Biochemical Pharmacology, Vol. 26, pp. 1901–1907. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF ORGANOPHOSPHATE INSECTICIDES ON ADRENAL CHOLESTERYL ESTER AND STEROID METABOLISM

MORTON CIVEN, CHARLESTA B. BROWN and ROBERT J. MORIN

Medical Research Programs, Veterans Administration Hospital, Long Beach, CA 90822 (M.C.), Department of Physiology, College of Medicine, University of California, Irvine, CA 92664 (C.B.B.), and Harbor General Hospital, Torrance, CA 90509, and Department of Pathology, UCLA School of Medicine, Los Angeles, CA 90024 (R.J.M.), U.S.A.

(*Received* 30 *August* 1976; *accepted* 11 *February* 1977)

Abstract—The organophosphate insecticides, dichlorvos and chlorpyrifos oxon, inhibit adrenal cholesterol esterification and hydrolyses *in vitro* at dose concentrations that correlate closely with their inhibition of adrenal steroidogenesis. The results *in vivo* show that dichlorvos, given in the drinking water at 120 ppm, produced a statistically significant depression of plasma corticosterone during the rising phase of the normal diurnal rhythm. Both the control and treated adrenal free and esterified cholesterol levels show marked diurnal changes. In the control group there is a close parallelism between the diurnal changes in adrenal esterified cholesterol and plasma corticosterone levels. There is little difference between the diurnal curves of plasma corticosterone of the 20 ppm chlorpyrifos oxon-fed animals and their controls. When chlorpyrifos oxon-fed animals were stressed, the expected fall in adrenal cholesteryl ester levels was blocked, as was the activation of adrenal cholesteryl ester hydrolase.

It has been previously shown that a wide variety of organophosphate and carbamate insecticides inhibit adrenal corticosterone formation in the isolated rat adrenal cell *in vitro* [1]. The precursor for the synthesis of corticosterone in rat adrenal is free cholesterol, which is stored as the long chain fatty acid ester [2]. Injection of adrenocorticotrophic hormone (ACTH) into hypophysectomized rats caused a marked depletion in adrenal cholesteryl esters with a simultaneous increase of corticoid output [3]. It has also been shown that in the perfused rat adrenal gland, ACTH or adenosine 3′,5′-cyclic monophosphate (cAMP) stimulates cholesteryl ester hydrolysis [4]. Trzeciak and Boyd [5] have shown that ether stress causes a marked depletion of cholesteryl ester in adrenal lipid droplets. Cholesteryl esterase (sterol-ester hydrolase, EC 3.1.1.13) and protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activities were significantly higher in the 105,000 *g* rat adrenal supernatant fraction prepared from rats subject to ether stress. Cholesteryl ester hydrolase was significantly stimulated by the addition of cAMP, ATP and theophylline in the presence of cyclic AMP-dependent protein kinase. Since organophosphate insecticides have been found to inhibit lecithin-cholesterol acyltransferase (EC 2.3.1.43) [6], it was of interest to examine their effects on cholesteryl ester metabolism in the adrenal and attempt to correlate these results with their inhibitory effects on corticosterone formation both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA weighing 300–400 g were used. The animals were housed in an isolated room with controlled illumination 0800–2000 hr and temperature (25 ± 1°). For the diurnal study the rats were individually housed and were handled daily. For all other studies the rats were maintained in colony cages. The animals were sacrificed in an area removed from the animal room, and a period of no more than 10 sec between picking up the rat and decapitation was maintained in order to minimize the rise in endogeneous ACTH levels as a result of stress. The time of sacrifice was at 0900 hours except where otherwise noted in the diurnal study.

