Biochemical Pharmacology, Vol. 20, pp. 1679–1688. Pergamon Press, 1971. Printed in Great Britain

EFFECT OF 1-(*o*-CHLOROPHENYL)-1-(*p*-CHLOROPHENYL)-2,2-DICHLOROETHANE ON ADRENOCORTICOTROPIC HORMONE-INDUCED STEROIDOGENESIS IN VARIOUS PREPARATIONS *IN VITRO* OF DOG ADRENAL CORTEX*

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(*Received* 11 *May* 1970; *accepted* 31 *August* 1970)

Abstract—Studies in the intact dog revealed that the intravenous administration of 60 mg/kg of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) inhibited ACTH-induced steroid production by greater than 90 per cent within 2 hr. Similar results were noted in perfused adrenals and adrenal slices obtained from *o,p'*-DDD-treated dogs *in vivo*. In these preparations, baseline steroid production was unaffected by *o,p'*-DDD treatment. Incubation of adrenal slices from control dogs in either Krebs–Ringer bicarbonate-glucose buffer (KRBG) of plasma containing *o,p'*-DDD for as long as 16 hr did not block the adrenal responsiveness to ACTH. Ligation of the blood supply to the liver or gastrointestinal tract had no effect on the ACTH-inhibitory actions of the drug when administered *in vivo*. A 2-hr retrograde perfusion of adrenals with KRBG or plasma containing *o,p'*-DDD almost completely abolished the response to ACTH. In adrenals perfused with plasma containing both a maximally stimulating concentration of ACTH and *o,p'*-DDD, steroid production fell at approximately the same rate as was observed in the intact dog. Thus, *o,p'*-DDD was found to be effective in an adrenal preparation *in vitro*. The drug is not effective in the adrenal slice, probably because it does not penetrate to the interior of the slice. These studies suggest that either *o,p'*-DDD *per se* is the active principle in inhibiting ACTH-induced steroidogenesis or the active principle is formed within the adrenal gland.

1-(*o*-Chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane‡ (*o,p'*-DDD) has been shown to exert three adrenal-related actions in man: two direct effects on the adrenal cortex to inhibit ACTH-induced steroid production[1–4] and to cause atrophy of its inner zones,[2,5] and a peripheral extra-adrenal action to increase cortisol metabolism.[6,7] Because of the nonavailability of human specimens, studies of the direct effects of *o,p'*-DDD on the human adrenal cortex are lacking. *o,p'*-DDD possesses similar adrenocorticolytic effects in the dog.[8,9] However, initial studies by Carzola and Moncloa[10] revealed that the drug had no effect on ACTH-induced steroidogenesis

* Research supported by USPHS Research Grant GM-13749 from the National Institute of General Medical Sciences, National Institutes of Health. From a dissertation presented to the Department of Pharmacology, The George Washington University in partial fulfillment of the requirements for the Ph.D.

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‡ *o,p'*-DDD was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisc.

when added *in vitro* to dog adrenal slice preparations, yet when the drug was administered *in vivo*, ACTH-induced steroid production in slices obtained 2 hr after drug administration was completely inhibited. This observation cast some doubt on the assumption that *o,p'*-DDD is the active principle in causing the direct adrenal effects.

The experiments described in this paper were designed to explore the possibilities that: (1) ACTH-induced steroid production is inhibited in the preparation *in vivo* because *o,p'*-DDD is metabolized to an active metabolite which then inhibits steroidogenesis; or (2) ACTH-induced steroid production is not inhibited when the drug is added *in vitro* to adrenal slice preparations because only a small percentage of the adrenal cells come into contact with the drug, i.e. those on the outside of the slice.

MATERIALS AND METHODS

Animals. Male, mongrel dogs (5–13 kg) obtained from Zartman Farms were maintained on standard Purina dog chow. Animals were fasted overnight before an experiment, but received water *ad lib.* Anesthesia was induced by a slow intravenous injection of 30 mg/kg of pentobarbital sodium (Abbott). Control animals received 1 ml/kg of drug solvent (1 : 1 propylene glycol–95% ethanol) intravenously, and drug-treated dogs *in vivo* received 60 mg/kg of *o,p'*-DDD in a similar amount of solvent.

