Effects of Naturally Occurring Food Plant Components on Insecticide Degradation in Rats

Tom W. Fuhremann, E. Paul Lichtenstein,* and Fredrick W. Stratman

The effects of the naturally occurring, insecticidal, food plant components myristicin and *d*-carvone on insecticide degradation by subcellular fractions of rat livers or by intact liver cells (hepatocytes) were evaluated. The naturally occurring compounds were incorporated into rat diets to determine their in vivo effects on insecticide degradation by subcellular fractions or hepatocytes. To determine their in vitro effects myristicin and *d*-carvone were added simultaneously with the insecticides to subcellular fractions or hepatocytes. Insecticides studied were [^{14}C]parathion, [^{14}C]paraoxon, and [^{14}C]fonofos. Results indicated that both myristicin and *d*-carvone interacted with rat liver components to either increase insecticide degradation to detoxified metabolites or to block degradation as measured by an increased stability of the parent insecticide. Effects varied depending on the particular natural compound, the route of administration (in vivo or in vitro), and the particular liver cell fraction. The effects of feeding myristicin and *d*-carvone were in most cases different from effects observed after their simultaneous in vitro addition with the insecticides. Data indicated that the effects observed with these naturally occurring compounds in the living organism are not necessarily the same as those observed after their addition to subcellular liver fractions. Hepatocytes were found to be a useful alternative technique for investigating insecticide degradation.

In recent years we have become increasingly aware of the presence of biologically active substances in our environment. The presence of synthetic chemicals in our food supply, for example, has been a particular cause for concern. Many plants containing naturally occurring toxic substances have been recognized and avoided by man for centuries; however, we are less knowlegeable about biologically active compounds in some plants which are a routine part of our diet and effect biological systems in more subtle ways. These components of our diet could, for example, act as modifiers of enzymatic activities or even as carcinogens (Miller, 1973). They may also have the potential to interact with synthetic chemicals such as drugs or pesticides and could produce undesirable effects.

In our laboratory, numerous crop plants were originally screened for insecticidal activity. As a result of these studies 2-phenylethyl isothiocyanate was isolated from and found to be responsible for the insecticidal properties of turnips (Lichtenstein et al., 1964). Myristicin (5-allyl-1-methoxy-2,3-methylenedioxybenzene), an insecticide and synergist, was isolated from the edible parts of parsnips (Lichtenstein and Casida, 1963). More recently, a number of compounds were isolated from dill plants which had insecticidal properties. These compounds were *d*-carvone (*p*-mentha-6,8-dien-2-one) and the methylenedioxyphenyl compounds, apiol, dill apiol, and myristicin (Lichtenstein et al., 1974).

Because these food plant components had insecticidal and/or insecticide synergistic activity with several insect species, we were interested in determining if they would also exhibit biological activity in mammals. For this reason we selected myristicin and *d*-carvone for further study, using rats as the test animal.

Myristicin is known to occur in nutmeg, mace, black pepper, and several members of the plant family Umbelliferae including parsnip, parsley, celery, dill, and carrots (Hall, 1973). Nutmeg is used in baked goods (2000 ppm), meats (670 ppm), beverages (700 ppm), and ice cream (550 ppm) (National Academy of Sciences, 1965). In addition

to these foods and flavorings, essential oils containing myristicin are used in soaps, hair tonics, perfumes, dentifrices, and tobacco flavoring (Fishbein and Falk, 1969). *d*-Carvone has been isolated from dill, caraway, and mandarin peel (Merck Index, 1968). Dill plants are used in baked goods (4800 ppm), meats (1200 ppm), and pickles (8200 ppm), while dill oil is used in ice cream (5.8 ppm), candy (9.9 ppm), meats (51 ppm), and condiments for seasoning (150 ppm). Carvone itself is used in beverages (850 ppm), ice cream (120 ppm), candy (180 ppm), baked goods (110 ppm), and liquors (130 ppm) (National Academy of Sciences, 1965).

Methylenedioxyphenyl compounds, including myristicin, are known to be substrates for microsomal mixed function oxidases and thus compete with other xenobiotics for enzyme. A product of their metabolism is known to bind with cytochrome P-450, forming an inhibited complex. They may also act as inducers of microsomal enzymes (Franklin, 1976). *d*-Carvone has been reported to increase the synthesis and excretion of ascorbate in rats (Longenecker et al., 1939; Ritz et al., 1940).

The present study was designed to determine the effects of these two naturally occurring dietary components on the activation and degradation of some organophosphorus insecticides in rats.

MATERIALS AND METHODS

Chemicals. [^{14}C]Parathion (97% radiopurity) labeled in the 2,6-phenyl positions (ICN Corporation, Irvine, Calif.) was diluted with nonradioactive parathion to a specific activity of 0.58 mCi/mmol. [^{14}C]Paraoxon was prepared by oxidation of [^{14}C]parathion with bromine as previously described (Lichtenstein et al., 1973). Parathion, paraoxon, *p*-nitrophenol, aminoparathion, aminoparaoxon, and *p*-aminophenol were obtained through the courtesy of Farbenfabriken-Bayer, Leverkusen, W. Germany. [*ethoxy*-^{14}C]Fonofos (Dyfonate), fonofos, and fonofos oxygen analogue were obtained through the courtesy of the Stauffer Chemical Company, Mountain View, Calif. [*ethoxy*-^{14}C]Fonofos was diluted with nonradioactive fonofos to a specific activity of 0.49 mCi/mmol.

NADPH$_2$ was purchased from Sigma Chemical Company, St. Louis, Mo. *d*-Carvone was purchased from Aldrich Chemical Company, Milwaukee, Wis. Myristicin

Department of Entomology (T.W.F., E.P.L.) and Institute for Enzyme Research (F.W.S.), University of Wisconsin, Madison, Wisconsin 53706.

was isolated from nutmeg spice powder (Black Diamond Brand, Archibald and Kendall, Inc., Chicago, Ill.) as described below.

Solvents used were redistilled acetone, benzene, carbon tetrachloride, chloroform, ethyl acetate, hexane, and absolute AR ethanol, diethyl ether, and methanol.

