Biochemical Pharmacology, Vol. 29, pp. 635–641.
Pergamon Press Ltd. 1980. Printed in Great Britain.

EFFECTS OF LOW LEVEL ADMINISTRATION OF DICHLORVOS ON ADRENOCORTICOTROPHIC HORMONE SECRETION, ADRENAL CHOLESTERYL ESTER AND STEROID METABOLISM

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(*Received* 15 *January* 1979; *accepted* 6 *August* 1979)

Abstract—The effects of the organophosphate insecticide dichlorvos [*O,O*-dimethyl-*O*-(2,2-dichlorvinyl phosphate)] on diurnal changes in the rat pituitary–adrenal axis have been studied. At 2 p.p.m. in the drinking water, it did not inhibit erythrocyte or plasma acetylcholinesterase activity. Dichlorvos ingestion markedly increased plasma adrenocorticotrophic hormone (ACTH) levels at the 9:00 a.m. sampling period, but did not further enhance the diurnally elevated ACTH levels at 5:00 p.m. Adrenal acyl-CoA:cholesterol acyltransferase (ACAT) and neutral cytosolic cholesterol ester hydrolase (CE hydrolase) were significantly inhibited at 9:00 a.m. while at 5:00 p.m. the enzymes were stimulated. Adrenal cholesterol ester levels were depressed at 9:00 a.m. and increased at 5:00 p.m. Adrenal free cholesterol levels did not change. Neither adrenal nor plasma corticosterone levels were significantly altered after dichlorvos. The results indicate that significant alterations in adrenal cholesterol ester levels may take place without corresponding changes in plasma and adrenal corticosterone levels.

Several organophosphate and carbamate insecticides inhibit adrenal steroidogenesis in the isolated rat adrenal cell *in vitro* [1]. Further studies in this system have shown that the organophosphates also inhibit cholesterol ester formation and hydrolysis at concentrations which inhibit steroidogenesis. Feeding the organophosphate chlorpyrifos oxone at 20 p.p.m. blocked the stress-induced increase in rat adrenal neutral cytosolic cholesterol ester hydrolase (sterol-ester hydrolase, EC 3.1.1.13) and the corresponding fall in adrenal cholesterol ester concentration [2].

In vivo, dichlorvos,* when fed to rats at 120 p.p.m., produced enhanced adrenal cholesterol ester levels and depressed plasma corticosterone levels during the rising phase of the diurnal cycle of this hormone [2].

The primary aim of the present study was to determine whether or not dichlorvos, administered at a dose which approaches that which might be absorbed from the environment, could have inhibitory effects on adrenal cholesterol ester and corticosteroid metabolism. In our previous work, we had not investigated the effects of the organophosphates on ACTH output. Acetylcholine causes the synthesis and release of corticotrophin releasing factor from rat hypothalamus *in vitro*, and corticotrophin releasing factor, in turn, causes the release of ACTH from the rat pituitary [3]. It was thus of interest to determine whether or not dichlorvos, through its cholinergic action, could bring about increases in the circulating ACTH levels.

* Abbreviations: CE hydrolase, cholesterol ester hydrolase; ACTH, adrenocorticotrophic hormone; ACAT, acyl-coenzyme A:cholesterol acyltransferase; dichlorvos, *O,O*-dimethyl-*O*-(2,2-dichlorovinyl phosphate).

MATERIALS AND METHODS

Male Sprague–Dawley rats (300–400 g) (Charles River Breeding Laboratories, Inc., Wilmington, MA) were housed in an isolated room with controlled illumination (from 8:00 a.m. to 8:00 p.m.) and temperature (25 ± 1°). In the dichlorvos feeding experiment six to seven animals per experimental group were used. Rats were individually housed and handled daily. The dilute aqueous dichlorvos solutions used in the feeding experiments were administered in the drinking water and were prepared daily from 99$^+$ per cent dichlorvos (provided by the Shell Development Corp., Modesto, CA). Since dichlorvos is volatile, control animals were kept at a distance in a separate room. Animals were guillotined at 9:00 a.m. or 5:00 p.m. One week elapsed between the deaths of the 9:00 a.m. and the 5:00 p.m. animals. Blood was collected and assayed for erythrocyte, plasma acetylcholinesterase and corticosterone as described previously [2]. ACTH was determined by radioimmunoassay [4,5] using the Amersham RIA Kit (Amersham Corp., Arlington Heights, IL). Neutral cytosolic CE hydrolase was determined by the method of Pittman and Steinberg [6]. Acylcoenzyme A: cholesterol acyltransferase (EC 2.3.1.26) activity was determined by the method of Balasubramaniam *et al.* [7]. For determination of adrenal corticosterone and free and esterified cholesterol, rat adrenal extracts were prepared and extracted as described previously [2]. cAMP phosphodiesterase was assayed

