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A Comparative Study of Ethylene Oxidation in *Vicia faba* and *Mycobacterium paraffinicum*

Fred B. Abeles

United States Department of Agriculture, Agricultural Research Service, Appalachian Fruit Research Station, Kearneysville, West Virginia 25430 USA

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Abstract. *Vicia faba* L. 'Herz Freya' (fababean) cotyledons and *Mycobacterium paraffinicum* Bardane strain (MPB) cells were studied to describe and compare physiological and biochemical factors regulating ethylene oxidation. Both organisms demonstrated a linear rate of ethylene uptake as a function of concentration from 1 ppm to 1,000 ppm. CO_2 did not influence ethylene oxidation by either organism. Zero degree temperatures and CO inhibited ethylene oxidation by fababeans but not by MPB.

An N_2 gas phase blocked ethylene consumption by fababeans. In contrast, MPB continued to consume ethylene at a reduced rate under anaerobic conditions. Hydrocarbon oxidation was limited to alkenes. Alkanes were not oxidized by either organism. Both organisms were sensitive to diethyldithiocarbamic acid, o-phenanthroline, carbonyl cyanide *m*-chlorophenyl hydrazone, and CS_2. The possibility that CS_2 acted as a suicide substrate is discussed. Evidence is presented that hydrocarbon gas oxidation by fababeans is not a part of, or reflection of, the way ethylene acts as a hormone.

The oxidation of ethylene to ethylene oxide by ethylene monooxygenase has been reported to occur in the actinomycete *Mycobacterium paraffinicum* (De Bont 1975, DeBont and Albers 1976, DeBont et al. 1979, De Bont and Harder 1978, Wiegant and DeBont 1980), a number of plants (Beyer 1980, Dodds et al. 1979, Jerie and Hall 1978), and mice (Ehrenberg et al. 1977). In microorganisms, only one pathway of ethylene oxidation has been described, while in

Abbreviations. CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; MPB, *Mycobacterium paraffinicum* Bardane strain; SKF-525A, (2-diethylamino ethyl-2, 2-diphenyl valerate hydrochloride); DIECA, diethyldithiocarbamic acid

plants two are known to occur, one of which forms CO_2 and the other ethylene oxide (Beyer 1975). Dual pathways of ethylene oxidation have been described for *Pisum sativum* (pea) (Beyer 1975), *Dianthus caryophyllus* (carnation) (Beyer 1977), and *Ipomoea tricolor* (morning glory)(Beyer and Sundin 1978). On the other hand, *Vicia faba* has only the ethylene oxide (ethylene monooxygenase) system and does not produce ^{14}C-CO_2 from ^{14}C-ethylene (Beyer 1980, Dodds et al. 1979, Jerie and Hall 1978).

The function of ethylene oxidation by *M. paraffinicum* is to provide carbon for the growth of this soil microorganism. Many strains that oxidize ethylene and other alkenes and alkanes have been described (Davis et al. 1956). It is thought that *M. paraffinicum* is responsible for the uptake of atmospheric hydrocarbons by the soil (Abeles 1982).

The following are possible explanations for the presence of an ethylene monooxygenase in plants: a vestigial enzyme inherited from microorganisms; a method of synthesizing ethylene oxide; a method of removing ethylene from tissues; an analog of some other substance that is the normal substrate (for example, hydrocarbon oxidases are used in cutin biosynthesis), [Croteau and Kolattukudy 1974]); and part of the site of ethylene action. This paper reports experiments designed to test the last hypothesis. There are two advantages in using fababeans as a model system. Fababeans have a single ethylene oxidation pathway, and the ethylene monooxygenase is active enough to measure directly, making it possible to use unlabeled substrates. This paper describes studies on: (1) the effects of inhibitors of ethylene action on ethylene oxidation; (2) comparisons between ethylene analogs and their rate of oxidation; and (3) the effect of inhibitors of ethylene oxidation on ethylene action.