Isolation of Myristicin from Nutmeg. Myristicin was isolated from nutmeg because this chemical is present at a concentration of 1% w/w (Fishbein and Falk, 1969) and nutmeg is available as a commercial product. For isolation purposes, 500 g of powdered nutmeg were extracted by refluxing with 800 mL of chloroform for 90 min. The cooled chloroform extract was filtered through Whatman No. 1 filter paper under vacuum and the extracted nutmeg was discarded. The chloroform was removed by flash evaporation at 45 °C and the remaining oily residue was dissolved in 700 mL of ethanol. The ethanol solution was stored at –10 °C for 30 min. This resulted in a white precipitate (Trymyristin; Ikan, 1969) which was discarded. After storing the ethanol filtrate overnight at –10 °C, it was again filtered and the ethanol was removed by flash evaporation at 45 °C. The red, oily residue was transferred to a 1-L separatory funnel with 75 mL of ethanol and partitioned between 300 mL of 2% aqueous Na_2SO_4 and 3 × 200 mL of hexane. The aqueous phase and the red resin (which is insoluble in both hexane and water) were discarded. The hexane phase was evaporated to 100 mL, stored at –10 °C for 4 h, and then centrifuged at 1500 g for 2 min. The hexane supernatant was then passed through a 4-cm diameter glass column containing an upper 4-cm layer of Florisil (Fisher No. F-100) and a lower 4-cm layer of alumina (Fisher No. A-540). This column was eluted with 500 mL of hexane, and the hexane and more volatile oils were removed under vacuum (15 mmHg) with a flash evaporator at 60 °C. The yellow, oily residue (ca. 15 mL) was transferred to a 3-cm diameter glass column containing a 25-cm layer of dry silica gel, Brockman activity III (ICN-Woelm No. 04526). This column was eluted with hexane, and 15-mL fractions were collected. The fractions were analyzed by flame-ionization gas–liquid chromatography (GLC) as described below. When only myristicin was detected in the fractions, the column was further eluted with 300 mL of carbon tetrachloride. The fractions containing pure myristicin were pooled and the solvents were removed by flash evaporation at 45 °C. The average yield from this procedure, repeated several times, was 5 mL of myristicin. It was determined to be at least 98% pure by GLC, thin-layer chromatography (TLC), and IR spectroscopy, as compared with samples of myristicin which had been synthesized or isolated from parsnips (Lichtenstein and Casida, 1963).

Feeding Treated Diets to Rats. To test the potential in vivo effects of myristicin or d-carvone, diets containing these compounds were fed to rats. For this purpose 1000 g of powdered Purina rat chow were thoroughly mixed with 100 mL of corn oil (controls) or 100 mL of corn oil mixed with either 10 mL (11.4 g) of myristicin or 10 mL (9.6 g) of d-carvone (1% v/w). These treated and control diets were fed to male rats (200–225 g, Sprague-Dawley, Madison, Wis.) ad libitum for 8 days prior to decapitation. During this feeding period an average of 2.2 g of d-carvone or 1.3 g of myristicin was calculated to have been consumed per rat based on the consumption by three rats which were fed together in one cage.

Preparation of Liver Cell Fractions. Rat liver microsomes and solubles (105 000g supernatant) were prepared in Tris–KCl–MgCl$_2$ buffer (pH 7.5) from three pooled rat livers per diet treatment and adjusted to 26 ±

1 mg of protein/mL prior to incubation as described by Lichtenstein et al. (1973).

Preparation of Hepatocytes. Suspended rat hepatocytes were prepared according to the methods of Zahlten and Stratman (1974), except that the rats were not fasted prior to surgery. Briefly, this procedure involves the surgical removal of livers from anesthetized rats, oxygenated perfusion of the liver with collagenase at 37 °C to digest connective tissues and isolation of viable, intact hepatocytes suspended in Krebs-Henseleit buffer (pH 7.4), containing 1.5% gelatin, by centrifugation. Hepatocyte concentration was determined with a Neubauer hemocytometer. The total volume of suspended cells was then adjusted as required.

Incubation of Cell Fractions and Hepatocytes. Triplicate reaction mixtures were incubated in open, 10-mL Erlenmeyer flasks at 37 °C in a Dubnoff Shaker (120 rpm) for various time periods as indicated in the footnotes to the tables. Hepatocytes were incubated with vigorous shaking (120 rpm) under a gassing hood with a flow rate of 5 L/min of 95% O_2/5% CO_2. Subcellular fractions were not gassed. Each 2-mL incubation mixture contained 52 ± 2 mg of microsomal or soluble protein or ca. 5 million hepatocytes, except where effects of hepatocyte concentration were studied. $NADPH_2$ was added at 1.2 × 10^{-4} M, only to microsomal preparations incubated with [^{14}C]parathion or [^{14}C]fonofos. Preliminary experiments indicated that added $NADPH_2$ was not required in soluble preparations or in microsomal preparations incubated with [^{14}C]paraoxon. [phenyl-^{14}C]Parathion, [phenyl-^{14}C]paraoxon, or [ethoxy-^{14}C]fonofos (50 μg, 0.1 μCi) were each added in 10 μL of ethanol to the appropriate incubation mixtures. When the in vitro effects of naturally occurring food components were studied, 10 μL of ethanol containing 100 μg of d-carvone or 100 μg of myristicin or 10 μL of ethanol only (controls) were also added to incubation mixtures containing one of the insecticides. Reactions were terminated by adding 5 mL of acetone to each incubation mixture, followed by freezing at –15 °C until extraction.

Extraction and Analysis. The incubation mixtures were quantitatively transferred with two 5-mL portions of water and 10 mL of acetone into 60-mL separatory funnels. Each mixture was partitioned with three 10-mL portions of hexane, which were pooled and dried with anhydrous Na_2SO_4. Extraction with hexane primarily removes parathion, paraoxon, and fonofos while detoxification products of these compounds remain in the water extraction phase. Thus the amount of radiocarbon in the water extraction phase after partitioning with hexane provides an estimate of the degradation of the insecticides. In order to quantitatively extract p-nitrophenol from water extraction phases of enzyme preparations incubated with [^{14}C]parathion or [^{14}C]paraoxon, these water phases were acidified to pH 1.5 with concentrated HCl and reextracted with three 10-mL portions of chloroform–ether (2:1). After the organic and water extraction phases had been adjusted to volume, they were analyzed by liquid scintillation counting (LSC) as described by Fuhremann and Lichtenstein (1978).