Table 1. Effects of dichlorvos administration on adrenal weight, body weight and fluid consumption*

	Adrenal weight (mg)	Body weight gain (g/2 weeks)	Fluid consumption (ml/24 hr)
Control		49.7 ± 18.3	45.2 ± 5.2
9:00 a.m.	23.80 ± 4.96		
5:00 p.m.	25.36 ± 5.94		
Dichlorvos-treated†		26.9 ± 21.5‡	54.5 ± 9.0§
9:00 a.m.	27.27 ± 6.80		
5:00 p.m.	24.65 ± 5.95		

* The data presented in Tables 1–4 are from a single experiment with six to seven animals in each group.
† Dichlorvos solution (2 p.p.m) in distilled drinking water for 2 weeks.
‡ Significantly different from control ($P < 0.01$).
§ Significantly different from control ($P < 0.005$).

by the method of Pöch [8] in the delipidated 100,000 *g* supernatant fraction of a 0.04% adrenal homogenate in 40 mM Tris, pH 8.0. All sample analyses were carried out at least in duplicate. Statistical comparisons were made using student's unpaired, two-tailed *t*-test, and data are expressed as mean ± standard deviation.

RESULTS

The experimental group of rats received dichlorvos in their drinking water *ad lib.* at a concentration of 2 p.p.m. for 2 weeks. Water intake was monitored in order to calculate the daily intake of dichlorvos. The average daily consumption of dichlorvos was 30.9 μg/100 g body wt/24 hr. At this dose level, dichlorvos did not produce any gross morphological or biochemical changes. Table 1 shows that dichlorvos produced a small but significant inhibition of whole body weight gain.

In previous studies, it was found that dichlorvos given in the drinking water at 120 p.p.m. for 14 days (1.09 mg/100 g body wt/24 hr) produced 54 and 57 per cent depression of plasma and erythrocyte acetylcholinesterase activity, respectively. In contrast, dichlorvos at 2 p.p.m. did not produce any significant inhibition of either of these two measures of organophosphate intoxication.

Dichlorvos is a chloinesterase inhibitor, and such compounds have been shown to induce the release of ACTH by a direct central nervous system mechanism [9]. The effects of dichlorvos administration on the concentration of circulating ACTH are shown in Table 2. The control animals which were killed at 9:00 a.m. had the lowest ACTH levels, while in the control animals killed at 5:00 p.m., the ACTH levels were significantly higher. Dichlorvos-fed rats, killed at 9:00 a.m., showed significantly elevated ACTH levels, compared with the 9:00 a.m. control animals. There was no difference, however, between the ACTH levels of the 9:00 a.m. and the 5:00 p.m. dichlorvos-fed rats, nor between the 5:00 p.m. dichlorvos and the control animals.

Table 3 shows that dichlorvos did not significantly affect plasma and adrenal corticosteroid levels at either 9:00 a.m. or 5:00 p.m. Adrenal cholesterol ester in the control showed a difference between 9:00 a.m. and 5:00 p.m. that was not statistically significant. In our previous work, a larger and statistically significant diurnal increase in adrenal cholesterol ester was observed [2]. Dichlorvos treatment produced a 29 per cent depression in cholesterol ester levels, compared to the control at 9:00 a.m. This was of borderline significance ($P < 0.06$). At 5:00 p.m. dichlorvos produced a 24 per cent elevation in cholesterol ester levels, compared to the controls. The combined decrease of cholesterol ester at 9:00 a.m. and increase at 5:00 p.m. after dichlorvos resulted in a significant (111 per cent) diurnal change ($P < 0.005$) in cholesterol ester levels after dichlorvos. Adrenal free cholesterol levels did not change significantly after dichlorvos.

