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Measurement of Ethylene Binding in Plant Tissue[1]

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ABSTRACT

Tobacco leaves were exposed to ^{14}C-labeled ethylene (3.7×10^{-2} microliters per liter) in the presence and absence of unlabeled ethylene and other compounds. Most of the [^{14}C]ethylene appears to be bound to displaceable sites. Lineweaver-Burk plots for a one-half maximum response in a tobacco leaf respiration test gave a value of 0.3 microliter per liter for ethylene, 50 microliters per liter for propylene, and 266 microliters per liter for carbon monoxide. Scatchard plots for displacement of [^{14}C]ethylene from the site gave 0.27 microliters per liter for ethylene, 42 microliters per liter for propylene, and 746 microliters per liter for carbon monoxide. At 2%, CO_2 displaces about 35% of the bound ethylene, but increasing the concentration to 10% does not displace the remaining [^{14}C]ethylene. A value of 3.5 nanomolar was calculated for the concentration of ethylene-binding sites available to exogenous ethylene. This does not account for the sites occupied by endogenous ethylene, and the total number of binding sites is probably somewhat higher. Using tissue culture material, the system was shown to be stable to freezing and thawing; and the π-acceptors, carbon monoxide, cyanide, *n*-butyl isocyanide, phosphorous trifluoride, and tetrafluoroethylene, were shown to compete with ethylene for binding.

For many years it has been known that ethylene causes a response in plants; and although the mechanism of action has remained obscure, some suggestions of its mode of action have been made. Burg and Burg (4), comparing the binding of olefins to Ag^+ ions to a biological response, have suggested that a metal is involved in the latter. Sisler (12) has pointed out that the types of compounds which give an ethylene-like biological response are π-acceptors, and has suggested the possibility of a *trans* mechanism since the types of compounds which give an ethylene response are also strong *trans* effectors in inorganic coordination complexes. Beyer (3) has considered the possibility that ethylene action and metabolism may be related. Proof of the mechanism of ethylene action probably cannot be obtained without isolating the ethylene-binding complex.

The general range of concentrations of ethylene which gives a saturating response is usually on the order of 1 to 100 μl/l. Abeles (1), using a value of 0.1 μl/l, has calculated that this would correspond to a 4.43×10^{-10} M solution and, since this gives a half-maximum response in some systems, has suggested about 500 attachment sites per cell. However, such calculations do not take into account the binding of ethylene *per se*.

Any attempt to isolate the ethylene-binding complex requires a suitable means for detecting bound ethylene. The concentration of the binding complex is probably too low to permit detection by ordinary chemical means but should not be below levels detectable by radioisotopic means. It is apparent that radioactive ethylene should readily be displaced from the binding sites by unlabeled ethylene or other compounds which can bind to the ethylene-binding site (8). However, at low levels, unlabeled ethylene should not effect the distribution of unbound ethylene since the distribution between gaseous phase and aqueous (or other) phase is determined by Henry's law (5) which is a ratio. These considerations have been applied in developing a sensitive quantitative method for detecting bound ethylene. A preliminary report of this work has been given (14).

MATERIALS AND METHODS

Plant Material. Tobacco plants (*Nicotiana tabacum* L.) were grown in a greenhouse either in nutrient solution (7) or soil. Leaves 20 to 30 cm long were harvested and the midrib removed. They were allowed to stand overnight for wound-generated ethylene to subside (13) before treatment.

Tissue Culture. Suspension cultures of the hybrid *Nicotiana glauca* × *Nicotiana langsdorfii* were grown on the media of Murashige and Skoog (10) without hormones. Cultures were grown on a rotary shaker at approximately 140 rpm.

Labeled Ethylene. [^{14}C]Ethylene (120 mCi/mmol) was obtained from Amersham/Searle, Arlington Heights, Ill., and trapped with mercury perchlorate (18). The ethylene-mercury perchlorate complex was stored at −15 C. When gaseous ethylene was desired, it was released by adding 1 ml of saturated LiCl. The ^{14}C-labeled product appeared in the ethylene peak when subjected to gas chromatographic analysis (13).

