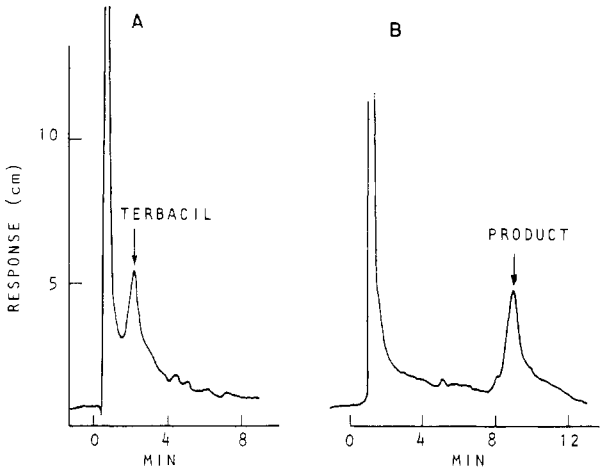

Figure 3. Confirmation of terbacil in corn (0.1 ppm) by alkylation: (A) 400-mg sample injected before reaction; (B) same extract after alkylation. Conditions are as described in Table I; attenuation 2×.

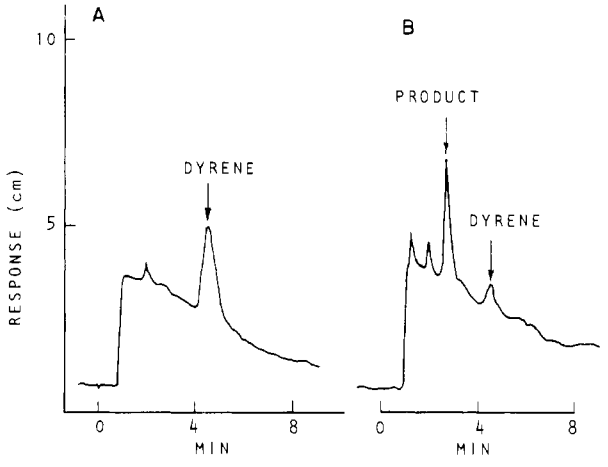

Figure 4. Confirmation of dyrene in corn (0.1 ppm) by trifluoroacetylation: (A) 400-mg sample injected before reaction; (B) same extract after acetylation. Conditions are as described in Table I; attenuation 2×.

1971). Figure 1 shows the reaction scheme for the methoxylation of the thiolcarbamates, EPTC, and diallate. The thiocarbamate linkage was broken and the corresponding methylcarbamates were formed which eluted from the GLC much faster than the parents. The methoxylation of dichlobenil resulted in both ring chlorine atoms being replaced with methoxyl substituents (*m/e* parent, 172; *m/e* product, 163).

The alkylation reactions proceeded well for atrazine, chlorpropham, dichloran, dyrene, linuron, propanil, and terbacil. The N–H substituent in all cases was converted to N–CH_3. The reaction mechanism has been discussed earlier for N–H containing compounds in general (Greenhalgh and Kovacicova, 1975; Lawrence and Laver, 1975).

Trifluoroacetylation was carried out as described in the literature (Drozd, 1975; Cochrane, 1975; Khan, 1975). The reactions proceeded in the normal manner resulting in the N–H moiety being converted to N–CO–CF_3. The derivatives eluted earlier than the parents in all cases.

The confirmation techniques were applied to sample extracts of corn spiked at 0.1 ppm. Figures 2–4 compare sample extracts containing three of the herbicides studied before and after derivatization. All pesticides eluted from the Florisil in the 15% acetone/hexane fraction with the exceptions of terbacil (50% acetone/hexane fraction) and diallate and dichlobenil which eluted in the 30% methylene chloride fraction. All could be confirmed at 0.1 ppm in corn by at least one of the derivatization reactions described herein.

