AN INVESTIGATION INTO THE OCCURRENCE OF ALDOSTERONE AND CORTICOSTERONE IN THE PLASMA OF *RANA CATESBEIANA*

EBER JANE SINE

August 19, 1966

To The Graduate Committee:

I am submitting a thesis written by Eber Jane Sine entitled "An Investigation into the Occurrence of Aldosterone and Corticosterone in the Plasma of Rana catesbeiana." I recommend that it be accepted for eight quarter hours credit in partial fulfillment of the requirements for the degree Master of Arts in Education, with a major in Biology.

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AN INVESTIGATION INTO THE OCCURRENCE OF ALDOSTERONE AND CORTICOSTERONE IN THE PLASMA OF *RANA CATESBEIANA*

An Abstract

Presented to

The Graduate Committee of

Austin Peay State College

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Education

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ABSTRACT

Double isotope dilution methods are described for the isolation and quantitation of aldosterone and corticosterone in the plasma of the Bullfrog, Rana catesbeiana.

The procedure employs descending paper chromatography and is accurate for measurement of small quantities of steroids. In this assay, the aldosterone level in R. catesbeiana plasma was found to be approximately .7 micrograms per cent, while the corticosterone level was approximately .5 micrograms per cent. These apparently high values may be attributable to high plasma ACTH levels as a result of stress prior to sacrificing the specimens.

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CHAPTER I

INTRODUCTION

Investigations of adrenocortical secretions have indicated a phylogenetic relationship of the biosynthetic pathways of these compounds in various vertebrates. Such is suggested for example by the identification of identical adrenocorticoids in Amphibia and Mammalia (Bern and Nandi, 1964; Jones et al., 1962; Jones et al., 1959; Phillips and Bellamy, 1963). However, qualitative and quantitative attempts to measure amphibian adrenocorticoid secretion have been limited in scope and involved difficult procedures. Most such attempts have been made within the last decade (Gorbman, 1964).

Procedures used in the identification of amphibian corticoid compounds have included interrenal incubation with the identification of hydroxylating enzymes (Hanke and Weber, 1964), labeling of corticoid precursors (Crabbe, 1963; Kraulis and Birmingham, 1964), and spectrometric and fluormetric analyses of urinary, plasma, and in vitro extracts (Caligaris and Astrada, 1963; Carstensen et al., 1961; Jones et al., 1959). The lack of microchemical techniques suitable for measuring minute quantities of hormones in the blood, and the small total blood volumes of the individual

specimens, have imposed limitations on previous studies.

The specific purpose of this investigation is to isolate, identify, and quantify aldosterone and corticosterone in the plasma of the Bullfrog, Rana catesbeiana. This animal was selected for this investigation on the basis of previous studies in which the principal adrenocorticoids had been identified in vitro (Macchi, 1956; Carstensen et al., 1961), and the availability and size of the species. The in vitro studies indicated the principal adrenocorticoids to be aldosterone and corticosterone (Carstensen et al., 1961), suggesting that these would also be the adrenocorticoids detectable in Rana catesbeiana plasma.

The biochemical techniques utilized in this investigation included the extraction and solvent-partitioning of fractions taken from large quantities of pooled plasma. Radioisotope double dilution and descending paper chromatography were employed in isolation, identification, and quantification of the compounds.

CHAPTER II

REVIEW OF THE LITERATURE

Macchi (1956) found that adrenocorticosteroids were present in *in vitro* media of adrenal incubates of *R. catesbeiana*, as shown by the presence of blue tetrazolium-reducing substances. In a similiar study, Carstensen *et al.* (1961) showed that aldosterone and corticosterone were the only Δ^4-3-ketosteroids produced in significant amounts. Furthermore, in the presence of purified adrenocorticotrophic hormone (ACTH), the ratio of aldosterone to corticosterone was approximately 4:1. This was the first animal in which aldosterone was demonstrated to be the principal adrenocorticosteroid by *in vitro* studies.