Gas–Liquid Chromatography (GLC) was employed for the quantitative and qualitative analysis of myristicin isolated from nutmeg. A Tracor Model 550 gas chromatograph equipped with a flame ionization detector and a 183 cm × 4 mm i.d. glass column packed with 10% Dow Corning 200 Silicone grease on 80/100 Gas-Chrom Q was used. The column was operated with a 60 cm^3/min nitrogen flow at 145 °C, inlet at 225 °C, and detector at 200

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°C. Under these conditions myristicin had a retention time of 3 min.

Thin-Layer Chromatography (TLC) was employed to qualitatively and quantitatively assess the degradation of [¹⁴C]parathion, [¹⁴C]paraoxon, and [¹⁴C]fonofos and to determine the purity of isolated myristicin. All TLCs were done with E. Merck Laboratories precoated silica gel "60" plates. Plates spotted with organic solvent extracts containing myristicin were developed in benzene, those with [¹⁴C]parathion or [¹⁴C]paraoxon and metabolites were developed in benzene–chloroform–methanol (6:3:1), while plates spotted with extracts containing [¹⁴C]fonofos and metabolites were developed in chloroform–ethyl acetate (1:1).

Myristicin (R_f 0.57) was visualized as a reddish-brown spot by spraying with 3–4 mL of a solution prepared by mixing 25 mL of glacial acetic acid, 0.5 mL of H_2SO_4, and 0.25 mL of anisealdehyde and then heating the plate at 100 °C for 20 min (Sengupta et al., 1973).

The ¹⁴C insecticides and their metabolites were visualized by spraying the plates successively with $PdCl_2$ in 0.25 N HCl and then with 5 N NaOH, or by autoradiography using Kodak medical X-ray film. In order to quantitate the compounds produced from the applied ¹⁴C insecticides in the hexane or chloroform–ether extracts, 3 mL of each triplicate 50 mL of hexane or 50 mL of chloroform ether extract were pooled, concentrated, and spotted. After development, spraying, and autoradiography, each ¹⁴C compound was scraped from the plate, dissolved in 5 mL of 0.1 N NaOH, and sonicated for 1 min. Two 1-mL aliquots were analyzed by LSC in the previously referenced scintillator which also contained 3.5% (w/v) Cab-o-sil suspension agent. The distribution of radiocarbon in each sample was then used to determine the amounts of each insecticide and its metabolites in the organic extraction phases in percent of the applied ¹⁴C insecticide.

EXPERIMENTAL SECTION

Basically three experimental approaches were used to investigate the effects of the naturally occurring plant components, myristicin and d-carvone, on the ability of rats to degrade the ¹⁴C insecticides.

Effects of Feeding Diets Containing Myristicin or d-Carvone to Rats on the Degradation of ¹⁴C Insecticides by Rat Liver Cell Fractions. In order to determine the potential in vivo effects of natural compounds on insecticide degradation, three male rats were fed chow diets (controls) or chow diets containing either 1% (v/w) myristicin or d-carvone for 8 days prior to decapitation. Microsome and soluble liver cell fractions were prepared from these rats and incubated with [¹⁴C]parathion, [¹⁴C]paraoxon, or [¹⁴C]fonofos (myristicin fed rats) or with [¹⁴C]parathion or [¹⁴C]paraoxon (carvone fed rats) as described. After extraction, the water and organic extraction phases were analyzed by LSC. The organic phases were also analyzed by TLC as described.

Effects of in Vitro Addition of Myristicin or d-Carvone on the Degradation of ¹⁴C Insecticides by Rat Liver Cell Fractions. In these experiments, microsome or soluble liver cell fractions were prepared from the pooled livers of three rats previously fed regular rat chow diets. These cell fractions were then incubated with [¹⁴C]parathion, [¹⁴C]paraoxon, or [¹⁴C]fonofos plus 100 μg of myristicin, 100 μg of d-carvone, or no natural compound (controls). After extraction, the water and hexane phases were analyzed by LSC to determine the extent of degradation of the ¹⁴C insecticides to water-soluble compounds under the various experimental conditions.

Degradation of ¹⁴C Insecticides by Rat Hepatocytes as Affected by in Vitro Addition of Myristicin or d-Carvone. Although rat liver hepatocytes have been used to study various biochemical processes, to our knowledge they have not been utilized to investigate the degradation of insecticides. These intact cells could be a valuable tool for these investigations because their use allows testing a number of factors with cells from a single liver. Moreover, some of the disadvantages of using subcellular fractions such as releasing inhibitory substances during cell disruption and the need for exogenous cofactors may be eliminated.

Preliminary experiments were performed to determine the concentration of hepatocytes which could degrade ca. 50% of a given insecticide to water-soluble compounds so that stimulation or inhibition of degradation due to the presence of the natural compounds could be assessed. For this purpose, hepatocytes were prepared as described and diluted with Krebs-Henseleit buffer so that final triplicate 2-mL incubation mixtures contained 5, 25, 50, and 500 × 10⁴ liver cells. These hepatocyte preparations were then incubated with [¹⁴C]parathion or [¹⁴C]fonofos. After extraction, the organic and water extraction phases were analyzed by LSC.

Once the optimal concentration of hepatocytes for insecticide degradation had been determined, they were used to study the effects of myristicin or d-carvone. For this purpose, triplicate hepatocyte preparations containing 5 million cells/2 mL of Krebs-Henseleit buffer were incubated with [¹⁴C]parathion, [¹⁴C]paraoxon, or [¹⁴C]fonofos plus 100 μg of either myristicin, d-carvone, or no natural compound (controls). After extraction, the organic and water phases were analyzed by LSC. The organic extraction phases were also analyzed by TLC as described. To study in vivo effects, similar studies were also conducted with hepatocytes prepared from rats which had been fed diets containing 1% v/w myristicin or d-carvone.