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LITERATURE CITED

Cochrane, W. P., *J. Chromatogr. Sci.* **13**, 246 (1975).
Drozd, J., *J. Chromatogr.* **113**, 303 (1975).
Greenhalgh, R., Kovacicova, J., *J. Agric. Food Chem.* **23**, 325 (1975).
Khan, S. U., *Residue Rev.* **59**, 21 (1975).
Lawrence, J. F., *J. Agric. Food Chem.* **22**, 936 (1974).
Lawrence, J. F., *J. Chromatogr.* **121**, 85 (1976).
Lawrence, J. F., Laver, G. W., *J. Agric. Food Chem.* **23**, 1106 (1975).
Lawrence, J. F., McLeod, H. A., *J. Assoc. Off. Anal. Chem.* **59**, 637 (1976).
Lawrence, J. F., Sen, N. P., *Anal. Chem.* **47**, 367 (1975).
Mendoza, C. E., Wales, P. J., Hatina, G. V., *J. Agric. Food Chem.* **19**, 41 (1971).

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Induction of Paraoxon Dealkylation by Hexachlorobenzene (HCB) and Mirex

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The induction of paraoxon dealkylation activity was measured in the microsomal fraction from livers of rats fed for 2 weeks on diets containing hexachlorobenzene (HCB) or Mirex. Formation of deethylparaoxon was increased significantly at 2 ppm of HCB and 1 ppm of Mirex. Other parameters, body weight gain, liver weight, microsomal protein, P_{450}, and aminopyrine *N*-demethylase, were not significantly affected by these dose levels, but they were increased after feeding 10 and 40 ppm of HCB or 5 ppm of Mirex.

Dealkylation of organophosphate insecticides is believed to occur via the microsomal mixed function oxidase system and a cytoplasmic glutathione transferase (Appleton and Nakatsugawa, 1972). Ku and Dahm (1973) have reported that O-dealkylation is increased considerably after administration of phenobarbital and other inducers, while the glutathione-requiring system is unaffected. Donninger (1971) also reported a 600-fold increase in specific activity

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toward chlorfenvinphos in rats pre-treated with dieldrin.

Recently, Tonkelaar and van Esch (1974) determined the no-effect levels of 12 organochlorine pesticides, including hexachlorobenzene (HCB), based on induction of microsomal liver enzymes. Compared with the other compounds, HCB was a relatively poor inducer, and the no-effect level was judged to be 20 ppm, a value similar to that proposed by Grant et al. (1974). The no-effect level for Mirex appears to be lower, since Baker et al. (1972) found that 1 ppm of Mirex caused a significant increase in P_{450} levels.

In view of the potentially large increase in dealkylation activity of induced microsomal preparations, the present investigation was begun to determine the effect of HCB and Mirex induction on paraoxon dealkylation, and to compare this parameter with others that have been routinely used for this purpose. Both HCB and Mirex are polyhalogenated, persistent pesticides with a potential for contamination of food. The choice of paraoxon was governed by background data and its availability in radiolabeled form.

MATERIALS AND METHODS

Male Sprague Dawley rats (200 ± 20 g) were obtained from Bio-Breeding, Ottawa, and housed in banks with free access to food and water for a 1-week acclimatization period. Corn oil solutions of Mirex [dodecachlorooctahydro-1,3,4-metheno-1*H*-cyclobuta[*c*,*d*]pentalene] (Technical, purity 98%, Hooker Chemical & Plastics Corp., Niagara Falls, N.Y.) or hexachlorobenzene (analytical standard, British Drug House, Montreal) were mixed thoroughly in the diets (prepared from ground fox cubes, Maple Leaf Mills Ltd., Toronto). The diets were fed for 2 weeks and contained the following levels: HCB, 0, 2, 10, and 40 ppm; Mirex, 0, 0.25, 1, and 5 ppm. There were 6 animals at each dose level. Other rats that had been maintained for 9 months on a diet containing 0 and 40 ppm of HCB were also sacrificed. Twelve rats were used from each dose level.

