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*EFFECT OF CHEMICAL CONTROL OF STOMATA ON TRANSPIRATION
AND PHOTOSYNTHESIS**

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The water transpired from leaves and the CO₂ fixed in them are assumed to pass mainly through the microscopic pores, the stomata, that provide a ready path for diffusion of gases between the surrounding atmosphere and the leaf interior.

It was recently reported¹ that many enzyme inhibitors close stomata in the light, when they are normally open, and the relative closure by different compounds was evaluated by a leaf disk assay. An effective substance of this kind, which brings about closure of stomata when *sprayed* on whole leaves, has now been found. We report here the relation between the results obtained by the leaf disk assay and those obtained by foliar sprays on intact plants. The lack of translocation of the compound within the tissues and the long duration of the induced stomatal closure are also shown. The induced closure permitted a demonstration of the relation between aperture and both transpiration and CO₂ assimilation, and also indicated that the diffusion rate is a limiting factor in these processes.

Relation between Standard Leaf Disk Assay and Effect of Foliar Spray.—In the previous work, leaf disks were floated on solutions of various metabolic inhibitors, and the dimensions of the stomata were subsequently measured. If this rapid assay method is to be used as an indicator of field performance, it must be correlated with the effect of spraying these compounds on a number of species of plants.

A conservative test of the correlation was made with three compounds of known ability to close stomata. The results (Table 1) show that similar concentrations are effective in the disk assay and as foliar spray.

Another test of the correlation was made by assaying and spraying the same compound on different species. In a standard disk assay, 3.3×10^{-5} M phenylmercuric acetate closed 15 per cent of tobacco and 87 per cent of maize stomata. When this compound was applied as a spray at a concentration of 10^{-4} M, the stomata were closed to the extent shown in Tables 2 and 3. Thus the standard leaf disk assay can be employed with different subclasses of plants and is an advantageous means of discovering useful compounds.

Translocation and Duration of Effect.—The use of phenylmercuric acetate was suggested to us by the report² that phenylmercuric chloride, sprayed on tomato and potato foliage, diminishes transpiration and increases growth. Phenylmercuric

TABLE 1

COMPARISON OF THREE COMPOUNDS IN LEAF DISK ASSAYS AND AS SPRAYS ON TOBACCO LEAVES

Compound	Concentration (<i>M</i>)	Percentage of Stomata Closed	
		Disk assay	Leaf spray
Phenylmercuric acetate	10×10^{-5}	86	80
	3.3×10^{-5}	28	33
8-Hydroxyquinoline	10×10^{-4}	30	54
	1.0×10^{-4}	7	
Sodium α -hydroxydecanesulfonate	10×10^{-4}	64	41
	5.0×10^{-4}	31	

In the standard disk assay, disks from leaves kept in the dark were floated on water (control) or on the solution to be tested. The disks were illuminated and maintained at 25° for 90 min when impressions in silicone rubber were made. The proportion closed was: 100 less the ratio of the proportion of stomata at least 2 μ wide in the treated disks to the proportion of stomata in the control disks at least 2 μ wide. Normally, 80-100% of the stomata in the control were open. The α -hydroxysulfonate was dissolved in potassium acetate or tartrate buffer (0.005 *M*) at pH 4.4. On a sunny day, upper surfaces of individual tobacco leaves on a single plant were sprayed with the solutions (all contained 0.02% Triton X-100) and control leaves were sprayed with water or buffer solution. Later, the entire plant was covered with a transparent plastic bag to raise the environmental humidity, and, about 4 hr after spraying, leaf impressions were made.

TABLE 2

PERCENTAGE OF HAVANA SEED TOBACCO STOMATA OPEN AFTER SPRAYING THE LEAVES WITH PHENYLMERCURIC ACETATE

Leaf	Surface	Treatment	Day 5		Day 14	
			Plant A	Plant B	Plant A	Plant B
1	U	0	98	100	100	100
	L	0	100	100	100	100
2	U	10^{-5} <i>M</i>	88	84	76	96
3	U	10^{-4} <i>M</i>	36	32	44	24
	L	0	100	100	100	100
4	U	10^{-4} <i>M</i>	36	28	48	20
	L	10^{-4} <i>M</i>	70	76	80	60

In the greenhouse, four leaves on two plants were chosen at random and sprayed on the upper (U) or lower (L) surface with 0, 10^{-5} or 10^{-4} *M* phenylmercuric acetate in 0.02% Triton X-100. The plants then stood for 5 and for 14 days. About two hr before impressions were made on clear days, the plants were covered with a plastic bag to raise the environmental humidity.