RESULTS AND DISCUSSION

Effects of Feeding Diets Containing Myristicin or d-Carvone to Rats on the Degradation of ¹⁴C Insecticides by Rat Liver Cell Fractions. Livers from rats fed diets containing 1% v/w myristicin were larger and had a darker colored, granular surface texture than livers from control rats. Livers from myristicin fed rats were 7.8% of the body weights, while livers from control or d-carvone fed rats amounted to only 4.0 and 4.7% of the body weights, respectively.

For clarity purposes, the effects of myristicin are discussed first (Tables I and II) and those of d-carvone later (Tables III and IV). After rat liver microsome and soluble cell fractions had been incubated with the ¹⁴C insecticides, various effects due to feeding myristicin were evident. Based on the partitioning of radiocarbon between organic solvent and water extraction phases, feeding myristicin had no apparent effect on the microsomal degradation of [phenyl-¹⁴C]parathion (Table I). However, degradation by soluble enzymes was stimulated as evidenced by a significant reduction in hexane-soluble radiocarbon (from 69 to 45% of applied) and a concomitant increase in water-soluble radiocarbon. Also, soluble preparations from myristicin fed rats contained only 55% of the undegraded parathion recovered from soluble liver preparations of control rats (Table II). Conversely, feeding myristicin resulted in an increased production by the soluble fraction of paraoxon from parathion (activation) and of the degradation products p-nitrophenol and unidentified water-soluble compounds as compared with control rats which had been fed regular diets without myristicin.

Insecticide Degradation in Rats

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Table I. Effects of Myristicin in Rat Diets on the Detoxification of ¹⁴C Insecticides by Subcellular Fractions of Rat Livers (Data Are Means ± SD for Three Replicate Incubation Mixtures)

¹⁴C insecticide applied[b]	extraction phase	radiocarbon recovered in % of applied ¹⁴C insecticides from liver cell fractions prepared from rats fed chow diets plus[a]			
		microsomes		solubles	
		none	myristicin	none	myristicin
parathion	hexane	88.5 ± 0.3	87.8 ± 1.0	68.9 ± 1.4	45.3 ± 0.4[e]
	water	11.1 ± 0.4	10.6 ± 0.1	28.5 ± 1.2	51.2 ± 1.5[e]
	total	99.6 ± 0.6	98.5 ± 1.0	97.3 ± 0.3	96.5 ± 1.3
	CHCl₃-ether[c]	8.3 ± 0.4	5.8 ± 0.6[f]	3.8 ± 0.4	10.9 ± 0.5[e]
paraoxon	hexane	67.1 ± 2.2	51.7 ± 6.2[f]	54.9 ± 1.0	43.3 ± 1.2[e]
	water	34.7 ± 2.4	50.9 ± 3.1[e]	48.2 ± 2.0	58.0 ± 1.9[f]
	total	101.9 ± 0.9	102.6 ± 3.5	103.1 ± 1.0	101.3 ± 3.1
	CHCl₃-ether	31.5 ± 2.6	43.9 ± 3.8[f]	12.5 ± 1.3	17.9 ± 1.4[e]
fonofos	hexane	83.8 ± 0.8	87.2 ± 2.8	94.0 ± 0.9	76.1 ± 3.2[e]
	water	20.8 ± 0.1	17.3 ± 1.1[e]	11.0 ± 0.4	25.8 ± 0.4[e]
	total	104.5 ± 0.8	104.5 ± 1.8	105.0 ± 0.4	101.9 2.8
	CHCl₃-ether	ND[d]	ND	ND	ND

[a] Rats were fed chow diets only (none) or diets containing 1% v/w myristicin for 8 days before preparing subcellular fractions. [b] Subcellular fractions were incubated with 50 μg (0.1 μCi) of [*phenyl*-¹⁴C]parathion for 2 h, [*phenyl*-¹⁴C]paraoxon for 15 min, or [*ethoxy*-¹⁴C]fonofos for 1 h. [c] Incubation mixtures were extracted three times with hexane, acidified to pH 1.5, and then extracted three times with chloroform-diethyl ether (2:1). [d] ND = not determined. [e,f] Data are significantly different from the respective controls (none) at the 0.1% (e) or 1% (f) level (Student's test).

Table II. Effects of Myristicin in Rat Diets on the Detoxification of ¹⁴C Insecticides by Subcellular Fractions of Rat Livers

¹⁴C insecticide applied[b]	¹⁴C compounds recovered[c]	R_f	liver cell fractions prepared from rats fed chow diets containing[a]			
			recovered by TLC in % of applied ¹⁴C insecticides			
			microsomes		solubles	
			none	myristicin	none	myristicin
parathion	parathion	0.90	79.6	80.0	66.1	36.7
	paraoxon	0.81	1.8	1.5	2.8	6.6
	p-nitrophenol	0.57	15.4	12.2	3.8	12.9
	unextracted ¹⁴C[d]		1.3	2.7	22.9	35.9
	total		98.1	96.4	95.6	92.1
paraoxon	paraoxon	0.81	60.4	21.2	64.1	48.6
	p-nitrophenol	0.57	38.2	74.5	3.4	12.6
	unextracted ¹⁴C		1.9	4.1	31.6	36.3
	total		100.5	99.8	99.1	97.5
fonofos	fonofos	0.86	83.0	86.3	93.1	72.3
	-oxon	0.54	0.8	0.9	0.9	3.8
	unextracted ¹⁴C		20.8	17.3	11.0	25.8
	total		104.6	104.5	105.0	101.9

[a] Rats were fed chow diets only (none) or diets containing 1% v/w myristicin for 8 days before preparing subcellular fractions. [b] Subcellular fractions were incubated with 50 μg (0.1 μCi) of [phenyl-¹⁴C]parathion for 2 h, [*phenyl*-¹⁴C]paraoxon for 15 min, or [*ethoxy*-¹⁴C]fonofos for 1 h. [c] Data for parathion, paraoxon, *p*-nitrophenol, fonofos, and its oxygen analogue were obtained by LSC of eluted TLC spots, from pooled hexane or pooled chloroform-ether extracts of triplicate incubation mixtures, which cochromatographed with authentic reference compounds. [d] Unextracted ¹⁴C compounds remaining in incubation mixtures after hexane and CHCl₃-ether extractions were determined by LSC.

