QUANTITATIVE AND QUALITATIVE ANALYSIS OF THE CORTICOSTEROIDS
PRESENT IN THE PERIPHERAL PLASMA AND ADRENAL CORTEX OF
THE COLLARED PECCARY DICOTYLES TAJACU

by

James Peyton Hughes

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SIGNED: James P. Hughes

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

I. M. Lytle  6-20-74
Professor of Biological Sciences
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ABSTRACT

Cortisol was isolated and identified as the principal corticosteroid present in the plasma of the male collared peccary (Dicotyles tajacu). Cortisone was tentatively characterized on the basis of Rf values in three separate solvent systems.

Cortisol, cortisone, and dehydrocorticosterone were identified in extracts of whole adrenal glands from the collared peccary. Two ultraviolet absorbing spots found on the thin-layer chromatoplate had Rf values corresponding to aldosterone and deoxycorticosterone, but these could not be confirmed using sulfuric acid chromogen spectra.

Deletion of water and base washes during partitioning, and the addition of more extensive thin-layer chromatographic techniques proved valuable to corticosteroid recovery and purification. Quantitative analyses of purified cortisol were performed with colorimetric techniques.
INTRODUCTION

Taxonomically, the peccaries are placed with the domestic pigs, hippopotami, and extinct giant hogs as a branch of the order Artiodactyla. In this offshoot or branch, the domestic pigs of the family Suidae form the only group readily accessible for steroid studies; therefore, it has previously been the only group studied. Cortisol, corticosterone, and small amounts of aldosterone were identified as the endogenous corticosteroids (Gorbman and Bern 1962, Dvorak 1967). Comparative studies are available only from the more distant representatives of the order Artiodactyla such as cattle and sheep. Estergreen and Venkataseshu (1967) reported cortisol and corticosterone to be the major products of bovine corticosteroid synthesis. Minute quantities of dehydrocorticosterone and aldosterone have also been reported (Gorbman and Bern 1962). Sheep are predominantly cortisol secretors, but they also synthesize identifiable amounts of cortisone and corticosterone (Chester Jones et al. 1964, Saba 1964).

This study offers an efficient method for corticosteroid isolation, and utilizes this method to present qualitative and quantitative analyses of the corticosteroids in the peripheral plasma of the collared peccary (Dicotyles
This isolation procedure was also used to identify products in extracts of whole adrenal glands. From these analyses, a useful framework of endocrine comparisons was initiated within the "pig branch" of the order Artiodactyla. Hypotheses were postulated to delineate possible biochemical pathways and associated enzyme systems. Comparisons to more distantly related families within the order were utilized only to illustrate major similarities and dissimilarities.
MATERIALS AND METHODS

Solvents

Volatile analytical grade reagents were distilled before use, while water was both distilled and demineralized. Spectro-quality dichloromethane was used without further purification. Steroids used as reference standards were checked for homogeneity on thin-layer chromatoplates. All remaining chemicals were analytical reagent quality, and were used in the original form.

Animals

Males peccaries (Dicotyles tajacu) were obtained from the Arizona Cooperative Wildlife Research Unit of The University of Arizona courtesy of Dr. L. K. Sowls and Dr. N. S. Smith. The animals were anesthetized with chloroform, and blood was withdrawn by cardiac puncture. Within one hour of collection, the plasma was separated from the cell fraction by centrifuging the whole blood at 15,000 RPM for ten minutes. The plasma was stored at minus ten degrees centigrade for later assay.

Plasma Extraction

One milliliter of 1N HCl was added for each 25 milliliters of plasma, and the mixture was shaken gently. The
plasma, cooled to ten degrees centigrade, was extracted five times using two volumes of dichloromethane cooled to zero degrees centigrade. Cold dichloromethane as suggested by Truscott and Idler (1972) prevented some emulsions without sacrificing recovery. Extractions were performed by shaking the separatory funnel containing the medium for five minutes. All emulsions were centrifuged at 15,000 RPM for five minutes. The combined dichloromethane phases were taken to dryness in vacuo at 38 degrees centigrade.