Materials and Methods

Seeds of *Vicia faba* 'Herz Frey' or 'Diana' (fababeans) were placed in Chlorox:water (1:10, v/v) for 5 min, rinsed with running water and grown in the dark in moist vermiculite at room temperature. A greenhouse was used to grow plants for photosynthesis experiments. Seeds were purchased from Roy Legumex, Incorporated, St. Jean, Manitoba, Canada ROG 2BO. Other varieties tested were a gift from Dr. P. McVetty, Plant Science Department, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. *Mycobacterium paraffinicum* Bardane strain (MPB) was isolated from a soil sample from the Appalachian Fruit Research Station. Procedures for isolating and culturing MPB were those described by De Bont (1976). The appearance and staining characteristics of MPB, namely gram-positive, acid-fast, were identical to those of *M. paraffinicum* E44 provided by De Bont.

Ethylene and other hydrocarbon gases were measured on a GC fitted with a flame ionization detector and a Porapak Q (0.63 cm × 1.8 m) column. CS_2 was measured using a flame photometric detector. Oxygen consumption was measured with a Yellow Springs Instrument oxygen electrode. Experiments were run in either 10-ml plastic syringes fitted with rubber vaccine stoppers, 25-ml Erlenmeyer flasks sealed with silicone rubber stoppers, or 4-l plastic paint cans. Because methane and ethane were not oxidized by fababeans or MPB, these gases were added to the gas phase of containers to detect gas

Table 1. Ethylene oxidation by *V. faba* 'Diana' seedlings. Tissues from five 6-day-old seedlings were incubated in a syringe at room temperature for 2 h except for cotyledons, in which case only three cotyledons were used. Initial ethylene concentration was 2.5 ppm. Each syringe contained approximately 1 g of tissue.

Seedling portion	Ethylene oxidation nl/g h ± S.D.
Epicotyl tip, 1 cm	3.8 ± 0.4
Remaining epicotyl tissue	4.1 ± 1.0
Cotyledons	4.0 ± 0.5
Upper root, 4 cm	7.0 ± 1.2
Root tip, 4 cm	−3.1 ± 4.8*

* Negative sign indicates ethylene production.

leakage during experiments. Flasks were incubated at 25°C on a water bath shaker. Except where noted, all chemicals and enzymes were purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

Gaseous CS_2 (10% v/v) was prepared by evaporating 14 µl CS_2 in a 50-ml glass syringe and drawing the plunger back to the 50 ml mark. Dilutions of CS_2 and other gases were made by transferring volumes of gases between glass syringes fitted with rubber vaccine stoppers. Analyzed mixtures of ethylene and ethane in air were used as calibration standards.

Results

Initially, four varieties of fababeans ('Ackerperle,' 'Aladin,' 'Diana,' and 'Herz Freya') were examined for their ability to oxidize ethylene. The variety 'Diana' was used in experiments described in Tables 1 and 7 and 'Herz Freya' was used in the other experiments. For most experiments, 3-day-old seedlings were used, since the rates of ethylene oxidation in the presence of 10 ppm ethylene for 1-, 2-, 3-, and 4-day-old seedlings were 3.5, 5.9, 7.3, and 4.0 nl/g h, respectively.

The rate of ethylene oxidation by cotyledons was found to be linear within a 4-h incubation period (data not shown). Cotyledons stored at 5°C between moist paper towels loosely covered with aluminum foil retained their ethylene oxidizing activity for at least 4 days. For example, in the presence of 10 ppm ethylene, ethylene oxidation by freshly harvested cotyledons was 4.3 nl/g h and 5.9 nl/g h after 4 days. Ethylene oxidation was lost if seeds were either frozen or stored anaerobically for more than 2 h. Cells of *M. paraffinicum* could also be refrigerated for at least 4 days without loss of activity.

The relative rates of ethylene oxidation by various portions of fababean seedlings is shown in Table 1. Except for root tips, all portions of the seedlings oxidized ethylene. It is not known if the root tips oxidize ethylene or whether the rate of ethylene production exceeds the rate of ethylene oxidation.

