A Rapid Method for Estimating Microsomal Oxidase Activity in the Housefly with [^{14}C]Parathion as Substrate

Ki Soon Rhee and Frederick W. Plapp, Jr.*

A sensitive method was developed for rapid estimation of microsomal oxidase activity in the housefly using [^{14}C]parathion as the substrate. The method measures both activation and degradation of parathion *in vitro*. The procedure is also applicable to enzyme preparations from other insects. In addition, it can be used to measure induction of microsomal activity in the housefly. Microsomal metabolism measured by this method was enhanced by a low concentration of bovine serum albumin, but higher concentrations resulted in a decrease in metabolism. Relative activities determined by this procedure were comparable with those obtained by the more commonly used but slower method of cyclodiene epoxidase measurement.

A great variety of structurally unrelated pesticides are oxidized by animal microsomes. This system is probably the most important detoxication mechanism available to animals to dispose of insecticides. Microsomal oxidase activity can be determined *in vitro* by measuring various chemical reactions, such as aromatic hydroxylation, O- and N-dealkylations, *N*-methyl hydroxylation of aliphatic hydrocarbons, oxidation of phosphorothionates, and epoxidation of cyclodienes.

The sensitivity of methods utilizing these reactions can be increased by using radioactive substrates. The measurement of epoxidation of [^{14}C]aldrin is one of the most common procedures. This method, however, is very time consuming and laborious because it includes, in addition to an incubation step, repeated extraction of the incubate with a solvent, evaporation of pooled solvent extracts, thin-layer chromatography of the residue, radioautography of the developed plate to visualize substrate and metabolite, and scraping of the radioactive regions followed by scintillation counting.

To improve the efficiency of procedures for measurement of oxidative metabolism, we have developed a technique utilizing [^{14}C]parathion as the substrate. The method is of equal sensitivity to standard assays employing [^{14}C]aldrin and is considerably faster.

Nakatsugawa and Dahm (1965) first reported the oxidative microsomal metabolism of parathion in insects using the American cockroach fat body. Major products of *in vitro* parathion metabolism by insects, in the presence of NADPH, are paraoxon, diethyl phosphorothioic acid (DEPTA), and diethyl phosphoric acid (DEPA) (Nakatsugawa *et al.*, 1968, 1969). The same microsomal oxidase system which catalyzes the activation of parathion to paraoxon degrades parathion and paraoxon to DEPTA and DEPA, respectively. 4-Nitrophenol is the accompanying degradation product. In rabbit liver microsomes the ratio of *in vitro* activation to degradation of parathion was found to range from 1:1 to 1:2 (Nakatsugawa and Dahm, 1967). No report of *in vitro* degradation–activation ratio has been made for insect microsomes.

When an incubate of microsomal oxidases, parathion, and NADPH is partitioned with a nonpolar organic solvent, the organic phase contains unmetabolized parathion and the activation product paraoxon, while the water-soluble degradation products DEPTA and DEPA partition into the aqueous phase. With [^{14}C]parathion as substrate, *in vitro* microsomal metabolism is estimated either by determining paraoxon content of the organic phase by thin-layer chromatography or by direct radiocarbon counting of the aqueous layer to determine degradation products.

Department of Entomology, Texas A&M University, College Station, Texas 77843.

The method we have developed estimates both activation and degradation of parathion *in vitro*. It is based on the fact that paraoxon is much more readily degraded by an alkali treatment to DEPA than parathion, which eventually degrades to DEPTA.

MATERIALS AND METHODS

Materials. *Reagents.* Ethyl-[1-^{14}C]parathion (1.54 mCi/mmol, 98% purity) was obtained from Mallinckrodt Chemical Co., St. Louis, Mo. Phenyl-^{14}*C*-labeled paraoxon (22.5 mCi/mmol) was a gift from Dr. T. Nakatsugawa, State University of New York, Syracuse, N. Y. It was synthesized from [1-^{14}C]-4-nitrophenol and purified by column chromatography (Appleton and Nakatsugawa, 1972). NADPH was purchased from Sigma Chemical Corp., St. Louis, Mo.