The major product of parathion degradation in microsomal preparations was *p*-nitrophenol (Table II) while the major product with soluble fractions was an unidentified phenyl-¹⁴C-labeled water-soluble compound which could not be extracted with chloroform-ether at pH 1.5. It is possible that this water-soluble compound is a conjugate of glutathione and *p*-nitrophenol which was reported to be formed by a soluble aryltransferase under similar conditions (Hollingworth et al., 1973).

With [*phenyl*-¹⁴C]paraoxon, feeding rats with diets containing myristicin stimulated degradation by both microsomal and soluble cell fractions based on the amount of radiocarbon recovered in hexane extraction phases (Table I) and on the actual [¹⁴C]paraoxon remaining (Table II). It was found, however, that the hexane-soluble radiocarbon is not a very accurate indicator of paraoxon

degradation, especially with microsome fractions, since about two-thirds of the paraoxon in incubation mixtures partitioned into hexane (three extractions) and the remainder partitioned into chloroform-ether (at pH 1.5). Conversely, about 20% of the *p*-nitrophenol partitioned into hexane and 80% into chloroform-ether. When large amounts of *p*-nitrophenol are formed by microsomes, estimates of paraoxon remaining based on hexane-soluble radiocarbon are too large because of the presence of radiocarbon due to *p*-nitrophenol in the hexane extraction phase.

The effects of feeding myristicin on the degradation of [¹⁴C]paraoxon with both microsomal and soluble cell fractions were indicated by the increased production of *p*-nitrophenol and a decrease in the persistence of paraoxon (Table II). The major product formed from paraoxon

Table III. Effects of d-Carvone in Rat Diets on the Detoxification of ^{14}C Insecticides by Subcellular Fractions of Rat Livers (Data Are Means ± SD for Three Replicate Incubation Mixtures)

^{14}C insecticide applied[b]	extraction phase	radiocarbon recovered in % of applied ^{14}C insecticides from liver cell fractions prepared from rats fed chow diets plus[a]			
		microsomes		solubles	
		none	d-carvone	none	d-carvone
parathion	hexane	82.6 ± 1.0	63.7 ± 2.6[d]	59.0 ± 2.0	56.5 ± 1.8
	water	15.5 ± 0.8	33.1 ± 2.5[d]	38.4 ± 2.5	39.5 ± 2.6
	total	98.1 ± 0.9	96.8 ± 0.6	97.4 ± 2.1	96.0 ± 1.0
	CHCl$_3$–ether[c]	13.4 ± 0.7	24.8 ± 2.4[d]	1.8 ± 0.2	2.3 ± 0.1[e]
paraoxon	hexane	26.0 ± 2.6	16.8 ± 1.5[e]	15.6 ± 0.2	25.7 ± 1.0[d]
	water	72.9 ± 3.0	80.5 ± 1.4[e]	84.0 ± 2.5	70.6 ± 0.8[d]
	total	98.9 ± 0.5	97.3 ± 0.5	99.6 ± 2.3	96.3 ± 0.3
	CHCl$_3$–ether	67.1 ± 2.7	62.4 ± 1.2	10.1 ± 0.8	16.0 ± 0.3[d]

[a] Rats were fed chow diets only (none) or diets containing 1% v/w d-carvone for 8 days before preparing subcellular fractions. [b] Subcellular fractions were incubated with 50 μg (0.1 μCi) of [phenyl-^{14}C]parathion for 2 h or [phenyl-^{14}C]paraoxon for 45 min. [c] Incubation mixtures were extracted three times with hexane, acidified to pH 1.5, and then extracted three times with chloroform-diethyl ether (2:1). [d,e] Data are significantly different from the respective controls (none) at the 0.1% (d) or 1% (e) level (Student's t test).

Table IV. Effects of d-Carvone in Rat Diets on the Detoxification of ^{14}C Insecticides by Subcellular Fractions of Rat Livers

^{14}C insecticide applied[b]	^{14}C compounds recovered[c]	R_f	liver cell fractions prepared from rats fed chow diets containing[a]			
			recovered by TLC in % of applied ^{14}C insecticides			
			microsomes		solubles	
			none	d-carvone	none	d-carvone
parathion	parathion	0.90	81.4	59.8	58.6	56.4
	paraoxon	0.81	0.3	0.4	0.5	0.4
	p-nitrophenol	0.57	14.3	28.3	1.7	2.0
	unextracted ^{14}C[d]		2.1	8.3	36.6	37.2
	total		98.1	96.8	97.4	96.0
paraoxon	paraoxon	0.81	16.7	4.1	21.0	37.5
	p-nitrophenol	0.54	76.4	75.0	4.7	4.3
	unextracted ^{14}C		5.8	18.2	73.9	54.5
	total		98.9	97.3	99.6	96.3

[a] Rats were fed chow diets only (none) or diets containing 1% v/w d-carvone for 8 days before preparing subcellular fractions. [b] Subcellular fractions were incubated with 50 μg (0.1 μCi) of [phenyl-^{14}C]parathion for 2 h or [phenyl-^{14}C]paraoxon for 45 min. [c] Data for parathion, paraoxon, and p-nitrophenol were obtained by LSC of eluted TLC spots, from pooled hexane or pooled chloroform-ether extracts of triplicate incubation mixtures, which cochromatographed with authentic reference compounds. [d] Unextracted ^{14}C compounds remaining in incubation mixtures after hexane and CHCl$_3$-ether extractions were determined by LSC.

