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ALDRIN EPOXIDASE FROM SOYBEAN ROOT NODULES

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Key Word Index—*Glycine max*; Leguminosae; root nodules; NADPH, aldrin; dieldrin; epoxidase.

Abstract—Soybean (*Glycine max* cv Forrest) root nodule homogenates oxidized aldrin to its epoxide, dieldrin. In crude tissue brei, addition of an NADPH-generating system was inhibitory to epoxidation. However, anaerobic gel filtration and sucrose density separation removed factors required for inhibition by NADPH, allowing a normal stimulation by the NADPH-generating system. In fractions from sucrose density gradients, activity was found predominantly at a density containing rough microsomes, with additional activities in the soluble and other fractions. Epoxidase activity was 2–4-times greater in the nitrogen-fixing nodules than in roots. This demonstration of active epoxidation indicates the capacity of nodules to detoxify other pesticides and xenobiotics.

INTRODUCTION

Oxidation of aldrin to its epoxide dieldrin has been well studied in mammalian, aquatic and arthropodian tissues [1] since the initial studies [2] with animal tissue. Early work done with soil microbes [3–5] showed the capacity soil micro-organisms have for this biological conversion, later confirmed with microbial isolates in laboratory culture [6–8]. Studies have also been carried out [9] on the inhibitory effect of high doses of aldrin on the growth and oxygen uptake in *Rhizobium* species.

Aldrin metabolism in plants was first demonstrated by Lichtenstein and Schulz [10]. Later, the general *in vivo* pesticide metabolism for a wide variety of plants was reviewed [11]. There are relatively few references available on this epoxidation *in vitro* with plant tissues [12–16]. However, these studies suggested that epoxidation in plant extracts required NADPH and oxygen, as required for the animal mixed function oxidase reaction. The addition of polyvinylpyrrolidone (PVP) during tissue preparation enhanced epoxidation activity as a general rule. Inhibitory actions on the system by oxygen during preparation, and by cyanide and various metal ions, were reported [13, 15, 16].

In the case of nitrogen-fixing root nodules, the *Rhizobium* bacteria have a close relationship with the plant tissue, being enclosed within vacuoles in the plant cell cytoplasm. While the biochemistry related to nitrogen fixation is relatively well studied, little is known of how the nodule tissues cope with the entry of xenobiotics or toxicants from the exterior. Here, the aldrin epoxidation reaction is considered as one model for mono-oxygenase activity in functional nitrogen fixing nodules.

RESULTS AND DISCUSSION

General properties

Conversion of aldrin to dieldrin was studied with respect to substrate level using soybean root nodule crude brei (Fig. 1). There was a linearly increasing dieldrin production for different substrate levels up to 30 μg aldrin per assay (0.3 mg protein/assay). Only above 40 μg aldrin/assay did stimulation of the rate of reaction from additional aldrin diminish. At each level of aldrin, the total conversion to dieldrin was *ca* 1.8%.

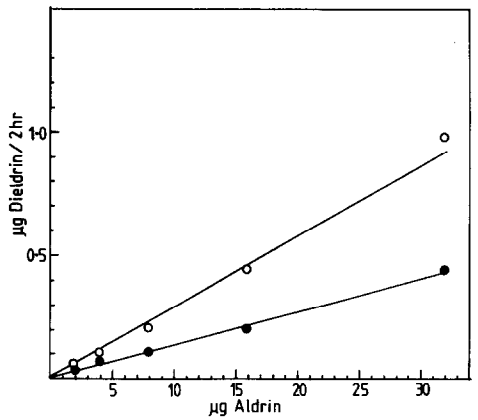

Fig. 1. Effect of aldrin concentration on the rate of dieldrin formation. Reaction conditions were as described in the Experimental using the equivalent of 40 mg nodule tissue/assay. The rates with added NADPH (●) and without NADPH (○) are shown.

The determination of an exact Michaelis constant for aldrin epoxidase is difficult. The concentration of lipid-soluble aldrin at the site of enzyme activity depends on the aldrin supplied and the proportion of the total lipid phase material in the preparation represented by the active membrane tissue. To ensure reproducible activity in assays of crude material, a standard ratio of aldrin–nodule tissue was necessary.

