Plant Physiol. (1970) 46, 36–39

A Lack of Specificity for Ethylene-induced Mitochondrial Changes[1]

Received for publication January 13, 1970

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ABSTRACT

A critical evaluation was made of the hypothesis that the primary mode of action of ethylene in inducing physiological responses is by changing the permeability of cell organelles. The parameter investigated was the evaluation of the influence of ethylene and other gases on mitochondrial oxidation and swelling. Spectrometric evidence demonstrated that mitochondria prepared with good respiratory control can be induced to swell more rapidly with ethylene and other aliphatic gases (ethane, propene, propane, 1-butene) in test solutions of 0.125 M KCl. The fact that saturated as well as unsaturated hydrocarbon gases elicited similar changes provides evidence that ethylene does not directly alter membrane permeability as its mechanism of action.

The specificity of ethylene as a growth regulator was demonstrated by the inhibition of elongation of pea stems from seedling plants (5, 12) and through the study of accelerants of abscission (1). Any test of hypotheses on the mechanism of ethylene action must show the same specificity.

One theory on the mechanism of ethylene action often proposed invokes the concept that ethylene directly causes some alteration of membrane permeability (2, 9–11, 17, 19, 23). Studies aimed at elucidation of this mechanism have frequently employed the mitochondrion as an experimental tool and have shown that membrane characteristics can be altered by ethylene treatment (8, 17, 20, 21). For example, an increased rate of swelling, suggesting a change in permeability, of rat liver and cauliflower bud mitochondria when treated with 100 μl/liter and higher concentration of ethylene has been shown (17). The process was reversible in that the mitochondria contracted upon the addition of ATP, magnesium, and BSA.[3] At 10 μl/liter, ethylene retarded mitochondrial swelling as did 50 μM dinitrophenol, but uncoupling of phosphorylation was not evident in the ethylene-treated particles. Also, ethylene at 20 and 100 μl/liter induced swelling of bean cotyledon, yeast, and rat liver mitochondria in 0.15 M KCl, 0.5 M mannitol, and sucrose, when ADP was added to the system *in vitro* (20, 21). A contraction was observed upon the addition of ATP. This volume change was not observed in the presence of the cardiac glycoside, ouabain, known to inhibit ATPase. It was also noted that ethylene increased the rate of ATP hydrolysis and that ouabain inhibited the hydrolysis in mitochondria from bean cotyledon and yeast, suggesting that the ATPase was activated by ethylene.

Despite this positive evidence on the influence of ethylene in altering membrane characteristics, no tests were reported as to the specificity of ethylene in inducing these changes. The experiments reported here were carried out with mitochondria isolated from mature green tomato fruit with the aim of comparing the influence of other aliphatic hydrocarbons and ethylene as to their ability to induce changes in oxidation or swelling. The results show that these other gases do elicit the same effects as ethylene, and therefore extrapolation of the results from studies *in vitro* to the mechanism of ethylene action is tenuous.

MATERIALS AND METHODS

Plant Material. Tomatoes (*Lycopersicon esculentum* cv. FM 428) were harvested at the mature green stage from plants grown in the greenhouse. The fruits were grown according to the methods described by Lyons and Pratt (16) and were harvested approximately 40 to 42 days after anthesis.

Mitochondrial Preparations. The procedures employed were similar to those described by Ku *et al.* (13), with the exception that an Oster juice extractor was employed to macerate the tissue instead of hand slicing with a razor blade. The fruits, chilled in ice for 1 hr, were sliced, and the jelly-like locular tissue was removed. The sliced fruit (500 g) was washed with the isolating buffer medium and macerated into 400 ml of buffer with the Oster juice extractor. Miracloth (Chicopee Mills, New York) was used to line the revolving basket to filter cell debris. The tissue was macerated, and the pH of the filtered juice in the isolating media was adjusted to 7.6 within 3 sec.

The isolation media consisted of the following: 0.4 M sucrose, 0.05 M tris, 0.01 M EDTA, 0.5 mg/ml BSA (fraction V, fatty acid-poor, Calbiochem), 4 mM cysteine hydrochloride, 1 mM KCl, 1 mM $MgCl_2$. The crude homogenate was centrifuged at 2,000g for 15 min, the pellet was discarded, and the supernatant was centrifuged at 11,000g for 20 min. The resultant pellet was resuspended in 200 ml of the following media: 0.4 M sucrose, 1 mM KCl, 1 mM $MgCl_2$, 1 mM tris, 10 mM K_2HPO_4, 10 mM KH_2PO_4 0.5 mg/ml BSA, pH 7.2. The washed mitochondrial suspension was centrifuged at 2,500g for 10 min, the pellet was discarded, and the supernatant was centrifuged at 10,000g for 15 min. The final pellet was resuspended in 1 to 2 ml of the same media and held in ice until used. Protein was estimated by the method of Lowry *et al.* (15).