Fasiculata-reticularis adrenal cells were harvested by a modified method [1] of Haning *et al.*[7] using collagenase (type 1, Worthington Biochemical Corp., Freehold, NJ) dispersion techniques. The method of Brecher *et al.* [8] was followed in the studies on incorporation of oleic[1-^{14}C] acid (Applied Science Laboratories, Inc., State College, PA) into the cholesteryl oleate fraction. The incubation medium consisted of Krebs Ringer bicarbonate glucose solution containing 0.1% bovine serum albumin (Pentex Fraction V, Miles Laboratories, Kankakee, IL) and an elevated Ca^{2+} concentration of 7.65 mM (KRBGA-Ca^{2+}). A cell count of 5.3×10^5 cells/incubate in a total volume of 1 ml was used. For stimulation of corticosterone formation and oleic[1-^{14}C] acid uptake, 1 ng ACTH (Cortrosyn, Organon Inc., West Orange, NJ)/incubate was added. Dichlorvos [*O,O*-dimethyl *O*-(2,2-dichlorovinyl phosphate)], obtained from Shell Development Co., Modesto, CA, and chlorpyrifos oxon (*O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphate), obtained from Dow Chemical, Midland, MI, were the insecticides tested as inhibitors at concentrations of 10^{-5} and 5×10^{-8} M respectively. Oleic[1-^{14}C] acid (3.5×10^5 cpm) was added to each sample. Incubation was for 2 hr at 37° in closed containers that had been gassed for 5 sec with an O_2:CO_2 (95:5) mixture. After incubation, the samples were centrifuged at room temperature for 10 min at 100 *g*, and the supernatant fraction was

removed and extracted with methylene chloride. Corticosterone formation was measured fluorometrically (Turner Associates model 111, Palo Alto, CA) by the method of Danellis *et al.* [9].

The cellular fraction was resuspended in 2 ml chloroform–methanol (2:1), unlabeled oleic acid and cholesterol were added as carriers, and the cells were extracted by vortexing. The cellular extract was evaporated to dryness under N_2, the tubes were washed twice with ether, and twice more evaporated to dryness. The extract was dissolved in benzene and applied to Silica gel plates (Prekote Silica gel GF, Applied Science Laboratories Inc., State College, PA). The chromatograms were developed following the protocol of Behrman and Armstrong [10]. Radioactivity of the fractions was determined in a Beckman scintillation spectrophotometer.

Cholesteryl ester hydrolase activity (see Tables 2 and 4) was determined by a modification of the method of Pittman *et al.* [11]. A 5% (w/v) homogenate of decapsulated adrenal glands was prepared in 50 mM sodium phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin. The 100,000 *g* supernatant fraction was used as the enzyme source. The substrate, cholesteryl[1-^{14}C]oleate (5 μCi) and unlabeled cholesteryl oleate (10 mg) (Applied Science Laboratories, Inc., State College, PA), was purified on an amberlite (Dowex 1-×8, 100–200 mesh) column 0.5 × 10 cm, and was stored at −20° as a benzene solution. Just prior to use in the assay system, an aliquot was evaporated to dryness under N_2 and resuspended in ethyl alcohol. This was heated to 70°, taken up in a 26–28 gauge needle and extruded into the buffer. The emulsified substrate was kept at 30° until used. The enzyme assay system contained, in a final volume of 0.5 ml, 1 mM cholesteryl oleate, 0.088 mg of enzyme protein, and the indicated amount of insecticide. Incubation was for 60 min at 30°. The reaction was stopped by the addition of 3 ml of benzene–choloroform–methanol (1:0.5:1.2) containing 0.1 mM oleic acid as carrier. The aqueous layer was made to 0.9 ml with a sodium hydroxide solution to give a final concentration of 200 mM sodium hydroxide. The samples were vortexed for 30 sec and centrifuged for 10 min at 1500 *g*. An aliquot of the aqueous layer was counted in a Beckman liquid scintillation spectrometer using tritosol [12]. In the activation experiment (see Table 4), the enzyme was preincubated with 5 mM magnesium acetate, 0.5 mM ATP and 10 μM cAMP in a total volume of 0.1 ml for 10 min at 23°. The assay of cholesteryl ester hydrolase activity in the adrenals from the chlorpyrifos oxon–ether stress experiment *in vivo* (see Table 3) was performed as previously described by Chen and Morin [13]. Protein content of the incubates was determined by the method of Lowry *et al.* [14].