The effect of ethylene concentration on the rate of oxidation for both organisms is shown in Fig. 1. In both cases, there appears to be a linear relationship between the log of the ethylene concentration and the log of the rate of oxi-

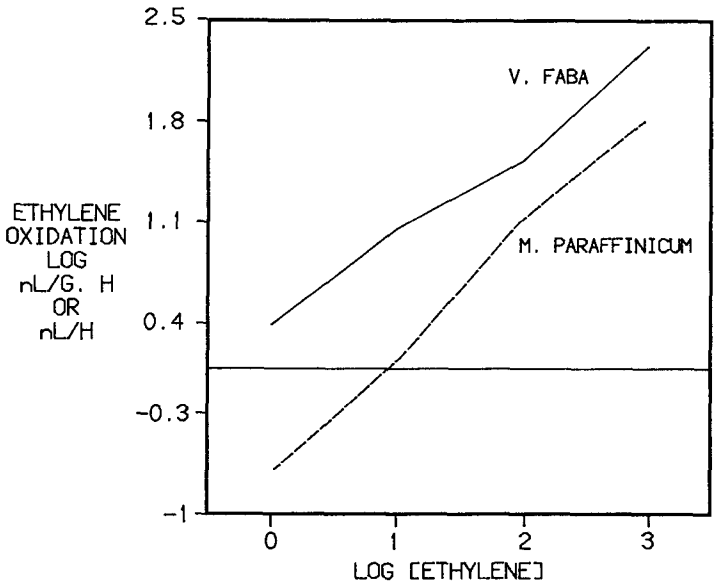

Fig. 1. A log log plot of ethylene oxidation, expressed as the log of ethylene uptake in nl/g h for *V. faba* (———) or nl/h for *M. paraffinicum* (-----) as a function of the log of ethylene concentration in ppm. Each syringe contained four 3-day-old seedling cotyledons and was incubated for 4 h. For *M. paraffinicum*, each flask contained 2 ml of mineral medium with cells yielding an $OD_{650} \approx 0.2$.

Table 2. Effect of O_2 on ethylene oxidation by *V. faba* and *M. paraffinicum*. For *V. faba*, each syringe contained two cotyledons from 2-day-old seedlings and a 5.1 ppm ethylene gas phase. For *M. paraffinicum*, each Erlenmeyer flask contained 2 ml of cells ($OD_{650} = 0.2$) and 100 ppm ethylene.

Oxygen %	Ethylene oxidized, % maximum rate	
	V. faba	*M. paraffinicum*
0	−1.1*	53
2	19	91
5	53	98
10	78	100
20	100	83

* Ethylene production.

dation. In the absence of added ethylene, fababeans produced ethylene at a rate of 0.53 nl/g h. The actinomycete did not evolve ethylene in mineral media or when methionine or 1-aminocyclopropane-1-carboxylic acid was added to the medium.

Table 2 presents data on the effect of O_2 on the rate of ethylene oxidation. An N_2 gas phase inhibited ethylene uptake by fababeans by 100% and by MPB by 50%.

CO_2 (10%) had no effect on the rate of ethylene oxidation by either organism (data not shown).

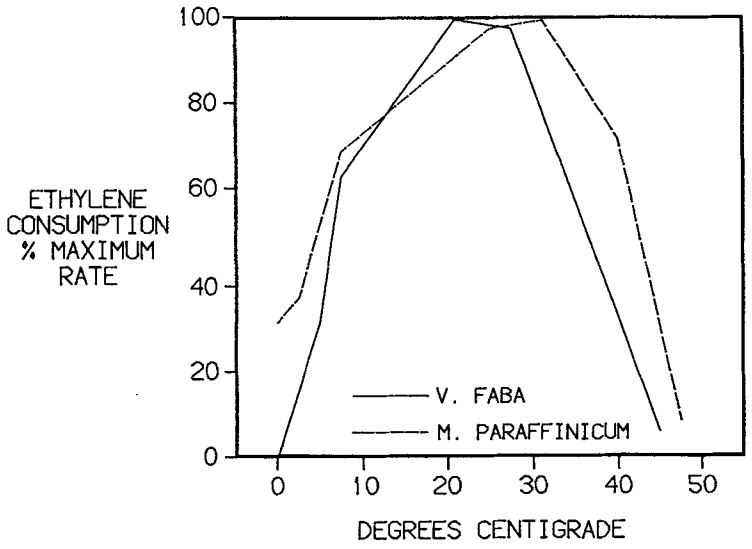

Fig. 2. Effect of temperature on the rate of ethylene oxidation. Experimental conditions were similar to Fig. 1, except ethylene concentration was 100 ppm and temperature varied as shown on the abscissa.

