Reduction of Transpiration of Leaves through Stomatal Closure Induced by Alkenylsuccinic Acids

Abstract. *Low concentrations of long-chain alkenylsuccinic acids and certain of their derivatives inhibit stomatal opening in a tobacco-leaf disk assay, and when used as a spray. The monomethyl ester of decenylsuccinic acid is presently the most effective of this class of compounds. Stomatal closure induced with this substance is accompanied by a significant reduction in the rate of transpiration. These inhibitors probably close stomata by altering the permeability of the membranes of the guard cells.*

The amount of water transpired from plant leaves may be significantly reduced by preventing the opening of leaf stomata with certain metabolic inhibitors (*1*, *2*, *3*). A standard assay of stomatal closure was developed by using tobacco leaf disks (*1*), and a number of these inhibitors prevented the opening of stomata in excised leaves under environmental conditions where opening would normally occur (*1*, *2*, *4*). More recently, Shimshi (*5*) has examined the effect of soil moisture and of controlled stomatal aperture on transpiration and photosynthesis in intact maize plants.

Alkenylsuccinic acids and certain of their derivatives have now been found to be effective agents for the control of stomatal opening. These substances have the structure

$$CH_3\text{-}(CH_2)_n\text{-}CH{=}CH\text{-}CH_2\text{-}CH(COOH)\text{-}CH_2COOH$$

Waggoner, Monteith, and Szeicz (*6*) report the use of one of these substances. They observed from 20 to 30 percent reduction of transpiration in barley and thereby demonstrated the importance of stomatal resistance under field conditions.

A modified disk assay of stomatal closure was used to investigate these compounds. Leaf disks were floated on water in the light for 90 minutes at 30°C to permit the stomata to open fully (*4*). Disks were then blotted and transferred to water or to the solution of alkenylsuccinic acid to be tested, and the experiment was continued in the light for an additional 30 minutes. Impressions of the lower epidermis were then cast in silicone rubber, and the mean stomatal apertures were measured (*1*).

Figure 1 shows that the maximal closure is obtained in the free acid series with dodecenylsuccinic acid ($n = 8$). Under the conditions of the disk assay, complete closure was achieved at a concentration of $5 \times 10^{-5}M$. Because of the limited solubility of these compounds in water, the experiments with the free acids were carried out in 0.5 percent ethyl alcohol, a solvent that has no effect on stomatal opening. Similar experiments showed that dodecylsuccinic acid (the saturated analog) was considerably less effective than the corresponding alkenylsuccinic acid at the same concentration. It was also shown that straight-chain dodecenylsuccinic acid is much more effective in stomatal closure than the branched-chain 12-carbon derivative. Free carboxyl groups are also apparently essential for maximal activity, because even partial neutralization of these compounds to form sodium, calcium, or ammonium salts resulted in a great loss of stomatal closing activity. Accordingly, distilled water was always used to prepare the solutions.

Monomethyl esters of alkenylsuccinic acids dissolved in water are also effective in the disk assay (Fig. 1). With these derivatives, there is a sharper optimal effect of chain length, the decenyl derivative ($n = 6$) being the most active. The nonenylsuccinic acid ($n = 5$) used in this experiment and by Waggoner *et al.* (*6*) had a branched methyl-substituted chain rather than a straight chain, and, as indicated previously, straight-chain derivatives are superior in bringing about stomatal closure.

As with our earlier experiments (*2*), the effect of stomatal closure on transpiration and photosynthesis was investigated in excised leaves that had previously been treated on both leaf surfaces with sprays of 0.1 percent Triton in distilled water or with these new compounds. The free dodecenylsuccinic acid ($n = 8$) did not always bring about stomatal closure when used as a spray. The monomethyl ester of decenylsuccinic acid ($n = 6$), however, was completely reliable. It closed stomata and significantly reduced transpiration at the concentrations shown in Table 1.