Tissue Treatment with Ethylene. Twenty-five g of leaves or tissue culture material were placed in a 2.5-liter desiccator with 2 ml of 40% NaOH to absorb CO_2 and 0.1 ml of ethylene-mercury complex in a 25-ml Erlenmeyer flask. [^{14}C]Ethylene was released from the mercury perchlorate complex (18) by addition of 1 ml of saturated LiCl. The LiCl was injected by a hypodermic needle through a silicone rubber stopper. Under these conditions approximately 80% of the [^{14}C]ethylene is in the gas phase in 1 h, and in excess of 99% is in the gas phase in 3 h. If a magnetic stirrer is included, 99% is released into the gas phase in 10 min. When ethylene was released without stirring, equilibrium between the leaf and gas phase was established in 4 h. If a magnetic stirrer was used, 2 h were sufficient. Except as otherwise indicated, leaves or tissue culture material were allowed to stand 8 h in the presence or absence of unlabeled ethylene, propylene, or CO, and then were removed, shaken in air, and placed in a second desiccator containing 1 ml mercury perchlorate in a counting vial. A 2.5-cm^2 piece of fiber glass filter was included in the vial to increase surface area. Approximately 25 s were required for the transfer. The competing compound was usually injected at the same time as the [^{14}C]ethylene was released, although sometimes it was injected later; in either case, the results were the same. When the effect of CO_2 was studied, the NaOH was omitted and the CO_2 was released from $NaHCO_3$. After 16 h the counting vial was removed, filled with 15 ml of 25% Triton X-100 in toluene and 4 g/l of scintillator (Omnifluor), and samples counted. This time interval collects approximately 95% of the labeled ethylene present

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in the leaf. After 24 h less than 1% of the ethylene remains in the leaf, as evidenced by the amount collected between 24 and 48 h with a counting vial containing 1 ml of mercury perchlorate. The leaves were used a maximum of three times and then discarded. Results the second and third times were the same as the first, indicating that the action of ethylene had no effect on binding. The radioactive material released and trapped from the leaves was identified as ethylene by paper chromatography (6). The amount of [^{14}C]ethylene in the gas phase was determined by withdrawing 30 ml of gas from the desiccators and injecting it into a 1-liter vessel containing a counting vial with 1 ml of mercury perchlorate.

Respiration Measurements. Tobacco leaf respiration measurements were made as previously described (13).

RESULTS AND DISCUSSION

***In Vivo* Measurement of Binding to the Ethylene-binding Site(s).** Although there are a number of assays available for measuring effects of ethylene binding, there are none which directly measure ethylene binding itself. Tobacco leaves respond to ethylene (13), and leaves exchange gases readily.

It should be possible to measure binding to sites by displacing [^{14}C]ethylene with unlabeled ethylene or by allowing unlabeled ethylene or some other compound to compete for the site. After tissue is exposed to a small but measurable amount of [^{14}C]-ethylene, the tissue should contain ethylene which is bound to specific sites, and, in addition, some ethylene in solution which is not bound.

The ethylene which is not bound should be distributed according to Henry's law (5), which states that at a fixed temperature, the weight of a gas dissolved in a given quantity of liquid is proportional to its partial pressure, *i.e.* $K = P/N$ (P is pressure in mm of Hg, N is the mole fraction in solution, and K is a constant which is equal to 8.6×10^6 for ethylene in water). Henry's law holds only in dilute solution; but, even at one atmosphere, deviates only 1 to 3% for many gases (5).

If some unlabeled ethylene is now applied, it should distribute itself by the same ratio as does labeled ethylene, *i.e.* $K = P/N = 8.6 \times 10^6$ in water. It should not alter the distribution of unbound ^{14}C-labeled ethylene in solution since, according to Dalton's law, in a mixture of gases every gas exerts the same pressure that it alone would if it alone were confined to the same volume; and the total pressure is simply the sum of the partial pressures. Dalton showed that the solubility of the individual gases is directly proportional to their partial pressures, the solubility of each gas being nearly independent of the presence of the others (5). Since partial pressures are additive, ^{14}C-labeled ethylene and unlabeled ethylene should behave as separate gases unless specific binding is involved. Figure 1 is a diagrammatic representation of these rules and is presented to assist in visualizing the distribution of ethylene in each case.

