dyloxy function replacing the methyl ester. In addition, the most active analogue has a propyl substituent on the epoxide terminus. The lipophilic extension no doubt decreased the polarity and probably also protected the epoxide from hydration. It also increased the molecular chain length from 15 to 18 atoms. JH mimics of similar structure but containing a phenoxy substituent instead of a pyridyloxy one were found to possess optimal activity against the silkworm, *Bombyx mori* (L.), when their chain length was lengthened to 17 or 18 atoms (Kiguchi et al., 1974). The three-carbon addition to the 15-atom-long pyridyl ether compound III increased the insect growth regulating activity against stored-product insects by more than an order of magnitude.

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Biologically Active Components of Anise: Toxicity and Interactions with Insecticides in Insects

Craig Marcus and E. Paul Lichtenstein*

The biological activity of components of anise tops was studied with insects. *trans*-Anethole was found to be the major insecticidal agent present in anise oil (56% by weight) derived from anise tops, with an LD_{50} of 75 μg/fly when topically applied to houseflies. The toxicity of nine other anise components (anisaldehyde, estragole, anisyl alcohol, anisic acid, *p*-cresol, *p*-creosol, eugenol, hydroquinone, and acetaldehyde) to houseflies was also studied. Anethole and anisaldehyde were both found to increase the toxicity to houseflies when applied simultaneously with parathion, paraoxon, carbaryl, carbofuran, DDT, or pyrethrum. Also, anethole fed to houseflies as 0.5% of their diet resulted in increased insect mortalities due to topically applied parathion or paraoxon in comparison to flies fed a diet without anethole. Further experiments with houseflies which had been fed with anethole as part of their diet indicated that the increased toxicity of paraoxon resulted apparently from an increased penetration of the insecticide into the insect body and a retardation of its degradation to nontoxic, water-soluble metabolites.

The existence of naturally occurring insecticidal plant components has been known for centuries. However, relatively few of these compounds are actually used in crop protection today. Increasing problems concerning the use of modern synthetic insecticides (Hayes, 1975), including insect resistance, persistence of residues, effects on nontarget organisms and human health hazards, has produced renewed interest in these naturally occurring compounds. Since these compounds are often less toxic and less persistent than their synthetic counterparts, and are in some instances already a component of mammalian diets, they are assumed to be environmentally more acceptable and less hazardous to humans. Of special interest, however, are those biologically active compounds which are natural components of food plants. Thus, insecticidal compounds were isolated in our laboratory from turnips (Lichtenstein et al., 1962), parsnips (Lichtenstein and Casida, 1963), and from dill plants (Lichtenstein et al., 1974).

A survey conducted in this laboratory in 1965 indicated that organic solvent extracts of anise plants (*Pimpinella anisum* L.), a widely used spice and flavoring agent, were toxic to fruit flies (*Drosophila melanogaster* M.). Water extracts of anise were also shown to have insecticidal activity when tested with mosquito larvae (*Aedes aegypti* L.). Additional work (Carter, 1976) suggested that two anise compounds, anethole and anisaldehyde, were toxic to fruit flies. The present study was conducted to further investigate the biological activity of anise plants.

MATERIALS

Chemicals. Analytical grade parathion and paraoxon were obtained through the courtesy of Fabenfabriken-Bayer, Leverkusen, West Germany. [^{14}C]Parathion labeled in the 2,6-phenyl positions (sp act., 2.2 mCi/mmol) was

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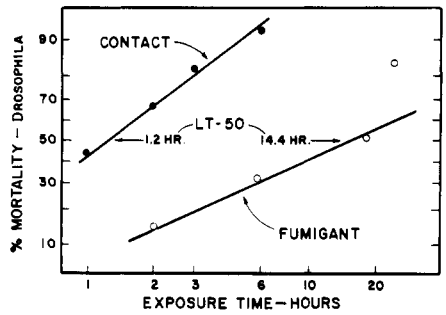


Figure 1. Mortality of fruit flies (*Drosophila melanogaster* M.) exposed directly to 3 g of macerated anise plants (contact) or to the vapors emanating from these plants (fumigant).

obtained from ICN Corp. [^{14}C]Paraoxon was prepared by oxidation of [^{14}C]parathion as described by Lichtenstein et al. (1973). Other insecticides used were analytical grade carbaryl (Union Carbide Co.), carbofuran (FMC Corp.), *p,p'*-DDT (Ciba-Geigy Corp.), and technical grade pyrethrum (S. B. Penick Co.).

Anethole, estragole, eugenol, anisyl alcohol, *p*-cresol, hydroquinone, and acetaldehyde were all purchased from the Aldrich Chemical Co. Anisic acid and *p*-creosol were purchased from Eastman Organic Chemicals. Bovine serum albumin (BSA) and $NADPH_2$ were purchased from the Sigma Chemical Corp. Solvents used were redistilled acetone, benzene, chloroform, and hexane as well as ethanol, diethyl ether, and methanol.