Other species of Ranidae in which positive identifications of adrenocorticoids have been made are the larval forms of *R. sylvatica* and *R. pipiens* (cortisol and possibly corticosterone), (Dale, 1962). *In vitro* studies of *R. pipiens* have demonstrated the presence of corticosterone and 18-OH-deoxycorticosterone (Macchi, 1956).

On the basis of this data, and the availability of the species and size of the specimens, a study was designed to detect and measure the major adrenocorticoids (specifically aldosterone and corticosterone) in the plasma of *R. catesbeiana*.

CHAPTER III

METHODS AND MATERIALS

Terminology

Because of the vast amount of hormone research that has been conducted on mammals by workers in different countries, and the sometimes poor communication existing between these workers, as many as three or four names for the same compounds have evolved in some cases. Names most widely accepted in the United States, such as cortisol and Kendall's letter compounds, as designated in The Chemistry of the Steroids (Klyne, 1960), have been used in this thesis. Groups of steroid compounds, pituitary secretory products, and steroid-acting drugs are referred to as designated by Klyne (ibid.), and Williams (1962).

Terminology used in biochemical techniques is as designated by Williams (ibid.), and Sunderman and Sunderman (1960).

Classification and identification of species is according to Conant's A Field Guide to Reptiles and Amphibians (1950).

For synonymy of steroid terminology, see the Appendix.

Animals

Male and female Bullfrogs, R. catesbeiana, were

collected from ponds in Houston, Stewart and Montgomery Counties, Tennessee. Additional specimens from the Louisiana coastal plain were obtained through Carolina Biological Supply Company. These latter animals were considerably larger than the specimens taken in Tennessee and differed further in being very melanistic and heavily patterned with dark brown blotches. Individual weights of the Tennessee animals ranged from 72 to 379.5 grams. It was not possible to obtain meaningful weights for the Louisiana animals because they had been force-fed, and they were sacrificed as soon as possible to prevent dehydration which would have resulted in a possible accompanying decrease in total blood volume. However, all Louisiana animals were estimated to weigh four hundred grams or more and to have been six inches or more in snout to vent length.

Collection and Preliminary Treatment of Blood

All specimens were anesthized with ethyl ether and the total peripheral blood volume collected by decapitation and drainage of peripheral blood into centrifuge tubes containing heparin as an anticoagulant. A large animal yielded approximately five milliliters (ml.) of whole blood or three ml. of plasma. All specimens combined yielded a total of 190 ml. of plasma, which was stored at -60° centrigrade until analysis was made.

Double Isotope Dilution Method

The method of measuring aldosterone in this investigation approximates Peterson's (1964) method for peripheral plasma in man, and the method used by Island at Vanderbilt University (personal communication), in the measurement of the 3-oxo-conjugate urinary metabolite of aldosterone. Essentially, the method is one of double isotope dilution involving solvent-partitioning and extraction and descending chromatography.

This procedural method was tested by a trial run using 100 ml. of human plasma to determine running rates in chromatography systems and the approximate amount of H^3 (tritiated) compounds to be added. The recovery rate was approximately ten per cent for aldosterone.

In a double isotope dilution method a known amount of the same compound that is to be measured may be added in the tritiated form and the D ring acetylated with C^{14}. In this procedure a known amount of D-aldosterone-1,2-H^3 was added to the sample mixture and the D ring acetylated at the 21 position with acetic anhydride-1-C-12. Changing the compound from monoacetate to diacetate and back to monoacetate form was instrumental in purification of the compound (Figure I).