Oxidative Activity. Oxidative rates were determined at 25 C by a polarographic oxygen sensor (YSI model 53, oxygen monitor, Yellow Springs Instrument Co.). The reaction media consisted of 0.1 ml of mitochondria and 2.9 ml of the media used to wash the mitochondria. Additions of succinate and ADP

[1] This work was supported in part by a National Science Foundation Traineeship and University of California Fellowship.

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[3] Abbreviation: BSA: bovine serum albumin.

Table I. *Oxidative Activity of Mitochondria Isolated from Mature Green Tomato Fruit*

Reaction media consisted of 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 10 mM tris, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 0.5 mg/ml BSA, pH 7.2. Final volume was 3.0 ml with 0.1 ml of mitochondrial suspension (1.1 to 1.4 mg protein) and 5 mmoles of succinate and 220 μmoles of ADP added.

	Oxidation of Succinate		ADP/O	Respiratory Control Ratio[1]
	State 3	State 4		
	mμmoles O_2/mg protein·min			
Average	256	137	1.3	1.8
Range	249–260	122–145	1.2–1.5	1.7–1.9

[1] Calculated as the ratio of the state 3 (ADP not limiting) to the state 4 (ADP limiting) rates.

Table II. *Oxidative Activity of Tomato Fruit Mitochondria Aged for 1 hr in Reaction Media at 25 C with Various Concentrations of Ethylene*

Reaction media and substrate as shown in Table I.

Ethylene Concn	ADP/O Ratio	Respiratory Control Ratio
μl/liter		
0	1.2	1.8
10	1.5	2.4
100	1.5	2.1
1000	1.2	1.9
10%	1.4	2.1
33%	1.2	2.5

were made following a 3-min temperature equilibrium interval. Respiratory control ratios were calculated according to the method of Chance and Williams (6) as the state 3 rate divided by the state 4 rate.

To test the influence of hydrocarbon gases, the mitochondria (0.1 ml) were added to the reaction media (0.9 ml) in a 17-ml test tube. A serum cap sealed the tube, 5 ml of air were withdrawn, and 5 ml of the test gas were added with a syringe. The tube was incubated 1 hr at 25 C and then transferred to the oxygen sensor cell which contained 2.0 ml of the untreated reaction media previously brought to 25 C.

Mitochondrial Swelling. The technique used for estimating volume changes spectrophotometrically was similar to that used by Lyons and Pratt (17). Light scattering of the mitochondrial suspension was measured as a change in optical density at 520 nm with a Beckman DB recording spectrophotometer.

The test solution consisted of 0.125 M KCl, 50 mM tris (pH 7.4). Three milliliters of test solution were placed in a photometer cuvette, and enough of the mitochondrial suspension was added to bring the absorbance to 0.45 to 0.50. The mitochondria were added and rapidly mixed by inverting the cuvette, and the start of the recording was begun within 15 sec. Swelling was followed for 15 to 18 min. The test solutions were allowed to equilibrate with the different gases and gas concentrations before the mitochondria were added.

The gases used were CP grade obtained from Matheson Chemical Co. with a purity of 99.0 to 99.5. While the purity of these gases is not absolute, contamination by any physiologically active gases would be insignificant. The purity of the gases was checked by flame-ionization gas chromatography (Beckman GC-4), and no ethylene contamination was found.

RESULTS

Oxidative Activity. The oxidative activity, ADP/O ratio, and respiratory control ratio for mitochondria isolated from mature green tomato fruit used in these studies are shown in Table I. One criterion for demonstration of the presence of quality mitochondria is coupling of oxidation to phosphorylation, which can be most easily measured by an oxygen electrode (6) where the respiratory control ratio can be measured. Respiratory control ratios for tomato mitochondria used in these studies are not as high (infinity) as those obtained by Ku *et al.* (13), where the tissue was carefully macerated with razor blades, but they are in the range reported for other tissues and fruits (7, 14, 25). The oxidation of succinate and ADP/O ratio are also in the usual range reported for mitochondrial preparations from plant tissues. While biochemical purity (as shown by respiratory control) is not conclusive evidence for the presence of high quality mitochondria, it is the best single criterion for the purpose of the studies reported here.

