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Effect of dichlorvos (DDVP) on mouse liver glutathione levels and lack of potentiation by methyl iodide and TOTP

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Dichlorvos (DDVP) [2,2-dichlorovinyl dimethyl phosphate] is an organophosphate insecticide widely used in pet flea collars, veterinary anthelmintics, and volatile resin strips. It is generally accepted that organophosphate toxicity results from inhibition of the critical nervous system enzyme, acetylcholinesterase. DDVP, however, is regarded as less toxic than most organophosphates since prolonged exposure of humans to insecticidal concentrations of DDVP did not lead to significant depression of blood cholinesterase activity [1–4].

The comparatively low mammalian toxicity of DDVP is thought to result from its rapid detoxification to metabolites which do not inhibit acetylcholinesterase activity. Of the several biotransformation reactions demonstrated for DDVP, hydrolysis of the phosphorus-vinyl bond has been suggested as the single most important metabolic pathway [5–11]. The esterases catalyzing this reaction are termed A-esterase (E.C. 3.1.1.2) [12] and are located primarily in the liver but are also found in other mammalian tissues.

In addition to A-esterase hydrolysis, binding to carboxylesterases (E.C. 3.1.1.1) may represent an important detoxification mechanism in that any organophosphate bound to and inhibiting carboxylesterases would not be available to inhibit the critical enzyme, acetylcholinesterase. Binding has been suggested as an important detoxification mechanism for malaoxon and paraoxon since inhibition of binding by prior treatment with triorthotolylphosphate (TOTP) decreased binding and subsequently increased the toxicity of these organophosphates [13, 14]. A previous investigation in this laboratory [15] demonstrated that DDVP was inactivated *in vitro* by mouse liver under assay conditions which were consistent with a binding mechanism of inactivation. In contrast to results obtained with malaoxon and paraoxon inhibition of binding by TOTP had no effect on the toxicity of DDVP. These findings suggested that other mechanisms play a more important role in DDVP detoxification in mice.

Hutson [10] suggested that, in the mouse, glutathione (GSH)-dependent dealkylation was also an important detoxification pathway for organophosphates, like DDVP, which contained at least one *O*-methyl group. Other investigations demonstrated that organophosphates with *O*-methyl substituents reduced liver GSH levels, suggesting that they were metabolized by GSH-dependent pathways [16–18]. The excretion of *S*-methyl metabolites after administration of labeled DDVP to rats and mice suggested that GSH-alkyl transferase was responsible for at least part of the dealkylation of this organophosphate [5, 6]. Hollingworth [18] demonstrated that methyl iodide treatment depleted liver GSH and thereby impaired GSH-dependent detoxification of sumithion and sumioxon and increased their anticholinesterase toxicity. The present investigation was undertaken to determine the effect of DDVP on mouse liver GSH levels and to determine if methyl iodide pretreatment would increase the toxicity of DDVP. In addition we studied the effect of combined inhibition of binding and depression of GSH concentration on DDVP's anticholinesterase action.

METHODS

Adult male Charles River mice (CD-1, 24–36 grams) were used for this study. They were kept in air-conditioned (25) animal quarters and given Purina Laboratory Chow and water *ad lib*. Mice were housed six per cage in stainless steel cages. The cages had a grid flooring which was suspended 2.5 inches above waste pans containing sawdust. Animals quarters were on a 12 hr (7–7) light-dark cycle. All injections were times to permit sacrifice between 8 and 11 AM. DDVP, 90% was supplied by Shell Chemical Co, TOTP, practical grade, was purchased from Eastman, and methyl iodide, 99%, was purchased from Aldrich Chemical Company. All compounds were dissolved in sufficient corn oil to provide the appropriate dose in an injection volume of 5 ml/kg. DDVP and TOTP were injected intraperitoneally and methyl iodide was administered orally. Control animals were given 5 ml/kg of corn oil by the appropriate route. Animals were sacrificed by decapitation and exsanguination and 10% (w/v) tissue homogenates were prepared in sodium phosphate buffer (0.1 M, pH 8) for cholinesterase determinations, or in sodium bicarbonate buffer (0.026 M, pH 7.6) for carboxylesterase and A-esterase determinations. Homogenates were kept on ice until used the same day or frozen and used within 8 days. Experiments in this laboratory have indicated that enzyme activities were unchanged over this period of time.