The method employed in purification of free cortico-

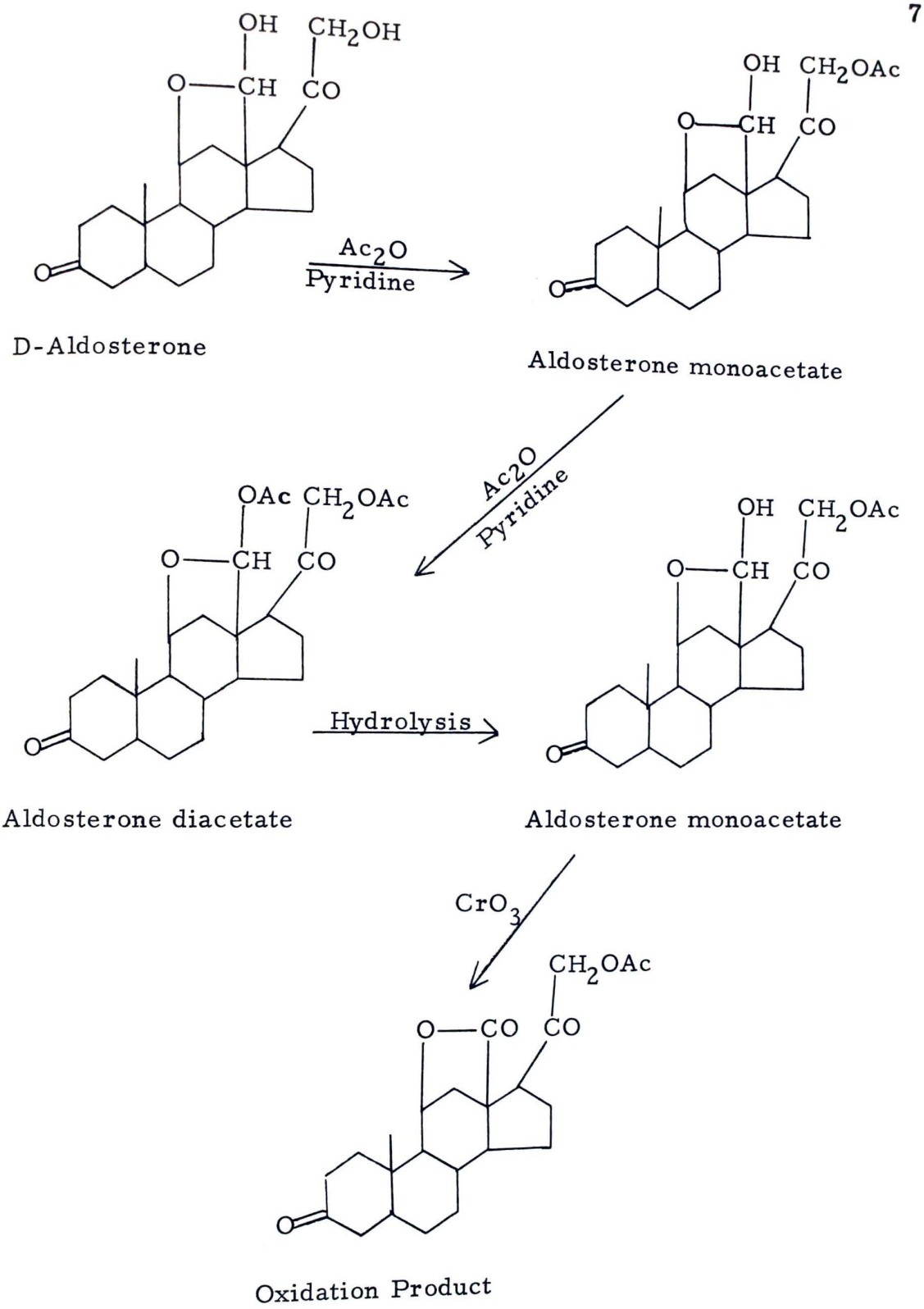

Figure 1. Characterization of Aldosterone.

sterone was also tested by a trial run with human plasma. It was difficult to obtain a pure compound due to acetylation of impurities, and four chromatography systems beyond acetylation were found necessary.

The reference standards used in this investigation are listed in the Appendix.

Before fractionation, the total plasma volume (190 ml.) was pre-extracted twice with four volumes of n-hexane. The hexane was discarded and the remaining plasma volume carefully measured. This plasma (185 ml.) was extracted twice with four volumes of carbon tetrachloride (hereafter designated as Fraction II). The plasma volume was again measured (177 ml.) and extracted with six volumes of dichloromethane (hereafter designated as Fraction I).