Housefly Abdomen Homogenates and Microsomes. Four housefly (*Musca domestica* L.) strains were used: Orlando-DDT and Orlando-Regular, with low microsomal oxidase activity, and R-Baygon and R-Fc with high oxidase activity (Plapp and Casida, 1969; Schonbrod *et al.*, 1968).

Female flies at 2 to 4 days after emergence were killed by freezing and their abdomens were cut off on Dry Ice. The abdomens were homogenized in 0.2 *M* potassium phosphate buffer (pH 7.8) using an ice bath and a Teflon pestle (Hansen and Hodgson, 1971). The homogenate was filtered through a compact layer of glass wool, maintained on ice, and used within 15 min of preparation.

Where microsomes were used instead of a homogenate, the homogenate was centrifuged at 18,000 × *g* for 15 min and the precipitate was discarded. The supernatant was recentrifuged at 74,000 × *g* for 60 min and the microsomal pellet resuspended in phosphate buffer.

For measuring induction of microsomal oxidase activity, 15 female flies, 4- to 6-days old, were exposed in glass jars to a film of Aroclor 1254. This chemical, a polychlorinated biphenyl preparation with 54% chlorine (Monsanto Chemical Co., St. Louis, Mo.), was used as described by Rhee and Plapp (1973). The flies were supplied with water and food. After 7 days of exposure to the chemical, abdomen homogenates were prepared as previously described.

Other Insect Microsomal Oxidase Preparations. Microsomes were prepared as described for houseflies from last instar larvae of the tobacco budworm (*Heliothis virescens* F.) and the bollworm (*Heliothis zea* Boddie). The former species is known to be resistant to organophosphate insecticides and to contain a high level of microsomal oxidases (Plapp, 1973).

Recommended Method. A typical 3-ml incubation mixture contained 2 ml of microsomal enzyme preparation in 0.2 *M* potassium phosphate buffer (pH 7.8), 1 mg of bovine serum albumin (BSA) in 0.9 ml of distilled water, 4 nmol

Table I. Conversion of Parathion and Paraoxon to Water-Soluble Metabolites in the Presence of Alkali[a,b]

Treatment time, min	% radioactivity in the aqueous layer					
	0.1 *N* NaOH		0.5 *N* NaOH		1.0 *N* NaOH	
	Parathion	Paraoxon	Parathion	Paraoxon	Parathion	Paraoxon
5	0.9	9.7	3.2	27.0	9.6	57.7
10	1.3	10.8	5.5	39.8	20.0	81.0
15	1.7	12.7	7.4	48.2	27.2	91.6
30	2.5	18.2	13.1	72.0	46.8	98.4
45	3.1	25.9	18.8	74.5	59.5	99.3
60	3.8	29.1	23.2	86.7	62.8	99.4

[a] Parathion; 4 nmol, ethyl-*1*-^{14}*C*-labeled. [b] Paraoxon; 4 nmol, phenyl-^{14}C-labeled.

of parathion (ethyl-*1*-^{14}*C*) in 20 μl isopropyl alcohol, and 0.5 μmol of NADPH in 0.1 ml of phosphate buffer (0.2 *M*, pH 7.8). The final concentration of the phosphate buffer was 0.14 *M*.

All incubation components except NADPH were placed in a 25-ml erlenmeyer flask on ice and the flask contents equilibrated in a shaking incubator at 32° for several minutes before initiating the enzymatic reaction by NADPH addition. Incubation was carried out aerobically for 15 min with constant shaking.

The reaction was terminated by the addition of 1 ml of NaOH (0.1 *N*), followed by 15 min of shaking at 32° to obtain cleavage of paraoxon. After alkali treatment, 5 ml of chloroform was added, the contents shaken vigorously for a few minutes, and the flask placed on ice. Upon cooling, the flask contents were transferred to a 10-ml stoppered centrifuge tube, shaken again, and placed on ice.

