Loss of β-carotene was monitored from spectra over the range 350–520 nm of solutions of aliquots (about 100 mg) in light petroleum (10 mL). Pigment concentration was calculated from the absorbance at 450 nm assuming $E_{1cm}^{1\%}$ = 2500. Control experiments were carried on simultaneously by storing trays in the dark. Antioxidant effect of ethoxyquin (0.02%) was determined in methyl oleate solutions exposed to the light.

RESULTS AND DISCUSSION

The rates of loss of β-carotene, presented in Figure 1 as averages of the duplicate experiments, reveal several significant points regarding the nature of the autoxidation process. It is clear that, with the superimposed effect of the fluorescent light, unsaturation in the fatty acid solvents is not a necessity for oxidative loss of pigment. Indeed the distinct and consistently slower rates of loss show that a protective effect is being introduced with greater efficiency as unsaturation is increased.

The autocatalytic nature of the curves with marked induction periods and the inhibition given by the antioxidant suggest that free radical reactions are involved in loss of the pigment. In this respect results are in accord with those previously obtained from oxidations in toluene solution at higher temperatures with azoisobutyronitrile as added initiator (El-Tinay and Chichester, 1970), but present results differ from those at the higher temperature which show no lag phase.

In the present instances sensitizers are absent so that the involvement of singlet oxygen must be ruled out and the effect of the light must be attributed to absorption by the β-carotene itself. The control experiments in the absence of light gave no measurable loss of pigment. It is important, therefore, to realize that reaction with oxygen is probably being promoted by absorption of light in the visible region as distinct from the more usual ultraviolet source of activation particularly in view of the screening given by the plate glass cover to the sample holder. The site of attack of oxygen has been open to much discussion although it has been claimed that β-ionone has been identified as a product of photooxygenation without sensitizer (Isoe et al., 1969).

In the event that degradation occurs as a free radical process, the protective effect of the unsaturated fatty acid esters is explained in providing substrates for diversion of sequence in a chain process, with the tendency to diversion enhanced as unsaturation increases.

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Toxicity of *O,O,S*-Trimethyl and Triethyl Phosphorothioate to the Rat

O,O,S-Trimethyl phosphorothioate administered orally to rats caused mortality at doses as low as 15 mg/kg. At the lower doses of 15–80 mg/kg death occurred 4–22 days following treatment. The animals which died after treatment at the low doses appeared to be physically normal before death except for loss of weight. *O,O,S*-Triethyl phosphorothioate was slightly less toxic than the trimethyl analogue.

In a recent study concerned with the identification of impurities present in technical malathion and the effect of the impurities on the toxicological properties of malathion, we reported the presence of *O,O,S*-trimethyl phosphorothioate as an impurity, along with other sulfur-containing trimethyl esters (Umetsu et al., 1977). The acute oral LD_{50} of this compound was reported as 260 mg/kg based on 24–48 h mortality. In continuing studies on the potentiating and toxicological properties of these impurities in rats and mice, we have discovered that *O,O,S*-trimethyl phosphorothioate is a far more hazardous material than estimated from 48-h mortality data.

EXPERIMENTAL SECTION

Rat oral LD_{50} determinations were made with 95–120-g female albino rats (Sprague-Dawley derived) obtained from Simonsen's Laboratories, Gilroy, CA. Solutions of the toxicants in corn oil were administered orally at 0.2 mL/100 g rat. The animals were fasted for 6 h before treatment and kept under observation for 25 days. The ranges given for the LD_{50} values were estimated from the mortality data given in Tables I and II.

Malathion carboxylesterase activity in rat serum was assayed by coupling the hydrolysis of malathion to the reduction of a tetrazolium dye according to Talcott (1979). Groups of rats received graded oral doses of test compound, and blood samples were obtained from each rat at different time intervals after dosing. Aliquots of the sera were assayed for malathion carboxylesterase and cholinesterase activities (Talcott et al., 1979) and the results of each time point were expressed as percentages of the control activity (sera from untreated animals). Rat serum

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Table I. Time of Death of Rats Treated Orally with Different Amounts of *O,O,S*-Trimethyl Phosphorothioate

dose, mg/kg	no. of rats treated	time (days) of occurrence of death									total rats killed	% killed
		1	2	3	4	5	6	7	8	9-25		
300	4		4								4	100
200	4		2	2							4	100
80	4				2		1		1		4	100
60	4				2		1		1		4	100
40	4				2		1		1		4	100
30	4				2	1	1				4	100
20	4						1		1	1[a]	3	75
15	4				1					1[b]	2	50
10	4										0	0
0	4										0	0

[a] Died on 17th day. [b] Died on 22nd day.

