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Sterol Molecular Modifications Influencing Membrane Permeability[1]

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ABSTRACT

Various sterols and related steroids were tested for their ability to influence ethanol-induced electrolyte leakage from *Hordeum vulgare* roots. Cholesterol had the greatest influence and, depending on concentration, it stimulated or inhibited the loss of electrolyte. Cholesterol, however, was ineffective if the roots were pretreated with ethanol. These data suggest that sterols protect rather than restore membrane structure. First, modifications in the cholesterol perhydrocyclopentanophenanthrene ring system suggest that at least one double bond is required for membrane activity. Second, increasing the bulkiness of the C_{17} side chain of cholesterol, as shown with campesterol, stigmasterol, and sitosterol, decreased its activity. Apparently for maximum effectiveness the sterol molecule should have a relatively flat configuration. Third, the C_3-hydroxyl group is required for membrane activity since cholesteryl methyl ether, cholest-5-ene-3β-thiol and cholesteryl halogens were without activity. Exception to the foregoing rule was cholestane which was slightly active but which has neither a C_3-hydroxyl group nor a double bond in the ring system.

In previous publications it was reported that exogenously applied cholesterol, and certain other higher plant sterols, modified the permeability of plant cells (9, 11, 15). To explain the action of sterols on plant membranes, a hypothesis which incorporated the results of animal and phospholipid film experiments (5–7, 13, 16, 21, 26, 27) was proposed. Briefly, it was suggested that cholesterol, or other sterols, insert themselves into the phospholipid layer of the membrane, with the C_3-hydroxyl group interacting by weak ion-dipole- and hydrogen-bonding with the polar nitrogenous base moiety of the phospholipid. In this model, the C_{17} side chain of cholesterol would align itself along the nonpolar terminal of the phospholipid, filling the phospholipid cavity and in turn stabilizing the lipid configuration. According to this hypothesis, the C_3-hydroxyl group of the sterol molecule is an important functional group. This hypothesis would explain why only the free sterols were active and not the steryl esters and steryl glycosides (9, 11), all of which are common higher plant constituents. However, sterol analogs with a small polar group in the C_3 position, other than a hydroxyl, have never been tested. The foregoing hypothesis of sterol action also assumes that for effectiveness, the sterol molecule must have a flat configuration similar to that of cholesterol (7, 26). This requirement was confirmed partially with sterols that differed in branching in the C_{17} side chain (9, 11), but in this respect sterols with modifications in the perhydrocyclopentanophenanthrene ring system have not been studied.

For purposes of studying the mechanism of sterol action on plant membranes, cholesterol can be taken as the basic molecule, and this molecule can be divided into three sites of possible physiological importance (Fig. 1). The cholesterol nucleus consisting of ring A, B, C, and D with a double bond of C_5—C_6, and methyl groups at C_{10} and C_{13} can be considered the first site. The C_{17} side chain can be taken as the second site of possible physiological importance, especially since many naturally occurring higher plant sterols have variation in the structure of this side chain. The secondary hydroxyl group at C_3, which occurs in plant sterols, can be considered the third site of importance in influencing membrane permeability. It is possible that modification in any one of the three sites changes the biological activity of the sterol molecule.

The purpose of the present communication was to investigate further the requirements for sterol action on plant membranes. In this respect, molecules of similar shape, size, solubility, and polarity are of interest and, therefore, were used in the present investigation.

MATERIALS AND METHODS

The steroids used in this study were obtained from the following sources: cholesterol, sitosterol, campesterol, and stigmasterol from Applied Science Laboratories Inc.; cholestane, cholestanol, cholesteryl methyl ether, ergosterol, cholesteryl 3β-chloride from Sigma Chemical Co.; and cholest-5-ene-3β-thiol from Aldrich Chemical Co. The purity of all steroids was checked by GLC and after recrystallization the purity was 99% or better, except for sitosterol (95%), ergosterol (93%), and cholest-5-ene-3β-thiol (90%).

Barley (*Hordeum vulgare*, L. var. Barsoy) seeds were germinated in the dark at room temperature over continuously aerated 0.5 mM $CaSO_4$. The roots of 3-day-old plants were excised, transferred to aerated 0.5 mM $CaSO_4$ for 30 min, and then allowed to accumulate KCl from an aerated 5 mM solution for 60 min. Before use, the roots were washed free of excess salt solution with distilled water.