Table 4 demonstrates the effects of dichlorvos feeding on neutral cytoplasmic CE hydrolase and

Table 2. Effect of dichlorvos administration on plasma ACTH levels

Treatment	Sampling time	ACTH (pg/ml serum) Individual samples	Mean ± S.D.
Control	9:00 a.m.	20, 22, 15, 96	38.1 ± 38.7
Control	5:00 p.m.	295, 245, 450, 212	300.5 ± 105.3*
Dichlorvos	9:00 a.m.	71, 305, 232	202.7 ± 119.7†
Dichlorvos	5:00 p.m.	230, 165	197.5 ± 46.0

* The 9:00 a.m. control is significantly different from the 5:00 p.m. control at $P < 0.01$.
† The 9:00 a.m. control is significantly different from the 9:00 a.m. dichlorvos-treated at $P < 0.05$.

Table 3. Effects of dichlorvos administration on plasma and adrenal corticosterone, and adrenal free and esterified cholesterol

	Control		Change	Dichlorvos (2 p.p.m.)		Change	Change (%) Control vs dichlorvos	
	9:00 a.m.	5:00 p.m.	(%)	9:00 a.m.	5:00 p.m.	(%)	9:00 a.m.	5:00 p.m.
Plasma corticosterone (μg/dl plasma)	2.72 ± 4.03	15.6 ± 10.9*	+473	4.61 ± 5.85	22.02 ± 7.05†	+378	+69	+41
Adrenal corticosterone (μg/100 mg tissue)	2.87 ± 1.10	9.06 ± 3.70‡	+216	3.07 ± 0.80	8.12 ± 0.96†	+164	+7	−10
Adrenal cholesterol ester (mg/g tissue)	24.61 ± 5.28	29.80 ± 8.16	+21	17.45 ± 7.25	36.80 ± 9.02‡	+111	−29	+24
Adrenal free cholesterol (mg/g tissue)	4.29 ± 0.67	4.61 ± 0.28	+8	3.94 ± 1.2	4.45 ± 0.43	+13	−8	−4
$\frac{\text{Cholesterol ester}}{\text{cholesterol}}$ ratio	5.96 ± 1.98	6.42 ± 1.51	+8	4.82 ± 1.51	8.20 ± 1.50*	+70	−19	+28

* Significant difference ($P < 0.025$) between 9:00 a.m. and 5:00 p.m.
‡ Significant difference ($P < 0.005$) between 9:00 a.m. and 5:00 p.m.
† Significant difference ($P < 0.001$) between 9:00 a.m. and 5:00 p.m.

Table 4. Effects of dichlorvos administration on CE hydrolase and ACAT activities

	CE hydrolase				
	Basal (nmoles/hr/mg protein)	Activated* (nmoles/hr/mg protein)	Activation(%)	ACAT (pmoles/hr/mg protein)	ACAT/CEase $\times 10^3$
Control					
9:00 a.m.	180.6 ± 48	254.9 ± 78	42.3 ± 24.8	141.3 ± 71.3	0.78
5:00 p.m.	61.1 ± 14.0	89.9 ± 24.5	46.6 ± 17.0	422.2 ± 179.0	6.91
Change(%)	−66.2†	−64.7†		199.0†	
Dichlorvos-treated (2 p.p.m.)					
9:00 a.m.	100.2 ± 44.4	138.3 ± 70.7	32.5 ± 37.1	99.4 ± 61.2	0.99
5:00 p.m.	101.4 ± 17.2	137.1 ± 18.8	38.1 ± 25.1	474.7 ± 225.1	4.68
Change(%)	1.1	0		378‡	
Control vs dichlorvos (% change)					
9:00 a.m.	−44.5§	−45.7		−29.6‖	
5:00 p.m.	66.0¶	52.5¶		12.4	