Plant Material. Anise plants (*Pimpinella anisum* L.) were grown on insecticide-free Plano silt loam soil at the University of Wisconsin Experimental Farm near Madison, WI. The mature plants were harvested when approximately half the seeds had ripened. The aerial portions of the plants ("tops") were macerated with a Hobart 215 food cutter and frozen.

EVIDENCE OF INSECTICIDAL ACTIVITY

Initial tests for the study of the biological activities of anise tops were conducted by exposing insects directly to macerated anise, to vapors emanating from this ground material, or to extract of the plant tissues. The insects used were fruit flies (*Drosophila melanogaster* M.), houseflies (*Musca domestica*, L.; CSMA 1948 strain), and third instar mosquito larvae (*Aedes aegypti* L.).

With Macerated Plant Tissue. The insecticidal activity of the anise tops was investigated by placing 3 g of each of the freshly macerated materials on wet filter paper in each of five bioassay jars (5 cm diameter and 6.3 cm deep). Fifty fruit flies (*Drosophila melanogaster* M.) were then introduced into each jar. As a control, flies were exposed to wet filter paper only. In addition, the same experiment was repeated except that cylindrical screen inserts were placed into the bioassay jars. Flies were then introduced into the inserted screen containers which prevented the insects from direct contact with the plant material. In this way fumigant toxicity could be tested. The mortality of fruit flies exposed directly to 3 g of fresh, macerated anise tops or to vapors emanating from this plant material was plotted as a function of time (Figure 1). It was found that anise tops were highly toxic when fruit flies were exposed directly to them (contact), resulting in 50% mortality of the insects after 1.2 h. Fruit fly mortalities resulting from exposure to vapors emanating from the macerated anise tops (fumigant) indicated that the toxicant was volatile, although the time necessary to kill 50% of the fly population was 14.4 h. Anise plants frozen for 6 months retained their insecticidal activity.

With Plant Extracts. Macerated anise tops were extracted with hexane or water at a ratio of 2 mL/g of plant material. After organic solvent extractions, the dried hexane was concentrated and adjusted to volume. Aliquots of these fractions were then pipetted into bioassay jars, and the solvent was evaporated in a fume hood. Contact and fumigant toxicity of residues to fruit flies was then determined as described.

Contact exposure of fruit flies to the dry residue of hexane extracts representing 0.5 g of anise tops resulted after 30 min in an apparent 70% mortality of fruit flies, yet after 24 h all the flies had revived. Hexane extracts representing 1.0 g of anise tops resulted in 98% mortality of fruit flies after 30 min of exposure, and these flies did not revive. Thus the hexane extract of anise had a potent "knock-down" effect, followed by eventual recovery of the flies at the lower doses tested.

Fruit flies exposed only to the vapors of dry residues of a hexane extract representing 0.5 g of anise tops did not die, while flies exposed to the fumigant action of hexane extracts representing 1.0 g showed 18% mortality in 30 min and 75% mortality after 24 h of exposure.

Water extracts from anise tops were tested for insecticidal components by introducing ten third instar mosquito larvae (*Aedes aegypti* L.) into triplicate 10-mL diluted aliquots representing 2.0 g of plant material. This resulted in 80 and 100% mortalities of mosquito larvae after an exposure for 0.5 and 24 h, respectively.

After anise tops had been extracted with hexane, they were no longer toxic to fruit flies exposed directly to them. However, extraction of anise tops with water did not decrease the toxicity of the macerated and water-extracted anise tops. This indicates that the insecticidal agent(s) present in anise plants are primarily hexane-soluble, nonpolar compounds.

With Anise Oil. Anise plants were also steam distilled. For this purpose, 300 g of anise greens and 300 mL of water were placed into a 2-L, two-necked distillation flask. While steam was passed through this material for 3–4 h, the distillate was collected in a round-bottomed flask which was immersed in dry ice and contained 50 mL of benzene. After 800–900 mL of the distillate had been collected, it was extracted twice with 150-mL portions of benzene. Flash evaporation of the benzene extract of the steam distillate at 40 °C yielded anise oil. Acetone solutions of the oil were prepared and minute amounts representing 0.28 or 0.56 g of anise tops were then tested for contact toxicity by exposing fruit flies to thin oil films. This resulted after 24 h in 28 and 100% mortalities, respectively.

For tests with houseflies, appropriate dilutions of acetone solutions of anise oil were prepared. Two microliters of acetone, containing equivalents of 17–35 mg of anise tops (114–228 μg of oil), was then topically applied with an Isco Model M-420 microapplicator onto the abdominal sternites of 2–3-day-old female houseflies anesthetized with CO_2. After treatment, three replicates each consisting of 15 flies were held in glass jars for 24 h, when mortality counts were performed. Results showed that 9, 60, 73, and 82% of the fly population had died after a 24-h exposure to anise oil, representing 17, 23, 29, or 35 mg of anise tops, respectively. Since anise oil is widely used as a flavoring agent in food, candies, liquors, and dentifrices (NAS, 1965), some of these toxicants are therefore present in human foodstuffs.

