

# Metabolism and Rate of Secretion of Aldosterone in the Bullfrog

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**ABSTRACT** A study of the metabolism of aldosterone in the bullfrog was undertaken to provide a measurable metabolite for the indirect isotope dilution technique for measuring secretory rates. The rate of excretion of labeled aldosterone was considerably slower in the frog than in man and made necessary the collection of excretory products for 5 days to insure reasonably complete recovery of metabolites. The major identifiable metabolite was a tetrahydro derivative subsequently identified as  $3\beta$ -hydroxy- $5\beta$ -tetrahydroaldosterone. This metabolite was excreted partly in the free form and partly as a glucuronic acid conjugate. The pH 1-hydrolyzable conjugate of aldosterone was not detected.

For the measurement of secretory rates, aldosterone- $^3\text{H}$  was injected into the dorsal lymph space and the animal placed in a bath to provide an environment of constant electrolyte composition and intake and a means of collecting excretory products. Urine and bath fluid were collected for 5 days, tetrahydroaldosterone was isolated, and its specific activity determined for the calculation of the aldosterone secretory rate. The rate of secretion of aldosterone in the bullfrog was increased by a tap water bath and by bovine adrenocorticotrophic hormone, decreased by a saline bath and by dexamethasone, and unchanged by valine-5-angiotensin amide.

## INTRODUCTION

Aldosterone is a quantitatively minor component of the mammalian adrenal cortex. Interest in its

secretion and regulation in certain lower vertebrates was stimulated by the finding of Carstensen, Burgers, and Li (1, 2) that aldosterone was a major corticosteroid in bullfrog interrenal incubates. Subsequent studies of the biosynthetic pattern of the tissue, which showed a predominance of aldosterone and its potential biogenetic precursors and absent  $17\alpha$ -hydroxylation (3, 4), appeared to support the implication of Carstensen and co-workers' findings (2) that the bullfrog interrenal was functionally homologous with the zona glomerulosa of the mammalian adrenal cortex.

The regulation of aldosterone secretion in mammals is predominantly extrapituitary. Earlier observations demonstrated the role of the pituitary in supporting adrenocortical function in certain anuran amphibians particularly during periods of increased metabolic activity (5). The existence of extrapituitary regulatory factors as well was suggested by the evidence of persistent electrolyte-active hormonal activity in hypophysectomized frogs (6, 7). It was of interest therefore to investigate the evolution of the aldosterone regulatory mechanism in a species in which efficient sodium conservation was crucial to its adaptation to a semiaquatic fresh water habitat.

The present report describes a method to measure the rate of aldosterone secretion in the bullfrog and the application of the technique to controlled studies of the effects of environmental salinity and potential regulatory factors. The method is based on the single injection isotope dilution technique used to measure the secretory rate of the hormone in man (8), modified by prolonging the period of collection of excretory products to 5 days because of the bullfrog's slower rate of me-

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tabolism and excretion of the radioactive steroid. The technique utilizes the semiaquatic habitat of the bullfrog to provide simultaneously an environment and intake of controlled electrolyte composition and a means of collecting metabolic products. Since the presentation of our preliminary report (9), the results of another technique using post-caval phlebotomy in a pithed preparation of *Rana catesbeiana* (10) are available for comparison.

## METHODS

Bullfrogs (*Rana catesbeiana*) of both sexes, weighing from 180 to 550 g, were in the fasting state and were kept in a sink under running tap water when not used for experiments. For the experimental periods which required control of external environment and collection of excretory products, the animals were kept in 12 inches  $\times$  6 inches  $\times$  5 inches plastic cages containing approximately 200 ml of bath fluid and covered with a wire mesh lid. This procedure allowed partial immersion of the frog and contact of the bath with the underside of its body when the frog was in a normal resting position. At the end of each 24-hr period, bath fluid was replaced and combined with contents of the urinary bladder, emptied by means of No. 205 polyethylene tubing inserted into the cloaca. Two bath fluids were used: tap water and 60 meq of NaCl per liter. When hypotonic saline was used, estimated daily evaporation from the plastic cage was replaced by adding distilled water to maintain the sodium concentration constant within  $\pm 10\%$ .