The conversion of aldrin to dieldrin exceeded 3% for the same tissue level (equivalent to 20 mg fresh nodule tissue) when the incubation was carried out in the absence of an NADPH-generating system. Table 1 shows the relation between the amount of tissue vs the net dieldrin

Table 1. Effect of tissue level and NADPH generating system on aldrin epoxidation

Nodule tissue (g)	pmol dieldrin/2 hr +NADPH	−NADPH
0	0	0
0.02	52	100
0.04	40	106
0.08	44	106
0.10	42	104
0.02 (root tissue)	36	27

Dieldrin produced from 1 μg aldrin in 2 hr incubation with a ±NADPH generating system.

production using crude soybean brei. The amount of dieldrin (pmol) produced was constant for the substrate (1 μg) at different tissue levels tested (20–100 mg). Apparently, the increase in active tissue was just compensated by the decreased aldrin concentration.

To establish an oxygen requirement for the enzyme reaction, assays were carried out in the presence and absence of atmospheric oxygen. Anaerobic conditions were maintained in the vials using rubber septa and flushing with argon by prior evacuation. No dieldrin production was obtained without oxygen, as expected for mono-oxygenase activity.

The time-course for aldrin epoxidation is shown in Fig. 2. Without added NADPH, a continuing dieldrin production with time was obtained, whereas with added NADPH the rate declined following the initial 60 min burst. In experiments under the same conditions with added dieldrin (20, 40 pmol) little or no disappearance of dieldrin was observed from the system for up to 3 hr. This finding suggests that the NADPH-generating system was responsible for an inhibition of dieldrin formation and not for accelerated removal of the dieldrin produced.

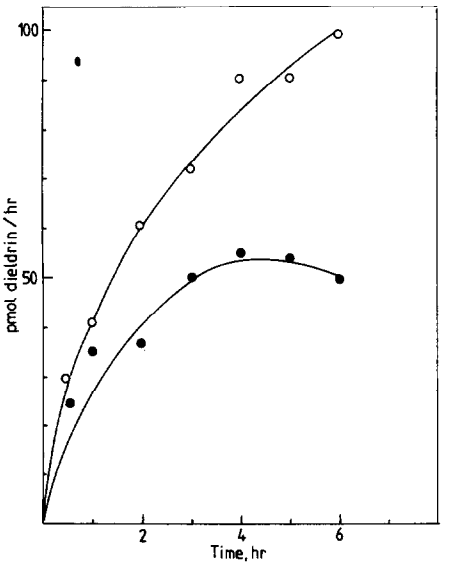

Fig. 2. Time course of aldrin epoxidase activity. Reaction conditions were as described in the Experimental with 1 μg of aldrin/0.3 mg protein (equivalent to 20 mg fr. wt of nodule tissue). Activity with added NADPH (●) and without NADPH (○) are shown.

The capacity of *Rhizobium* bacteroids to metabolize aldrin was tested. The result suggested that bacteroids were inactive in epoxidation in the standard assays.

NADPH inhibition

The action of an added NADPH-generating system with varying substrate level, time of reaction and level of nodule brei is shown in Figs 1 and 2 and in Table 1. With these nodule breis, added NADPH unexpectedly inhibited dieldrin formation.

An inhibitory action of added NADPH was noted earlier [13, 14] for root homogenates only at low tissue levels (< 500 mg/6 ml of assay medium). However, we could not find any decrease in the inhibition from NADPH for up to 500 mg nodule tissue/ml of assay medium. It was suggested earlier [13] that an excess of cofactors (NADPH and NADH) over plant tissue was inhibitory in some way, but we consider it more likely that high tissue levels depleted the amount of oxygen required for expression of the inhibition in the earlier work.

To show that the inhibitory action was due to NADPH, experiments were conducted with components of the NADPH-generating system in the standard assays. There was no difference in dieldrin formation with $NADP^+$ or glucose-6-phosphate separately when compared with the control (−NADPH) (Table 2). However, when mixed in the brei with the glucose-6-phosphate dehydrogenase, the activity dropped to nearly half that of the control, apparently as a result of NADPH formation. NADPH itself was added to the brei at three concentrations, producing a proportional decrease in the activity (Table 2). Thus, NADPH was the apparent source of inhibition.