Steroid extraction and determination. Steroid concentrations in plasma and Krebs–Ringer bicarbonate-glucose (KRBG) buffer were determined by the acid fluorescence assay of Zenker and Bernstein.[11] Development of maximal steroid-dependent fluorescence was measured in an Aminco fluoro-microphotometer (primary filter 472λ and secondary filter 522λ). Under these conditions cortisol (F) and corticosterone (B) are the only two steroids produced in the dog adrenal cortex which fluoresce to any significant extent.[12] The fluorescence of B is about 10 times greater than that of F. However, the dog adrenal produces primarily F and the ratio of B to F remains constant after ACTH stimulation. Consequently, total fluorescence calculated as F was used as a measure of response to ACTH.

Sampling of adrenal venous blood. Samples of adrenal venous blood from a cannula installed in the right lumboadrenal vein were collected[13] during a 2- to 3-hr period after the intravenous injection of 60 mg/kg of *o,p'*-DDD. Maximal stimulation of steroidogenesis was maintained by a constant intravenous infusion of ACTH (Corticotropin injection U.S.P.) at a rate of 0·05 units/min. The rate of steroid production (μg/min) was calculated as the product of right adrenal blood flow (ml/min) and steroid concentration (μg/ml).

Adrenal slice preparation in vitro. At a specific time after intravenous drug administration, the adrenal glands were surgically removed, cleaned, sliced, preincubated and incubated as described previously.[14] Incubation of slices from control dogs was carried out for various periods of time (1–16 hr) in 3 ml of one of the following media: (1) KRBG; (2) KRBG plus 0·1 unit/ml of ACTH*; (3) KRBG plus *o,p'*-DDD (10^{-2} M) in suspension; (4) plasma from control dogs; (5) plasma obtained from dogs 2 hr after receiving *o,p'*-DDD *in vivo*.

o,p'-DDD was added to the incubation medium by forceful injection of the drug in 1:1 propylene glycol–95% ethanol. After incubation in media 3–6, the slices were washed with KRBG and reincubated for 1 hr in either medium 1 or 2.

* U.S.P. Corticotropin reference standard.

Slices of adrenal cortex from *o,p'*-DDD-treated dogs *in vivo* were preincubated for 1 hr in Krebs–Ringer bicarbonate (KRB) and for another hour in either medium 1 or 2. Reactions were terminated by placing the flasks on ice and removing the incubation medium which was stored at 0° for later steroid analysis. Steroid production rates during incubation were calculated as micrograms of fluorescent steroids (as F) per 100 mg wet weight of adrenal per hour of incubation.

Liver-bypassed and eviscerated dogs. A bypass of the blood circulation of the liver was accomplished in a three-step procedure. First, one end of a 2-ft long, clamped polyethylene cannula (PE 360) filled with heparinized saline was inserted into the left external jugular vein. The portal vein was ligated immediately adjacent to its entrance into the liver; then, in less than 2 min, it was cannulated at a point distal to the ligature but proximal to the point of convergence of the smaller abdominal veins.

Evisceration of the dog was performed by placing ligatures at both ends of the gastrointestinal tract. The first closed off the esophagus adjacent to the esophageal-stomach sphincter, and the second occluded the rectum about 4 in. from the anal sphincter. All branches of the arterial and venous blood supply to the tied-off segment of the gastrointestinal tract were ligated. The blood supply to the liver and spleen was occluded, but these organs were not removed.

Immediately after completion of surgical procedures, drug-treated animals received *o,p'*-DDD intravenously, while control animals received only drug solvent. After a 2-hr waiting period, adrenal slices were prepared and incubated in either medium 1 or 2 above.

Isolated perfused adrenal. Adrenals from control dogs were surgically removed under pentobarbital anesthesia. After cannulation of the lumboadrenal vein, each adrenal was retrogradely perfused[15] with either KRBG or plasma by a constant flow model 600-930 quadruple syringe infusion/withdrawal pump (Harvard Apparatus Company). The rate of perfusion (0·8 ml/min) was found to be sufficient, since the adrenals were responsive to ACTH for as long as 5 hr. The temperature of the perfusion fluid was maintained at 37° by looping the cannula through a constant temperature, recirculating water bath which surrounded the glass tube containing the suspended adrenal. To oxygenate and maintain the pH of the perfusion medium, 95% oxygen–5% carbon dioxide was bubbled into the perfusion fluid and water bath.