The [^{14}C]ethylene bound to specific sites should be readily displaced, and the amount of unlabeled ethylene needed to displace one-half of the labeled ethylene can be obtained from a Scatchard plot (9, 11) of the data obtained using a number of concentrations. This is considered to be the best of the linear transformations of the saturation equation (9). The per cent of labeled ethylene displaced should be equal to the per cent of sites occupied by unlabeled ethylene. Typical results of a saturating amount of unlabeled ethylene are shown in Table I.

A Scatchard plot of the data for ethylene (Fig. 2) indicates that 0.27 μl/l of unlabeled ethylene is needed to displace one-half of the [^{14}C]ethylene. The amount of ethylene needed for one-half maximum activity in the tobacco respiration test is 0.3 μl/l obtained from a Lineweaver-Burk plot (8) of activity data. This plot is used in an empirical way but has been used previously for this type of data (4).

This method also provides a satisfactory means for measuring the binding of other compounds to the ethylene-binding sites. For propylene, 50 μl/l is needed for one-half maximum activity, and 42 μl/l displaces one-half of the ^{14}C-labeled ethylene (Fig. 3). For CO, 266 μl/l are needed for one-half maximum activity in the tobacco leaf respiration test; but 746 μl/l are needed to displace one-half of the bound ethylene (Fig. 4), suggesting a weaker binding of CO to the sites than that of ethylene. The fact that the one-half maximum activity values obtained for ethylene and propylene are both very close supports the view that this method is, in fact, valid for determining binding to ethylene-binding sites; although it is possible that some binding to nonactive sites may also occur. There is evidence that hydrocarbon gases may bind to

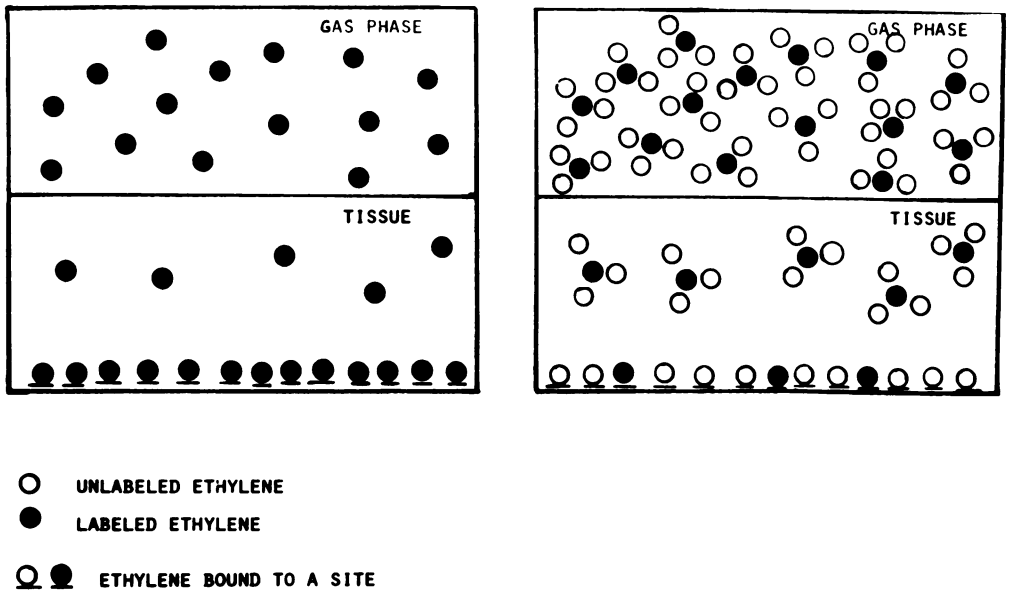

FIG. 1. Diagrammatic representation of [^{14}C]ethylene distribution between plant tissue and gas phase in the absence and presence of unlabeled ethylene. (It is not intended to represent all of the sites, but only those sites occupied by [^{14}C]ethylene in the absence of unlabeled ethylene. In the leaf experiments, this amounted to 13% of the total number of sites.) Note that the distribution of unbound [^{14}C]ethylene is the same in both cases and that unlabeled ethylene has distributed itself by the same ratio. As the unlabeled ethylene concentration is increased, the remainder of the ^{14}C-labeled ethylene would be displaced.