Animals were sacrificed by decapitation; the livers were removed, weighed, and packed in crushed ice. Within 30 min after sacrificing, the livers were homogenized in 4 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 9000*g* for 20 min and the microsomal fraction subsequently sedimented twice, with washing at each time, at 100 000*g* for 1 h. The washed microsomal pellet was resuspended in the buffer and aliquots removed for measurement of the various parameters. Aminopyrine *N*-demethylase was determined in a total volume of 5.0 ml containing: glucose 6-phosphate (3 mM), NADP (0.6 mM), magnesium chloride (3 mM), buffer (50 mM), pH 7.4, glucose-6-phosphate dehydrogenase (2 units), and microsomes to give approximately 1 mg of protein/ml of incubate. Incubation was for 30 min in air at 37 °C. The formaldehyde formed was determined according to Cochin and Axelrod (1959) and P_{450} according to Omura and Sato (1964). Protein was measured by the Biuret method of Gornall et al. (1949). Deethylparaoxon formation was determined in a total volume of 1.0 ml containing the co-factors listed above, in the same final concentration, except the amount of microsomes was increased to approximately 10 mg of protein/ml of incubate. Paraoxon was 1 mM.

Preliminary work on the deethylparaoxon formation was carried out using ethoxy-^{14}C-labeled paraoxon (New England Nuclear, Montreal). The metabolites were separated with silica gel thin-layer chromatography (TLC) in acetonitrile–water (88:12). Only two metabolites were readily noted: diethylphosphoric acid (DEP) and deethylparaoxon (DEO). As indicated by Ku and Dahm (1973), the yield of DEO decreased as the incubation time increased. We found the yield of DEO was maximal after 10 to 20 min of incubation and dropped to 60% of maximum after 60-min incubation. Total DEO formation increased with increasing protein concentration reaching a maximum at 5 mg of microsomal protein per ml of incubate and declining to 85% of peak value at 10 mg/ml. Although the formation of DEO was decreased at the higher protein concentration this level was employed in order to remain consistent with the procedure of Ku and Dahm (1973), while providing a measurable amount of control activity. The mean error associated with all of the DEO measurements made in this investigation was 9.3 ± 1.8%.

Liver residues of HCB and Mirex were determined by the GLC methods given by Grant et al. (1974) and Khera et al. (1975), respectively.

To increase the utility of the method, an extraction procedure was established to enable gas–liquid chromatography (GLC) estimation of DEO. The procedure was based on the alkylation method of Shafik et al. (1973). In this case, the remaining paraoxon is extracted and the DEO is realkylated to produce paraoxon. The final procedure was as follows: after 15-min incubation protein is precipitated with 1.0 ml of 10% trichloroacetic acid (Cl_3CCOOH) and centrifuged for 10 min at 3000*g*. A 1.5-ml aliquot of the clear supernatant is removed and extracted twice with 1.0 ml of hexane. A 1.0-ml aliquot of the aqueous layer is removed and the metabolite is extracted and alkylated according to Shafik et al. (1973) using diazoethane prepared from *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Montreal). The hexane extraction removes all traces of paraoxon as determined by GLC, and no paraoxon peak was found unless alkylation occurred.

The extraction procedure for DEO was verified using [^{14}C]DEO prepared from ethoxy-^{14}C-labeled paraoxon by the method of Hollingworth (1967). The recovery of DEO averaged 97%. Incubation of [^{14}C]paraoxon and TLC scanning revealed again that the parent compound is completely removed by hexane extraction. GLC was performed on a Varian 2100 equipped with a rubidium sulfate detector and a 3-ft glass column of 5% DEGS on Chromosorb W-HP, 80–100 mesh. The column temperature was 155 °C, nitrogen 35 ml/min, air 200 ml/min, and hydrogen 27 ml/min. Under these conditions, the peak height of paraoxon (retention time 4.3 min) was linear with concentration over a range equivalent to DEO formation of 0.075 nM min^{-1} mg^{-1} to 1.8 nM min^{-1} mg^{-1}. This is a range at least twofold greater than the highest level measured. Derivatization to form lower or higher alkyl homologues resulted in either a loss of sensitivity or a decrease in retention time such that there was an interference with the desired peak.

