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Short Communication

Effects of Free Sterols, Steryl Ester, and Steryl Glycoside on Membrane Permeability[1]

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The physiological importance of sterols in higher plants has never been fully established. It has been suggested that they are structural components of membranes (7, 11), and that they might be involved in controlling the permeability of membranes (6). Cholesterol was found to be more effective than $CaCl_2$, the classic membrane stabilizer, in preventing the methanol-induced leakage of betacyanin from red beet disks (6). Other plant sterols, such as β-sitosterol and stigmasterol, were found to be less effective. It was suggested that only those sterols that have a flat molecular configuration, similar to that of cholesterol, are physiologically active because only they can penetrate the phospholipids of the membranes (6). Sterols in higher plants occur in at least three different forms: as free sterols, steryl esters, and steryl glycosides, and the steryl glycosides may or may not be acylated (1, 4). Quantitatively, these sterol forms vary with tissue and species. In potato tuber the steryl glycosides are the major form (4), while in barley shoots the free sterols are highest in concentration (1). Only the free sterols have been tested for their membrane stabilizing effectiveness (6). It, therefore, seemed of interest to compare the effectiveness of free sterol, steryl ester, and steryl glycoside on membrane permeability.

MATERIALS AND METHODS

Barley Root Material. Barley (*Hordeum vulgare,* var. Barsoy) was germinated on cheesecloth over continuously aerated 0.5 mM $CaSO_4$ at room temperature in the dark. Three-day-old barley roots were harvested and placed in 0.5 mM $CaSO_4$ for 30 min, transferred to 5 mM KCl for 1 hr, and washed with 500 ml of distilled water in a funnel layered with cheesecloth. The $CaSO_4$ and KCl solutions were continuously aerated.

Chemicals. Cholesterol, campesterol, stigmasterol, β-sitosterol, and cholesteryl palmitate were purchased from Applied Science Laboratories, Inc., State College, Pa. The synthesis of cholesteryl glucoside was by the method of Meystre and Miescher (13). Five grams of cholesterol and 6 g of silver carbonate were dissolved in 200 ml of benzene. This mixture was brought to boiling under continuous stirring, and 13.3 g of acetobromo-D-glucose dissolved in 300 ml of benzene were added drop-wise with continuous boiling and stirring. The mixture was filtered, taken to dryness under vacuum, and redissolved in 200 ml of methanol. It was cooled in an ice bath and 250 ml of sodium alcoholate were added. The mixture was allowed to stand for 3 hr and then neutralized with dilute HCl. The cholesteryl glucoside precipitated. The precipitate was washed with alcohol and ether, boiled in water, cooled, filtered, and washed with cold water. The residue was dissolved in 50 ml of pyridine, heated, and filtered, and water was added till a precipitate was obtained. The precipitate was washed with alcohol and ether, and applied in *n*-hexane to a 5-g silica gel column (1-cm diameter). The column was washed with 150 ml of 40% benzene in *n*-hexane, 50 ml of benzene, and 100 ml of chloroform; all were discarded. The cholesteryl glucosides were eluted with 150 ml of 5% methanol in chloroform. This fraction gave a single spot by thin layer chromatography.

Analysis of Sterols. About 50 g of fresh barley roots were homogenized in acetone and extracted in a Soxhlet apparatus for 24 hr. At the same time three samples were removed for dry weight determination. One gram of fresh roots equaled 0.0543 g of dry weight. The acetone extract was taken to dryness under vacuum, redissolved in 50 ml of acetone, and dried under vacuum in the presence of 5 g of silica gel. This procedure allowed for a more exact transfer of the *n*-hexane suspended sample to the silica gel column. A 1.5-cm diameter column packed with 25 g of silica gel (70 to 325 mesh) in *n*-hexane was used for serial elution (5, 14). Passed through the column were 150 ml of 10% benzene in *n*-hexane. This fraction was discarded. The steryl esters were eluted with 700 ml of 40% benzene in *n*-hexane. The free sterols were eluted next with 150 ml of 100% benzene followed by 800 ml of chloroform. The acylated steryl glycosides were eluted with 700 ml of 2% methanol in chloroform, and the steryl glycosides were eluted with 600 ml of 5% methanol in chloroform. The four sterol fractions were taken to dryness under vacuum. The esters were hydrolyzed with 5% KOH in 95% methanol for 30 min, and the glycosides were hydrolyzed with 0.5% H_2SO_4 in 95% methanol for 12 hr. The above fractions were neutralized and the sterols were extracted 3 times with *n*-hexane, dried, and precipitated with digitonin (7). The qualitative and quantitative sterol analysis was performed by gas chromatography using a 1.80-m U-shaped glass column, 6-mm internal diameter, packed with 5% OV-101 on Anakrom ABS 80/90 mesh. The column temperature was 255 C and the nitrogen carrier flow rate was 60 ml/min. Cholestane was used as the internal standard. For more details refer to a previous report (8).

