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Activity of solutions of methanol, ethanol and *n*-butanol on stomatal opening in presence or absence of carbon dioxide

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Of the three alcohols studied, *n*-butanol was the most effective. At 0.15 M it prevented stomatal opening whereas methanol has the same effect at 0.5 M and ethanol at 0.9 M. Cytoplasmic movement is not affected by *n*-butanol but the stomatal plastids are sometimes altered. It is supposed on the basis of numerous reports in the literature, that the lipophilic nature of this alcohol must have a major effect on the lipid constituents of the cell membranes and thus render impossible the maintenance of the low osmotic potential required for stomatal opening. This conclusion is supported by the fact that the stomata did not reopen in CO_2 free air.

Another possible effect is a disturbance of the redox equilibrium of the cells, particularly the $NADH_2$:NAD ratio, with the result that the active pumping system cannot perform the work required to drive in the cations. The lipid components of ATPase can also be damaged.

It is concluded that when alcohols are used as solvents, care must be taken to avoid causing subtle membrane lesions.

Introduction

Many chemicals of biological interest are insoluble or only slightly soluble in water. To use them experimentally they must first be dissolved in a solvent such as an alcohol and then diluted with water.

It is generally supposed that methanol or ethanol at a concentration of 3–4 % do not damage plant cells. However, in experiments conducted some years ago with inhibitors (Mouravieff 1971) it was established that some which had no inhibitory effect on stomatal opening became active if they were solubilized in alcohol. This interesting observation led to the present study of the action of alcohols on stomatal cells.

The number of publications concerning the effects of alcohols on cells is considerable. The major part of the literature is devoted to animal cells. As far as the author is aware, there have been no studies of the effects on stomata. Nevertheless, responses of stomata could provide a test of high value in the examination of the toxicity of various chemicals. The opening mechanism consists of a delicate cation pumping system, and the delivery of metabolic energy is necessary to accomplish this work. From this point of view researches on stomatal behaviour are obviously of more general biological interest.

Materials and methods

These were described in detail by Mouravieff (1971) and are only summarized here. The leaves of *Plantago lanceolata* L., *Rumex acetosa* L., *Veronica beccabunga* L. and *Baldellia ranunculoides* Parl. were collected in the greenhouse in the evening and placed for 13 h on the alcohol solutions, but were not immersed; the control leaves were placed on water. Next morning they

were rapidly washed, the basal ends were cut off and then each leaf was cut into two along the midrib, one half being placed in normal air, the other in CO_2-free air. Each piece was placed in a small cup with its basal end in water but with the major part of the leaf in air. The cups with the leaves were then put into a glass container, with the abaxial epidermis facing the side to be illuminated. The illumination continued for 3 h and consisted of blue light from a fluorescent lamp (Sylvania blue, 20 W) with an intensity of 13 W/m². The leaf portions exposed to CO_2-free and normal air were in different containers but received the same illumination.

Stomatal aperture was measured microscopically on intact leaves and on paradermal sections after placing them in glucose at 0.3 or 0.5 M to study plasmolysis. The control leaves always displayed well open stomata.

TABLE 1. INHIBITION OF STOMATAL OPENING IN LEAVES PRETREATED WITH ALCOHOLS AND ILLUMINATED 3 h IN NORMAL AIR

+++, stomata well open; ++, stomata half open; 0, stomata closed.

	concentration of alcohol/M											
	methanol			ethanol				*n*-butanol				
plant species	0.25	0.5	0.75	0.25	0.5	0.75	1.0	0.03	0.05	0.1	0.15	control
Plantago lanceolata	++	0	0	+++	++	+	0	+++	++	0	0	+++
Rumex acetosa	++	0	0	++	+	0	0	++	++	0	0	+++
Veronica beccabunga	++	0	0	+++	++	+	0	+++	++	0	0	+++
Baldellia ranunculoides	++	+++	+++	+++	+++	++	+	+++	+++	++	+	+++

TABLE 2. INHIBITION OF STOMATAL OPENING IN LEAVES PRETREATED WITH ALCOHOLS AND ILLUMINATED 3 h IN CO_2 FREE AIR

+++, stomata well open; ++, stomata half open; 0, stomata closed.

