Interactions of Naturally Occurring Food Plant Components with Insecticides and Pentobarbital in Rats and Mice

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The interaction of several spice plant oils and some of their components with pentobarbital or insecticides in mice and rats was investigated. Ames tests were also conducted. *trans*-Anethole, anisaldehyde, estragole, *d*- and *l*-carvone, and oils from anise, fennel, nutmeg, and spearmint all significantly increased pentobarbital sleeping times in mice but only when injected simultaneously with pentobarbital. *trans*-Anethole injected (ip) into rats with sublethal doses of [^{14}C]fonofos or [^{14}C]parathion had no significant effects on the metabolism and excretion of the insecticides. After administration of *trans*-anethole in the rat diet (1%), the degradation of [^{14}C]parathion by rat liver microsomes was increased. Simultaneous treatment of microsomal incubation mixtures with [^{14}C]parathion and anethole, however, had no effect on the insecticide degradation as compared to controls without anethole, while anethole added with [^{14}C]parathion to the 15000*g* supernatant reduced the degradation of the insecticide. This in vitro degradation by the 15000*g* supernatant was also inhibited by elemicin, safrole, myristicin, and apiol, the rate of inhibition increasing as the number of methoxy substituents increased on a (methylenedioxy)phenyl ring. Anise oil and fennel oil were slightly mutagenic when tested as bacterial mutagens with *Salmonella typhimurium* mutants and a liver activation system (Ames test).

Man is continually exposed to countless chemicals in his daily environment. Many are newly developed synthetic chemicals, but most are naturally occurring organic or inorganic molecules. In the recent past, much time and effort have been spent to study the toxicities of the rapidly expanding numbers of synthetic organic chemicals. There are, however, a vast number of naturally occurring compounds, many of them present in food plants, that have yet to be identified or characterized regarding their biological activities. In many cases the prevailing attitude has been that naturally occurring compounds which have been eaten by humans and livestock for centuries with no obvious harmful effects must therefore be safe for consumption. In terms of marked acute toxic responses, this may well be true. In reality, however, man is exposed to constantly fluctuating combinations of chemical compounds. As the number of synthetic compounds to which man is exposed in his environment and his diet such as preservatives, dyes, flavoring agents, pesticide residues, etc. increases, so does the potential for undesirable interactions between these synthetic chemicals and their naturally occurring counterparts.

Past studies in our laboratory have examined the toxicity of various naturally occurring compounds in food plants and their interactions with insecticides (Lichtenstein et al., 1974; Fuhremann et al., 1978; Fuhremann and Lichtenstein, 1979; Marcus and Lichtenstein, 1979). Many of these naturally occurring compounds are used as flavoring agents. These are of particular interest because of their synergistic activity with synthetic insecticides and because some of them possess relatively weak insecticidal activities.

trans-Anethole is one of these widely used flavoring agents and is commonly found in many spice plants, especially anise and fennel. The acute LD_{50} of *trans*-anethole has been determined to be 2090 mg/kg when administered orally to rats (Jenner et al., 1964), and the long-term effects of *trans*-anethole consumption have been reported on by Taylor et al. (1964) and Le Bourhis (1973). Furthermore, the metabolic pathways of *trans*-anethole degradation in several species including man have been defined (Le Bourhis, 1970; Solheim and Scheline, 1973) and the mutagenicity of this compound has also been reported (Miller and Miller, 1979). The general conclusion from these studies has been that *trans*-anethole is a relatively harmless compound in the quantities normally encountered by man. However, since previous work in our laboratory (Marcus and Lichtenstein, 1979) established the existence of substantial interactions in insects between *trans*-anethole derived from anise plants and synthetic insecticides, it was decided to continue these investigations with mammalian systems. Several additional compounds contained in spice plants as well as some plant-derived flavoring oils were also investigated. This study therefore reports on the interactions of some naturally occurring flavoring agents (Figure 1), especially *trans*-anethole, with insecticides or pentobarbital in rats and mice as well as on the possible mutagenicity of these materials.

EXPERIMENTAL SECTION

Materials. Anisaldehyde, *trans*-anethole, *d*- and *l*-carvone, estragole, and safrole were all obtained from Aldrich Chemical Co., Milwaukee, WI. Anethole (cis/trans = 90%/10%), apiol, dill apiol, and elemicin were all provided courtesy of Dr. J. A. Miller, University of Wisconsin. Myristicin was isolated from nutmeg oil by vacuum distillation and column chromatography (Fuhremann and Lichtenstein, 1979). All compounds were of greater than 96% purity at the time of use.