Table I. *Effect of Unlabeled Ethylene on Amount of ^{14}C-labeled Ethylene in Tobacco Leaves*

Treatment was with 0.5 μCi of [^{14}C]ethylene (120 mCi/mol) in a 2.5-liter container. This would amount to about 0.04 μl/l of ethylene. Leaves were allowed to stand 24 h in the presence of labeled ethylene and 24 h in the presence of mercury perchlorate to trap the labeled ethylene.

Unlabeled Ethylene (1,000 μl/l)	[^{14}C]Ethylene in Gas Phase	[^{14}C]Ethylene Diffusing from Tissue	Δ
	dpm/25 ml	*dpm/25 g*	
−	9499	1860	
+	9481	92	1768

some proteins; however, such interactions are thought to involve nonspecific sites, and probably the hydrocarbons are "dissolved" within the protein molecules (2, 17). Methane at 4,000 μl/l was without effect on ^{14}C-labeled ethylene binding. In six separate experiments using 100-g amounts of tissue, no binding could be demonstrated in mushrooms, although binding could readily be demonstrated in a number of other higher plant tissues. The fact that mushrooms do not bind ethylene strengthens the view that binding is specific.

CO_2, reported to be a competitive inhibitor of ethylene's action (4), does not appear to displace half of the [^{14}C]ethylene even at a concentration of 10%. About 60% of the ethylene bound in the

