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EFFECTS OF 1-(*o*-CHLOROPHENYL)-1-(*p*-CHLOROPHENYL)-2,2-DICHLOROETHANE* AND PUROMYCIN ON ADRENOCORTICOTROPIC HORMONE-INDUCED STEROIDOGENESIS AND ON AMINO ACID INCORPORATION IN SLICES OF DOG ADRENAL CORTEX†

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Abstract—In adrenal slices from control dogs, ACTH *in vitro* produced a 3-fold increase in steroid production but had no effect on incorporation of ^{14}C-labeled amino acids into adrenal cortical protein. Puromycin *in vitro* partially inhibited the steroidogenic response to ACTH and almost completely inhibited amino acid incorporation. Administration *in vivo* of 1-(*o*-chlorophenyl)-l-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD), 2–48 hr prior to adrenalectomy, completely blocked the steroidogenic response to ACTH and had the paradoxical effect of producing a marked increase in incorporation of labeled amino acids into protein. Possible implications of these data are presented.

THE EFFECTS of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) on the dog adrenal gland are characterized by rapid loss of responsiveness to ACTH[1] and slow progressive adrenal cortical atrophy.[2] In contrast, other inhibitors of steroid production, such as aminoglutethimide,[3,4] cyanotrimethylandrostenolone,[5,6] tranylcypromine,[7] amphenone,[8,9] and metyrapone,[10,11] block all endproduct steroid production and cause progressive hypertrophy of the adrenal cortex. Treatment of animals with inhibitors of cholesterol biosynthesis, such as AY-9944 [*trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride] have also been shown to decrease steroid production and cause adrenal hypertrophy.[12] In the case of all these drugs, including *o,p'*-DDD, the ACTH titer in the bloodstream is elevated. However, only with *o,p'*-DDD is the adrenal growth effect of ACTH blocked.[13] The inhibitors of steroid production mentioned here (not including *o,p'*-DDD) block steroidogenesis as far back in the biosynthetic pathway as the aminoglutethimide-sensitive step in the conversion of cholesterol to pregnenolone, but do not block the growth effect due to the increased ACTH concentration in the blood. *o,p'*-DDD has also been shown to prevent the ACTH-stimulated conversion of cholesterol to pregnenolone.[14,15] Thus it might be assumed that the action of *o,p'*-DDD occurs prior to the site of action of

* 1-(*o*-Chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wis.

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aminoglutethimide. This assumption implies a common mechanism or two interdependent mechanisms for the action of ACTH on steroidogenesis and adrenal growth. Recent evidence suggests that the growth effect of ACTH may exert significant control over the steroidogenic response to ACTH.[16–18]

It has been suggested that the steroidogenic effect of ACTH may be mediated through or require the synthesis of new protein.[19–21] Ferguson[20] and later Garren *et al.*[21] reported that, when protein synthesis was blocked with puromycin in normal rat adrenals, the steroidogenic effect of ACTH was also inhibited. Farese[22] showed that chloramphenicol partially inhibited protein synthesis in rat adrenals and completely abolished ACTH-induced steroidogenesis. In these experiments it did not seem likely that the inhibitory effect on ACTH-stimulated steroid production was due to general nonspecific cellular damage, since NADPH addition resulted in increased steroidogenesis.[19,20]

These findings suggested to us that *o,p'*-DDD may exert its effect on the adrenal cortex by inhibiting protein synthesis essential to the action of ACTH. The resulting decrease in ACTH-induced steroid production, decrease in the activity of NADPH producing glucose 6-phosphate dehydrogenase,[1,23] and atrophy of the adrenal cortex[24,25] might be attributed to such an effect. The experiments described in this paper were designed to allow comparison of the effects of *o,p'*-DDD and puromycin on ACTH-induced steroidogenesis and labeled amino acid incorporation into adrenal cortical protein.

