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# METABOLISM OF [$^3$H]-TESTOSTERONE BY THE ADRANAL OF THE DOG: THE ACTION OF ACTH*

G. F. WASSERMANN,† A. BALDI, E. del CASTILLO and E. CHARREAU†
Laboratorio de Esteroides, Instituto de Biología y Medicina Experimental, Obligado 2490-Buenos Aires-Argentina, and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires

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## SUMMARY

The metabolism of [$^3$H]-testosterone in perfused dog adrenals and in adrenal minces of a normal dog has been studied.

Three principal metabolites were studied and identified by isotope dilution and gas–liquid chromatography–mass spectrometry: androstenedione, 11$\beta$-hydroxyandrostenedione and 11 $\beta$-hydroxytestosterone.

The last of these metabolites is obtained in high yield which is further increased after ACTH administration.

## INTRODUCTION

CHANG *et al.*[1] studied the *in vitro* metabolism of testosterone by minces of human adrenal glands and found androstenedione, adrenosterone, 11 $\beta$-hydroxy-androstenedione and 11 $\beta$-hydroxytestosterone as the major conversion products. Bardin *et al.*[2] have reported that the metabolic clearance rate of testosterone is increased in patients with adrenal tumors suggesting an active metabolism of the hormone by the tumor.

In 1969 Wassermann and Eik-Nes[3] reported the lack of a measurable secretion of testosterone by the adrenal of the dog. On the contrary, the data presented in this paper suggest a catabolism, i.e. uptake and modification of the testosterone molecule by this gland.

To our knowledge, neither the metabolism of testosterone by the adrenal gland of the dog, nor the action of ACTH on this metabolism has been investigated. Such a study, however, seems to be important in view of the relation existing between adrenal function and androgen metabolism[4].

In this paper we investigated the metabolism of [1,2-$^3$H]-testosterone by isolated perfused adrenals of normal and hypophysectomized dogs and by adrenal minces of a normal dog, as well as the effect of ACTH upon these preparations.

## MATERIAL AND METHODS

Two different sets of experiments were devised for this study:

(1) *Perfusion of isolated dog adrenals.* The experiments were carried out on seven mature, healthy mongrel male dogs ranging from 15–20 kg.

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†Established investigator from the Consejo Nacional de Investigaciones, Científicas y Técnicas, (Argentina).

The dogs were divided in two groups: normal (5) and hypophysectomized (2). All experiments were conducted with animals anesthetized by means of intravenous (i.v) injections of sodium pentobarbital (30 mg/kg). Heparin was administered intravenously prior to collection of blood and removal of the adrenals. The dogs were intravenously maintained with 0·9% sodium chloride solution and injected during surgery. The hypophysectomized dogs were orally treated with dexamethasone (8 mg/day) from the day of hypophysectomy and a supplementary dose of 4 mg of the drug diluted in the saline solution was given during surgery.

*Surgical preparation:* (a) *Hypophysectomy:* was performed 4 days before the perfusion experiments(5).

The animals were maintained in separate cages. Food and water were administered "ad libitum".

(b) *Adrenal gland preparation:* The anesthetized dogs were abdominally eviscerated and the kidneys removed. The adrenal glands with an adjoining piece of aorta, vena cava and renal arteries were taken out. The dissecting technique was similar to the one described by Feldberg[6] for cats. It differed in that both adrenals were used in the same preparation and that the perfusion was carried out through the aorta instead of the coeliac artery. At the end of the dissection the adrenals were attached by vascular connections to the aorta, vena cava and renal arteries. At this moment the distal and proximal end of the aorta and vena cave were ligated and the isolated preparation immediately transported to a chamber at 37°C.

*Perfusion of the gland*

A total of 150–300 ml of blood required for the perfusion was collected from the femoral artery, and diluted with 50% saline solution.

The diluted blood was then oxygenated with $O_2 : CO_2$ (95 : 5 v/v) and kept at 37°C. All connections and pumps were maintained in the chamber in order to prevent temperature losses.