For cholesterol determination, portions of rat adrenals were homogenized in 10 times their weight of chloroform–methanol (2:1) at 45,000 rev/min for 2 min using a Vir Tis homogenizer. The homogenates were centrifuged at 3000 *g* for 10 min, after which aliquots of the supernatant solutions were evaporated to dryness under N_2. Total cholesterol concentrations in these rat adrenal extracts were analyzed by gas chromatography as follows [15]: One ml of alcoholic KOH (6 ml of 33% KOH and 94 ml ethanol) was added to each extract, followed by heating at 60° in an aluminum block for 1 hr. Water (1 ml) was added, after which 3.0 ml of a stigmasterol internal standard, 50 μg/ml in hexane, was added to each. The mixtures were shaken automatically in a Vir Tis Extractomatic for 10 min and then allowed to stand for 5 min or more to separate the phases. Aliquots of 1 ml of each top layer were evaporated to dryness, redissolved in 20 μl carbon disulfide, and aliquots then injected into a Barber–Colman model 5,000 gas chromatograph with flame ionization detection, using a 3% QF-1 liquid phase on Gaschrom Q 100/120 (Applied Science Laboratories), at 240° and a nitrogen carrier gas flow rate of 45 ml/min. Free cholesterol was analyzed in a similar manner except that the potassium hydroxide was omitted from the reagent, and the mixture was not heated. Esterified cholesterol was calculated by subtracting free cholesterol from total choles-

Table 1. Comparative effects of two organophosphate insecticides on incorporation of oleic[1-^{14}C] acid into cholesteryl oleate and on formation of corticosteroid by decapsulated adrenal cells

Treatment	Per cent esterification/ 10^5 cells/2 hr*	Per cent change from basal group	Per cent change from ACTH-stimulated group	Corticosteroid formed (μg/ml/2 hr)*	Per cent change from basal group	Per cent change from ACTH-stimulated group
Basal	3.72 ± 1.28			0.058 ± 0.028		
ACTH†	2.00 ± 0.354	−46.2‡		0.996 ± 0.337	+1617.0§	
Basal + 10^{-5} M dichlorvos	2.14 ± 0.503	−42.5‡		0.099 ± 0.056	−70.7	
ACTH + 10^{-5} M dichlorvos	1.66 ± 0.762	−55.4‡	−17.0	0.312 ± 0.096	+437.9§	−68.7‖
Basal + 5×10^{-8} M chlorpyrifos oxon	0.40 ± 0.283	−89.2‡		0.029 ± 0.010	−50.0	
ACTH + 5×10^{-8} M chlorpyrifos oxon	0.45 ± 0.212	−87.9‡	−77.5‖	0.184 ± 0.019	+217.0§	−81.5¶

* Means ± S.D. of three replicate experiments done in duplicate.
† Cortrosyn (1 ng) in an incubation volume of 1 ml.
‡ Significantly different from basal group at $P < 0.01$.
§ Significantly different from basal group at $P < 0.001$.
‖ Significantly different from ACTH-stimulated group at $P < 0.01$.
¶ Significantly different from ACTH-stimulated group at $P < 0.05$.

terol concentrations. Erythrocyte and plasma acetyl cholinesterase levels were determined by the method of Ward and Hess [16].

RESULTS

Table 1 shows the effect of two organophosphate compounds, dichlorvos (DDVP) and chlorpyrifos oxon (CPO) on the incorporation of oleic[1-^{14}C] acid into cholesteryl oleate and on the formation of corticosterone in fasiculata-reticularis rat adrenal cells. Addition of ACTH produced a significant depression of cholesteryl esterification. DDVP and CPO strongly inhibited esterification of both control and ACTH-treated adrenal cells. A lower inhibition of esterification of ACTH-stimulated cells was observed with DDVP as compared to CPO.

ACTH produces a marked stimulation of steroidogenesis in the isolated adrenal cell. The basal levels of steroid are negligible. It can be seen that there is a significant inhibition of ACTH-induced steroidogenesis produced by the two insecticides.

Table 2 shows that the hydrolysis of cholesteryl esters by the 100,000 *g* rat adrenal supernatant fraction is markedly inhibited by DDVP and CPO. Parallel experiments indicated that these insecticides also inhibited cAMP-induced steroidogenesis in rat adrenal cells to an extent comparable to the inhibition of cholesteryl ester hydrolase.

It was of interest to determine if the inhibitory effects of the two organophosphates on cholesteryl ester metabolism and on corticosteroidogenesis in the isolated rat adrenal cell also occur *in vivo*. The adrenal gland has been shown to have a functional diurnal rhythm in the rat and many other species [17, 18]. This has been characterized by diurnal variations in both the adrenal and plasma corticosterone concentrations. If organophosphate insecticides are inhibiting the synthesis of cholesteryl esters and corticosteroids *in vivo*, alterations in the diurnal patterns of these metabolites might be observed.

