH_3(371, 33%), M^+-H_2O(368, 43%), M^+-CH_2-H_2O (353, 30%), M^+-85(301, 40%), M^+-SC (side chain)(273, 28%), M^+-SC-H_2O(255, 25%), M^+-SC-C_2H_6(231, 30%), M^+-SC-C_3H_8(229, 12%), M^+-SC-C_3H_6-H_2O(213, 38%), M^+-SC-C_3H_8-H_2O(211, 13%)	N.E.[c]
Campesterol (24α-methylcholesterol)	1.29	1.33	1.29	1.14	1.20	M^+(400, 100%), M^+-CH_3(385, 31%), M^+-H_2O(382, 58%), M^+-CH_2-H_2O (367, 24%), M^+-85(315, 25%), M^+-SC(273, 19%), M^+-SC-H_2O(255, 26%), M^+-SC-C_2H_6(231, 17%), M^+-SC-C_3H_6(229, 6%), M^+-SC-C_3H_6-H_2O(213, 28%), M^+-SC-C_3H_8-H_2O (211, 1%)	N.E.[c]
Stigmasterol (24α-ethylcholesta-5,22-dienol)	1.40	1.47	1.32	1.14	1.20	M^+(412, 67%), M^+-CH_3(397, 6%), M^+-H_2O(394, 19%), M^+-CH_2-H_2O (379, 6%), M^+-C_3H_5(369, 14%), M^+-85(327, 2%), M^+-C_7H_{10}-H_2O(300, 28%), M^+-SC(273, 12%), M^+-SC-2H (271, 29%), M^+-SC-H_2O(255, 46%), M^+-SC-C_2H_6(231, 6%), M^+-SC-C_3H_8 (229, 7%), M^+-SC-C_3H_6-H_2O(213, 15%)	N.E.[d]
Sitosterol (24α-ethylcholesterol)	1.61	1.68	1.54	1.32	1.42	M^+(414, 100%), M^+-CH_3(399, 23%), M^+-H_2O(396, 58%), M^+-CH_2-H_2O (381, 25%), M^+-85(329, 27%), M^+-SC(273, 17%), M^+-SC-H_2O(255, 25%), M^+-SC-C_2H_6(231, 14%), M^+-SC-C_3H_6-H_2O(213, 23%)	nmr of sitosterol C-18 (0.68s), C-19(1.01s), C-21(0.92d, J=6.0 Hz), C-26 and C-27(0.84d, 0.82d, J=6.0 Hz), C-29 (0.85t, J=7-8 Hz) ppm.

[a]RRT_c = Retention time relative to cholesterol.
[b]α_c = k'/k' for cholesterol.
[c]N.E. = not examined due to insufficient material, therefore the absolute configuration at C-24 of this isolated sterol is equivocal.
[d]N.E. = not examined but probably α-ethyl at C-24 because [14C]-stigmasterol is synthesized from foliar applied [14C]-sitosterol (Heupel and Nes, unpublished).
[e]Major significant ions at high mass end.

For hplc, an Altex model 110 A pump (Altex, Berkeley, CA) was connected through a model 7125 (Rheodyne) injector to a new 250 x 4 mm i.d. stainless steel chromatography tube (Altex) packed with ultrasphere-ODS (5 μm dp). The eluent for reversed-phase chromatography was 96% (system I) or 90% (system II) aqueous MeOH. The flow rate was 1.6 ml/min. The detector was a Hitachi variable-wavelength spectrometer (Altex model 155) set at 205 nm. Hplc retention volumes (and times) were expressed relative to that of cholesterol, which was either added as an internal standard or chromatographed immediately before or after the sample.

As shown in equation 1, the k' values of the compounds in the sample(s) divided by that of cholesterol give α_c, which is a measure of selectivity relative to cholesterol. The k' values were calculated as shown in equation 2 where V_R and t_R were the retention volumes and retention times of the various compounds, V_o was the void volume of the column, and t_o was the retention time of a compound not retained by the reversed-phase column. The elution time for cholesterol was observed to be in solvent system I, 24 min and in solvent system II, 75 min.