The effect of temperature on ethylene oxidation is shown in Fig. 2. The maximum activity occurred over a broad range from 20°C to 30°C. As mentioned above, a temperature of 0°C inhibited ethylene oxidation by fababean cotyledons. In contrast, MPB retained 30% of its activity at 0°C.

Methane and ethane were not oxidized by cotyledons. In addition, these gases did not inhibit the oxidation of ethylene. As shown in Table 3, cotyledons oxidized ethylene, propylene, vinyl bromide, vinyl fluoride, and allene at essentially similar rates. In all cases, CS_2 inhibited hydrocarbon oxidation. The action of CS_2 is described more fully below.

The effect of inhibitors on ethylene oxidation is shown in Table 4. Both organisms were sensitive to the action of DIECA, o-phenanthroline, and CCCP. Cotyledons were more sensitive than MPB to 8-hydroxyquinoline, fluoroacetic acid, and NaN_3. Cotyledons treated with CCCP produced ethanol and an unknown volatile (data not presented).

CO inhibited ethylene oxidation by cotyledons (Table 5). The inhibition occurred only when CO was present during the experiment. No inhibition was observed when cotyledons were pretreated with CO for 1 h followed by an air flush. In addition, light did not reverse the inhibitory effect of CO. In contrast, up to 20% CO had no effect on ethylene oxidation by MPB (data not shown).

As reported by Beyer (1977, 1980), CS_2 and COS inhibited ethylene oxidation (Table 6). Unlike the effect of CO, the inhibitory effects of CS_2 and COS were irreversible. A 10-min 10 ppm CS_2 treatment completely and irreversibly blocked ethylene oxidation (Table 6).

The metabolic effects of CS_2 on a number of major physiological processes in fababeans were measured. CS_2 had no effect on respiration of cotyledons or photosynthesis of 4-week-old fababean leaves as measured by a Gilson

Table 3. Oxidation of 10 ppm hydrocarbon gases by *V. faba* cotyledons. Each syringe contained four 3-day-old seedling cotyledons and was incubated for 4 h. Cotyledons were pretreated with 100 ppm CS_2 for 5 min and then flushed with air. Methane and ethane were not oxidized by the cotyledons nor did they inhibit ethylene oxidation.

Treatment	Hydrocarbon consumption nl/g h ± S.D. Control rate	+ 100 ppm CS_2
Ethylene	16.8 ± 3.8	0.9 ± 3.4
Propylene	12.2 ± 4.0	0.2 ± 1.1
Vinyl bromide	23.5 ± 2.5	2.8 ± 7.8
Vinyl fluoride	13.7 ± 0.4	0.9 ± 0.9
Allene	14.7 ± 2.4	4.0 ± 4.8

Respirometer (data not shown). In addition, CS_2 had no effect on either endogenous or indoleacetic acid stimulated ethylene production by *Phaseolus vulgaris* L. 'Red Kidney' seedlings. CS_2 was not readily metabolized by fababean cotyledons. Using a gas chromatograph equipped with a Porapak Q column and a flame photometric detector, we failed to observe any difference in CS_2 uptake by heat-killed or viable cotyledons. Both heat-killed and viable cotyledons readily absorbed CS_2 (70% of a 10-ppm gas phase in 1 h). The data shown in Table 7 indicate that growth of fababean seedlings was not changed even though ethylene oxidation was inhibited by CS_2.

Since CS_2 was an effective inhibitor of ethylene metabolism in fababean, the experiment summarized in Table 7 was performed to compare the effect of CS_2 on both ethylene oxidation and action. The data in Table 7 indicate that CS_2 inhibited the oxidation of ethylene by epicotyls but had no effect on ethylene action.