* Cofactor mixture for CE hydrolase activation: 0.5 mM ATP, 10 μM cAMP and 5mM Mg acetate.
† The 9:00 a.m. control is significantly different from the 5:00 p.m. control ($P < 0.005$).
‡ The 9:00 a.m. dichlorvos-treated is significantly different from the 5:00 p.m. dichlorvos-treated ($P < 0.005$).
§ The 9:00 a.m. control is significantly different from the 9:00 a.m. dichlorvos-treated ($P < 0.01$).
‖ The 9:00 a.m. control is significantly different from the 9:00 a.m. dichlorvos-treated ($P < 0.025$).
¶ The 5:00 p.m. control is significantly different from the 5:00 p.m. dichlorvos-treated ($P < 0.005$).

Table 5. Effects of dichlorvos *in vitro* on basal and activated CE hydrolase and ACAT activities

Dichlorvos (M)	CE hydrolase (nmoles/hr/mg protein)				Dichlorvos (M)	ACAT (pmoles/hr/mg protein)	
	Basal	Inhibition(%)*	Activated	Inhibition(%)		Basal	Inhibition(%)
Control	52.6		86.1		Control	10.5	
1×10^{-8}	45.3	14	84	3	5×10^{-9}	10.0	5
1×10^{-7}	46.8	11	81	6	5×10^{-8}	9.2	12
1×10^{-6}	26.4	50	47.2	45	5×10^{-7}	7.4	30
1×10^{-5}	7.0	82	18.6	80	5×10^{-6}	6.5	38
					5×10^{-5}	0.8	92

* Cholesterol ester hydrolase inhibition was conducted for 10 min at 24° before the addition of substrate.

microsomal ACAT. In the control animals there were highly significant diurnal changes in the activities of both of these enzymes. CE hydrolase activity decreased 66 per cent between 9:00 a.m. and 5:00 p.m. At 9:00 a.m. dichlorvos-fed animals showed significant inhibition of CE hydrolase. The diurnal variation in CE hydrolase activity in these animals, judged by these two time points, therefore had been abolished. The *in vitro* activation of CE hydrolase by the cofactor mixture (0.5 mM ATP, 10 μM cAMP, and 5 mM Mg acetate) was approximately the same, although there was a tendency for the extracts from dichlorvos-fed rats to show a lower, but not statistically significant, activation than did the extracts from the control animals.

The basal activity of ACAT was about 1000-fold lower than that of CE hydrolase. ACAT increased in activity 199 per cent between 9:00 a.m. and 5:00 p.m. in the controls. Dichlorvos produced a 30 per cent decrease in activity at 9:00 a.m., and a 12 per cent increase at 5:00 p.m. The net effect of dichlorvos, therefore, was to increase the 9:00 a.m. to 5:00 p.m. diurnal variation from 199 per cent to 378 per cent.

Table 5 shows the *in vitro* inhibitory effects of dichlorvos on ACAT and CE hydrolase. The data

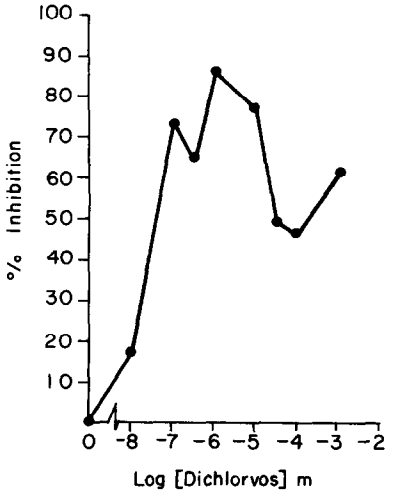

Fig. 1. Effect of dichlorvos on cAMP phosphodiesterase activity in the 100,000 *g* supernatant fraction of 0.04% rat adrenal homogenate in 40 mM Tris, pH 8.0.

indicate that both enzymes are capable of being inhibited by dichlorvos directly, with CE hydrolase being more sensitive than ACAT to dichlorvos in the *in vitro* assay. Thus, the inhibitory effects seen in the *in vivo* experiments were probably due to direct effects on the individual enzyme systems. Our previous studies showed that cAMP-stimulated steroidogenesis in the isolated rat adrenal cell was inhibited 50 per cent by 10^{-5} M dichlorvos [1,2], which is approximately the same concentration at which these enzymes are inhibited.