After a 60-min equilibration period (perfusion with KRBG), fluid samples were serially collected. The experiment began with a 30-min control perfusion with KRBG to measure baseline steroid production. A 3- to 5-min perfusion with ACTH (1·5–2·0 mU) was followed by another 30-min control perfusion with KRBG during which the steroid response to ACTH was monitored. Then the gland was perfused for 2 hr with either KRBG or control plasma containing either drug solvent or *o,p'*-DDD (5×10^{-3} M). After washing out the gland for 20 min with KRBG, the gland was rechallenged with ACTH and steroid production was monitored for the next 60 min of perfusion with KRBG.

Steroid production rates (μg/min) were calculated as the product of the flow rate (ml/min) and steroid concentration (μg/ml).

Before and after each experiment, the extent of adrenal perfusion was checked by examining the pattern of distribution of perfused heparinized blood or a green ink. Results from glands not shown to be completely perfused were discarded.

RESULTS AND DISCUSSION

Time course of action of o,p'-*DDD in the intact dog.* Samples of adrenal venous blood from a cannula in the right lumboadrenal vein were collected during the 2- to 3-hr period after the intravenous injection of *o,p'*-DDD. The patterns of steroid production in typical control and *o,p'*-DDD-treated dogs are shown in Fig. 1. During the first 40 min, the adrenal blood flow and steroid production stabilized. When ACTH infusion was begun, there was no change in adrenal blood flow and usually no noticeable increase in steroid production. This was not unexpected, as the dog was under surgical stress. When the solvent was injected into the control dog, both adrenal blood flow and steroid production remained at control levels. However, when *o,p'*-DDD was injected, the adrenal blood flow dropped immediately, concomitant with a drop in systemic blood pressure, but returned to normal within 40 min. Steroid production, on the other hand, began to fall slowly and reached one-half of the control level 51 min after finishing drug injection. Within 88 min, steroid production was 90 per cent inhibited.

In four *o,p'*-DDD-treated dogs the slight, transient decrease in systemic blood pressure (36·3 mm Hg) and adrenal blood flow (1·0 ml/min) disappeared within 20 $\pm$ 6 (mean $\pm$ 1 S.E.M.) and 35 $\pm$ 9 min respectively. ACTH-induced steroid production decreased slowly and reached one-half control levels within 58 $\pm$ 11 min after injection. Greater than 90 per cent inhibition occurred within 106 $\pm$ 9 min. It appears, therefore, that the inhibition of steroid production following *o,p'*-DDD administration is not due to any effects of the drug on systemic blood pressure or adrenal blood flow.

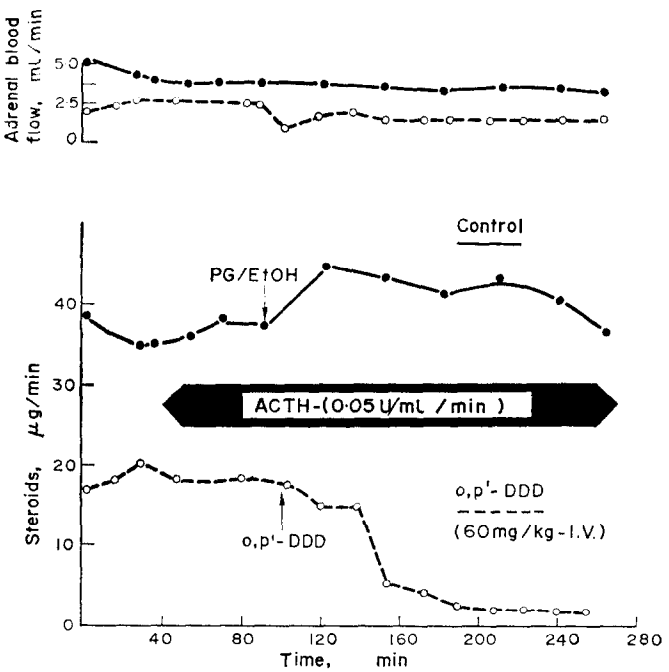

FIG. 1. Effect of *o,p'*-DDD (60 mg/kg, i.v.) on adrenal blood flow and on steroid levels in dog right lumboadrenal venous plasma. The solid line represents a typical control dog and the broken line a typical *o,p'*-DDD-treated dog.

Since ACTH-induced steroid production was greater than 90 per cent inhibited within 2 hr, subsequent studies were carried out in adrenals from dogs pretreated with *o,p'*-DDD for 2 hr.