The nature of the NADPH inhibition was further investigated by partitioning nodule brei into particulate and soluble fractions by centrifugation at 10 000 *g*. This is sufficient to sediment large organelles (bacteroids, mitochondria, plastids and microbodies) but not membranous materials, such as microsomes. The NADPH inhibition

Table 2. Effect of cofactors on epoxidase activity

Substrate (μmol)	pmol dieldrin min/mg protein
−NADPH generating system	2.2
NADP (0.175), glucose-6-phosphate (3.5), glucose-6-phosphate dehydrogenase	1.0
NADP (0.175)	2.4
NADP (0.35)	2.0
NADP (0.70)	1.8
Glucose-6-phosphate (3.5)	2.2
Glucose-6-phosphate (7.0)	2.2
NADP (0.35), glucose-6-phosphate (3.5), glucose-6-phosphate dehydrogenase	1.0
NADPH (0.35)	0.9
NADPH (0.70)	0.4
NADPH (1.40)	0.2

Dieldrin produced from 1 μg aldrin in 1 hr incubation at varying components of NADPH generating system.

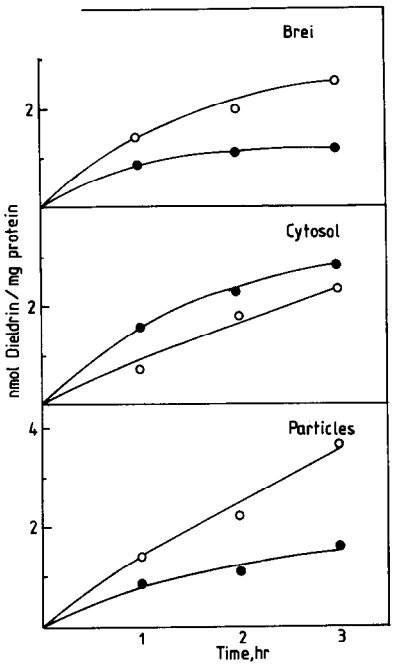

Fig. 3. NADPH inhibition of aldrin epoxidase activity in fractions of nodule brei. Brei was prepared as described in the Experimental and partitioned into particle and cytosol fractions by centrifugation for 15 min at 10000 *g*. Epoxidase activity in the presence (●) and absence of NADPH (○) are shown.

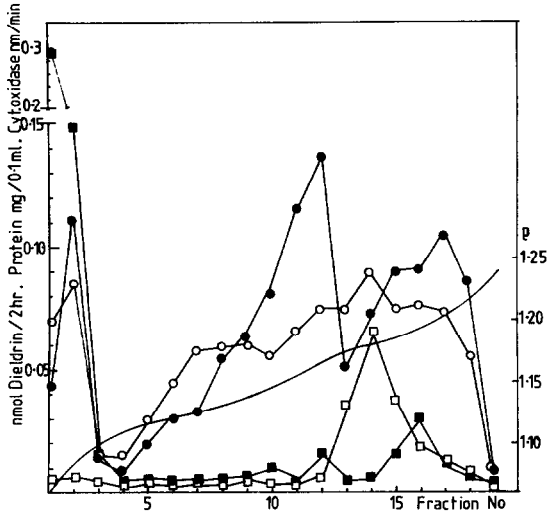

Fig. 4. Sucrose density gradient fractions of aldrin epoxidase activity. Brei (5 ml) equivalent to 1 g of nodule (fr. wt) was layered on a 20–50% (w/w) sucrose gradient prepared as described in the Experimental. The distributions of epoxidase [(●), +NADPH; (○), −NADPH], cytochrome oxidase (□), protein (■) and density (——) of 2 ml fractions are shown. Peaks for mitochondria and bacteroids are indicated by high values for cytochrome oxidase (tube 14) and protein (tube 16), respectively.

was most severe when both soluble and particulate fractions were involved, but was replaced by activation in the case of NADPH added to the soluble fraction (Fig. 3). This is suggestive that further metabolism of added NADPH is required to cause the inhibition, perhaps involving conversion of other soluble molecules. NADPH inhibition was much less or absent with root homogenates (Table 1).