Since only a few studies regarding the influence of ethylene on isolated mitochondria have been reported (8, 17, 20, 21), and none of these with mitochondria exhibiting respiratory control, it was of importance to test this factor in the present study. Results of such an experiment are shown in Table II. It is readily apparent that ethylene had essentially no influence on these mitochondria under the conditions tested.

It has been suggested (17) that perhaps sucrose might exert some influence on the mitochondria and mask any effect of ethylene. To investigate this aspect, oxidative activities of the tomato fruit mitochondria were assayed in reaction media of varying sucrose concentrations. Sucrose concentrations varying over a wide range had very little and inconsistent influence over either the ADP/O ratio or respiratory control (Table III). Again, ethylene at a concentration as high as 33% exerted no significant influence.

Mitochondrial Swelling. Results from experiments to evaluate mitochondrial swelling are presented in Figures 1 and 2. It is readily apparent from these data that the aliphatic gases can accelerate the rate of mitochondrial swelling in this test system, but that there is no particular specificity shown toward ethylene.

With test solutions saturated with various aliphatic gases (Fig. 1) it can be shown that both saturated and unsaturated gases can induce a more rapid swelling when compared to the untreated control. An important criterion in any study to evaluate the influence of ethylene on subcellular particles or biochemical systems is to establish specificity for ethylene. In experiments where attempts are made to relate the results of an ethylene treat-

Table III. *Influence of Sucrose Concentration in the Reaction Media on Oxidative Activity of Ethylene-treated Mitochondria*

Reaction media and substrates as shown in Table I.

Sucrose Concn	ADP/O Ratio		Respiratory Control Ratio	
	Control	Ethylene-treated[1]	Control	Ethylene-treated[1]
M				
0.00	1.2	1.2	2.5	1.8
0.05	1.4	1.2	2.2	2.2
0.10	1.1	1.2	2.5	2.1
0.20	1.3	1.2	1.3	1.3
0.30	1.0	1.1	1.2	3.4
0.40	1.5	1.3	1.9	2.9
0.50	1.0	1.1	1.6	1.5

[1] Aged 1 hr in reaction media at 25 C with 33% ethylene in the atmosphere.

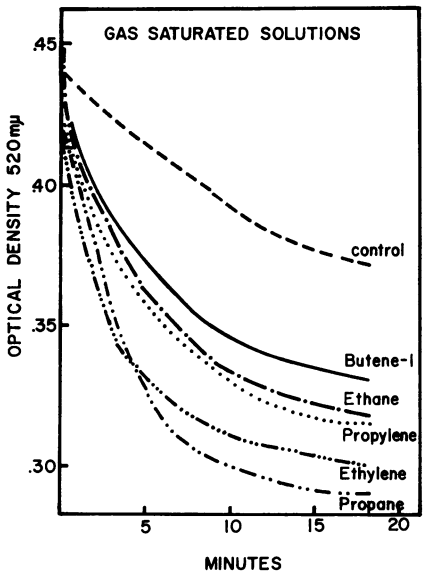

FIG. 1. Effect of saturated solutions of ethylene, ethane, propylene, propane, and butene-1 on swelling of mitochondria isolated from green tomato fruit. Swelling is indicated by a change in absorbance at 520 nm.

ment to biochemical systems *in vitro* and the physiology one observes when treating intact plant tissues, it is important to point out again that the saturated hydrocarbons are completely inactive, and the next higher homologue than ethylene in the unsaturated hydrocarbons (propylene) requires 130 times the concentration, with 1-butene requiring 140,000 times the concentration (5). Rather than fulfilling the above criteria, the results in Figure 1 demonstrate a significant lack of specificity, and, instead, the influence of each gas is exerted in the approximate order of their solubilities in water (18).

Since the concentrations of the gases used above are far beyond any physiological significance, it was important to ascertain any effect at lower concentrations. In Figure 2, the effects of various concentrations of ethylene are compared with those of like concentrations of ethane—a saturated hydrocarbon gas with no physiological significance (5). While slight differences exist between the two gases in their ability to induce swelling in isolated tomato fruit mitochondria, it is readily apparent that in the range of physiologically significant concentrations (10–1000 μl/liter) there is essentially no difference between ethylene and ethane; in fact, neither gas exerts any profound influence except at very high concentrations.

DISCUSSION AND CONCLUSIONS

Since one of the most immediate effects observed when many plant tissues (and particularly preclimacteric fruit) are exposed to ethylene is an increase in respiratory activity, the mitochondrion offers an attractive site to search for the mechanism of ethylene action. Furthermore, many workers have implicated membrane permeability as the controlling mechanism in ethylene effects, as well as fruit ripening (3, 4, 17, 24), which also makes the mitochondrion a useful experimental tool. Although it is perhaps not representative of all cellular membrane types, the mitochondrion is biological and possesses a double membrane which must be maintained intact for complete biochemical integrity. From the results presented here, it is clearly established that ethylene does not exert any specific influence on oxidative activity (Tables II and III) or swelling of mitochondria (Figs. 1 and 2), at least as isolated from mature green tomatoes.