With Isolated Anise Components or Commercially Available Compounds. To isolate insecticidal components of anise plants, 100 μL of steam-distilled anise oil, representing 14.8 g of anise tops, were streaked onto a 20 × 20 cm silica gel plate containing fluorescent indicator.

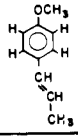
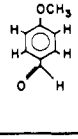
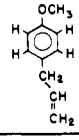
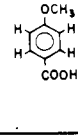
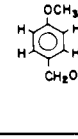
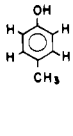
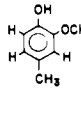
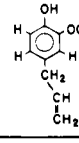
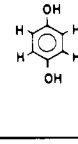
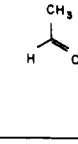
trans-ANETHOLE	p-ANISALDEHYDE	ESTRAGOLE	ANISIC ACID	ANISYL ALCOHOL
LD-50[a] HF 75 R 2090 GP 2160	LD-50 HF 105 R 1510 GP 1260	LD-50 HF 84 R 1820	LD-50 HF NT[b]	LD-50 HF 240 R 1200
$C_{10}H_{12}O$	$C_8H_8O_2$	$C_{10}H_{12}O$	$C_8H_8O_3$	$C_8H_{10}O_2$
mp 21.4°C	mp 0°C	mp —	mp 184°C	mp 24-25°C
bp 234-237°C	bp 248°C	bp 216°C	bp 275-280°C	bp 259°C
				
p-CRESOL	p-CREOSOL	EUGENOL[c]	HYDROQUINONE	ACETALDEHYDE
LD-50 HF 80 R 1800	LD-50 HF 72	LD-50 HF 72 R 2680 GP 2130	LD-50 HF NT R 320	LD-50 HF NT R 1900
C_7H_8O	$C_8H_{10}O_2$	$C_{10}H_{12}O_2$	$C_6H_6O_2$	C_2H_4O
mp 35°C	mp 170°C	mp -9°C	mp 170-171°C	mp -123°C
bp 201°C	bp 220°C	bp 255°C	bp 285-287°C	bp 21°C
				

Figure 2. Naturally occurring components of anise plants (*Pimpinella anisum* L.) [a]HF = housefly, topical, μg/fly; R = rat, oral, mg/kg; GP = guinea pig, oral, mg/kg. [b]NT = nontoxic. [c]Occurs in Japanese star anise (*Illicium verum* H.). Toxicity data for HF determined in this study. Toxicity data for rat and guinea pig from Merck Index, 9th ed, or Toxic Substances List, HEW, NIOSH, 1973 ed.

After development with benzene, five regions (R_f 0.00–0.27, 0.27–0.43, 0.43–0.70, 0.70–0.88, 0.88–1.00) containing UV–visible bands were scraped from the plate, and the five silica gel regions were extracted with hexane–acetone (1:1). After concentration to 5 mL by flash evaporation (40 °C), replicate 1-mL aliquots of each of the five samples were tested for contact toxicity with fruit flies. Results of these tests are reported below in the Identification and Quantitation of Isolated Anise Components section.

In addition to anethole, nine compounds reported by Karrer (1958) to occur in anise plants were obtained commercially. Their particular configurations are shown in Figure 2. To determine their potential insecticidal activity, they were tested with houseflies by applying topically increasing amounts of each compound. After dose–mortality curves had been obtained, LD_{50} values as shown in Figure 2 were determined.

EXTRACTION AND ISOLATION PROCEDURES

Extraction procedures of plant material have been described above under procedures describing the testing of insecticidal activities "With Plant Extracts" and "With Anise Oil". For isolation of individual anise components, TLC was used as described above under procedures for testing insecticidal activities "With Isolated Anise Components".

IDENTIFICATION AND QUANTITATION OF ISOLATED ANISE COMPONENTS

Five regions of the thin-layer chromatogram of 100 μL of steam-distilled anise oil were visualized under UV light and then eluted with hexane–acetone (1:1) from the plate and tested for their insecticidal activity as described. Only the sample of material eluted from the region R_f 0.70–0.88 proved to be toxic to fruit flies. Exposure of these insects for 15 min to the dry residues of one-fifth of this isolate resulted in a 100% mortality. Comparison of this material with authentic reference compounds by TLC indicated