Rats were given DDVP (120 ppm) or CPO (20 ppm) in their drinking water *ad lib.* for a period of 2 weeks. Water intake was monitored in order to calculate the daily intake of the organophosphate compounds. The average daily consumption of DDVP was 1.09 mg/100 g of body weight when the animals were given it at a concentration of 120 ppm. At this dose level, DDVP did not produce any obvious toxic effects. There were no gross morphological or behavioral changes observed and no changes in adrenal and body weights. It did produce an inhibition in erythrocyte and plasma acetyl cholinesterase levels for the four time points tested. The average depression of acetyl cholinesterase was 54 per cent in the erythrocyte fraction and 57 per cent in plasma. For all four time points, the per cent changes were significant at either $P < 0.01$ or <0.001. This indicates the dosage of DDVP used had the anticipated pharmacologic effect.

Figure 1 reports the results of the DDVP feeding experiment. Variations in plasma corticosterone and adrenal corticosterone, esterified cholesterol, and free cholesterol are shown.

Adrenal free cholesterol. Two peak levels of adrenal free cholesterol concentration appeared, one at 1600 hours and another at 2300 hours. The changes from the adrenal free cholesterol level at 0900 hours are statistically significant for both groups, with $P < 0.05$ for the 0900–1600 period (26 per cent difference) and $P < 0.001$ for the 0900–2100 period (57 per cent difference).

Adrenal esterified cholesterol. Adrenal esterified cholesterol shows a more marked diurnal fluctuation than does adrenal free cholesterol, with the maximum level occurring at 1600 hours and the minimum level at 0900 hours in both groups. The 81 per cent increase for the control group and the 89 per cent increase for the DDVP group from minimal to maximal levels are both statistically significant at $P < 0.05$. These percentage increases are similar in magnitude to those seen in the adrenal free cholesterol and

Table 2. Effect of dichlorvos and chlorpyrifos oxon on adrenal cholesteryl ester hydrolase activity and cAMP-stimulated adrenal steroidogenesis*

Treatment	Cholesteryl ester hydrolase activity†	Per cent change	Corticosteroid formed (μg)/2 hr	Per cent change
Basal	55.72 ± 8.49		0.16 ± 0.01	
cAMP			1.10 ± 0.01	+588‡
Basal + 10^{-6} M dichlorvos	47.16 ± 9.74	−15.36§	0.06 ± 0.02	−62.5‡
cAMP + 10^{-6} M dichlorvos			0.98 ± 0.01	+512‡
Basal + 10^{-5} M dichlorvos	19.29 ± 1.94	−65.38§	0.48 ± 0.02	+200‡
cAMP + 10^{-5} M dichlorvos			0.42 ± 0.03	+200‡
Basal	113.24 ± 21.50		0.16 ± 0.03	
cAMP			1.10 ± 0.11	+586‖
Basal + 7 × 10^{-8} M chlorpyrifos oxon	28.22 ± 6.23	−75.08‡	0.12 ± 0.01	−25.0
cAMP + 7 × 10^{-8} M chlorpyrifos oxon			0.44 ± 0.01	+175

* Concentration of cAMP is 10 mM. Values are the means ± S.D. of duplicate experiments.
† Cholesteryl oleate (nmoles hydrolyzed/mg of protein/60 min).
‡ Significantly different from basal group at $P < 0.05$.
§ Significantly different from basal group at $P < 0.001$.
‖ Significantly different from basal group at $P < 0.01$.