Anise oil, coriander oil, and sesame oil were obtained from Ruger Chemical Co., Inc., Irvington, NJ. Caraway oil, fennel oil, and spearmint oil were obtained from Amend Drug and Chemical Co., Lincoln Park, NJ. $NADPH_2$ and Tween-80 were purchased from Sigma Chemical Corp., St. Louis, MO.

Solvents used were redistilled acetone, benzene, hexane, and chloroform and analytical-grade methanol, diethyl ether, and 95% ethanol. [^{14}C]Parathion labeled in the 2,6-phenyl positions, 2.2 mCi/mM, was purchased from ICN Corp., Irvine, CA, and [^{14}C]fonofos, uniformly ring labeled, 17.1 mCi/mM, was provided courtesy of Stauffer Chemical Co., Mountain View, CA. [^{14}C]Paraoxon was

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Table I. Effects of Naturally Occurring Compounds on Pentobarbital-Induced Sleeping Times in Mice

	mice sleeping time, min, for pentobarbital injected with			
		test compd		
test compd	none (controls)	simultaneous	0.5 h prior[a]	% of controls
trans-anethole	62.8 ± 14.3	118.7 ± 46.9[c]		186
trans-anethole	47.5 ± 10.8		74.3 ± 14.2[c]	156
anisaldehyde	62.8 ± 14.3	87.4 ± 19.3[b]	NS	139
estragole	62.8 ± 14.3	102.3 ± 43.8[b]		163
estragole	47.5 ± 10.8		69.3 ± 13.8[b]	146
d-carvone	62.8 ± 14.3	121.6 ± 30.8[d]	NT[f]	194
l-carvone	62.8 ± 14.3	98.0 ± 29.5[c]	NT	156
anise oil	73.9 ± 15.4	143.0 ± 55.3[c]		193
anise oil	47.5 ± 10.8		73.4 ± 17.2[b]	154
coriander oil	68.2 ± 19.5	NS[e]	99.9 ± 42.7[b]	146
fennel oil	68.2 ± 19.5	99.8 ± 35.5[b]	NS	146
nutmeg oil	73.9 ± 15.4	104.0 ± 22.7[c]	NT	141
spearmint oil	73.9 ± 15.4	117.0 ± 57.3[b]	NT	158
caraway oil	73.9 ± 15.4	NS	NT	
sesame oil	73.9 ± 15.4	NS	NT	

[a] Injection of naturally occurring compounds 72, 48, and 24 h prior to the administration of pentobarbital had no effects on mice sleeping times. [b-d] Results are means ± SD obtained from six mice each. Significantly different from controls at (*b*) 5, (*c*) 1, and (*d*) 0.1% (Student's *t* test). [e] NS = results were not significantly different from those of the control. [f] NT = not tested.

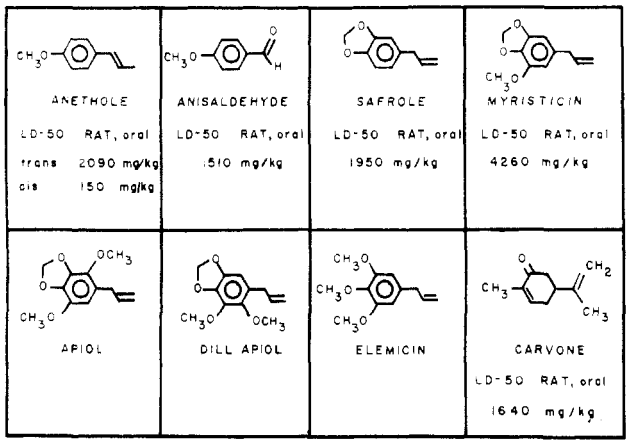

Figure 1. Biologically active naturally occurring compounds.

prepared from the [^{14}C]parathion as described by Lichtenstein et al. (1973). All radiochemicals were purified to greater than 98% radiopurity prior to use and diluted with unlabeled analytical-grade insecticide as required for various experiments.

Effects of Naturally Occurring Plant Compounds on Pentobarbital-Induced Sleeping Time with Mice. To screen for biologically active naturally occurring plant products, we conducted tests with mice employing a pentobarbital-induced sleeping time assay. Barbiturates, pentobarbital included, are thought to be representative of a large number of drugs and other compounds all known to be metabolized primarily by microsomal enzymes. Therefore, substances which interact with these microsomal enzymes might be expected to alter the duration of the sleeping time induced by these drugs by altering their metabolism (Kamienski and Murphy, 1971).