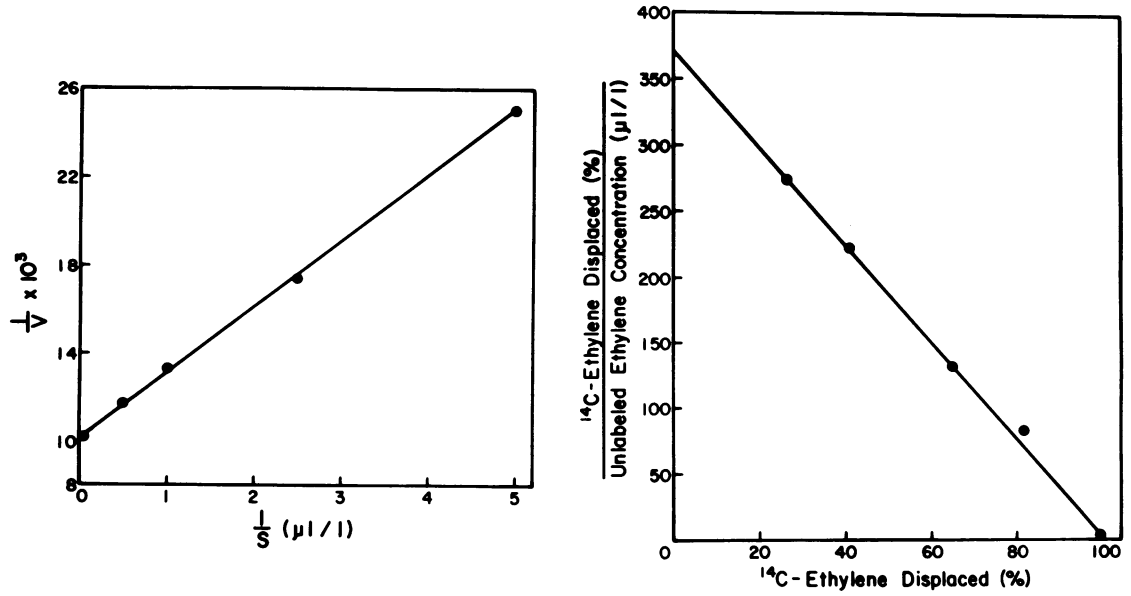

FIG. 2. Lineweaver-Burk plot for ethylene activity in a tobacco leaf respiration test and a Scatchard plot for displacement of [^{14}C]ethylene by unlabeled ethylene. In the Lineweaver-Burk plot, 1/V is the reciprocal of the activity (13) and 1/S is the reciprocal of the ethylene concentration. In the Scatchard plot, dividing the ordinate by the abscissa gives the reciprocal of the ethylene concentrations. The value for one-half maximum activity is 0.3 μl/l and 0.27 μl/l for one-half maximum displacement. Each experiment was run a minimum of three times.

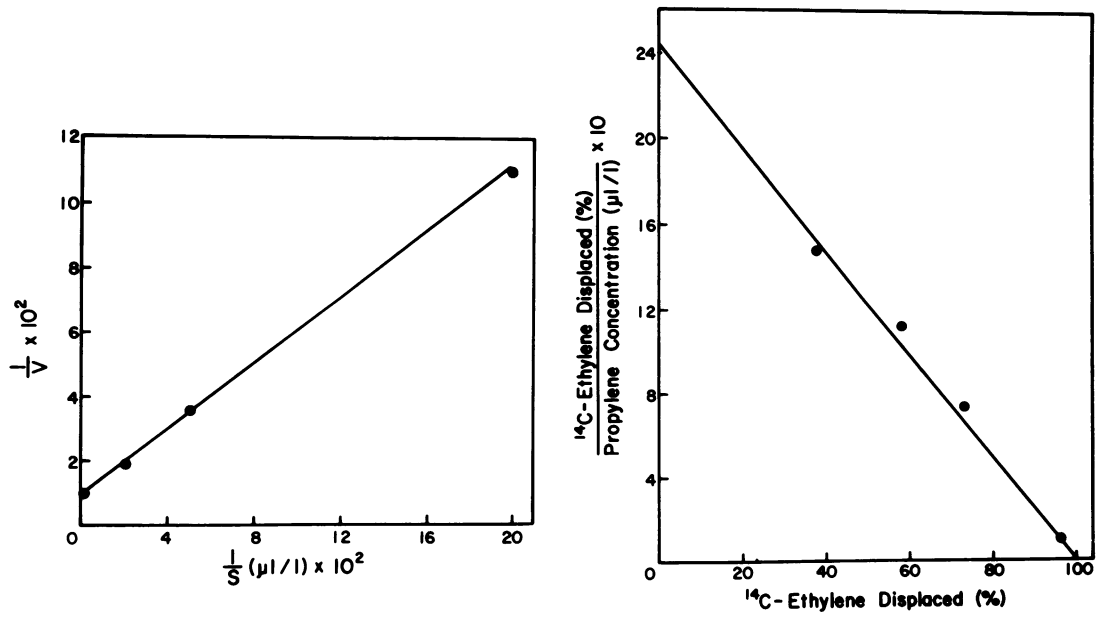

FIG. 3. Lineweaver-Burk plot for propylene activity in a tobacco leaf respiration test and a Scatchard plot for displacement of [^{14}C]ethylene by propylene. In the Lineweaver-Burk plot, 1/V is the reciprocal of the activity (13) and 1/S is the reciprocal of the propylene concentration. The value for one-half maximum activity was 50 μl/l and 42 μl/l for one-half maximum displacement. Each experiment was run a minimum of three times.