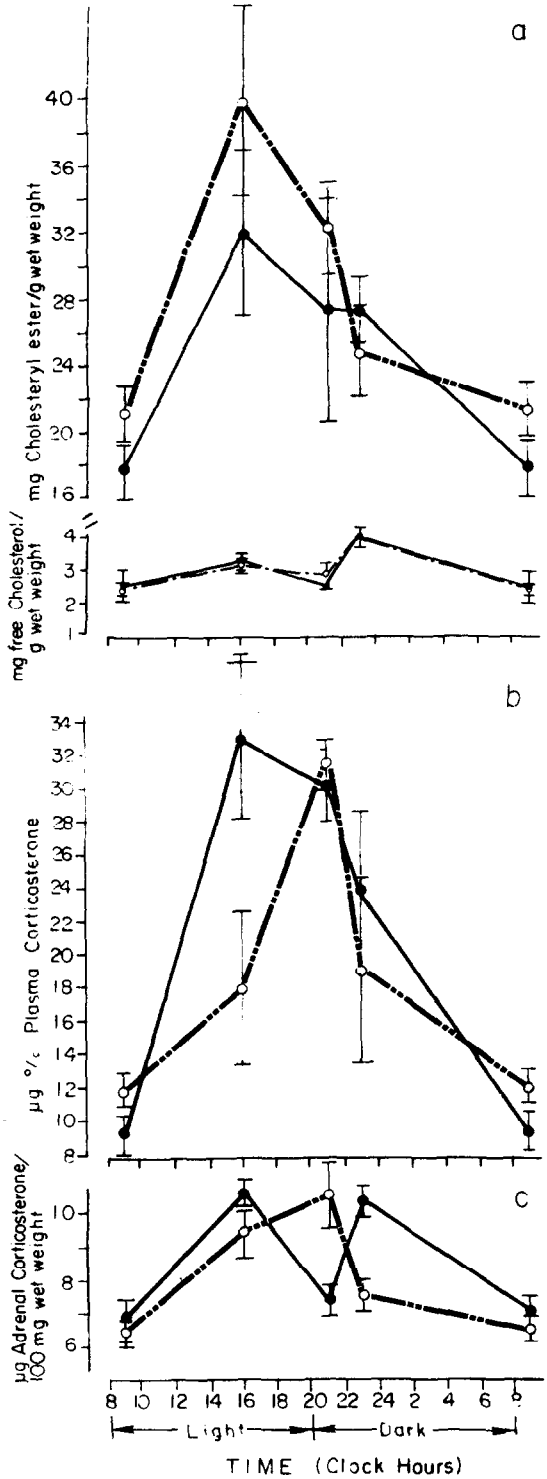

Fig. 1. Effect of feeding rats 120 ppm dichlorvos in their drinking water for a 2-week period. The parameters examined were adrenal free and esterified cholesterol (a), plasma corticosterone (b), and adrenal corticosterone (c). The solid lines represent the control groups and the broken lines represent the treated groups. The points represent the mean values ± S.D. of six animals per group. The experiment was performed three times with duplicate samples per experiment. All three experiments resulted in a similar shift in the diurnal rhythm of the DDVP-treated group and showed a significant difference from the control group each time at the point of maximal plasma corticosterone production by the control group.

adrenal corticosteroid diurnal rhythms. In the control groups there is a close parallelism between adrenal esterified cholesterol and plasma corticosterone levels. The DDVP-treated group shows somewhat increased esterified cholesterol levels at three of the four time points sampled, but the differences are statistically insignificant.

Plasma corticosterone. The presence of the well-known diurnal rhythmic changes in plasma corticosterone is seen. In the control group the minimum level occurs at 0900 hours and the maximum level at 1600 hours. The increase observed is 266 per cent. In the DDVP-treated groups, the minimum level again is seen at 0900 hours, but the maximum level is reached 5 hr after that of the control group, at 2100 hours, and the increase is 166 per cent. At 1600 hours, at the time or near the time of maximal plasma corticosterone production by the control animals, the plasma corticosteroid levels of the dichlorvos-treated rats are significantly depressed ($P < 0.05$) by 45 per cent.

Adrenal corticosterone. The adrenal corticosterone levels of the control and DDVP-treated groups do not show as marked variation during the 24-hr period as do the plasma corticosterone levels. The maximal–minimal differences in the control and DDVP-treated animals are 51.8 and 62.3 per cent respectively. However, it is seen that the maximum level of the control group is reached at 1600 hours, corresponding to the

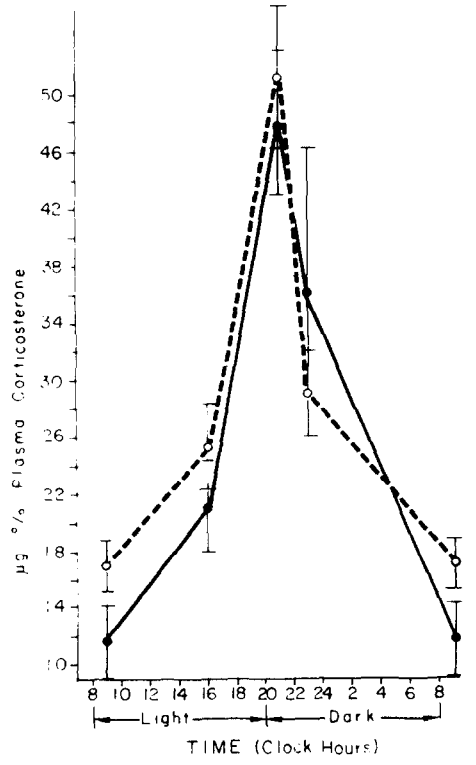

Fig. 2. Variations in plasma corticosterone levels in rats fed 20 ppm chlorpyrifos oxon in their drinking water for a 2-week period. The solid lines represent the control groups and the broken lines represent the treated groups. The points represent the mean values ± S.D. of six animals per group, done in duplicate.