Brain and liver cholinesterase activities were determined using the spectrophotometric method of Ellman, *et al.* [19]. Acetylthiocholine iodide (0.001 M, SIGMA) served as the substrate for hydrolysis by 0.8 mg brain or 2 mg liver during 30 min at 27°. Mean ± S.E. cholinesterase activities from 10 or more control mice were 11.4 ± 0.2 and 5.9 ± 0.2 micromoles of substrate hydrolyzed/minute/gram (wet wt) of brain and liver, respectively. For assays of carboxylesterase activity, hydrolysis of 0.0067 M diethylsuccinate by 2.5 mg, 0.027 M triacetin by 5.0 mg, and 0.02 M procaine HCl by 100 mg of liver was determined manometrically as described previously [20, 21]. Mean ± S.E. carboxylesterase activity of 5 control mice was 182 ± 4, 242 ± 14, and 1.1 ± 0.7 micromoles of substrate hydrolyzed per gram (wet wt) of liver/minute for triacetin, diethylsuccinate, and procaine, respectively. All assays were performed in duplicate and tissue levels and substrate concentrations employed were selected to provide optimum conditions at which enzyme activity of the tissue would be the rate-limiting factor in the assays. Liver GSH concentrations were determined based on extinction (412 nm) of a trichloroacetic acid treated homogenate supernatant reacted with the thiol reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma), as described previously [16]. The Mean ± S.E. level of GSH in livers from 5 control mice was 750.0 ± 22.5 nmoles per 100 mg (wet wt) of tissue.

Hydrolysis of DDVP by A-esterase was determined manometrically [22]. The incubate consisted of 40 mg liver homogenate, 0.001 M Mn, 0.01 M DDVP, and sufficient bicarbonate buffer (0.026 M, pH 7.6) for a total volume of 3.0 ml. After gassing with 5% carbon dioxide in nitrogen and equilibrating at 37°, DDVP was added to liver and carbon dioxide production was measured for the next 30 min as an indication of DDVP hydrolysis. All data in this study were analyzed by the Student's *t* test. Probability values of 0.05 or less were considered statistically significant.

RESULTS

Effect of DDVP on liver glutathione levels. To determine if DDVP would reduce liver GSH concentration, groups

Table 1. Effect of DDVP on mouse liver glutathione (GSH) levels

DDVP mg/kg, i.p.	Time after injection (min)	GSH† nmoles/100 mg	% Reduction
0*	30	750 ± 19	—
15	30	675 ± 35	10
30	30	610 ± 29‡	19
30	120	700 ± 68	7

* Controls received corn oil 5 cc/kg, i.p.
† Mean ± S.E. of 5 or more mice.
‡ Significantly different from control ($P < 0.05$).

of mice were given corn oil or DDVP and sacrificed 30 or 120 min later for GSH determinations. Results are given in Table 1. Significant depression of GSH concentration was observed only ½ hr after 30 mg of DDVP per kg. Two hr after DDVP, GSH levels were not significantly different from control. Fifteen mg/kg did not significantly affect liver GSH concentration. After 35 mg of DDVP per kg all mice died within 10 min, yet liver GSH concentrations were not significantly different from controls (not shown in Table 1).

Effect of glutathione depletion on DDVP toxicity. The results in Table 1 are consistent with the hypothesis that GSH-dependent demethylation has a role in DDVP detoxification [7, 8]. If this biotransformation mechanism played a major role in DDVP inactivation in the mouse, one would expect that prior depression of hepatic GSH concentrations by methyl iodide would enhance the toxicity of subsequently administered DDVP. To test this, groups of mice were pretreated with corn oil or with methyl iodide (135 mg/kg orally) and one hr later half the mice in each group were sacrificed without challenge. The remaining mice in each group were challenged with DDVP (30 mg/kg, i.p.) and sacrificed 30 min later. Results are shown in Table 2. Brain cholinesterase activity was inhibited to the same degree by DDVP in both control and methyl iodide pretreated mice. Methyl iodide alone did not inhibit liver hydrolysis of triacetin yet it appeared to enhance the inhibition of this enzyme activity by DDVP. In contrast, additive inhibitory effects of methyl iodide and DDVP were observed for liver hydrolysis of diethyl succinate and procaine. Liver GSH concentrations were decreased less in the mice given both methyl iodide and DDVP than would be predicted from simple addition of the effects of the two agents when given alone.