The detached leaves were allowed to equilibrate in the chamber so that the stomata in the control leaves could open fully. The experiment was conducted for 1 hour under high light intensity, 0.3 cal cm^{-2} min^{-1}, and high relative humidity at 30°C with turbulence in the atmosphere of the chamber. Transpiration was determined in each pair of leaves by weighing, and the mean stomatal apertures were measured from impressions of the central portions of both surfaces of each leaf. The closure of stomata resulted in significant reductions of transpiration in the equally turgid leaves, ranging from 22 to 37 percent.

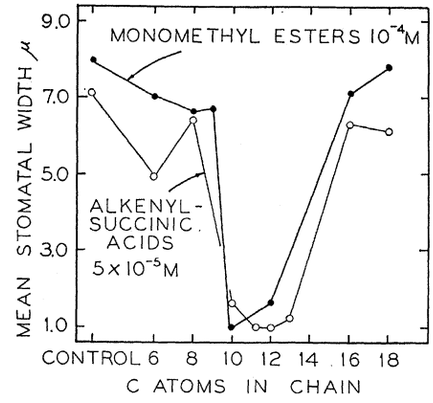

Fig. 1. Effect of length of hydrocarbon chain of alkenylsuccinic acids and their monomethyl esters on stomatal opening in a standard leaf disk assay.

In other experiments similar to those in Table 1, the monoethyl ester of decenylsuccinic acid was almost as effective as the monomethyl ester, but the *n*-propyl and isopropyl esters were less reliable. It would appear that the methyl ester derivative penetrates the guard cells more efficiently, and spraying the upper surface of a tobacco leaf with solutions of the monomethyl ester of decenylsuccinic acid at $1 \times 10^{-3}M$ was generally effective after 5 days and

Table 1. Effect of sprays containing the monomethyl ester of decenylsuccinic acid on stomatal width and on transpiration. Pairs of adjacent tobacco leaves on a given plant were sprayed with 0.1 percent Triton B-1956 in distilled water or the concentration indicated of monomethyl ester of decenylsuccinic acid in Triton. The leaves were sprayed from 1 to 23 hours before being excised and placed in the chamber.

Pair	Concentration	Mean stomatal width (μ)	Transpiration (mg H_2O per cm^2 leaf surface per hr)
1	Control	9.3	6.7
	$1 \times 10^{-3}M$	2.9	4.5
2	Control	7.4	4.8
	$1 \times 10^{-3}M$	2.8	3.4
3	Control	6.5	5.3
	$1 \times 10^{-3}M$	3.0	4.0
4	Control	7.9	4.2
	$1 \times 10^{-3}M$	3.8	3.2
5	Control	9.5	5.9
	$0.33 \times 10^{-3}M$	6.7	4.6
6	Control	7.2	4.9
	$1 \times 10^{-3}M$	1.0	3.1

sometimes after 7 days. No toxic effects have ever been observed with any of the alkenylsuccinic acids. As indicated (*2, 5*), closure of stomata by these compounds usually results in a greater inhibition of transpiration than of photosynthesis.

Opening of stomata can be prevented by inhibiting any of the biochemical reactions concerned with the increase in turgor of guard cells in the light, or by affecting the permeability of their membranes (*7*). The alkenylsuccinic acids which exhibit optimal activity for stomatal closure have also been shown by Kuiper (*8*) to be most effective in increasing the permeability of cell membranes of roots to water. The parallelism suggests that these long-chain unsaturated dibasic fatty acids behave as inhibitors of stomatal opening because of their effects upon the lipid layers of the membranes of the guard cells. A model of the effect of certain herbicides upon the lipids of plant cell membranes has previously been outlined by van Overbeek and Blondeau (*9*). However, the alkenylsuccinic acids are highly specific in their action, and apparently penetrate guard cells more readily than other epidermal cells. Thus these compounds provide an example of still another biochemical approach to the reduction of transpiration in the field and forest through stomatal control.