The assays described in this study were conducted in the laboratory of Dr. June Dahl, Department of Pharmacology, University of Wisconsin—Madison, and were similar to those reported by Seto and Keup (1969). Preweighed groups of six Sprague-Dawley male white mice (ca. 25 g each) were injected intraperitonealy (ip) with sodium pentobarbital in 0.1 mL of 0.5% Tween-80 in sterile saline. Concentrations were adjusted such that a single 0.1-mL injection contained pentobarbital doses of 50 mg/kg or pentobarbital plus one of the test compounds, 50 mg/kg each. Naturally occurring materials (Table I) were injected either simultaneously with pentobarbital or 3 times prior to (at 72, 48, and 24 h) or only once (0.5 h) before the injection of the drug. The second and third tests were conducted to determine whether any changes occurred in the activity of pentobarbital due to the metabolism or distribution of these compounds in mice, before the drug was injected. Such changes might be caused by inhibition, activation, or induction of pentobarbital metabolizing enzymes. In all tests, "control" mice were injected with pentobarbital (or carrier) only. Pentobarbital-induced sleeping times were determined for control and test mice concurrently.

Effect of *trans*-Anethole on the Metabolism and Excretion of Insecticides by Rats. Since naturally occurring plant products are likely to be consumed along with pesticide residues on or in food products, two experiments were conducted to test for potential effects of *trans*-anethole on the metabolism and/or excretion of two organophosphate insecticides ([^{14}C]parathion; [^{14}C]fonofos) by rats. *trans*-Anethole is a widely used naturally occurring flavoring agent commonly found in spices, candy, ice cream, baked goods, meats, and liquors and hence would often have the opportunity to interact with inadvertently consumed pesticide residues.

Eight male King white rats (ca. 200 g each) were used for each experiment. Four control rats were injected (ip) with a previously established sublethal dose of [^{14}C]parathion (1.5 mg/kg), while four test rats were injected with a mixture of [^{14}C]parathion (1.5 mg/kg) and *trans*-anethole (100 mg/kg). All compounds were injected in 0.2 mL of 3% Tween-80 in sterile saline. Following injections, each rat was held in an individual stainless steel metabolism cage for the duration of the experiment. Pulverized Purina rat chow and water were provided ad libitum. Urine was collected on ice over 6- or 12-h intervals and then diluted with a deionized water rinse of the collection apparatus to a final volume of 100 mL. Replicate 0.5-mL aliquots of the urine were first analyzed for radiocarbon content by LSC. Then 50-mL aliquots of each urine sample were extracted. Urine from rats treated with [^{14}C]parathion was adjusted to pH 1.5 with HCl and then extracted with three 25-mL portions of chloroform–ether (2:1). The aqueous and organic phases were then analyzed by LSC.

For comparison purposes an identical experiment was conducted, except that a previously determined sublethal dose of [*ring*-^{14}C]fonofos (2 mg/kg) was used instead of

parathion. For extraction, urine from these rats was not acidified and only extracted with three 25-mL portions of benzene.

Effects of *trans*-Anethole or Myristicin in the Diet on the in Vitro Insecticide Metabolism by Rat Liver Cell Fractions. Experiments conducted in our laboratory with houseflies (Marcus and Lichtenstein, 1979) indicated that *trans*-anethole affected the in vitro degradation of [^{14}C]paraoxon by 10000*g* housefly supernatants when administered in the housefly diet but not when added in vitro with the [^{14}C]paraoxon to the incubation mixtures. To study these effects with mammalian systems, we conducted similar tests with subcellular fractions from rat livers. A group of four male King white rats (ca. 200 g each) were fed a diet consisting of pulverized Purina rat chow (90%), corn oil (9%), and *trans*-anethole (1%) for 7 days prior to sacrifice and cell fractionation. Four rats were fed an identical diet but without anethole. Cell fractions (15000*g* supernatants, microsomes, and 105000*g* supernatants) were prepared from the combined livers of the control or anethole-fed rats as described by Lichtenstein et al. (1973). Aliquots of each fraction were incubated for 2 h at 37 °C, pH 7.5, as described by Fuhremann et al. (1978) and Lichtenstein et al. (1973) with 50 μg of [*ring*-^{14}C]parathion or with 50 μg of [*ring*-^{14}C]paraoxon. Each 2-mL incubation mixture was prepared in such a way that it contained 50 mg of liver cell protein. For comparison of potential in vivo with in vitro effects of *trans*-anethole on the degradation of the insecticides by the liver cell fractions, *trans*-anethole was also added in vitro simultaneously with [^{14}C]parathion or [^{14}C]paraoxon to rat liver cell fractions prepared from control rats. Thus [^{14}C]parathion or [^{14}C]paraoxon was incubated with 15000*g* supernatants prepared from control rats, with 15000*g* supernatants prepared from control rats to which *trans*-anethole was also added, or with 15000*g* supernatants prepared from rats fed anethole. Identical tests to those just described were also conducted utilizing rat liver microsomes or 105000*g* supernatants. For each test, control and test cell fractions were prepared and incubated simultaneously in triplicate. Following incubation, reactions were stopped by adding 5 mL of acetone to each flask. Reaction mixtures were extracted and analyzed by LSC, TLC, and autoradiography as described by Furhemann et al. (1978), to determine to the amounts of ^{14}C-labeled insecticides and ^{14}C-labeled metabolites present in the reaction mixtures.