Discussion

Ethylene monooxygenase of fababeans appears to be a constitutive enzyme and, with the exception of root tips, distributed throughout the plant (Table 1). Jerie and Hall (1978) reported that fababeans did not convert ^{14}C-ethylene to ^{14}C-CO_2. This is unlike ethylene oxidation in other plants in which ethylene is converted to both CO_2 and ethylene oxide (Beyer 1980). This means that conclusions derived from work reported here pertain only to the ethylene oxide forming system (equivalent to the tissue incorporation system described by Beyer) and not to the CO_2 producing system (denoted as the oxidative system by Beyer).

As reported by Beyer (1979a), the rate of ethylene oxidation by plants was directly proportional to its concentration. Fig. 1 shows that the same observation also holds true for the MPB system. This oxidation dose response curve is unlike the one observed when the biological effect of the gas is measured (Abeles 1973). For most ethylene-mediated processes, the dose response curve is saturated between 1 and 10 ppm, and additional quantities of the gas have no further biological effect. If ethylene oxidation is a link between action and

Table 4. Effect of inhibitors on ethylene oxidation by *V. faba* and *M. paraffinicum*. Each flask contained 12 cotyledons sliced into 2-mm sections. Prior to ethylene uptake studies, the slices were incubated for 5 min in 10^{-3}M (CCCP = 10^{-4}M) inhibitor after which time they were were blotted dry. Ethylene = 10 ppm. Data are an average of at least two experiments consisting of three replicates per treatment and are expressed as the % inhibition when compared to the untreated control. Each 30-ml flask contained 2 ml *M. paraffinicum* cells with an OD_{650} = 0.2. Flasks were shaken for 2 h at 25°C and contained 100 ppm ethylene. Dashed lines (—) indicate experiment not done.

Compound	Average % inhibition	
	V. faba	*M. paraffinicum*
KCN	2.0	28
$AgNO_3$	3.5	—
SKF 525-A	8.3	92
DIECA	38	95
O-phenanthroline	44	98
CCCP*	48*	82
8-hydroxyquinoline	52	22
Fluoroacetic acid	70	3
NaN_3	96	13
CS_2	99	95

* 10^{-4}M

metabolism, then the dissimilarities between the two kinds of curves must be explained. One such explanation is that the limiting reaction between ethylene application and action occurs at some point after the oxidative step and that the action step is the limiting one; the oxidative is not.

As reported by Beyer and Blomstrom (1979) and Dodds et al. (1979), anaerobicity blocked ethylene oxidation in higher plants (Table 2). In addition, 2 h of anoxia irreversibly destroyed the fababean ethylene oxidation system. In contrast, the actinomycete continued to metabolize ethylene at a reduced rate under anaerobic or fermentative conditions. While care was taken to exclude O_2 from these experiments, it is possible that some leakage still occurred. On the other hand, MPB may be able to consume ethylene by an alternative anaerobic pathway.

CO_2 had no effect on ethylene oxidation by either the fababeans or MPB (data not shown). Beyer (1979a) also reported that CO_2 had no effect on the tissue incorporation (ethylene oxide formation) system but did inhibit CO_2 production from ethylene. The ability of 5% to 10% CO_2 to inhibit or reverse a number of ethylene-mediated effects is well known and often used as a diagnostic test of ethylene involvement in physiological processes. The observation that CO_2 had no effect on ethylene uptake suggests that the ethylene oxide forming system is not linked to ethylene action.

Another difference between the fababean and MPB system was the effect of temperature on the rate of ethylene oxidation (Fig. 2). The ability of MPB to retain a portion of its biological activity at 0°C may reflect the adaptation of this organism to the soil environment, which is at or below 0°C for a part of the year. The inhibition of ethylene oxidation by fababeans at 0°C may be due to cellular disruption by freezing temperatures.

