Plant Physiol. (1968) 43, 484–488

Effect of Sterols on the Permeability of Alcohol-Treated Red Beet Tissue[1, 2]

C. Grunwald

Department of Agronomy, University of Kentucky, Lexington, Kentucky 40506

Received October 3, 1967.

Abstract. **Alcohols and hydrogen peroxide altered the permeability of membranes of *Beta vulgaris* root cells. Generally alcohols increased the permeability of membranes without going through an induction period except methanol which required a 10- to 15-hour induction period. The membrane effect of methanol could be inhibited with $CaCl_2$, cholesterol, β-sitosterol, and stigmasterol. Cholesterol was the most effective inhibitor, followed by β-sitosterol and stigmasterol; and at the same concentration, the sterols were more effective than $CaCl_2$, the classic membrane stabilizer.**

Ergosterol increased the methanol-initiated betacyanin leakage. Since none of the tested sterols reversed the betacyanin efflux induced by hydrogen peroxide, the sterols do not apparently act as antioxidants. The results are explained in terms of sterol-phospholipid interaction, based on stereochemistry and charge distribution.

In 1930 Schoenheimer *et al.* (12) suggested that sterols in plants are merely waste products. This assumption had not been questioned until recently when Heftmann (9) expressed the view that steroids in plants may act much the same way as in animals. Steroids are said to act as hormones on cells and cell constituents (16). Steroid hormones may control the metabolic processes by affecting 1 or more of the following processes: altering the rate of enzyme production, altering the enzyme activity, being involved as a coenzyme, or altering the permeability of the cell membrane or subcellular membrane system.

To develop insight into known physio-chemical properties of the cell membrane, it is important to consider the chemical characteristics and molecular organization of biological membranes. It is well established that the physical properties of monomolecular layers formed by mixed lipids may be quite different from those formed by single lipid components (10). Although phospholipids appear to be the major, and probably the dominant lipid component of cell membranes, large quantities of cholesterol have also been isolated (5). Most lipid molecules in aqueous dispersion can be accompanied by phospholipid molecules in mixed lipid layers or mixed lipid micelles, as long as the proportion of ionic lipids remains high (10). Of course, the lipid molecules will modify to some extent the physical properties of the system such as the charge distribution at ionic surfaces and the micellar size, but few observations of such effects have been published. At the moment the most detailed information of this kind is concerned with the cholesterol-phospholipid interaction (10).

It has been suggested that the transformation of the biological membrane from the open to closed configuration is mainly due to the micellar transformation of the lipid components, which in turn is dependent upon the distribution of cations and the nature of the micelles (10). Leathes (11) reported that cholesterol causes a decrease of the area occupied by the phospholipid (lecithin) molecule and Winkler *et al.* (19) visualized cholesterol as a stabilizer of the phospholipid molecule in the red blood cell membrane. It is generally assumed that the open lipid configuration is relatively unstable (metastable), while the closed configuration is relatively stable.

Hechter and Lester (8) suggested that certain steroids may alter the permeability of excised rat uteri. Willmer (18) proposed a theory for steroid action based on the interaction of the steroid with the lipid of the plasma-membrane. He suggested that only steroids which have the flat configuration similar to cholesterol can penetrate the lipids of the membrane and act as hormones. However, Gershfeld and Heftmann (6) using monolayer models were unable to find film penetration by the steroids tested. They suggested that nonspecific adsorption of steroids occurred at the monolayer interphase.

The work reported in this paper is part of an investigation dealing with the physiological effects of sterols in plants. The results presented here suggest that certain plant sterols may act as mem

---

[1] The investigation reported in this paper (No. 67-3-116) is in connection with a project of the Kentucky Experiment Station and is published with approval of the Director.

[2] This work was supported by Crops Research Division, Agricultural Research Service, United States Department of Agriculture Contract (No. 12-14-100-8945-34).

brane stabilizers in much the same way as calcium but other sterols may destabilize the membrane. It is suggested that sterols in plants may control the permeability of the plasma-membrane.

Materials and Methods

Cylinders of beet root (*Beta vulgaris* L.) were cut with a No. 3 cork borer and further subdivided into 3 mm uniform disks by an equally spaced razor blade assembly (14). The disks were washed in cold running tap water for 14 to 17 hours and pre-incubated in buffer, without the test compounds, for 1 hour.