Effect of combined inhibition of binding and lowered hepatic glutathione concentrations on the toxicity of DDVP. The preceding experiments suggest that GSH dependent metabolism plays only a minor role in the detoxification of DDVP in the mouse. Previous studies [15, 21, 23] suggested that DDVP was inactivated by binding to carboxylesterases but that such binding played only a minor role in DDVP detoxification. In spite of the fact that both mechanisms seem to be of minor importance *in vivo*, in mice, it is possible that simultaneous impairment of these two apparently minor pathways might result in enhanced DDVP toxicity. To test this, groups of mice were pretreated with corn oil or with TOTP (125 mg/kg i.p., 18 hr prior to DDVP) to inhibit binding or with methyl iodide (135 mg/kg orally, 1 hr prior to DDVP) to decrease GSH levels, or with both methyl iodide and TOTP (at the above dosages and times before DDVP). Half the mice in the methyl iodide pretreated group and half in the methyl iodide and TOTP treated group were challenged with DDVP (30 mg/kg i.p.) and sacrificed 30 min later for determination of liver and brain cholinesterase activities and liver GSH levels. The remaining mice were sacrificed without DDVP challenge. TOTP reduced DDVP binding to zero. Results are presented in Table 3. DDVP's anticholinesterase action was not altered by the combination pretreatment.

Hydrolysis of DDVP by A-esterases. *In vitro* hydrolysis of DDVP by mouse liver A-esterase has not been reported. However, Hutson and Hoadley [6] suggested that this enzyme may be responsible for 50 to 85 per cent of DDVP metabolism in mice. Their estimate was based upon measurement of the amount of dimethyl phosphate metabolite recovered after DDVP exposure. If their estimates were correct, the extensive metabolism of DDVP by mouse liver A-esterase might have been sufficient to prevent poisoning when minor detoxification pathways were blocked by TOTP and methyl iodide. Thus, we felt that it would be worthwhile to measure mouse liver A-esterase

Table 2. Effect of methyl iodide pretreatment on DDVP's antiesterase action

	Per cent inhibition*		
	Methyl iodide† alone	DDVP‡ alone	Methyl iodide§ and DDVP
Brain			
Cholinesterase (CHE)	0	52 ± 4	56 ± 4
Liver hydrolysis			
Triacetin (TA)	0	30 ± 5	47 ± 3
Diethylsuccinate (DES)	11 ± 3	58 ± 3	69 ± 2
Procaine (PROC)	8 ± 5	16 ± 4	25 ± 9
Liver			
Glutathione (GSH)	53 ± 4	21 ± 4	48 ± 9

* Mean ± S.E. of 5 mice. Values of corn oil controls were 11.4 ± 0.2, 182 ± 4, 242 ± 14, and 1.1 ± 0.7 micromoles substrate hydrolyzed/min/gm for CHE, TA, DES and PROC respectively. GSH = 750 ± 19 nmoles/100 mg.
† Methyl iodide 135 mg/kg, P.O. 60 min before sacrifice or DDVP challenge.
‡ DDVP 30 mg/kg, i.p. 30 min before sacrifice.
§ Methyl iodide pretreated, DDVP challenged mice.