Table V. Effects of in Vitro Addition of Myristicin or d-Carvone on the Detoxification of ^{14}C Insecticides by Subcellular Fractions of Rat Livers (Data Are Means ± SD for Three Replicate Incubation Mixtures)

^{14}C insectic. applied[b]	extraction phase	radiocarbon recovered in % of applied ^{14}C insecticides from liver cell fractions also incubated with[a]					
		none	myristicin	d-carvone	none	myristicin	d-carvone
		microsomes			solubles		
parathion	hexane	85.4 ± 0.6	89.2 ± 0.8[d]	88.6 ± 0.4[c]	62.0 ± 2.2	78.2 ± 1.1[c]	87.2 ± 0.4[c]
	water	14.5 ± 0.6	11.3 ± 1.0[d]	10.9 ± 0.5[c]	37.4 ± 0.8	25.2 ± 0.4[c]	12.2 ± 0.6[c]
	total	99.9 ± 0.6	100.5 ± 0.3	99.5 ± 0.9	99.4 ± 1.5	103.4 ± 1.0	99.4 ± 0.7
paraoxon	hexane	58.6 ± 2.7	57.9 ± 1.3	56.7 ± 0.5	40.6 ± 0.3	40.3 ± 0.9	69.5 ± 0.4[c]
	water	42.2 ± 1.8	43.0 ± 1.3	43.0 ± 1.8	55.4 ± 0.6	55.2 ± 0.5	30.4 ± 0.5[c]
	total	100.8 ± 1.5	100.9 ± 2.6	99.7 ± 1.4	96.0 ± 0.9	95.5 ± 1.3	99.9 ± 0.2
fonofos	hexane	73.3 ± 3.7	71.7 ± 0.4	76.5 ± 0.4	95.0 ± 1.0	97.3 ± 1.7	95.3 ± 1.0
	water	27.5 ± 2.1	26.1 ± 1.3	24.8 ± 1.7	9.2 ± 0.1	8.7 ± 0.7	9.2 ± 0.1
	total	100.8 ± 1.6	97.8 ± 1.1	101.3 ± 2.1	104.2 ± 1.0	106.0 ± 2.4	104.5 ± 1.0

[a] One hundred micrograms of myristicin or d-carvone was added to each incubation mixture immediately before adding ^{14}C insecticides and starting incubation. [b] Subcellular fractions were incubated with 50 μg (0.1 μCi) of [phenyl-^{14}C]parathion for 2 h, [phenyl-^{14}C]paraoxon for 15 min, or [ethoxy-^{14}C]fonofos for 1 h. [c,d] Data are significantly different from the respective controls (none) at the 0.1% (c) or 1% (d) level (Student's t test).

by soluble cell fractions was an unidentified water-soluble metabolite, but its production was only slightly affected by feeding myristicin.

Feeding myristicin to rats had little or no apparent effect on the microsomal degradation of [ethoxy-^{14}C]fonofos. However, degradation of this insecticide by soluble cell fractions was stimulated by feeding myristicin as evidenced by a decrease in the amounts of hexane-soluble radio-

carbon (Table I), and a more than two-fold increase in the production of water-soluble compounds. Feeding myristicin was also responsible for an increased formation of fonofos oxygen analogue (Table II). The nature of the degradation products of [14C]fonofos (Dyfonate) in the rat has been reported by McBain et al. (1971).

The effects of *d*-carvone in rat diets on the degradation of [14C]parathion and [14C]paraoxon by subcellular fractions of rat livers are summarized in Tables III and IV. The effects of feeding carvone on the degradation of fonofos was not tested. As shown in Table III, feeding *d*-carvone to rats had the opposite effect with parathion than that observed after feeding myristicin; microsomal degradation was stimulated, while degradation by soluble cell fractions was unaffected. The stimulation of microsomal degradation of [14C]parathion due to feeding *d*-carvone is shown by the significantly lower recovery of hexane-soluble radiocarbon and concomitant higher recoveries of water-soluble radiocarbon (Table III). Results (Table IV) indicate that due to the in vivo effects of *d*-carvone, the total amount of paraoxon, *p*-nitrophenol, and water-soluble radiocarbon was 37% of the insecticide applied to the microsomal fraction, but was only 16.7% with controls. Degradation of [14C]parathion with soluble fractions was not affected by feeding *d*-carvone.

Feeding *d*-carvone to rats also increased the microsomal degradation of [14C]paraoxon, but inhibited degradation of the insecticide by soluble enzymes (Table IV). Carvone did not affect the production of *p*-nitrophenol by microsome or soluble cell fractions. However, the production of unidentified, water-soluble compounds was stimulated in microsomal preparations and was inhibited in soluble preparations, thereby accounting for the effects of *d*-carvone on [14C]paraoxon degradation.

The foregoing data indicate that the degradation of parathion, paraoxon, and fonofos by soluble liver cell fractions is stimulated by feeding rats with diets containing myristicin. The microsomal degradation of paraoxon is also stimulated while microsomal degradation of parathion and fonofos are not affected. Feeding diets containing *d*-carvone to rats is responsible for a stimulation of the microsomal degradation of both parathion and paraoxon and an inhibition of paraoxon degradation by soluble cell fractions.

Effects of in Vitro Addition of Myristicin or *d*-Carvone on the Degradation of 14C Insecticides by Rat Liver Cell Fractions. In these experiments the insecticides had been added with or without (controls) the naturally occurring compounds to subcellular liver fractions from rats which had been fed a regular diet. Data obtained after analyses of the incubation mixtures are presented in Table V. The microsomal degradation of parathion was slightly inhibited by addition of either myristicin or *d*-carvone based on larger recoveries of hexane-soluble and smaller recoveries of water-soluble 14C compounds. The microsomal degradation of [14C]paraoxon and [14C]fonofos were not affected by in vitro addition of either myristicin or *d*-carvone. The degradation of [14C]parathion was inhibited in soluble fractions by addition of either myristicin or *d*-carvone while soluble enzyme degradation of [14C]paraoxon was inhibited only by addition of *d*-carvone. The degradation of [14C]fonofos by soluble liver fractions was unaffected by addition of either myristicin or *d*-carvone.