Ten beet disks were placed in 9 cm petri dishes containing 10 ml of medium. The standard reaction medium contained 9 ml of 0.1 M phosphate buffer pH 6.6 with 0.1 M sucrose and 1 ml of methanol with or without the test compounds. Generally the disks were incubated for 24 hours on a reciprocating shaker at 25°. No difficulty was encountered in maintaining the buffer solution at pH 6.6 during incubation. All experiments were run at room temperature.

The betacyanin efflux was followed, using a Spectronic-20 spectrophotometer at 535 mμ. Colorimetric values beyond the usable range of the instrument were obtained from suitably diluted aliquots of the buffered medium. Three samples were used for each determination and each experiment was repeated at least 10 times. One can not compare the absolute optical density values (OD) of 1 experiment with those of another because the amount of betacyanin leakage varied from beet to beet.

Results

Red beet root disks incubated in 10 % methanol at pH 6.6 had a 10- to 15-hour induction period before an efflux of betacyanin could be observed (fig 1A). However, if the beet disks were incubated at the same pH in 10 % tert-butanol betacyanin leakage started within 15 minutes and without going through a long induction period (fig 1A). Other tested alcohols, including ethanol, propanol, butanol and iso-butanol also showed an immediate efflux of betacyanin. The membrane effect of alcohols is related to the structure of the alcohols and could be reversed with 5 mM $CaCl_2$ (fig 1B). A 0.5 mM $CaCl_2$ concentration was not effective in reversing the methanol effect. Calcium ions reversed the membrane effect of methanol not only in short term experiments but also in relatively long term experiments.

Beet disks incubated in 0.1 M H_2O_2 had only a very short induction period. Generally betacyanin leakage occurred within the first 30 minutes (fig 1C). The betacyanin efflux usually reached its maximum within 7 to 10 hours and did not change

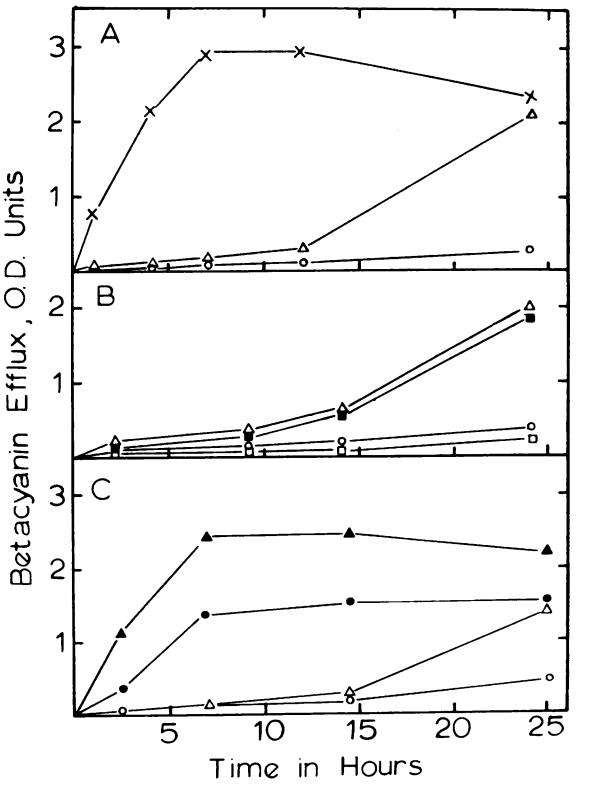

FIG. 1. Betacyanin efflux at various times with different treatments. A) Effect of alcohols on betacyanin efflux. B) Effect of $CaCl_2$ on alcohol-treated tissue. C) Effect of H_2O_2 on betacyanin efflux. Control (○——○), 10 % methanol (△——△), 10 % tert-butanol (X——X), 10% methanol plus 0.5 mM $CaCl_2$ (■——■), 10 % methanol plus 5 mM $CaCl_2$ (□——□), 0.1 M H_2O_2 (●——●), and 10 % methanol plus 0.1 M H_2O_2 (▲——▲).

during the following 15 hours. During the methanol induction period the oxidant H_2O_2 had a synergistic effect with the alcohol on the membrane (fig 1C) and the maximum betacyanin efflux was also reached within 7 to 10 hours. The methanol did not alter the basic H_2O_2 betacyanin leakage pattern.