Table 5. CO inhibition of ethylene oxidation by *V. faba* cotyledons. Each 30-ml flask contained 12 cotyledons. In experiment 1, flasks were incubated for 2 h. In experiment 2, CO pretreatment was for 1 h. In experiment 3, 22,000 lux of light was supplied by a combination of incandescent and fluorescent lamps.

% carbon monoxide		Ethylene oxidation
Experiment		nl/g h ± S.D.
1	0.0%	16.1 ± 3.2
	0.5%	13.0 ± 3.0
	1.0%	8.0 ± 2.0
	2.0%	5.8 ± 3.4
	5.0%	0.2 ± 2.0
2	Control	25.5 ± 2.7
	5%, 1-h pretreatment	27.0 ± 1.8
	5% during experiment	6.1 ± 1.4
3	Dark control	20.6 ± 2.5
	1% CO dark	7.9 ± 1.4
	Light control	28.4 ± 3.8
	1% CO light	9.8 ± 0.6

A comparison (Table 3) of the relative activity of various hydrocarbon gases as substrates for ethylene monooxygenase was performed to assess the relationship between oxidation and action. The relative biological effect of ethylene and its analogs has been summarized earlier (Abeles 1973), and in general, ethylene is the most active gas of the series, followed by propylene, carbon monoxide, acetylene, vinyl fluoride, vinyl bromide, and allene. The relative activity of ethylene versus allene is 10,000-fold. We observed that fababean cotyledons consumed ethylene, propylene, vinyl fluoride, vinyl bromide, and allene with only a 2 fold difference in uptake rates (Table 3). CO, on the other hand, inhibited ethylene oxidation. The absence of a correlation between biological and biochemical activity also suggests that ethylene monooxygenase activity is not linked to ethylene action.

The data in Table 4 are a summary from a series of studies on the action of inhibitors on ethylene oxidation. Both organisms were sensitive to the action of the metal ion chelators, 8-hydroxyquinoline and DIECA. These observations suggest that Fe(III) or Cu(II) may be an essential component of this enzyme or some other enzymatic system that contributes to ethylene oxidation. The inhibition of ethylene oxidation by CCCP suggests that electron transport or one of its products contributes to ethylene oxidation. Other inhibitors of respiration, fluoroacetic acid and NaN_3, had a greater effect on the fababean cotyledon system than on the microbial one. Inhibitors of ethylene production, Co(II) (Yu and Yang 1979), and of ethylene action, Ag(I) (Beyer 1976), did not inhibit ethylene oxidation by fababean cotyledons (data not shown).

The possibility that ethylene monooxygenase might be a cytochrome P-450 enzyme was tested by exposing seeds to SKF-525A and CO. SKF-525A, a drug often used as an inhibitor of cytochrome P-450 (Testa and Jenner 1976), had no effect on ethylene oxidation by fababeans, but it did inhibit ethylene oxidation by MPB. However, the ethylene oxidizing system may respond differently to SKF-525A than insect or mammalian systems.

Table 6. Inhibition of ethylene oxidation by CS_2 and COS. Cotyledons from 3-day-old seedlings were exposed to concentrations of the gas indicated for 10 min and then vented. Ethylene was added to yield 10 ppm and oxidation measured for 4.5 h.

	Ethylene consumption	
	nl/g h ± S.D.	
PPM gas	CS_2	COS
0	11.7 ± 2.1	
1	2.1 ± 1.9	8.1 ± 0.7
10	0.6 ± 1.2	8.1 ± 1.3
100	−0.5 ± 0.5	6.1 ± 2.1
1000	0.1 ± 2.3	3.4 ± 4.3

Table 7. Effect of CS_2 on ethylene oxidation and growth of *V. faba* 'Diana' epicotyls. Twenty 3-day-old seedlings growing in 8 cm × 11 cm diam. cups containing 40 g moist vermiculite were placed in 4-l containers and treated with 100 ppm CS_2 for 10 min. The containers were then flushed with air for 15 min, and filled with ethylene as indicated; both epicotyl elongation and ethylene uptake measured after 3 days. The letters following data are the mean separation by Duncan's Multiple Range Test, 5% level. A negative sign indicates ethylene production.

