Plant Physiol. (1980) 66, 395-399
0032-0889/80/66/0395/05/$00.50/0

The Influence of Aliphatic Alcohols on Leaf Senescence[1]

Received for publication January 2, 1980 and in revised form March 31, 1980

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ABSTRACT

Because of the effects of ethanol used as a solvent in other experiments, the action of aliphatic alcohols on leaf senescence in the dark has been studied systematically. These compounds both maintain chlorophyll and prevent proteolysis in the dark, much as do the cytokinins and other senescence-delaying substances. The activity of the straight-chain alcohols increases in a log-linear fashion with increasing chain length up to 1-octanol. Introduction of a branch in the chain or of a second OH group greatly decreases, or in some cases annuls, the antisenescence activity. In all cases, the action on senescence is closely (although not always exactly) paralleled by opening of the stomata. Abscisic acid and exposure to high concentrations of osmoticum, both of which close the stomata, antagonize the action of the alcohols. Some interactions with other agents are noted. The effects are compared with reported effects on seed germination, on hemolysis and animal membranes, and especially on permeability to K^+ ions, and a tentative basis for the mechanism of action is advanced.

The ability of a number of chemically unrelated compounds to exert striking modifications of the progress of senescence in the detached leaves of oat seedlings has been reported elsewhere (22, 24, and literature cited therein). Besides the cytokinins, at least five different compounds act to delay senescence in the dark, whereas three, as well as two physical treatments, promote the slower rate of senescence in the light, thus antagonizing the delaying effect of light. We found that in all cases the action on senescence was paralleled by an action on the stomatal aperture; stomatal closure paralleled promotion of senescence and stomatal opening paralleled its delay or inhibition.

In studying these effects, it was necessary on several occasions to use alcohol as a solvent. Although the ethanol was diluted down to a maximum of 1% before application of the solution to the leaves, we noted that controls floated on this aqueous alcohol in darkness behaved very differently from those floated on H_2O. An example, with two concentrations of ethanol, is shown in Table I. Figures 1 and 2 of reference 24 also showed alcohol effects, but these were superimposed on the effects of FC[2]. It is clear from these data that ethanol exerts an effect on senescence in darkness like that of the several senescence inhibitors, although more weakly. Repeated observations of this alcohol effect led to a study of the action of a number of aliphatic alcohols on senescence. In this paper we report the considerable activity of a series of aliphatic alcohols in preventing or delaying leaf senescence in the dark. Disappearance of Chl and proteolysis responded similarly in all cases examined. The relation between structure and activity among the alcohols is closely parallel to reported effects on hemolysis, enzyme inhibition, and permeability changes, and a suggestion will be made as to the possible underlying mechanism.

MATERIALS AND METHODS

Oat seedlings (*Avena sativa* cv. Victory) were grown in Vermiculite under continuous "Daylite" fluorescent lights as previously described (22, 24). At the age of 7 days (at 25 C), the first leaves were removed and 3-cm subapical segments were cut and floated on test solutions. After 3 or 4 days in total darkness or 4 or 5 days in the same light as for growth, the fresh weights were determined after a standardized blotting, to compare with the diffusion resistance values. The latter were measured at the outset and from time to time during the experiments, using the Diffusion Porometer of Lambda Instruments (23), and the r values calculated as indicated by Kanemasu *et al.* (7). (Note that, in the formula cited in reference 23, the stroke was omitted after the bracket.) The leaves were then analyzed for Chl, amino nitrogen, proteins, and reducing and nonreducing sugars (11, 22).

In experiments previously described, 2 drops of Tween 80 have been added to 10 ml test solution, but in the present work detergents were not added, except in the few cases indicated. At the concentrations used (0.25% maximum), the Tween 80 had no detectable effect on senescence. Temperature was held at 25 C throughout. With the lower boiling alcohols, the solution was changed after 2 days in case evaporation had altered the concentrations.

