Oxidative Metabolism of Aldrin and Isodrin by Bean Root Fractions

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Homogenates prepared from the excised roots of bean seedlings oxidized aldrin to its epoxide, dieldrin, and to small amounts of a compound chromatographically similar to aldrin diol (5,6,7,8,9,9-hexachloro-1,2,3,4,4a,5,8,8a-octahydro-*trans*-1,4-5,8-dimethano-naphthalene-2,3-diol). The system also oxidized isodrin, producing a compound corresponding chromatographically to endrin ketone (1,8,9,10,-11,11-hexachloro-pentacyclo [6.2.1.1^{3,6}.0^{2,7}.0^{4,10}] dodecan-5-one). No metabolism of heptachlor was detected. Similar results were obtained with pea seedling root homogenates but not with corn. On an equal weight basis, pea root homogenates were less than one-half as active as bean. The oxidase system was most active at pH 6.5 (phosphate) and the optimum seedling age was 9 to 21 days. NADPH stimulated the reaction at high tissue levels, but was inhibitory at low tissue levels. The active fraction of the bean root homogenates was stable for 44 days when stored at sub-zero temperatures. There was no evidence that dieldrin is the intermediate in the formation of aldrin diol.

Recent studies of enzyme systems isolated from plant tissue have shown that the common plants can perform many of the pesticide metabolizing reactions exhibited by animal systems. These reactions include hydroxylation (Vaughan and Butt, 1969), *N*-demethylation (Frear *et al.*, 1969), epoxidation (Lichtenstein and Corbett, 1969), and the "NIH shift" (Russell *et al.*, 1968).

The use of *in vitro* methods in the study of pesticide metabolism by plants has certain advantages which warrant further development of the methods. The reactions can be conducted in the dark, thus eliminating nonenzymatic light-induced products, and losses of substrate and product by evaporation and other physical factors can be controlled. The time required for a study is short, a few hours compared to days when intact plants are used. The products of the reactions are more readily removed from the system and there is less likelihood of the presence of secondary products such as glucoside conjugates. Perhaps the main advantage of the *in vitro* system is the opportunity it affords to study the primary or initial steps in a metabolic process that may ultimately result in several products. Thus, as a supplement to traditional studies with intact plants, *in vitro* methods seem to have considerable merit.

Lichtenstein and Corbett (1969) were able to show only one product (dieldrin) when aldrin was incubated with various fractions of pea root homogenates. The system used did not epoxidize isodrin or heptachlor and did not require NADPH. We report additional work with bean root homogenates which has resulted in a more complete understanding of these enzymes, and shows that they are more active than pea root homogenates and that they produce at least two metabolites of aldrin and one of isodrin.

MATERIALS AND METHODS

Chemicals. Analytical grade samples of aldrin, dieldrin, isodrin, endrin, aldrin diol (5,6,7,8,9,9-hexachloro-1,2,3,4,-4a,5,8,8a-octahydro-*trans*-1,4-5,8-dimethano-naphthalene-2,3-diol), and endrin ketone (1,8,9,10,11,11-hexachloro-pentacyclo [6.2.1.1^{3,6}.0^{2,7}.0^{4,10}] dodecan-5-one) were supplied by the Shell Chemical Co. and heptachlor and heptachlor epoxide by Velsicol Corp. The purity of these compounds was confirmed by the absence (except in the case of endrin ketone, as discussed later) of extraneous peaks in gas chromatograms of standards or fortified tissue controls. SKF 525-A (β-diethylaminoethyl α-diphenyl β-dimethyl propionate HCl) was obtained from Smith, Kline and French Laboratory, sesamex [2-(2-ethoxyethoxy)ethyl 3,4-methylenedioxyphenyl acetal of acetaldehyde] from Shulton, Inc., and safrole (3,4-methylenedioxyallylbenzene) from Morton Beroza, U.S. Department of Agriculture, Beltsville, Md. Glucose 6-phosphate (G-6-P), G-6-P dehydrogenase, NADP, NADH, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. All other chemicals and solvents used were analytical reagent grade.