**Adrenal Extraction**

Whole adrenal glands were cleaned of adhering connective tissue, and were weighed to the nearest hundredth of a gram. These glands were minced and then homogenized with the addition of ten milliliters of water per gram of tissue. The homogenate, cooled to ten degrees centigrade, was extracted five times using two volumes of dichloromethane cooled to zero degrees centigrade. Extractions were performed by shaking the medium in a separatory funnel for five minutes. All emulsions were centrifuged at 15,000 RPM for five minutes. The combined dichloromethane phases were taken to dryness in vacuo at 38 degrees centigrade.

**Solvent Partitioning**

The residue from the primary extraction was taken up in 25 milliliters of 70% MeOH, and was partitioned twice.
against an equal volume of hexane. This partitioning was utilized by Idler, Ronald, and Schmidt (1959) to remove fats and other less polar material. Partitioning was performed by continuously inverting a separatory funnel containing the medium for one minute. The washed 70% MeOH was saved while the hexane from the first partitioning was backwashed with a second 25 milliliter aliquot of 70% MeOH. This first hexane phase was discarded, and the MeOH backwash was partitioned against a new 25 milliliter aliquot of hexane before being combined with the first MeOH fraction. Hexane phase number two was treated in the same manner as the previous hexane phase producing a third 70% MeOH fraction. This final fraction was partitioned against 25 milliliters of hexane, and was combined with the first two fractions. All 70% MeOH fractions were taken to dryness in vacuo at 60 degrees centigrade. Pressure must be reduced to 150 torr to remove the aqueous phase.

**Centrifugation**

The semi-washed residue was taken up in five milliliters of absolute EtOH, and was transferred to a conical tube. This tube was centrifuged at low speed for five minutes to remove denatured proteins and other insoluble impurities. A second conical tube was provided for the supernatant. Two more EtOH aliquots were used in an identical manner to extract the residue from the drying flask resulting
in a final supernatant volume of fifteen milliliters. The supernatant phases were reduced to 100 microliters in vacuo at 60 degrees centigrade using a rotary evaporator.

**Preliminary Chromatography**

Chromatoplates (20 x 20 cm) were coated with a thin layer (300 μ) of Silica Gel G. The uniformity of application was determined by holding the plates up to a bright light. With a 100 microliter pipet, the supernatant and 100 microliters of EtOH washings were applied to the chromatoplate as a seven centimeter band. This band was chromatographed for a distance of six centimeters utilizing EtOAc as a solvent. Reference standards were chromatographed alongside the band as markers for the positions of suspected steroids. Short ultraviolet light with a peak wavelength of 234 millimicrons was used to illuminate alpha, beta-unsaturated ketosteroids as suggested in a report by Lisboa (1963).

**Elution**

Matthews, Pereda, and Aguilera (1962) described a method for removing unknowns from silica gel chromatoplates by using a zone extractor and vacuum pump suction. This method was utilized to remove the entire area between the original band and the front running lipids on preliminary chromatoplates. The collector was eluted twice with two milliliter aliquots of absolute EtOH. The solvent was
allowed to pass through the collector under the influence of gravity without any assistance from pump suction. In a rotary evaporator, the EtOH was reduced to 50 microliters in vacuo at a temperature of 60 degrees centigrade.

**Final Chromatography**

The 50 microliter eluate plus a 50 microliter EtOH wash were plated as a spot approximately 0.5 centimeters in diameter. In a report by Bennett and Heftmann (1962), the solvent system CHCl₃-MeOH-H₂O (90:10:1) was deemed suitable for separating cortisol and cortisone. The separation was ample, but the two corticosteroids tended to diffuse to a point unacceptable for quantitation. In this study, cortisol and cortisone traveled a shorter distance, diffused less, and maintained individual resolution when chromatographed for eight centimeters in the solvent system CHCl₃-MeOH-H₂O (180:10:1). After air drying under a hood, the plate was chromatographed for eight centimeters in the solvent system EtOAc-CHCl₃-H₂O (90:10:1) which was reported by Bennett and Heftman (1962). This second system was chromatographed perpendicularly to the first.