Fraction I

Fraction I contained the aldosterone and was analyzed by the following steps:

Addition of isotope. Approximately twenty-six thousand counts per minute (cpm.) of D-aldosterone-1,2-H^3 were added to this fraction (1,082 ml.). A duplicate aliquot was dried in a vial for scintillation counting.

Washing. The fraction was washed first with one-tenth

volume of .1N sodium hydroxide, then one-tenth volume of distilled water, and finally with one-tenth volume of one-tenth per cent acetic acid. The remaining volume (1,032 ml.) was then evaporated to dryness with a Buchler Flash Evaporator (temperature maintained below 40° centrigrade).

Chromatography of D-aldosterone-1,2-H^3 and free aldosterone in System I. The dried fraction was eluted with approximately three ml. of water-washed dichloromethane, transferred to a disposable tube, and dried under a dry air stream in a hood. The dried material was eluted with three drops of redistilled (100 per cent) ethanol and six drops of water-washed dichloromethane and applied to Whatman #3 MM chromatographic paper with Compound E (cortisone) and Compound F (cortisol) side standards. The paper was allowed to equilibrate for two hours and then developed in System I (Table I) for five hours. The sample strip was scanned with a Vanguard Automatic Chromatogram Scanner and the area between E and F eluted at the radioactive peak with methanol (100 per cent), (one ml. per square centimeter). The insolubles were centrifuged and the methanol dried in a ground-glass stoppered tube under dry air stream. The tube was placed in a calcium chloride desicator overnight to insure complete dryness.

TABLE I

PAPER CHROMATOGRAPHY SYSTEMS USED IN ASSAY OF PERIPHERAL PLASMA LEVEL OF ALDOSTERONE AND CORTICOSTERONE

System Number	Components of System (v:v ratios)
System I[a]	benzene: methanol: water (4:2:1)
System IIR,S[a]	cyclohexane: benzene: methanol: water (4:2:4:1)
System E-IV[b]	iso-octane: t-butanol: methanol: water (12:5:3:2)
System E2R[b]	iso-octane: t-butanol: water (500:250:400)
System CDMW[c]	cyclohexane: dioxane: methanol: water (10:5:10:1)
System P-20[d]	cyclohexane: benzene: methanol: water (5:10:10:2)
System P-21[d]	cyclohexane: benzene: methanol: water (2:2:2:1)

a. Bush Systems (Bush, 1961)

b. Eberlein Systems (Bush, 1961)

c. Donald Island, Vanderbilt University (personal correcpondence)

d. Ralph E. Peterson, Cornell University (personal correspondence)

Acetylation. Acetic anhydride-C^{14} (concentration of three microcuries per millimole, twenty per cent in benzene) was used to acetylate the steroid. Redistilled pyridine (0.05 ml.) and acetic anhydride-C^{14} (0.03 ml.) were added to the dry tube in a nitrogen atmosphere. The top was sealed and taped and the reaction allowed to proceed at room temperature for four hours. Simultaneously, fifty micrograms of cortisol were acetylated with the sample to determine the specific activity of the acetic anhydride-C^{14}. Acetylation was stopped by the addition of one ml. of twenty per cent ethanol. The ethanol was extracted into ten ml. of dichloromethane, and washed twice with one-half ml. of distilled water. The dichloromethane was then dried under a dry air stream in a ground-glass stoppered tube.

Hydrolysis. Ninety-five per cent ethanol (0.1 ml.) and 0.5 ml. of an acid mixture (.222M hydrochloric acid, .053M potassium chloride in water) were added to the dry tube. Care was taken to elute all dried material from the sides of the tube and the reaction allowed to proceed for thirty minutes at room temperature. The reaction was stopped by the addition of one-half ml. of distilled water, which was extracted with ten ml. of dichloromethane. The dichloromethane was washed twice with one-half ml. of

distilled water, transferred to a disposable tube and dried under a dry air stream.