In reviewing the influence of other gases which will induce physiological events similar to those of ethylene, Burg and Burg (5) have delineated the molecular requirements for ethylene, or ethylene-like effects. These requirements include only unsaturated aliphatic compounds with a terminal double bond. They further noted that active compounds meeting these requirements possessed an activity inversely related to their molecular size.

The influence of ethylene on mitochondrial activity has been reported previously by other workers. Hall *et al.* (8) were able to induce swelling in mitochondria extracted from castor beans although there were no apparent effects on oxidative phosphorylation. Lyons and Pratt (17), with mitochondria isolated from both cauliflower buds and rat livers, were able to demonstrate that ethylene at concentrations of 100 μl/liter and higher could induce a more rapid swelling as compared with the controls. They also showed that the swelling effect was reversible since in the presence of Mg^{2+}, ATP, and BSA, the mitochondria would contract. More recently, Olson and Spencer (20, 21) have reported that, in a closed system with ethylene at 100 or as low as

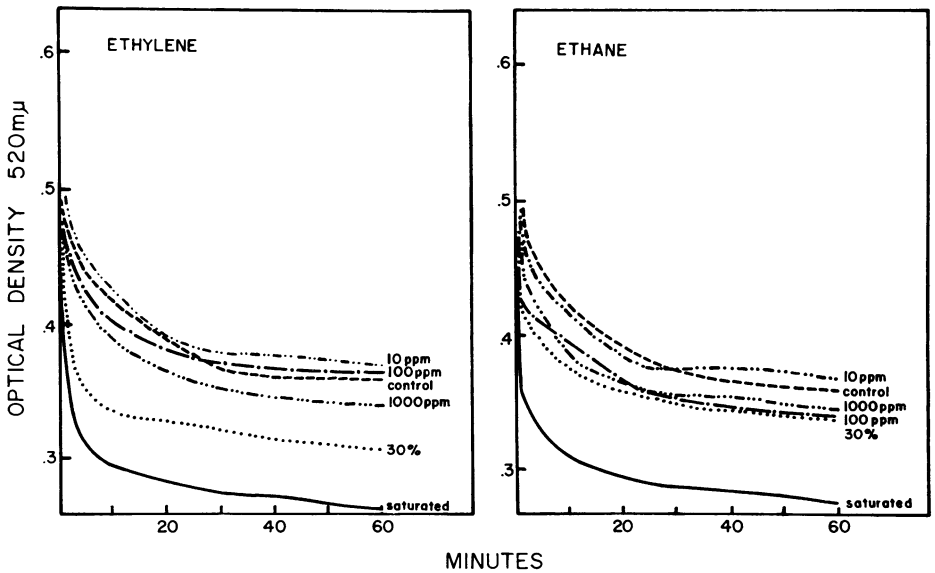

FIG. 2. Effect of various concentrations of ethylene and ethane on swelling of mitochondria isolated from green tomato fruit. Swelling is indicated by a change in absorbance at 520 nm.

20 μl/liter, mitochondria swell when ADP is added and contract when ATP is added, as did happen in the absence of ethylene. They further reported that the effect of ethylene was to stimulate activity of an ATPase by approximately 5%. They were able to show (22) that with proper mathematical treatment a 5% increase in ATPase could account for the magnitude of response in respiratory activity following ethylene treatment and to propose that the influence of ethylene is exerted through its effects on ATPase.

Each of these studies (8, 17, 20, 21), while in agreement with the fact that ethylene can have an influence on mitochondrial swelling (which can be interpreted by a multitude of discussion as an effect on membrane integrity), is singularly lacking in the most important criterion of demonstrating any specificity toward ethylene. The results of the present study clearly show that indeed ethylene and other aliphatic gases can induce changes in mitochondrial swelling, but these changes only occur at concentrations of 100 μl/liter and higher. Furthermore, the fact that other aliphatic gases, and particularly the saturated ones having no physiological activity, could cause similar effects at the same concentrations points to the conclusion that the influence of these gases is exerted through some nonspecific surface phenomenon which has no real relationship with the mechanism of ethylene action in intact plants. This does not mean that the primary mechanism of ethylene action cannot mediate through some change in membrane integrity; but it does provide clear evidence that if ethylene does exert its effect through altering membranes it is not by means of a simple physical effect.

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