Table 3. Effect of ether anesthesia on free and esterified adrenal cholesterol and on cholesteryl ester hydrolase activity in normal and chlorpyrifos oxon-treated rats*

Treatment	N†	Esterified cholesterol (mg/g wet wt)	Free cholesterol (mg/g wet wt)	Esterified cholesterol/ free cholesterol ratio	Cholesteryl ester hydrolase activity‡
Normal rats					
Control	3	18.25 ± 1.99	1.62 ± 0.05	11.26	25.77 ± 1.72
Ether anesthesia	3	13.77 ± 1.23§	1.83 ± 0.21	7.56§	37.67 ± 2.74§
Chlorpyrifos oxon-treated rats‖					
Control	4	15.29 ± 3.80	1.63 ± 0.10	9.44	22.30 ± 2.36
Ether anesthesia	4	16.03 ± 2.12	1.61 ± 0.10	9.96	22.78 ± 2.86

* Rats ether-stressed for 2 min and sacrificed 3 hr later. Values are the means ± S.D.
† Number of animals.
‡ Cholesteryl oleate (nmoles hydrolyzed/mg of protein/60 min).
§ Significantly different from control group at $P < 0.05$.
‖ Chlorpyrifos oxon (20 ppm) in the drinking water for a 2-week period.

plasma corticosterone maximum, while the DDVP-treated group reached a maximum adrenal corticosterone level at 2100 hours, again corresponding to the DDVP plasma corticosterone maximum.

CPO was administered in the drinking water at 20 ppm. The average daily consumption was 0.225 mg/100 g of body weight. CPO was used at a 6-fold lower level than DDVP since *in vitro* it is a 1000 times more potent inhibitor of steroidogenesis than is DDVP [1]. The results are reported in Fig. 2 and in Table 3.

Figure 2 shows a close parallelism in plasma corticosterone levels between the control and chlorpyrifos oxon groups, with the minimum occurring at 0900 hours and maximum at 2100 hours for both groups. The per cent increase from minimal to maximal levels is 315 per cent for the control group and 200 per cent for the chlorpyrifos oxon-fed group. Although at three of the four time points tested there appear to be increased corticosterone levels in the chlorpyrifos oxon-treated group, the differences are not statistically significant.

Table 3 reports the effect of ether anesthesia on the levels of free and esterified adrenal cholesterol and on cholesteryl ester hydrolase activity in control rats and rats fed 20 ppm CPO in their drinking water for a 2-week period. In the non-stressed group treated with chlorpyrifos oxon, adrenal concentrations of free and esterified cholesterol were not significantly different from the control group; cholesteryl ester hydrolase activity, although slightly lower, was also not significantly different from the control. In the control animals, stress by ether anesthesia resulted in a significant decrease ($P < 0.05$) in esterified cholesterol, a slight increase in free cholesterol and a significant increase ($P < 0.05$) of 46 per cent in cholesteryl ester hydrolase activity. In the chlorpyrifos oxon group subject to ether stress, in contrast to the effects of ether stress in the control group, cholesteryl ester concentrations did not fall and cholesteryl ester hydrolase activity did not increase.

Table 4 shows the effects of chlorpyrifos oxon *in vitro* on the basal and activated cholesteryl ester hydrolase activities. The basal enzyme activity is significantly depressed by the insecticide at concentrations of 7×10^{-8} M (75.1 per cent, $P < 0.05$) and 5×10^{-8} M (60.9 per cent, $P < 0.05$). The statistically significant inhibition of the activated enzyme activity at these concentrations is 77 and 70 per cent respectively ($P < 0.001$).