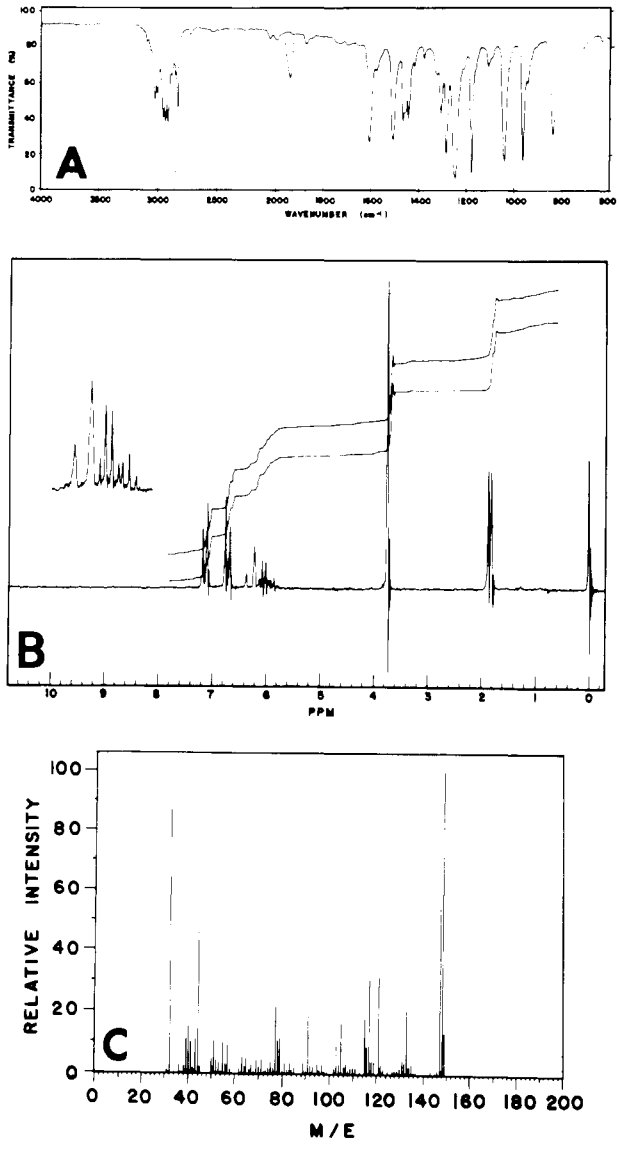


Figure 3. Infrared (A), NMR (B), and mass spectra (C) of naturally occurring anethole isolated from anise tops. UV λ_{max} in ethanol was 259 nm.

that the insecticidal compound was either anethole or estragole, which had the same R_f (0.78) and developed the same deep maroon color when sprayed with acetic acid–sulfuric acid–anisaldehyde (50:2:1) (Sengupta et al., 1973). Analysis by GLC performed as described by Fuhremann et al. (1978), indicated the insecticidal compound to be anethole (t_R 1.32 min) with trace amounts (less than 1.0%) of estragole (t_R 0.8 min). GLC was also used for quantitative analyses using synthetic anethole as a standard. The identity of the insecticidal compound was also confirmed by UV, IR, NMR, and MS (Figure 3) to be *trans*-anethole. This is also one of the two compounds isolated by Carter (1976). Only traces of anisaldehyde, the second compound isolated by Carter, were detected in these samples of anise oil.

Anethole was also isolated from anise oil by high-pressure liquid chromatography (LC). To that effect, a Varian 8500 liquid chromatograph equipped with a 50 cm × 8 mm Micropak SI-5 preparative column was employed. Ten milliliters of crude anise oil was dissolved in 10 mL of hexane, and 15 separate 1.0-mL injections of this anise oil–hexane solution were made on the preparative column. The column was eluted with 250 mL/h of hexane. Effluent

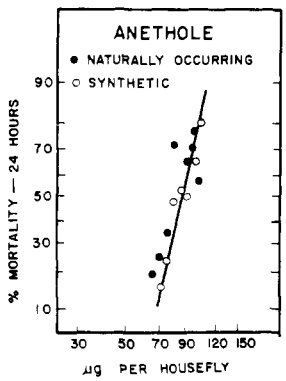


Figure 4. Toxicity of naturally occurring and synthetic anethole after topical application to houseflies (*Musca domestica* L.).

from the column was passed through the detector flow cell and collected in fractions of 8.3 mL at 2.0-min intervals utilizing a Gilson FC-100 Microfractionator. Although the absorbance maximum for *trans*-anethole is 259 nm, effluent absorbance was monitored at 265 nm to eliminate solvent interferences. Column fractions containing only anethole were pooled. Removal of the hexane by flash evaporation at 40 °C yielded 2.5 g of anethole.

Experiments were conducted to compare the insecticidal activity of naturally occurring anethole, isolated by LC from anise oil, and that of synthetic anethole. To that effect, houseflies were topically treated with increasing dosages (50–100 μg) of naturally occurring or synthetic anethole. The resulting dose–mortality curves obtained are shown in Figure 4. Since the toxicity of anethole from the two sources was identical (LD_{50} 75 μg/fly), the more readily available synthetic anethole was employed in subsequent experiments.

As shown in Figure 2, estragole, *p*-cresol, *p*-creosol, and eugenol had LD_{50} values similar to that obtained with *trans*-anethole. However, their concentration in anise is considerably less than that of anethole. GLC analyses of the anise oil obtained by steam distillation indicated an anethole content of 56% (by weight). Based on Karrer (1958) the concentration of anethole in anise oil ranges from 60 to 70%. All these data suggest that anethole is the major insecticidal substance present in anise oil. Although both anethole and anisaldehyde exhibited insecticidal properties with houseflies, anethole was almost 1.5 and 4 times more toxic than anisaldehyde by contact (topical application) and fumigant exposure, respectively. This is further evidence that anethole appears to be the most significant toxicant present in anise.

