population with increased carbon dioxide levels has been shown by previous investigators (Baran et al., 1970; Clark and Lentz, 1969) to be produced mainly by the reduction of growth of pseudomonads on the meat surface. Pseudomonads, although aerobic, can grow effectively at oxygen concentrations of 0.8%, but are inhibited by increasing concentrations of carbon dioxide, with 10% carbon dioxide causing 44% inhibition in the growth of *Pseudomonas* 1482 (Ledward et al., 1971). As demonstrated by these authors, it is easier under industrial conditions to increase the carbon dioxide concentration to 10% than to decrease the oxygen concentration to less than 0.4%. The inhibition by carbon dioxide is also more effective at a given concentration as temperature is decreased.

King and Nagel (1967) investigated various mechanisms and factors regulating the growth of pseudomonads that could be influenced by carbon dioxide levels. The inhibition did not appear to be produced by alterations in oxygen tension, pH, or ionic strength of the substrate solutions. It appeared that specific enzymes involved in the catabolism of the various substrates examined were influenced to different degrees by carbon dioxide. More recently (King and Nagel, 1975), these authors concluded that the action of carbon dioxide on *Pseudomonas aeruginosa* was to limit the rate of growth by a mass action inhibition on certain decarboxylating enzymes, particularly isocitric and malate dehydrogenases. Incubation temperature has also been shown to alter the proportional utilization of the Entner–Doudoroff and hexose monophosphate pathways of glucose catabolism in *Pseudomonas fluorescens* through regulation of the growth limiting concentration of glucose (Palumbo and Witter, 1969). Although the substrates in meat that are actually utilized by the pseudomonads are unknown and may vary between meat samples, similar types of regulation of growth by carbon dioxide concentration may have occurred in these experiments.

As a result of inhibited pseudomonads growth, the slower growing lactobacilli and microbacterium flora which can grow in low oxygen and high carbon dioxide are predominant (Ledward et al., 1971). These species appear to have little effect on the formation of metmyoglobin during storage and are not a major concern in fresh meat spoilage.

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Synthesis and Carbon-13 Studies of Malathion Acid Derivatives

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Malathion [*O,O*-dimethyl *S*-(1,2-dicarbethoxy)-ethyl phosphorodithioate] is anticipated to undergo chemical and biological degradation in the aquatic environment to give malathion monoacid derivatives. Convenient synthetic routes to give selectively the α- and β-malathion monoacids were devised. Unambiguous structural assignments of the monoacids were made on the basis of chemical shifts and carbon–phosphorus coupling constants employing ^{13}C nuclear magnetic resonance (NMR). The ^{13}C NMR assignments for malathion and six of its related compounds are reported.

Malathion [*O,O*-dimethyl *S*-(1,2-carbethoxy)ethyl phosphorodithioate] is a widely used organophosphorus pesticide that exhibits low mammalian toxicity. Its high degree of biological selectivity has prompted extensive investigation of its comparative metabolism.

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Chen et al. (1969) reported that carboxylesterase isolated from rat liver degraded malathion to *O,O*-dimethyl *S*-(1-carboxy-2-carbethoxy)ethyl phosphorodithioate (α-monoacid). Welling et al. (1974), carrying out pesticide metabolism studies with houseflies, found that malaoxon was degraded to malaoxon β-monoacid. In the biological degradation of malathion by soil organisms, five metabolites were found, one of which was identified only as a malathion monoacid (Walker, 1972). Working with a heterogeneous bacterial population in aqueous medium, Paris et al. (1975) report that malathion is degraded to *O,O*-dimethyl *S*-(1-carbethoxy-2-carboxy)ethyl phosphorodithioate (β-monoacid). Work in our laboratory has disclosed that under pH

values and temperatures common to the aquatic environment, malathion undergoes competing carboxyl ester hydrolysis and *O,O*-dimethyl phosphorodithioic acid elimination (Wolfe et al., 1975).

Although malathion monoacids may be environmentally important, their correct structural assignments were still not clear. Chen et al. (1969) made tentative structural assignments for the α- and β-monoacids based on their proton nuclear magnetic resonance (NMR) chemical shifts. The infrared spectra of the α- and β-monoacids are very similar except for a single absorption at 1350 cm^{-1} reported to be indicative of the α isomer, but structural assignments could not be confirmed (Welling et al., 1970).