TABLE 3

PERCENTAGE OF MAIZE STOMATA OPEN AFTER SPRAYING WITH PHENYLMERCURIC ACETATE

Leaf	Surface	Treatment	Day 5				Day 14			
			Plant				Plant			
			A	B	C	D	A	B	C	D
1	U	0	88	88	84	86	98	92	88	96
	L	0	94	92	100	80	94	100	78	94
2	U	10^{-5} <i>M</i>		76	86			100	84	
3	U	10^{-4} <i>M</i>	66			70	68			12
	L	0	94			94	98			44
4	U	10^{-4} <i>M</i>		66		62		80		2
	L	10^{-4} <i>M</i>		88		44		84		58

In the greenhouse, 4 leaves were chosen at random on 4 maize plants. They were sprayed on the upper (U) or lower (L) surface with 0, 10^{-5} or 10^{-4} *M* phenylmercuric acetate in 0.1% Triton X-100. Subsequent procedure was as in Table 2.

acetate is more convenient than the chloride because of its equal effectiveness and greater solubility in water. Mercuric chloride and acetate are ineffective in the standard disk assay. Presently, phenylmercuric acetate is our most effective compound and was employed in the remaining experiments of this paper.

Tables 2 and 3 show that the effect of this substance sprayed on leaves is observed only on the surface treated. Obviously, the substance is not translocated even from one surface of the leaf to the other. However, the stomata of the upper surface of tobacco and maize leaves are more readily closed than those of the lower surface. Furthermore the effect is a prolonged one. At least 14 days after the

leaves of tobacco or maize were sprayed with a solution as dilute as $10^{-4} M$, the stomata were still closed.

Effect of Stomatal Closing upon Transpiration and Photosynthesis.—The role of stomatal opening in transpiration and photosynthesis can be analyzed in terms of Fick's first law of diffusion:

$$T = D(X_f - X_p)/(L + S); \quad (1)$$

$$P = D'(X'_f - X'_a)/(L + S + M). \quad (2)$$

T and P , in $\text{gm cm}^{-2} \text{sec}^{-1}$, are rates of transpiration and photosynthesis per unit of leaf surface, and D and D' , in $\text{cm}^2 \text{sec}^{-1}$, are the coefficients of diffusion of H_2O and CO_2 in air. The X_f and X_p , in gm cm^{-3} , are the concentrations of H_2O in the surrounding air and at the evaporating surface, i.e., the surfaces of spongy parenchymatous cells about the substomatal cavities. The X'_f and X'_a are the concentrations of CO_2 in the free air and at the site of an acceptor within the chloroplasts.

The L and S are apparent path lengths for the diffusion in the surrounding atmosphere and through the stomata. These terms do not, of course, correspond to any actual lengths. Rather, L/D and S/D represent the resistance of the atmosphere and of the stomata and substomatal cavities to the diffusion of water. The M is the apparent length of the aqueous path, which encompasses the surface of the mesophyll cells about the substomatal cavities, to the sites of the CO_2 assimilation; hence, the M appears in the equation defining the diffusion of CO_2 in photosynthesis, but not in that for transpiration.

The usefulness in botany of equations (1) and (2) has often³⁻⁵ been demonstrated. We shall use it both to relate transpiration and photosynthesis to stomatal aperture, and to predict the relative changes in transpiration and CO_2 assimilation.

We may calculate S from observations of stomatal width, a . We chose Penman and Schofield's⁴ relation between S and a :

$$S = [l/(\pi ab) + 1/(2\sqrt{ab})] \div n, \quad (3)$$

where n is number of stomata per cm^2 (12,000 per cm^2 of leaf surface for tobacco), l is depth of stomatal opening (10μ) and b is length of aperture (20μ). The mean number and width were obtained from both leaf surfaces.