Ethylene treatment, ppm	Epicotyl length, mm after 3 days' growth		Total ethylene consumed, µl ethylene/20 seedlings	
	Control	CS_2	Control	CS_2
Initial	11E			
Air	66 A	66 A	−2	−3
0.1	46 B	40 BC	2	−3
1.0	30 CD	20 DE	52	−7
10.0	16 E	18 E	466	4

CO inhibited ethylene oxidation by fababeans (Table 5). However, the effect was not light reversible, another characteristic of cytochrome P-450 (Cooper et al. 1965). We observed that the CO enzyme complex was freely dissociable (Table 5) as evidenced by the fact that ethylene oxidation activity was regained when CO was flushed from the system. Ethylene oxidation by MPB was not inhibited by up to 20% CO (data not shown).

The inhibiting effect of CS_2 and COS was originally described by Beyer (1977, 1980) and confirmed here (Table 6). CS_2 was the more active of the two and concentrations as low as 10 ppm for 10 min irreversibly blocked the enzyme. The order of activity of the double bond carbon inhibitor series is CS_2, COS, CO, CO_2. Beyer (1977) suggested that the inhibiting action of CS_2 was due to a chelation reaction with Cu(II). However, Dodds et al. (1979) reported that Cu(II) did not serve as a cofactor in an impure cell-free ethylene monooxygenase isolated from fababean cotyledons. In order to test the idea of Cu(II) chelation, the Cu(II) containing enzymes ascorbic acid oxidase, galactose oxidase, and uric acid oxidase were tested for activity after exposing them to 10^{-3}M CS_2. None of these Cu(II) enzymes was inhibited by CS_2, while the known Cu(II) chelator DIECA blocked enzyme activity (data not shown).

Some biochemical effects of CS_2 include denaturation of cytochrome P-450 (Obrebska et al. 1980), inhibition of endoplasmic reticulum Ca(II) pumps (Moore 1982), and inhibition of nitrification by *Nitrosomonas* (Kudeyarov and Jenkinson 1976, Powlson and Jenkinson 1971). CS_2 also binds with amino acids and Cu(II) to form dithiocarbamates, and it inhibits cytochrome oxidase, monoamine oxidase, and alkaline phosphatase (See Brieger[1967] for a review).

In plants, the effect of low concentrations of CS_2 appears to be specific. Except for the inhibition of ethylene oxidation, CS_2 did not appear to inhibit major biochemical or physiological systems such as growth, photosynthesis, and respiration. As shown in Table 4, CS_2 also inhibited ethylene oxidation by MPB.

The action of CS_2 on ethylene monooxygenase is unknown, but the structural similarity among $S=C=S$, $O=C=S$, and $H_2C=CH_2$ suggest it may act as a suicide substrate in much the same way ethylene acts as a suicide substrate for cytochrome P-450 (Oritz De Montellano et al. 1981).

Evidence that ethylene oxidation is a part of, or a reflection of, ethylene action has been presented by Beyer (1979b, 1980). He demonstrated that changes in ethylene metabolism are correlated with abscission, ripening, and senescence and that Ag(I) ions reduce both ethylene action and its conversion to CO_2. The observations reported here suggest that the fababean ethylene monooxygenase does not behave as if it were a part of the site of ethylene action. A typical ethylene-action dose response curve levels off between 10 and 100 ppm. In contrast, the ethylene monooxygenase was not saturated at concentrations up to 1,000 ppm. Though biological activity of hydrocarbons with double bonds may vary by 10- to 1,000-fold, their rate of removal is essentially similar. CO, another ethylene analog, inhibited ethylene oxidation. The inhibitors of ethylene action, CO_2 and Ag(I), did not inhibit ethylene oxidation. Conversely, CS_2, a potent inhibitor of ethylene oxidation, did not block ethylene action or appear to have any effect on plant growth (Table 7). Nevertheless, these observations concern a species that has an unusually active enzyme. While there is no reason to believe that ethylene monooxygenase from cotyledons is different from the stem tissue enzyme, some of the results presented here were obtained with tissue that does not normally respond physiologically to ethylene.

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