Plant Materials. Seeds of bean (*Phaseolus vulgaris*, Dwarf Horticulture variety), pea (*Pisum salivum*, Freezer variety) and corn (*Zea mays*, Golden Bantam variety) were surface sterilized by soaking in a 0.5% aqueous solution of sodium hypochlorite for 10 min. After washing with distilled water they were planted in vermiculite flats in the greenhouse and supplied with Hoagland's solution. Water was added as necessary. Unless otherwise indicated, the seedlings were used when the stems were about 15 cm tall, approximately 2 weeks after planting. The roots were cleaned with running tap water before use. They were then washed in ice-cold glass-distilled water and partially dried with paper towels.

To ascertain if the enzyme activity was due to microorganisms associated with the plant roots, some seeds were germinated under asceptic conditions. Two-liter Erlenmeyer flasks containing 110 g of vermiculite and 300 ml of half-strength nutrient solution containing chloramphenical ($5 \times 10^{-6}M$) were sterilized in an autoclave for 1 hr. They were sterilized once more. Fifteen surface sterilized bean seeds were then placed on the vermiculite in each flask. The flasks were again plugged with cotton and kept in the laboratory under cool white fluorescent lamps (10 hr of light daily) for 2 weeks. Nonsterilized vermiculite was used as control treatment.

Tissue Preparations. Ten grams of roots were homogenized in 15 ml of 0.1M sodium phosphate buffer, pH 6.5 in a Virtis 45 homogenizer, operated at maximum speed for 30 sec. The crude homogenate was squeezed through cheesecloth and the filtered homogenate was centrifuged at 22,000 $\times$ G for 25 min in a Servall refrigerated centrifuge. The pellet was discarded and the supernatant, termed the 22,000 $\times$ G supernatant, was recentrifuged in the Spinco Model L ultracentrifuge at 105,000 $\times$ G for 2 hr. The final supernatant was termed the soluble fraction. The pellet obtained from each preparation was resuspended in an appropriate volume of 0.1M sodium phosphate buffer, pH 6.5, to make a final concentration equivalent to 0.5 g of plant tissue per ml. The

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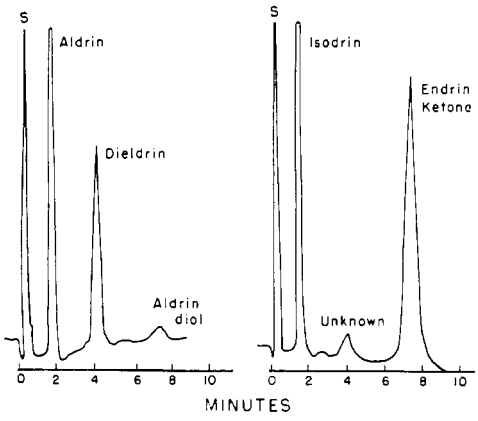

Figure 1. Gas chromatograms of products of isodrin and aldrin metabolizing systems. Glc on QF-1/DC-11 at 190° C

above procedures were conducted at 0° to 2° C. All fractions were used immediately after preparation.

The protein concentration of each preparation was determined according to the method described by Lowry *et al.* (1951), employing bovine serum albumin as a standard.

Incubation Procedure. The standard 6 ml incubation mixture consisted of 2 ml of 22,000 × G supernatant (equivalent to 1 g of plant tissue); 0.1*M* sodium phosphate buffer, pH 6.5; 1.8 μmoles of NADP; 18 μmoles of glucose 6-phosphate; and 1 unit of glucose 6-phosphate dehydrogenase. The reaction was initiated by addition of 20 μg of the appropriate substrate in 0.2 ml of methyl cellosolve and incubations were carried out with shaking (120 oscillations per min) at 37° C in an atmosphere of air for 2 hr. Enzyme preparations incubated without substrate and boiled preparations incubated with substrate were used as controls. All incubations were in duplicate.

To determine if the products obtained were light-induced by exposure to laboratory fluorescent lights, one experiment was also carried out in the dark. The incubation flasks were wrapped in aluminum foil and the experiments were conducted as before.

Analysis. The reaction was stopped by shaking with 10 ml of 2-propanol-hexane (2 to 3, v/v) mixture. This phase was retained and the incubates extracted twice with hexane (10 ml). The combined extracts were then dried over anhydrous sodium sulfate and routinely analyzed for metabolites in a Varian Aerograph Model 1200 gas chromatograph equipped with an electron capture detector. The standard column was a 1/8 in. by 4-ft aluminum column packed with a 2.4 to 1 mixture of 7% QF-1 and 7% DC-11 on 100 to 120 mesh High Performance Chromosorb W. The operating conditions were: column, 190° C; injector port, 200° C; detector, 200° C; and nitrogen carrier gas, 50 ml per min.