Table 3. Effect of TOTP and methyl iodide pretreatments on DDVP's anticholinesterase (CHE) action

	Per cent inhibition*				
	DDVP† alone	TOTP‡ alone	Methyl iodide and DDVP§	TOTP and DDVP‖	TOTP, methyl iodide, and DDVP¶
Brain CHE	47 ± 3	2 ± 3	47 ± 8	67 ± 4	58 ± 5
Liver CHE	27 ± 5	85 ± 5	21 ± 7	93 ± 1	93 ± 1
Liver GSH	19 ± 4	0	36 ± 3	—	51 ± 9

* Mean ± S.E. of 5 mice. Values of corn oil controls were 11.4 ± 0.2 and 5.9 ± 0.2 micromoles substrate hydrolyzed/gm/min for brain and liver CHE, respectively. Gluthathione (GSH) = 750 ± 19 nmoles/100 mg.
† DDVP, 30 mg/kg, i.p., 30 min before sacrifice.
‡ TOTP 125 mg/kg, i.p., 18 hr before sacrifice or DDVP challenge.
§ Methyl iodide 135 mg/kg, p.o., 1 hr before DDVP challenge.
‖ TOTP pretreated, DDVP challenged mice.
¶ TOTP and methyl iodide pretreated, DDVP challenged mice.

hydrolysis of DDVP. Furthermore, it has been reported that TOTP did not affect rat liver A-esterase hydrolysis of paraoxon and methyl paraoxon [24, 25]. Therefore, for comparison, we also measured DDVP hydrolysis by livers from mice which had been sacrificed 18 hr after 125 mg of TOTP/kg. Control mouse liver hydrolyzed 1100 ± 60 nmoles of DDVP/gm/min (mean ± S.E.; $n = 6$). Livers from TOTP-treated mice hydrolyzed 990 ± 60 nmoles/mg/min. The difference between the groups was not statistically significant ($P > 0.05$).

DISCUSSION

This investigation has demonstrated that DDVP decreased mouse liver GSH concentrations. It is interesting to note that a significant decrease was detected only after 30 mg of DDVP per kg and not after 15 mg/kg. Hutson [10] proposed that GSH-dependent metabolism of organophosphates becomes important only after hydrolytic pathways are 'overloaded.' The observation that 35 mg of DDVP per kg killed all mice in less than 10 min suggests that the lower, non-lethal 30 mg/kg dose of DDVP may have approached an 'overload' situation. The rapid return of liver GSH levels observed after DDVP is consistent with the reported rapid resynthesis of mouse liver GSH [26].

It should be noted that the degree and duration of GSH reduction observed after DDVP was much less than that reported after sumithion and sumioxon [18]. The toxicity of the latter compounds was potentiated by prior depression of liver GSH subsequent to methyl iodide pretreatment. In this study we used the same methyl iodide pretreatment and observed similar depletion of GSH, yet, methyl iodide did not potentiate DDVP toxicity. Of course, it remains possible that further reduction of hepatic GSH levels by more potent compounds might alter DDVP toxicity. However, these observations suggest that, in contrast to sumithion and sumioxon, GSH-dependent metabolism does not represent a major detoxification pathway for DDVP in the mouse. Lack of DDVP potentiation in mice pretreated with sufficient TOTP to completely inhibit hepatic DDVP binding suggested that binding also did not represent a major detoxification pathway in the mouse [15, 21]. This was further emphasized in this study in that combined inhibition of binding and depression of hepatic GSH concentrations by TOTP and methyl iodide, respectively, did not alter DDVP's anticholinesterase action.

The mouse liver hydrolysis of DDVP observed in this study was somewhat lower than that reported for rat liver [11, 22] and as has been reported for rat liver [17, 24] DDVP hydrolysis by hepatic A-esterases was not inhibited in TOTP-pretreated mice. The present study considered along with those cited above suggest that the low mammalian toxicity following exposure to DDVP results from rapid metabolism by A-esterases.

Summary. DDVP (30 mg/kg, i.p.) caused 50 per cent inhibition of mouse brain cholinesterase activity and 20 per cent decrease in mouse liver GSH concentration 30 min after injection. Methyl iodide (135 mg/kg, i.p.) reduced liver GSH concentration to 50 per cent of control within one hr. However, this methyl iodide treatment did not alter the anticholinesterase action of subsequently administered DDVP. This suggested that GSH-dependent metabolism does not represent an important detoxification mechanism in the mouse. Combined treatment with TOTP and methyl iodide inhibited DDVP inactivation by binding and depressed liver GSH levels yet the toxicity of subsequently administered DDVP was not altered. Results suggest that low DDVP toxicity and lack of toxic interaction with TOTP and methyl iodide result from the rapid hydrolysis by A-esterases.

