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EPOXIDASE/EPOXIDE HYDRASE ACTIVITY IN CELL CULTURES OF *PHASEOLUS VULGARIS*

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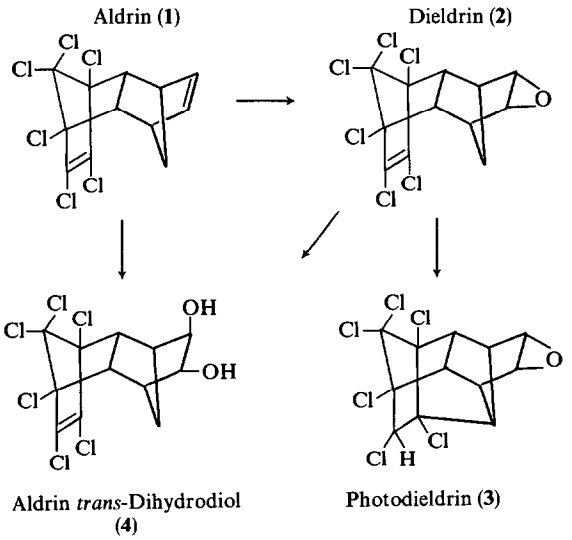
Key Word Index— *Phaseolus vulgaris*; Leguminosae; dwarf bean; cell suspension cultures; epoxidase/epoxide hydrase enzymes.

Abstract—The presence of epoxidase and epoxide hydrase enzymes in cell suspension culture of *Phaseolus vulgaris* is demonstrated. Results indicate high levels of enzyme activity using stilbene and stilbene oxide as substrates.

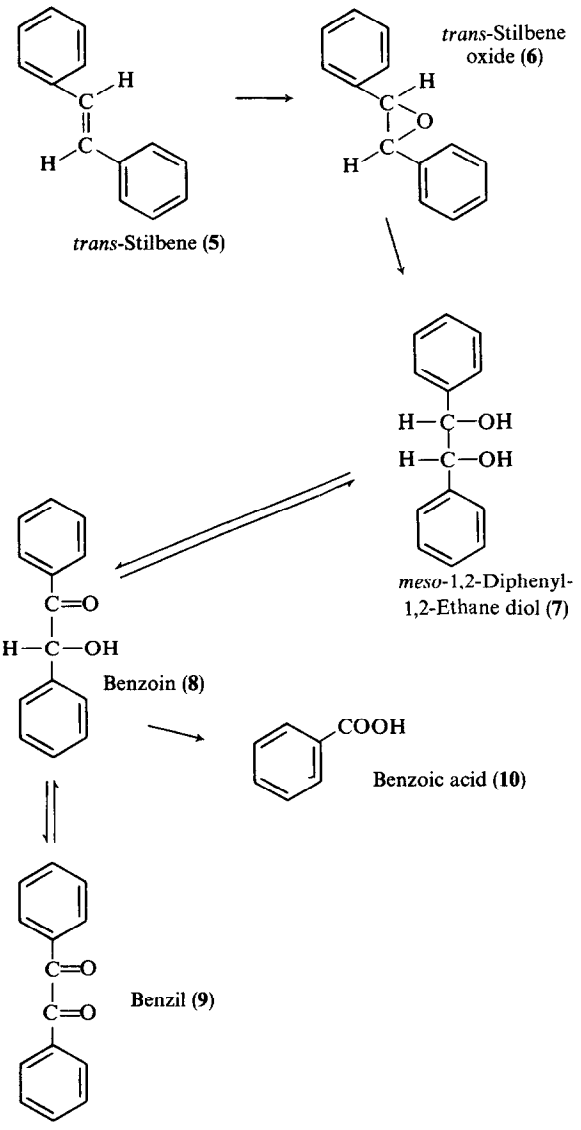
INTRODUCTION

The metabolic pathways of the cyclodiene insecticides aldrin and dieldrin have been extensively studied in plants both *in vivo* and *in vitro* [1]. Aldrin (**1**) is converted to its epoxide (**2**) (dieldrin), photodieldrin (**3**) and other, more polar products (Scheme 1). The production of dieldrin indicates the presence of an epoxidase enzyme and this has been demonstrated in various fractions derived from pea and bean root homogenates [2–5]. There is some indication that more than one enzyme system is involved [5].

Aldrin *trans*-dihydrodiol (**4**) should, logically, be the result of epoxide hydrase activity on dieldrin. However, it has been shown by a number of workers using various plant systems that only aldrin is capable of conversion to aldrin *trans*-dihydrodiol [1]. In our previous work on aldrin and dieldrin metabolism in plant cell suspension cultures of *Phaseolus vulgaris* cv Canadian Dwarf we have also shown that the *trans*-dihydrodiol is produced from aldrin, the only identifiable metabolite afforded by dieldrin being photodieldrin [6].