Hormones and labeled aldosterone were dissolved in sterile distilled water containing 0.9% benzyl alcohol as preservative, and 0.5 ml was injected into the dorsal lymph sac. Aldosterone-1,2- $^3\text{H}$  (50 mc/ $\mu\text{mole}$ ) was prepared biosynthetically (11).

Radioactivity was measured in a liquid scintillation spectrometer equipped with external standardization. The phosphor solution consisted of 3.0 g of 2,5-diphenyl-oxazole and 6 ml of ethanol per liter of toluene. For the counting of aqueous solutions, a 0.5-ml aliquot was mixed with 5 ml of absolute ethanol and 10 ml of toluene phosphor solution.

**Tritium- $^{14}\text{C}$  ratios.** The previous (12) simplification of the equation of Okita, Kabara, Richardson, and Leroy (13)

$$^3\text{H}/^{14}\text{C} = \frac{N_1 - N_2/b}{N_2 - aN_1} \quad (1)$$

where  $N_1$  = net counts per minute in tritium channel;  $N_2$  = net counts per minute in  $^{14}\text{C}$  channel;  $a = N_2/N_1$  for pure tritium and  $b = N_2/N_1$  for pure  $^{14}\text{C}$  was based on the assumption that constant  $a$  was negligible compared to  $b$ . With improved liquid scintillation instrumentation better discrimination between the two isotopes could be attained. Typical values for the overlap constants were  $a = 10^{-4}$  and  $b = 5.0$ . Under these circumstances constant  $a$  could be neglected in a further simplification of

the equation,

$$^3\text{H}/^{14}\text{C} = N_1/N_2 - 1/b \quad (2)$$

The  $N_1/N_2$  ratio was first calculated and then corrected by subtracting  $1/b$ . When  $N_1/N_2$  was greater than 5.0, and  $1/b$  was 0.2, the error introduced by neglecting  $1/b$  was less than 4%. Under these circumstances,  $N_1/N_2$  was a reasonable approximation of the  $^3\text{H}/^{14}\text{C}$  ratio

$$^3\text{H}/^{14}\text{C} = N_1/N_2. \quad (3)$$

**Preparation of urinary fractions.** After the injection of aldosterone- $^3\text{H}$ , a 5 day pool of bath fluid and urine was extracted with ethyl acetate and the extract washed with dilute sodium hydroxide and water to obtain the free fraction. The combined washings and urine were adjusted to pH 5 and incubated with approximately 250 U/ml of mammalian glucuronidase at  $45^\circ\text{C}$  for 2 days. A neutral ethyl acetate extract of the incubate represented the glucuronidase-hydrolyzable fraction. The combined washings and extracted urine from the preceding step were adjusted to pH 1 with HCl, allowed to remain at room temperature for 24 hr, and a neutral ethyl acetate extract, the pH 1-hydrolyzable fraction, was prepared.

**Aldosterone secretory rates.** When the bath composition was changed, sodium concentration in catheterized bladder urine was monitored daily and secretory rates determined when equilibration occurred. Measurements were made during all seasons of the year but were preceded by a control determination to minimize variation due to seasonal fluctuations (14) in adrenal activity. 5  $\mu\text{C}$  of aldosterone- $^3\text{H}$  was injected, and pooled bath fluid and urine were collected for 5 days. An aliquot of the pool was counted to verify recovery of at least 65% of the injected dose. In a few instances when recovery was less, the experiment was discarded. Tetrahydroaldosterone was isolated from the pool and its specific activity measured as described below. The 5 day secretory rate was calculated by dividing the counts per minute of aldosterone injected by the specific activity of the metabolite, and the average daily secretory rates shown in the Figures and Tables were obtained by dividing by 5.