To avoid the risk that the observed effects might be due to toxicity, the turgor or rigidity of the leaves was tested with a simple device at the close of each experiment. Limpness had been found to be a sensitive indicator of toxicity. In none of the experiments reported were the leaves limp.

RESULTS

Influence of Straight-chain Alcohols. Figure 1 shows the Chl contents and the free amino acids of the leaves after 3 days in darkness on aqueous solutions of the alcohols. The concentration scales are successively condensed as the mol wt of the alcohol is increased (note also that two of the scales are logarithmic). However, the scales for propanol, butanol, and pentanol are the same, and it will be seen that each alcohol above C_3 at its optimal level can maintain the Chl at close to its initial value and can reduce the proteolysis to about 10% of that which occurs in the dark controls. In the case of heptanol, solubility (even in the presence of 0.25% Tween 80) limited the effectiveness, but the higher activity of octanol allowed almost complete repression of proteolysis well within the solubility limits. Table I includes additional data for ethanol.

Figure 2 summarizes the effects of the alcohols C_3 to C_7 on the free amino nitrogen released. From the curves in Figure 1, the concentrations of each alcohol needed for 50% maintenance of Chl were read off, and the logs of these values are shown in Figure 3. Each additional CH_2 group decreases the log of the effective

[1] This work was supported in part by Grant BMS 7683126 from the National Science Foundation (to K. V. T.).

[2] Abbreviations: FC: fusicoccin; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone.

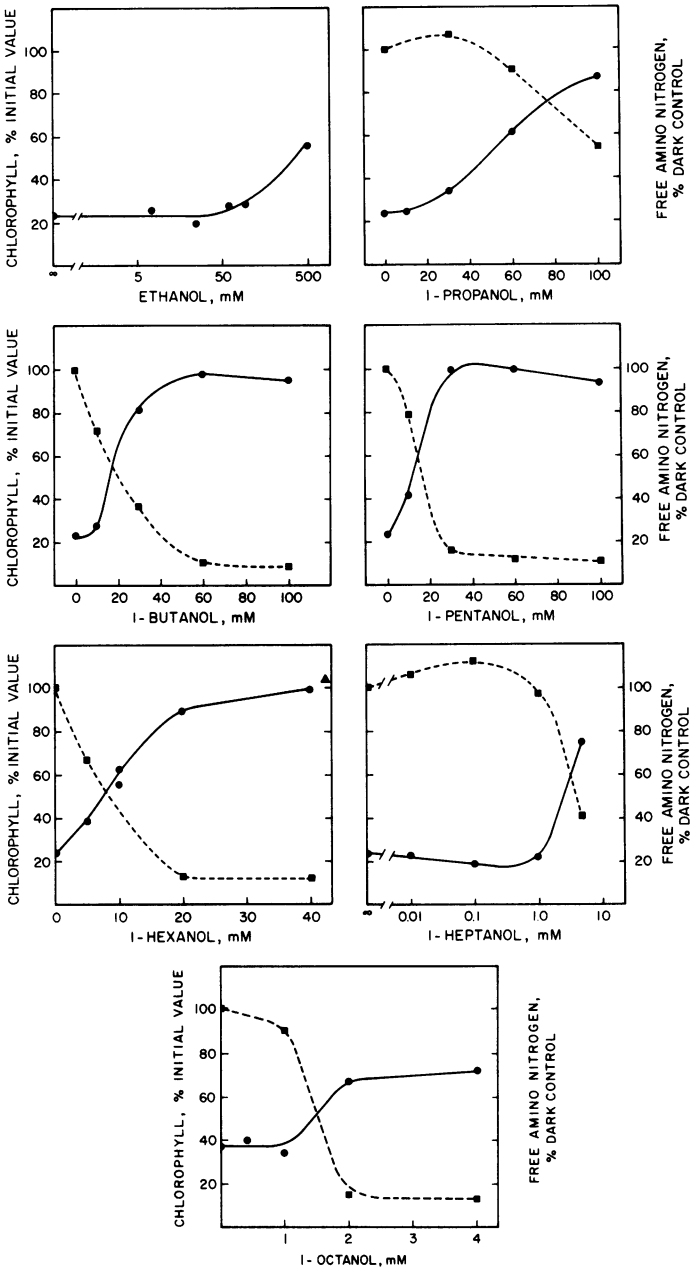

FIG. 1. The influence of alcohols on the senescence of oat leaves after 3 days in the dark. (——): Chl as per cent of the initial value; (- - -): free amino nitrogen as per cent of the dark control. Note the varied scales for abscissas; those for ethanol and heptanol are logarithmic.