The adrenal preparation was kept on a nylon mesh and perfused at a constant rate of 5 ml/min/g of gland[7].* The effluent from the adrenals was collected in beakers through cannulae tied to the adrenal veins. After 5 min of perfusion to allow the preparation to equilibrate, the infusion of [$^3$H]-testosterone began, and the effluent blood was collected for 30 min (in dog 1, only a 10 min sample was collected). The [1, 2-$^3$H]-testosterone (specific activity 1 mCi/22·2 n moles) was diluted in saline and infused at constant flow of 0·2 ml/min (0·366 $\mu$Ci/min) into the preparation.

When adrenals from hypophysectomized dogs were used, three consecutive samples corresponding to a blood collection of 25 min were taken. ACTH† saline solution was injected into the preparation in a continuous flow (0·2 ml/min; 3·42 U/min) after the collection of the first 25 min blood sample.

*Extraction and purification procedure*

Two hundred $\mu$g of each of the metabolites (11$\beta$-hydroxyandrostenedione, adrenosterone, testosterone and androstenedione) were added to the blood

*The weight of the adrenal glands was calculated according to Hechter *et al.*[8] (57 mg/kg body weight).

†ACTH Nordic–Nordic Lab. Montreal, Canada (s.a. 63 U/mg).

samples. In the first three experiments (dogs 1, 2, 3) blood was centrifuged immediately and the plasma removed. The red cell layer was washed with 50 ml saline solution and the washed fraction added to the plasma.

The combined plasma was extracted with ether. The red cell layer and the aqueous phase were processed for conjugated steroids. Due to the small amounts of radioactivity present, this procedure was omitted in the subsequent samples.

In all other experiments, whole blood was extracted with ether. After defatting the ether extracts [9], the samples were chromatographed on thin layer plates coated with Silica Gel in the solvent system chloroform: ether (4: 1 v/v).

After the end of the perfusion the adrenals were cleaned, weighed, homogenized in saline, and processed by the same method as above.

(2) *Incubation of adrenal gland minces.* Both adrenals of a 20 kg dog were minced and divided into twelve samples of 150 mg each. After a 60 min preincubation, the 5 ml of buffer solution was discarded and the samples were reincubated in 5 ml of fresh Krebs–Ringer bicarbonate buffer (plus glucose) pH 7·4 in an atmosphere of $O_2$: $CO_2$ (95:5 v/v) at 37°C using a Dubnoff shaking incubator.

To each sample, 5 $\mu$Ci of [1,2-$^3$H]-testosterone (S.A. 1 mCi/22·2 n moles) and 10 $\mu$g of unlabelled testosterone dissolved in ethanol were added. The incubation was started after the addition of 15 I.U. of ACTH dissolved in 0·2 ml of saline to the treated flasks. Saline (0·2 ml) was added to controls.

After a 20 or 60 min incubation,* the samples were mixed with 5 ml of ethyl acetate and kept in the freezer until use.

The samples were extracted with ethyl acetate, dried under nitrogen and chromatographed in a thin layer system using chloroform: ether (4: 1 v/v) as solvent.

A parallel incubation of 2·8 g of a pool of minced adrenals from 3 dogs was carried out. In this case, 5 mg of unlabelled testosterone were used as substrate. The incubation lasted 3 h and was performed under similar conditions using 10 ml of Krebs–Ringer bicarbonate buffer.

*Determination of added 4-ene-3-ketosteroids*

The amounts of the steroids were determined by U.V. (240 nm) absorption using the Allen correction in a Beckmann DB Spectrophotometer.

*Radioactivity determination:* The chromatograms were scanned in a Packard (model 7201) Radiochromatogram scanner and the radioactivity counted in a Packard (model 3320) Tri-Carb Liquid scintillator spectrometer.

*Derivative formation:* Acetylation was performed according to Zaffaroni and Burton [10] and oxidation according to V.R. Mattox *et al.* [11].

*Corticoid measurements:* The determination of corticoids in an aliquot of the total extract of the blood on adrenal samples was done as follows: 0·2 ml of redistilled ethyl alcohol, 0·15 ml of a solution of blue tetrazolin (0·15% in ethanol) and 0·15 ml of tetramethylamonium hydroxide (0·3 ml of a 10% aqueous solution diluted to 10 ml with ethanol) were added to the residue. The tube was mixed, incubated for 20 min at 28°C in the dark and measured within 20 min in a Beckman spectrophotometer at 480, 510 and 600 nm. Cortisol was used as standard [12].

*See table.