Mortalities observed 24 h after topical application of anise oil to houseflies indicated an LD_{50} of 135 μg of oil/fly. Analysis of the oil by GLC indicated that 135 μg of anise oil contained 76 μg of anethole. Since the LD_{50} obtained with synthetic anethole was determined to be 75 μg/fly, it appears that the insecticidal activity of anise oil is due to its anethole content.

EXPERIMENTAL SECTION

Synergistic Activity of Anise Components Applied Simultaneously with Synthetic Insecticides. Preliminary toxicity tests using fruit flies and houseflies indicated that nontoxic concentrations of both anise oil and hexane extracts of anise tops increased the toxicity of selected synthetic insecticides. To investigate potential synergistic activities of anise components, nine commercially available compounds (Figure 2), except eugenol, were applied at close to sublethal dosages with parathion, paraoxon, carbofuran, DDT, or pyrethrum. For this purpose, houseflies were topically treated with one of the

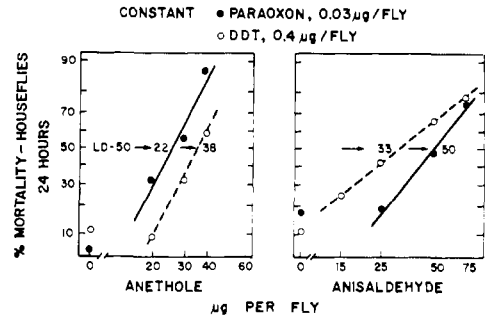


Figure 5. Effect of anethole or anisaldehyde on the toxicity of a constant dose of topically applied paraoxon or DDT with houseflies (*Musca domestica* L.).

anise components (control) or with a particular insecticide (control) or with both. Anethole and anisaldehyde were tested with parathion, paraoxon, carbofuran, carbaryl, DDT, and pyrethrum. Estragole, anisic acid, anisyl alcohol, *p*-creosol, *p*-cresol, hydroquinone, and acetaldehyde were tested with parathion, carbofuran, and DDT. Mortalities observed 24 h after treatment were compared with those observed with control flies.

Anethole and anisaldehyde were also tested for fumigant synergistic activity with insecticides. For this purpose, houseflies were topically treated with close to sublethal doses of 0.03 μg of parathion, 0.12 μg of carbofuran, or 0.4 μg of DDT/fly and then exposed for 24 h to vapors emanating from either 6 mg of anethole or 25 mg of anisaldehyde. After that mortalities were recorded and compared with those observed with control flies treated with insecticide only or with those observed with control flies exposed only to vapors of anethole or anisaldehyde.

To also investigate the effects of increasing dosages of anethole and anisaldehyde on the toxicity of synthetic insecticides, houseflies were topically treated with a constant dose of paraoxon (0.03 μg/fly) or DDT (0.4 μg/fly) plus increasing, but nontoxic doses of anethole or anisaldehyde. Control flies were treated with the insecticides only.

Results of these experiments showed that anethole and anisaldehyde were the only anise compounds that significantly increased the toxicity of all six insecticides after topical application to houseflies. Only two other anise components, estragole and *p*-creosol, increased the toxicity of carbofuran. (Eugenol, a component of star anise, was not tested for synergistic activity.) Since anethole and anisaldehyde increased the toxicity of the organophosphorus and carbamate insecticides as well as of DDT and pyrethrum (Table I), it appears unlikely that anethole and anisaldehyde affected a substrate-specific enzyme which could have been responsible for a particular detoxification mechanism. These naturally occurring anise compounds could also have affected the penetration of the insecticides into the insect body after their topical application to houseflies.

Vapors of anethole and anisaldehyde also synergized the synthetic insecticides. Thus, vapors of anethole increased the toxicity of topically applied carbofuran or DDT but not that of parathion, while vapors of anisaldehyde increased the toxicity of carbofuran but not that of parathion or DDT.

The synergistic effects of increasing, but sublethal doses of anethole and anisaldehyde applied simultaneously with a constant dose of either paraoxon or DDT to houseflies is shown in Figure 5. Results indicate that the synergistic activity of these naturally occurring compounds is dose dependent. The potency of the two compounds as synergists seems to be a function of the insecticide being

Table I. Increase in Insecticidal Activity of Commercial Insecticides by Topical Application of Anise Plant Components to Houseflies

	% mortality, 24 h after treatment		
		synergist	
insecticide	none	anethole,[a] 40 μg	anisaldehyde,[a] 75 μg
none	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 3.8
parathion (0.03 μg)	4.4 ± 7.6	31.1 ± 3.8	
parathion (0.03 μg)	4.4 ± 3.8		66.6 ± 6.6
paraoxon (0.03 μg)	11.1 ± 3.8	75.5 ± 7.6	
paraoxon (0.03 μg)	2.2 ± 3.8		53.3 ± 6.6
carbofuran (0.12 μg)	8.9 ± 3.8	88.8 ± 10.2	
carbofuran (0.12 μg)	6.6 ± 6.6		86.6 ± 6.6
carbaryl (0.25 μg)	0.0 ± 0.0	44.4 ± 19.2	
carbaryl (0.25 μg)	4.4 ± 3.8		57.7 ± 3.8
DDT (0.4 μg)	11.1 ± 7.7	62.2 ± 13.8	
DDT (0.4 μg)	11.1 ± 3.8		75.5 ± 10.2
pyrethrum (0.5 μg)	11.1 ± 7.7	55.5 ± 10.2	
pyrethrum (0.5 μg)	2.2 ± 3.8		84.4 ± 7.7

