Mechanism of Ethylene Action

BIOLOGICAL ACTIVITY OF DEUTERATED ETHYLENE AND EVIDENCE AGAINST ISOTOPIC EXCHANGE AND CIS-TRANS-ISOMERIZATION

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ABSTRACT

Deuterated ethylene was used to study the mechanism of ethylene action in etiolated pea seedlings (Pisum sativum L. cv. Alaska). No apparent differences were observed in the biological activity of tetradeuteroethylene (C,D4) and ordinary ethylene (C2H4) using the pea stem straight growth assay. The absence of an isotopic effect is discussed in relation to the possibility that ethylene binds to a metal or that carbon to hydrogen bonds of ethylene are broken during its mechanism of action.

Analyses by gas chromatography of gas samples obtained from chambers containing intact etiolated pea plants exposed to 2 microliters of C,D4 per liter of air for up to 5 days resulted in no detectable exchange between the deuterium atoms of C,D4 and the hydrogen atoms of the tissue. Similarly, infrared spectra of gas samples obtained from chambers containing plants exposed to either cis or trans-C,D4H8 indicated that no conversion had occurred to the corresponding trans or cis isomer. These results suggest that the mechanism of ethylene action does not involve an intermediate ethylene complex resulting in hydrogen exchange or cis-trans isomerization during a possible catalytic activation of the receptor site(s).

The biological effects of ethylene are well known (see reviews 4, 11, 12, 15); however, the initial events that occur at the ethylene receptor site(s) which cause these effects are entirely unknown. Likewise, the location, chemical nature, and the type of bonding which occurs between the receptor site(s) and ethylene (e.g., covalent, coordinate, van der Waals) and the subsequent molecular changes, if any, that ethylene undergoes during receptor site activation are unknown. The experiments reported in this paper deal with the use of deuterated ethylene (C2D4, cis-C2D4H8, trans-C2D4H8) in an attempt to learn about the ethylene receptor site and its activation.

Burg and Burg (5) have proposed on the basis of indirect evidence that ethylene binds to a metal-containing receptor site. Deuteration, especially where the deuterium is directly bound to an unsaturated carbon atom as in ethylene, increases the stability of silver ion-olefin complexes (1, 10). Therefore, C2D4 might be expected to be slightly more biologically active than C2H4, and the demonstration of such a difference would strengthen the concept that ethylene binds to a metal. If C2D4 were less active, this would also provide some clue as to the chemistry involved in receptor site(s) activation. For example, the formation of an activated complex which involves the cleavage of a carbon-to-hydrogen bond of ethylene would be expected to form more easily with C2H4 than C2D4, because of differences in zero-point energies of the C—H and C—D bonds (16).

Molecular changes (e.g., hydrogen exchange, cis-trans-isomerization) may occur in the ethylene molecule itself during receptor site activation because of intermolecular stresses. The detection of such changes, if they occur, would not only aid in the understanding of the biological chemistry involved in ethylene action but would also provide an assay for the isolation of the ethylene-receptor molecule(s) and a technique to explore other questions such as the apparent weak competition between CO2 and ethylene for the receptor site (5).

MATERIALS AND METHODS

Biological Activity of Deuterated Ethylene. Pea seeds (Pisum sativum L. cv. Alaska), soaked in distilled water for 3 hr, were planted in moist vermiculite in plastic trays and were germinated in the dark at 27 C and 70% relative humidity. Seven mm long cut from directly below the plumular hook of 7-day-old etiolated seedlings were placed in Petri dishes with 10 ml of a medium containing 2% sucrose, 0.2 µM IAA and 20 mM potassium phosphate buffer, pH 6.8, in double distilled water. Two Petri dishes containing 10 sections each in 200-mm sealed glass desiccators were fumigated with the desired concentration of C2D4 or C2H4. The length of the sections was measured following a 20-hr fumigation period at 27 C in the dark.

Isotopic Exchange and cis-trans Isomerization. Approximately 200 pea seeds were germinated in 160-mm glass desiccators as described for the pea straight-growth test. When the seedlings were approximately 3 cm long, the desiccators were sealed and C2D4, cis-C2D4H8, or trans-C2D4H8 was added to four such desiccators to give a concentration of 2 to 10 µl/l. The desiccators were connected in a series with Tygon tubing. Controls were four similar desiccators which contained no seedlings. An air pump was used to circulate the air in each series of four desiccators through Ascarite to keep CO2 levels below 0.2%. O2 was added to the system to restore atmospheric pressure. Following a 4- to 5-day fumigation period, the ethylene was collected in a liquid nitrogen trap. Ethylene was analyzed in a Perkin-Elmer 900 gas chromatograph using a flame ionization detector with helium as the carrier gas. Separations were made at −20 C on a 63-foot by one-eighth inch stainless steel column packed with Chromosorb R which had been

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coated with a saturated silver nitrate-ethylene glycol solution (1). cis- and trans-Dideuteroethylenes were analyzed in a Perkin-Elmer Model 21 infrared spectrophotometer using a 3.3-cm micro gas cell.