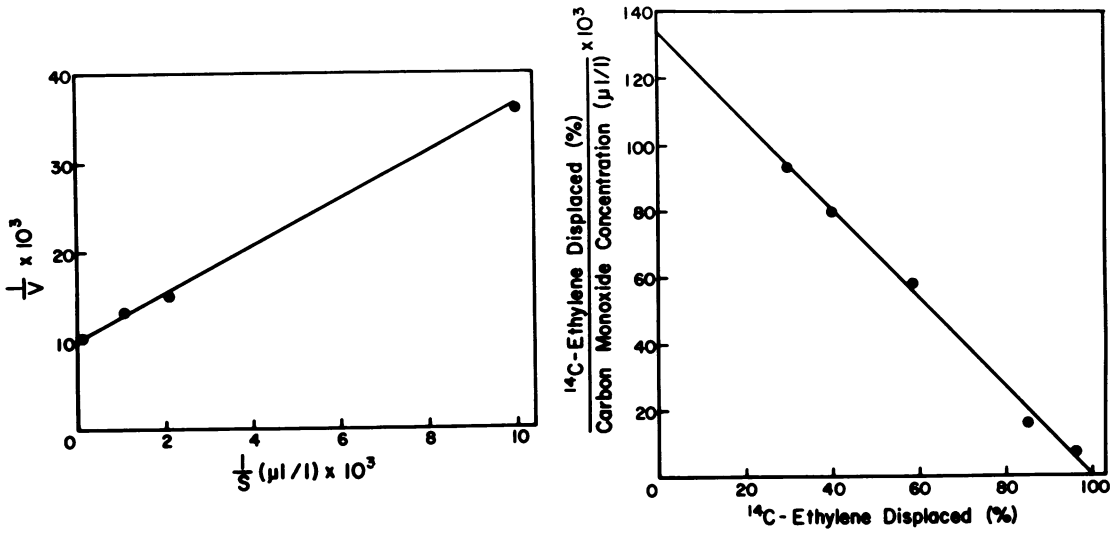

FIG. 4. Lineweaver-Burk plot for CO activity in a tobacco leaf respiration test and a Scatchard plot for displacement of ethylene by CO. In the Lineweaver-Burk plot, 1/V is the reciprocal of the activity (13) and 1/S is the reciprocal of the CO concentration. The value for one-half maximum activity was 266 µl/l and 746 µl/l for one-half maximum displacement. Each experiment was run a minimum of three times.

Table II. *Effect of CO_2 on Ethylene Binding*

Each experiment was run a minimum of three times.

CO_2 Concentration[a]	Ethylene Displaced
%	%
2	34
3	40
4	36
5	36
10	41

[a] 0.8% of the CO_2 assumed to originate from respiration of leaves.

absence of CO_2 remains bound even at such concentrations (Table II). This suggests that CO_2 acts, not by direct competition which prevents most of the ethylene from binding, but through some indirect manner.

^{14}C-Displacement as an Assay Method for the Ethylene-binding Component. When a saturating amount of unlabeled ethylene is applied to a leaf sample, there is a large difference in the amount of [^{14}C]ethylene which remains in it and that which remains in a comparable sample which has not been treated with unlabeled ethylene (Table I). The difference should be equal to the amount bound to specific sites. This should provide a suitable assay procedure for determining the presence of the ethylene-binding component(s).

Also, using this value, an estimate can be made of the number of ethylene-binding sites present in tissue. Abeles (1) made a calculation, based on solubility data of ethylene in water, and arrived at a value of 8.86×10^{-10} mol of ethylene/l of plant material. His calculations do not take into account how much ethylene is bound. Displacement data using the values of Table I give 70,720 dpm/kg of ^{14}C-labeled ethylene bound to specific sites. This is 3.2×10^{-2} µCi. The activity of the [^{14}C]ethylene was 8.4×10^{-9} mol/µCi. This gives 2.68×10^{-10} mol of ethylene/kg of tissue bound to specific sites. The concentration of [^{14}C]ethylene in the gas phase was 3.7×10^{-2} µl/l.

From a Scatchard plot of the data, a value of 13% displacement is obtained at this concentration of ethylene in the gas phase. This value of 13% should be equal to the per cent of the total sites occupied by the ^{14}C-labeled ethylene initially. Using 13% as the number of occupied sites, $(2.68 \times 10^{-10})/0.13 = 2.06 \times 10^{-9}$ mol/kg of tissue should be the amount of ethylene bound when all of the sites are occupied.