DISCUSSION

The results *in vitro* reported in Tables 1 and 2, showing that both cholesterol esterification and hydrolysis are inhibited, point to a primary site of action of DDVP and CPO at the level of cholesteryl ester metabolism. At a given dose concentration there is a close correlation with steroidogenic inhibition and cholesteryl ester hydrolase inhibition. However, since

Table 4. Effect of chlorpyrifos oxon *in vitro* on basal and activated cholesteryl ester hydrolase activity

	Basal		Activated*	
Chlorpyrifos oxon (M)	Cholesteryl ester hydrolase activity†	Per cent inhibition	Cholesteryl ester hydrolase activity†	Per cent inhibition
0	113.2 ± 21.5		152.0 ± 1.5	
5×10^{-10}	113.2 ± 14.8	0	164.4 ± 11.4	0
7×10^{-9}	94.3 ± 9.2	16.7	135.4 ± 8.4	10.9
1×10^{-8}	74.3 ± 1.6	34.4	123.4 ± 11.6	18.8
5×10^{-8}	44.3 ± 8.1	60.9‡	44.9 ± 1.5	70.5§
7×10^{-8}	28.2 ± 6.2	75.1‡	34.9 ± 0.6	77.0§

* Preincubation for 10 min at 23° of the enzyme (0.088 mg protein) with 5 mM magnesium acetate, 0.5 mM ATP and 10 μM cAMP in a total volume of 0.1 ml. Values are the means ± S.D. of duplicate experiments.
† Cholesteryl oleate (nmoles hydrolyzed/mg of protein/60 min).
‡ Significantly different from the control at $P < 0.05$.
§ Significantly different from the control at $P < 0.001$.

the inhibitors of cholesteryl ester hydrolase activity would tend to decrease the hydrolysis of newly formed cholesteryl oleate in our esterifying assay system *in vitro*, and the decreased adrenal steroid concentrations might stimulate esterification [19], the levels of organophosphate compounds necessary to inhibit cholesterol esterification to the same extent may appear to be considerably greater than actually are required. To prove this it will be necessary to isolate and assay the cholesteryl ester synthetase in a cell-free system under conditions comparable to the assay for cholesteryl ester hydrolase. It is possible that the two enzyme systems may exist as a multienzyme complex under a coordinated regulatory system.

The study *in vivo* (Fig. 1) shows that DDVP, given in the drinking water at 120 ppm, produces a statistically significant depression of plasma corticosterone during the rising phase of the normal diurnal rhythm. These results *in vivo* correlate with the inhibition *in vitro* of ACTH- and cAMP-stimulated steroidogenesis in the isolated adrenal cell.

Of the adrenal biochemical parameters measured, the diurnal changes in esterified cholesterol were of the greatest magnitude, whereas free cholesterol shows minimum changes. The rapid return of the adrenal corticosterone level of the control group to the maximum level between 2100 and 2300 hours suggests that there is possibly a more rapid oscillation in these levels than the diurnal oscillation. To confirm this it will be necessary to examine adrenal corticosterone fluctuations at shorter time intervals during the diurnal cycle. The closest temporal parallelism is in the diurnal curves of the adrenal esterified cholesterol and the plasma corticosterone levels in the control group. In the DDVP-fed group, although it is not statistically significant, there is a suggestion of an inverse relationship between adrenal cholesteryl ester and plasma corticosterone levels. It is possible that the rates of formation and/or degradation of cholesteryl ester *in vivo* are rate limiting to the synthesis and secretion of corticosteroids, or as suggested by Flint *et al.* [19] the local concentrations of steroids may acutely regulate the activity of cholesterol esterifying enzymes. It will be of interest to observe the diurnal changes in the cholesteryl ester synthetase and hydrolase systems to further elucidate the relationship.

In contrast to the lack of difference in the diurnal changes in plasma corticosterone in the 20 ppm CPO-fed animals and their controls (Fig. 2), it is clear that ether stress-induced activation of adrenal cholesteryl ester hydrolase (Table 3) is blocked by prior CPO treatment. The partially blockaded cholesteryl ester-metabolizing system does not affect the diurnal plasma corticosteroid changes, indicating that sufficient reserve precursor of free cholesterol substrate may be available to maintain corticosterone synthesis at normal levels in the resting state. It will be important to examine the levels of adrenal and plasma corticosterone in the highly stressed animal (in which presumably the adrenal cortex is maximally stimulated) in order to determine whether this partial blockade in cholesteryl ester hydrolysis is reflected in decreased corticosterone output.

