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Communication

The Dependence of Stomatal Closure on Protein Synthesis[1]

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ABSTRACT

Seven different inhibitors of the synthesis of protein and RNA, all of which are found to delay the senescence of detached oat leaves in darkness, also cause the opening of the stomata in the dark. The concentration ranges for activity on the two processes agree closely. Four other compounds of similar effects on RNA and protein synthesis, but which are inactive on senescence, correspondingly fail to open the stomata. This not only strengthens the relationship between stomatal closure and senescence, but—more important—provides strong evidence that continued protein synthesis is necessary to keep foliar stomata closed.

It was shown in 1972 (8) that the rapid senescence of oat (*Avena sativa*) leaves when placed in darkness was strongly delayed by CHI.[3] Since the CHI also caused up to 90% inhibition of the synthesis of protein in the leaves, the effect was interpreted as due to blockage of the synthesis of proteases. The normal increases in acid and neutral proteases in the leaf, as well as the synthesis of total protein, were in fact largely prevented by the CHI (8).

This action of CHI on oat leaf senescence has since been extended to many other species (5–8, 10, 12, 14) and to other inhibitors of the synthesis of RNA and protein (11, 14). However, we subsequently discovered an additional aspect of this action, namely that the delay of senescence by CHI was accompanied by the opening of the stomata in darkness (9). The full significance of this second effect was not appreciated at the time, because several other agents, not known to affect protein synthesis, had comparable effects on stomatal opening. But reconsideration of the early evidence of Yoshida (13), that the presence of the nucleus promotes senescence in the cells of *Elodea*, focused our attention on the role of protein synthesis not only on leaf senescence itself but also on the changes in the stomatal opening that seem to accompany it. As a result a study has been made of the action of a number of inhibitors of protein and RNA synthesis, both on senescence proper and on stomatal aperture, and the results lead to a rather unexpected conclusion.

MATERIALS AND METHODS

Seeds of oats (*Avena sativa* cv Victory), from Svalöv, Sweden, were grown in vermiculite in continuous 'daylight' fluorescent light of intensity 30 μE $m^{-2}s^{-1}$. Ten segments 3 cm long, cut 3 mm below the tip of the 7-d-old first leaf, were floated on 10 ml of test solution in a 10 cm petri dish in darkness. Stomatal aperture was measured as diffusion resistance, usually on the 3rd day, with a Delta-T Automatic Porometer (Decagon Devices, Pullman, WA). For these measurements the leaves (10 per dish) were rapidly blotted dry between two filter papers and inserted into the holder of the porometer within 1 to 2 min. The holder, as well as the dish with the remaining segments, was kept in darkness during the measurement. Five consistent readings were taken and averaged for each segment. The leaf segments were subsequently extracted in boiling 80% ethanol and the Chl, free amino acids and protein determined as before (8).

RESULTS AND DISCUSSION

In the study by Yoshida and Kao (13), RIF was shown to prevent the action of the nucleus in causing senescence of *Elodea* cells. More recently Yu and Kao (14) have shown RIF to prevent Chl loss by soybean leaf discs in darkness. Table I shows that in fact RIF prevents all three of the typical aspects of the senescence of oat leaves, namely Chl loss, accumulation of free amino acids and proteolysis. But more importantly, the last column shows that the two concentrations fully effective on senescence also clearly open the stomata. The opening takes place slowly and is superimposed on the gradual opening that occurs on water or buffer (9). Comparable experiments in white light showed no appreciable effect of RIF, either on senescence or on the stomatal aperture. It was this finding that led to the study of other inhibitors.

The selection of inhibitors for comparative study was limited by the need for the following properties: (a) solubility in water, because alcohols have their own strong effects on senescence; (b) small molecular size, *i.e.* ability to enter undamaged leaf cells, since peeling and scraping have special effects on leaf senescence

Table I. *Action of RIF on Senescence and on Stomatal Aperture of Detached Oat Leaves in Darkness*

Data the mean of two complete experiments, with 10 leaf segments in each test solution.