Table I. *Effects of Two Concentrations of Ethanol on Four Different Criteria of Senescence in Darkness*

All values are given after 108 h in darkness, except diffusion resistance, which is given as r values after 48 h.

	Chl	Amino-N	Reducing Sugars	Resistance (r)
	A_{660}	*μmoles/segment*	*μg/segment*	*s/cm*
Water control	0.032	2.48	28.0	109.0
Ethanol 1%	0.097	0.38	4.5	79.2
Ethanol 5%	0.210	0.10	0.6	3.0

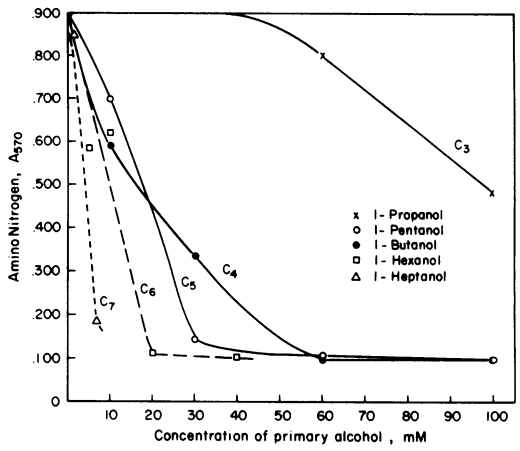

FIG. 2. Plot of the free amino nitrogen after 3 days in the dark with the straight chain alcohols C_2 through C_7, all on the same concentration scale.

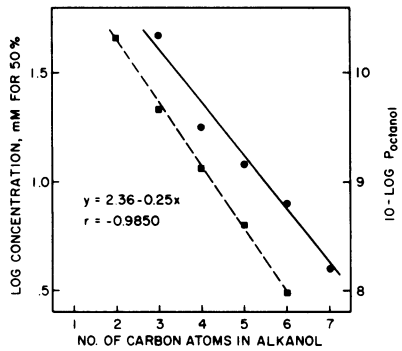

FIG. 3. (——): The logarithms of the concentrations of the alcohols giving 50% maintenance of the Chl, after 3 days in the dark; (- - -): 10–log of the partition coefficient of the alcohols between octanol and H_2O (from ref. 10).

Table II. *Action of Vapors of Aqueous Alcohol Solutions on Senescence of Oat Leaves after 4 Days in Darkness*

	Concentration	Chl	Amino Nitrogen
	mM	A_{660}	A_{570}
Water		0.035	1.240
1-Propanol	100	0.257	0.472
1-Butanol	60	0.240	0.260
1-Pentanol	40	0.267	0.288
1-Hexanol	20	0.195	0.424
Initial value		0.276	0.177

mM concentration by 2.9. The high correlation coefficient (0.985) suggests a straightforward physicochemical mechanism. The plot of the relative solubilities of the alcohols in H_2O and octanol supports this conception.

Advantage was taken of the volatility of these lower alcohols to test the effect of exposing the leaves only to the vapor. For this purpose, the leaves were placed on a small platform above the liquid, in a closed Petri dish. This treatment (Table II) is as effective as floating on the solution, for with the lower mol wt compounds, about 90% of the Chl is maintained. Both treated and control leaves lost a minimum of fresh weight. The data for each alcohol in Table II are given for the concentration that had been optimal for floating segments. The Chl in this system was somewhat better preserved than the protein. Since the vapor pressures of pentanol and hexanol at 25 C are very low, this experiment demonstrates that the actual volumes of the alcohols required to delay senescence almost completely must be quite small.