**Identification**

Preliminary identification of unknowns relied on their ability to run with standards in three separate solvent systems: CHCl₃-MeOH-H₂O (180:10:1), EtOAc-CHCl₃-H₂O (90:10:1), and cyclohexane-EtOAc-toluene (10:10:1). In cases where
unknowns could be isolated in quantities approaching ten micrograms, final confirmation depended on a procedure developed by Zaffaroni (1950) in which steroids were characterized on the basis of sulfuric acid chromogen spectra. Isolated unknowns were residues from two milliliter eluates of chromatoplate spots, and blanks were residues from two milliliters of absolute MeOH.

**Assay**

Unknown steroids were located with short wave ultraviolet light with a peak wavelength at 254 millimicrons. This method was sensitive to approximately 0.5 micrograms per square centimeter. Due to its limited quantity in the peripheral plasma, cortisone was estimated immediately by the darkness of its spot on the final chromatoplate. Cortisol and a suitable blank were removed from the thin-layer chromatoplate as 1.5 centimeter squares using the method of Matthews et al. (1962). Each collector was eluted twice with one milliliter aliquots of absolute MeOH. Each eluate was combined with 0.5 milliliters of 2,4-dinitrophenylhydrazine solution as suggested in a method reported by Gornall and MacDonald (1952). The sample tubes were incubated for 90 minutes in a water bath adjusted to 59 ± 2 degrees centigrade. After incubating for 90 minutes, the tubes were removed from the water bath and were cooled to room temperature. A volume of 0.5 milliliters of 4N NaOH was added to each tube, and the
reaction was allowed 30 minutes at room temperature to develop full color. Samples were read at 475 millimicrons on a Beckman DB spectrophotometer utilizing 0.5 milliliter microcells. Absorbance was compared to a standard curve prepared from twenty observations ranging from 1 to 20 micrograms of a cortisol standard. The regression line for the observations was $Y = 0.037X + 0.042$ where $Y$ equals the absorbance at 475 millimicrons, and $X$ equals the micrograms of cortisol corresponding to that absorbance. The coefficient of correlation was 0.9997.
RESULTS

Extraction and Recovery

Previous research (Bush 1952, Silber and Porter 1954, Zenker and Bernstein 1958, deRoos 1961, Benraad and Kloppenberg 1964) has suggested the use of water and base washes to purify primary tissue extracts. In preliminary experiments employing these washes, the mean recovery rate of cortisol was less than 60 percent while final experiments without aqueous washes demonstrated a cortisol recovery rate of 77.3 percent. This seventeen percent deficit in cortisol recovery forced a permanent removal of water and base washes from extraction procedures. Idler et al. (1959) suggested an ether extraction of the aqueous medium resulting from hexane-70% methanol partitioning as a means to recover the corticosteroids. This extraction step was both time-consuming and unnecessary since the 60 degree centigrade temperature required to evaporate the aqueous medium did not appear detrimental to steroid structure. Chemical alteration could not be demonstrated in control experiments even when corticosteroids were subjected to temperatures of 75 degrees centigrade. A lack of structural alteration was inferred from the similarity of migration between heated and unheated standards when chromatographed on thin-layer plates.
Due to the limited supply of plasma from the experimental animals, standard recoveries (see Table 1) were calculated with human plasma donated by the Veteran's Administration Hospital. Reliance on values derived from human plasma could lead to considerable error so one experiment involving "percent recovery" was performed entirely with some extra plasma from the first experimental animal. Plasma cortisol from this animal was determined to be 6.1 micrograms per 100 milliliters. In Table 2 this value has been corrected for "percent recovery" and reads as 7.9 micrograms per 100 milliliters of plasma. Assay of a 100 milliliter aliquot of plasma to which 15 micrograms of cortisol had been added rendered a value of 16.9 micrograms of cortisol. Subtraction of the endogenous amount of 6.1 micrograms from 16.9 micrograms, and division of the resultant figure by the 15 micrograms of cortisol originally added leads to the recovery figure of 72.0 percent.