Additional columns used to compare unknown metabolites with standard compounds were of borosilicate glass and were 1/8 in. × 4 ft, packed with QF-1 and DC-11 (as above), and operated at 175° C; 1/8 in. × 5 ft, packed with 2.7% QF-1 on 80/100 acid washed Chromosorb G operated at 195° C; and 1/8 in. × 5 ft, packed with 11% DC-200 (2,500,000 centistokes) and 0.01% Versamid 900 on 60/80 Gas Chrom Q operated at 211° C.

In addition, the procedure of Hancock (1969) was used to prepare the trimethylsilyl derivatives of the aldrin metabolites. These were compared with the trimethylsilyl derivative of authentic aldrin diol, using the QF-1/DC-11 column.

The more abundant isodrin metabolite (Figure 1) was also characterized by tlc. The systems used were: stationary phase, silica gel G; solvent system I, benzene:ethyl acetate, 3 to 1; II, hexane:acetone 19 to 1. The spots were detected with Kovacs reagent (Kovacs, 1966).

RESULTS

Nature of Metabolites. Typical chromatograms of glc resolution of aldrin and isodrin incubation extracts are illustrated in Figure 1. The unknown peaks were identified by gas chromatographic comparisons in three columns (see Methods) with authentic standards. The two aldrin metabolites corresponded exactly with dieldrin and dihydroaldrin transdiol in the three systems. The chromatograms of the trimethylsilyl derivative of aldrin diol and the second aldrin metabolite were also identical. These two metabolites, when both were present, were always found in approximately the same proportions. No further confirmation of their identity was attempted.

Recovery and substrate balance studies indicated that a small amount (2 to 5%) of the substrate was converted to water soluble or nondetectable products during the incubations. For example, 91 to 93% of the aldrin (20 μg) added to a typical incubation mixture containing inactivated (boiled) enzyme was recovered by the standard extraction procedures. With an active system, however, aldrin recoveries, including that accounted for as dieldrin and aldrin diol, were slightly less, about 88%. Also, when ^{14}C-labeled aldrin was the substrate in incubations containing active and inactive enzyme preparations, there was a slight difference in the amount of radioactivity present in the aqueous phase, 6.4% ±0.29 with the active samples (average of three incubations) and 4.6% ± 0.36 with the boiled samples (average of three). No attempts were made to isolate or identify these products.

No aldrin-metabolizing activity could be seen when the enzyme source was placed in boiling water for 15 min. When light was eliminated during incubation and extraction procedures, there was no change in the amount or nature of the metabolites. There was no significant lowering of enzyme activity in root homogenates from asceptically reared plants, indicating that the metabolism was not due to microorganisms associated with the roots.

The two glc peaks (Figure 1) obtained when isodrin was the enzyme substrate coincided exactly with those obtained when endrin ketone was chromatographed. This result occurred with each of the three glc columns tested. The smaller peak is probably due to an artifact of gas chromatography.

The other isodrin unknown was produced in quantities sufficient for tlc comparisons. In the two systems used (see Methods) this material coincided exactly with endrin ketone. No further confirmation of identity was attempted.

In tests with boiled tissue fractions, 92% of added isodrin was recovered. The sum of isodrin and the two metabolites after typical incubations accounted for 95% of the added substrate. The aqueous fraction was not examined, since these calculations indicated that only negligible amounts of unknown metabolites could be present.

Plant Source of the Enzymes. The aldrin-metabolizing activity observed in several root homogenate fractions obtained by differential centrifugation is shown in Table I. The results clearly indicate that maximum activity is associated with the 22,000 × G supernatant. This fraction contained an average of 4.9 mg of protein per g of root tissue. The lowest activity was found in the soluble fraction. This result differs from that of Lichtenstein and Corbett (1968), who studied pea

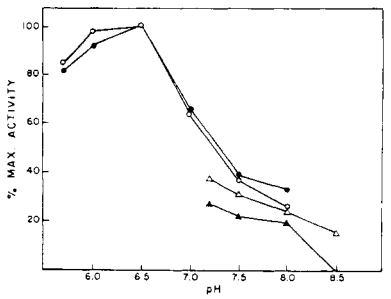

Figure 2. Effect of pH on aldrin-metabolizing activity of 22,000 × G supernatant of bean root homogenate. ○, dieldrin (phosphate buffer); ●, aldrin diol (phosphate buffer); △, dieldrin (Tris buffer); ▲, aldrin diol (Tris buffer)