**Isolation of frog tetrahydroaldosterone.** The 5 day pool of urine and bath fluid collected after a single injection of labeled aldosterone was incubated with mammalian glucuronidase, extracted with ethyl acetate, and the extract washed with dilute sodium hydroxide and water. The extract which represented the combined free and glucuronidase-hydrolyzable fractions was chromatographed on the ethylene dichloride-formamide system for 24 hr with cortisol as reference steroid. Tetrahydroaldosterone<sup>1</sup> was located for elution by radioactive scanning and by its migration at 0.85 the rate of cortisol. The eluate was dissolved in methylene chloride, washed

<sup>1</sup> The chromatographic mobilities of frog tetrahydroaldosterone differed slightly from those of the major metabolite in human urine, the  $3\alpha$ -hydroxy- $5\beta$  isomer (10). The frog metabolite has subsequently been identified as the  $3\beta$ -hydroxy- $5\beta$  isomer:  $3\beta,11\beta,21$ -trihydroxy-20-keto- $5\beta$ -pregnan-18-al (H. C. Rose and S. Ulick; data in preparation).

with water, dried and evaporated, and acetylated with acetic anhydride- $^{14}\text{C}$  (0.8 mc/mole):toluene (1:4) in pyridine at  $60^\circ\text{C}$  for 18 hr and excess reagents removed by partition (11). The triacetate was chromatographed on the Bush A and methylcyclohexane-formamide systems in which it migrated at approximately 1.1 times the rate of deoxycorticosterone acetate. After elution, the dried residue was shaken mechanically with 5 ml of 0.1 N HCl for 30 min at room temperature in a glass-stoppered test tube. The aqueous phase was extracted with methylene chloride and the extract washed with 0.1 N NaOH and water, dried, and evaporated. The resulting 3,21-diacetate was isolated after chromatography in methylcyclohexane:toluene (9:1)-formamide in which it migrated at 0.55 the rate of deoxycorticosterone. The 18-acetoxy group was reintroduced by acetylation with unlabeled acetic anhydride in pyridine in the usual manner at  $80^\circ\text{C}$  in an aluminum test tube heating block and the methylcyclohexane-formamide system was used to re-isolate the triacetate. When this specific procedure of selective removal and regeneration of the C-18 acetoxy group was applied, the  $^3\text{H}/^{14}\text{C}$  ratios of diacetate and triacetate agreed within 5%. Specific activity was calculated in the usual manner from the product of the  $^3\text{H}/^{14}\text{C}$  ratio of purified metabolite, the specific activity of the acetylating reagent, and the number of  $^{14}\text{C}$ -labeled acetoxy groups (two) in the derivative.

An alternate procedure for purifying tetrahydroaldosterone triacetate used the previously described chromatographic sequence (8) except that an alumina column was substituted for Celite in the final step. The triacetate was applied to a 10 g column of neutral alumina, prepared as described (11, 15) in ligroin:benzene (4:1) and the column developed with 25 ml of this solvent. The eluting solvent was changed to benzene, and 5-ml fractions were collected automatically. Peak concentrations of the triacetate usually appeared in fractions 16-20. Specific activity was calculated from the  $^3\text{H}/^{14}\text{C}$  ratio of the  $^{14}\text{C}$  peak fraction to minimize the error of isotope effect, provided the criteria (11) for radiochemical purity were met. Tetrahydroaldosterone could also be isolated as the etiolactone derivative by direct periodic acid oxidative cleavage (11) of the pool of urine and bath fluid, followed by saponification and relactonization. Because the doubly labeled derivative was a monoacetate, the sensitivity of the procedure was somewhat less.