Peripheral Plasma of *Dicotyles Tajacu*

Cortisol proved to be the major corticosteroid in the peripheral plasma of male peccaries. Assays of 100 milliliter aliquots of plasma from five separate animals, ranging in age from three to five years, produced values of 5.8 to 10.7 micrograms of cortisol. The mean value was 7.5 micrograms of cortisol per 100 milliliters of plasma. Cortisone, tentatively identified on the basis of Rf values in three
TABLE 1. Recovery of Cortisol from Human Plasma

<table>
<thead>
<tr>
<th>Experiment</th>
<th>µg Cortisol Recovered</th>
<th>Mean Recovery</th>
<th>Standard Deviation</th>
</tr>
</thead>
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<tr>
<td>Control plasma without the addition of exogenous cortisol</td>
<td>-2.7 -2.8 -2.6</td>
<td>2.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Control plasma with the addition of 15 µg of cortisol</td>
<td>12.1 12.9 12.7 13.9</td>
<td>14.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>
separate solvent systems, appeared as a barely visible spot when the final chromatoplate was viewed with short wave ultraviolet light. This spot was produced by each of the five experimental animals. Table 2 summarizes the results of the five assays.

Cortisol was partially identified on the basis of Rf values in each of the following solvents: chloroform-methanol-water (180:10:1), ethyl acetate-chloroform-water (90:10:1), and cyclohexane-ethyl acetate-toluene (10:10:1).

Conformation depended on the correlation between sulfuric acid chromogen spectra of a cortisol standard, and that of the unknown (Figure 1). Absorbance was read every five millimicrons between 220 and 600 millimicrons. The unknown demonstrated absorption maxima (240, 285, 390 and 475 μm) which were identical to the cortisol standard treated in an identical manner. For a final identification, a methanol eluate of the unknown was read every two millimicrons between 220 and 320 millimicrons. The unknown revealed an absorption maximum at 242 millimicrons (Figure 2) which corresponded to a cortisol standard.

**Whole Adrenal Glands of Dicotyles Tajacu**

Extracts of 10.63 grams of whole adrenal glands were subjected to thin-layer chromatography for final isolation and preliminary identification. Nine ultraviolet absorbing spots were discernable on the chromatoplate. Five of the
TABLE 2. Cortisol and Cortisone in the Peripheral Plasma of *Dicotyles tajacu*

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age (years)</th>
<th>Date of Collection</th>
<th>Cortisol (μg/100 ml plasma) Corrected for % Recovery</th>
<th>Cortisone</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10/4/73</td>
<td>7.9</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>10/4/73</td>
<td>6.1</td>
<td>+</td>
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<tr>
<td>3</td>
<td>5</td>
<td>10/12/73</td>
<td>10.7</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2/1/74</td>
<td>5.8</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2/1/74</td>
<td>7.0</td>
<td>+</td>
</tr>
</tbody>
</table>

* = Steroid identified but not quantified.
Figure 1. Sulfuric Acid Spectra of a Cortisol Standard and of Material from Peripheral Plasma of *Dicotyles tajacu*.
Figure 2. Ultraviolet Spectra of a Cortisol Standard and of Material from Peripheral Plasma of *Dicotyles tajacu*. 
spots corresponded to cortisol, cortisone, dehydrocortico-
sterone, deoxycorticosterone, and aldosterone standards when
chromatographed in the solvent systems: chloroform-methanol-
water (180:10:1), ethyl acetate-chloroform-water (90:10:1),
and cyclohexane-ethyl acetate-toluene (10:10:1). The four
additional ultraviolet absorbing spots were chromatographed
in the three solvent systems, but no positive identification
could be made. The corticosterone standard did not corre-
spond to any of the four spots. The relative strengths of
the various spots, judged by their spot darkness under ultra-
violet light, were as follows: cortisol > cortisone > de-
hydrocorticosterone and aldosterone > deoxycorticosterone >
four unidentified spots.