Since cAMP is involved in the activation of CE hydrolase, it was of interest to determine the effect of dichlorvos on adrenal cAMP phosphodiesterase. The data in Fig. 1 show that dichlorvos maximally inhibited this enzyme at 10^{-6} M, in the adrenal 100,000 *g* supernatant fraction. Other data (not shown) indicate that this enzyme activity in the 15,000 *g* fraction was also inhibited in the same concentration range of dichlorvos.

DISCUSSION

Dichlorvos-induced inhibition of CE hydrolase and ACAT activities and stimulation of ACTH release are seen only in the morning. Presumably, this reflects the fact the dichlorvos, which was administered in the drinking water, was consumed almost entirely during the dark periods, when the rats do most of their feeding. The enzymatic inhibitions at this level of dichlorvos must be completely reversible, or new enzyme is formed to replace the inactivated enzyme, since both enzyme activities were increased considerably above control levels at 5:00 p.m.

Studies on the reaction mechanism indicate that organophosphate insecticides phosphorylate a reactive serine group at the active center of acetylcholinesterase [10,11]. In the case of CE hydrolase, this phosphorylation could take place at the active center of the enzyme or at sites at which it would interfere with the activational phosphorylative process via the protein kinase–cAMP mediated transfer of ATP phosphate to the enzyme. The phosphoryl-enzyme is subject to a slow spontaneous hydrolysis in aqueous medium [12]. The rate of de-inhibition is conditioned by the chemical structure of the phosphoryl group. The rates of decomposition of a series of phosphoryl-substituted acetylcholinesterases are in the order: dimethyl phosphoryl > diethyl phos-

phoryl > di-*n*-propyl phosphoryl. Vandekar and Heath [13] have determined that the half-life of dimethylphosphorylated rat erythrocyte acetylcholinesterase is 2 hr *in vivo*. Since dichlorvos is a dimethylphosphoryl-substituted compound, it might be expected that dimethylphosphorylated CE hydrolase and/or ACAT might also regenerate at a significant rate, if similar reactions are involved between the organophosphates and these enzymes. The fact that at 5:00 p.m. both enzyme activities in the dichlorvos-treated animals were above control levels suggests that the morning inhibition may stimulate the synthesis of new enzyme. Possibly, the observed lowering of cholesterol ester levels in the 9:00 a.m. dichlorvos-treated rats enhances the synthesis of ACAT, which in turn, after cholesterol ester levels have risen, triggers the synthesis of CE hydrolase. A combination of stimulated synthesis and regeneration of inhibited enzyme might account for the supranormal levels observed in the 5:00 p.m. dichlorvos-treated rats.

The inhibitory effects of dichlorvos on both enzyme systems are direct, as shown by the *in vitro* dose–response studies on CE hydrolase and ACAT in Table 5, and in previous studies with isolated rat adrenal cells [2]. Both enzymes were inhibited to approximately the same extent. The net result of the inhibition of the two enzymes, CE hydrolase and ACAT, was to decrease somewhat the level of adrenal cholesterol ester. While inhibition of ACAT alone would produce lower intracellular cholesterol ester levels, inhibition of CE hydrolase would tend to favor cholesterol ester accumulation. Thus, the opposing effects of dual inhibition of the two enzymes would tend to keep cholesterol ester levels constant. The fact that cholesterol ester levels were low at 9:00 a.m. and were elevated at 5:00 p.m. seems to indicate that accumulation of cholesterol esters via the ACAT reaction is the rate-limiting step in the control of cholesterol ester levels. Furthermore, in the dichlorvos-treated rats, CE hydrolase did not change from a.m. to p.m. and ACAT showed a 2-fold greater diurnal increase than the control animals (199 per cent vs 378 per cent). Thus, the greater increase in adrenal cholesterol ester concentration in the dichlorvos group from a.m. to p.m. was most likely a result of the greater elevation of ACAT activity. From the measurements made in this study, it appears that ACAT in the adrenal gland is roughly 3000 to 4000-fold lower than CE hydrolase in activity. This would also fit with the concept that ACAT is the rate-limiting enzyme in the control of adrenal cholesterol ester levels [14].