MATERIALS AND METHODS

Male, mongrel dogs weighing 7–13 kg were fasted overnight before an experiment, but received water *ad lib.* To suppress endogenous ACTH blood levels, 20 mg methylprednisolone acetate (Upjohn), a long-acting glucocorticoid, was administered intramuscularly to each dog 18 hr prior to anesthetization. To further supplement this action 2 mg of dexamethazone sodium phosphate (Merck, Sharpe & Dohme) was administered intravenously after induction of anesthesia with 30 mg/kg pentobarbital sodium. Drug-treated animals received slow (10 min) intravenous infusions of 60 mg/kg *o,p'*-DDD dissolved in 1:1 propylene glycol–95 per cent ethanol at a concentration of 60 mg/ml. Control dogs were injected with drug solvent (1 ml/kg).

Two hr after *o,p'*-DDD injection, the adrenal glands were surgically removed, cleaned of adhering connective tissue, and sliced on a Stadie–Riggs hand microtome. Slices containing large areas of capsule or medulla were discarded. Each slice was cut in half and the halves placed in separate, paired, tared 25-ml Erlenmeyer flasks containing 3 ml Krebs–Ringer bicarbonate buffer (KRB).[26] Each flask contained 50–150 mg tissue.

Incubations were carried out at 37° under an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide in a MagniWhirl constant temperature shaker. A 1-hr preincubation was performed to deplete endogenous ACTH. The supernatant was removed and the slices were washed with 5 ml KRB. Adrenal slices were then incubated in 3 ml of one of the following media: (1) KRB containing 200 mg/100 ml glucose (KRBG); (2) KRBG plus ACTH (0·1 unit/ml)*; (3) KRBG plus puromycin (10^{-3} M);

* This concentration of U.S.P. corticotropin reference standard produces a maximal response in the system described.

or (4) KRBG plus puromycin (10^{-3} M) plus ACTH (0·1 unit/ml). Each flask contained 0·1 μc (0·066 μg) of uniformly labeled ^{14}C-L-leucine or 0·1 μc (0·0478 μg) of uniformly labeled ^{14}C-L-lysine. After 30 or 60 min of incubation, the medium was removed and replaced with 3 ml of 10% trichloroacetic acid to terminate the reaction. Steroid concentrations in the incubation media were determined by the fluorimetric assay of Zenker and Burnstein[27] and quantitated as cortisol. Even though the fluorimetric assay measures only 11-oxygenated steroids, it effectively monitors the effect of *o,p'*-DDD on the ACTH-induced conversion of cholesterol to pregnenolone, since the major site of inhibition with this drug is at this step.[14,15] Protein was purified by the method of Siekevitz.[28] An aliquot of the dried protein was weighed and dissolved in 0·5 ml of 1 M Hyamine hydroxide. Ten ml of scintillation mixture* was added and the ^{14}C content was determined using a Nuclear Chicago model 724 controlled temperature liquid scintillation spectrometer. Specific activity of adrenal protein was calculated as disintegrations per minute (dis./min) per milligram of dried adrenal protein.