The five spots corresponding to known standards were
converted to sulfuric acid chromogens, and their spectra were
read every five millimicrons between 220 and 600 millimicrons.
The unknown identified as cortisol demonstrated absorption
maxima (240, 285, 390 and 475 mp) identical to those of a
cortisol standard (Figure 3). The spot identified as corti-
sone was divided into two separate sections before it was re-
moved from the chromatoplate. This was done because half of
the spot appeared to be slightly contaminated. Section num-
ber one produced maxima (285, 345 and 420 mp) which corre-
sponded to a standard cortisone (Figure 4) treated in an
identical manner. Section number two demonstrated maxima at
Figure 3. Sulfuric Acid Spectra of a Cortisol Standard and of Material from the Adrenal Glands of *Dicotyles tajacu*.
Figure 4. Sulfuric Acid Spectra of a Cortisone Standard and of Material from the Adrenal Glands of *Dicotyles tajacu*.

The two unknown curves represent two different sections of the same spot as removed from the chromatoplate.
285, 345 and 410 millimicrons. The contamination was assumed to be minor. Absorption maxima for the spot identified as dehydrocorticosterone (285, 355 and 415 mp) corresponded exactly with a standard dehydrocorticosterone treated in the same manner (Figure 5). Unknown spots identified as deoxycorticosterone and aldosterone did not correlate well with their respective standards when converted to sulfonic acid chromogens (Figures 6 and 7). Deoxycorticosterone produced a single peak at the correct absorption maximum, but contamination caused deviations between 310 and 350 millimicrons. Aldosterone, as with cortisone, was split into two sections before being removed from the chromatoplate. Section number one produced a single peak with an absorption maximum five wavelengths removed from the standard. Section number two demonstrated absorption maximum at 225, 268 and 285 millimicrons. The portions of both sections that remained on the chromatoplate were sprayed with a 35 percent aqueous solution of o-phosphoric acid according to a method described by Bolliger et al. (1965). After the chromatoplate was incubated for ten minutes at 120 degrees centigrade, the unknown fluoresced a brilliant green when viewed with long wave ultraviolet light. The green coloration was also characteristic of an aldosterone standard treated in an identical manner.
Figure 3. Sulfuric Acid Spectra of a Dehydrocorticosterone Standard and of Material from the Adrenal Glands of *Dicotyles tajacu*. 
Figure 6. Sulfuric Acid Spectra of a Deoxycorticosterone Standard and of Material from the Adrenal Glands of *Dicotyles tajacu*. 
Figure 7. Sulfuric Acid Spectra of an Aldosterone Standard and of Material from the Adrenal Glands of *Dicotyles tajacu*.

The two unknown curves represent two different sections of the same spot as removed from the chromatoplate.
DISCUSSION

Water and base washes suggested by previous research (Bush 1952, Silber and Porter 1954, Zenker and Bernstein 1958, deRoos 1961, Benraad and Kloppenburg 1964) were responsible for cortisol losses greater than 17 percent when incorporated into the preparatory experimental procedures of this study. Due to this low cortisol recovery, aqueous washes were removed from partitioning techniques before the final experiments were initiated. Resolution was not sacrificed to promote the higher "percent recovery" because aqueous washes accomplished little more than removal of gross contaminants. Hexane-70% methanol partitioning as reported by Idler et al. (1959) did not prove detrimental to recovery, and the purified extract could be applied directly to preliminary thin-layer chromatoplates following centrifugation. Preliminary thin-layer techniques, in which the steroids were applied as a band, served to eliminate both the front running nonpolar contaminants and the very polar materials which remained at the origin. This technique replaced the function of the aqueous washes and purified the extract to a far greater extent. The eluate from the preliminary chromatography could be applied to the final chromatoplate as a
single spot approximately 0.5 centimeters in diameter. A small spot was necessary for good resolution and final quantification.