Four closely related plant sterols: cholesterol, β-sitosterol, stigmasterol, and ergosterol were tested for their ability to reverse the methanol and hydrogen peroxide effects. Cholesterol was quite effective in reversing the methanol effect even at very low concentrations, 10^{-6} M (fig 2A). The widely distributed plant sterols, β-sitosterol and stigmasterol also reversed the methanol effect (fig 2B and 2D) at concentrations much below calcium ions (fig 1B). A calcium concentration of 5 mM was required to obtain a complete reversal of the methanol effect, while only 0.1 mM cholesterol was almost as effective, but β-sitosterol and stigmasterol were somewhat less effective than cholesterol. On the other hand, the most important of the provitamin D-series, ergosterol greatly stimu-

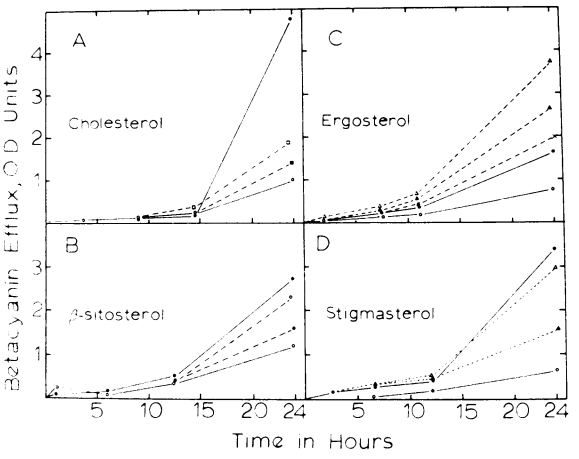

FIG. 2. Betacyanin efflux at various times with different treatments. A) Effect of cholesterol on alcohol-treated beet tissue. B) Effect of β-sitosterol on alcohol-treated beet tissue. C) Effect of ergosterol on alcohol-treated beet tissue. D) Effect of stigmasterol on alcohol-treated beet tissue. Control (○——○), 10 % methanol (●——●) cholesterol 1 μM (□——□), 0.1 mM (■——■), β-sitosterol 1 μM (○ - - ○), 0.1 mM (●--●), ergosterol 1 μM (△--△), 10 μM (▲--▲), 0.1 mM (X--X), stigmasterol 1 μM (△----△), 0.1 mM (▲----▲).

lated the betacyanin efflux at very low concentration, 1 μM, and had almost no effect at higher concentration, 0.1 mM (fig 2C).

As already pointed out hydrogen peroxide had no apparent induction period (fig 1C) and the betacyanin efflux pattern was not altered in any way by the addition of cholesterol, β-sitosterol, stigmasterol or even ergosterol (fig 3).

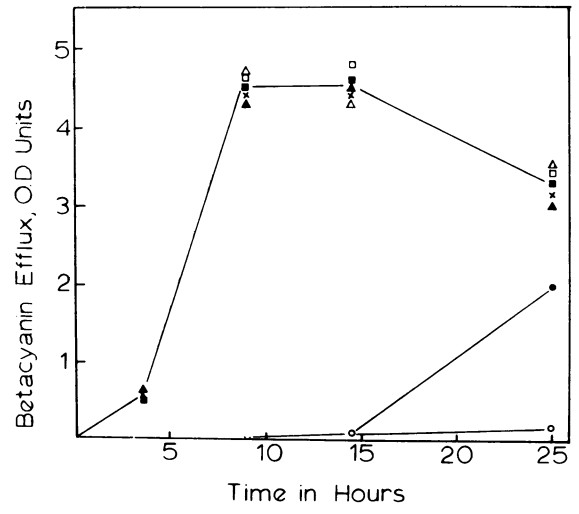

FIG. 3. Betacyanin efflux at various times with different treatments. Control (○——○), 10 % methanol (●——●), 0.1 M H_2O_2 and 10 % methanol (X——X). All other samples contained 0.1 M H_2O_2 and 10 % methanol in addition to 1 of the following sterols at 0.1 mM: cholesterol (■——■), β-sitosterol (▲——▲), ergosterol (□——□), stigmasterol (△——△).

Discussion

Gudjonsdottir and Burstrom (7) described some stimulating effects of low molecular weight alcohols on excised wheat roots and proposed that the effect was on the plasma-membrane. Siegel and Halpern (13) observed that the leakage of betacyanin from red beet root tissue and the inhibition of rye germination could be induced by certain alcohols. They observed further that with alcohols the membrane permeability (leakage of red pigment) increased with lengthening of the carbon-chain from C_1 to C_5 and decreased with branching on the alcoholic C-1. The rapidity of the response and its inhibition by calcium ions (fig 1B), the classic membrane stabilizer (2), is cited as evidence that the alcohol effect is at the membrane level (13).