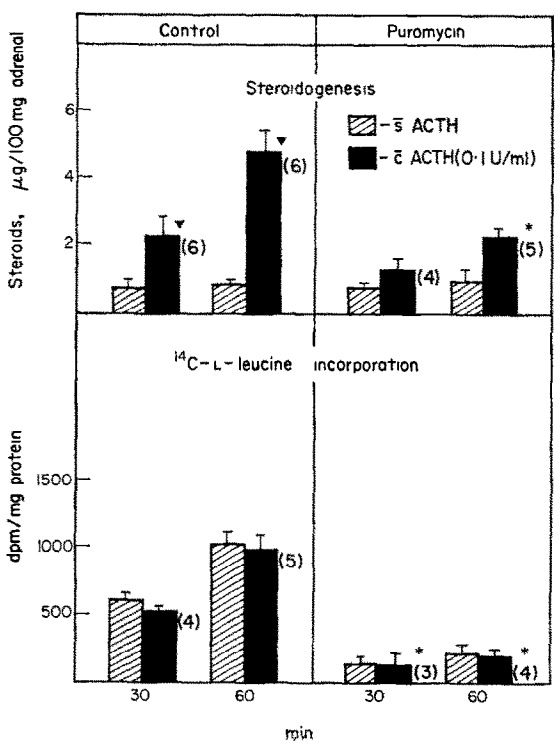

FIG. 1. Effects of ACTH and puromycin (10^{-3} M) on steroid production and ^{14}C-L-leucine incorporation in slices of dog adrenal cortex. The number of dogs is shown in parentheses and the vertical lines represent one standard error of the mean (S.E.M.). The solid triangle (▼) indicates a significant difference ($P < 0.05$) between flasks incubated with (c̄) and without (s̄) ACTH, and the asterisk (*) indicates a significant difference between control and puromycin-treated slices as determined by the *t*-test using the method of paired comparisons.

* The mixture consists of 3·0 g 2,5-diphenyloxazole (PPO) and 100 mg 1,4-bis-2-(4-methyl-5-phenlyoxazolyl) benzene (dimethyl POPOP).

RESULTS AND DISCUSSION

Figure 1 shows the effect of 10^{-3} M puromycin on steroidogenesis and ^{14}C-L-leucine incorporation in adrenal slices from normal dogs. In control slices, ACTH-stimulated steroid production was linear over the time period studied. ACTH treatment resulted in a 3- to 4-fold increase in steroid production. ^{14}C-L-leucine incorporation was also linear for at least 60 min, but ACTH had no effect on incorporation. This has been a consistent finding in our studies. Other investigators have published results agreeing with these data.[20,21,29,30] Agreement is not universal however. Halkerston *et al.*[31] and Ferguson *et al.*[32] reported that ACTH *in vitro* inhibited incorporation of labeled amino acids into protein of quartered rat adrenals. These observations seem to be at variance with observations where administration of relatively large doses of ACTH *in vivo* caused an increase, both *in vivo* and *in vitro*, in amino acid incorporation into adrenal protein.[19,33]

In Fig. 1, 10^{-3} M puromycin reduced the adrenal steroidogenic response to ACTH and inhibited ^{14}C-L-leucine incorporation by 75–85 per cent. In puromycin-treated slices, ACTH caused only a 0·5 to 2-fold increase in steroid production, as compared