In similar studies with rat liver microsomes and 105000*g* supernatants conducted in our laboratory (Fuhremann et al., 1978), the effects of myristicin after being fed to rats had been different in most cases from those observed after its simultaneous in vitro addition with the insecticides. To compare our results with anethole with those obtained by Fuhremann et al., we conducted experiments with myristicin but utilizing the 15000*g* supernatant from rat livers. As with anethole, rats were fed a control diet or a control diet amended with 1% myristicin for 7 days prior to preparation of 15000*g* liver cell supernatants. Supernatants from control rats were incubated with 50 μg of [^{14}C]parathion or with 50 μg of [^{14}C]parathion plus 100 μg of myristicin added in vitro, and supernatants from myristicin-fed rats were incubated with 50 μg of [^{14}]parathion. All procedures and subsequent analyses were identical with those described above for anethole.

Structure–Activity Relationships of Naturally Occurring Compounds as in Vitro Inhibitors of [^{14}C]Parathion Metabolism. A series of experiments was conducted to ascertain the potency of *trans*-anethole relative to that of other naturally occurring food plant components as in vitro inhibitors of the metabolism of [^{14}C]parathion. Kerr (1951) and Lichtenstein et al. (1974) had shown that with increasing numbers of methoxy groups, the insecticidal and synergistic activity of this type of compound increased. Therefore, safrole, myristicin, apiol, dill apiol, and elemicin were selected for testing because of their increasing numbers of methoxy groups in the molecule (Figure 1). In addition, *trans*- and *cis*-anethole and anise oil were each also incubated at quantities from 0 to 200 μg with a 15000*g* rat liver cell supernatant (2 mg of protein) and 50 μg of [^{14}C]parathion. Incubation mixtures were incubated, extracted, and analyzed as described previously.

Mutagenicity of Naturally Occurring Compounds. In his environment, however, man is generally exposed to relatively low levels of many different compounds simultaneously rather than to single doses of individual compounds. Therefore, a series of experiments was undertaken to study the mutagenicity of various plant oils from anise, nutmeg, spearmint, caraway, fennel, or coriander, commonly used as flavoring agents. Such oils are complex, relatively undefined mixtures of numerous compounds. These flavoring extracts were tested in the laboratory of Dr. J. A. Miller, Department of Oncology, University of Wisconsin, Madison, by utilizing the Ames test, a sensitive microbial bioassay capable of detecting a wide variety of chemical mutagens (Ames et al., 1975; Swanson et al., 1979).

Cultures of *Salmonella typhimurium* mutants, strain TA-100 (for detection of base-pair substitution type mutagens) and strain TA-98 (for detection of frame shift type mutagens) were tested with the oils. For comparison purposes, highly purified individual components of these oils (anisaldehyde, *d*- or *l*-carvone, myristicin, and *trans*-anethole) were also tested. All oils and compounds were tested with both *Salmonella* strains, each at a low (2 mg of protein/plate) and a high (7 mg of protein/plate) level of activation by 13000*g* rat liver supernatant (S-13), as well as without activation. Test compounds were added in 5 μL of Me_2SO or 20 μL of 95% ethanol, depending on their solubility. Each test material was assayed over the dose range from zero (controls) up to the level of cell toxicity.

RESULTS AND DISCUSSION

Effects of Naturally Occurring Plant Compounds on Pentobarbital-Induced Sleeping Times with Mice. The effects of plant extracts and some of their components on pentobarbital-induced sleeping times with mice are summarized in Table I. When the test compound was injected simultaneously along with pentobarbital, the most effective agents in prolonging sleeping times were *d*-carvone (0.1% level, Student's *t* test) and *trans*-anethole, anise oil (90% *trans*-anethole), nutmeg oil (which contains myristicin), and *l*-carvone (1.0% level, Student's *t* test). Estragole, spearmint oil, fennel oil (70% *trans*-anethole), and anisaldehyde were only marginally effective in doing so (5% level). Sesame oil, caraway oil, and coriander oil did not significantly increase sleeping times.