## RESULTS

**Rate of excretion of labeled aldosterone and metabolites.** Fig. 1 shows the rate of appearance of radioactivity in urine and bath fluid after the injection of  $10\text{ }\mu\text{C}$  of aldosterone- $^3\text{H}$  in four frogs (shaded area) compared to data obtained in human subjects (11). The frogs continued to excrete appreciable amounts of radioactivity over the first 3 days. Since there was negligible increment from the 4th to the 5th day, a 5 day collection period was chosen for the subsequent secretory rate

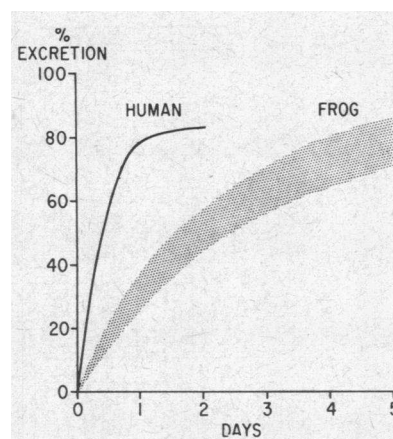


FIGURE 1 Cumulative rate of excretion of radioactivity (expressed as per cent of dose) into urine and bath fluid in four bullfrogs after the injection of  $10\text{ }\mu\text{C}$  of aldosterone- $^3\text{H}$  into the dorsal lymph space. Comparative data in human subjects are from reference 11.

determinations. The cumulative 5 day excretion was 65-78% of the dose.

**Pattern of radioactive metabolites.** 5-day pools of urine and bath fluid were analyzed for free, glucuronidase-hydrolyzable, and pH 1-hydrolyzable radioactivity in each of four animals. Table I shows that a mean of 42% of injected radioactivity remained in the aqueous phase after removal of the three fractions. This material could not be rendered extractable by solvolysis (16), incubation with sulfatase, or oxidation with periodic acid (11).

The free fraction contained the largest portion of extractable radioactivity. Chromatographic analysis of this fraction showed that a major component was an isomer of tetrahydroaldosterone. A

TABLE I  
Pattern of Radioactive Metabolites of Aldosterone  
in the Bullfrog

Bullfrog weight	Per cent of injected aldosterone- $^3\text{H}$ ( $10\text{ }\mu\text{C}$ ) excreted in fraction				
	Aqueous (nonextractable)	Free	Glucuronidase hydrolysis	pH 1 hydrolysis	Total recovered
<i>g</i>					
220	43.5	13.3	11.1	1.5	69.4
235	39.1	21.5	14.2	2.0	76.8
440	35.5	19.5	8.0	1.0	64.0
530	49.0	19.4	7.2	0.85	76.5
Mean	41.6	18.4	10.1	1.4	71.5

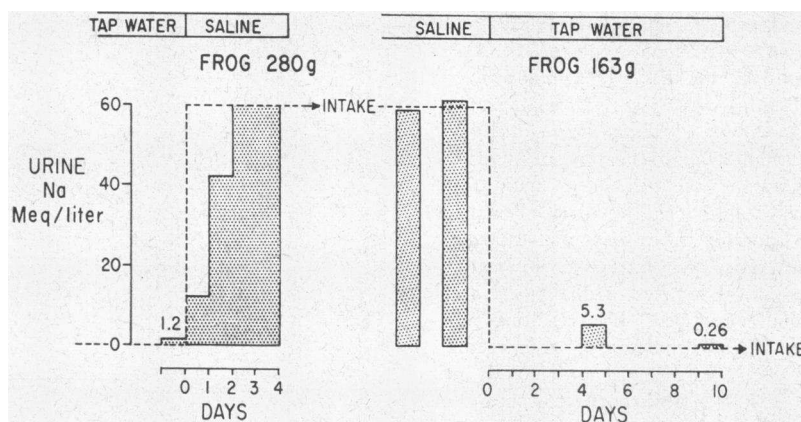


FIGURE 2 Effect of a change in bath fluid composition on the sodium concentration of catheterized frog bladder urine.

minor component of the free fraction, generally less than 10%, consisted of unmetabolized aldosterone. Tetrahydroaldosterone was also excreted in conjugated form. It was a major component of the extractable radioactivity liberated by incubation with mammalian glucuronidase. For the subsequent isolation and analysis of the tetrahydro metabolite the combined free and glucuronide fraction was used.