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DDE-Induced microsomal mixed-function oxidases in the puffin (*Fratercula arctica*)

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Hepatic microsomal mixed-function oxidases of different avian species respond to organochlorine insecticides in various ways. Davison and Sell [1] found, for example, that DDT pretreatment decreased aniline hydroxylase activity in chicken liver microsomes, but increased this activity in duck liver microsomes. Although a considerable volume of work has been done concerning enzyme induction of hepatic mixed-function oxidases in birds [2–5], it has been largely confined to domesticated species. The sole exception is a study on the American kestrel (*Falco sparverius*) [6]. In view of the observed species-to-species variation it is desirable that studies designed to elucidate possible environmental effects be carried out on the species concerned rather than extrapolating from others. This report is part of a series of studies undertaken to evaluate the effect of toxicants on a variety of physiological mechanisms in seabirds [7]. In this note the effects of DDE, a metabolite of DDT that occurs as a widespread environmental contaminant, on some xenobiotic-metabolizing enzyme activities of microsomes prepared from puffin liver are reported. The puffin is a fish-eating pelagic species of the auk family for which large declines in population have recently been noted on both sides of the North Atlantic [8, 9]. This report describes the response of puffin hepatic microsomal aniline hydroxylase and benzphetamine demethylase activities to oral dosing of puffins with DDE.

Immature puffins (approximately 40-days-old) were dosed orally by intubation with DDE dissolved in corn oil (experimental birds) or with corn oil alone (control birds) for 16–21 days before sacrifice. The DDE administered was selected to approximate 50 ppm in the diet, based on a total daily intake of 120 g fish per day. (Thus, each treated bird received approximately 6 mg daily). Birds were maintained in artificial burrows made of tile until the pre-fledging starvation phase had started when they were brought into the laboratory. This pre-fledging starvation phase is a normal physiological change that occurs a few days before fledging and is not influenced by the presence of food. This lack of feeding caused mobilization of DDE previously stored in the fat and this release was enough to cause toxic manifestations in the treated birds.

After decapitation of puffins, an aliquot of liver, minus the gall bladder, was immediately removed and frozen. Liver was assayed for mixed-function oxidase activity within 48 hr of sacrifice. Use of this procedure has not resulted in significant loss of mixed-function oxidase activity in any of the mammalian or marine species we have tested.

Prior to microsome preparation, the liver aliquots were placed in ice-cold 0.15 M KCl adjusted to pH 7.5 with HEPES-NaOH buffer and were allowed to thaw slowly. All subsequent steps were carried out at 0–4°C. The livers were minced with scissors and homogenized in a glass Pot-

Table 1. Effect of DDE administration on the hepatic microsomal mixed-function oxidase system of the Puffin and on tissue residues.

	Puffin		
	Control	DDE-fed[1]	P[2]
Yield Microsomal Protein (mg/g liver)	22.2 ± 2.2 (4)[3]	24.2 ± 1.0 (5)	N.S.
Aniline Hydroxylase (nmoles/min/mg protein)	0.19 ± 0.05 (4)	0.70 ± 0.10 (5)	< 0.05
Benzphetamine Demethylase (nmoles/min/mg protein)	1.05 ± 0.19 (4)	5.46 ± 0.45 (5)	< 0.05
Residual DDE in liver (ppm dry weight)	4.9 ± 1.8 (3)	2037 ± 586 (4)	< 0.05

[1] Puffins dosed orally with DDE in corn oil for 16–21 consecutive days (amount selected to approximate 50 ppm in diet). Controls received only corn oil.
[2] Statistical comparisons made using the nonparametric Mann-Whitney U test [14].
[3] Mean ± S.E.M. (*n*).