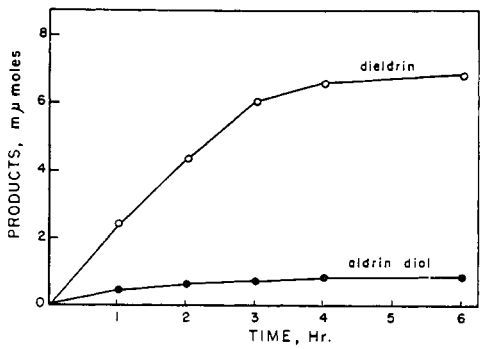

Figure 3. Rate of production of aldrin metabolites by 22,000 × G supernatant of bean root homogenate

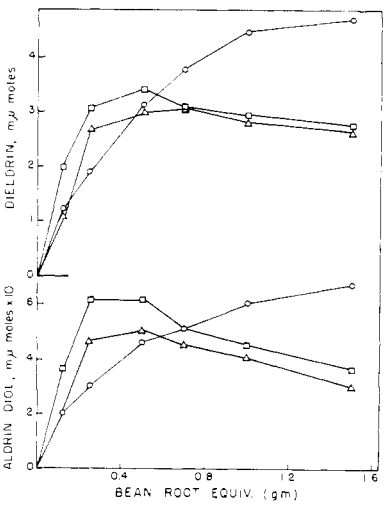

Figure 4. Effect of tissue level and cofactors on aldrin metabolism by 22,000 × G supernatant of bean root homogenate. Dieldrin production, upper graph; aldrin diol production, lower graph; ○, with NADPH; □, minus NADPH; △, with NADH

root homogenates and obtained the most enzyme activity from the soluble fraction.

In comparing roots, stems, and leaves of bean plants for epoxidase activity, using the 22,000 × G supernatant as enzyme source, it was found that roots were highest, about sixfold that of stems and tenfold that of leaves (Table II). There was no aldrin diol detected in the preparations from stems and leaves.

Pea root fractions were less than one-half as active as bean roots and produced relatively less aldrin diol. There was no detectable metabolism of aldrin in the corn root fraction.

Table I. Distribution of Aldrin-Metabolizing Enzyme Activity in Bean Root Homogenates

Homogenate fraction	Products, mμ moles/g roots/2 hr[a] dieldrin	aldrin diol
Total homogenate	3.40	0.63
22,000 × G pellet	3.36	0.62
22,000 × G supernatant	4.44	0.75
105,000 × G pellet	2.21	0.48
105,000 × G supernatant	1.80	0.27

[a] Average of two experiments, each incubation in duplicate.

Table II. Plant Source of Aldrin-Metabolizing Enzymes

Enzyme source[a]	Products, mμ moles/g roots/2 hr dieldrin	aldrin diol
Bean roots	4.36	0.85
Bean stem	0.72	0
Bean leaves	0.40	0
Pea roots	1.82	0.24
Corn roots	0	0

[a] 22,000 × G supernatant as enzyme source.

Addition of BSA (0.166%) to these incubation mixtures to remove inhibitors did not improve the results.

pH, Reaction Rate, and NADPH Requirements. The 22,000 × G supernatant of bean roots was used as enzyme source in these experiments. The effect of pH on the activity of the system was determined with two buffer systems, sodium phosphate and Tris (both 0.1*M*) over the pH range 5.7 to 8.5. The same buffer solution was employed for the preparation of the fractions. The aldrin-metabolizing enzyme showed less activity in Tris buffer than in phosphate buffer (Figure 2). The maximum enzyme activity, for both metabolites, was at pH 6.5 in phosphate buffer, declining rapidly as the pH was increased.

As indicated in Figure 3, the rate of aldrin epoxidation is linear up to approximately 3 hr. The amounts of substrate converted to dieldrin and aldrin diol in this experiment were 12.4 and 1.6%, respectively, after 6 hr incubation.