In a constant environment, equation (1) can be rewritten as a regression equation

$$1/T = \text{constant} + \text{constant}'(S), \quad (4)$$

where S is estimated from observations of stomatal width and equation (3). In the same way, $1/P$ can be related linearly to S .

The easy demonstration of the effect of chemically induced stomatal closure requires that we experiment in the region where change in transpiration per change in path length S is greatest.

The changes in transpiration and photosynthesis per change in S are:

$$\frac{dT}{dS} = -\frac{D(X_f - X_p)}{(L + S)^2}, \quad \frac{dP}{dS} = -\frac{D'(X'_f - X'_a)}{(L + S + M)^2}.$$

The diffusion coefficients and concentrations of H_2O and CO_2 , in the surrounding air, will be unaffected by stomatal closure.

If the concentration of water vapor X_p is near saturation, (about 30×10^{-6} gm cm $^{-3}$ for a leaf at 30°) and the stomata are open, this concentration might increase to 37×10^{-6} when the stomata close, evaporation decreases, and the leaf temperature rises to, say, 34° . The effect of closure upon X'_a , on the other hand, cannot be clearly predicted. Thus some of the effect of closure upon transpiration might be moderated by a greater gradient ($X_f - X_p$), while the corresponding effect upon photosynthesis is indefinite. We expect, however, that the effects upon the diffusion gradients will be slight compared with other effects that we will now discuss under the assumption that the numerators $D(X_f - X_p)$ and $D'(X'_f - X'_a)$ are essentially constant.

Under environmental circumstances c , the transpiration and photosynthesis are labeled T_c and P_c . In that neighborhood, the changes in these two quantities caused by changes in stomatal opening can be simplified by substituting from equations (1) and (2):

$$dT/dS = -T_c/(L + S); \quad (5)$$

$$dP/dS = -P_c/(L + S + M). \quad (6)$$

Thus a change in both quantities T and P will be obtained most dramatically when the initial levels T_c and P_c are high; for example, when plants are active because of their heredity or history, or are exposed to a high light intensity, great change in transpiration or photosynthesis will follow induced stomatal closure.

The effect of atmospheric turbulence is less obvious intuitively, but is clearly shown algebraically: if turbulence is great and L is therefore small, the changes in loss of water and gain of CO_2 will be greatest for a given change in stomatal resistance, S . Similarly, the changes will be greatest if the stomata are initially wide open and S is small. All of these factors have been considered in the design of the experiments.

The presence of M , in the equations for photosynthesis, suggests that the fraction of photosynthesis remaining after the stomata close will be greater than the fraction of transpiration remaining. This can be seen in equations (5) and (6): M reduces the change in P below that in T . From equations (1) and (2), one can show that the ratios of the processes in untreated (subscript 0) and treated (subscript t) leaves are

$$(P_t/P_o)/(T_t/T_o) = [(L + M + S_o)/(L + S_o)](L + S_t)/(L + M + S_t). \quad (7)$$

This index, the fraction of photosynthesis remaining after treatment relative to the fraction of transpiration remaining, is greatest when S_o is zero and S_t is infinitely large. Thus the most pronounced effect of M will be seen if we compare untreated plants whose stomata are widest and treated plants whose stomata are most tightly closed. A further use of the relative values of T_t/T_o and P_t/P_o is in the estimation of L and M . The regression equation, (4), summarizes all observations of S and $1/T$, or S and $1/P$. With use of these regressions, first T_t/T_o can be written as a function of S , which is calculated from observations of stomatal width by means of equation (3). This permits a calculation of L . Similarly, the regression of $1/P$ on S permits a calculation of M .

All of these predictions are valid if we deal with the simple physical system specified by our mathematics. Since transpiration is generally conceded to be

understandable in the rather simple physical terms of potentials and resistances, it is unlikely that we have gone astray in our specification of this process. Photosynthesis, however, involves many steps beyond the supply of CO_2 to the acceptor, and should any of these steps be hindered by the treatment designed for inducing closure of stomata alone, photosynthesis would be lower than our prediction.

From this we concluded that the compound which closes stomata should be applied as directly to the guard cells and to as few other cells as possible. Accordingly, the spraying technique was used. We also concluded that a compound that is not translocated was to be desired; hence, the failure of phenylmercuric acetate to move from upper to lower guard cells (Tables 2 and 3) was an advantage.