Because of the environmental interest in and significance of malathion degradation products, an investigation of the synthesis and structural assignments of the malathion monoacids and the corresponding malaoxon derivatives was begun. Reaction sequences were devised for selective synthesis of each monoacid. Liquid chromatography was used for quantitative separation of the products and permitted optimization of reaction conditions to give the desired monoacid. Unambiguous structural assignments were made employing ^{13}C NMR on the basis of chemical shifts and coupling constants. The ^{13}C NMR assignments for malathion and six of its related compounds used in this study are reported and discussed.

EXPERIMENTAL SECTION

General. All melting points were obtained on a Fisher Johns melting point apparatus and are uncorrected. All infrared spectra (ir) were taken on a Perkin-Elmer 621 grating infrared spectrophotometer. The ultraviolet spectra (uv) were obtained on a Perkin-Elmer 602 recording spectrophotometer.

Gas-liquid chromatography (GLC) was carried out on a Tracor MT-220 gas chromatograph equipped with a hydrogen flame ionization detector and fitted with a 6 ft × 0.25 in., 4% SE-30 on Chromosorb W column. Injection port, column, and detector temperatures were 235, 210, and 250°, respectively, and the flow rate was 60 cm^3/min.

Liquid chromatographic (LC) analyses were performed on a DuPont Model 820 liquid chromatograph equipped with an ultraviolet photometric detector (254 nm). The column was a Micropak S1-10, 50 cm × 2.2 mm (i.d.). The mobile phase was 5% methanol in methylene chloride at a pressure of 1000 psi and a 1 ml/min flow. For the malathion monoacids ϵ = 152 (water) at 254 nm.

NMR. Samples for proton NMR spectra were prepared in standard 5 mm sample tubes at a concentration of 0.3 *M* in $CDCl_3$. Tetramethylsilane (Me_4Si) was used as an internal reference and lock signal source. Proton NMR spectra were obtained on a Varian Associates HA-100 spectrometer operating in the frequency sweep mode with a probe temperature of 30°. A sweep width of 1000 Hz with a sweep time of 500 sec was used to record the spectra. Spectra were calibrated utilizing the frequency difference network.

Proton, noise-decoupled, natural abundance, carbon-13 spectra were recorded on a JEOL PFT-100 spectrometer with the JEOL EC-100 20K data system. Samples were prepared in 10-mm tubes to a concentration of ~0.75 *M* in $CDCl_3$ with Me_4Si (3%) added as an internal reference. The sample of malathion diacid was prepared in acetone-d_6 and the sample of *O,O*-dimethyl phosphorodithioic acid was run as a neat liquid with Me_4Si and acetone-d_6 added as reference and lock signal, respectively. Fourier transform spectra were obtained using a spectral width of 5000 Hz with either 8,192 or 16,384 data points. A pulse angle of ~40° was used with a repetition rate of 3 sec. Approximately 500 accumulations were used to obtain each spectrum with the exception of that of *O,O*-dimethyl phosphorodithioic acid for which 25 scans were sufficient. Chemical shifts are reported in parts per million downfield from Me_4Si and are considered accurate to ±1.2 and 0.6 Hz as indicated. The carbon–phosphorus coupling constants reported are considered accurate within similar limits.

Syntheses. *O,O-Dimethyl Phosphorodithioic Acid.* Phosphorus pentasulfide (Eastman) (35.2 g, 0.158 mol) was added to 40 ml of benzene. The slurry was stirred and maintained at 40°; 28.8 ml (0.713 mol) of absolute methanol was added dropwise over a period of 3 hr. The reaction mixture was filtered and the solvent removed by flash evaporation. The *O,O*-dimethyl phosphorodithioic acid was purified by distillation (12-in. Vigreux column), and the fraction distilling at 34–35° (0.15 mmHg) [lit. 42–44° (0.5 mmHg)] was collected. The ir spectrum compared with the literature spectrum (Nyquist, 1969).

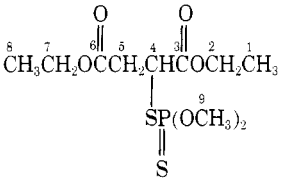
O,O-Dimethyl S-(1-Carbethoxy-2-carboxy)ethyl Phosphorodithioate (β Isomer). Following a procedure similar to that of Chen et al. (1969), 0.550 g (3.5 mmol) of *O,O*-dimethyl phosphorodithioic acid and 0.434 g (3.0 mmol) of ethyl hydrogen maleate (Dahlgren and Long, 1960) in the presence of pyridine (ca. 1 mg) were maintained at 60° for 2.5 hr. The reaction mixture was cooled and 10 ml of water was added. The mixture was slowly titrated with sodium hydroxide (1 *M*) until the residue dissolved (pH below 6). The solution was washed with 10 ml of chloroform and separated; the resulting aqueous layer was acidified to pH 2.5 (10% HCl) and extracted with three 50-ml portions of chloroform. The organic extracts were dried and evaporated, yielding 0.78 g (86%) of a colorless oil that slowly crystallized on standing. Analysis by LC showed the mixture to consist of the β isomer (97%) and the α isomer (3%). Recrystallization from chloroform–hexane gave white crystals: mp 42–45° (lit. 51–52°). The ir (Welling et al., 1970; Chen, 1967) and proton NMR (Chen et al., 1969) compared with those reported in the literature.