$$\alpha_c = \frac{k' \text{ of compound}}{k' \text{ of cholesterol}} \qquad \text{Eq. 1}$$

$$k' = \frac{V_R - V_o}{V_o} = \frac{t_R - t_o}{t_o} \qquad \text{Eq. 2}$$

Glc retention times were also expressed relative to that of cholesterol; the retention times of the compounds in the sample were divided by the retention time of cholesterol added as an internal standard to give RRT_c. The retention time of cholesterol was approximately 10 min for each of the three packed columns; i.e., 3% SE-30, 3% OV-17, and 1% SP-1000 operated at 235°, 235°, and 255°, respectively. A plot of increasing amounts of cholesterol (glc) and [^{14}C]-cholesterol (hplc, system I) was linear between 64 ng and 4 μg (glc) and 1.0 μg and 70.0 μg (hplc). Recovery of [^{14}C]-cholesterol from the hplc column was >97%. For hydrogenation, the campesterol/stigmasterol fraction (containing 314 μg stigmasterol and 113 μg campesterol) was diluted with authentic campesterol (119 μg) and cholesterol (302 μg). The fraction containing carrier material was hydrogenated under 1 atm of H_2 gas for 4.75 h with PtO_2 catalyst (11.24 mg) and EtOH (8 ml). Because the catalyst coagulated, an additional amount of PtO_2 (7.89 mg) was added and stirring continued for an additional 3.5 h. The mixture was filtered to remove the catalyst and then evaporated to dryness under N_2. This hydrogenated sample was dissolved in EtOH and examined further by hplc. Acid and base hydrolyses were performed as described in the literature (14).

RESULTS

Preliminary studies indicated that the sterol content of leaf tissue in developmental stages equivalent to that of the leaf tissue at the time of [2-^{14}C]-acetate treatment and at the time of harvest (seven days later) did not vary to a measurable extent. Thus, it was necessary to feed a common radio-labeled precursor, [2-^{14}C]-acetate, to determine the turnover rates of the individual sterols relative to each other by determining the mass of each isolated sterol and the radioactivity associated with each.

The leaf tissue was treated with 5.55 x 10^8 dpm (58.9 mCi/mMol) [2-^{14}C]-acetate. The treated leaf tissue, after sectioning, weighed 10.11 g fresh weight (1.95 g dry weight). The dried and ground leaf material was extracted with refluxing Me_2CO for 18 h in a Soxhlet apparatus. The specific activity of the dried Me_2CO extract (total lipid fraction, tlf) was 2.4 x 10^7 dpm/87.1 mg. An aliquot (8.3% by volume) of the tlf was saved to analyze for steryl esters and steryl glycosides. The other 91.7% tlf was saponified with 10% methanolic KOH under reflux. The nonsaponifiable lipid fraction (nlf) was partitioned into the organic phase by extraction with Et_2O and H_2O (specific activity, 1.15 x 10^7 dpm/36.2 mg-gravimetric determination). The nlf was chromatographed on 3% deactivated Al_2O_3 column eluted with increasing amounts (10% increments of 100 ml each) of Et_2O in hexane followed by elution with more polar solvents, i.e., MeOH, n-BuOH, and glacial HOAc.

The 4,4-desmethyl sterols (specific activity 4.67 x 10^5 dpm/2.4 mg-gravimetric determination) were found to elute in the fraction that contained Et_2O-hexane in the

proportions 60:40, respectively. Quantitation by glc and hplc of this fraction showed four sterols: cholesterol 18.25 μg, campesterol 248 μg, stigmasterol 658 μg, and sitosterol 470 μg. An aliquot of this material was analyzed by glc-ms, and the identities of the four sterols were confirmed. The 4,4-desmethyl fraction was purified by adsorption tlc on a silica gel G plate developed with C_6H_6-Et_2O (9:1). We injected an aliquot of the purified fraction (to which carrier cholesterol was added) into the hplc (system I) and determined the radioactivity in each of 60 one-minute fractions (Figure 1). The rest of the sample was separated by preparative hplc (same system) into (I) cholesterol, (II) a mixture of campesterol and stigmasterol, and (III) sitosterol (Table 1).
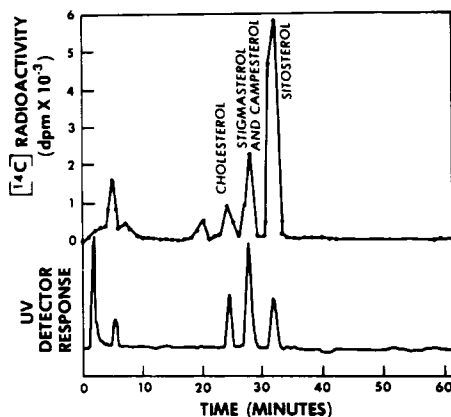

FIGURE 1.   Hplc-rc of 4,4-desmethyl sterols from sorghum leaves treated with [2-^{14}C]-acetate.