We observed, therefore, the changes in transpiration and photosynthesis after spraying phenylmercuric acetate upon tobacco leaves. The untreated leaf had open stomata. Photosynthesis was active, turgidity and illumination were high, and ventilation was rapid for both leaves. If the photosynthesis was diminished by an equal or smaller amount than transpiration, the compound was evidently acting on little else but guard cells, and our analysis in the physical terms of diffusion is reasonable.

Methods.—On Havana Seed tobacco plants growing in a subirrigated greenhouse bench, two adjacent leaves, 300 to 400 cm^2 in projected area, were chosen for similarity of size. One leaf, chosen at random, was sprayed with a 0.02% Triton X-100 solution, and the other was sprayed with phenylmercuric acetate dissolved in the Triton solution. The pair of leaves was excised from 1 hr to 3 days after being sprayed. They were then stood in small beakers of water upon the flooded floor of a transparent chamber ($28 \times 36 \times 46$ cm) and illuminated by 0.3 $\text{cal cm}^{-2} \text{min}^{-1}$ for 1 hr, conditions which were found to insure the opening of normal stomata as determined from silicone rubber impressions. The leaves were supported at an angle of 49° above the horizontal. Radiation is given as energy below 3,000 μ and perpendicular to the leaf. The radiation came from water-cooled incandescent bulbs 60 cm above the chamber floor;⁶ they delivered two-thirds as many quanta per calorie as does the sun.

With no turbulence in the chamber and radiation of 0.7 $\text{cal cm}^{-2} \text{min}^{-1}$, a mean difference of 2.2° was measured between a 30 gauge copper-constantan thermocouple touching the upper surface of the leaf and a reference junction 1 cm above. The difference was reduced to about 0.3° by spinning a 22 cm fan inside the chamber at an arbitrary speed. During the experiments, the fan was rotated at this same speed, thereby reducing L to about 0.12 of its value without the fan. The CO_2 concentration in the air was maintained by two 2.5 cm diameter openings in the chamber.

The air within the chamber was kept at 27° to 30° by blowing cool air on the thin transparent plastic film of which the chamber was constructed. This maintained the dewpoint at about 22° by condensation of water inside the film.

At time zero, which was one hour after the leaves were placed in the chamber, the beakers and leaves were weighed, radiation was increased to 0.7 $\text{cal cm}^{-2} \text{min}^{-1}$, the fan was turned on, and the measurements of transpiration were begun. After 40 min, the two openings in the chamber were stoppered, and 10 μ moles of CO_2 containing 4 μc of C^{14} were released into the chamber. This increased the CO_2 concentration in the air by no more than 5 ppm. After exposure of both leaves to C^{14} for 2 min, the ventilators were opened and fresh air was swept through the chamber. At the expiration of 60 min, the leaves and beakers were removed and weighed. Stomatal opening was determined from silicone rubber impressions and the radioactivity of leaf homogenates was measured. The observation that the leaves were fully turgid both at the beginning and end of the experiment was confirmed by the negligible change in fresh weight of the leaves and in the stomatal condition.

Results.—The uniformity of the leaves and of the environment within the chamber was attested by the behavior of untreated leaves: their transpiration ranged only from 6.1 to 11.4 mg cm^{-2} , their photosynthesis from 1,140 to 1,950 counts min^{-1}