RIF Concn.	Percentage of Initial Values[a] after 4 d			Stomatal Diffusion Resistance after 3 d
	Chl.	Free amino nitrogen	Protein	
μM				$S\ cm^{-1}$
0	32	341	55	128
12	52		59.5	137
36	72.5	261	62.5	72
110	97.5	50	87	6

[a] Initial values, per segment: Chl 23 μg; amino N 0.40 μmol; protein 380 μg; fresh weight of segment 17.2 mg.

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[3] Abbreviations: CHI, cycloheximide; RIF, rifampicin.

Table II. *Action of Puromycin and CHI on Senescence and on Stomatal Aperture of Detached Leaves in Darkness*

Concn.	Percentage of Initial Values[a] after 3 d		Stomatal Diffusion Resistance after 3 d
	Chlorophyll	Free amino nitrogen	
			$S\ cm^{-1}$
Puromycin (μM)			
0	31	418	158
330	60	330	110
500	66	300	47
CHI (μM)			
100	96	50	10
300	100	78	11

[a] Initial values as for Table I.

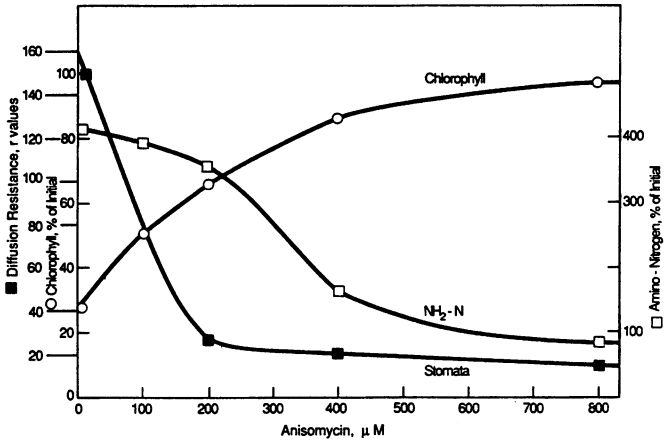

FIG. 1. Action of anisomycin on stomatal aperture of detached oat leaves in darkness, together with the action on senescence, as shown by gain in Chl and loss of amino-nitrogen. Mean of two complete experiments. Initial values as given below Table I.

Table III. *Action of Emetine on Senescence and Stomatal Aperture of Detached Oat Leaves in Darkness*

Data the mean of three complete experiments.

Concn.	Percent of Initial Values[a] after 3 d		Stomatal Diffusion Resistance after 3 d
	Chl	Free amino nitrogen	
μM			$S\ cm^{-1}$
0	26	438	61.5
300	37.5	357	70
500	40	342	75

[a] Initial values as for Table I.

(4); and (c) known activity in the nucleus of eukaryotes. From the literature (*e.g.* Ref. 3) the following molecules were therefore selected:

1. Anisomycin, inhibiting the peptidyl-transferase on 80S ribosomes (and probably inactive on prokaryotes);
2. Aurin-tricarboxylic acid, reported as a general inhibitor of the process;
3. Azaguanine and azaadenine, inhibiting the total synthesis of nucleic acids in general;
4. Emetine, inhibiting the translation process at an unreported site;
5. Ethidium bromide, a planar ring that intercalates between base pairs of DNA and thus blocks the RNA polymerase activity;
6. Puromycin, acting as an analog of aminoacyl-*t*-RNA in both pro- and eukaryotes;
7. The tetracyclines, well known as antibiotics, and binding to 70S or 80S ribosomes to inhibit their function.