Sucrose density gradients

To locate the site(s) of activity in the nodules, the crude brei was fractionated with a 20–50% linear sucrose density gradient [16]. The findings, shown in Fig. 4, were as follows. (1) Dieldrin formation was catalysed by both soluble and particulate fractions of soybean. A pronounced shoulder of activity at density *ca* 1.13 corresponds to smooth microsomes derived from the endoplasmic reticulum. The marker enzyme NADH–cytochrome *c* reductase was used to confirm the microsomal nature of this activity [16]. (2) Nodules contained most of the activity in higher density fractions, possibly corresponding to rough microsomes ($\rho = 1.17$) or bacteroids (Fig. 4). However, the activity in the bacteroid region could also be due to the peroxisomal ghosts of microbodies reported to have an equilibrium density of 1.21 [19]. (3) NADPH inhibition of epoxidation was less in gradient-fractionated extracts. In the main particulate fractions, added NADPH stimulated epoxidation. This is consistent with diminished NADPH inhibition when soluble and particulate material is separated, as suggested in Fig. 3. (4) A heat-labile enzyme was present in the soluble fractions capable of converting aldrin to its epoxide, dieldrin, but not requiring NADPH. This corresponded to *ca* 12% of the nodule aldrin epoxidase activity. (5) Although it has been shown that soil bacteria can catalyse dieldrin formation from aldrin [8, 9], it is not yet clear if bacteroids have any endogenous activity. Possibly an induction process would be required.

Anaerobic gel filtration

Anaerobic gel filtration of crude nodule brei removed factors which otherwise reduced aldrin epoxidase activity in the presence of added NADPH. This method [15] was effective in the case of soybean root nodules, removing low MW material and smaller proteins including peroxidases and leghaemoglobin. As seen in Fig. 5, the turbid fractions excluded from the gel showed higher activity with added NADPH. This finding confirms that the inhibition caused in the crude nodule brei with added NADPH also required other low MW materials, as suggested earlier. Turbidity was a useful indicator for the particulate membrane material containing epoxidase activity (Fig. 5). Some aldrin epoxidase activity also occurred in material not excluded by Sephadex G-100 and, therefore, soluble but this activity was not stimulated by NADPH, in agreement with results from the sucrose density gradients.

CONCLUSION

It is concluded here that the major aldrin epoxidase activity of soybean nodule is a mono-oxygenase activity behaving similarly to that in legume roots reported elsewhere. However, nodules showed a 2–4-times greater activity per unit fr. wt of tissue than the roots. In addition,

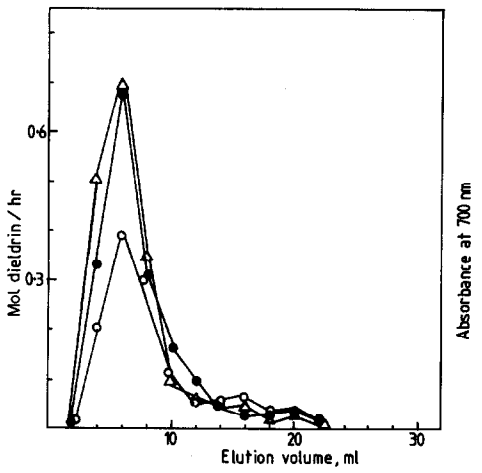

Fig. 5. Anaerobic gel filtration (Sephadex G-100) of crude brei. Brei (5 ml) was prepared with 1 g nodules (fr. wt) and eluted through a Sephadex column as described in the Experimental. Epoxidase activity in the particulate and soluble fractions [(●) +NADPH; (○), −NADPH], and turbidity (△) are shown.

breis from nodules showed substantial activity in the absence of added NADPH; apparently some other source of electrons was supplying aldrin epoxidase in this case, as suggested earlier for pea roots [15]. A small amount of heat-labile soluble activity (12%) was found in the nodule density gradients and by gel filtration. Further studies are in progress on the detailed location of enzymes for detoxification in nodules.