Effect of o,p'-*DDD* in vivo *and* in vitro *on ACTH-induced steroidogenesis in adrenal slices.* The ability of *o,p'*-DDD to block ACTH-induced steroidogenesis was tested in two adrenal slice preparations: (1) adrenal slices from dogs pretreated *in vivo* with *o,p'*-DDD 2 hr prior to adrenalectomy; and (2) adrenal slices from control dogs which were then incubated for varying periods of time with *o,p'*-DDD in the medium. Results of 1-hr steroid production in these experiments are shown in Fig. 2. In control slices, ACTH caused a 3-fold increase in steroid production. When anesthetized dogs were given *o,p'*-DDD *in vivo* and their adrenals were incubated 2 hr later, ACTH did not stimulate steroidogenesis. These results are in agreement with those seen in the intact dog.

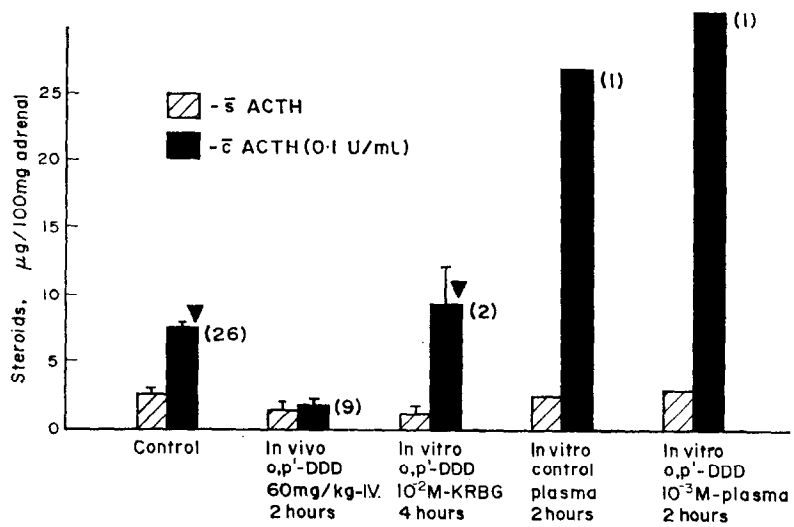

FIG. 2. Effect of *o,p'*-DDD *in vivo* and *in vitro* on ACTH-induced steroidogenesis in slices of dog adrenal cortex. The number of dogs is shown in parentheses. Vertical lines represent one standard error of the mean. The triangle (▼) indicates a significant difference between with and without ACTH ($P < 0{\cdot}05$) using the *t*-test by the method of paired comparisons.

If it is assumed that the injected *o,p'*-DDD is initially distributed only to the plasma, then the maximal concentration of the drug passing through the adrenal is 4×10^{-3} M. When adrenal slices from control dogs were incubated in KRBG containing 10^{-2} M *o,p'*-DDD (in suspension) for up to 4 hr and then reincubated for 1 hr in KRBG, there was the same 3- to 4-fold increase in ACTH-induced steroidogenesis as was seen in control slices. In other words, *o,p'*-DDD (10^{-2} M) *in vitro* was not effective in inhibiting steroid production in this slice preparation, even when incubated twice as long as was necessary for 90 per cent inhibition in the *o,p'*-DDD-treated animal *in vivo*.

It seemed possible that the inhibitory action of *o,p'*-DDD *in vivo* might be due to the ability of plasma to either (1) increase the solubility of the compound, or (2) metabolize

the drug to an active metabolite. Plasma does, in fact, result in the solubilization of various chlorinated hydrocarbons.[16] *o,p'*-DDD is soluble in plasma at a concentration of 10^{-2} M and is almost completely insoluble in KRBG.

Control adrenal slices were incubated for 2 hr in plasma containing 5×10^{-3} M *o,p'*-DDD. As can be seen in Fig. 2, *o,p'*-DDD added in plasma had no effect on the response to ACTH. Only one animal was used in the above experiment; however, two pairs of flasks were incubated for each condition shown in Fig. 2, and confirmation of this observation was obtained in another experiment (Table 1). Incubation of adrenal slices for 16 hr in control plasma slightly decreased the ability of the slices to respond to ACTH. However, incubation in slices in plasma containing 5×10^{-3} M *o,p'*-DDD did not result in any inhibition of the remaining ACTH-inducible steroid production.