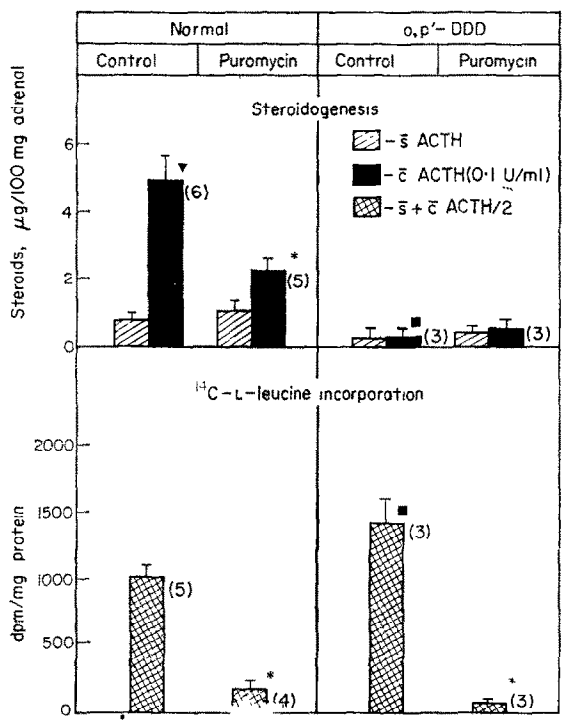

FIG. 2. Effect of *o,p'*-DDD *in vivo* and puromycin *in vitro* on ACTH-induced steroidogenesis and ^{14}C-L-leucine incorporation in adrenal slices obtained 2 hr after drug treatment and incubated for 60 min, as described in Methods. The number of dogs is shown in parentheses and the vertical lines represent one S.E.M. The cross-hatched bars represent the mean ^{14}C-L-leucine incorporation obtained from paired flasks without (s̄) and with (c̄) ACTH. The solid triangle (▼) indicates a significant difference between flasks with (c̄) and without (s̄) ACTH, and the asterisk (*) indicates a significant difference between control and puromycin-treated slices, using the *t*-test by the method of paired comparisons. The solid square (■) indicates a significant difference between control and *o,p'*-DDD-treated dogs as determined by analysis of variance. In all cases $P < 0{\cdot}05$ was accepted as significant.

to a 3- to 4-fold increase in control slices. Baseline levels of steroid production were unaffected by puromycin. It appears, therefore, that the effects of puromycin on dog adrenal slices are similar to those reported for the rat.[20]

In Fig. 2, the effects of puromycin and *o,p'*-DDD may be compared. Amino acid incorporation is expressed as the pooled values from paired ACTH-stimulated and nonstimulated adrenal slices. ACTH-induced steroidogenesis was completely inhibited by *o,p'*-DDD. Amino acid incorporation, on the other hand, was actually increased by 37 per cent.

o,p'-DDD completely inhibited ACTH-induced steroidogenesis at a time when amino acid incorporation was increased, whereas puromycin only partially inhibited ACTH-induced steroidogenesis at a time when amino acid incorporation was almost entirely inhibited. It would appear that these two drugs are not working similarly in the adrenal cortex of the dog and that *o,p'*-DDD does not block protein synthesis.

Since *o,p'*-DDD does cause adrenal cortical atrophy, it seemed likely that sooner or later protein synthesis must be decreased. With this thought in mind, the effect of *o,p'*-DDD on amino acid incorporation was examined in adrenal slices from dogs receiving intravenous *o,p'*-DDD for longer time periods prior to adrenalectomy. The results of these studies are shown in Fig. 3. Twenty-four hr after a single intravenous dose of *o,p'*-DDD, steroid production was still not significantly stimulated by ACTH and amino acid incorporation was increased by 120 per cent. It was thought

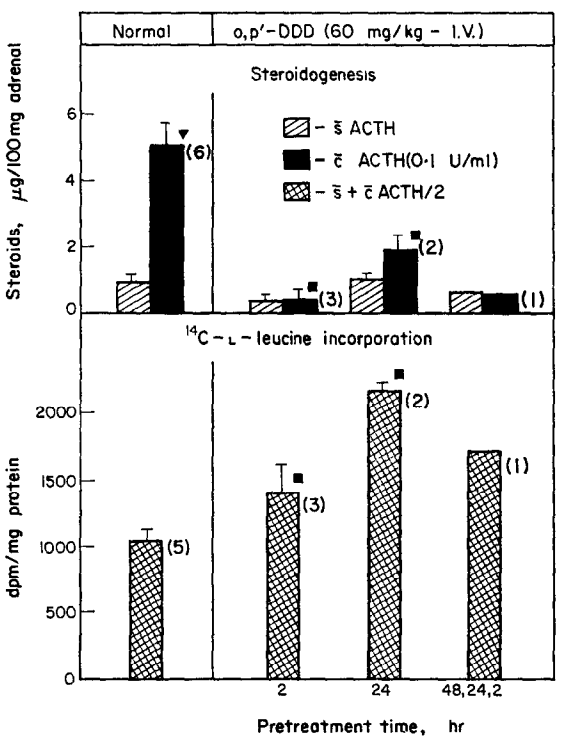

FIG. 3. Effects of *o,p'*-DDD on ACTH-induced steroidogenesis and ^{14}C-L-leucine incorporation in adrenal slices obtained at various time periods after drug treatment. Conditions and symbols are identical to those described in Fig. 2.

that the increase in incorporation might represent recovery from the effects of a single dose of *o,p'*-DDD and an attempt at cellular repair. In order to be certain that the adrenals of the dog were maximally suppressed with *o,p'*-DDD, one animal received three doses of *o,p'*-DDD at 48, 24 and 2 hr before adrenalectomy. Amino acid incorporation remained 75 per cent above normal, while ACTH-induced steroidogenesis remained completely blocked.