Chromatography of Aldosterone Monoacetate in System II-S. The sample was eluted with three drops of redistilled ethanol and six drops of dichloromethane and applied to Whatman #2 paper with aldosterone-21-acetate internal standard and 17α OH,11-ketoprogesterone (HKP) side standard, and developed eighteen hours in System II-S (Table I). The sample strip was scanned and the ultraviolet spot and radioactive peak area eluted with methanol. The methanol was evaporated in a long ground-glass stoppered tube and later placed in the calcium chloride desicator for one hour to insure complete dryness.

Reacetylation. Redistilled pyridine (0.2 ml.) and redistilled acetic anhydride (0.1 ml.) were then added to the dry tube. The reaction was allowed to proceed sixteen hours at 30^{o} centrigrade. Acetylation was stopped by the addition of one ml. of twenty per cent ethanol. The ethanol was extracted into fifteen ml. of dichloromethane. The dichloromethane was washed twice with one-tenth volume of water and the sample volume concentrated by partial drying. The sample was then transferred to a disposable pointed-bottom tube and completely dried under a dry air stream.

Chromatography of Aldosterone Diacetate in System II-R. The sample was eluted as previously with ethanol-dichloromethane and applied to Whatman #1 paper with aldosterone diacetate as an internal standard and adrenosterone as a side standard. The paper was allowed to equilibrate for one hour and then developed approximately six hours in System II-R (Table I). The sample was scanned and isolated by radioactive peak and ultraviolet light and then eluted with methanol and dried in a ground-glass stoppered tube.

Hydrolysis. An ethanol-acid hydrolysis was carried out as before (see page 11).

Chromatography of Aldosterone Monoacetate in System P-21. The sample was applied to Whatman #1 paper with ethanol-dichloromethane using an aldosterone monoacetate internal standard and 17αOH,11-ketoprogesterone as a side standard. The paper was equilibrated for one hour and allowed to develop approximately five hours in System P-21 (Table I). The sample was scanned and isolated by radioactive peak and ultraviolet light and then eluted with methanol (three ml.). One-fifth of the total volume (0.6 ml.) was removed for counting and dried in a scintillator counting vial. The remaining amount was dried in a ground-glass stoppered tube under a dry air stream.

Oxidation. Chromium trioxide (one microgram per ml.) in redistilled glacial acetic acid (0.25 ml.) was added to the dried sample. The reaction was allowed to proceed for five minutes at room temperature, then stopped by the addition of one-half ml. of twenty per cent ethanol, and extracted into ten ml. of dichloromethane. The dichloromethane was washed twice with one-half ml. of distilled water and dried under a dry air stream.

Chromatography in System E2R. The sample was eluted with ethanol-dichloromethane and applied to Whatman #1 paper with cortisol side standard. The paper was equilibrated for one hour and allowed to develop six hours in System E2R (Table I). The sample was scanned and isolated by radioactive peak and ultraviolet spot and then eluted with ethanol (ninety-five per cent). One-half of the total volume was dried in a counting vial.

Counting. Seven milliliters of scintillator counting solution were added to each dried aliquot of sample to be counted. Ten minute counts for H^3 and C^{14} were then made in a Tri-Carb Liquid Scintillator Counter.

Fraction II

The carbon tetrachloride fraction contained the

corticosterone and was analyzed by the following steps:

Addition of isotope. Approximately twelve thousand counts per minute of corticosterone-H^3 were added to this fraction. A duplicate aliquot was dried in a vial for scintillation counting.

Washing. The fraction was washed first with one-tenth volume of .1N sodium hydroxide, then one-tenth volume of distilled water, and finally with one-tenth volume of one-tenth per cent acetic acid. The total volume (fifteen hundred ml.) was then evaporated to dryness.

Chromatography of Corticosterone-H^3 in System P-20. The dried fraction was eluted with water-washed dichloromethane, transferred to a disposable tube, and dried under a dry air stream. The dried material was eluted with ethanol-dichloromethane and applied to Whatman #2 paper with corticosterone and tetrahydrocorticosterone side standards. The paper was allowed to equilibrate for one hour and then developed in System P-20 (Table I) for four hours. The sample strip was scanned and the radioactive peak eluted with methanol. The methanol was dried in a ground-glass stoppered tube and then desicated for six hours to insure complete dryness.