*Recrystallization:* The steroids were recrystallized to constant specific activity after the addition of 30 mg of authentic standards, according to Axelrod *et al.* [13].

*Preparation of derivatives for mass spectrometry:*

Acetylated steroids were formed in the following manner: The steroids were adsorbed on stainless steel mesh and left at 37°C overnight in an atmosphere of acetic anhydride and pyridine. Trimethylsilyl derivatives were prepared under comparable conditions using bis(trimethysilylacetamide).

Combined gas–liquid chromatography–mass spectrometry were carried out on a LKB 9000 instrument with the solid injection technique of Menini and Norymberski. (14) The stationary phase of the column was 2% $OV_1$ on gaschrom A (Applied Science Laboratories, State College, Penn.). The mass spectrometer ionizing current was kept at 50 $\mu$A, and the ionizing energy was maintained at 70 eV. The source temperature was 310°C; column temperature was kept at 207°C.

## RESULTS

*Identification of* [$^3$H]*-testosterone metabolites formed in both types of experiments*

The radioscans of the thin layer chromatograms (chloroform:ethyl ether 4:1 v/v) of the samples from the perfusions and incubation experiments revealed a very constant qualitative pattern of steroid metabolites formed.

From the 4 well-defined radioactive peaks detected, the chromatographic mobility of 3 areas coincided with that of the cold steroids added. The remaining peak corresponded to one or several steroid metabolites of higher polarity. The findings are summarized in Fig. 1. The four eluted areas were processed as follows:

*Peak No.* 1: (androstenedione area) was acetylated and chromatographed in t.l.c. using benzene:ethyl acetate (5:1 v/v) as a solvent system. After elution of the U.V. absorbing zone corresponding to androstenedione, aliquots were taken

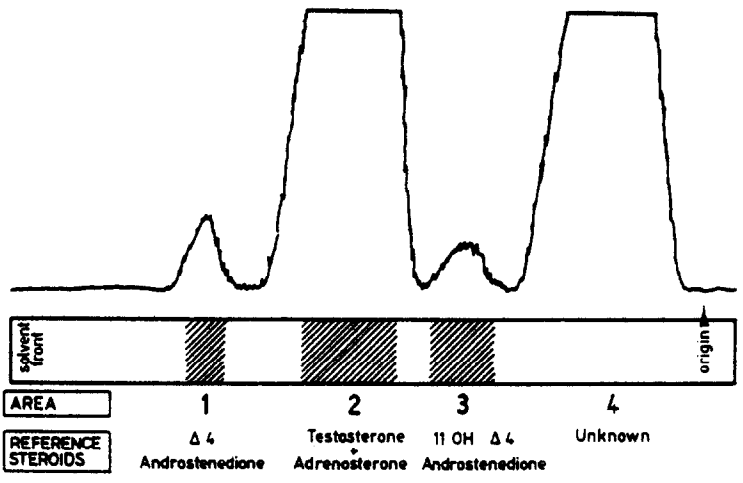


Fig. 1. Radioscan of a t.l.c. (chloroform-ethyl ether 4:1 v/v) of the ether extract of effluent blood from dog adrenal perfused with [$^3$H]-testosterone as a precursor.

for recovery and radioactivity counting. Its identity was proved by recrystallization to constant specific activity (Table 1).

*Peak No.* 2: (testosterone and adrenosterone area). The eluted sample was acetylated and chromatographed in t.l.c. (benzene: ethyl acetate 3 : 2 v/v) and the areas of testosterone acetate and adrenosterone eluted. From both steroids, aliquots were taken for recovery and radioactivity determination. The adrenosterone sample had completely lost radioactivity on recrystallization.

*Peak No.* 3: (11$\beta$-hydroxyandrostenedione area). After additional t.l.c. in benzene: ethyl acetate (3 : 2 v/v), the extract from this area was oxidized to adrenosterone and chromatographed in chloroform: ethyl ether (4 : 1 v/v). From the eluted steroid, aliquots were taken for recovery and radioactivity determination. The samples were recrystallized using an authentic adrenosterone as standard. As seen in Fig. 1, constant specific radioactivity was achieved after three successive recrystallizations (Table 1).

*Peak No.* 4: This area was eluted and rechromatographed (3 consecutive runnings) in the system benzent: ethyl acetate (3 : 2 v/v) in which it showed to be composed of several peaks; one of these contained almost all the radioactivity and was further studied. No attempt was made to identify the other peaks.