One of the most interesting results of this study was the observation of different effects of myristicin or *d*-carvone in the living organism as compared to effects observed after their in vitro addition to liver cell fractions. Figure 1
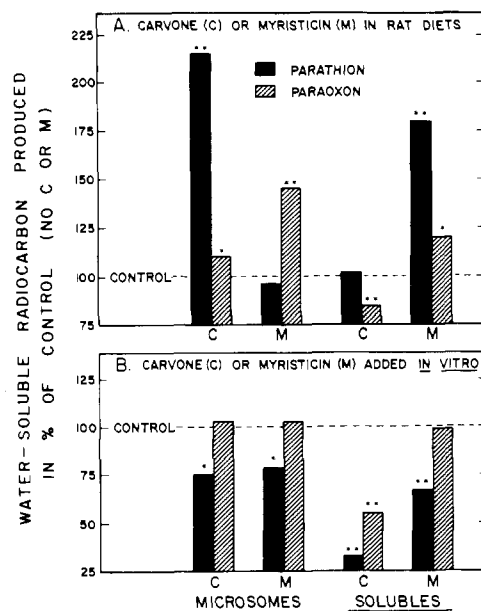

Figure 1. Effects of *d*-carvone or myristicin on the detoxication (water-soluble radiocarbon remaining in incubation mixtures after extraction with hexane) of [14C]paraoxon or [14C]parathion by rat liver cell fractions. Carvone or myristicin were (A) fed to rats (1% of the diet) or (B) added directly to the microsome or soluble cell fractions (100 μg/2 mL of incubation mixture). Results are expressed in percent of controls conducted without carvone or myristicin. Data are significantly different from the respective controls at the 0.1% (**) or 1% (*) level.

depicts these differences as a function of the amounts of water-soluble radiocarbon produced in controls (=100%). Some of the major points are the following: The degradation of [14C]parathion by microsomes was increased by *d*-carvone added in vivo (diets), but was inhibited by in vitro addition. Degradation of [14C]parathion by the soluble cell fraction was not affected by *d*-carvone added in vivo, but was inhibited by in vitro addition. Myristicin added in vivo increased [14C]parathion degradation by the soluble cell fraction but inhibited degradation after in vitro addition.

The degradation of [14C]paraoxon by the soluble cell fraction was inhibited by both in vivo and in vitro addition of *d*-carvone. The in vivo addition of myristicin stimulated paraoxon degradation by both microsome and soluble cell fractions. These results, therefore, indicate that data obtained after addition of compounds to subcellular fractions do not necessarily reflect the effects of these compounds in the living organism.

Degradation of 14C Insecticides by Rat Hepatocytes as Affected by the in Vitro Addition of Myristicin or *d*-Carvone. In order to evaluate the potential use of rat hepatocytes for studying the degradation of 14C insecticides, a preliminary test was run to determine the optimal hepatocyte concentration. Based on the amounts of water-soluble radiocarbon produced from [14C]fonofos or [14C]parathion, rat hepatocytes could degrade the 14C insecticides, and the degradation was a function of hepatocyte concentration (Figure 2). Approximately 5 million hepatocytes in 2 mL of buffer caused a 51 and 34% degradation of applied [ethoxy-14C]fonofos and [phenyl-14C]parathion to water-soluble 14C compounds, respectively. Therefore, this concentration of hepatocytes was used in subsequent experiments.

Results obtained after in vitro addition of myristicin or *d*-carvone and incubation of hepatocytes with the 14C insecticides are presented in Tables VI and VII. Based

Table VI. Effects of in Vitro Addition of Myristicin or d-Carvone on the Detoxification of ^{14}C Insecticides by Suspended Rat Hepatocytes (Data Are Means ± SD for Three Replicate Incubation Mixtures)

^{14}C insectic. applied[b]	extraction phase	radiocarbon recovered are in % of applied ^{14}C-insecticides from rat hepatocytes also incubated with[a]				
		none (CK)	myristicin	% CK	d-carvone	% CK
parathion	hexane	77.1 ± 2.4	87.2 ± 0.7[f]		81.7 ± 2.1	
	water	19.0 ± 0.6	11.0 ± 0.4[e]	57.9	15.9 ± 0.4[e]	83.7
	total	96.1 ± 1.8	98.2 ± 0.6		97.6 ± 2.1	
	CHCl$_3$-ether[c]	3.5 ± 0.5	3.6 ± 0.9		3.5 ± 0.4	
paraoxon	hexane	49.5 ± 3.3	63.2 ± 0.8[f]		65.5 ± 3.5[f]	
	water	47.1 ± 2.1	38.5 ± 0.2[e]	81.7	35.3 ± 2.3[f]	74.9
	total	96.6 ± 3.6	101.7 ± 0.6		100.8 ± 1.2	
	CHCl$_3$-ether	30.1 ± 1.4	24.7 ± 1.1[f]		22.1 ± 3.7	
fonofos	hexane	72.2 ± 1.9	80.9 ± 1.2[f]		75.5 ± 0.4	
	water	27.1 ± 1.2	19.1 ± 0.4[e]	70.4	24.6 ± 0.2	90.7
	total	99.3 ± 1.1	100.0 ± 1.3		100.1 ± 0.5	
	CHCl$_3$-ether	ND[d]	ND		ND	

^{a} One hundred micrograms of myristicin or d-carvone was added to each incubation mixture immediately before adding ^{14}C insecticides and starting incubation. ^{b} Approximately 5 million hepatocytes were incubated with 50 μg (0.1 μCi) of [phenyl-^{14}C]parathion for 45 min, [phenyl-^{14}C]paraoxon for 15 min, or [ethoxy-^{14}C]fonofos for 30 min. ^{c} Incubation mixtures were extracted three times with hexane, acidified to pH 1.5, and then extracted three times with chloroform-diethyl ether (2:1). ^{d} ND = not determined. ^{e,f}Data are significantly different from the respective controls (none) at the 0.1% (e) or 1% (f) level (Student's t test).