It is possible that elevated intracellular cAMP levels may also play a role in increasing the activity of one or both of these enzymes. It was found previously that another organophosphate, diazinon [15], had an inhibitory effect on ACTH-induced increases in cAMP levels in isolated rat adrenal cells at high diazinon levels (10^{-2} M), and a potentiating effect at low diazinon levels (10^{-7} M). Rat adrenal cAMP phosphodiesterase activity has also been found to be strongly inhibited by dichlorvos at 10^{-7} M (Fig. 1). At concentrations of 10^{-7} M, dichlorvos does not markedly inhibit either ACAT or CE hydrolase (Table 5), but still inhibits cAMP phosphodiesterase. Thus, it is possible that lower levels of dichlorvos found during the day may enhance intracelluar cAMP levels. This, in turn, could result, directly or indirectly, in the elevation of CE hydrolase activity. Table 4 shows that there is slightly less *in vitro* activation of CE hydrolase in the 5:00 p.m. dichlorvos animals than in the controls, although the differences are not statistically significant. Trzeciak and Boyd [16] have shown that stressing of rats produces a decrease in the percent activation of adrenal CE hydrolase *in vitro*. With stress, elevated ACTH [17] markedly enhances cellular cAMP levels [18] resulting in prior *in vivo* activation of the enzyme and, therefore, a smaller *in vitro* activation by the cAMP-containing activation mixture.

Since metabolic studies have indicated that dichlorvos is extremely rapidly metabolized *in vivo*, measurable levels of dichlorvos must have been maintained for only short periods after ingestion ceased. [^{14}C]Dichlorvos inhalation exposure of pigs for 3–4 weeks to 2×10^{-7} moles/kg/day yielded detectable levels of only metabolites in the tissues of the animals. Due to the specific activities of the labeled compounds, these methods would have detected 5×10^{-9} mole/kg of dichlorvos [19]. Similar results were obtained in rats exposed to labeled dichlorvos orally and by inhalation [20].

Our results also show that the administration of dichlorvos at a dose level which is not detectable by plasma and erythrocyte acetylcholinesterase inhibition is capable of strongly inhibiting adrenal CE hydrolase and ACAT. Certain rat liver esterases have also been shown to be inhibited by organophosphates administered at levels below which they inhibit plasma and erythrocyte acetylcholinesterase [21].

Finally, our results show that, at these low levels of dichlorvos, marked changes in adrenal cholesterol ester metabolism occurred without any concomitant effects on plasma and adrenal corticosteroid levels. In our previous work [1], when rats were exposed to 60-fold higher concentrations of dichlorvos for the same time period, significant depression of the normal diurnal increase in plasma steroid levels resulted. ACTH has been shown to cause an increased association between cholesterol and rat adrenal cytochrome $P450_{scc}$ which correlated with an increased rate of cholesterol side chain cleavage to yield pregnenolone from endogenous cholesterol [22]. Previously, it had been found that dichlorvos has no effect on purified adrenal cholesterol side chain cleavage enzyme [12]. ACTH also stimulates the uptake of cholesterol independently of the intracellular conversion of cholesterol to corticosteroids [23]. The effect of dichlorvos on this process is unknown. Thus, at lower dose levels of dichlorvos, the enhancement of ACTH levels might counterbalance the partial inhibition of cholesterol ester metabolism by stimulation of one of the above processes. At higher dichlorvos levels, the inhibition of cholesterol ester metabolism would become more complete and the other adrenal stimulatory effects of enhanced ACTH might not be able to override the cholesterol ester blockade by dichlorvos.

Acknowledgements—We should like to thank Dr. Harry

Mersmann of the Shell Development Corp., Modesto, CA, for carrying out cholinesterase determinations and for a supply of highly purified dichlorvos. We would also like to thank Mrs. Pauline Morris for her excellent technical assistance.

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