In the second experiment (incubation of 2·8 g of adrenals with 5 mg of testosterone) the identification of this unknown compound was attempted. In this incubation a clear U.V. spot with a similar $R_F$ was observed. This area was eluted and mixed with peak No. 4 from dog No. 5.

The U.V. absorbing material and peak No. 4 showed to have identical mobilities in the following systems: benzene: ethyl acetate (3 : 2 v/v); hexane: ethyl acetate: acetic acid (20 : 75 : 5 by vol.) and in paper chromatography (Bush $B_3$).

The area was finally eluted from paper, evaluated using a U.V. spectrophotometer (220, 240, 260 nm) and an aliquot was used for identification by mass spectrometry.

The mass spectra of the free compound had a molecular im (M +) of 304 and main fragmentation peaks m/e, 286, 271, 227, 164 characteristic of 11$\beta$-hydroxytestosterone.

Table 1. Recrystallizations of steroids formed by adrenal glands of dogs using [$^3$H]-testosterone as a precursor* (specific activity in d.p.m. mg)

| Crystallization | Solvent system | Androstenedione | 11$\beta$-hydroxy-androstenedione† | 11$\beta$-hydroxy-testosterone |
|---|---|---|---|---|
| 1 | Acetone–water | 3076 | 2110 | 3156 |
| 2 | Ethanol–water | 2972 | 2016 | 4984 |
| 3 | Methanol–water | 2953 | 1934 | 4846 |
| 4 | Acetone–water | 2947 | 1898 | 4804 |

*Samples were recrystallized using 30 mg of authentic standard.
†Recrystallized as adrenosterone.

After acetylation, the GLC pattern and the mass spectra were indistinguishable from those of authentic 11 β-hydroxytestosterone acetate, showing a molecular im (M+) 346 and fragmentation peaks m/e 328, 280, 268, 253. As a TMS derivative the compound showed a molecular im (M+) 376 and main fragmentation peaks m/e 350, 343, 333, 320, 305, 286, 262, 253. The retention time and mass spectra corresponded to that of the TMS derivative of 11 β-hydroxytestosterone.

After the identification by mass spectrometry, the remainder of the sample was recrystallized with 30 mg of authentic 11β-hydroxytestosterone and constant specific activity was obtained, again confirming the identity of the compound synthetized by the adrenals (Table 1).

*Amounts of radioactive steroids found in blood and adrenal glands in the perfusion experiments*

(a) *Normal dogs.* The amounts of radioactive steroids isolated and identified in the effluent blood of the perfused adrenals as well as those found in the glands at the end of the perfusion are shown in Table 2. A striking feature of the data obtained is the large variation in the amounts of metabolites found in the different dogs under quite uniform experimental condition. In all dogs but No. 1, the main quantitative metabolite was 11β-hydroxytestosterone, in spite of the fact that losses have not been taken into account for this latter steroid.

(b) *Hypophysectomized dogs.* In Table 3 the levels of [$^3$H]-steroids found in the blood samples of the hypophysectomized dogs before and after ACTH administration are expressed, as well as the amounts found in the adrenal gland after perfusion. As in normal dogs, the principal metabolite of infused [$^3$H]-testosterone was 11β-hydroxytestosterone.

The concentration of this steroid rises after administering ACTH to the

Table 2. Radioactive steroids in the effluent blood and in adrenal glands after perfusion experiments in normal dogs (in d.p.m. × $10^4$)

| Dog No | Body weight (kg) | Sample | Vol. of perfusion or adrenal weight (pair) | Androstenedione | 11β-hydroxy-androstenedione | 11β-hyroxy-testosterone† |
|---|---|---|---|---|---|---|
| 1* | 15 | blood | 38·8 ml | 251 | 48 | 99 |
| | | adrenal | 0·88 g | 1·6 | 7 | 12 |
| 2 | 16 | blood | 120 ml | 8 | | 28 |
| | | adrenal | 0·90 g | 2 | | 4 |
| 3 | 20 | blood | 171 ml | 11 | | 75 |
| | | adrenal | | 0·1 | | 1 |
| 4 | 25 | blood | 153 ml | 11 | 10 | 85 |
| | | adrenal | 2·25 g | negligible | | 20 |
| 5 | 26 | blood | 160 ml | 41 | 25 | 128 |
| | | adrenal | 1·98 g | negligible | 3 | 16 |

†Values not corrected for losses.