TABLE 4
EFFECT OF PHENYLMERCURIC ACETATE SPRAY ON STOMATAL WIDTH, TRANSPIRATION AND PHOTOSYNTHESIS IN TOBACCO LEAVES

Pair	Treatment		Mean stomatal width, μ		Transpiration (mg cm ⁻² hr ⁻¹)	Photosynthesis (counts min ⁻¹ cm ⁻²)	Pair	Treatment		Mean stomatal width, μ		Transpiration (mg cm ⁻² hr ⁻¹)	Photosynthesis (counts min ⁻¹ cm ⁻²)
	U	L	U	L				U	L	U	L		
1	0	0	3.1	6.4	7.7	1270	10	0	6.3	8.6	10.6	1230	
	10	10	1.0	4.4	6.2	1120	0	10	7.4	8.2	10.9	1290	
2	0	0	1.4	4.6	6.1	1290	11	0	5.5	8.3	8.3	1140	
	3.3	3.3	1.0	2.3	5.8	1030	0	10	1.1	5.3	7.8	1010	
3	0	0	1.7	6.1	6.9	1410	12	0	6.4	7.7	9.7	1750	
	10	10	1.1	1.3	3.9	870	0	10	1.0	9.3	7.9	1480	
4	0	0	1.9	6.7	6.8	1160	13	0	10.0	11.4	10.5	1690	
	10	10	1.0	1.2	3.1	740	0	10	2.0	2.7	7.3	1610	
5	0	0	2.1	6.8	6.7	1540	14	0	5.5	6.8	10.2	1580	
	3.3	3.3	1.0	4.8	7.0	1490	0	10	1.0	2.4	7.3	1270	
6	0	0	4.7	9.1	8.4	1480	15	0	1.8	2.1	7.8	1210	
	3.3	3.3	1.5	6.2	7.0	1430	0	10	1.0	1.3	5.8	960	
7	0	0	1.8	8.5	8.2	1510	16	0	4.4	6.4	9.4	1870	
	10	10	1.2	2.2	5.6	1030	33	33	1.1	1.1	4.6	920	
8	0	0	2.9	7.8	8.5	1390	17	0	6.8	9.0	10.3	1950	
	10	0	1.1	5.1	6.3	1290	33	33	1.2	1.1	7.1	1360	
9	0	0	2.2	10.2	6.7	1340	18	0	7.9	10.6	11.4	1710	
	0	10	2.5	1.8	4.9	930	33	33	1.4	1.2	6.9	1070	

Pairs of leaves on greenhouse-grown Havana Seed tobacco plants were sprayed with 0.02% Triton X-100 (0) and phenylmercuric acetate solution in Triton at a concentration of 3.3, 10, or 33 times 10⁻⁴ M respectively on the upper (U) or lower (L) leaf surface as indicated. The observations were made as described under *Methods* and are expressed in terms of cm² of projected leaf area. There were 2.16 times as many stomata on the lower surface as on the upper surface of the leaves of this variety of tobacco.

cm⁻², and in only one of 18 experiments were the stomata of the lower surface of control leaves smaller than 4.6 μ in width (Table 4). The leaves identified as pairs 1 to 9 were taken from the same lot of plants, were examined on nearly consecutive days, and were exposed to aliquots of the same radioactive solution. They provide a uniform set.

The phenylmercuric acetate sprayed upon the leaves closed the stomata of these plants as in our earlier experiments, providing stomatal resistances or apparent lengths S , which were estimated from the measurements of stomatal width (Table 4) by means of equation (3). The S ranged from 0.05 to 0.21 cm. The clear linear relation between S estimated from stomatal widths and the reciprocals of transpiration (Fig. 1) and of photosynthesis (Fig. 2) verify anew the theory introduced to botany by Brown and Escombe (equations (1), (2), and (4)) and Penman and Schofield's relation between aperture and S (eq. (3)). The statistical suitability of relating $1/P$ or $1/T$ to S is apparent from the uniformity of the deviations from the calculated curves.

The observations from different plants and days within the first nine pairs show the surprising uniformity of the material and provide an explanation of the variability in terms of stomatal behavior.

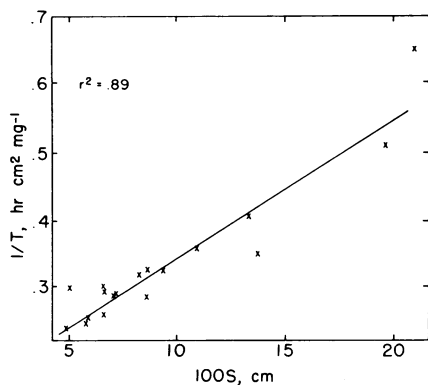


FIG. 1.—The $1/T$, reciprocal of transpiration, as a function of S , apparent stomatal length, for leaf pairs 1 to 9.