	concentration of alcohol/M											
	methanol			ethanol				*n*-butanol				
plant species	0.25	0.5	0.75	0.25	0.5	0.75	1.0	0.03	0.05	0.1	0.15	control
Plantago lanceolata	++	+	0	+++	++	+	0	+++	++	+	0	++++
Rumex acetosa	++	0	0	+++	+	0	0	++	++	0	0	++++
Veronica beccabunga	+++	+	0	+++	++	+	0	+++	++	+	0	++++
Baldellia ranunculoides	+++	++	+	+++	+++	++	+	+++	+++	++	+	++++

RESULTS AND DISCUSSION

As seen in the tables the three alcohols exert very different effects. Whereas ethanol inhibits the opening of stomata at 0.9 M, methanol is as effective at 0.5 M and the lipophilic *n*-butanol at 0.15 M. This last alcohol is much more toxic. It is significant that CO_2-free air does not lead to opening of the stomata after treatment with alcohols (table 2).

Saubert (1937) found that methanol and butanol exerted a much greater effect on *Chara* cells than ethanol, increasing the water permeability. It is now well known that *n*-butanol causes disorganization of the lipoprotein constituents of membranes (Glinka & Reinhold 1972) and

thus raises the permeability. This property has been useful in various physiological studies (Puth & Lüttge 1973).

Recent researches on stomata have clearly demonstrated that the opening is tied to a substantial movement of K^+ ions into the cells which leads to a fall in osmotic potential (Humble & Raschke 1971; Willmer & Pallas 1973). This amount of potassium can only be held in the cells if the membranes are not damaged. Furthermore, it can be assumed that photosynthesis will be inhibited by alcohols so that the concentration of carbon dioxide remains high. However, the experiments with CO_2-free air showed that this is not the only cause of stomatal closure. If it is assumed that ATPase is one of the active contributors of the pumping capacity of cells, the lipophilic components of this enzyme could be altered by *n*-butanol. It is suggestive that stomata which remain closed in *n*-butanol and methanol do not accumulate K^+ as tested with cobaltinitrite solution, and the amount of starch is not diminished. Evidently the pumping mechanism remains inactive.

Besides the plasmalemma and tonoplast, the e.r. membrane and the various cell organelles are also affected by alcohols. In animal cells which have been well studied in this respect, the mitochondria are particularly sensitive (David 1970) and it is known that mitochondria are abundant in stomatal cells (Mirimanov 1971). Chloroplasts are also damaged by alcohols. Liebald (1913) found that chloroplasts of *Vallisneria* were 'agglutinated' if placed in a 5% ethanol solution for 24 h, and Lepeschkin (1926) observed the formation of 'vacuoluls' in plastids of *Bryopsis* treated with alcohol at 5–10%. In the present work the breaking of plurilocular stomatal chloroplasts has been observed in *n*-butanol at 0.15 M. Of particular interest here was the fact that the movement of cytoplasm continued in the closed stomata, and the cells could be plasmolysed normally.

Many enzymes are affected by alcohols. They inhibit phosphorylases and various transglycosidases, important factors in carbohydrate metabolism. Cellular metabolism can be disturbed by oxidation of alcohols in cells. Most important is the shift of the $NADH_2$:NAD ratio (Forsander 1970; Zambotti & Albertini 1971) which leads to the accumulation of reduced enzyme and makes the cellular environment more reducing (Mezey, Potter & Read 1973). The turnover of malate is also modified and it is well known that malate plays an important part in ionic movements (Osmond & Laties 1969).

Although methanol and ethanol are less liposoluble, they are nevertheless wetting agents and are 'membrane active', and indeed can influence the architectural arrangement of molecules and so affect ion flow. It is noteworthy that methanol is more effective in preventing stomatal opening than is ethanol. Perhaps the latter is more readily converted by alcohol dehydrogenase to aldehyde which does not affect membrane permeability (Puth & Lüttge 1973).

The present work has shown clearly that alcohols affect subtle factors such as ion flow, and so care must be taken when using them. Only the use of ethanol can be advised, and the concentration should not exceed 0.4 M.

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