Although the pH 1-hydrolyzable conjugate of aldosterone, a probable 18-glucuronide (17), accounts for approximately 10% of the secreted hormone in man (18), this metabolite was a negligible excretory product in the frog. Hydrolysis at pH 1 yielded a mean of 1.4% of the dose. Pooled extracts of this fraction were analyzed by the addition and reisolation of aldosterone- $^{14}\text{C}$ . The  $^3\text{H}/^{14}\text{C}$  ratio of purified product indicated that less than 0.06% of injected aldosterone was in this form.

*Effect of environmental sodium on frog's uri-*

*nary sodium concentration and aldosterone secretory rate.* Fig. 2 shows the effect of a change in bath fluid composition on the sodium concentration of catheterized bladder urine. The change from tap water to saline resulted in a prompt increase in bladder urinary sodium concentration in the 280 g frog to values equal to (and in other experiments slightly exceeding) that of the external environment. The adaptation from saline to tap water shown for a 163 g frog was associated with a fall in urinary sodium concentration to a value of 0.26 meq/liter after 10 days.

Fig. 3 correlates the changes in urinary sodium concentration observed in Fig. 2 with the measurement of aldosterone secretory rate after each animal had come into equilibrium with its environment. The very low urinary sodium concentrations observed when the frogs were kept in the tap water bath were associated with the secretion of 11 and 19  $\mu\text{g}$  of aldosterone per kg per day. Conversely, the high urinary sodium concentrations

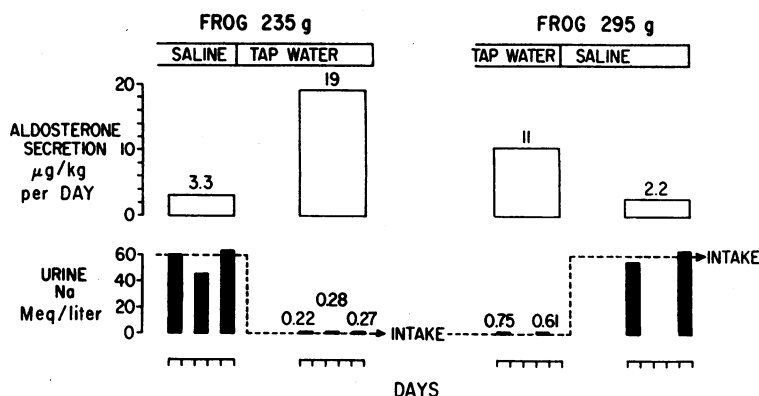


FIGURE 3 Correlation of the changes in urine sodium concentration (Fig. 2) with the measurement of frog aldosterone secretory rate in response to changes in bath fluid composition.

TABLE II  
Effect of Angiotensin, ACTH, and Dexamethasone  
on Aldosterone Secretion in Bullfrogs

Bullfrog weight	Rx/kg per day	Aldosterone secretory rate, $\mu\text{g/kg per day}$					
		Bath. NaCl 60 meq/liter	NaCl, 60 meq/liter	Tap water	Tap water	Tap water	Tap water
		0	Angiotensin, 40 $\mu\text{g}$	0	Angiotensin, 40 $\mu\text{g}$	ACTH, 1.0 U	Dexamethasone, 140 $\mu\text{g}$
g							
220		3.6	4.5	22	19	64	
235		4.0	3.8	16	21	37	
440		4.6	3.9	20	22	53	
530		5.9	4.9	19	14	47	
230				10.9			2.3
260				10.4			1.9

ACTH, adrenocorticotrophic hormone; Armour bovine ACTH, valine-5-angiotensin amide (Hypertensin-Ciba).