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References and Notes

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Histone Staining with Ammoniacal Silver

Abstract. *Under controlled conditions, ammoniacal silver (A-S) stains the bands of Dipteran salivary gland chromosomes in a precise and selective manner. Such staining is dependent upon the histone content of the band, as shown by the effects of selective extraction and blocking of the histones. The effects of acid extraction and HONO solution on the A-S staining of different bands suggest that specific genes may have particular histones associated with them.*

Histone rather than DNA appears to be the cytochemical covariable in many instances of functional change and cell development (*1*). However, even the chemical definition of histones is still speculative; as yet it has only been demonstrated that they are basic proteins of the nucleus intimately associated with DNA and extractable in dilute acid or acid salt solutions. At a recent symposium on histones, one conclusion reached was that a method is needed that is sufficiently delicate and sensitive to demonstrate whether histones do, indeed, differ in different functional states and stages of development (*2*).

In 1958 we reported that under controlled conditions ammoniacal silver (A-S) stained the nuclear chromatin of a variety of cells (*3*). This was followed by a series of reports in which the A-S staining of nuclear chromatin under different functional conditions was described. Data were presented which suggested that the A-S was staining the histone component of nuclear chromatin (*4*). It was found that A-S stained the nuclear chromatin of formalin-fixed smears of all types of cells tested, including mammals, fowl, invertebrates, and plants. The cytoplasm of such cells was stained faintly or not at all, while nucleoli were unstained. To test further the use of A-S as a cytochemical stain for histones, we decided to apply it to the giant chromosomes of the salivary glands of various Diptera, including those of *Sciara coprophila* (*5*).

The salivary glands were squashed in 45 percent acetic acid, a step which, while not at all essential to the A-S reaction, is essential to good spreading of the chromosomes in question. The coverslip was removed after gentle heating and the preparation was air dried. Where histones were to be extracted before staining, it was found best to extract the larvae being dissected in a drop of 0.25*N* HCl before squashing, and to transfer the glands to a vial with several changes of acid in the cold over a period of 1 hour. The extraction of histone by this method is remarkably complete and cannot be improved by changing the time in the acid or its concentration. The slide to be stained is fixed in 10 percent acetate-buffered neutral formalin for at least 15 minutes, rinsed in five changes of distilled water, placed in the A-S solution, and agitated for 5 to 10 seconds. Longer periods do not increase the staining significantly. It is then washed well in five changes of distilled water and developed in 3 percent formalin for 2 minutes, washed again, and run through the alcohols, cleared in xylol, and mounted permanently. The A-S solution, which should be fresh, is prepared by adding a 10 percent solution of $AgNO_3$ to concentrated NH_4OH until the first permanent turbidity appears (approximately 10 : 1, vol/vol, respectively). The exposure to formalin before staining is an obligatory step whether the material has been previously fixed in formalin or not; only the brief rinsing in five changes of distilled water should intervene.

These procedures were controlled by staining similar preparations by the Feulgen reaction, both before and after extraction, and also by the alkaline Fast Green method of Alfert and Geschwind (*6*) for histones. In addition, duplicate preparations were blocked with HONO before staining with A-S. The HONO solution is prepared by mixing equal parts of a 10 percent trichloroacetic acid (TCA) and a 10 percent sodium nitrite solution. After exposure to formalin and rinsing

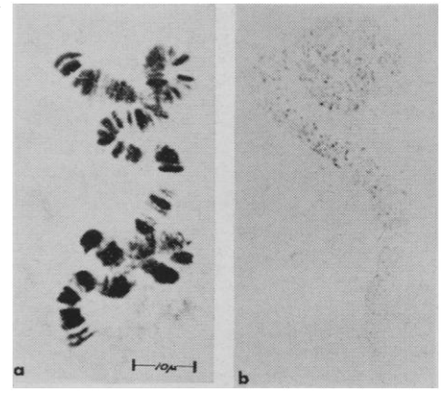

Fig. 1. Chromosome IV, of *Sciara coprophila*, stained with A-S. (*a*) Control; (*b*) extracted in cold, dilute HCl. While extraction of histone removes ability to stain with A-S, an intact banded structure is disclosed by Feulgen staining.