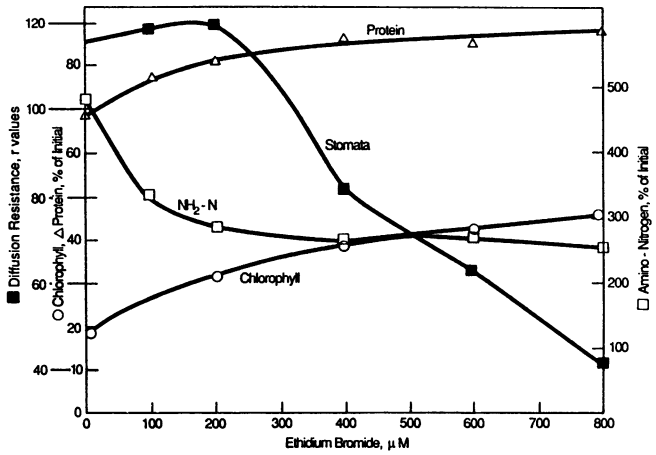

FIG. 2. Action of ethidium bromide. Same conditions as in Figure 1. Mean of three complete experiments.

Chlortetracycline and oxytetracycline had a small but rather variable effect on senescence, which was improved somewhat by adding 1 mM $MgSO_4$ (*cf.* p. 449 of Ref. 3). But there was a consistent opening of stomata in the dark. At 100 μM, chlortetracycline lowered the stomatal resistance on d 3 from 139S cm^{-1} in dark controls to 50S cm^{-1}; similarly, oxytetracycline at 100 μM lowered it from 138 to 60. Comparable opening by benzylaminopurine requires a concentration of about 10 μM.

In Table II the weak but real effects of puromycin are compared with the drastic effects of CHI, which can actually lower the free amino acid level below the initial value, thus perhaps promoting the synthesis of some specific protein(s). However that may be, the concentrations active in delaying senescence, with both inhibitors, are just those that are active on stomatal aperture.

Anisomycin clearly delays senescence (Fig. 1). Its effects on stomatal aperture are even stronger than those on Chl and protein, but both are exerted over comparable concentration ranges.

The action of emetine is shown in Table III. The proportionality between Chl protection and stomatal opening is not perfect, but it is clear that again a protein synthesis inhibitor opens the stomata, and that the concentrations active on the stomata are the same as those that prevent senescence.

Finally, the action of ethidium bromide is shown in Figure 2. In all four experiments stomatal opening began only above 200 μM, while protection of Chl and protein was detectable at 100 μM or less. Thus, unlike anisomycin, the action on stomatal aperture is a little weaker than that on senescence, but it is reproducible.

The concentrations that are active in opening stomata are comparable for all the seven compounds. This supports the idea that all are acting in a similar way. As against these seven positive effects, four negative effects are notable. The purine derivatives, azaguanine and azaadenine, which inhibit nucleic acid formation in general, had no effect on either senescence or stomatal aperture. The concentration needed for a positive effect, however, may well be too high to be safely applied to the leaves. Aurin-tricarboxylic acid had no effect, but its uptake may be limited by its high dissociation at cytoplasmic pH. Chloramphenicol, which was earlier found inactive on oat leaf senescence, and had

only a slight action on soybean leaf discs (14), is reported to act only on prokaryotic systems. Thus the lack of effect on senescence in three of the four cases parallels lack of effect on stomatal aperture.

All the active compounds act in eukaryotes on the formation of RNA or on the steps of its translation into proteins. It is of course not entirely excluded that they all exert some secondary effect which relates more directly to the movement of guard cells. However, because the compounds are so structurally unrelated, that explanation is extremely improbable. The action would have to be coupled in each case to the delaying action on senescence. That these compounds act by inhibiting protein synthesis is certainly the simplest explanation. Then, since inhibiting protein synthesis thus causes stomata to open, it would follow that the continued synthesis of one or more proteins must be needed to close them, or more likely to keep them closed. Perhaps this finding may help to explain some of the peculiarities of guard cell behavior. For instance, there is often a marked discrepancy in timing between the onset of stomatal closure and the appearance of an increase in the ABA content of the leaf (*e.g.*, Ref. 1, and Fig. 1 of Ref. 2). Although explanations based on ABA redistribution have been put forward, the intervention of a macromolecular synthetic process might provide a broader basis for explanation. In any event, it presents a new aspect of guard cell physiology that needs exploring.

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