The three fractions were again purified by tlc to remove residual hplc bleed, and their specific activities were assessed by glc and radio-counting. Their specific activities were as follows: (I) 1036 dpm/μg, (II) 214 dpm/μg, (III) 416 dpm/μg. Because our reversed-phase hplc system fails to separate stigmasterol from campesterol, i.e., (II), this fraction—together with added carrier cholesterol and campesterol—was hydrogenated with PtO_2 catalyst and the reduced products rechromatographed by hplc (system I, Figure 2). The uv detector set at 205 nm does not detect the saturated sterol molecule; therefore, the radioactive peaks eluting between 20 and 40 min; at α_c 1.00, α_c 1.14, α_c 1.32, and α_c 1.49, were examined by glc. A 1% SP-1000 packed glc column was used to analyze these hplc fractions, because this liquid phase readily separates Δ^0- from Δ^5-sterols.

As expected, the first component contained some unreduced cholesterol; the second peak was a mixture of cholestanol and campesterol; the third peak, a mixture of campestanol and sitosterol (SP-1000, RRT_c 1.19 and 1.54, respectively); and the fourth peak, relatively pure, >99%, stigmastanol (SP-1000, RRT_c 1.41). Because we added a mixture of radiolabeled campesterol and stigmasterol, we expected to find no radioactivity in the α_c 1.14 peak corresponding to cholestanol (SP-1000, RRT_c 0.92). Had there been significant radioactivity present in cholestanol, we should have seen more radioactivity in the eluent corresponding to cholesterol (α_c 1.00). Thus, by knowing the radioactivity corresponding to α_c 1.49 and α_c 1.14 coupled with our determining the mass in each of the eluted fractions by glc, we were able to calculate the specific activities of campesterol and stigmasterol in the original sterol mixture, which was 389 dpm/μg in the former and 39 dpm/μg in the latter.

An aliquot of the tlf (3.8%) was chromatographed on silica gel G plates developed with C_6H_6-Et_2O (9:1). Three principal radioactive zones were detected corresponding
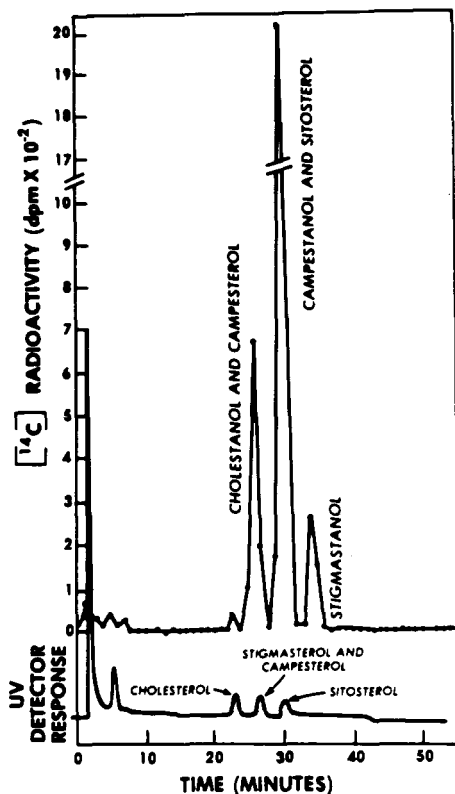
FIGURE 2. Hplc-rc of stigmasterol/campesterol mixture from sorghum leaves treated with [2-^{14}C]-acetate after hydrogenation. (Cold cholesterol was added prior to hydrogenation. Stanols not detected by uv detector. Hydrogenation process failed to reduce completely Δ^5 to Δ^0-sterols.)