Siegel and Halpern (13, 14) reported that they never observed an induction period in methanol-treated tissue and that methanol had no apparent effect on the cell membrane. Such behavior for methanol was not observed in the present investigations (fig 1A). The methanol effect always occurred after a 10- to 15-hour induction period. Siegel and Halpern used relatively short test periods (20 mins) and, therefore, never observed the induction period, and the methanol effect on the membrane. Furthermore, in the present investigation somewhat higher methanol concentrations were used in order to dissolve the sterol test compounds and this may also account for part of the disagreement on betacyanin leakage.

It is suggested that alcohol involves not only the anionic sites (15) but also the interaction of the sterol with the phospholipid causing the membrane to assume the open configuration. Two ways of changing the sterol-phospholipid interaction are the partial or complete removal of the sterol component or the removal of water from the hydrated sterol-phospholipid system. Bear *et al.* (1) reported that certain sterol-lipid systems became unstable and underwent phase separation when the water of hydration was removed. Dervichian (3) suggested that with different sterol-lipid proportions the lipophilic segments of the phospholipid layer lead to different molecular associations or structural arrangements which result in an area change of the phospholipid molecule.

The addition of alcohol results in a dehydration of the membrane, which leads to an open membrane configuration and, in turn, to a betacyanin efflux (fig 1A). Increasing the sterol-lipid ratio by adding cholesterol, β-sitosterol or stigmasterol results in a more condensed structural arrangement of the membrane and a decrease in betacyanin efflux (fig 2A, B, and D). On the other hand, the addition of low concentrations of ergosterol further opens the structural arrangement, resulting in an even greater betacyanin efflux (fig 2C). Increasing the ergosterol concentration apparently condenses the structural arrangement of the mem-

brane and thereby decreases the betacyanin efflux (fig 2C).

Willmer (18) proposed that only those steroids which have a flat configuration similar to that of cholesterol are physiologically active because only they can penetrate the phospholipids of the membrane. It appears that the flat configuration, as found in cholesterol, is important but that the charge distribution of the molecule also plays an important part in determining the membrane configuration. Cholesterol, β-sitosterol and stigmasterol which have very similar effects on the cell membrane (fig 2A, B, and D) are flat molecules. Ergosterol also is a flat molecule but its physiological effect is opposite to that of the above mentioned 3 sterols (fig 2C). All 4 sterols have the 3β-hydroxy group and the characteristic 5,6-double bond of cholesterol. Stigmasterol and β-sitosterol differ from cholesterol in having an extra ethyl group at C_{24}, while ergosterol has a methyl group at this position. Stigmasterol and β-sitosterol are C_{29} sterols and differ in only 1 double bond at C_{22}-C_{23} (trans). Ergosterol is a C_{28} sterol possessing a second nuclear double bond, C_7-C_8, conjugated with the 5,6-double bond characteristic of cholesterol. These small differences in the chemical structure of the sterol molecules are enough to influence the structural arrangement of the membrane.

Ergosterol greatly stimulated the betacyanin efflux, especially at very low concentrations (fig 2C). This sterol is structurally very similar to stigmasterol which reversed the methanol effect the least. The second nuclear double bond of ergosterol apparently changes the van der Waals' forces in such a way that the micelle configuration becomes less condensed which produces relatively large membrane pores. In Finean's model (4), as modified by Vandenheuvel (17), the interaction between the phospholipid head-group and the protein envelope is of major importance in determining the micelle configuration. The interaction of the phospholipid head-group and the protein envelope is in turn controlled by the special arrangement of the sterol and phospholipid molecule, and any small shift in van der Waals' forces can produce a very pronounced effect in the membrane configuration.

The membrane effect of hydrogen peroxide is probably related to an oxidation of the phospholipids and, therefore, cannot be reversed by either Ca-ions (15) or sterols (fig 3). The oxidation of the phospholipids causes the cell membrane to open; however, the resulting membrane configuration is not the same as that of the open membrane form induced by alcohol which is calcium ion reversible (compare fig 2 and 3). Apparently the phospholipid residues, after the oxidation of the phospholipids, take on a new arrangement which is not due to a simple rearrangement of van der Waals' interaction forces. These results agree with the finding of Siegel and Daly (13) that the methanol and peroxide effects are different and independent. The methanol activity can be reversed by Ca-ions (fig 1B) and certain sterols (fig 2A, B, and D), while the peroxide effect can only be inhibited with antioxidants, α-tocopherol, thiols, and some vinyl ethers (14).