The permeability measurements were carried out as described previously (11). Briefly, 0.25 ± 0.01 g of barley roots dried on filter paper were transferred to 2-cm round glass chambers fitted with circular magnetic stirrers and microtubes for continuous aeration. The sample chambers were mounted in a 30 C constant temperature water bath. The test medium

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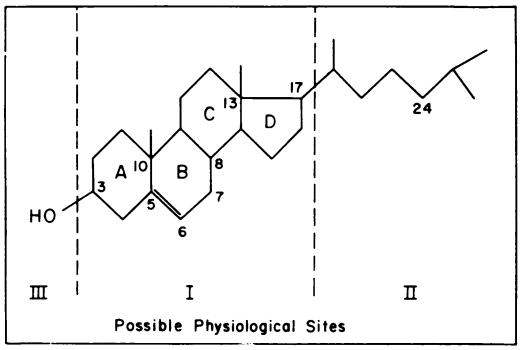

FIG. 1. Cholesterol structure: shown are three sites which may have possible physiological importance in controlling membrane permeability.

consisted of 14.25 ml of distilled water (conductance <5 micromhos) and 0.75 ml of 95% ethanol. The compound to be tested was dissolved in the ethanol at 20 times the final required concentration. Each setup included one water control (15 ml of distilled water) and one ethanol control (14.25 ml of water plus 0.75 ml of 95% ethanol).

The pretreatment experiments were carried out as follows: barley root samples were incubated in the sample chambers with 15 ml of distilled water (water control) or 14.25 ml of distilled water and 0.75 ml of 95% ethanol. After 30-min preincubation, 75 μl of 95% ethanol containing 200 times the final concentration of the test compound were added with a syringe. No ethanol was added to the water control sample; however, ethanol was added to the ethanol control sample. The complete experiment was carried out at 30 C with continuous aeration.

Cell permeability was determined by the measurement of the leakage of electrolytes from the excised barley roots. The specific conductance of the test medium was followed with a microdipping type conductivity cell (Yellow Spring, model 3403) connected to a conductivity bridge (Serfass, model RCM 15 B1). The conductivity cell had a constant of 1.0 and the specific conductance is expressed in mhos (reciprocal ohms). All experiments, even if the data are not shown, were carried out at 0.1, 1.0, 10, and 100 μM steroid concentrations. The values presented are the means of three replications.

RESULTS

As previously reported (11), cholesterol at relatively low concentrations decreased the alcohol-induced permeability of excised barley roots and enhanced it at a higher concentration (Fig. 2). The most effective cholesterol concentration tested to inhibit leakage of electrolytes was 10 μM. Lower cholesterol concentrations were less effective in the inhibition of electrolyte leakage. Cholesterol at 100 μM increased the permeability of barley root. If the roots were incubated for 30 min in 5% ethanol before cholesterol was added no changes in permeability were observed (Fig. 3). Other plant sterols, sistosterol, stigmasterol, and campesterol, gave the same pattern of results in ethanol-preincubation studies (data not shown).

The first modification of the cholesterol molecule to be tested was the elimination of the C_5—C_6 double bond in the perhydrocyclopentanophenanthrene ring system (site I). The results with 100 and 10 μM cholestanol are shown in Figure 4. Saturation of the C_5—C_6 double bond completely destroyed all biological activity in this test system. Another interesting modification in the sterol ring system is the introduction of a second double bond, *e.g.* in the C_7—C_8 position. Ergosterol is a naturally occurring plant sterol which has the C_5—C_6, C_7—C_8 conjugated ring system; however, ergosterol differs from cholesterol also in its C_{17} side chain. Ergosterol, just as cholesterol, inhibited and stimulated the loss of electrolytes (Fig. 5); but in no way did the permeability pattern resemble that obtained with cholesterol. Ergosterol at low concentration (0.1 and 1 μM) increased permeability, whereas at higher concentration (10 and 100 μM) inhibited the loss of electrolytes. Exactly how much of this leakage pattern is caused by the second double bond in ring B and how much is caused by the modification of the C_{17} side chain is difficult to say.