Some of the above compounds were also administered to the mice 30 min prior to the pentobarbital, thus possibly giving time for either pentobarbital-degrading enzyme induction or enzyme blocking. Results indicated that of the six materials tested (coriander oil, fennel oil, anise oil, *trans*-anethole, anisaldehyde and estragole), *trans*-anethole was still the most effective agent in increasing sleeping times (1% level), although to a lesser extent than when administered simultaneously with the drug. Anise oil and coriander oil were only marginally active (5% level) in this test. Anisaldehyde and fennel oil became ineffective

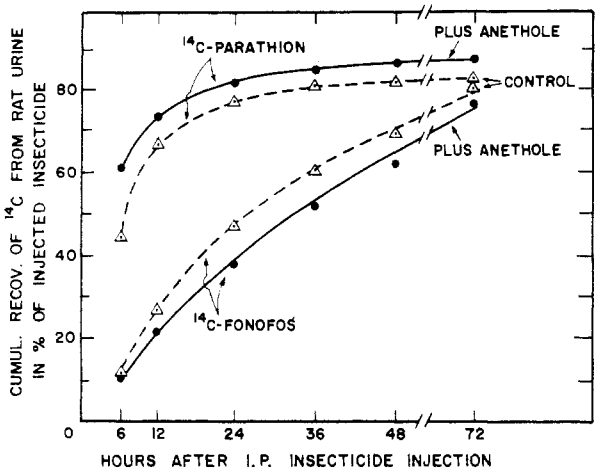

Figure 2. Effects of *trans*-anethole on the urinary excretion of radiocarbon by rats, after ip injections of ^{14}C-labeled insecticides with or without *trans*-anethole.

during the 30-min delay and no longer significantly increased sleeping times. The test compounds were most likely partially or completely metabolized by the mice during the 30-min delay, thus becoming ineffective, except for coriander oil which may have been slightly bioactivated.

There was no evidence for the occurrence of enzyme induction caused by these compounds, since none of the plant materials tested (*trans*-anethole, anisaldehyde, or anise oil) had any effect on sleeping times when administered daily for 3 days prior to pentobarbital, the last injection of test material being 24 h before pentobarbital was injected. The results, therefore, show that the effect of these naturally occurring compounds in blocking the metabolism of pentobarbital was only of short duration.

In general, these results suggest that these naturally occurring compounds do have the capability of interacting with drug-metabolizing enzymes, although the interaction does not seem to be of sufficient magnitude or duration to be of practical concern. These effects may be caused by the naturally occurring compounds serving as alternate substrates for the mixed-function microsomal enzymes, thus competing directly with pentobarbital detoxication, or by blocking the degradation of the drug by some other mechanism. This would explain why the test materials had little or no effect unless administered simultaneously with the pentobarbital.

Effect of *trans*-Anethole on the Metabolism and Excretion of Insecticides by Rats. Results from LSC analyses of urine samples for their radiocarbon content collected from rats injected with ^{14}C-labeled insecticides or ^{14}C labeled insecticides plus anethole are shown in Figure 2. It is evident that radiocarbon derived from [^{14}C]parathion was eliminated by rats more rapidly than that derived from [^{14}C]fonofos, especially during the first 12 h after injection. However, by 72 h after injection of the ^{14}C-labeled insecticides, approximately the same amount of radiocarbon (77–87% of applied) had been eliminated by all rats. After urine samples had been extracted and partitioned into organic-soluble and water-soluble fractions, it was found that throughout the experiment significantly greater quantities of water-soluble radiocarbon were recovered from the urine of rats injected with [^{14}C]parathion than from rats injected with [^{14}C]fonofos. This was especially apparent during the first 12 h after injection. Thus, [^{14}C]parathion was metabolized and excreted by rats faster than was [^{14}C]fonofos. This was due to the more rapid conversion of the [^{14}C]parathion to more readily excretable water-soluble metabolites.

Table II. Effect of *trans*-Anethole in the Diet or Added in Vitro on the Metabolism of [^{14}C]Parathion by a 15000*g* Rat Liver Cell Supernatant

	radiocarbon recovered in % of applied [^{14}C]parathion added to incubation mixtures containing 15000*g* liver cell supernatants prepared from		
	control rats		
extraction phase	(A) [^{14}C]-parathion	(B) [^{14}C]-parathion plus anethole	rats fed anethole, (C) [^{14}C]-parathion
organic soluble (O)[a]			
parathion	12.5 ± 0.5	20.2 ± 3.3[d]	1.6 ± 0.7[f]
paraoxon	23.6 ± 2.5	21.9 ± 1.2	22.9 ± 0.8
p-nitrophenol	28.9 ± 6.8	29.4 ± 3.3	29.1 ± 1.8
other[b]	14.1 ± 2.5	12.2 ± 2.3	23.0 ± 0.6[e]
total	79.1 ± 2.2	82.7 ± 1.2	76.7 ± 1.2
water soluble (W)[c]	18.5 ± 0.6	15.7 ± 1.0[e]	20.7 ± 0.9[d]
total (O + W)	97.6 ± 2.8	98.4 ± 0.8	97.3 ± 2.0
% parathion degraded	87.5	79.8	98.4