Relationship between Structure and Activity. In a limited number of tests, alcohols with branched chains were found much less active than those with straight chains; several were quite inactive (Table III). However, the long-chain compound farnesol (racemic mixture), with unsaturation as well as a branched chain, was quite active (Fig. 4). This result is of interest because this compound is naturally present in leaves and thus may exert some endogenous influence on senescence.

Table III. *Relative Effects of Straight and Branched Isomers of Butanol*
All are at 60 mM. The data given are after 4 days in darkness.

Configuration	Chl	Amino Nitrogen	Protein	Change in Fresh Weight
	A_{660}	A_{570}	A_{500}	*mg/100 mg*
C—C—C—C—OH	0.220	0.120	0.308	−1.03
C—C(OH)—C—C	0.035	0.392	0.252	+2.10
C—C(OH)—C	0.037	0.863	0.218	+2.53
C—C—C—OH (C)	0.088	1.056	0.200	+0.85
Water control	0.040	0.985	0.197	+2.35

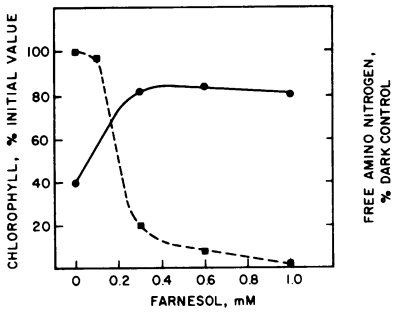

FIG. 4. The influence of farnesol on senescence after 3 days in the dark. Legend as for Fig. 1.

Table IV. *Relative Effectiveness of Primary and Secondary Carbinols and of Di- and Trihydric Alcohols on Senescence of Oat Leaves*
The data given are after 4 days in darkness.

Alcohol	Concentration	Chl	Amino Nitrogen	Protein	Change in Fresh Weight
	mM	A_{660}	A_{570}	A_{500}	*mg/100 mg*
Water control		0.037	0.952	0.205	+1.87
1-Propanol	100	0.162	0.418	0.300	+0.48
2-Propanol	100	0.035	1.030	0.177	+2.81
Glycerol	100	0.065	1.140	0.275	+0.74
1-Butanol	60	0.220	0.120	0.308	−1.03
2-Butanol	60	0.032	0.390	0.250	+2.10
1,4-Butanediol	60	0.042	0.763	0.240	+2.67
1-Pentanol	30	0.252	0.125	0.325	−2.02
1,5-Pentanediol	30	0.050	0.940	0.215	+2.67
Initial value		0.282	0.150	0.837	

Results with two examples of dihydric alcohols and one with glycerol are presented in Table IV. It is clear that a second hydroxyl group, which lowers the lipophilia and increases the solubility in water, greatly decreases or even annuls the activity in preventing senescence. Table IV also provides additional data on the lower activity of isomers with branched chains.

Neither lactic nor stearic acid had any effect on senescence.

Parallelism with Effects on Stomatal Aperture. Since the prevention of senescence in the dark is always accompanied, to a greater or lesser degree, by stomatal opening in the dark (24), it was of importance to determine whether this rule holds also for the aliphatic alcohols. Table V shows the diffusion resistance for four of the alcohols after 3 days in the dark. In addition, Table VI brings together, for a series of 1-butanol concentrations, the values for three chemical assays and two measures of stomatal aperture. It is clear that the parallelism, although perhaps not quite exact, holds very well. Stomatal opening by three alcohols was recently reported by Mouravieff (16).

Modification of Alcohol Effects by Other Reagents. Calcium ions were shown by Poovaiah and Leopold (18) to exert some effect in preventing the senescence of leaves of *Zea* and *Rumex*. Table VII shows that the effect on oat leaves under our conditions

Table V. *Diffusion Resistance Values of Leaves on Primary Alcohols after 4 Days in Darkness*

Alcohol	Concentration	Chl[a]	r Values
	mM	*% of initial*	*s/cm*
None (water)	—	35	156
1-Propanol	100	45	60
1-Butanol	60	85	20
1-Pentanol	20	60	26
1-Hexanol	10	61	8

[a] From other experiments.