From Figure 4 it can be seen that an increase in enzyme level up to 1 g equivalent of plant tissue resulted in a corresponding increase in aldrin metabolism, although the increase was not proportional. The results also indicate the presence of endogenous NADPH. The addition of an NADPH-generating system enhanced the activity only when higher levels of the enzyme were used (above 0.52 g for aldrin epoxidation and 0.75 g for aldrin diol formation). When tissue levels were low, the addition of the NADPH-generating system was inhibitory. As seen in Figure 4, for example, at 0.2 g equivalent of root tissue, nearly twice as much dieldrin was produced without added NADPH as in its presence. This result was obtained consistently in many experiments, reversal of the situation occurring only at 0.5 g of root tissue and above. Apparently, the excess of cofactors at low tissue levels is inhibitory in some way. NADH was slightly inhibitory at all tissue levels. Because of these effects the standard incubation mixture was prepared to contain 1 g of tissue and an NADPH-generating system.

Effect of Storage. The stability of the aldrin metabolizing enzymes was investigated by storing the active fraction at 0° and −10° C for periods up to 44 days. Periodic assays indicated the level of activity compared to that of the fresh preparation (Figure 5). Storage at −10° C prolonged the enzyme activity and resulted in a significant increase (up to 45%) during the first 3 weeks. Activity remained at or above

Table III. Inhibition and Enhancement of Aldrin-Metabolizing Activity in Bean Root Homogenates

Addition to standard incubation mixture, conc.[a]		Activity, % of control: dieldrin	aldrin diol
None		100	100
$CuSO_4$	$10^{-3}M$	8	0
$CuSO_4$	$10^{-4}M$	17	25
$FeCl_3$	$10^{-3}M$	121	125
$FeSO_4$	$10^{-3}M$	77	50
$MnCl_2$	$10^{-3}M$	55	75
$MgSO_4$	$10^{-3}M$	125	125
$CaCl_2$	$10^{-3}M$	133	125
$CaCl_2$	$10^{-4}M$	118	113
KCN	$10^{-3}M$	73	50
Sesamex	$10^{-3}M$	61	60
Sesamex	$5 \times 10^{-4}M$	77	82
Sesamex	$5 \times 10^{-5}M$	91	98
Safrole	$10^{-3}M$	14	0
Safrole	$10^{-4}M$	97	87
SKF 525-A	$5 \times 10^{-4}M$	18	18
SKF 525-A	$5 \times 10^{-5}M$	69	68
N_2-atmosphere[b]		21	19
BSA	0.083% (w/v)	121	125
BSA	0.166% (w/v)	143	140

[a] 22,000 × G supernatant as enzyme source. [b] Flasks were purged with nitrogen for several minutes prior to incubation.

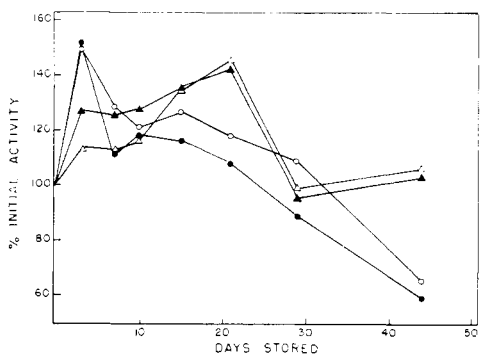

Figure 5. Storage stability of aldrin-metabolizing enzyme from bean root homogenate (22,000 × G supernatant). ○, dieldrin, 0°C; △, dieldrin −10° C; ●, aldrin diol, 0° C; ▲, aldrin diol −10° C. LSD ($p < 0.05$) for dieldrin production = 8.63; for aldrin diol production = 13.33

its initial value for 44 days. The enzyme stability was less at 0° C, showing an increase at 3 days, then declining to approximately 60% of the initial activity by the 44th day. Factorial analysis of variance also indicated a time-temperature interaction under these storage conditions. These results are similar to those obtained with aldrin epoxidase of rat liver microsomes (Chan and Terriere, 1969).

Effect of Metal Ions, Inhibitors, and Enhancers. It can be seen from Table III that, of those ions evaluated, the most effective inhibitor was $CuSO_4$ which, at $10^{-3}M$, resulted in more than 90% inhibition of the reactions. Some inhibition was observed with Fe^{+2}. These results differ from those of Lichtenstein and Corbett (1969), who found both of these ions to be quite stimulatory to their pea root epoxidase system. In agreement with these authors we find that Mn^{+2} is inhibitory. We also agree with their finding that Fe^{+3} and Mg^{+2} stimulate the plant epoxidase system. Other enzyme enhancers included Ca^{+2} and BSA. The latter result suggests that enzyme inhibitors may be present in the system (Tsukamoto and Casida, 1967).