in Rf values (13) to steryl glycosides (2.4 x 10^5 dpm), free sterol (0.39 x 10^5 dpm), and steryl esters (2.05 x 10^5 dpm). The steryl glycoside and the steryl ester fractions were hydrolyzed (acid hydrolysis for the glycosides and base hydrolysis for the esters), and the hydrolysates were extracted with Et$_2$O. We then chromatographed these extracts in the tlc system described to obtain free sterols. The "free sterol" fractions liberated from the glycoside and ester forms contained considerably less radioactive material than the free sterol fraction from the tlf. The proportion of radioactive sterols in the glycoside (5.0 x 10^3 dpm) and ester (2.46 x 10^3 dpm) forms relative to that in the free form (0.39 x 10^5 dpm) was found to be in the ratio 11:5:84. Glc analysis of each 4,4-desmethyl zone after chromatography of the hydrolysate extracts showed cholesterol, campesterol, stigmasterol, and sitosterol to be components of the esters and glycosides. Cholesterol (10 dpm/0.5 μg, which essentially represents background) was not labeled in the hplc-radio activity counting (system I) chromatogram of the sterol mixture liberated by acid hydrolysis from the glycosides, while the other sterols were labeled: campesterol/stigmasterol mixture 321 dpm/1.5 μg and sitosterol 1088 dpm/2.6 μg. The same four sterols were detected in the sterol fraction (specific activity of the total sterol mixture was 2.4 x 10^3 dpm/3.07 μg) liberated from the ester form by base hydrolysis. The ratio of cholesterol, campesterol, stigmasterol, and sitosterol as determined by glc on 3%

SE-30 (packed column) was 12:14:36:38. The low level of the labeled sterols liberated by hydrolysis of both the glycoside and ester fractions coupled with the lack of finding labeling associated with cholesterol liberated from the glycoside form would indicate the derivatization of 24-desalkylsterols is operational primarily at an earlier stage in the leaf's development.

DISCUSSION

While the mechanism and sequence leading to C-24 reduction and alkylation of the sterol side chain in higher plants is well known (2) (Figure 3), less is understood about the relative turnover rates of these compounds as free or C-3 derivatized products. On the assumption that the levels of each sterol in the cytoplasmic pool of the leaf did not change during the seven-day treatment, then, in the present study, the observed differences in specific activities may reflect a continued turnover of the molecules, but at significantly different rates. A long half-life (low specific activity when isolated from feeds) for sterols in a plant's development, as recently shown by Doireau *et al.* (15), would be presumptive evidence for sterols having a structural role (16), while a short half-life (high specific activity when isolated from feeds) may indicate a hormonal (17, 18) or regulatory role.[3] In few studies has there been separation of the sterol mixture into individual components from which specific activities were determined.

In the present study, we have demonstrated that the endogenous pool size of the 24-desalkylsterols is minimally one order of magnitude less than the mixture of 24-alkylsterols. Although more data are required, this relative pool size apparently does not change throughout the life history of the plant (8-10). The small amount of cholesterol found may have been due to laboratory-introduced contamination during the extraction of the leaf tissue, or, quite conceivably, to the dealkylation of the 24-alkylsterols by known sorghum fungal or insect pathogens (6, 7). However, the fact that the ratio of radioactivity to mass, *i.e.*, its specific radioactivity, found in the hplc fractions corresponding to cholesterol (1036 dpm/μg) was of the same order of magnitude as those corresponding to radiochemically pure campesterol (389 dpm/μg) and sitosterol (416 dpm/μg), indicates that sorghum is definitely capable of synthesizing cholesterol. A precursor-product relationship of cholesterol with the 24-alkylsterols involving desmosterol as an intermediate, as suggested by Jacobsohn and Frey (19), to account for differences between cholesterol and the 24-alkysterols specific activities in a tracheophyte was ruled out in the present study inasmuch as [^{14}C]-cholesterol applied to the sorghum was recovered 24 h later in unchanged form (no 24-alkysterols were detected by hplc-radio counting Me$_2$CO extracts of the leaf material (20, also Heupel and Nes, unpublished data).

The small, and probably constant, pool size and high specific activity of cholesterol imply that its biosynthesis is tightly regulated throughout the plant's development. This implies that the $\Delta^{24(25)}$-reductase is a heretofore unrecognized key enzyme in sterol biosynthesis whose regulation plays an important role in the alkylation-reduction bifurcation (Figure 3) in sterol biosynthesis. While the specific activity of cholesterol is approximately twofold higher than the specific activities of sitosterol and campesterol, the difference is not sufficient to warrant a hormonal role for this sterol and not the other two. Alternatively, the large pool size of the stigmasterol and its low specific activity (39 dpm/μg) indicate that this sterol may play the major non-metabolic structural role in the lipid leaflet throughout the life history of the plant and that most of the stigma-