Table II. Toxicological Symptoms in Rats Treated with *O,O,S*-Trimethyl Phosphorothioate

dose mg/kg	rat	symptons[a-c] after treatment (days)																					
		1			2			3			4			5			6			9		
		a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
200	rat I	10	++	+	17	++	+	18	(died)													
	rat II	8	++	+	11	(died)																
	rat III	15	+	+	21	++	−	24	(died)													
	rat IV	10	+	+	14	(died)																
30	rat I	15	−	+	24	+	+	31	−	−	37	(died)										
	rat II	16	−	+	23	−	−	29	−	−	37	(died)										
	rat III	14	−	±	23	−	−	30	−	−	35	−	−	40	−	−	46	(died)				
	rat IV	12	−	+	22	−	+	27	−	−	32	−	−	35	(died)								
15	rat I	8	−	−	15	−	−	11	−	−	6	−	−	5	−	−	+1	−	−	+7	−	−
	rat II	1	−	−	3	−	−	3	−	−	+1	−	−	+2	−	−	+7	−	−	+3	−	−
	rat III	10	−	−	14	−	−	10	−	−	8	−	−	+2	−	−	+6	−	−	+2	−	−
	rat IV	6	+	−	17	−	−	25	−	−	30	(died)										
0 (corn oil only)	rat I	+4	−	−	+10	−	−	+16	−	−	+16	−	−	+21	−	−	+25	−	−	+37	−	−
	rat II	+9	−	−	+16	−	−	+21	−	−	+22	−	−	+30	−	−	+35	−	−	+55	−	−

[a] Percent weight loss from initial weight. [b] Diarrhea or urination: ++, heavy; +, observed; −, not observed. [c] Bleeding from mouth, nose, or ear: ++, heavy; +, observed; −, not observed.

cholinesterase activity was determined spectrophotometrically using acetylthiocholine as the substrate according to Ellman et al. (1961). Enzyme assays were conducted at 37 °C.

O,O,S-Trimethyl phosphorothioate (Hilgetag et al., 1960) was prepared by reacting the potassium salt of *O,O*-dimethyl phosphorothioic acid with dimethyl sulfate. The product was purified by silica gel column chromatography using benzene and benzene–ethyl acetate (9:1) as the eluting solvents. The purity of the product was checked by TLC (solvent system: benzene–ethyl acetate, 1:1). Only a single TLC spot was evident using 2,6-dibromo-*N*-chloro-*p*-benzoquinoneimine spray reagent (Menn et al., 1957). Triethyl phosphorothioate was prepared by reaction of ethylsulfenyl chloride with triethyl phosphite according to Morrison (1955). The product was purified by vacuum distillation. The structures of both compounds were confirmed by NMR and mass spectroscopy.

RESULTS AND DISCUSSION

In contrast to intoxication by an anticholinesterase organophosphorus insecticide, e.g., parathion, death of rats treated with *O,O,S*-trimethyl phosphorothioate required substantially longer holding periods, particularly at the lower doses. Table I provides rat mortality data at different doses and posttreatment periods. Based on the data, the estimated 48-h LD_{50} is ~200 mg/kg, in line with our previous value. However, the LD_{50} value at an extended holding period of 25 days is between 15 to 20 mg/kg. Therefore, *O,O,S*-trimethyl phosphorothioate is almost as toxic to the rat as parathion (rat oral LD_{50} = 3–30 mg/kg) (Kenaga and End, 1974), although death occurs in a slower, more insidious manner. Rats treated at 30–80 mg/kg were alive 3 days after treatment but died during the fourth–eighth day.

Table II summarizes symptoms observed at different time intervals after oral treatment of rats with 15, 30, and 200 mg/kg of *O,O,S*-trimethyl phosphorothioate. Symptoms indicated are weight loss (a), diarrhea and excessive urination (b), and bleeding from mouth, nose, and ear (c). At the high dose of 200 mg/kg, diarrhea, excessive urination, and bleeding were common among the animals treated, and in all cases diarrhea was heavy. These symptoms of poisoning also were observed at doses as low as 40 mg/kg but were slight at 30 mg/kg. Diarrhea, excessive urination, and bleeding were observed only during the first 2 days after treatment. The most prevalent symptom of poisoning was weight loss and this occurred in all animals, including those treated at 15 mg/kg. In some cases weight loss was severe, e.g., rat III treated at 30 mg/kg lost almost half its weight in 6 days (at the time when death occurred), although from outward appearances it did not seem to be in a distressed state. Virtually all of the rats treated at the low dose of 15 mg/kg appeared to be normal in all aspects except for weight loss. One of these rats, however, died on the 4th day and one on the 22nd day.

O,O,S-Triethyl phosphorothioate also was examined for delayed toxicity since this material may be present in technical diethyl phosphorothioate insecticides. Rat mortality data at different doses and posttreatment periods are given in Table III. While the same kinds of intoxication symptoms were observed with this ester, i.e., diarrhea, excessive urination, etc., the time pattern of the occurrence of death was somewhat different than that for the trimethyl analogue. At the higher doses (85–110

Table III. Time of Death of Rats Treated Orally with Different Amounts of *O,O,S*-Triethyl Phosphorothioate

dose, mg/kg	no. of rats treated	time (days) of occurrence of death									total rats killed	% killed
		1	2	3	4	5	6	7	8	9-25		
110	4	3					1				4	100
100	5	4									4	80
85	4	1	1								2	50
75	4		2								2	50
50	4				1				1		2	50
30	4										0	0