Evidence presented in Fig. 2 and Table 1 appears to have eliminated the plasma and adrenal as primary metabolizing systems for *o,p'*-DDD. The possibility still existed that the drug was metabolized to an active compound by other tissues and transported in the bloodstream to the adrenal, thus accounting for the lack of activity *in vitro*. Control adrenal slices were incubated for 16 hr in plasma obtained from dogs 2 hr after receiving *o,p'*-DDD intravenously. Steroid production in these slices showed the usual increase with ACTH (Table 1). This experiment does not support the possibility that the plasma carries an active metabolite of *o,p'*-DDD to the adrenal cortex.

TABLE 1. EFFECT OF *o,p*-DDD ON STEROID PRODUCTION BY ADRENAL SLICES INCUBATED FOR 16 hr IN PLASMA*

Incubation medium	Time (hr)	Adrenal steroid production (μg/100 mg adrenal) Without ACTH	With ACTH	Increase (%)
KRB	1	3·13	10·80	240
Control plasma	16	4·94	8·21	70
Control plasma + *o,p'*-DDD (5×10^{-3} M)	16	4·69	8·62	80
Plasma from *o,p'*-DDD-treated dog (2 hr)	16	3·60	11·90	230

* At the end of each incubation period, the slices were washed and reincubated in KRBG for 1 hr. One of each set of paired flasks had 0·1 unit/ml of ACTH.

Effect of o,p'-*DDD* in vivo *on ACTH-induced steroidogenesis in control, liver-bypassed and eviscerated dogs.* Based on work by other investigators, it appeared that the most likely tissues in which *o,p'*-DDD metabolism might occur were the liver[17] and gastrointestinal tract.[18] Figure 3 shows the effect of ACTH on the steroid production by adrenal slices prepared from normal, liver-bypassed, and eviscerated dogs 2 hr after they had received an intravenous injection of solvent or *o,p'*-DDD. In normal dogs *o,p'*-DDD completely blocked the usual 3-fold increase in steroid production with ACTH. A similar response to *o,p'*-DDD was seen in both the liver-bypassed and eviscerated animals. These data suggest that *o,p'*-DDD does not act through a metabolite formed in either of these tissues.

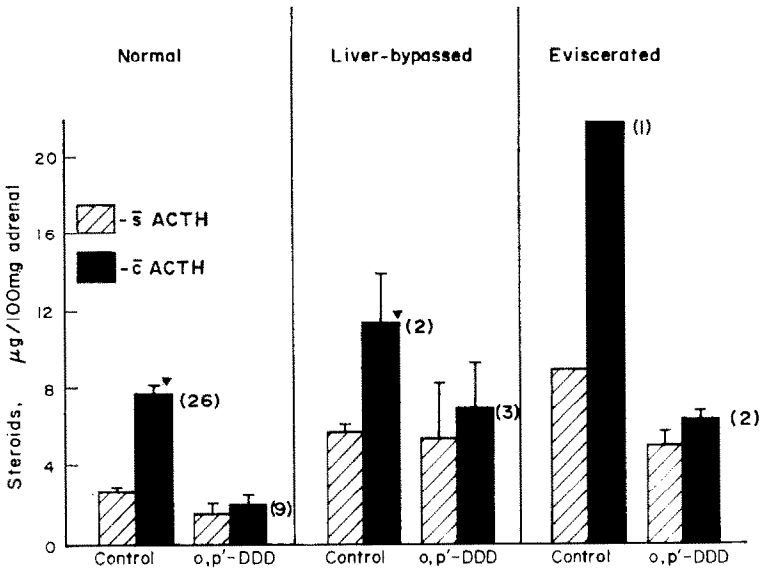

FIG. 3. Effect of *o,p'*-DDD (60 mg/kg, i.v.) *in vivo* on ACTH-induced steroidogenesis *in vitro*. The number of dogs is shown in parentheses. Vertical lines represent one standard error of the mean. The triangle (▼) indicates a significant difference between with and without ACTH ($P < 0.05$) using the *t*-test by the method of paired comparisons.