EXPERIMENTAL

Plant material. Soybean (*Glycine max*; Forrest 78-6003) seeds were surface-sterilized by soaking for 15 min in 70% EtOH and then washed several times with tap and distilled H_2O. Seeds were inoculated with *Rhizobium japonicum* (CB 1809) and planted in Perlite medium and provided with the following N-free nutrient soln at pH 6; $MnSO_4$ (0.004 mM), K_2SO_4 (0.080 mM), $MgSO_4$ (0.060 mM), $CaCl_2$ (0.040 mM), KH_2PO_4 (0.040 mM), $NaNO_3$ (0.070 mM), sequestrene 138 (Fe-chelate) 7 mg/l.), H_3BO_3 (0.700 μM), Na_2MoO_4 (0.160 μM), $ZnSO_4$ (0.060 μM), $CuSO_4$ (0.025 μM) and $CoCl_2$ (0.015 μM) [17] while growing in a greenhouse. Plants were removed at 4 weeks and the roots washed under running tap H_2O. Nodules were detached by hand and used for aldrin epoxidase preparations.

Tissue extract. Nodules were partially dried with paper towelling and homogenized in a deoxygenated sucrose (0.3 M)–KPi (0.1 M) buffer pH 7 (5 ml/g fr. wt) with 2% w/v Polyclar AT (insol. PVP) under N_2 in a glass mortar kept below 4°. The resulting slurry was squeezed through cheese-cloth and centrifuged in a refrigerated IEC B20A centrifuge at 1000 *g* for 10 min. The pellets were discarded and the supernatant (crude brei) was used for epoxidase assays.

Epoxidase assay. To a 12 mm diameter glass vial (3 ml) was added 0.35 μmol NADP, 3.5 μmol glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase and crude brei (equivalent to 20 mg nodule fr. wt), and the reaction was initiated by adding aldrin (1–10 μg) in methyl cellosolve (1–10 μl). The total vol. was made to 1 ml with 0.1 M KPi buffer. Reaction mixtures were shaken at 28° for a specified time and the reaction was terminated with 2 ml of redistilled hexane–isopropanol (1 : 1) by shaking on a vibrator, samples were stored below −10°. The clear supernatant of the extracting solvent was injected (1 μl) into a GC equipped with ^{63}Ni (8 mCi) e.c. detector (Varian model 3700). The conditions for GC were: column temp. 181°, injection temp. 199°, detector temp. 228°, N_2 flow 70 ml/min. A glass column (1.6 M × 3 mm i.d.) was packed with 5% OV-210 on Chromosorb W HP, 80–100 mesh.

Sucrose density gradient (20–50%, *w/v*). Sucrose (50%) in KPi buffer (5 ml) was transferred to centrifuge tubes by syringe. Solns (5 ml) of sucrose in KPi buffer (45%, 40%, 35%, 30%, 25% and 20%) were successively layered and the tubes allowed to stand for 15–20 hr. at 2° before use. Fresh homogenate (2 ml) was layered on to the top of the gradients and the tubes were centrifuged at 25 000 rpm for 150 min in a Beckman L2-65 ultracentrifuge using the swing-out rotor (SW-27). Sucrose gradients were fractionated into 2 ml samples by displacement with 60% sucrose soln, the refractive index of the samples was measured and the density determined.

NADH–cytochrome c reductase. NADH–cytochrome *c* reductase activity was measured by the NADH-dependent increase in *A* at 550 nm [20] using an extinction coefficient of 21/mM · cm.

Cytochrome oxidase. Cytochrome oxidase activity was determined from the decrease in *A* at 550 nm [16] after reduced cytochrome *c* (0.02 μmol) and buffer alone had been incubated for 5 min at 30°.

Protein. Protein was measured by the Coomassie brilliant blue dye binding assay [18].

Anaerobic gel filtration. Crude filtrate (5 ml) was transferred to a small Sephadex G-100 column (1.6 × 26 cm) equilibrated in the correct degassed buffer under Ar. Fractions (2 ml) were collected and each tube assayed for aldrin epoxidation ± NADPH.

Turbidity. The *A* at 700 nm was measured against KPi buffer.

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