RESULTS

No apparent differences were observed in the biological activity of C,D, and C,H, using the pea straight-growth test (Fig. 1). A third experiment using 0.1 μl/l C,D, and C,H, and a larger number of replications (five replications with 10 sections per replication) also failed to show any significant differences.

Analyses by gas chromatography of gas samples obtained from chambers containing pea plants exposed to 2 μl/l of C,D, for 5 days and showing typical ethylene effects (i.e., growth inhibition, stem swelling, transverse geotropism) failed to show any exchange between the deuterium atoms of C,D, and the hydrogen atoms of the tissue (Fig. 2). This experiment was repeated three times using 2, 5, and 10 μl/l C,D, The sensitivity of the gas chromatographic system was such that a 2 to 3% conversion of C,D, to C,D,H, could have been detected (Fig. 2A). An attempt was made to detect the presence of C,D,H, since it would be formed before C,D,H, provided that one deuterium was exchanged at a time. The retention time required to achieve separation of C,D,H, from C,D, however (>200 min), reduced the resolution to such an extent that only a relatively large conversion of C,D, to C,D,H, could be detected (>15%). Under these conditions no C,D,H, was observed.

A considerable quantity of C,H, was detected in the samples collected from the pea plants, and a small but significant amount was also collected from the controls. This was apparently due to the fact that the pea seedlings were producing ethylene during the experiment, and that a small amount was produced by fungi growing in the moist vermiculite.

Infrared spectra of gas samples from chambers containing plants exposed to 10 μl/l cis-C,D,H, for 4 days and exhibiting typical ethylene effects showed no detectable amounts of the trans-isomer (Fig. 3). Conversion of less than 10% of the cis-C,D,H, to the trans-isomer could have been detected. In a second experiment 2 μl/l of trans-C,D,H, was applied to pea seedlings for 4 days and again no isomerization was observed.

DISCUSSION

Few papers have dealt specifically with the ethylene receptor site and the initial events that occur during its activation.

**Fig. 1.** Effect of C,H, and C,D, on the percentage of increase in growth of etiolated pea epicotyl sections. Sections incubated in medium containing 0.2 μM IAA, 2% sucrose, and 20 mM potassium phosphate buffer, pH 6.8, for 20 hr. Bars through data points represent standard deviations.

**Fig. 2.** Gas chromatograms of A: a standard mixture of C,H, and C,D, (solid line) and a similar mixture containing one part of cis-C,D,H, to 10 parts of C,D, (dotted line); B: a standard mixture of C,H, and C,D, (500 μl/l each) chromatographed immediately prior to samples shown in C and D; C: a sample obtained from the chambers containing etiolated pea seedlings exposed to 2 μl/l C,D, for 5 days; D: a sample from the control chambers. Retention times: C,H, 87 min; cis-C,D,H, 93 min; C,D, 101 min. Column, 63-foot × one-eighth-inch stainless steel packed with Chromosorb K coated with a solution of ethylene glycol saturated with silver nitrate; temperature, −20°C; inlet pressure, 60 p.s.i.; flow rate, 16 cc per min.

This is undoubtedly due to the inherent difficulties involved in studying a process about which so little is known and the technical difficulties encountered when using physiological concentrations of the gas.

Burg and Burg (5) studied the molecular requirements for ethylene action using the pea-stem-section assay. A comparison of the relative biological activity of several unsaturated compounds with ethylene using this assay indicated that the molecular requirements for ethylene action were similar to those governing silver-olefin complex formation. This idea was supported by the fact that a close correlation was found to exist between biological activity and the stability constants of several olefin-silver complexes as determined by gas chromatography. These and other observations lead to the suggestion that ethylene binds to a metallic receptor site in the tissue.

On the basis of gas chromatography data, deuteration has been shown to increase the stability of the silver ion-ethylene complex (1, 10). Therefore, if ethylene does bind to a metallic receptor site, deuterated ethylene might be expected to be slightly more effective than ordinary ethylene. No evidence was obtained to support ethylene to metal binding, however, since deuterated ethylene and ordinary ethylene had essentially the same inhibitory effect on pea stem elongation (Fig. 1). While the absence of an isotopic effect fails to support ethylene to
In contrast to studies with fruit tissues, all studies involving vegetative tissues have reported some incorporation of radioactivity. Hall et al. (6) found that cotton and Coleus plants absorbed and fixed a significant amount of radioactivity during a 15-hr exposure period to 1 ml/l of 14C-labeled ethylene. Detailed studies were conducted to isolate radioactive metabolites, and at least 18 were found. The plant material used in these detailed studies was exposed to ethylene-14C which had been regenerated from a solution of mercuric perchlorate, since such treatment was found to enhance greatly the amount of label fixed. The extent of enhancement was 25-fold following a single trapping-regeneration procedure and 1000-fold following a second such procedure. Gas chromatographic analysis of regenerated samples indicated ethylene to be the main constituent.