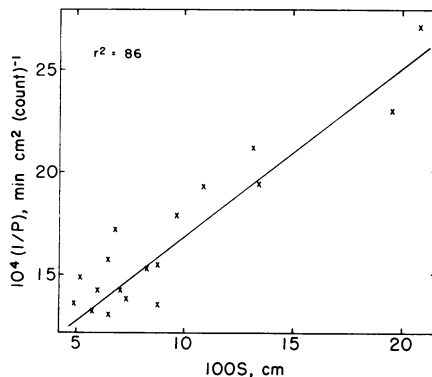


FIG. 2.—The $1/P$, reciprocal of photosynthesis, as a function of S , apparent stomatal length (estimated from widths and equation (3)), for leaf pairs 1 to 9.

An index of the fraction of photosynthesis remaining after treatment relative to the fraction of transpiration remaining is provided by equation (7). The index was expected to be greatest when the change in S is large and the remaining transpiration is small. Therefore, we selected from Table 4 those experiments where the remaining transpiration, i.e., transpiration after treatment, was no more than three-quarters of the control. The index (equation (7)) for these 11 experiments ranged from 1.40 to 0.96 with a median of 1.07. Since this index was greater than 1.00 in 9 out of 11 experiments, we conclude that the fraction of photosynthesis remaining after treatment is greater than the fraction of transpiration remaining. (The probability of observing 9 out of 11 in the predicted direction by chance alone is 0.03.)

The regressions (Figs. 1 and 2) lead to estimates of L and M , as explained earlier. The 18 observations of S and $1/T$ correspond to $L = 0.07$ cm. This estimate and the 18 observations of S and $1/P$ correspond to $M = 0.05$ cm. Thus the ratio of $L:S:M$ is 7:5:5 in a control leaf and 7:20:5 in a sprayed leaf.

Others^{3, 4} have observed L in a chamber without a fan to be 1.3 to 1.6 cm. The change in temperature gradients in our chamber showed L was reduced to about one-eighth of that in still air when the fan was operating. Therefore, the estimate $L = 0.07$ is reasonable.

As stated earlier, our measurements of the index, equation (7), and estimate of M will be reduced to the extent that phenylmercuric acetate injures biochemical events in photosynthesis. In a sense M indicates injury as well as resistance to diffusion. Since the median of our observations corresponds to values of M lower than others have estimated,^{4,5} a possibility exists that compounds more specific for stomatal closure will be found that will reduce transpiration with even less disadvantage to CO_2 assimilation.

Reducing Transpiration from Intact Plants.—The preceding theory and observa-

tions on excised leaves led to experiments in transpiration control in whole plants grown in containers in the greenhouse. (Lack of space prevents our reporting here the experiments in which a foliar spray diminished the water loss significantly; these results will appear in the August issue of these PROCEEDINGS.)

Summary.—The standard leaf disk assay showed that at concentrations about 10^{-4} M phenylmercuric acetate would close stomata. The effectiveness revealed by the disk assay has been correlated with the results of foliar spraying and, hence, the assay is advantageous for revealing useful compounds. Phenylmercuric acetate sprayed on tobacco or maize leaves closed stomata only on the leaf surface sprayed, and closure persisted for at least 14 days.

A high correlation of the reciprocals of the rates of transpiration and photosynthesis with resistance of diffusion through stomata, which was calculated from stomatal width, was predicted from diffusion theory and has been verified by observation. The variation in stomatal resistance was attained by spraying phenylmercuric acetate on the tobacco leaves. In 9 out of 11 experiments, induced closure of stomata reduced transpiration relatively more than CO₂ assimilation.

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THE ISOLATION OF DEGRADATION PRODUCTS FROM THE ANTIBIOTIC, ACTINOSPECTACIN

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The antibiotic, actinospectacin^{1, 2} has recently³ been shown to contain a 1,3 bis(methylamino)tetrahydroxycyclohexane unit called actinamine (I). Results obtained in these laboratories are in agreement with the proposed structure, and in this paper we present evidence for the nature of the other part of the molecule.

Experimental.—The N.M.R. spectra were obtained with a Varian V-4300B spectrometer operating at 60 Mc/s. All spectra were run in deuterochloroform solution. The apparatus and method described by Freeman⁴ were used for the decoupling experiments.

A Wilkens Aerograph gas chromatographic instrument fitted with a Wheelco recorder was used for the separation of the liquid products. A 4' × ³/₈" column packed with 10 per cent silicone on fluoropak was used.