RESULTS

Figure 1 illustrates a GLC recording for the blank (microsomes added after Cl_3CCOOH), control, and HCB-induced incubations. This illustrates the lack of response with no enzymatic activity and the low level of activity in control animals. A Cl_3CCOOH scan of an incubate using [^{14}C]paraoxon with microsomes from HCB-induced animals is presented in Figure 2. When the cofactors were omitted, only DEP and paraoxon were present, confirming that DEO production is dependent on mixed function oxidase activity, as reported by Appleton and Nakatsugawa (1972). Table I lists the results of HCB induction at 40 ppm for 9 months. Every parameter was

Table I. Effect of Feeding 40 ppm of HCB for 9 Months on Selected Biochemical Parameters; $n = 12$; $^{**}P < 0.05$

	Liver, % body wt	Protein, mg/g of liver	*N*-Demethylase, nM min^{-1} mg^{-1} of protein	P_{450}, nM/mg of protein	Deethylparaoxon, nM min^{-1} mg^{-1} of protein
Control	3.01	20.95	3.42	0.890	0.141
	±0.10	±0.87	±0.15	±0.025	±0.007
HCB	3.38**	24.75**	5.76**	1.071**	0.798**
	±0.07	±0.81	±0.19	±0.035	±0.056

Table II. Effect of HCB on Selected Biochemical Parameters after Feeding for 2 Weeks; $n = 6$; $^{*}P < 0.10$; $^{**}P < 0.05$

	HCB, ppm in diet			
	0	2	10	40
Body wt gain, g	97.7	95.5	100.3	97.3
	±7.4	±5.2	±4.9	±6.5
Liver, % body wt	4.78	4.94	5.05	5.15
	±0.06	±0.17	±0.16	±0.18
Microsomal protein, mg/g of liver	20.18	20.10	18.98	18.28
	±1.02	±0.66	±0.87	±0.70
P_{450}, nM/mg of protein	0.84	0.80	0.95	1.08**
	±0.05	±0.02	±0.08	±0.06
N-Demethylase, nM min^{-1} mg^{-1} of protein	2.74	2.75	3.08*	3.78**
	±0.12	±0.09	±0.14	±0.030
Deethylparaoxon, nM min^{-1} mg^{-1} of protein	0.138	0.219**	0.372**	0.887***
	±0.017	±0.014	±0.042	±0.085

Table III. Effect of Feeding Mirex in the Diet for 2 Weeks on Selected Biochemical Parameters; $n = 6$; $^{*}P < 0.10$; $^{**}P < 0.05$

	Mirex, ppm in diet			
	0	0.25	1	5
Body wt gain, g	85.3	78.3	78.0	71.6
	±4.8	±6.0	±4.1	±3.4
Liver, % body wt	4.58	4.48	4.22	4.45
	±0.13	±0.14	±0.10	±0.13
Microsomal protein, mg/g of liver	18.98	20.4	17.9	21.9
	±0.85	±0.65	±0.68	±1.04
P_{450}, nM/mg of protein	0.886	0.905	0.975*	1.19**
	±0.037	±0.032	±0.030	±0.039
N-Demethylase, nM min^{-1} mg^{-1} of protein	2.90	3.14	2.75	3.28*
	±0.12	±0.10	±0.11	±0.15
Deethylparaoxon, nM min^{-1} mg^{-1} of protein	0.125	0.104	0.218**	0.427**
	±0.026	±0.007	±0.024	±0.040