*Dog No 1 was perfused 10 min; in other dogs the perfusion lasted 30 min.

Table 3. Radioactive steroids found in the effluent blood and in glands from the adrenal preparation of hypophysectomized dogs*

| Dog | Sample | Volume perfused | Androstenedione (d.p.m. × $10^4$) | 11$\beta$-hydroxy-androstenedione (d.p.m. × $10^4$) | 11$\beta$-hydroxy testosterone (d.p.m. × $10^4$)† | Corticoids‡ ($\mu$g) |
|---|---|---|---|---|---|---|
| No. 1 weight 25 kg | Basal | 152 | 7·5 | 10 | 24 | 4·8 |
| | 1st. ACTH infusion | 157 | 9·9 | 23 | 90 | 7·2 |
| | 2nd. ACTH infusion | 161 | 11 | 16 | 218 | 12·0 |
| | Adrenal gland (weight 1·45 g) | | | 5 | 6 | 1·8 |
| No. 2 weight 10 kg | Basal | 92 | 8 | 2 | 29 | 8·0 |
| | 1st. ACTH infusion | 50 | 8 | 2 | 27 | 6·7 |
| | 2nd. ACTH infusion | 97 | 10 | 3 | 53 | 15·2 |
| | Adrenal gland (weight 1·15 g) | | 0·7 | 3 | 27 | 7·5 |

*Adrenals were isolated and perfused as described in Materials and Methods. [1,2-$^3$H]-testosterone (22·2 n moles, 1·00 m Ci) was infused at a rate of 0·366 Ci/min. After a 25 min collection (basal sample), the infusion of ACTH starts and two more 25 min samples were collected. Values of adrenal glands are the ones found at the end of perfusion.

†Values not corrected for losses.

‡Corticoids were measured in an aliquot of the total extract by the Blue Tetrazolium method according to Elliott F. H. *et al.*[12].

perfused adrenals. In dog No. 1 the increase was clearly seen in both samples taken; in dog No. 2, difficulties during perfusion prevented the observation of the increase during collection of ACTH sample No. 1 (only 50 ml were collected). The increase was visible however in ACTH sample No. 2. The increase of corticoids secreted by the perfused adrenals and detected by the Blue Tetraxolium reaction, corresponded fairly well to the rise in the 11$\beta$-hydroxytestosterone concentration (See table).

*Production of* [$^3$H]*-testosterone metabolites by adrenal gland minces*

The qualitative metabolism of [$^3$H]-testosterone by adrenal gland minces was similar to that produced by the perfused gland.

For both time intervals studied, 20 and 60 min, 11$\beta$-hydroxytestosterone was the principal metabolite. Androstenedione and its 11$\beta$-hydroxylated derivative were also isolated.

In the samples containing ACTH a significant increase ($p < 0{\cdot}05$) of 11$\beta$-hydroxytestosterone was observed. The yield of this steroid was quite high in stimulated and control samples although: (a) no correction for losses was made, and (b) the specific activity of [$^3$H]-testosterone was lowered by adding unlabelled testosterone (10 $\mu$g).

Table 4. Radioactive steroids found in incubation of dog adrenal gland minces with [$^3$H]-testosterone* (d.p.m. × $10^{-4}$)

| Sample | Number of cases | 11β-hydroxy-testosterone | % of conversion | 11β-hydroxy-androstenedione | % of conversion | Androstenedione | % of conversion |
|---|---|---|---|---|---|---|---|
| 20 min control | 3 | 103 ± 7 | 9 | 44 ± 4 | 4 | 9 ± 0·1 | 0·8 |
| 20 *min* ACTH 15 I.U. | 3 | 139 ± 9† | 13 | 58 ± 10 | 5 | 9 ± 0·7 | 0·8 |
| 60 min control | 3 | 218 ± 7 | 20 | 41 ± 5 | 4 | 6 ± 0·3 | 0·5 |
| 60 min ACTH 15 I.U. | 3 | 372 ± 12† | 34 | 51 ± 8 | 5 | 6 ± 1·7 | 0·5 |

*Adrenal glands minces (150 μg) were incubated in 5 ml of Krebs–Ringer Bicarbonate buffer (with glucose) at pH 7·4 in an atmosphere of $O_2$:$CO_2$ (95:5 v/v) for 20 or 60 min. To each sample, 5 μCi of [1,2-$^3$H]-testosterone (S.A. 1 mCi/22·2 n moles) and 10 μg of unlabelled testosterone were added. All samples were preincubated during 60 min.