When *o,p'*-DDD was given orally (200 mg/kg) 40 hr prior to adrenalectomy (and ^{14}C-L-lysine was used as the labeled amino acid), similar results were obtained (Table 1). Kaminsky *et al.*[25] reported histological evidence of severe degenerative changes in the adrenal cortex of the dog as early as 12 hr after the oral administration of the same dose of drug. Even though ACTH-induced steroidogenesis was blocked, amino acid incorporation was stimulated by almost 300 per cent in the 30-min incubation slices and by more than 150 per cent in the 60-min incubation slices. These data suggest that if the increased amino acid incorporation is associated with attempts at cellular repair, it persists at a time when histological examination shows extensive cellular degenerative changes.

TABLE 1. EFFECT OF ORAL *o,p'*-DDD (200 mg/kg) ON ACTH-INDUCED STEROIDOGENESIS AND ^{14}C-L-LYSINE INCORPORATION INTO ADRENOCORTICAL PROTEIN IN SLICES OBTAINED 40 hr AFTER DRUG TREATMENT

Incubation time (min)	ACTH (units/ml)	Normal (3)	*o,p'*-DDD (2)
		Steroids (μg/100 mg adrenal)*	
30		1·96 ± 0·61	0·34 ± 0·12
	0·1	4·75 ± 1·28†	0·66 ± 0·10
60		2·83 ± 0·34	1·59 ± 0·48
	0·1	11·63 ± 0·95†	1·80 ± 0·36
		^{14}C-L-lysine incorporation (dis./min/mg protein)‡	
30		299 ± 57	1135 ± 75§
60		978 ± 63	2610 ± 63§

* Values represent mean ± S.E.M. The number of dogs is shown in parentheses.
† Significant difference ($P < 0·05$) between with and without ACTH.
‡ Mean and S.E.M. calculated using pooled values for with and without ACTH.
§ Significant difference ($P < 0·05$) between normal and *o,p'*-DDD treated dogs.

As shown in the above experiments, *o,p'*-DDD, administered both intravenously and orally, resulted in an increased rate of amino acid incorporation into adrenal cortical protein concomitant with a complete blockade of ACTH-induced steroidogenesis. The following hypotheses are offered as possible explanations for these results. (1) The primary action of *o,p*-DDD may be unrelated to protein synthesis and the increased amino acid incorporation reflects an attempt at cellular repair. The chronic administration experiments presented here do not seem to support this hypothesis. (2) The primary site of action of *o,p'*-DDD may be on the protein-synthesizing mechanisms, causing the biosynthesis of large quantities of nonfunctional protein. (3) The primary site of action may be on the protein metabolizing systems, resulting in increased degradation of protein and a concomitant secondary increase in amino acid

incorporation. (4) *o,p'*-DDD may act to decrease the pool size of amino acids in the adrenal cortex. This would produce a higher specific activity of labeled amino acid in the pool, possibly resulting in a greater rate of incorporation of label when no change or a decrease in protein synthesis occurs. Reddy and Streeto[34] and Bransome[13] have suggested that, among other things, changes in pool size in adrenal slices may account for the apparent lack of stimulation of amino acid incorporation with ACTH *in vitro*. However, a decrease in pool size would be expected to result in an increase in incorporation of label in puromycin-treated slices. Such was not the case. Figure 2 shows that a combination of *o,p'*-DDD and puromycin actually results in incorporation of less label than does puromycin alone.

Further evaluation of these paradoxical findings is being sought through the use of adrenal cell suspensions where amino acid pool size can be regulated and protein synthesis and degradation can be more closely examined.

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