O,O-Dimethyl S-(1-Carboxy-2-carbethoxy)ethyl Phosphorodithioate (α Isomer). To 0.158 g (1.0 mmol) of *O,O*-dimethyl phosphorodithioic acid in 10 ml of 50% benzene–hexane (v/v) was added 0.144 g (1.0 mmol) of ethyl hydrogen maleate (Dahlgren and Long, 1960). 1′,1′,1′-Triphenylbenzeneazomethane (Eastman) (1 mg) was added; the solution was cooled to 0° and irradiated through Pyrex with a Hanovia medium-pressure 450-W lamp for 1 hr. The malathion monoacid product mixture contained the α isomer (95%) and the β isomer (5%) as shown by LC analysis. The reaction mixture was worked up as described above, yielding 0.120 g (40%) of a white solid. Crystallization from chloroform–hexane gave white crystals, mp 55–57° (lit. an oil). The ir and NMR spectra compared with the literature spectra (Welling et al., 1970; Chen, 1967; Chen et al., 1969).

O,O-Dimethyl S-(1,2-Dicarboxy)ethyl Phosphorodithioate (Diacid). Following the above procedure, 23.7 g (0.150 mol) of *O,O*-dimethyl phosphorodithioic acid was added to 14.7 g (0.150 mol) of maleic anhydride (Aldrich, recrystallized) and 1 drop of pyridine. The reaction was maintained at 60° for 3 hr. The residue was dissolved in 150 ml of chloroform, washed with three 100-ml portions of water, and dried (Na_2SO_4). To 11.8 g (0.046 mol) of the anhydride was added 40 ml of water; the resulting solution was heated at 70° for 1 hr. The aqueous solution was washed with 20 ml of chloroform, acidified to pH 2 (concentrated HCl), and extracted with four 50-ml portions of ether. Evaporation of solvent yielded a white solid, which crystallized from chloroform to give 9.8 g (78%) of the diacid: mp 115–118°. Recrystallization from chloroform yielded crystals with a melting point of 127–129°. The ir spectrum matched that reported in the literature (Walker, 1972).

O,O-Dimethyl S-(1-Carbethoxy-2-carboxy)ethyl Phosphorothiolate. A 10% solution of bromine in water was added dropwise with stirring to malathion β-monoacid (1 g, 3.3 mmol) dissolved in 10 ml of 50% aqueous ethanol (v/v) (27°) until a faint yellow color persisted. The aqueous solution was extracted with three 150-ml portions of chloro-

Table I. Carbon-13 Chemical Shifts for Malathion and Related Compounds[a]



Compound	ν_1[b]	ν_2	ν_3	ν_4	ν_5	ν_6	ν_7	ν_8	ν_9
Malathion[c]	14.05	62.08	169.95	45.26	37.95	169.80	61.11	14.05	54.26
			(4.8)	(3.7)	(3.1)				(3.6)
Malathion diacid[d]			171.58	45.54	38.12	171.58			54.60
			(4.9)	(3.7)	(3.6)				(4.9)
Malathion monoacid[d] (β isomer)	13.97	62.27	169.83	44.99	37.69	175.76			54.40
			(6.2)	(3.1)	(4.3)				(5.5)
Malathion monoacid[c] (α isomer)			175.51	45.09	37.70	169.98	61.40	14.11	54.24
			(4.9)						
Malaoxon[d]	14.17	62.18	169.9	42.68	38.24	169.78	61.11	14.17	54.43
			(6.1)	(3.7)	(4.9)				(6.1)
Malaoxon monoacid[d] (β isomer)	13.98	62.27	169.97	42.49	37.95	173.09			54.50
			(4.9)	(4.7)	(4.9)				(4.8)
O,O-Dimethyl phosphorodithioic acid									54.21
									(6.1)

[a] Values in parentheses are for the carbon-phosphorus coupling constants. [b] In parts per million downfield from internal Me_4Si. [c] ±1.22 Hz. [d] ±0.61 Hz.

form, and the organic layer dried (Na_2SO_4). Concentration gave 0.91 g (95%) of a colorless oil. The GLC retention time, after methylation (diazomethane), was less than the retention time of methylated starting material, and the ir spectra were consistent with the title compound. Attempts to crystallize or distill the oil under reduced pressure failed.