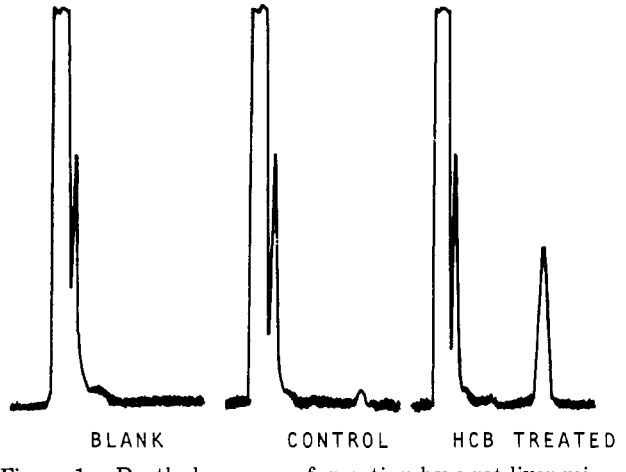

Figure 1. Deethylparaoxon formation by a rat liver microsomal fraction. A GLC tracing showing the absence of deethylparaoxon in the blank and the low control activity compared to an HCB-induced sample.

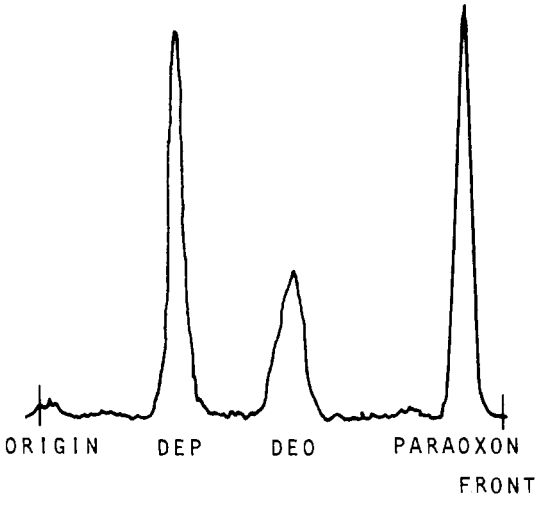

Figure 2. A tracing from a radiochromatographic scan of a TLC plate spotted with an unextracted incubate obtained with microsomes from HCB induced liver. Diethyl phosphate (DEP), deethylparaoxon (DEO), and the remaining substrate, paraoxon, are the only peaks discernible on the plate. The deethyloxon peak was not present when the cofactor was omitted from the incubation.

significantly different ($P < 0.05$) from controls with DEO formation exhibiting the largest increase. Body weight gain was not significantly greater in the treated group. The increase in formation of DEO in the HCB-treated group was confirmed using [^{14}C]paraoxon with scanning of the TLC plates and liquid scintillation counting of the DEO band which was scraped into vials containing 10 ml of cocktail.

Table II lists the effects of feeding 0, 2, 10, or 40 ppm of HCB for 2 weeks. At 2 ppm and higher, DEO formation was significantly greater than control levels ($P < 0.05$) while *N*-demethylase and P_{450} did not reach this level of

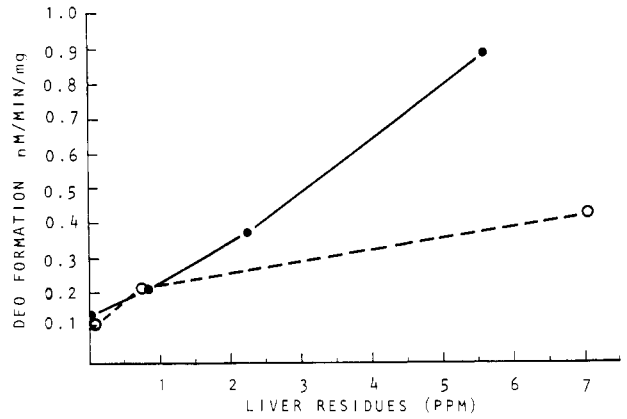

Figure 3. The relationship between liver levels of inducer and deethylparaoxon (DEO) formation. The open circles and the closed circles refer to HCB and Mirex, respectively. Each point represents the mean liver residue level from five or six animals.

significance except when 40 ppm of HCB was fed. Two additional confirmatory feeding studies were completed at the same levels of HCB, and for DEO formation, $P < 0.05$ at 2 ppm of HCB for the second study and $P < 0.10$ for the third.

Table III lists the results obtained with Mirex. The DEO response was the greater observed and the no-effect level based on this parameter would be 0.25 ppm. However, P_{450} was also increased (at a lower level of significance, $P < 0.1$) at this dietary level.