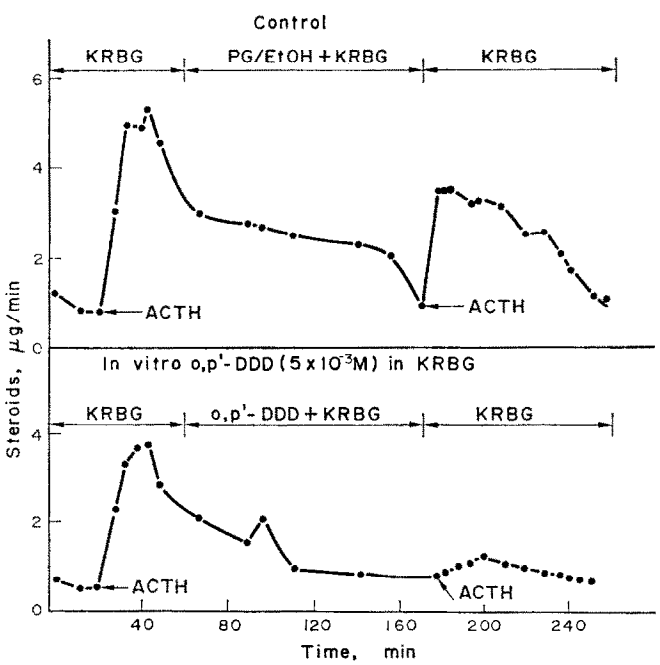

FIG. 4. Effect of *o,p'*-DDD (5×10^{-3} M) in KRBG on steroid production in the perfused dog adrenal. Each graph shows the results from a representative experiment.

Effect of o,p'-*DDD* in vitro *on ACTH-induced steroid production in the isolated perfused dog adrenal.* Thus far, none of the evidence supported the hypothesis that *o,p'*-DDD is metabolized to an active compound in the intact dog. Therefore, the possibility was entertained that the absence of activity *in vitro* was due to lack of penetration of *o,p'*-DDD into the adrenal slices. A system was developed which provided a greater contact between the drug and the individual adrenal cortical cells. Steroid production by retrograde, KRBG-perfused adrenal glands is shown in Fig. 4. The control gland responded to both doses of ACTH, but the treated gland was no longer responsive to ACTH after perfusion with KRBG containing *o,p'*-DDD (5×10^{-3} M in suspension).

TABLE 2. EFFECT OF *o,p'*-DDD *in vitro* IN KRBG ON STEROID PRODUCTION IN PERFUSED DOG ADRENALS

		Steroid production (μg/min) Control period			After treatment period		
		Baseline steroids	Peak ACTH steroids	Increase with ACTH	Baseline steroids	Peak ACTH steroids	Increase with ACTH
Drug solvent	1	0·59	5·19	4·60	0·52	3·02	2·50
	2	0·54	2·48	1·94	0·76	2·84	2·08
	3	0·72	5·33	4·61	0·78	3·49	2·71
	Av.	0·62	4·33	3·71*	0·69	3·12	2·43*
	S.E.M.	0·05	0·93	0·88	0·08	0·19	0·19
o,p'-DDD†	4	0·31	3·81	3·50	0·46	0·79	0·33
	5	0·55	3·78	3·23	0·75	1·23	0·48
	Av.	0·43	3·80	3·37*	0·61	1·01	0·40
	S.E.M.	0·12	0·02	0·14	0·15	0·22	0·08

* Significant difference between before and after ACTH values ($P < 0·05$).
† 5×10^{-3} M.

The results of three KRBG control and two *o,p'*-DDD-perfused adrenals are shown in Table 2. A 3·71 ± 0·88 μg/min increase in ACTH-induced steroid production was seen during the control period. ACTH still stimulated steroid production by 2·43 ± 0·19 μg/min in those adrenals perfused with KRBG containing solvent only, but no statistically significant stimulation was observed in adrenals perfused with KRBG containing 5×10^{-3} M *o,p'*-DDD.

In another series of experiments, basically the same experimental procedure was followed except that plasma from control dogs was used as the perfusate instead of KRBG. There are two reasons for repeating the above perfusion experiment using a different perfusion medium. (1) Since the plasma used for perfusion contained large quantities of endogenous ACTH, the effect of *o,p'*-DDD on the time necessary for steroid production levels to fall to half of control levels ($T_{1/2}$) could be measured. A comparison could then be made between the effects of *o,p'*-DDD in the systems *in vivo* and *in vitro*. (2) Because of the insolubility of the *o,p'*-DDD in KRBG, there was some question as to whether the decreased response to ACTH seen in the perfused gland was due to a direct effect of the drug on the cells of the adrenal cortex or to a capillary