[a] Results are significantly different from controls at the 1% level (Student's t). (Results are means ± standard deviation of triplicate tests).

synergized since anethole appears to be a somewhat more effective synergist with paraoxon than with DDT, but the reverse appears to be true for anisaldehyde.

Effect of Anethole in the Housefly Diet on Insecticide Toxicity. Since compounds administered orally could produce different effects than their topical applications to insects, the effect of anethole in housefly diets on insecticide toxicity was studied.

A population of approximately 1000 1-day-old adult houseflies was divided into two feeding groups. These flies were fed either a normal milk diet (controls) or a milk diet amended with 0.5% anethole (w/v) for 3 days. During that time no differences in food consumption could be observed since both groups appeared to be feeding at an equal rate. On the fourth day, female flies from both groups were topically treated with either parathion or paraoxon. The doses of the insecticides were near their LD_{50} values, so that increases or decreases in toxicity due to anethole could be detected. Mortalities were determined 24 h after treatment. Results indicated that feeding anethole indeed increased the insecticidal activity of topically applied doses of parathion or paraoxon. Mortalities of control flies fed for 3 days with milk only and then treated with 0.07 μg/fly of parathion were 10.0 ± 6.9% after 24 h of exposure to the synthetic insecticides, while mortalities of flies fed milk plus 0.5% anethole for 3 days and then treated with the same amount of parathion were 50.0 ± 21.8%, a fivefold increase in mortality. Control flies topically treated with the larger amount of 0.2 μg/fly of paraoxon showed a mortality of 42.2 ± 23.4%, while nearly all flies (98.8 ± 2.7%) fed with an anethole diet and then treated with the same amount of paraoxon were dead after 24 h of exposure. Both increases in toxicity were significant at the 0.1% level (Student's t). Anethole thus appears to act as an insecticide synergist either when topically applied (Table I) or after having been consumed in the diet.

The possibility exists that in some way anethole affected the penetration of the synethetic insecticides into the insect body and/or affected the detoxification of the insecticides within the organism. To obtain some information on the mode of action of anethole, the following experiments were conducted.

Effect of Anethole in the Housefly Diet on the Penetration and in Vivo Metabolism of [^{14}C]Paraoxon. To examine the effects of anethole administered with the insect food, an experiment was designed to determine the effects of anethole on the penetration and metabolism of [^{14}C]paraoxon in living houseflies. A population of approximately 1000 1-day-old adult flies was divided into two feeding groups and fed a milk diet only (controls) or a milk diet plus 0.5% anethole for 3 days as described above. On the fourth day, 200 female flies from each group were topically treated with 0.051 μg (4.07×10^{-4} μCi) of [^{14}C]paraoxon per fly. The two groups of flies were then held in 2-L glass jars covered with cheesecloth at 22 ± 2 °C for 1 h. At the end of the holding period the few dead flies were removed. After that, the radiocarbon content and the amount of [^{14}C]paraoxon on the outer surfaces of the insect, the inside of the insect, and on the inner glass surfaces of the beakers were determined. For this purpose, the flies of each group were anesthetized with CO_2 and then placed into 250-mL glass-stoppered Erlenmeyer flasks. To remove external residues, each group of flies was shaken for 1 min with 100-mL portions of acetone. These acetone rinses were quantitatively decanted through a Buchner funnel, pooled, and designated "external". The rinsed flies were then placed in a 250-mL Erlenmeyer flask containing 100 mL of hexane/acetone (1:1) and blended for 1 min with a Polytron homogenizer (Kinematica GmbH). This macerate was filtered under vacuum. The extracted fly pulp was rinsed successively with 25-mL aliquots of water, acetone, and hexane. It was then air-dried and combusted to release and trap $^{14}CO_2$ for the determination of unextracted ("bound") ^{14}C insecticide residues as described by Fuhremann and Lichtenstein (1978).

The fly extracts ("internal") were diluted with 100 mL of water and partitioned with 3 × 50 mL of hexane. The resulting water extraction phases were acidified to pH 1.5 with concentrated HCl and then reextracted with 3 × 50 mL of chloroform/ether (2:1). This procedure quantitatively extracts paraoxon and p-nitrophenol and minimizes troublesome chloroform emulsions (Fuhremann et al., 1978). Finally the residue on the inside walls of the beakers in which the treated flies had been held was removed by first scrubbing the beaker walls with 2 × 50 mL of water (using the cheesecloth beaker covers) and then with 2 × 50 mL portions of acetone. The water and acetone were combined and concentrated in a flash evaporator at 40 °C until most of the acetone was eliminated. The remaining water (and acetone) was then extracted with 3 × 50 mL of hexane, resulting finally in a water and hexane extraction phase. After adjustment to volume, the radiocarbon of each extraction phase was determined by LSC. Results obtained by this procedure were designated as "excreted" radioactivity.