The second site of possible physiological importance is the C_{17} side chain (Fig. 1). The side chain of cholesterol is C_8H_{17} and the major higher plant sterols differ from cholesterol in only the side chain. The common plant sterol campesterol differs from cholesterol in having one extra methyl group in the

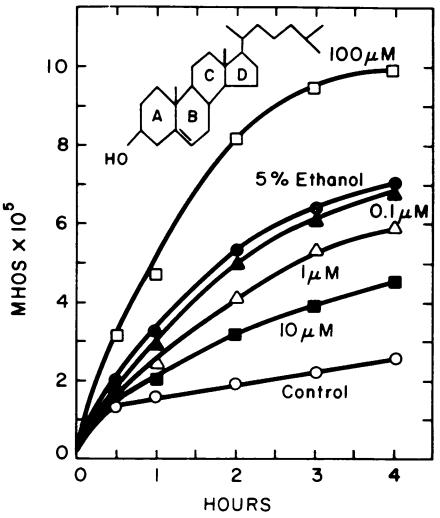

FIG. 2. Effect of cholesterol on the leakage of electrolytes from excised barley roots. Root samples of 0.25 g were incubated in 15 ml of test solution, which contained 14.25 ml of distilled water (< micromhos), 0.75 ml of 95% ethanol, and the desired sterol concentration. A control sample ○——○ containing no ethanol and no sterol was included. Final cholesterol concentrations: 0.1 μM ▲——▲, 1 μM △——△, 10 μM ■——■, 100 μM □——□, and ethanol control ●——●.

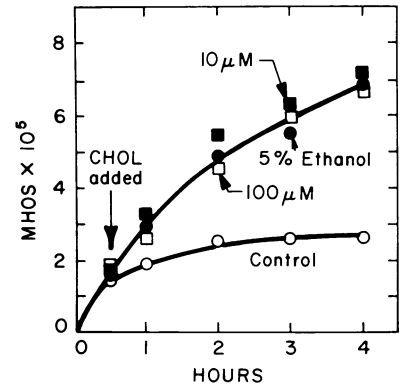

FIG. 3. Effect of cholesterol on the leakage of electrolytes from 5% ethanol preincubated excised barley roots. Roots were preincubated in 14.25 ml of distilled water (<5 micromhos) and 0.75 ml of 95% ethanol. After 30 min, 75 μl of 95% ethanol and the desired cholesterol content were added to all samples except the water control ○——○. Final cholesterol concentrations: 10 μM ■——■, 100 μM □——□ and ethanol control ●——●.

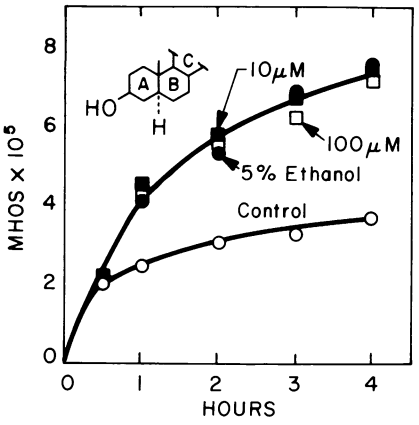

FIG. 4. Effect of cholestanol on the leakage of electrolytes from excised barley roots. Experimental conditions were as in Fig. 2. Final cholestanol concentrations: 10 μM ■——■, 100 μM □——□, water control ○——○, and ethanol control ●——●.

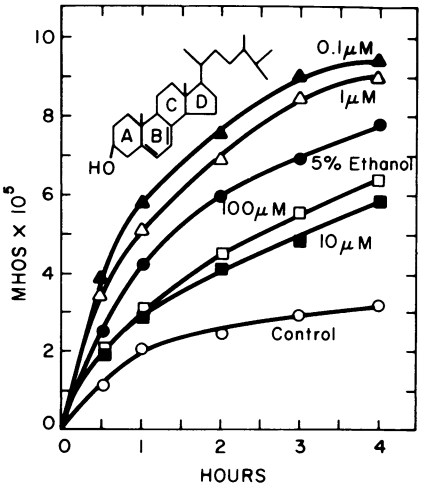

FIG. 5. Effect of ergosterol on the leakage of electrolytes from excised barley roots. Experimental conditions were as in Fig. 2. Final ergosterol concentrations: 0.1 μM ▲——▲, 1μM △——△, 10 μM ■——■, 100 μM □——□, water control ○——○, and ethanol control ●——●.