The condensing effect of sterols on phospholipids apparently depends upon several different actions, and further studies will be required to elucidate them. It appears that many different sterols are capable of forming the closed membrane configuration and their effectiveness depends upon their molecular configuration and charge distribution. If the sterols in the plant actually play a role in controlling the permeability of the membrane then the particular sterol or combination of sterols that is involved in any specific case will be determined by all the functions served by the membrane, rather than by any single function. The general impermeability of biological membranes can be attributed in part to the stabilizing influences of compounds like cholesterol, β-sitosterol or stigmasterol. If the suggested stabilizing and compacting influences on the lipid layer by the above mentioned factors were to be eliminated or reduced by antagonistic agents, such as alcohol, the membrane would be expected to undergo structural rearrangement and its permeability would be expected to change.

Acknowledgments

The author gratefully acknowledges the technical assistance of Mr. William Scrivner.

Literature Cited

1. Bear, R. S., K. J. Palmer, and F. O. Schmitt. 1941. X-ray diffraction studies of nerve lipids. J. Cellular Comp. Physiol. 17: 335–67.
2. Davson, H. 1951. Textbook of General Physiology. London. J. and A. Churchill, Ltd.
3. Dervichian, D. G. 1958. In: Surface Phenomenon in Chemistry and Biology. J. F. Danielli, K. G. A. Pankhurst, and A. C. Riddiford, eds. New York and London, Pergamon Press. p 70–87.
4. Finean, J. B. 1953. Phospholipid-cholesterol complex in the structure of mycelin. Experientia 9: 17–19.
5. Finean, J. B. 1966. The molecular organization of cell membranes. Progr. Biophys. Mol. Biol. 16: 145–70.
6. Gershfeld, N. L. and E. Heftmann. 1963. Steroid hormones and monolayers. Experientia 19: 2.
7. Gudjonsdottir, S. and H. Burström. 1962. Growth-promoting effects of alcohols on excised wheat roots. Physiol. Plantarum 15: 498–504.
8. Hechter, O. and G. Lester. 1960. Cell permeability and hormone action. Recent Progr. Hormone Res. 16: 139–86.
9. Heftmann, E. 1963. Biochemistry of plant steroids. Ann. Rev. Plant Physiol. 14: 225–48.

10. KAVANAN, LEE J. 1965. Structure and Function of Biological Membranes. I. and II. Holden-Day, Inc., San Francisco, California.
11. LEATHES, J. B. 1925. Role of fats in vital phenomena. Lancet 208: 853–56.
12. SCHOENHEIMER, R., H. VON BEHRING, AND R. HUMMEL. 1930. 4. Mitteilung: Untersuchung der Sterine aus verschiedenen Organen auf ihren Gehalt an gesättigten Sterinen. Z. Physiol. Chem. 192: 93–96.
13. SIEGEL, S. M. AND L. A. HALPERN. 1964. The effect of branching at C-1 on the biological activity of alcohols. Proc. Natl. Acad. Sci. U. S. 51: 765–68.
14. SIEGEL, S. M. AND L. A. HALPERN. 1965. Effects of peroxides on permeability and their modification by indoles, vitamin E, and other substances. Plant Physiol. 40: 792–96.
15. SIEGEL, S. M. AND O. DALY. 1966. Regulation of betacyanin efflux from beet root by poly-l-lysine, Ca-ion, and other substances. Plant Physiol. 41: 1429–34.
16. TEPPERMAN, J. AND H. M. TEPPERMAN. 1960. Some effects of hormones on cell and cell constituents. Pharmacol. Rev. 12: 301–53.
17. VANDENHEUVEL, F. A. 1963. Biological structure at the molecular level with stereomodel projections. I. The lipids in the mycelin sheath of nerve. J. Am. Oil Chem Soc. 40: 455–72.
18. WILLMER, E. N. 1961. Steroids and cell surfaces. Biol. Rev. Cambridge Phil. Soc. 36: 368–98.
19. WINKLER, K. C. AND H. G. BUNGENBERG DE JONG. 1941. Structure of the erthrocyte-membrane. Arch. Neerl. Physiol. 25: 431–508.