Table II. In Vivo Metabolism of [^{14}C]Paraoxon[a] by Houseflies Fed Anethole[b,c]

extraction phases	distribution of radiocarbon in % of total ^{14}C recovered from extraction phases							
	external[d] (E)		internal[d] (I)		excreted[d] (Ex)		E + I + Ex	
	P=O[b]	P=O + A[c]	P=O	P=O + A	P=O	P=O + A	P=O	P=O + A
organic	37.5	23.7	18.3	30.5	8.7	8.0	64.5	62.2
aqueous			23.8	24.0	11.2	13.2	35.0	37.2
bound[d]			0.5	0.6			0.5	0.6
total	37.5	23.7	42.6	55.1	19.9	21.2	100.0	100.0

[a] [*ring*-^{14}C]Paraoxon (0.051 μg; 4.07 × 10^{-4} μCi) applied topically in 1 μL of acetone to each of 200 female houseflies. Total recovery of ^{14}C in percent of applied was 99% for control and 89% for anethole-fed flies. [b] Control flies treated with [^{14}C]paraoxon only. [c] Flies fed for 3 days a milk diet plus 0.5% anethole and then treated with paraoxon. [d] External = acetone rinses of whole flies; internal = chloroform–ether extracts of homogenized flies after external rinses; bound = non-extractable ^{14}C determined by combustion of extracted flies; excreted = water–acetone rinses of fly holding jars after the 1-h holding period.

Results obtained after the "external", "internal", and "excreted" radiocarbon had been determined (Table II) indicate that 13.8% more organic-soluble radiocarbon remained on the outside ("external") of control flies than on flies fed anethole for 3 days. Concomitantly, there was 12.2% less organic-soluble radiocarbon recovered from the inside ("internal") of the control flies (18.3% of the total recovered) than from the flies fed anethole (30.5%). Thus, more organic-soluble radiocarbon was found within houseflies fed anethole for 3 days.

There was no difference in the total amount of water-soluble radiocarbon recovered from the control flies (35.0% of recovered ^{14}C) and from flies fed anethole for 3 days (37.2% of recovered ^{14}C). The distribution of water-soluble radiocarbon in the various extraction phases (E, I, Ex in Table II) was the same for control and anethole-fed flies. Since water-soluble radiocarbon represents the polar detoxification products of the parent insecticide, paraoxon, these data suggest that anethole in the housefly diet had no detectable effect on the degradation of paraoxon under these experimental conditions. However, since more "internal" organic-soluble radiocarbon was recovered from the anethole-fed flies, it appears that more paraoxon penetrated the housefly body when anethole was present in the diet.

In Vivo and in Vitro Effects of Anethole on the in Vitro Degradation of [^{14}C]Paraoxon in Houseflies. Experiments were also conducted to study whether anethole affected the in vitro degradation of [^{14}C]paraoxon by either being added to subcellular components prepared from houseflies or by being fed to houseflies prior to the preparation of subcellular components from these insects. To that effect flies were fed for 3 days a regular milk diet (control, Table III) or a milk diet containing also 0.5% anethole. After that, 10000*g* supernatants were prepared from female housefly abdomens as described by Fuhremann and Lichtenstein (1972). Three groups of experiments were then conducted: (A) [^{14}C]paraoxon (50 μg–0.1 μCi) was added in 10 μL of ethanol to 3 mL of supernatant prepared from control flies. (B) [^{14}C]Paraoxon was added the same way to supernatant prepared from control flies, but in addition 50 μg of anethole in 10 μL of ethanol was added to the incubation mixture. This was done to study the potential in vitro effect of anethole on the metabolism of [^{14}C]paraoxon. (C) [^{14}C]Paraoxon was added to supernatants prepared from flies, to which anethole had been administered in their diet. In this way we intended to compare the effects of anethole added either in vitro or via the fly diet on the in vitro degradation of [^{14}C]paraoxon. All tests were conducted in triplicate.

After incubation of the reaction mixtures for 4 h at 30 °C, they were quantitatively transferred with two 5-mL portions of water and 10 mL of acetone into a 60-mL separatory funnel. The contents of the funnel were acidified to pH 1.5 with concentrated HCl to ensure the complete extraction of *p*-nitrophenol and partitioned with 3 × 50 mL of hexane and then with 3 × 50 mL of diethyl ether. This procedure resulted in a hexane–diethyl ether and a water extraction phase. The organic solvent phases were pooled, dried over anhydrous Na_2SO_4, and analyzed by LSC and TLC, while the water phase was only analyzed for its radiocarbon content.