The relationship between liver residue levels of inducer and DEO formation is shown in Figure 3. There is a greater induction by HCB than with Mirex and this occurs at lower HCB levels. Mirex residues were greater than those of HCB, to the extent that a dietary level of 5 ppm of Mirex resulted in a liver residue that was greater (7.08 ± 0.45 ppm) than the HCB level (5.56 ± 0.03 ppm) accumulated from an HCB dietary level of 40 ppm. The Mirex tissue levels obtained in the present study were consistent with the accumulation pattern noted by Khera et al. (1975). Liver HCB levels in the rats fed for 9 months were 25.4 ± 2.1 ppm. Induction of DEO activity was no greater in these animals than occurred after feeding the same level (40 ppm) for 2 weeks.

DISCUSSION

The increase in paraoxon dealkylation activity appeared to be a sensitive indicator of induction by HCB, when compared to previous reports (Tonkelaar and van Esch, 1974; Grant et al., 1974). On the other hand, the increased activity noted with Mirex at 1 ppm is consistent with increased P_{450} levels noted by Baker et al. (1972) and confirmed in the present study. Although the increase in DEO formation elicited after 9 months feeding of HCB at 40 ppm was no greater than that after 2 weeks feeding, it is doubtful that this can be termed a maximum increase since prolonged induction can result in a decrease in activity (Kinoshita and DuBois, 1970).

The induction by compounds always raises a question of anomalies caused by the presence of inducers. In the present study, HCB levels in the homogenates were determined and equivalent amounts were added to control and phenobarbital induced liver microsomal fractions. There was no indication of inhibition or activation of DEO formation in either case. However, the observations of Kutt et al. (1971) on inducer effects on substrate–microsomal spectra suggested that effects may occur and are difficult to interpret. Since both HCB and Mirex are metabolized very little, this would lessen the concern of metabolite effects and suggest that the increase in DEO production is a valid measure of induction.

The fact that similar liver residue levels of HCB and Mirex did not result in a similar increase in DEO activity is probably the result of several phenomena, including a specificity exhibited for the inducer as well as a subsequent specificity for the substrate by the range of induced enzymes. This variation necessitates the use of a range of substrates in studies of this type, since the no-effect level determined may vary with the substrate used to measure the extent of induction.

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LITERATURE CITED

Appleton, H. T., Nakatsugawa, T., *Pestic. Biochem. Physiol.* **2**, 286 (1972).
Baker, R. C., Coons, L. B., Mailman, R. B., Hodgson, E., *Environ. Res.* **5**, 418 (1972).
Cochin, J., Axelrod, J., *J. Pharmacol. Exp. Ther.* **125**, 105 (1959).
Donninger, C., *WHO Bull.* **44**, 265 (1971).
Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.* **177**, 751 (1949).
Grant, D. L., Iverson, F., Hatina, G., Villeneuve, D., *Environ. Physiol. Biochem.* **4**, 159 (1974).
Hollingworth, R. M., Metcalf, R. L., Fukuto, T. R., *J. Agric. Food Chem.* **15**, 242 (1967).
Khera, K. S., Villeneuve, D. C., Terry G., Panopio, G., Nash, L., Trivett, G., *Food Cosmet. Toxicol.* **14**, 25 (1975).
Kinoshita, F. K., DuBois, K. P., *Toxicol. Appl. Pharmacol.* **17**, 406 (1970).
Ku, T., Dahm, P. A., *Pestic. Biochem. Physiol.* **3**, 175 (1973).
Kutt, H., Waters, L., Fouts, J. R., *J. Pharmacol. Exp. Ther.* **179**, 101 (1971).
Omura, T., Sato, R., *J. Biol. Chem.* **239**, 2370 (1964).
Shafik, T., Bradway, D. E., Enos, H. F., Yobs, A. R., *J. Agric. Food Chem.* **21**, 625 (1973).
Tonkelaar, E., van Esch, G. J., *Toxicology* **2**, 371 (1974).

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