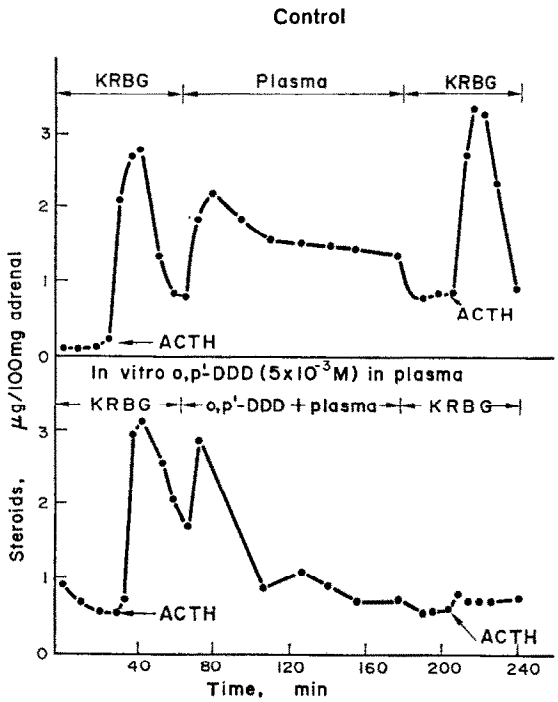

FIG. 5. Effect of *o,p'*-DDD (5×10^{-3} M) in plasma on steroid production in the perfused dog adrenal. Each graph shows the results from one representative experiment.

TABLE 3. EFFECT OF *o,p'*-DDD *in vitro* IN PLASMA ON STEROID PRODUCTION IN PERFUSED DOG ADRENALS

		Control period (μg/min)				After treatment period (μg/min)		
		Baseline steroids	Peak ACTH steroids	Increase with ACTH	Treatment period ($T_{1/2}$)	Baseline steroids	Peak ACTH steroids	Increase with ACTH
Drug solvent	1	0·18	2·76	2·58		0·83	3·37	2·25
	2	0·74	3·56	2·82		0·59	4·28	3·69
	3	0·13	1·30	1·17		0·57	1·40	0·83
	4	0·48	4·39	3·91		0·87	4·06	3·19
	Av.	0·38	3·00	2·62*		0·72	3·27	2·55*
	S.E.M.	0·14	0·66	0·56		0·08	0·65	0·63
o,p'-DDD	5	0·18	1·97	1·79	49	0·21	0·35	0·14
	6	0·50	3·09	2·59	49	0·54	0·71	0·17
	7	0·35	3·00	2·65	89	0·94	1·45	0·51
	8	0·27	4·19	3·92	45	0·84	1·10	0·26
	Av.	0·33	3·06	2·73*	58	0·63	0·90	0·27
	S.E.M.	0·07	0·45	0·44	10	0·16	0·24	0·80

* Statistically significant difference between before and after ACTH values ($P < 0·05$).

clogging effect of the precipitated drug, thus preventing proper perfusion. This possibility was excluded by solubilizing the *o,p'*-DDD in plasma.

Data from two typical plasma perfusion experiments are shown in Fig. 5. In the control gland, steroid production increased immediately and remained high throughout the 2-hr plasma perfusion because of the endogenous plasma ACTH. The same initial increase occurred in the *o,p'*-DDD-treated gland, but then the level began to fall off slowly. Table 3 shows that the time necessary for steroid levels to fall to half of the initial peak steroid level in four animals was 58 $\pm$ 10 min. This value *in vitro* for $T_{1/2}$ is roughly an approximation of that seen in the situation *in vivo*, 68 $\pm$ 11 min. In four control adrenals, the average increase with ACTH before plasma perfusion was 2·62 $\pm$ 0·56 μg/min and afterwards was 2·55 $\pm$ 0·63 μg/min. There is no statistical difference between these two values. After perfusion with plasma containing *o,p'*-DDD, the gland was no longer responsive to ACTH.

The data presented in this paper strongly suggest that *o,p'*-DDD is itself the active compound in causing inhibition of ACTH-induced steroidogenesis in the adrenal cortex of the dog. However, the possibility that the drug is metabolized to another active compound within the adrenal gland itself has not been conclusively excluded.

The most likely explanation for the lack of activity *in vitro* of *o,p'*-DDD in the adrenal slice preparation appears to be that the drug does not easily penetrate to the interior of the slice and cannot inhibit the action of ACTH. In the perfused gland, the drug comes into intimate contact with individual cells through the extensive circulatory system of the organ. Confirmation of this hypothesis might be obtained if it could be demonstrated that *o,p'*-DDD inhibited ACTH-stimulated steroid production in an adrenal cell suspension. This work is now in progress.

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