Some further factors need to be considered. The leaves themselves produce ethylene, and this ethylene would be occupying some of the sites. During the treatment time the leaves produce sufficient ethylene to give 0.2 µl/l in the gas phase. A value of 42% is obtained from a Scatchard plot for this concentration of ethylene. This would leave only 58% of the sites available for labeled ethylene, and the corrected value would give 3.5×10^{-9} mol/kg when 100% of the sites are available. This value of 3.5×10^{-9} mol/kg would be the number of sites occupiable by external ethylene. Assuming 1 molecule of ethylene bound per site, 3.5×10^{-9} mol of ethylene-binding material should be present per kg of plant material. This is almost eight times that calculated by Abeles and represents about 4,000 sites/cell. Some sites may be occupied by internally produced ethylene, and the true value may be higher; also, other binding sites which bind ethylene at a higher concentration could be present and not detected by this method. Although 24 h were allowed for complete equilibrium of the leaf with the gas phase, 3 h gave very similar results. Diffusion of ethylene from leaves is at least biphasic. Approximately 80% of the ethylene diffuses out rapidly with half of this emerging in 10 min. Approximately 20% of the total ethylene diffuses out much more slowly over a period of 24 h. This might suggest that binding occurs in two different locations.

Measurement of Ethylene Binding in Tissue Culture Material. Attempts to measure ethylene binding in tissue culture material have been made with some success. Using 25 g of living tissue culture material in a 2.5-liter desiccator and 0.5 µCi of ^{14}C-labeled ethylene, 2,956 dpm was obtained in the absence of ethylene, and 1,223 dpm was obtained in the presence of 1,000 µl/l of unlabeled ethylene with a difference of 1,733 dpm (see Table III). Using frozen and thawed tissue, values obtained were 2,642 dpm in the absence of ethylene and 1,155 dpm in the presence of 1,000 µl/l of unlabeled ethylene. The difference of 1,487 dpm is nearly as large as the difference of 1,733 obtained in living tissue. Boiling for 3 min eliminates binding. A value of 1,246 dpm was obtained in the presence of 2,000 µl/l of HCN, indicating it also interacts with the ethylene-binding site(s). Cyanide has been reported to cause an "ethylene response" in cyanide-resistant tissue (15, 16). Cyanide also appears to compete with labeled ethylene in living leaves, but some injury occurs making the results difficult to interpret. *n*-Butyl isocyanide, CO, phosphorous trifluoride and tetrafluoroethylene, all π-acceptor compounds previously shown to mimic ethylene action in the tobacco respiration test and in the bean petiole abscission test (12), also compete with ethylene in binding studies with frozen and thawed tissue.

Table III. *Effect of Some π-Acceptor Compounds on the Amount of ^{14}C-labeled Ethylene in Tissue Culture Material*

Each experiment was run a minimum of three times.

Tissue	Competing Component	[^{14}C]Ethylene[a] Diffusing From Tissue	Δ
		dpm/25 g	
Fresh	None	2,956	
	1,000 μl/l ethylene	1,223	1,733
Frozen and thawed	None	2,642	
	1,000 μl/l ethylene	1,155	1,487
	2,000 μl/l cyanide	1,246	1,396
	1,000 μl/l tetrafluoroethylene	1,874	768
	2,000 μl/l phosphorous trifluoride	1,294[b]	1,348
	1,000 μl/l *n*-butyl isocyanide	1,396	1,246
	10,000 μl/l carbon monoxide	1,491	1,151

[a] Exposure was to 0.5 μCi of ^{14}C-labeled ethylene in a 2.5-liter desiccator.
[b] Exposure was to 1,000 μl/l initially followed by another 1000 μl/l 3 h before removing from [^{14}C]ethylene.

Although living tissue culture material contains more labeled ethylene in the presence of a saturating amount of unlabeled ethylene and thus does not give as clear a result as living leaves, the killed tissue culture material handles much better than killed leaves and may be the tissue of choice for further studies. The results indicate the binding component(s) can be measured in nonliving tissue; and, presumably, conditions can be found for its extraction and subsequent purification.

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