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[3]Steroid hormones are oxygenated sterol metabolites presumably synthesized in one part of the plant and transported to some other target tissue. A regulatory sterol may be defined (30, 31, 33) as one that effects plant physiology by affecting membrane functions without its prior transport into and out of the cell (*cf.*, last paragraph in Discussion).
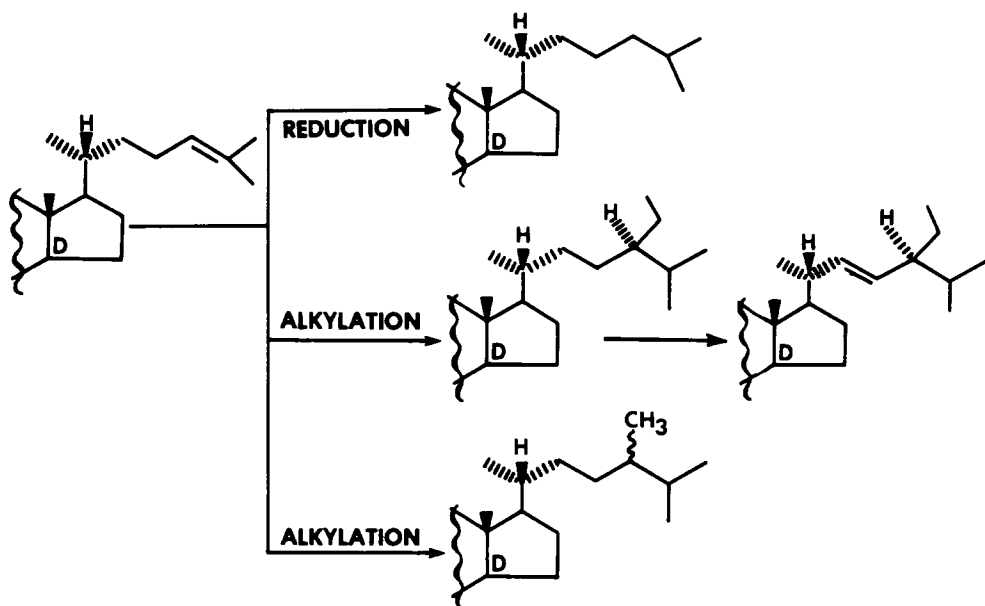
FIGURE 3.   C-24 alkylation-reduction bifurcation operative in sorghum.

sterol was synthesized prior to the feed. We have determined that foliar applied [^{14}C]-sitosterol is converted to [^{14}C]-stigmasterol by sorghum (Heupel and Nes, unpublished data). Thus, the differences in the specific activities and pool sizes of sitosterol and stigmasterol indicate that the Δ^{22}-dehydrogenase (21, 22) may be the regulatory enzyme that controls the level of membranous 24-ethylsterols in the mature leaf.

By monitoring the uptake of sterols into caged aphids that had been feeding at various intervals along the surface of a sorghum leaf, we have observed that cholesterol and sitosterol are actively translocated (20). Because the leaf fails to convert cholesterol into a glycoside at this point in its development, this metabolic derivative (steryl glycoside) cannot be the form of the molecule transported in the plant, as suggested by others (23). Similar findings have been reported earlier for translocation in *Solanum khasianum* (24) and *Pelargonium hortorum* (25). Cholesterol and a series of Δ^5-24-alkylsterols can be derivatized to steryl glycosides in a fungus unable to synthesize sterols (7) and in *in vitro* enzyme preparations from a variety of tracheophytes (26). Cholesterol glycosides and 24-alkylsteryl glycosides have been shown to co-occur with the free forms in higher plants (27 and references cited therein). The failure to observe in a mature sorghum leaf the active conversion of cholesterol to a glycoside may have to do with regulation of this process through compartmentalization *in situ*. Lack of transfer of this molecule from the endoplasmic reticulum [site of sterol biosynthesis (28)] to the plasmalemma [site of glycosylation (28, 29)] may account for its inability to be glycosylated.

Recently, there has been a demonstration of a multiplicity of roles for sterols in fungi (30-32). These different functions are not believed to involve metabolism but rather to involve, in the lipid leaflet of the membrane, the binding of sterol to protein (regulatory) (31, 33), on the one hand, and to fatty acyl lipids (structural interaction), on the other. The differences in the observed specific activities and pool size coupled with derivatization or lack thereof, between the 24-desalky- and 24-alkylsterols indicate that these two groups of sterols may also have different roles in sorghum.

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