Scheme 1. The metabolism of aldrin and dieldrin.



Scheme 2. The metabolism of *trans*-stilbene and *trans*-stilbene oxide.

The fact that aldrin *trans*-dihydrodiol is not found as a metabolite of dieldrin could be due to either the absence of an epoxide hydrase enzyme in the plant systems studied or to exceptional stability of the epoxide [7]. The fact that animal systems are capable of converting dieldrin to aldrin *trans*-dihydrodiol might be taken as supporting the former proposition [1].

Cell suspension cultures of *P. vulgaris*, known to be capable of converting aldrin to dieldrin, photodieldrin and aldrin *trans*-dihydrodiol were examined to ascertain whether they possessed both epoxidase and epoxide hydrase activity. A number of simple model compounds have recently been used as substrates for these enzymes in liver microsomal systems and for this study *trans*-stilbene (**5**) and *trans*-stilbene oxide (**6**) proved satisfactory. The metabolism of these compounds by rabbit liver microsomes has been reported recently by Watabe and Akamatsu [8], Scheme 2 represents the major oxidative metabolites of these compounds. All the compounds illustrated in Scheme 2 were available as standards and were capable of rapid analysis by HPLC.

RESULTS AND DISCUSSION

Cell suspension cultures of *P. vulgaris* maintained on Murashige and Skoogs medium [9] supplemented with 0.5 mg/l. 2,4-D and 0.5 mg/l. kinetin, were used in this study. Initial work on aldrin and dieldrin metabolism relied on incorporation of substrate into the medium at the time of inoculation with suspension culture and analysis was carried out after 28 days. It was anticipated that administration of substrate to established suspension cultures would result in more rapid metabolism and that the stilbene models would be more rapidly metabolized than the cyclodiene insecticides. Initial experiments indicated that over 50% of a 10 mg administration of *trans*-stilbene into 10-day-old cultures was metabolized within 8 days. An additional, not unexpected, bonus was that the cultures were able to tolerate far higher doses of *trans*-stilbene and *trans*-stilbene oxide than aldrin and dieldrin. Consequently metabolic studies were carried out using 25 mg substrate/100 ml of 20-day-old cultures and analyses were performed after 1,3 and 8 days. All studies were carried out in triplicate and controls utilized similar cultures in which the cells were killed by autoclaving before substrate incorporation.

The metabolism of *trans*-stilbene shows (Fig. 1), by the production of *trans*-stilbene oxide, that an epoxidase enzyme is present and in parallel with the metabolism of aldrin, the *meso*-glycol (**7**) is formed. However the rapid decrease in epoxide level from 5.2% at 3 days to 0.5% at 8 days indicates that *trans*-stilbene oxide

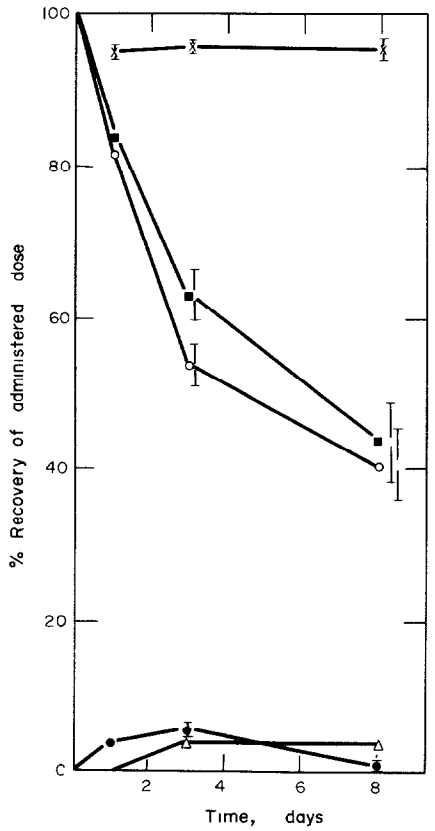


Fig. 1. The metabolism of *trans*-stilbene by *Phaseolus vulgaris* root cell suspension cultures. ×——× *trans*-stilbene control; recoveries from treated cultures; O—— —O *trans*-stilbene; ●——● *trans*-stilbene oxide; △——△ *meso*-1,2-diphenyl-1,2-ethane diol; ■——■ total level of recovered compounds. I 1 standard error either side of the mean