Acetylation. The sample was acetylated by the addition of redistilled pyridine (0.05 ml.) and acetic anhydride-C^{14} (0.03 ml.) in a nitrogen atmosphere. The top was sealed and the reaction allowed to proceed twenty-four hours at 30^{o} centrigrade. Acetylation was stopped by the addition of one ml. of twenty per cent ethanol. The ethanol was extracted into ten ml. of dichloromethane, and washed twice with one-half ml. of distilled water. The dichloromethane was then dried under a dry air stream in a disposable tube.

Chromatography Systems After Acetylation. Purification of the acetylated compound was obtained by applying the sample to Whatman #2 paper with corticosterone acetate side and internal standards and developing the paper in the following sequence of systems (Table I); CDMW (twelve hours), E-IV (five hours), CDMW (eleven hours), and E-IV (six and one-half hours).

Counting. Aliquots of the sample were taken for counting following each of the above four systems. Only data from the last two systems were used in calculations.

Specific Activity of Acetic Anhydride-1-C-14

Standard cortisol (fifty micrograms) was dried in a

ground-glass stoppered tube and acetylated as the samples at room temperature for twenty-four hours. The cortisol acetate was then applied to Whatman#2 paper with cortisol acetate side standard and allowed to develop for eighteen hours in System II-R. The cortisol acetate was easily seen under ultraviolet light (245 microns) and this spot was eluted and dried. The cortisol acetate was then applied to Whatman #2 paper and developed in System E-IV for fourteen hours. The ultraviolet spot was eluted with methanol and dried. The dried material was eluted with four ml. of ethanol and aliquots of 0.5 ml. and 1.0 ml. were removed for a Porter-Silber determination in order to determine micrograms per cubic centimeter (μg/cc), and 0.05 ml. and 0.10 ml. dried in counting vials in order to determine counts per minute per microgram (cpm/μg).

CHAPTER IV

RESULTS

The H^3 and C^{14} were counted in a Tri-Carb Liquid Scintillation Counter. Its efficiency for C^{14} was determined to be .2678 and for H^3, .1196. The sample crossover was calculated to be .4702.

The plasma aldosterone and corticosterone concentrations were calculated as follows (modified from Peterson, 1964):

$$\frac{C}{H_o - (z \times C)} \times \frac{H_i}{S_c} \times \frac{100 \text{ ml.}}{\text{vol. plasma extracted}} = \mu g/100 \text{ ml.}$$

C = counts per minute C^{14} in counting vial

H_i = counts per minute H^3 added to sample

H_o = counts per minute H^3 in counting vial

S_c = specific activity of cortisol monoacetate in counts per minute C^{14} per microgram

z = correction factor for crossover

The specific activity of cortisol monoacetate was found to be 1,755 cpm/µg. Calculation of aldosterone level in R. catesbeiana plasma after multiple purification is as follows:

After Chromatography System P-21:

$$\frac{128}{1,477 - (.4702 \times 128)} \times \frac{26,082}{1,755} \times \frac{100}{177} = .759 \text{ µg/100 ml.}$$

After Chromatography System E2R:

$$\frac{50}{624 - (.4702 \times 50)} \times \frac{26,082}{1,755} \times \frac{100}{177} = .699 \text{ µg/100 ml.}$$

Calculation of corticosterone in R. catesbeiana plasma after multiple purification is shown below.