Table III. In Vivo and in Vitro Effects of Anethole on the in Vitro Metabolism of [^{14}C]Paraoxon (Results Are Mean ± Standard Deviation of Triplicate Tests)

extraction phases	distribution of radiocarbon in % of total ^{14}C recovered from incubation mixture of 10000*g* supernatant from houseflies		
	incubation mixtures from:		
	control flies[a]		flies fed anethole[b]
	(A) plus [^{14}C]paraoxon[c]	(B) plus [^{14}C]paraoxon[c] plus anethole[d]	(C) plus [^{14}C]paraoxon[c]
organic soluble[e]	26.5 ± 15.8	22.5 ± 12.9	48.2 ± 6.8[g]
paraoxon	18.5	17.5	33.0
p-nitrophenol	4.2	2.0	7.9
other	3.5	2.8	7.3
water soluble[f]	73.5 ± 13.3	77.5 ± 12.9	51.8 ± 6.8[h]
	Total Recovery of ^{14}C in % of Applied		
	93.6 ± 15.8	98.1 ± 8.9	90.3 ± 9.6

[a] Flies fed milk only for 3 days. [b] Flies fed milk containing 0.5% anethole for 3 days. [c] Fifty micrograms (0.1 μCi) of [^{14}C]paraoxon added in 10 μL of ethanol to 3 mL of 10000*g* supernatant and incubated 4 h at 30 °C. [d] Fifty micrograms of anethole in 10 μL of ethanol added in vitro to incubation mixtures. [e] Determined by TLC, autoradiography, and LSC. [f] Determined by LSC. [g,h] Results were different from controls (without anethole) at *g* the 9% level or *h* the 7% level (Student's *t* test).

Results indicated (Table III) that anethole affected the degradation of [^{14}C]paraoxon only when administered via the fly diet (Table III, C), but not when added simultaneously with [^{14}C]paraoxon to the subcellular fraction (Table III, B). The in vivo effect of anethole resulted in a reduced degradation of the insecticide. This was evidenced by the higher recoveries of paraoxon (Table III, C) in comparison to controls without anethole (Table III, A). The amounts of paraoxon remaining after the addition of anethole in vitro were the same as those observed in the absence of anethole. The reduced degradation of paraoxon after in vivo administration of anethole was also evidenced by the reduced production of water-soluble radiocarbon

(Table III, C). This increased persistence of [^{14}C]paraoxon due to anethole could, therefore, be related to the increased toxicity of paraoxon.

In summary, four major results should be mentioned. (1) Topically applied anethole and anisaldehyde increased the toxicity of several classes of topically applied synthetic insecticides to houseflies (Table I). (2) The presence of anethole in the housefly diet increased the toxicity of topically applied parathion or paraoxon to houseflies. (3) The presence of anethole in the diet resulted in an increased penetration of [^{14}C]paraoxon derived radiocarbon into the housefly body (Table II). (4) The presence of anethole in the housefly diet reduced the degradation of [^{14}C]paraoxon by a cell-free supernatant prepared from houseflies fed anethole for 3 days (Table III). It appears, therefore, that the increased mortalities of houseflies fed anethole in their diet for 3 days prior to topical application of paraoxon was due to an increased penetration of paraoxon into the insect body and an increased stability of paraoxon due to a reduction in the degradation of paraoxon to nontoxic, water-soluble metabolites. Both of these effects would increase the amount of toxic paraoxon within the houseflies and hence increase mortalities.

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Stability of Epoxide-Containing Juvenoids to Dilute Aqueous Acid

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The morphogenetic activity on *Tenebrio molitor* and the stability in weak aqueous acid of 13 *p*-bromophenyl geranyl ether epoxides containing juvenile hormone mimics, radiolabeled juvenile hormone, and three additional nonhalogenated hormone mimics were examined. Although changes in the molecule remote to the epoxide caused variations in hydrolytic stability, major changes resulted from varying the substituents on the epoxide. Increasing the size of alkyl substituents on the epoxide resulted in increased hydrolytic stability, and mono- and disubstituted epoxides were more stable than tri- and tetrasubstituted epoxides. As a derivatization technique, conversion of a trisubstituted epoxide to its diol proved unreliable when sulfuric acid in aqueous tetrahydrofuran was used but quantitative when dilute aqueous buffers were used.

The high biological activity of some insect growth regulators with juvenile hormone-like activity (juvenoids) has resulted in a great deal of synthetic effort leading toward the development of juvenoids as pesticides. Two juvenoids are currently marketed in the United States and several others are under development for U.S. and/or world markets. These and other juvenoids have been found to have very low acute and chronic mammalian toxicity, high specificity for many target vs. nontarget organisms, and favorable environmental properties. As a class of compounds, juvenoids also possess limitations. These limitations often include the appearance of cross-resistance and resistance in target insects (for literature see Vinson and Plapp, 1974; Hammock and Quistad, 1976; Georghiou et al., 1978), a limited spectrum of biological activity, and such rapid environmental degradation that asychronous pest populations are not adequately controlled.

Various functionalities at the isopropylidene end of juvenoid molecules have been explored, and the epoxide, as in the natural hormone, was found to have high biological activity. Epoxide-containing juvenoids have received some attention for commercial development, but

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