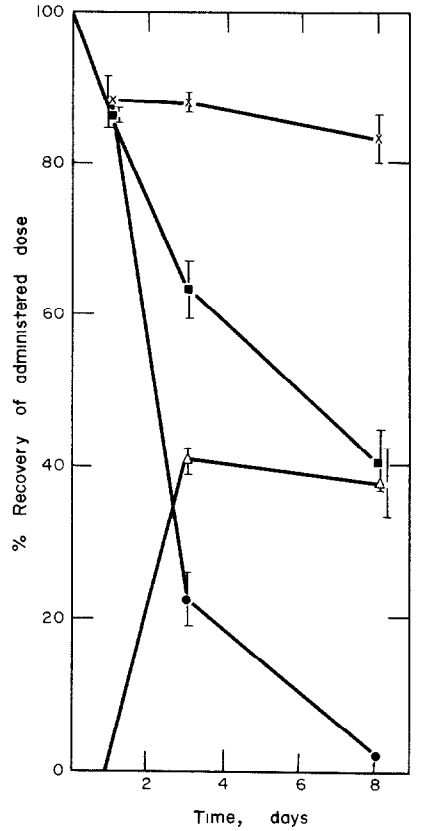


Fig. 2. The metabolism of *trans*-stilbene oxide by *Phaseolus vulgaris* root cell suspension cultures. ×——× *trans*-stilbene oxide control; recoveries from treated cultures ●——● *trans*-stilbene oxide; △——△ *meso*-1,2-diphenyl-1,2-ethane diol; ■——■ total level of recovered compounds I 1 standard error either side of the mean.

is not as resistant to enzymatic modification as is dieldrin. Of the other metabolites (Scheme 2) only benzoin (**8**) could be detected. *Trans*-stilbene oxide metabolism (Fig. 2) proved to be very different from that observed for dieldrin in that the predominant product was the *meso*-glycol (**7**) (40.6% after only 3 days) thus proving conclusively the presence of an epoxide hydrase enzyme. The only other detectable metabolite was benzoin (**8**).

The evidence from the metabolism of *trans*-stilbene and *trans*-stilbene oxide is that cell suspension cultures of *P. vulgaris* have both epoxidase and epoxide hydrase enzyme systems. This would indicate that the inability of plants to convert dieldrin to aldrin *trans*-dihydrodiol is not due to the absence of the requisite enzyme. The presence of a direct pathway from olefin to diol, which has been invoked to explain the production of aldrin *trans*-dihydrodiol [1] cannot be demonstrated by this method. It would appear to be an insignificant pathway for metabolism of the stilbene as the amount of diol produced at day 3 was 10 times higher for *trans*-stilbene oxide substrate than for *trans*-stilbene itself.

The extraction procedure adopted (see Experimental) allows the differentiation of the metabolites into cell and medium fractions. From an examination of the metabolite cell/medium ratio (Table 1) it can be seen that, in the stilbene metabolism, the cell/medium ratio for *trans*-stilbene oxide and *meso*-glycol both rise appreciably throughout the experiment whereas the substrate cell/medium ratio remains relatively constant. This would suggest cellular uptake of *trans*-stilbene followed by metabolism in which the epoxide is not the final product. The rapid drop in cell/medium ratio for *meso*-glycol from day 3 to day 8 with *trans*-stilbene oxide as precursor supports further metabolism within the cells. It is probable, in the absence of appreciable amounts of oxidative metabolites, that glycoside formation is an important pathway, as found in the cell suspension culture metabolism of cisanilide [10].

In our experiments with *trans*-stilbene and *trans*-stilbene oxide it was found that the plant system did not exactly model the rabbit liver microsomal studies of Watabe and Akamatsu [8] in that no trace of benzil (**9**) or benzoic acid (**10**) could be detected (it should be noted that benzil, being a better chromophore than any of the other compounds, was capable of detection at much lower levels than any of the reported metabolites). It remains to be seen why dieldrin may be relatively easily converted into aldrin *trans*-dihydrodiol in animal systems, a possible implication of this work being that the mammalian enzyme system is less specific than that of the plant.

Table 1. Time course metabolism of *trans*-stilbene and *trans*-stilbene oxide in *Phaseolus vulgaris* root suspension cultures

Compound	Day 1		Day 2		Day 3	
	t*	r†	t	r	t	r
Stilbene substrate						
trans-Stilbene (**5**)	80.3	0.09	53.3	0.07	39.8	0.11
trans-Stilbene oxide (**6**)	3.4	0.31	5.2	0.49	0.5	1.50
meso-1,2-Diphenyl-1,2-ethane diol (**7**)	0	—	4.1	0.37	3.1	2.88
Benzoin (**8**)	0	—	0.11	1.75	0	—
Stilbene oxide substrate						
trans-Stilbene oxide (**6**)	86.1	0.13	22.5	0.07	1.8	0.29
meso-1,2-Diphenyl-1,2-ethane diol (**7**)	0	—	40.6	0.86	38.0	0.10
Benzoin (**8**)	0	—	0.45	0.29	0.15	0.39

*t = total as % initial substrate.
†r = cell/medium concentration ratio.