[a] Determined by TLC and LSC. [b] Includes *p*-aminoparathion, *p*-aminoparaoxon, *p*-aminophenol, and some unidentified radiocarbon. [c] Determined by LSC. [d–f] Results are different from controls (Student's *t* test) at the (*d*) 5.0, (*e*) 1.0, or (*f*) 0.1% level.

As seen in Figure 2, *trans*-anethole injected with sublethal doses of [^{14}C]parathion or [^{14}C]fonofos had no significant effects on the metabolism and excretion of the insecticides. Although it appears in Figure 2 that anethole may have increased the rate of excretion of parathion, and slightly retarded the excretion of fonofos, these differences are not statistically significant. Any effect that anethole might have on the in vivo metabolism and excretion of these insecticides was not detected under the experimental conditions employed in this test.

Effects of *trans*-Anethole or Myristicin in the Diet on the in Vitro Insecticide Metabolism by Rat Liver Cell Fractions. Results obtained from tests with the 15000*g* supernatant clearly indicated that anethole affected the degradation of the insecticide. These effects were primarily noticed after anethole had been fed to rats, thus indicating an in vivo effect of anethole within the rat organism. As shown in Table II (C), the greatest degradation of [^{14}C]parathion occurred during incubation with the 15000*g* liver supernatant derived from rats fed anethole, since only 1.6% of the originally applied insecticide was recovered. In the absence of anethole [Table II (A)], however, 12.5% of the applied [^{14}C]parathion remained undegraded. Conversely, significantly more [^{14}C]parathion degradation products had been formed by liver fractions from anethole-fed rats, such as *p*-aminoparathion, *p*-aminoparaoxon, *p*-aminophenol ["other" in Table II (C)], and water-soluble ^{14}C-labeled compounds. These data suggest that feeding anethole to rats for 7 days induced the synthesis of parathion-degrading enzymes, resulting in the increased in vitro degradation of the insecticide.

However, addition of anethole directly to incubation mixtures prepared from control rats [Table II (B)] yielded opposite results: less parathion was degraded and less water-soluble ^{14}C-labeled compounds had been formed compared to untreated controls. These data confirm findings previously reported from our laboratory (Fuhremann et al., 1978) that the "effects observed with naturally occurring compounds in the living organism are not necessarily the same as those observed after their addition to subcellular liver fractions".

Table III. Effect of Myristicin in the Diet or Added in Vitro on the Metabolism of [^{14}C]Parathion by a 15000g Rat Liver Cell Supernatant

	radiocarbon recovered in % of applied [^{14}C]parathion added to incubation mixtures containing 15000g liver cell supernatants prepared from		
	control rats		
extraction phase	(A) [^{14}C]-parathion	(B) [^{14}C]-parathion plus myristicin	rats fed myristicin, (C) [^{14}C]-parathion
organic soluble (O)[a]			
parathion	10.9 ± 1.0	36.1 ± 3.2[e]	11.6 ± 1.0
paraoxon	14.8 ± 0.9	10.9 ± 0.2[d]	3.9 ± 0.2[e]
p-nitrophenol	42.2 ± 1.3	35.5 ± 4.1	60.5 ± 0.4[e]
other[b]	4.7 ± 0.7	3.0 ± 1.0	4.9 ± 0.8
total	72.1 ± 2.4	85.4 ± 2.2[d]	80.3 ± 1.8[d]
water soluble (W)[c]	18.3 ± 1.8	10.2 ± 1.1[d]	10.7 ± 1.3[d]
total (O + W)	90.4 ± 0.8	95.6 ± 3.2	91.0 ± 2.8
% parathion degraded	89.1	63.9	88.4

[a] Determined by TLC and LSC. [b] Includes p-aminoparathion, p-aminoparaoxon, p-aminophenol, and some unidentified radiocarbon. [c] Determined by LSC. [d,e] Results are different from controls (Student's t test) at the (d) 1.0 or (e) 0.1% level.