The samples were centrifuged in a clinical centrifuge for 15 min. A 0.5-ml aliquot of the aqueous (top) layer was counted for radiocarbon content with a liquid scintillation spectrometer (Packard) in 4.4% Packard "Permafluor™ (25×)" in toluene–ethylene glycol monomethyl ether (2:1, v/v) mixture. It was possible to count a greater volume, *i.e.*, 2 ml of aqueous layer using 15 ml of "Aquasol" liquid scintillator (New England Nuclear).

The reagent blank, containing all incubation components but NADPH, was also carried through the procedure. The blank counts were subtracted from those of samples.

Variation between duplicates averaged 3%, with a range of 0–7%.

RESULTS

Effect of Alkali Treatment on Parathion and Paraoxon. Parathion and paraoxon can be hydrolyzed nonenzymatically by alkali to DEPTA and DEPA, respectively. However, paraoxon is more sensitive to alkali than parathion. The data in Table I show this differential effect of alkali on parathion and paraoxon. The samples (3 ml) included all incubation constituents except enzyme preparation, together with 4 nmol of either parathion or paraoxon. The final concentration of phosphate buffer was 0.14 *M*, the same as the enzyme incubate. After adding 1 ml of 0.1, 0.5, or 1.0 *N* NaOH to the samples, they were shaken in the 32° water bath for 5 to 60 min and handled as previously described.

Paraoxon cleavage was nearly complete after 30 min of 1.0 *N* NaOH treatment. However, the same treatment also hydrolyzed half of the substrate, parathion, resulting in too high blank values in the actual enzyme assay. The 0.1 *N* NaOH treatment yielded about 30% cleavage of paraoxon in 60 min, whereas parathion hydrolysis was about 4%. For any given treatment time, the degradation of paraoxon by 0.1 *N* NaOH was over seven times that of parathion. The effect of 0.5 *N* NaOH was intermediate between those of 0.1 *N* and 1.0 *N* NaOH. Of all possible combinations of NaOH strength and treatment time, 15 to 60 min with 0.1 *N* NaOH seemed most reasonable for practical enzyme assays. Although a 15-min treatment was chosen in the recommended method, actual enzyme assays with 60 min of treatment with 0.1 *N* NaOH proved to be equally satisfactory.

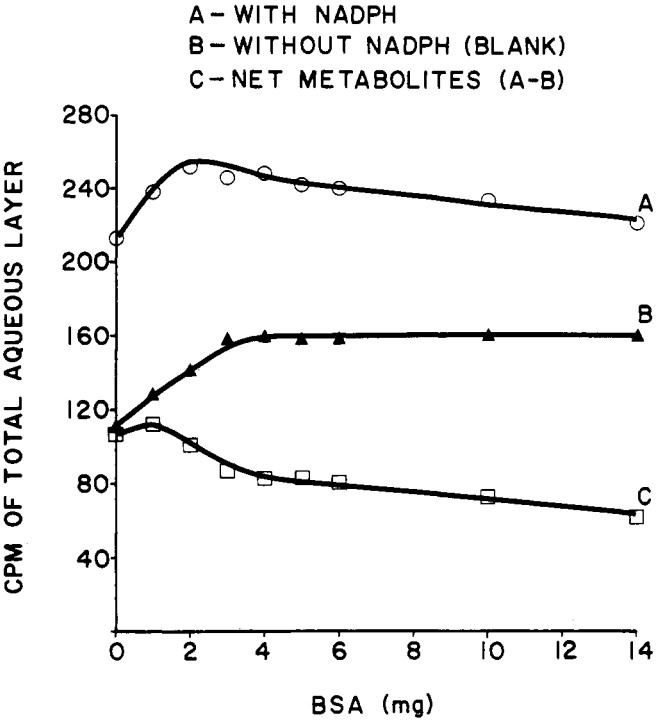

Figure 1. Effect of BSA concentration on *in vitro* parathion metabolism. The incubation mixture included crude homogenate equivalent to two 3-day-old adult Orlando-DDT female housefly abdomens.