Table VI. *Effects of 1-Butanol on Five Criteria of Senescence*
The data given are at start and after 4 days in darkness.

Butanol Concentration	Chl	Amino Nitrogen	Reducing Sugar	Diffusion Resistance r Values	Change in Fresh Weight
mM	A_{660}	A_{570}	A_{540}	*s/cm*	*mg/100 mg leaves*
(Initial)	0.292	0.407	0.118	(118)	
0	0.033	1.300	0.157	187	+2.35
10	0.083	0.997	0.115		+0.55
30	0.180	0.850	0.078	59	+0.29
60	0.265	0.238	0.060	54	−0.59
100	0.277	0.172	0.052		−0.59

Table VII. *Interactions of $CaCl_2$ and of CCCP with 1-Butanol*
All data given are initial and after 4 days in darkness; 1-butanol was 60 mM throughout.

Solution	Chl	Change in Fresh Weight
	A_{660}	*mg/100 mg leaves*
Initial value	0.276	
Water (dark control)	0.040	+2.28
$CaCl_2$ 100 mM	0.057	
1-Butanol	0.220	−0.94
1-Butanol + $CaCl_2$	0.165	
1-Butanol + CCCP 1 μM	0.107	+0.22
1-Butanol + CCCP 3 μM	0.132	+0.68
1-Butanol + CCCP 10 μM	0.085	+0.77
1-Butanol + CCCP 30 μM	0.087	+0.71

is barely significant. Instead, in the presence of an optimal concentration of 1-butanol, $CaCl_2$ exerts an opposite effect, somewhat promoting the loss of Chl.

In exploring further the relation between senescence and ATP level (12), several uncouplers of phosphorylation have been examined. Results with CCCP are included in Table VII. By itself, CCCP has no significant influence on Chl loss or change in leaf weight but, in the presence of optimal 1-butanol, it clearly promotes senescence, bringing the Chl content down to about one-half of that in butanol and antagonizing most of the loss in fresh weight, presumably by partially closing the stomata. CCCP and tertiary butanol show similar antagonism in modifying H_2O permeability in carrot tissues (2).

DISCUSSION

The present results have numerous apparent parallels in the literature. Although all but one of these concern inhibitions, there are plenty of grounds for considering the prevention of senescence as an inhibition. For example, cycloheximide, in concentrations that inhibit protein synthesis in these leaves, powerfully prevents senescence (15). The requirement for aerobic metabolism in dark senescence (15), as well as the large increases in respiration and ATP formation which accompany it (12, 22), all show that senescence is indeed a highly active process. However, as pointed out below, the action of the alcohols may be in fact mediated *via* an increase in permeability to K^+ ions. In addition, it is clear, not only from our rigidity tests but also from the ready reversal shown in Table VII, that the effects cannot be due to simple toxicity.

The most extensive data previously reported are those of Reynolds (19, 20) on the inhibition of germination of lettuce "seeds" (achenes). There the activity of the primary alcohols increased up to 1-nonanol and, as here, the diols and glycerol were much less active. In contrast to our data, the presence of branching did not consistently decrease activity. For each additional CH_2 group from C_2 to C_9, the concentration causing 50% inhibition dropped by about 3.2 times (*cf.* 2.9 times in our Fig. 3).