After separation of the 15000g rat liver supernatant into microsomal and soluble (105000g supernatant) fractions and their incubation with [^{14}C]parathion, it become evident that anethole added to the rat diet was increasing the degradation of parathion primarily by enzymes contained in the microsomal fraction. Thus, 56.5 ± 1.3% of the insecticide added to the microsomal incubation mixture from anethole-fed rats had been degraded as opposed to only 34.1 ± 1.4% by microsomes from control rats. Conversely, 2.4 times more paraoxon, 1.4 times more p-nitrophenol, 1.6 times more p-aminoparathion, p-aminoparaoxon plus p-aminophenol, and 1.6 times more water-soluble ^{14}C-labeled compounds had been formed by microsomes prepared from rats fed anethole. Simultaneous in vitro treatment of microsomal incubation mixtures with [^{14}C]-parathion and anethole, however, had no effect on the insecticide degradation as compared to controls without anethole added in vitro.

trans-Anethole administered in the rat diet or added in vitro had essentially no effect on the degradation of [^{14}C]parathion by 105000g rat liver supernatants. These results therefore show that the effects of anethole on the degradation of [^{14}C]parathion are primarily manifested on those enzymes associated with the microsomal cell fraction.

Experiments identical with those described above were also conducted with [^{14}C]paraoxon. Results indicated that *trans*-anethole had no effect on the metabolism of [^{14}C]-paraoxon by any of the three rat liver cell fractions, either when added in vitro or fed in vivo via the rat diet. This suggests that trans-anethole has a very specific effect on the enzymes responsible for the metabolism of parathion, while having no effect on the enzymes responsible for the metabolism of paraoxon. Since paraoxon is the activation product of parathion, *trans*-anethole may only affect the first step of the metabolic pathway that converts parathion to paraoxon and subsequently to other less toxic metabolites. Alternatively, *trans*-anethole may be affecting another metabolic pathway that does not employ paraoxon as an intermediate in the degradation of parathion.

For comparative purposes, the effects of myristicin on the degradation of [^{14}C]parathion were also investigated. Myristicin had similar effects as did anethole when added

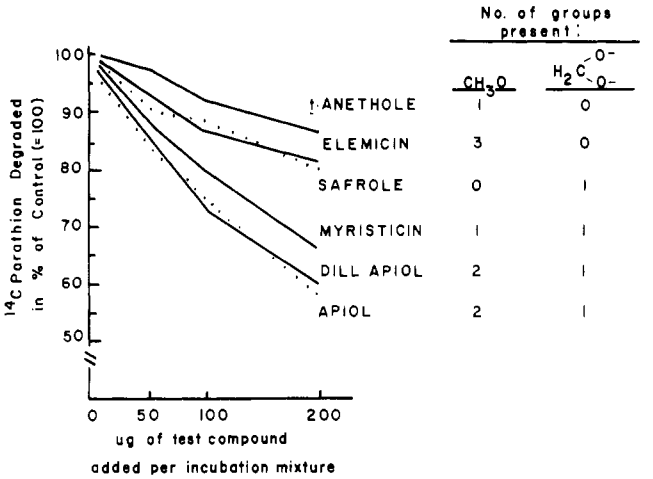

Figure 3. Inhibition of the degradation of [^{14}C]parathion by naturally occurring compounds added in increasing amounts to incubation mixtures (15000g rat liver supernatant) also containing the insecticide.

simultaneously with [^{14}C]parathion in vitro to 15000g supernatants, inhibiting the degradation of the insecticide [Table III (B)]. Thus, 36% of the applied insecticide was recovered undegraded at the conclusion of the incubation period when 100 μg of myristicin was added in vitro as opposed to only 11% with controls. Also, less paraoxon and smaller amounts of water-soluble radiocarbon had been formed. However, results with supernatants prepared from livers of myristicin-fed rats were very different from those observed with anethole-fed rats [Tables II (C) and III (C)]. While feeding anethole to rats caused an increase in [^{14}C]parathion degradation, the quantity of parathion degraded did not change when rats were fed myristicin [Table III (A and C)]. However, less paraoxon, considerably more p-nitrophenol, and smaller amounts of water-soluble radiocarbon had been formed by 15000g supernatants from myristicin-fed rats in comparison to both controls [Table III (A)] and to the comparable experiment with anethole [Table II (C)]. Since with the myristicin experiment the total amounts of radiocarbon recovered were similar under all three conditions [Table III (A–C)], a change in the pathway of [^{14}C]parathion degradation had occurred due to myristicin in the rat diet.