Third Chromatography System After Acetylation:

$$\frac{38}{197 - (.4702 \times 38)} \times \frac{11,361}{1,755} \times \frac{100}{185} = .742 \text{ µg/100 ml.}$$

Fourth Chromatography System After Acetylation:

$$\frac{19}{148 - (.4702 \times 19)} \times \frac{11,361}{1,755} \times \frac{100}{185} = .478 \text{ µg/100 ml.}$$

CHAPTER V

DISCUSSION AND CONCLUSIONS

Evaluation of Procedure

Fraction I. The procedure devised for measurement of the aldosterone in Fraction I is adequate and accurate for minute quantities. In relation to reference side and internal standards, the chromatography systems used were very effective in the separation and purification of the free aldosterone. The overall recovery of the D-aldosterone-1,2-H^3 indicator, from its addition to Fraction I through elution from the final chromatogram as aldosterone monoacetate was approximately three per cent.

The accuracies of the above methods depend on the purity of the labeled compound added, the ratio of the amount of labeled compound to unlabeled compound, and the precision of laboratory technique. The ratio of the amount of labeled to unlabeled compound was small and thus did not contribute a significant error to the method. Calculations of counts per minute to add were based on assumed plasma aldosterone and corticosterone concentrations of less than one microgram per 100 ml. The purity of the added compound was known to be greater than ninety-five per cent.

Fraction II. The procedure employed in the measurement of corticosterone was not as adequate as that used for aldosterone. The first elution from the dried carbon tetrachloride fraction would have been more efficient had ethanol-dichloromethane been used instead of dichloromethane. The acetylation with C^{14} was satisfactory, but a large amount of impurities was also acetylated, thereby requiring more chromotography systems, which compounded the loss of added H^3 compound. An alternate procedure could have included an oxidation of the compound to 11-dehydrocorticosterone after development in the cyclohexane: dioxane: methanol: water system (CDMW).

Conclusions Based on Values Obtained

These results demonstrate free aldosterone and free corticosterone to be present in measureable amounts in peripheral plasma of R. catesbeiana.

Furthermore, the plasma collected from these animals probably had a high ACTH level due to the stressful situation under which the animals were maintained prior to sacrificing, thus accounting for the apparently high values of aldosterone and corticosterone obtained. Such a conclusion is in agreement with Cartensen's (1961) in vitro work in which a fifteen-fold increase in aldosterone was shown in response

to the addition of 13.3 international units of ACTH to 1,218 milligrams of adrenal incubate.

In mammals, ACTH stimulates the secretion of aldosterone from the zona glomerulosa of the adrenal, although to a minor extent. In R. catesbeiana, there is no zonation of the adrenal cortex (Jones, 1959). This suggests a significant difference in steroid biosynthetic pathways between mammals and R. catesbeiana.

One possible explanation could be the lack of a 17α-hydroxylating enzyme in R. catesbeiana adrenals. Carstensen (1961) states:

> It is reasonable to assume that the 18-hydroxylating reaction proceeds relatively faster in relation to the other hydroxylating reactions in the amphibian adrenal than in the mammalian, so that some of the hydroxylating reactions which require TPNH as a co-factor become rate-limiting, since it has been shown that one of the functions of ACTH as it acts on the adrenal is the generation of TPNH.

CHAPTER VI

SUMMARY

A double isotope dilution method for the determination of aldosterone and corticosterone in R. catesbeiana peripheral plasma is described. The method is accurate for the measurement of even small amounts of these steroids.

Both free aldosterone and free corticosterone were shown to be present in measureable amounts in R. catesbeiana peripheral plasma. The apparent high levels may be attributable to high plasma ACTH levels due to the stress to which the animals were subjected prior to sacrificing.

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APPENDIX

APPENDIX

Nomenclatural Synonymy

Compound Name	Synonyms
17αOH, 11-ketoprogesterone *	HKP
Cortisol *	Kendall's Compound F
Cortisone *	Kendall's Compound E
Corticosterone *	
Corticosterone acetate *	
Aldosterone, 21-acetate *	
Aldosterone Diacetate *	
Adrenosterone *	
Aldosterone monoacetate *	
Tetrahydrocorticosterone *	
Tritiated Aldosterone	$AldoH^3$
Tritiated Corticosterone	BH^3
Adrenocorticotrophin Hormone	ACTH
18-OH-Deoxycorticosterone	

* Used as reference standards.