Table IV. In Vivo Inhibition of Rat Serum Malathion Carboxylesterase with Time by Trialkyl Phosphorothioates

compound	dose, mg/kg	rat	percent control activity						
			4 h	20 h	2 days	3 days	6 days	7 days	14 days
$(CH_3O)_2P(O)SCH_3$	15	1	38.7		73.1		55.6		100.0
		2	80.6		85.7		61.1		32.2 (died)
		3	58.1		75.6		66.7		100.0
		4	48.4		70.6 (died)				
	40	1	45.7					43.7 (died)	
	60	1	12.7	18.2		33.3 (died)			
		2	13.0	27.0		43.5 (died)			
$(C_2H_5O)_2P(O)SC_2H_5$	50	1	7.7		70.5		37.8		94.4
		2	7.7		85.7		100.0		100.0
		3	12.9		45.9 (died)				
		4	3.2		40.3		38.9 (died)		
	85	1	4.5		27.7		75.0		100.0
		2	3.9 (died)						
		3	6.5		50.4		77.8		100.0
		4	5.8		4.5 (died)				

Table V. In Vivo Inhibition of Rat Serum Cholinesterase with Time by Trialkyl Phosphorothioates

compound	dose, mg/kg	rat	percent control activity				
			4 h	2 days	3 days	6 days	14 days
$(CH_3O)_2P(O)SCH_3$	15	1	63.2	59.3		66.7	100.0
		2	61.1	48.2		51.9	68.4 (died)
		3	68.4	56.3		63.0	100.0
		4	73.7	60.7 (died)			
	60	1	43.8		75.0 (died)		
		2	42.7		80.0 (died)		
$(C_2H_5O)_2P(O)SC_2H_5$	50	1	29.5	55.6		44.4	100.0
		2	29.5	63.0		63.0	100.0
		3	31.6	49.6 (died)			
		4	26.3	49.6		37.0 (died)	
	85	1	13.7	32.6		40.7	82.1
		2	13.7 (died)				
		3	23.2	40.7		51.9	100.0
		4	21.1	19.3 (died)			

mg/kg) death occurred within 24 h after treatment. However, at the lower dose of 50 mg/kg death occurred on the fourth and eighth day. LD_{50} values for the triethyl ester after 48 h were estimated to be about 80 mg/kg, while a value of 45–90 mg/kg was estimated after 8 days. Thus, relatively little difference in LD_{50} was observed between the two time periods.

Little can be said at the present time concerning the mode of action of these trialkyl phosphorothioate esters. Concurrent studies on the mode of potentiating action of *O,O,S*-trimethyl phosphorothioate on malathion toxicity to rats show that both serum carboxylesterase and serum cholinesterase activities are significantly inhibited for extended periods after a single oral dose as low as 15 mg/kg of the trimethyl phosphorothioate. Data showing the decrease in serum carboxylesterase and serum cholinesterase activities at different time intervals following single oral doses of different amounts of either *O,O,S*-trimethyl or *O,O,S*-triethyl phosphorothioate are presented in Tables IV (carboxylesterase) and V (cholinesterase). The results show that serum carboxylesterase is more sensitive to inhibition than serum cholinesterase. Recovery of enzymatic activity, however, was slow with both enzymes. While the inhibition of carboxylesterase activity is probably responsible for the potentiating activity of these trialkyl esters, it is not clear whether there is any relationship between the inhibition of this enzyme and the delayed mortality observed. This problem is currently under investigation.

The point to be made is that simple trialkyl phosphorothioates, generally believed to be nontoxic compounds, can be highly toxic materials of an insidious nature. Of the two trialkyl phosphorothioates, the *O,O,S*-trimethyl ester was more toxic to rats than the corresponding triethyl ester. The trimethyl ester has been reported as a 0.04–0.1% impurity in technical malathion, 0.015–0.019% impurity in phenthoate and 0.2% impurity in acephate (Umetsu et al., 1977; Pellegrini and Santi, 1972). While the percentage of this compound in technical organophosphorus insecticides is small, it is possible that larger amounts may be formed when the technical materials are exposed to sunlight. For example, photolysis studies with the organophosphorus insecticide parathion have demonstrated yields of *O,O,S*-triethyl phosphorothioate as high as 39% following ultraviolet irradiation of parathion (Grunwell and Erickson, 1973). Further, the

photolytic formation of the triethyl phosphorothioate and its toxicity to rats raise the question of its possible role in the anomalous poisoning of workers who entered fields sprayed with parathion during periods when parathion levels were low and the fields were considered safe (Milby et al., 1964).

It should be pointed out that other sulfur-containing trialkyl organophosphorus esters also are present in technical organophosphorus insecticides (Umetsu et al., 1977). *O,S,S*-Trimethyl and *O,O,S*-trimethyl phosphorodithioates, two impurities present in technical malathion, however, did not elicit the delayed toxic effects caused by *O,O,S*-trimethyl phosphorothioate.

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