Effect of BSA Concentration. Experiments were carried out to determine the effect of different concentrations of BSA on this assay system. A crude homogenate prepared from abdomens of 3-day-old Orlando-DDT female flies was used as the enzyme preparation. Figure 1 illustrates the results. Net parathion metabolism was slightly enhanced by the addition of 1 mg of BSA per sample. Further increases in BSA concentration resulted in a decrease in the microsomal metabolism measured. Similar results were obtained when microsomes from abdomens of 3-day-old Orlando-Regular female flies were used as the enzyme preparation.

Effect of Enzyme Concentration and Incubation Time. Studies on enzyme concentration were pursued with microsomal enzyme preparations of different insects, *i.e.*, microsomes from houseflies, *H. virescens*, and *H. zea*. As shown in Figure 2, linearity was demonstrated over the entire enzyme concentration range studied with all test enzyme preparations. The differences in the oxidase activity between the two housefly strains and the two *Heliothis* species are similar to those observed in our laboratory by measuring the epoxidation of [^{14}C]aldrin.

A study was made on the time course of parathion metabolism with an abdomen homogenate of 6-day-old R-Fc female flies. The reaction was linear for 15 min and the rate slowly leveled off thereafter (Figure 3).

In Vitro **Activation** *vs.* **Degradation of Parathion by Housefly Microsomal Oxidase System.** We made a quantitative comparison between activation and degradation of parathion by the following procedure. Degradation products (A), DEPTA and DEPA, were determined by omitting the 0.1 *N* NaOH treatment from the described procedure. The activation product (B), paraoxon, was es-

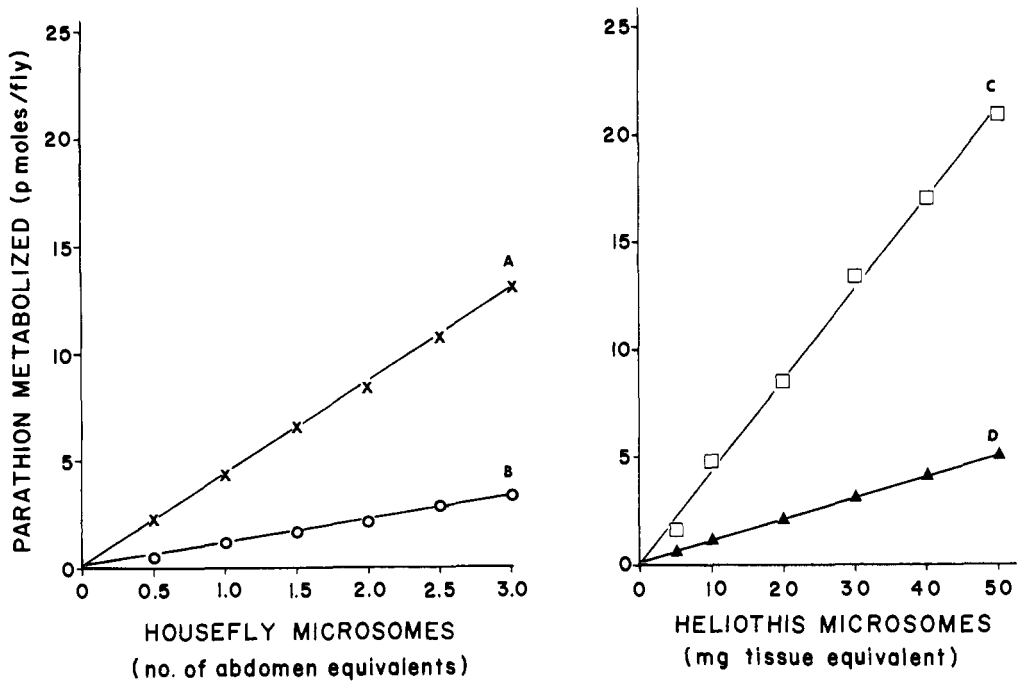

Figure 2. Effect of enzyme concentration on *in vitro* parathion metabolism. Two-day-old adult houseflies and last instar larvae of *Heliothis* were used for preparation of microsomes. (A) R-Baygon housefly; (B) Orlando-DDT housefly; (C) *Heliothis virescens;* (D) *Heliothis zea.*