Similarly Shimokawa and Kasai (13) and Shimokawa et al. (14) used ethylene-14C regenerated from mercuric perchlorate to study the fixation, translocation, and metabolism of ethylene in the Japanese morning glory. Intact seedlings were found to actively fix ethylene-14C, and the extent of fixation was enhanced by light. Exposure of a single leaf to ethylene-14C followed by autoradiography of the intact seedling indicated that 14C from the treated leaf was not transported to other parts of the plant. Isolation of labeled material following a 2-hr exposure to 100 ml/l of ethylene-14C indicated that ethylene had a high affinity for RNA. It was suggested that the binding of ethylene or its metabolite to RNA may induce a conformational change in RNA.

Jansen (9) studied the metabolic fate of "fresh" (direct from the manufacturer) and "aged" (trapped in mercuric perchlorate and then regenerated) radioactive ethylene and reported that the metabolism of aged ethylene bears little resemblance to that of fresh ethylene. Jansen concluded that ethylene metabolism is apparently obscured by the rapid metabolism of impurities in the aged ethylene.

In view of the reported difference between aged and fresh radioactive ethylene, it is possible that the incorporation which has been obtained may be an artifact due to impurities in the applied ethylene. This could arise from radiation decomposi- tion, trapping in mercuric perchlorate solutions, or inherent impurities in the original samples. It is therefore difficult at the present time to determine from these data whether or not the incorporated radioactivity has any physiological significance.

Although the results obtained in this study using deuterated ethylene do not completely resolve this question, they do provide evidence against carbon to hydrogen bond cleavage which might be expected if ethylene were covalently bound during its mechanism of action. If covalent bonds were broken in an activated complex, the effect of deuterium should be to reduce the rate of its formation (16). Reactions involving the breaking of a C—H bond in the rate determining step are often several times slower when deuterium rather than hydrogen is the atom involved. No differences, however, were observed in the biological activity of C2D4 and C2H4 (Fig. 1), indicating that bond breaking does not occur during the rate-determining step. Obviously, these results cannot completely rule out the possibility that a C—H bond of ethylene is broken, since no isotopic effect is to be expected if bond breaking occurs after the rate determining step.

No evidence was obtained for hydrogen exchange or cis-trans isomerization during a possible catalytic activation of the receptor site(s). These results were obtained under conditions designed to optimize the chances of obtaining the effects sought. In the experiments involving isotopic exchange, for example, over 800 seedlings (about 600 g) were exposed in a confined space (about 7 liters of air space) to less than 35 ml of deuterated ethylene for up to 5 days without any detectable

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**Fig. 3.** Infrared spectra of A: standard mixture of one part C2H4, C2D4, cis-C2D4H2 and trans-C2D4H2 per 100 parts room air; B: ethylene sample from chambers containing etiolated pea seedlings grown in 10 ml/l cis-C2D4H2 for 4 days; C: ethylene sample from control chambers.

Metal binding, its absence does not imply that such binding does not occur. The idea that deuterium might result in a more stable complex and therefore be more biologically effective is based principally on data derived from complexes of ethylene with the silver ion (1, 10). These data indicate that the stability of such complexes can be increased by about 10% by deuterating all four positions of ethylene. The effect of deuteration on the stability of ethylene complexes with other metals more likely to be involved in ethylene binding in biological systems, however, is largely unknown. For this reason the absence of an isotopic effect cannot disprove the ethylene to metal binding concept. It is possible that with other metals the effect of deuteration may be so small that differences could not be detected using the pea stem straight growth assay. Unfortunately, in this case only the presence of an isotopic effect would provide important insight into the mechanism of ethylene action.

A basic question concerning the primary mechanism of ethylene action is whether ethylene acts catalytically or whether it is permanently incorporated into the tissue during its mechanism of action. Conflicting results have appeared in the literature as to the extent of incorporation when radioactive ethylene is applied to fruit tissues. Buhler et al. (3) found that ripe avocados and green pear fruits exposed to 1 ml/l of 14C-labeled ethylene for several days incorporated 0.05% of the applied radioactivity. Jansen (7, 8) has reported a similar percentage of incorporation using mature green avocado fruit. In other experiments, however, Buhler et al. failed to obtain any incorporation of radioactivity into ripe oranges, limes, papayas, green apples, tomatoes, and grapes. Behmer (2) also failed to obtain any incorporation of radioactivity from ethylene-14C into apple tissue.
exchange (Fig. 2). These results and those obtained using cis-C6D4H2 (Fig. 3) suggest that no detectable molecular change occurs in the ethylene molecule itself when it accomplishes its biological function. This conclusion, of course, is dependent on the assumption that ethylene functions catalytically and is not permanently incorporated into cellular components.

It should be pointed out that the differences sought using deuterated ethylene would not necessarily be expected to be large. Therefore it is possible that analytical limitations and inherent variations in the growth test may have precluded the detection of an isotopic effect, hydrogen exchange or cis-trans isomerization. The methods used in this study were sufficiently sensitive, however, to clearly indicate that if such effects do occur they are so small that their detection will be extremely difficult and will probably severely limit their usefulness in future studies.

LITERATURE CITED