side chain at C_{24}. This sterol gave leakage patterns very similar to that for cholesterol (Fig. 6A), but the introduction of a methyl group stimulated less leakage at high concentration (100 μM). At a lower concentration (10 μM), campesterol was quite effective in decreasing permeability. Campesterol concentrations below 10 μM were less effective in decreasing membrane permeability (data not shown). The most widespread higher plant sterols, sitosterol and stigmasterol, differ from cholesterol in having an ethyl group at C_{24} side chain, and stigmasterol differs from sitosterol in an extra double bond at C_{22}—C_{23}. Sitosterol (Fig. 6B) and stigmasterol (Fig. 6C) were ineffective in changing membrane permeability.

The secondary hydroxyl group at C_3 of the cholesterol molecule is the third site that might influence membrane permeability (Fig. 1). A number of modifications can be made in this position, and one of the simplest is the substitution of a methyl group for the hydrogen atom. The methyl ether of cholesterol was ineffective in changing the loss of electrolytes (Fig. 7A). A second possibility to change the cholesterol molecule is to substitute a sulfhydryl group for the 3β-hydroxyl group. But cholest-5-ene-3β-thiol was also ineffective in changing membrane permeability (Fig. 7B). Still another modification in the C_3 position would be to replace the hydroxyl group with another polar group, such as a 3β-halogen. The results with cholesteryl chloride are shown in Figure 7C. Chloride substitution destroyed all sterol activity, and cholesteryl iodine and cholesteryl bromide were also inactive (data not shown). The elimination of the sterol hydroxyl group by substituting a hydrogen would make it a less polar molecule. Cholestane, which does not have a C_3 substitution, decreased the loss of electrolytes (Fig. 8). This steroid was most effective at 10 μM, and lower or higher concentrations were less effective. It must be pointed out that cholestane differs from cholesterol not only in the absence of the C_3-hydroxyl group but also in the absence of the C_5—C_6 double bond. It is interesting to note that cholesterol (Fig. 4), which has the C_5—C_6 position saturated and has the C_3-hydroxyl group, was ineffective in changing membrane leakage.

DISCUSSION

Sterols in higher plants occur in different forms, *e.g.* free, esterified, and glycosidated. A number of hypotheses have been proposed for the physiological function of the different sterols (1, 2, 8, 9, 11, 14, 18–20). Even though more and more research is carried out in the field of sterol physiology, generally

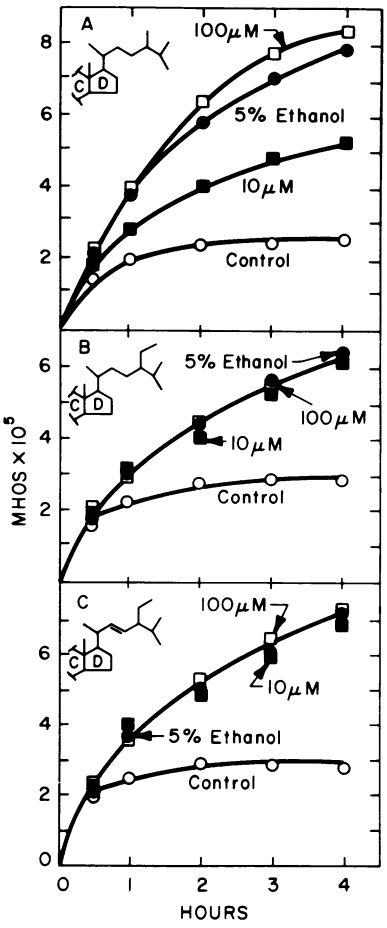

FIG. 6. Effect of campesterol (A), sitosterol (B), and stigmasterol (C) on the leakage of electrolytes from excised barley roots. Experimental conditions were as in Fig. 2. Final sterol concentrations: 10 μM ■——■, 100 μM □——□, water control ○——○. and ethanol control ●——●.