Permeability Experiments. Barley root samples of 0.25 ± 0.01 g were transferred to glass sample chambers (2-cm diameter) fitted with a circular magnetic stirrer. The samples were continually aerated in a 30 C constant temperature water bath. The test medium consisted of 14.25 ml of distilled water (conductance <5 micromhos) to which was added 0.75 ml of ethanol containing 20 times the desired sterol concentration. A

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Table I. *Sterol Composition of 3-Day Old Dark Grown Barley Roots*

Sterols were quantitated using gas chromatography. Cholestane was used as the internal standard. Column: 5% OV-101 on Anakrom ABS 80/90 mesh; temperature: column, 255 C, detector, 300 C; carrier gas: nitrogen at a flow rate of 60 ml/min. The sterol composition was normalized to 100% for the four components. Each figure is the mean of two replicate samples.

Sterol Fraction	Sterol	β-Sitosterol	Campesterol	Cholesterol	Stigmasterol
	mg/g dry wt	%	%	%	%
Free	3.57	76.4	23.3	0.3	0.0
Ester	0.11	59.6	24.6	10.2	5.6
Glycoside					
Nonacylated	0.42	74.7	23.6	0.7	1.0
Acylated	0.07	61.4	36.1	2.5	0.0

Table II. *Effect of Free Sterols on the Ethanol-Induced Leakage of Electrolytes from Barley Roots*

Dark-grown barley root samples of 0.25 g fresh weight were incubated for 3 hr in 15 ml of 5% ethanol containing indicated levels of sterols. The average conductance of the control (C_c) and 5% ethanol (C_{eth}) samples which did not contain sterol, were 25 and 47 micromhos, respectively. Each figure is the mean of three replicate samples.

Sterol Concentration	Sterol[1]			
	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
μM	%	%	%	%
100	+100	+15	−3	0
10	−73	−35	+6	−11
1	−9	−20	−12	+6
0.1	−5	−5	−13	−3

[1] Percentage stimulation (+) or inhibition (−) in conductance.

control sample, containing no ethanol, was also included in each run. Leakage of electrolytes, which was taken as a measure of permeability, was followed using a Serfass Conductivity Bridge (model RCM 15 B1) equipped with a Yellow Springs conductivity cell (microdipping type, model 3403) with a cell constant of 1.0. The specific conductance is expressed in reciprocal ohms (mhos). The percentage change in ethanol-induced conductance (change in leakage) in Table II was calculated as follows:

$$\frac{C_t - C_{eth}}{C_{eth} - C_c} \times 100$$

where C_t = conductance in test solution, C_{eth} = conductance in 5% ethanol, and C_c = conductance of control.

RESULTS AND DISCUSSION

It has been reported that green and etiolated barley shoots contained free sterols, steryl esters, steryl glycosides, and acylated steryl glycosides (1). Three-day-old roots from dark grown barley seedlings were analyzed for these sterol forms (Table I). The free sterols accounted for 85.6%, followed by the steryl glycosides (10.1%), steryl esters (2.6%), and acylated sterol glycosides (1.7%). The barley roots contained the same four sterols that were found in barley shoots (1), namely: β-sitosterol, campesterol, cholesterol, and stigmasterol; however, the percentage composition was quite different. In barley shoots, both green and etiolated, stigmasterol and β-sitosterol were the major sterols, while in barley roots β-sitosterol and campesterol were the major components. Stigmasterol accounted for less than 1% of the sterols in the roots.

Since excised barley roots from dark-grown seedlings had free sterols, steryl esters, and steryl glycosides it was decided that this tissue should be well suited to study the ability of the different sterol forms to change membrane permeability. The effectiveness of the four free sterols (β-sitosterol, campesterol, cholesterol, and stigmasterol) on membrane permeability is presented in Table II. Cholesterol at 10 μM was most effective in reducing the ethanol-induced leakage of electrolytes; however, 100 μM cholesterol greatly stimulated leakage. Campesterol was the second most effective sterol in changing the permeability of excised barley roots. However, the major higher plant sterols, β-sitosterol and stigmasterol (15), were virtually ineffective in influencing leakage.