EXPERIMENTAL

Tissue culture. P. vulgaris seeds (cv Canadian Dwarf) were surface sterilised with H_2O_2, washed with sterile H_2O and germinated aseptically. 1 cm sections of seedling root tissue were transferred to modified Murashige and Skoogs medium [9] (Flow Labs) supplemented with 6 mg/l. 2,4-D, 10% w/v coconut milk and 0.8% w/v Noble agar in 250 ml conical flasks. After callus induction, subculture in fresh medium was performed every 28 days. 20 weeks after initiation 5 g fr. wt callus portions were transferred to the same basal medium supplemented with 0.5 mg/l. 2,4-D and 0.5 mg/l. kinetin. Subculture of *ca* 2 g fr. wt (100 mg dry wt) samples of liquid culture into fresh 100 ml portions of medium (pH 5.5) was performed every 28 days. Liquid cultures were used for incorporation trials 76 weeks after initial induction.

Precursor incorporation. Trans-stilbene and *trans*-stilbene oxide were separately injected (25 mg in 0.5 ml portions of solns in Et_2O) 20 days after the 19th subculture. Control treatments were made into fresh 100 ml medium portions and medium containing cells autoclaved at 1 kg/cm^2 for 20 min. 3 flasks for each treatment were taken and analysed, 1,3 and 8 days after incorporation.

Extraction. Hexane (15 ml) was added to the flask contents and gently agitated. The suspensions were filtered under vacuum and the cells were washed with H_2O (10 ml) and 25% EtOAc–hexane (30 ml). The liquid phases were extracted with EtOAc–hexane (1:3) (3 × 50 ml) and EtOAc (50 ml). The organic phases were combined and dried (medium extract) The cells and filter pad were transferred to a Soxhlet thimble and the filtration apparatus was washed with MeOH (50 ml), the washing, the Soxhlet thimble and further MeOH (150 ml) were then transferred to a Soxhlet apparatus and extracted for 4 hr (cell extract). The medium and cell extracts were taken to near-dryness by evaporation under vacuum. H_2O was removed from the cell extract by repeated co-distillation with hexane. All extracts were then evapd to dryness.

Analysis. All extracts were made up to 5 ml with MeOH. A 1 ml aliquot of each extract was dried and made up to 1 or 5 ml with cyclohexane and assayed for *trans*-stilbene, *trans*-stilbene oxide, benzaldehyde and benzil using HPLC system I. A further 2 ml of each extract was dried, methylated with a Et_2O–CH_2N_2 and assayed for Me benzoate using HPLC system I. The remaining MeOH extract was dried, made up to 1 ml with EtOAc and assayed for benzoin and *meso*-1,2-diphenyl-1,2-ethane diol using HPLC system II. HPLC analyses of 5 μl samples was performed using a 10 × 0.5 cm stainless steel column packed with 10 μm Spherisorb S1OW (Jones Chromatography). The solvents were delivered at the rate of 2 ml/min by a Waters 6000 M solvent delivery system. Detection was with a Cecil CE 212 spectrophotometer, with a 10 μl flow cell, monitoring at 264 nm. Elution was with MeCN–2,2,4-triMepentane (0.1.19.9) (system I) or EtOAc–cyclohexane (3:7) (system II). R_fs (min) system I, *trans*-stilbene 0.2, *trans*-stilbene oxide 0.95, Me benzoate 1.3, benzaldehyde 2, benzil 3.3; system II, benzoin 0.45, *meso*-1,2-diphenyl-1,2-ethane diol 1.25.

REFERENCES

1. Kearney, P. C. (1976) *J. Assoc. Offic. Agr. Chemists* **59**, 866.
2. Mehendale, H. M., Skrentny, R. F. and Dorough, H. W. (1972) *J. Agr. Food Chem.* **20**, 398.
3. Yu, S. J., Kiigemegi, U. and Terriere, L. C (1971) *J. Agr. Food Chem.* **19**, 5.

4. Lichtenstein, E. P. and Corbett, J. R. (1969) *J. Agr. Food Chem.* **17**, 589.
5. McKinney, J. D. and Mehendale, H. M. (1973) *J. Agr. Food Chem.* **21**, 1079.
6. Brain, K. R. and Lines, D. S. (1976) *Proc. Conf. Biotech. Applic. of Plant Cell Culture.* Springer-Verlag, Munich, (in press).
7. Parsons, A. M. and Moore, D. J. (1966) *J. Chem. Soc. (C)* 2026.
8. Watabe, T. and Akamatsu, K. (1975) *Biochem. Pharmacol.* **24**, 442.
9. Murashige, T. and Skoog, F. (1962) *Physiol. Plantarum* **15**, 473.
10. Frear, D. S. and Swanson, H. R. (1975) *Pesticide Biochem. Physiol.* **5**, 73.