†Significative differences from controls ($P < 0·05$).

For statistical analysis a "t" test has been used.

## DISCUSSION

In the two experimental methods used: perfusion of isolated glands and incubation of minces, the adrenal gland of the dog metabolized the [$^3$H]-testosterone molecule to three principal metabolites: androstenedione; 11β-hydroxyandrostendione and 11β-hydroxytestosterone. Of these metabolites the latter appears to be the most important one, because of its high yield and the significant increases observed after ACTH administration. Some other radioactive peaks appear to be associated with it, but no attempts of identification were made. Adrenosterone was not synthetized in identifiable amounts. The metabolism of testosterone by the adrenal gland has been studied by other authors. Hydroxylation at C-11 has been reported after incubation with beef adrenal homogenates[15], human adrenal homogenate[1] and human adrenal cortex slices[16]. The 11β-hydroxylation of 17α-methyl testosterone by adrenal tissue of the rat has also been reported[17]. The same authors found a suppression of corticosteroidogenesis by 17α-methyl testosterone.

In 1963 Sharma *et al.*[18] described an *in vitro* inhibition of 11β-hydroxylation of desoxycorticosterone by the competitive action of androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and testosterone. The effect of testosterone was more pronounced than that of the other two compounds.

Recent reports have indicated that besides its action as a competitor of 11β-hydroxylation, the testosterone molecule has some additional effects on the adrenal gland that could explain the diminution of corticosteroidogenesis after its administration. In the adrenal mitochondria of the rat treated with testosterone, a fall in the levels of cytochrome P 450 has been reported[19], and an inhibition

of protein synthesis in rat adrenal quarters incubated in the presence of testosterone was found.

Griffiths and Glick in 1965 [20] obtained an increase in 11$\beta$-hydroxylase activity at the fascicular reticular border in the rat adrenal gland after 3 h of ACTH treatment, and Kowal in 1969 [21] using monolayer cultures of an ACTH responsive cell line from transplantable mouse adrenal tumor found a twofold or greater increase in the 11$\beta$-hydroxylation of added [$^3$H]-pregnenolone or [$^3$H]-progesterone after having treated the culture with ACTH for 12–72 h.

In 1970, Laury and McCarthy [22] in rats treated with ACTH 1 h before sacrifice found elevated levels of *in vitro* 11$\beta$-hydroxylation by adrenal mitochondria in the presence of malate or succinate.

In our experimental conditions, a significant increase of the amount of 11$\beta$-hydroxytestosterone formed by adrenal glands was seen upon ACTH administration.

Lantos *et al.* [23] reported that adrenal slices from male rats incubated with labelled corticosterone produced a polar metabolite tentatively identified as 3$\alpha$,5$\alpha$-tetrahydrocorticosterone. This conversion was inhibited by the addition of ACTH *in vitro* with a corresponding conservation of corticosterone.

Adrenal 5$\alpha$-metabolite formation was reported to be stimulated after hypophysectomy and inhibited upon ACTH replacement *in vivo*. The net result of this ACTH action is a diminution of the catabolism of corticosterone.

We did not discard a similar action of the ACTH on the 11$\beta$-hydroxytestosterone molecule formed. The catabolism of 11$\beta$-hydroxytestosterone has not been studied in the present experiments.

After 1 h of perfusion the isolated adrenals were still responding to ACTH, as was demonstrated by the increase in corticoid secretion during the collection of the second ACTH sample. Vogt [24], using a similar preparation to ours, described that the adrenal gland was viable and reactive to ACTH after 90 min of perfusion.

Dexamethasone, administered to the dogs, increased the basal levels of corticoids detected by means of the Blue tetraxolium reaction, but did not mask the response to ACTH (see Table 3).

It is concluded that the adrenal gland of dogs preferentially metabolized testosterone to 11$\beta$-hydroxytestosterone and that this conversion is stimulated by ACTH administration.

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