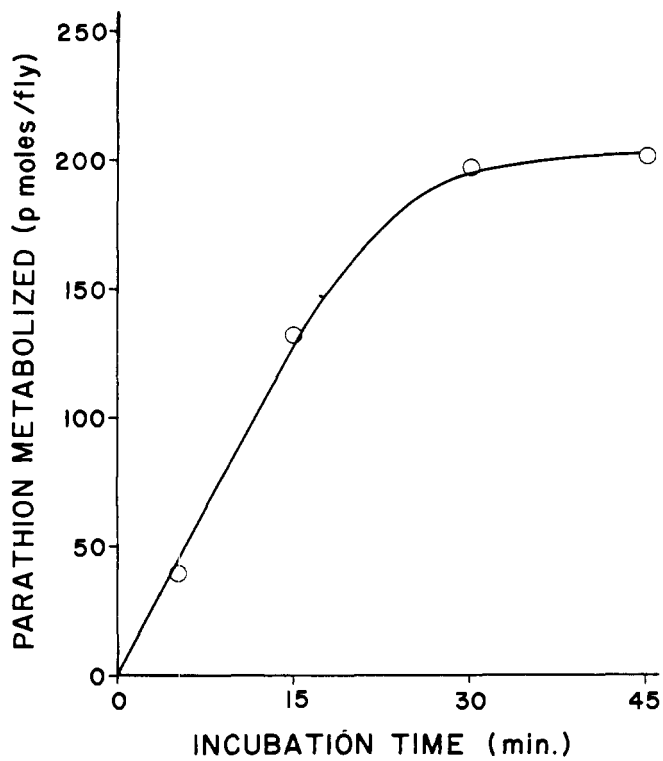

Figure 3. Effect of incubation time on *in vitro* parathion metabolism. The incubation mixture included crude homogenate equivalent to one 6-day-old adult R-Fc female housefly abdomen.

timated by subtracting the concentration of degradation products from that of total metabolites (C) measured and by incorporating the 12.7% cleavage factor of paraoxon under the alkali treatment condition. In other words, [B] = ([C] − [A]) × 100/12.7.

In experiments with crude homogenates from female flies of R-Baygon and Orlando-DDT strains, the ratio of *in vitro* activation to degradation ranged from 1:1 to 2:1. In other words, the activation either equaled or exceeded degradation in housefly preparations.

Table II. Induction of Microsomal Oxidases in the Housefly by Aroclor 1254[a]

Housefly strain	Activity as % of the untreated control[b]	
	Present method	Aldrin epoxidase assay[c]
Orlando-DDT	356	315
	296	294
Orlando-Regular	246	230
	164	154

[a] 1 mg of Aroclor 1254/jar for 7 days. [b] Two separate experiments for each. [c] Cited from Rhee and Plapp (1973).

Measurement of Microsomal Oxidase Induction. Microsomal oxidase induction in two strains of houseflies exposed to Aroclor 1254 was measured by this method to compare with our previous induction results obtained by determining aldrin epoxidase (Rhee and Plapp, 1973). The results are shown in Table II. Induction measured by the two methods was comparable and slightly greater with parathion as the substrate.

DISCUSSION

The effect of BSA concentration on this assay system is worthy of note. The decreased metabolism measured with high concentrations of BSA as observed in this study has not previously been reported for microsomal oxidase systems. A similar inhibition was found by Oppenoorth *et al.* (1972) in experiments on the glutathion-dependent degradation of parathion by soluble enzymes of houseflies. Inhibition by excess BSA of the soluble enzyme system was of similar magnitude to that in our experiments.