Structure–Activity Relationships of Naturally Occurring Compounds as in Vitro Inhibitors of [^{14}C]Parathion Metabolism. The relative potency of the natural products tested as inhibitors of the in vitro metabolism of [^{14}C]parathion by a 15000g rat liver supernatant is shown in Figure 3. The amounts of radiocarbon recovered in each test were expressed in percent of the applied [^{14}C]parathion, thus providing a basis of comparison between experiments. Results were then calculated as percent degradation in the presence of the test compound in comparison to untreated controls (Figure 3). Least-squares regression analysis of these data reveals that the inhibition of parathion metabolism caused by these compounds is essentially a linear function of the dose of test compound applied ($r \geq 0.966$) for all compounds over the dose range tested.

trans-Anethole, a propenylbenzene with a single methoxy ring substituent, proved to be a rather weak inhibitor of parathion metabolism by the 15000g rat liver cell supernatants. Since tests with *cis*-anethole (90% cis; 10% trans) and anise oil (90% *trans*-anethole) produced results very similar to those obtained with pure *trans*-anethole, they are not shown in Figure 3. As shown in Figure 1 and 3, various components tested contain none or different

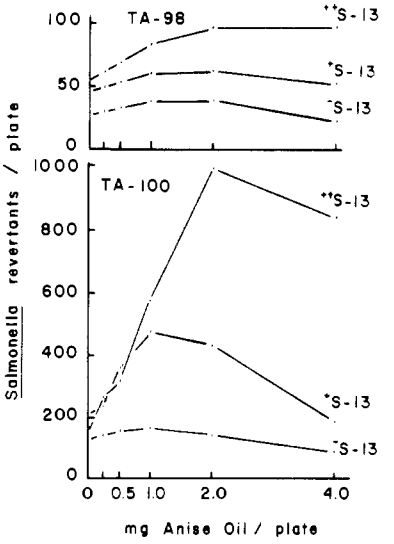

Figure 4. Mutagenicity of anise oil to *S. typhimurium* mutants (Ames test). Results are averages of duplicate tests. Anise oil was tested without bioactivation by a 13000*g* supernatant from rat liver homogenates ($^{-}$S-13) or with activation by 2 mg of protein of supernatant ($^{+}$S-13) or with 7 mg of protein of supernatant ($^{++}$S-13).

numbers of methoxy groups. Kerr (1951), utilizing eugenol (one methoxy group), methyl eugenol (two methoxy groups), and elemicin (three methoxy groups), found that the ability of these compounds to increase the toxicity of pyrethrins to houseflies increased with the number of methoxy groups present. Furthermore, he concluded that "adjuvant activity was found to be directly related to the number of methoxy groups present, while a methylenedioxy group is ineffective unless one or more methoxyl groups are also present". Tests conducted in our laboratory (Lichtenstein et al., 1974) with myristicin (one methoxy group) and apiol (two methoxy groups) seem to confirm this principle with fruit flies, since apiol was more toxic to insects than was myristicin and also had a more pronounced synergistic activity with parathion than did myristicin.

As is shown in Figure 3, our tests confirm this structure–activity relationship, since compounds with increasing numbers of methoxy groups caused an increasing inhibition of the degradation of [^{14}C]parathion but were much more effective inhibitors when associated with a (methylenedioxy)phenyl ring. Thus, elemicin with three methoxy groups but no methylenedioxy group was rather ineffective as an inhibitor of parathion degradation, although it was more effective than *trans*-anethole. In view of the finding that the inhibitory activity of apiol and dill apiol were essentially identical, the location of the methoxy group on the benzene nucleus does not seem to be as important a factor as is the actual number of groups.

Mutagenicity of Naturally Occurring Compounds. Results of the mutagenicity tests conducted with oils of anise, nutmeg, spearmint, caraway, fennel, or coriander and with anisaldehyde, myristicin, and *trans*-anethole indicated that only *trans*-anethole, anise oil (90% *trans*-anethole), and fennel oil (70% *trans*-anethole) exhibited any significant mutagenic activity in this assay. No compound or oil was judged to be mutagenic unless it was capable of inducing a mutation (reversion) rate of at least 3 times that of the incident background rate. As shown with anise oil in Figure 4, peak mutagenic activity occurred with 2 mg of anise oil/plate by utilizing TA-100 aand the highest level of liver supernatant activation. Fennel oil produced a maximal number of revertants (4.4 times background) at a dose of 2.5 mg/plate under the same conditions as above for anise oil. Among the isolated natural compounds tested, only *trans*-anethole was mutagenic, which confirms previous studies of naturally occurring compounds utilizing this assay (Swanson et al., 1979; Rockwell and Raw, 1979).

Although the results of these experiments indicate that many of the naturally occurring compounds studied are biologically active in mammalian systems, they do not seem to be potent enough or persistent enough to be of serious practical concern at this time.

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