RESULTS AND DISCUSSION

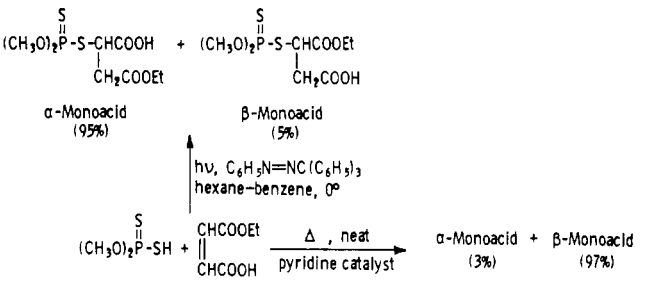
Synthesis. A synthetic scheme was needed to selectively synthesize each malathion monoacid. Following a procedure similar to that of Chen et al. (1969) gave on work-up of the reaction mixture an oil that crystallized on standing. The crystals consisted of two monoacids, as determined by LC analysis. Based on peak area ratios, one monoacid (t_R = 2.5 min) comprised 97% of the mixture, and the other (t_R = 4.2 min), 3%.

Recrystallization afforded a single monoacid (t_R = 2.5 min), the NMR of which compared to that reported by Chen et al. (1969) for the β-monoacid. Attempts to carry out the reaction in benzene solvent or without pyridine as an amine catalyst resulted in no reaction.

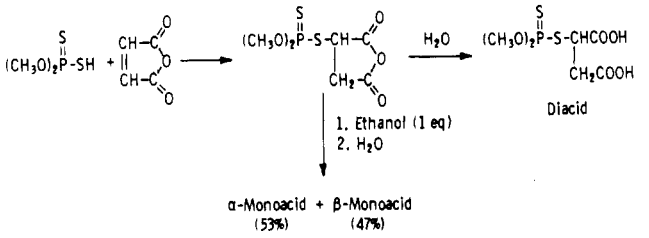
The predominance of β-monoacid formation is consistent with an ionic mechanism in which *O,O*-dimethyl phosphorodithioic acid undergoes acid-catalyzed nucleophilic addition to the carbon–carbon double bond. This addition pathway is favored in preference to electrophilic addition because of deactivation of the carbon–carbon double bond by the carboxy and carbethoxy groups.

We expected that the α-monoacid could be obtained by carrying out the addition reaction in the presence of a free-radical source. Bacon and LeSuer (1954) reported that *O,O*-dimethyl phosphorodithioic acid in the presence of peroxides added to alkenes in an anti-Markovnikov addition. In the absence of peroxides, normal Markovnikov addition was observed. Huang (1956) found that under homolytic addition reaction conditions, the trichloromethyl radical added β to the carbethoxy group of ethyl hydrogen maleate. We employed 1′,1′,1′-triphenylbenzeneazomethane (PAT) as a free-radical initiator. The reaction was carried out in a nonpolar organic solvent at 0° and the free radicals were generated by irradiation of PAT with Pyrex-filtered light (Scheme I). Liquid chromatographic analysis showed the product mixture to consist of the α-monoacid (95%) and the β-monoacid (5%). The isomer distribution is sensitive to reaction conditions. The reaction was repeated several times and the relative selectivity for the α-monoacid varied from 70% to a maximum of 95%.

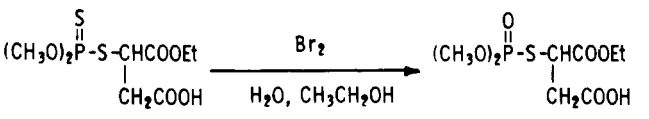
Scheme I



Scheme II



Scheme III



Malathion diacid [*O,O*-dimethyl *S*-(1,2-dicarboxyl)ethyl phosphorodithioate] was expected to be of value in making the ^{13}C NMR assignments in the monoacids (March et al., 1956). The addition of 1 equiv of ethanol instead of water to the intermediate anhydride yielded a mixture of α- and β-monoacids (α, 53%; β, 47%), as shown by LC (Scheme II). No attempt was made to separate the mixture by physical or chemical methods.