These experiments with barley roots (Table II) agree with previous results obtained with red beet disks (6). These data support the hypothesis of Finean (3) and Vandenheuvel (16) that only those sterols with a flat configuration, like that of cholesterol, can penetrate the phospholipids of the membranes. β-Sitosterol and stigmasterol have an ethyl group at C_{24} in the C_{17} side chain and this makes these molecules somewhat more bulky than cholesterol. The bulky β-sitosterol and stigmasterol molecules apparently were unable to penetrate the phospholipid layers deep enough and, therefore, failed to stabilize the membrane. Conversely, campesterol with only a methyl group at C_{24} has a less bulky C_{17} side chain and probably can penetrate the phospholipids to a greater degree, in turn influencing membrane permeability to a greater extent. Yet another important factor is the sterol concentration (Table II, Fig. 1A). Cholesterol at 10 μM greatly reduced the leakage of electrolytes,

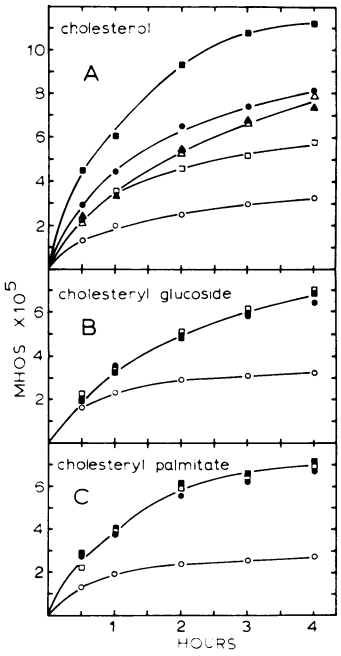

FIG. 1. Time course changes in conductance of test medium in the presence of (A) cholesterol (B) cholesteryl glucoside, and (C) cholesteryl palmitate. Barley root samples of 0.25 g were incubated in 15 ml of test solution, which contained 14.75 ml of distilled water (<5 micromhos), 0.25 ml ethanol, and the desired sterol concentration. A control sample ○—○ containing no ethanol was also included. Final sterol concentrations 0.1 μM △—△, 1 μM ▲—▲, 10 μM □—□, 100 μM ■—■, and 5% ethanol only ●—●. Each point is the mean of three replicate samples.

while at 100 μM it greatly stimulated the loss of electrolytes. As Dervichian (2) has pointed out, with different sterol-lipid proportions the lipophilic components of the phospholipid layer assume different molecular arrangements and as a result change the permeability of the membrane.

Barley roots, however, contain not only free sterols but also steryl glycosides and steryl esters (Table I). Since cholesterol was found to be the most effective sterol in influencing membrane permeability, cholesteryl palmitate and cholesteryl glucosides were selected for more detailed kinetic studies. As can be seen in Figure 1, cholesteryl glucoside (Fig. 1B) and cholesteryl palmitate (Fig. 1C) did not change the rate of electrolyte leakage over a 4-hr test period, even though free cholesterol (Fig. 1A) was quite effective. These results suggest that only the free sterols are involved in changing membrane permeability. Apparently the secondary hydroxyl group at C_3 must be free to interact with the phospholipids, probably by means of hydrogen bonding. According to the Finean (3) model, as modified by Vandenheuvel (16), the micelle configuration of the membrane is a function of phospholipid and protein interaction, and steroids which are capable of penetrating the phospholipids can change the micelle arrangement (18). Vandenheuvel (16) has predicted that the hydroxyl group interacts by weak ion-dipole and hydrogen bonding with the nitrogen of the nitrogenous base moiety of the phospholipid molecule. The results shown in Figure 1 certainly support this hypothesis because only free cholesterol was effective in changing membrane permeability. Cholesteryl palmitate and cholesteryl glucoside, which do not have a free hydroxyl group at the C_3 position of the cholestene nucleus, were ineffective. Further results in support of the above hypothesis have been presented by Long *et al.* (12). They reported that cholesterol increased the rigidity and order of egg lecithin lattice structure in the dry lipid film state as well as in the hydrated multilayers; however, the hydroxyl group at C_3 was necessary for the orientation and condensing effect.

Based on these observations it is suggested that only the free sterols actually play a role in controlling the permeability of membranes in plant tissue. Kemp *et al.* (10), studying changes in the levels and composition of the esterified and free sterols of corn seedlings during germination, concluded that the free sterols have a structural function. However, this leaves us with an important question. What is the physiological function of the steryl esters and steryl glycosides? Kemp *et al.* (10), by analogy with cholesteryl esters in animal tissue, postulated that the steryl esters may be involved in the transport of sterols. In corn, the nuclear fraction had the greatest proportion of steryl ester (11), while in tobacco the microsomal fraction contained the greatest amount of this sterol form (7). These results are difficult to explain in terms of sterol translocation. Furthermore, it has been reported that the steryl ester composition of greening barley shoots was quite different from the free sterols (1). However, in this same study the steryl glycosides were very similar in composition to the free sterols, making this sterol form the more likely for translocation. But it is also possible that the steryl esters and/or steryl glycosides are precursors to other steroids which may act as plant hormones (9, 17).

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