Other workers (Hook *et al.*, 1968) reported on the activity-enhancing effect of BSA on the housefly microsomal oxidase system. It has been a common understanding that BSA protects the oxidase enzymes from endogenous inhibitors which may be present in housefly heads (Schonbrod and Terriere, 1971a,b; Tsukamoto and Casida, 1967).

In our study of this assay system with a crude abdomen homogenate or microsomes, the concentration of BSA needed to obtain a beneficial effect is minimal, 1 mg/sample. In practice, the enzyme assay without BSA would be better than the assay with BSA at greater than 1 mg/sample.

The ratio of parathion activation to degradation appears to differ between insects and mammals. In our work with the housefly, activation equaled or exceeded degradation. In rabbit microsomes, degradation equaled or exceeded activation (Nakatsugawa and Dahm, 1967).

Neal (1967) reported that with rat liver microsomal metabolism of parathion, the effects of enzyme inhibitors, stimulators, and substrate concentration were quantitatively different for metabolism to DEPTA and to paraoxon. He concluded that the differences in the two reactions may either be due to two separate mixed-function oxidase enzyme systems or two different binding sites for parathion which share a common electron transport pathway. It is not known whether similar differences may also occur in insect microsomal metabolism of parathion.

There are also soluble enzyme systems metabolizing phosphorothionate insecticides. They are reduced glutathion (GSH)-dependent *S*-alkyl transferase (Fukami and Shishido, 1966) and *S*-aryl transferase (Shishido *et al.*, 1972), as well as esterases which require no cofactors. When a crude homogenate is used as a microsomal enzyme preparation in this assay procedure, the aqueous layer may contain a small amount of the reaction products of the above enzymes. The contribution of these reaction products to the aqueous layer count would, however, be the same for both the sample and blank which in this method also contains the enzyme preparation. Therefore, the net result of the microsomal parathion metabolism measured by this procedure will not be affected by the soluble enzymes.

Although the recommended method does not measure total microsomal parathion metabolism *in vitro,* the results obtained by the method still represent degradative metabolism plus activation in part. Relative activities determined by this procedure are comparable with those obtained by the more commonly used but slower method of measuring cyclodiene epoxidation.

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Comparative Study of Flavor Properties of Thiazole Derivatives

Alan O. Pittet* and Denis E. Hruza

A series of mono-, di-, and trisubstituted thiazoles containing alkyl, alkoxy, and acetyl functional groups was synthesized and their mass spectra, gc retention indices (I_E values), and organoleptic properties were determined. Significant flavor correspondence was found between the thiazoles and derivatives of pyrazine and pyridine possessing comparable functional groups. These similarities tended to be greater with the 2- and 4-substituted thiazoles than the 5-substituted thiazoles, which were more sulfury in character. The variations in odor quality in these N-containing heterocycles are discussed in terms of structure and charge distribution.

Although thiazole derivatives have been known to synthetic organic chemists for almost a century (Hoffman, 1879), little interest was shown in these heterocyclic compounds from a nutritional point of view until Williams (1935) demonstrated that vitamin B_1 contains a thiazole ring. The potential of thiazole derivatives as flavorants became evident through the work of Stoll *et al.* (1967a), who found that the strong nutlike odor of a basic fraction obtained from a cocoa extract was due to a trace amount of 4-methyl-5-vinylthiazole. Since then an increasing number of volatile thiazole derivatives has been isolated from a wide range of food products. Although some thiazoles, *e.g.*, 2-isobutylthiazole in tomato, probably arise biogenetically (Kazeniac and Hall, 1970), the majority of the thiazole derivatives to date have been isolated from foods which have undergone heat processing or Maillard-type reactions. These include cocoa (Stoll *et al.*, 1967a), coffee (Stoll *et al.*, 1967b), roasted peanuts (Walradt *et*

International Flavors and Fragrances, Inc., R&D Center, Union Beach, New Jersey 07735.