only two hypotheses have any experimental support. The first hypothesis proposes that sterols, directly or indirectly, act in some way as plant hormone(s) (1, 2, 14, 19, 20). The second hypothesis considers sterols as structural components of plant membranes (3, 9–11, 14, 15, 18). The latter hypothesis finds support in the observation that sterols in plants are in the membrane-containing subcellular fractions, which also contain the phospholipids (3, 10, 18). Based on permeability experiments, it has been suggested that only the free sterols play an important role in membrane structure (11); and of the free sterols, cholesterol was the most effective sterol (9, 11). As reported previously (11) and again found in this investigation (Fig. 2), cholesterol decreased (10 μM) and increased (100 μM) membrane permeability. This observation is in agreement with the hypothesis that the lipophilic side of the phospholipid membrane assumes different molecular associations featuring different sterol-lipid proportions and that these changes in structural arrangement can result in changes of the area of phospholipid molecules (6, 13). The hypothesis has also been confirmed with micelles formed with different proportions of phospholipids and phytosterols (28). The results suggest that the cholesterol concentration of the tissue is critical, and that slight changes in sterol content could result in changes in permeability and function of membranes. The physiological picture is further complicated by the fact that in higher plants cholesterol is generally only a minor sterol component and the major sterols are sitosterol and stigmasterol (14). For example, in barley roots, cholesterol accounts for only 0.3% of the free sterols (11), which is about 1 μg of cholesterol per g of fresh weight, whereas sitosterol and campesterol are the major sterols in this tissue (11).

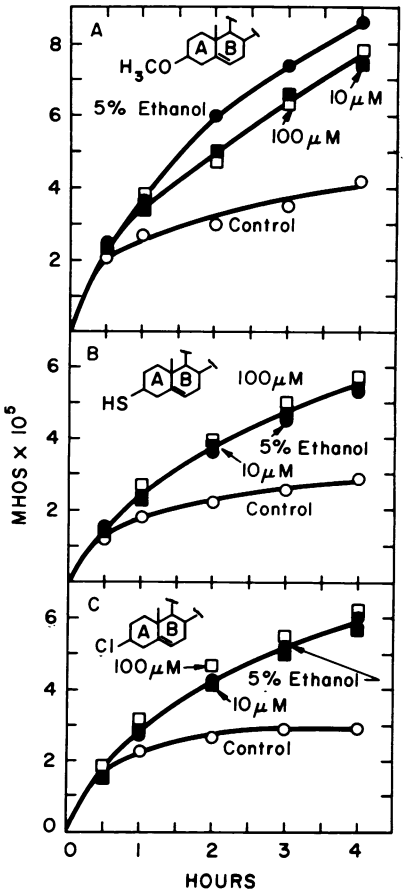

FIG. 7. Effect of cholesteryl methyl ether (A), cholest-5-ene-3β-thiol (B), and cholesteryl chloride (C) on the leakage of electrolytes from excised barley roots. Experimental conditions were as in Fig. 2. Final sterol concentrations: 10 μM ■——■, 100 μM □——□, water control ○——○, and ethanol control ●——●.

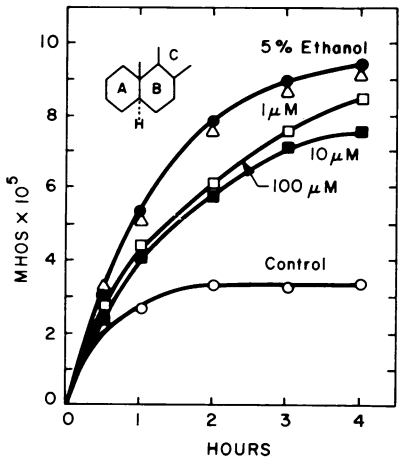

FIG. 8. Effect of cholestane on the leakage of electrolytes from excised barley roots. Experimental conditions were as in Fig. 2. Final cholestane concentrations: 1 μM △——△, 10 μM ■——■, 100 μM □——□, water control ○——○, and ethanol control ●——●.

If barley roots were preincubated for 30 min in 5% ethanol before cholesterol was added no change in permeability was observed (Fig. 3). It has been suggested that alcohol not only influences the anionic sites (23) but also the sterol-phospholipid interaction (6, 9). The rapidity of the leakage response and the inhibition of leakage by $CaCl_2$ are evidence that the alcohol effect is at the membrane level (9, 12, 24). Apparently, once the rearrangement of the sterol-lipid association has occurred no reversal can be induced with cholesterol; it appears therefore that sterols protect, rather than restore membrane structure.