Another effect of the aliphatic alcohols is exerted on phosphorylation by chloroplasts, chromatophores, and mitochondria (1, 26, 27). Here the straight-chain alcohols from C_1 to C_5 all inhibit, the potency increasing with chain length, and the branched chain compounds are all less active. Some diols had less activity, but a few were fully active. In the inhibition of a totally different system, the $(Na^+ + K^+)$-transport ATPase of plasma membranes of guinea pig kidney (6), the concentrations of alcohols for half-maximal inhibition dropped by an average of 2.5 times for each CH_2 group added. The increases in activities paralleled the increases in the partition coefficients, P, between octanol and H_2O. As with our data, branched chain alcohols were uniformly less active than those with straight chains. For comparison with these results of Hegivary (6), the partition coefficients of the alcohols between octanol and H_2O, which can be considered an index of lipophilia, have been plotted alongside our data in Figure 3. Although the slopes are not identical, the similarity is striking and certainly suggests a relation between the two phenomena. The rule of Hansch and Glave (5), which is expressed as

$$\log 1/C = a \log P + b,$$

where C is the effective concentration for a biological effect and P the above partition coefficient, fits our data equally well.

The ATPase of the mitochondrial inner membrane is also inhibited by aliphatic alcohols (9) and the effective alcohol concentrations similarly decrease in a log-linear fashion; but, in that work (9), all the concentrations were much higher than those active on senescence and, furthermore, at least up to C_8, the carboxylic acids were more active than the alcohols. Grisham and Barnett (3) with a comparable $(Na^+ + K^+)$-ATPase, found the effective concentration to fall off by about 3.7 times for each added CH_2 group, but this was an irreversible inhibition of the enzyme, ascribed to denaturation. In contrast to these inhibitions, the *total* ATPase activity of sarcoplasmic membranes is *increased* by low concentrations of the straight-chain alcohols (8). Finally, two studies deal with alcohol effects on permeability proper. In one, permeation of alcohols into mitochondria was found to decrease as the number of OH groups increases (17). In the other, with an artificially thin lipid membrane from sheep red cells, Gutknecht and Tosteson (4) reported that aliphatic alcohols lower the resistance of this membrane to ions and especially that they increase its permeability to K^+.

Reynolds' (19, 20) explanation of the importance of lipophilia was that this property controls penetration of the alcohol to the active site, but this seems improbable because the corresponding hydrocarbons are of very low activity. In a similar contrast, the pentanols inhibit photophosphorylation in chloroplasts but the hydrocarbon pentane is without effect (26). In the other researches cited above, inhibition of an ion-exchanging ATPase was the property studied, and it is difficult to associate such an inhibition directly with the maintenance of Chl and protein in our experiments. But if, as indicated with the lipid membranes (4), the alcohols increase the permeability to potassium ions, an effect on the rate of intake of K^+ into the guard cells, and hence on stomatal aperture, could readily be understood. In the absence of other evidence, this seems now the most attractive explanation of the influence of the alcohols on senescence. Most of the published work reaches the conclusion that the alcohol effects are exerted in some way upon membranes[3], and this would be consonant with the effectiveness of the small quantities which must be present as vapors (Table II). In the present connection, a heightened permeability would mean that, for a given ATP usage, K^+ could be taken up more readily. As Mansfield (13) notes, "any means of controlling K^+ transfer between the guard cells and their neighbours should be an effective means of imposing controls on stomatal movements." As pointed out earlier (24), such a conclusion in regard to the guard cells would point to the stomatal aperture as being the controlling agency in senescence and not simply a secondary result of the senescence syndrome.

The requirement for a straight chain with terminal hydroxyl is characteristic and holds for many other systems (1, 6, 21, 26, 27). A simple explanation would be that the alcohol becomes inserted with its lipophilic chain into the membrane, perpendicular to the surface, with the hydrophilic -OH at the interface. Siegel and Halpern's finding (21) that branching of the chain remote from the -OH has a smaller effect (on beet-root cell permeability) than branching at position 2 fits this concept well.

All of the present experiments concern senescence in darkness. Senescence in white light is slower (25) and here the alcohols actually exert a promoting effect; they are known to inhibit cyclic photophosphorylation (1, 26, 27). Similarly farnesol is reported to cause stomatal closure of Sorghum in light (14, 28) in contrast to its effect (above) in the dark. The alcohols are not the only reagents having these reversed effects, and a study of these reversals will be reported in a later communication.

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