Table VII. Effects of in Vitro Addition of Myristicin or d-Carvone on the Detoxification of ^{14}C Insecticides by Suspended Rat Hepatocytes

^{14}C insecticide applied[b]	^{14}C compounds recovered[c]	^{14}C compounds recovered by TLC in % of applied ^{14}C insecticides from hepatocytes also incubated with[a]			
		R_f	none	myristicin	d-carvone
parathion	parathion	0.90	71.9	83.8	75.0
	paraoxon	0.81	7.4	5.9	8.8
	p-nitrophenol	0.57	1.3	1.1	1.4
	unextracted ^{14}C		15.3	7.1	12.3
	total		95.9	97.9	97.5
paraoxon	paraoxon	0.81	62.3	78.7	79.0
	p-nitrophenol	0.57	17.3	9.2	8.6
	unextracted ^{14}C		17.9	14.9	14.6
	total		97.5	102.8	102.2
fonofos	fonofos	0.86	65.1	74.8	71.2
	-oxon	0.54	7.1	6.1	4.3
	unextracted ^{14}C		27.1	19.1	24.6
	total		99.3	100.0	100.1

^{a} One hundred micrograms of myristicin or d-carvone was added to each incubation mixture immediately before adding ^{14}C insecticides and starting incubation. ^{b} Approximately 5 million hepatocytes were incubated with 50 μg (0.1 μCi) of [phenyl-^{14}C]parathion for 45 min, [phenyl-^{14}C]paraoxon for 15 min, or [ethoxy-^{14}C]fonofos for 30 min. ^{c} Data for parathion, paraoxon, p-nitrophenol, fonofos, and its oxygen analogue were obtained by LSC of eluted TLC spots, from pooled hexane or pooled chloroform–ether extracts of triplicate incubation mixtures, which cochromatographed with authentic reference compounds. ^{d} Unextracted ^{14}C compounds remaining in incubation mixtures after hexane and CHCl$_3$-ether extractions were determined by LSC.

on larger recoveries of hexane-soluble radiocarbon (Table VI) and of the originally applied ^{14}C insecticides (Table VII) myristicin inhibited the degradation of [^{14}C]parathion, [^{14}C]paraoxon, and [^{14}C]fonofos by rat hepatocytes. Carvone, however, significantly inhibited only paraoxon degradation.

It is interesting to note that the major degradation product of [phenyl-^{14}C]parathion from these intact liver cells was unidentified water-soluble radiocarbon and not p-nitrophenol (Table VII). Similar results were obtained when whole livers were perfused with [phenyl-^{14}C]parathion or [phenyl-^{14}C]paraoxon (Fuhremann et al., 1974). Since these water-soluble compounds are primarily formed by soluble rather than particulate cell fractions (Tables II and III; Lichtenstein et al., 1973), it appears that these

soluble enzymes may be of greater in vivo significance than was previously suspected.

Preliminary experiments were also conducted by incubating ^{14}C insecticides with hepatocytes prepared from rats which had been fed diets containing myristicin, d-carvone, or no natural compound (controls). These experiments were repeated three times with three different groups of rats. The results, however, were erratic, i.e., inhibition, stimulation or no effect was noticed depending on the particular rat, even though they had received identically treated diets prior to hepatocyte preparation. These inconsistent results were probably due to the fact that great difficulty was encountered in determining the hepatocyte concentration from the livers obtained from differently fed rats. Hepatocytes prepared from rats fed
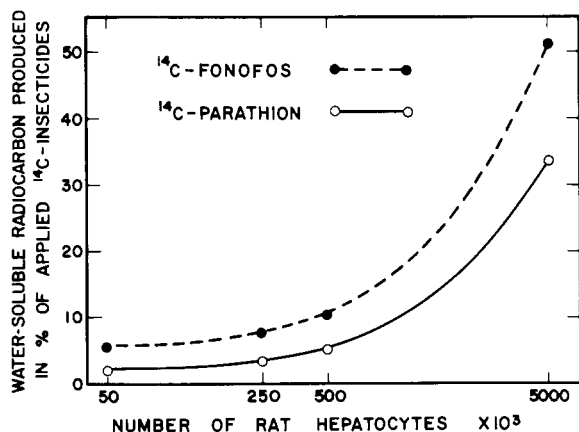
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Figure 2. Effects of the concentration of suspended rat hepatocytes on the degradation of [^{14}C]fonofos and [^{14}C]parathion to water-soluble ^{14}C compounds. The indicated numbers of hepatocytes were incubated in 2 mL of buffer (pH 7.4) for 45 min at 37 °C.

myristicin appeared to be much enlarged, while hepatocytes prepared from rats fed diets containing d-carvone tended to clump together. Thus we suspect that the varying results were due to inconsistent hepatocyte concentrations as compared with control hepatocytes. In the future, the concentration of hepatocytes could possibly be based on protein estimations rather than on cell counts. It is also possible that the three rats which fed together on the same diet in one cage did not receive the same amount of natural compound. Because only one of the three rats was selected for hepatocyte preparation, differences between experiments could have resulted. When subcellular fractions were prepared, the three rat livers were pooled in order to eliminate any individual variation.

The data presented in this paper must be considered to be preliminary. Any final assessment of the effects of these natural compounds awaits in vivo degradation studies because of the complicated patterns of their interaction with different cell fractions and insecticide degradation pathways. However, a number of important aspects of these investigations should be emphasized. First of all, it is clear that naturally occurring compounds in our food supply have the ability to interact with cellular components, thus affecting the fate of synthetic chemicals in mammals. Second, the effects of in vitro addition of compounds do not necessarily represent the effects these compounds may have in dietary sources or upon prolonged exposure. Third, isolated rat hepatocytes could be a useful alternative for studying pesticide degradation and activation. Finally, myristicin and d-carvone could be utilized as biochemical probes for investigating insecticide degradation pathways in vivo. For instance, feeding d-carvone stimulated the degradation of parathion by microsomal enzymes (Figure 1A) but had no effect on soluble enzymes.

Conversely, feeding myristicin had no effect on parathion degradation by microsomal enzymes but stimulated degradation by soluble enzymes. Thus, these natural compounds could be used to study the relative importance of these enzyme systems in vivo.

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