SKF 525-A, safrole, and sesamex, which are known inhibitors of hepatic microsomal enzymes, also inhibited the bean root system. The requirement for oxygen was demonstrated by an 80% reduction in metabolism when the reaction was carried out in a nitrogen atmosphere.

Age of Seedlings. The effect of age of the bean seedlings on aldrin metabolism was studied in an experiment in which planting dates were varied, so that all assays could be done on the same day. The age for maximum enzyme activity was between 9 and 21 days (the differences were not significant), but after 21 days the activity declined (significant at 5% level).

Substrate Specificity. Lichtenstein and Corbett (1969) were unable to detect any epoxidation of isodrin or heptachlor when these compounds were incubated with pea root homogenates. As indicated in Figure 1, bean root homogenates had considerable activity against isodrin, producing one metabolite resembling endrin ketone and the other unidentified. Identical chromatograms were obtained when the incubations and the extractions were conducted in the dark. The results, with isodrin as substrate, were somewhat different than with aldrin. No epoxide (endrin) could be detected, NADPH was less stimulatory, and the product yield (endrin ketone) was higher, up to 29% conversion occurring in a 6-hr incubation. Oxygen was required. The maximum conversion with aldrin as substrate was 12.4%. When endrin was the substrate, significant amounts of the ketone (about 8%) were produced, but this was found to be nonenzymatic.

Heptachlor, incubated at the same level as aldrin and isodrin, was not attacked by the enzyme systems. The possibility that this result was due to strong inhibition by heptachlor epoxide was checked in additional experiments in which mixtures of aldrin and heptachlor epoxide (20 and 2 μg, respectively) were incubated up to 2 hr with the active fraction of bean root homogenates. In this case, production of dieldrin and aldrin diol was normal, indicating that the added heptachlor epoxide was not inhibiting. Thus, the failure of the system to convert heptachlor to its epoxide is unexplained.

DISCUSSION

These results confirm those of Lichtenstein and Corbett (1969), who demonstrated aldrin epoxidation in pea root homogenates. Although the present investigation was limited to three plant species and to three substrates, the evidence suggests that plant enzymes oxidizing these pesticides may be more specific than the corresponding animal enzymes and less widely distributed. Thus, no metabolism could be detected in the corn root homogenates, nor were bean or pea root homogenates capable of converting heptachlor to its epoxide. These apparent differences in the nature and distribution of the enzymes may disappear when further studies of plant systems reveal the optimum conditions for each species.

It seems generally agreed that with animal systems (Brooks, 1969) and soil microorganisms (Matsumura and Boush, 1967; Wedemeyer, 1968; Matsumura *et al.*, 1968), dieldrin is the precursor of aldrin diol, in addition to other metabolites. With bean root homogenates, however, it seems unlikely that this is the case. This is concluded from the fact that, in several incubations in which dieldrin was the substrate instead of aldrin, we were unable to detect any metabolism. The levels used (from 0.25 μg to 5.0 μg) were in the range found in the normal incubations with aldrin as the substrate.

When aldrin was the substrate there was no evidence of a lag in the formation of aldrin diol, although incubation periods shorter than 1 hr were not tested. There was no evidence of accelerated production of the diol as the dieldrin levels in the

system increased (Figure 3). The evidence thus suggests that two different enzymes systems are involved, with aldrin as the common substrate—one producing dieldrin and the other producing aldrin diol.

If two independent systems attack aldrin, their conditions for optimum activity must be identical. This is indicated by the similarity of their pH optima, storage stability, NADPH and tissue level requirements, plant age for optimum activity, and the nearly constant ratio of the products obtained under various conditions of study.

In their report of their work with the epoxidase system of pea roots, Lichtenstein and Corbett (1969) do not indicate whether the pH used (7.0) was optimum for the system. If it was not, their failure to detect aldrin diol or to show a requirement for NADPH might be due to this factor. As shown in Table II, the amount of aldrin diol produced (at pH 6.5) is small and under more adverse conditions may be too low for detection.

It is clear from these experiments that the bean root homogenate, and to a lesser extent pea roots, contains sufficient enzyme activity of the oxidative type for practical use in the study of pesticide metabolism. The reactions are characteristic of those of the mixed function oxidases.

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