At this point the experiments by Stowe and his co-workers must be cited (17, 25). These workers postulated that certain oily substances, oleanimins, are active in the pea bioassay by forcing apart the lecithin molecules and thereby changing membrane properties (25). According to their rules for lipid activity, sterols should be active, but sitosterol (25) and stigmasterol (17) were not when tested by themselves. In fact, stigmasterol, when added with the active oily substance heptadecylbenzene, partially inhibited the full response produced by the oleanimin (17). In light of what is known about the stabilizing effect of sterols on plant membranes (9, 11), it appears that stigmasterol prevented the phospholipids from being forced apart by heptadecylbenzene. Filipin, which is known to change cell permeability strongly reduced the influx of K^+ and Hendrix and Higinbotham (15) found that cholesterol counteracted the inhibition of filipin. They suggested that filipin interfered with sterol stabilization of phospholipids. These interpretations would be in general agreement with the hypothesis of sterol action on plant membranes.

What are the structural requirements of the sterol molecule for its interaction with phospholipids of plant membranes? The sterol ring system (site I in Fig. 1), appears to have some requirements. Cholesterol has a C_5—C_6 double bond and showed activity (Fig. 2) but saturation of this position, as shown with cholestanol (Fig. 4), destroyed all activity. Ergosterol, which has a C_5—C_6 double bond conjugated with a C_7—C_8 double bond, was active (Fig. 5). Ergosterol also showed the inhibition and stimulation of electrolyte leakage characteristic of choles-

terol, even though at different concentrations. These data are in agreement with ergosterol permeability tests with red beet tissue (9). Similarly, experiments with human erythrocytes showed that cholestanol increased osmotic fragility but ergosterol did not (4). Apparently, at least one double bond in the B-ring is required for physiological activity on membranes.

The second site of possible physiological importance is the C_{17} side chain (Fig. 1). The structure of the C_{17} side chain influenced the sterol stabilizing effect on membranes (Figs. 2, 6A, B, and C). Campesterol, with an extra methyl group at C_{24}, was less potent than cholesterol in enhancing permeability at the high concentration of 100 μM (Fig. 6A). However, as with cholesterol, campesterol showed its greatest stabilizing effect at 10 μM. The addition of an ethyl group at C_{24} destroyed membrane activity as is shown with stigmasterol (Fig. 6C) and sitosterol (Fig. 6B). The latter two sterols are the major higher plant sterols (14), and their inability to influence membrane permeability was at first disturbing, especially because both sterols were active in a red beet system (9) and in the pea bioassay oleanimin test (17). But it is very possible that the phospholipid composition of barley roots is different from that of red beet root and pea tissue, creating phospholipid cavities that are too narrow for the bulky sitosterol and stigmasterol to penetrate. It may be presumed that the active compounds cholesterol (Fig. 2) and campesterol (Fig. 6A), which do not have the bulky side chain, were able to penetrate the phospholipid cavities. This explanation is in agreement with the fact that lecithin varies in fatty acid composition (5, 22) and, based on experiments with phospholipid film, the sterol condensing effect (16, 21) depends upon the fatty acid composition and pairing in the lecithin molecule (5). As previously pointed out (9), if sterols in higher plants actually play a role in the structure and function of membranes then the particular sterol, or combination of sterols, that are involved in any specific case will be determined by all the functions of the membranes and not by only one function. Of course, the molecular make-up of the phospholipids is also important in the physiological behavior of the membrane.

The third sterol site of possible physiological importance is the C_3-hydroxyl group (Fig. 1). It was found that the hydrogen of the hydroxyl group is required because the methyl ether of cholesterol was not active in the permeability test (Fig. 7A). Molecular polarity alone does not appear to be the whole requirement since the C_3-halogen analogs of cholesterol were also inactive (Fig. 7C). The weak ion-dipole and hydrogen-bonding requirements of the sterol molecule must be quite precise as to bond energy and bond angle because the C_3-sulfhydryl analog of cholesterol was unable to change membrane permeability (Fig. 7B). These observations are in agreement with phospholipid film experiments in which the importance of the hydroxyl group was also observed (16, 21). Somewhat surprising was the slight biological effect of cholestane on membrane permeability since this steroid does not have the C_3-hydroxyl group nor the C_5—C_6 double bond (Fig. 8). No explanation for this behavior can be given at the present time. In general, since a C_3-hydroxyl group was required, the present experiments supported the hypothesis (11) that only the free sterols are active in the control of plant membrane permeability.

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