In the present experiments *in vitro* the organophosphate compounds inhibited cholesteryl ester hydrolase activity in the adrenal supernatant fraction both with and without addition of exogenous ATP, cAMP and Mg^{2+}, suggesting a possible direct inhibition of the phosphorylated enzyme itself, rather than inhibition of the processes leading to enzyme activation. After administration of CPO *in vivo*, however, resting adrenal levels of cholesteryl ester hydrolase were not significantly inhibited, whereas the stress-induced activation of enzyme activity was blocked. These apparent differences between effects *in vitro* and *in vivo* may be attributable to conversion of the organophosphate compounds *in vivo* to derivatives with lesser inhibitory activity [20] or to less inhibitor–enzyme interaction due to possible lower cellular concentrations of inhibitor *in vivo*. Evidence from previous studies indicates that the inhibitory effects of organophosphate compounds on acetyl cholinesterase may be attributable to phosphorylation of the serine hydroxyl residue in the enzyme [21, 22]. The delayed neurotoxicity that occurs after administration of organophosphate compounds correlates with the gradual phosphorylation of a neural enzyme that hydrolyzes phenylphenyl acetate [23]. It is possible in the present experiments *in vivo* that a gradual binding of organophosphates to the cholesteryl ester hydrolase occurred, or that the enzyme was phosphorylated at sites which interfered with the activational phosphorylative process via the protein kinase–cAMP-mediated transfer of ATP phosphate to the enzyme. The time interval of the experiments *in vitro* may have been too short to permit a similar process to occur.

Acknowledgement—The authors wish to thank Dr. Ray C. Pittman of the Department of Medicine, University of California, San Diego, for making available to us his assay method for adrenal cholesterol ester hydrolase activity before publication.

REFERENCES

1. M. Civen and C. B. Brown, *Pestic. Biochem. Physiol.* **4**, 254 (1974).
2. H. L. Moses, W. W. Davis, A. S. Rosenthall and L. D. Garren, *Science, N.Y.* **163**, 1203 (1971).
3. W. W. Davis and L. D. Garren, *Biochem. biophys. Res. Commun.* **24**, 805 (1966).
4. D. T. Armstrong, *Recent Prog. Horm. Res.* **24**, 255 (1968).
5. W. H. Trzeciak and G. S. Boyd, *Eur. J. Biochem.* **37**, 327 (1973).
6. M. Nakagawa and M. Uchiyama, *Biochem. Pharmac.* **23**, 1641 (1974).
7. R. Haning, S. A. S. Tait and J. F. Tait, *Endocrinology* **87**, 1147 (1970).
8. P. Brecher, F. Braga-Illa and A. V. Chobanian, *Endocrinology* **93**, 1163 (1973).
9. J. V. Danellis, E. Anderson and L. Trigg, *Endocrinology* **79**, 624 (1966).
10. H. R. Behrman and D. T. Armstrong, *Endocrinology* **85**, 474 (1969).
11. R. C. Pittman, J. C. Khoo and D. Steinberg, *J. biol. Chem.* **250**, 4505 (1975).
12. U. Fricke, *Analyt. Biochem.* **63**, 555 (1975).
13. L. Chen and R. Morin, *Biochim. biophys. Acta* **231**, 194 (1971).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. R. J. Morin, *Clinica Chim. Acta* **71**, 75 (1976).

16. F. B. Ward and T. H. Hess, *Am. J. vet. Res.* **32,** 499 (1971).
17. F. Halberg, E. Halberg, C. P. Barnum and J. J. Bittner, in *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. R. B. Withrow), p. 803. A.A.A.S., Washington, D.C. (1959).
18. M. Civen, R. Ulrich, B. Trimmer and C. B. Brown, *Science N.Y.* **157,** 1563 (1967).
19. A. P. F. Flint, D. L. Grinwich and D. T. Armstrong, *Biochem. J.* **132,** 313 (1973).
20. M. Civen, E. Lifrak, and C. B. Brown, *Pestic. Biochem. Physiol.* **7,** 169 (1977).
21. E. Reiner and W. N. Aldridge, *Biochem. J.* **105,** 171 (1967).
22. R. M. Krupka, *Can. J. Biochem. Physiol.* **42,** 677 (1964).
23. W. N. Aldridge and M. K. Johnson, *Bull. Wld Hlth Org.* **44,** 259 (1971).