The mean value of 7.5 micrograms of cortisol per 100 milliliters of plasma found in *Dicotyles tajacu* compared with the 5.5 micrograms of 17-hydroxycorticosteroids per 100 milliliters of plasma reported for domestic pigs (Dvorak 1967), and the 7.2 micrograms of cortisol per 100 milliliters of plasma reported for cows (Estergreen and Venkataseshu 1967). Seasonal variation could not be demonstrated within the sampling period of October through January (see Table 2), but this period did not include the heat stress encountered in the summer months. Purity of the isolated cortisol could only be inferred from its sulfuric acid chromogen spectrum (Figure 1) and its ultraviolet spectrum (Figure 2). Both spectra correlated well with those of a cortisol standard and suggested good resolution using thin-layer techniques. Tentative identification of cortisone in the peripheral plasma of peccaries differed from domestic pigs where Gorbman and Bern (1962) reported a minor role for cortisone in comparison to corticosterone. In the literature pertaining to Artiodactyla, only Saba's (1964) reports on sheep demonstrated an animal with a greater quantity of cortisone than corticosterone. The appearance of significant amounts of cortisone in the peccary suggests that the steroid is the result of an active 11-dehydrogenase enzyme system (Figure 8) in the
Figure 8. Flow Chart Depicting a Hypothetical Pathway for the Synthesis of 17-Hydroxycorticosteroids in *Dicotyles tajacu*.
adrenal cortex rather than a degrading enzyme system in the liver. Active 11-dehydrogenase enzymes have been more commonly reported in teleosts (Idler et al. 1959, Idler, Freeman and Truscott 1963, Nandi and Bern 1965).

Whole adrenal glands were homogenized and extracted for the purpose of qualitatively confirming cortisol and cortisone as the major corticosteroids present in *Dicotyles tajacu*. Sulfuric acid chromogen spectra (Figures 3 and 4) coupled with qualitative determinations of spot darkness on chromatoplates illuminated with ultraviolet light demonstrated the prominence of cortisol and secondarily that of cortisone. Corticosteroid not previously identified in plasma extracts were present in adrenal extracts. A positive identification of dehydrocorticosterone (Figure 5) added strength to the proposal that the adrenal cortex of peccaries possesses an active 11-dehydrogenase enzyme system (Figure 9). The tentative identification of deoxycorticosterone and aldosterone, using Rf values and sulfuric acid chromogen spectra (Figures 6 and 7), further suggests the presence of a pathway involving corticosterone (Figure 8).
Figure 9. Flow Chart Depicting a Hypothetical Pathway for the Synthesis of 17-Deoxycorticosteroids in *Dicotyles tajacu*. 
CONCLUSION

Contrary to an earlier report (Nandi and Bern 1965), corticosteroids can be quantified using silica gel eluates without sacrificing resolution or "percent recovery". Two procedural modifications were primarily responsible for the increased purification and recovery of extracted corticosteroids. Chromatoplates were coated with a thicker layer of silica gel (300 μ) to prevent excessive steroid adherence, and aqueous washes were replaced by a preliminary thin-layer chromatography to prevent loss and increase resolution.

*Dicotyles tajacu* is predominantly a cortisol secretor with a mean value of 7.5 micrograms per 100 milliliters of plasma. Cortisone is maintained in the plasma in quantities approximately twenty-fold less than those of cortisol, and thus plays a secondary role in glucocorticoid activity. Corticosteroid could not be identified in either the plasma or adrenal extracts, but positive identification of dehydrocorticosterone and tentative identification of both aldosterone and deoxycorticosterone as adrenal intermediates strongly suggest that corticosterone is present in peccaries. The presence of relatively large amounts of cortisone and dehydrocorticosterone indicates an active 11-dehydrogenase enzyme system in the adrenal cortex of peccaries which has not
been reported for domestic pigs. Oxidation of corticosterone in the eleven position to form dehydrocorticosterone, and hydroxylation of corticosterone in the eighteen position to form aldosterone could account for the minute quantities of corticosterone in the adrenal glands.

Due to the small quantities of corticosterone produced in the 17-deoxycorticosteroid pathway, its synthesis can not reasonably be considered in the main purpose of the pathway. Maintenance of the pathway is probably more closely related to the need for an effective mineralocorticoid such as aldosterone to prevent excessive sodium loss during the hot summer months. Further studies will be required to resolved this question.
LIST OF REFERENCES