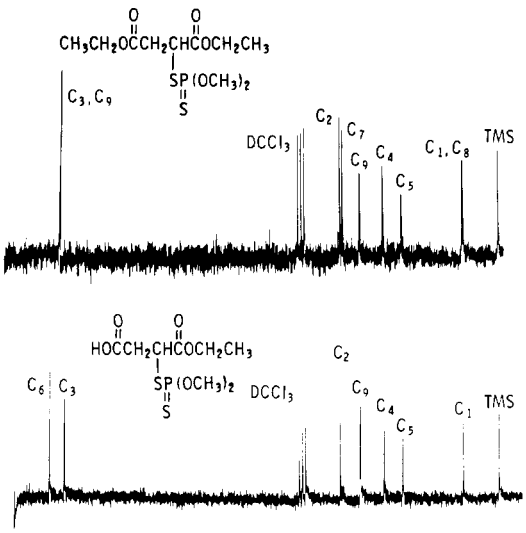


Figure 1. Carbon-13 spectra of malathion and malathion β-monoacid.

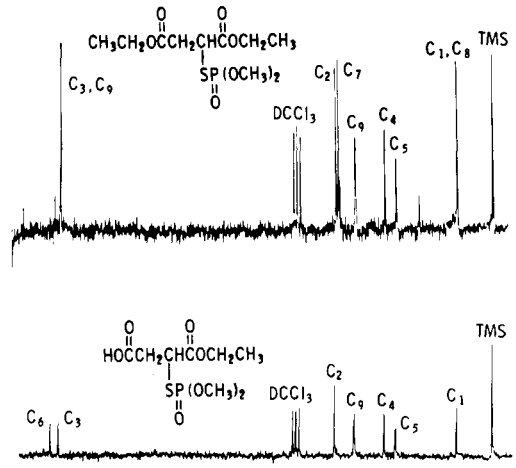


Figure 2. Carbon-13 spectra of malaoxon and malaoxon β-monoacid.

Malaoxon and malaoxon derivatives were obtained by oxidation of the corresponding malathion compound. Both malaoxon and the β-malaoxon monoacid were readily obtained by oxidation with bromine in aqueous ethanol (Fallscheer and Cook, 1956) (Scheme III).

NMR Studies. The carbon-13 chemical shifts and carbon–phosphorus coupling constants obtained for malathion and several related compounds are given in Table I. Representative spectra are reproduced in Figures 1 and 2. The proton noise-decoupled spectrum of malathion (Figure 1) exhibits nine carbon resonances as expected. Assignment of these peaks to the carbons of malathion was made from comparison with the spectra of the related compounds in Table I and from expected trends in the chemical shifts and phosphorus–carbon coupling constants.

The spectrum of *O,O*-dimethyl phosphorodithioic acid shows a doublet at 54.2 ppm for the methoxy carbons. The peak appearing at ~54 ppm in the spectra of the remaining compounds in Table I was therefore assigned to the methoxy carbons. The doublets observed for the methylene and methine carbons in the succinic acid chain were assigned based on off-resonance proton coupling. The two carbonyl resonances, one a doublet and the other a singlet, were assigned on the basis of previously reported trends in carbon–phosphorus coupling constants (Stothers, 1972).

Data from several phosphate esters show that J_{CCOP} is ca. 6 Hz, but more importantly for the present study, the data also show that J_{CCCOP} is ca. 0 Hz. The use of these trends allows an unambiguous assignment of the carbonyl resonances since only the carbonyl group three bonds removed from phosphorus is expected to show carbon–phosphorus coupling. Similar considerations were applied in the assignment of the resonances from malathion diacid.

Previous studies have shown that the carbon-13 resonance of an acid carbonyl is downfield from that of an ester carbonyl (Stothers, 1972). Using this trend along with that for the phosphorus–carbon coupling constants allows a straightforward assignment of the two monoacids obtained from the hydrolysis of malathion. Thus, the solid monoacid obtained from the reaction of *O,O*-dimethyl phosphorodithioic acid and ethyl hydrogen maleate exhibits a doublet at 169.8 ppm and a singlet at 175.7 ppm and is therefore consistent only with the β isomer. This assignment is identical with that reported by Chen et al. (1969), based on small differences in the proton chemical shifts of the two isomers. The carbon-13 spectra, however, allow a more straightforward and unambiguous assignment.

Considerations similar to those above were used to assign the carbons in the carbon-13 spectrum of malaoxon and establish that the monoacid obtained from the chemical hydrolysis of malaoxon is the β isomer (Figure 2).

We expect the malathion monoacids to form under certain environmental conditions. However, the impact of these monoacids on aquatic ecosystems has not been investigated. Both monoacids are now readily obtained through one-step synthetic schemes and are available for environmental studies. The unambiguous assignment of their structures is straightforward using carbon-13 NMR and illustrates the potential of this technique for structural studies involving environmental problems.

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