\* Hormones were administered every 12 hr for 6 days beginning 1 day before the determination of the aldosterone secretory rate.

of the frogs kept in hypotonic saline were associated with lowered secretion of 2.2 and 3.3  $\mu\text{g}$  of aldosterone per kg per day. This 5- to 6-fold difference between the sodium-depleted and sodium-repleted states is comparable to that observed in mammals. Thus an environment simulating the frog's natural habitat was associated with relative hypersecretion of aldosterone. Hypotonic saline suppressed the secretory rate to the range of the mean basal rate (2  $\mu\text{g/kg per day}$ ) in man determined by a similar method (11).

*Effect of angiotensin, ACTH, and dexamethasone on aldosterone secretion.* Angiotensin was administered to each of four animals both in tap water and in a hypotonic saline environment. Table II shows that the angiotensin-treated animals did not secrete significantly different amounts of aldosterone from the control values. The animals in the tap water bath which did not respond to angiotensin were then given ACTH. There was a 1.8–3.4-fold increase in aldosterone secretion in the four animals, indicating that they were not already maximally stimulated. This evidence for the role of pituitary factors was extended by the administration of ACTH-suppressive doses of dexamethasone. In two experiments, the elevated aldosterone secretory rates associated with a tap water environment were decreased to approximately one-fifth of the control value by dexamethasone.

## DISCUSSION

Application of the indirect isotope technique for the measurement of secretory rates required essentially complete excretion of radioactivity associated with the metabolite chosen for measurement. Completeness of excretion of the metabolite, 3 $\beta$ ,5 $\beta$ -tetrahydroaldosterone, as judged by the rate of excretion of total radioactivity, required 5-day collection periods. This relatively slow rate of excretion was probably a reflection of the hypometabolism of the poikilothermic state. The 5-day collection periods limited the application of the technique in the bullfrog to chronic experiments, but this limitation was offset by the precision and convenience of the method which allowed repeated measurements to be made in the conscious animal serving as its own control and avoided the introduction of uncontrollable variables such as surgery, blood loss, and adrenal blood flow.

Secretion of aldosterone by the bullfrog, suggested by the identification of the steroid in interrenal incubates (2), was established in vivo by the isotope dilution technique used in the present study. The observed reciprocal relationship between sodium intake and urinary sodium concentration on the one hand and the rate of secretion of aldosterone on the other agrees with the findings in man (8), dog (19), sheep (20), and rat (21). Earlier observations which anticipated this relationship showed that a measure of hormonally stimulated sodium transport, the isolated skin short circuit current, was increased when frogs were kept in distilled water (22, 23). A suppressive effect of a saline environment on plasma aldosterone has been shown in *Bufo marinus* (24). A saline infusion failed to decrease plasma aldosterone in a pithed preparation of *Rana catesbeiana* (10). Blood loss and dexamethasone treatment may account for the conflicting result in the latter report (10).

Earlier observations demonstrated the role of the pituitary in maintaining adrenocortical function in anuran amphibians, particularly during periods of increased metabolic activity (5). Specific effects of ACTH on the secretion of electrolyte-active hormone were suggested by the augmentation of skin short circuit current in ACTH-treated frogs (6). In vitro stimulation of aldosterone synthesis in bullfrog interrenal incubates

by the addition of mammalian ACTH and of frog pituitary extract was shown by Carstensen, Burgers, and Li (2). The present data confirm stimulation of aldosterone secretion in vivo by mammalian ACTH. The observed decrease in aldosterone secretion with ACTH-suppressive doses of dexamethasone suggest that the frog's endogenous ACTH supports aldosterone secretion in vivo. Although intramuscular ACTH did not significantly increase plasma aldosterone in a bled, pithed preparation of *Rana catesbeiana*, an intravenous infusion of the peptide did increase the plasma level in dexamethasone-treated animals (10).

The existence of extrapituitary mechanisms for regulating electrolyte-active hormone in the frog was suggested by observations after hypophysectomy that adrenocortical cell atrophy was incomplete (25) and that frogs kept in distilled water continued to excrete urine of very low sodium concentration (7) and showed increased skin short circuit current after an initial postoperative depression (6). Evidence for a role of the renin-angiotensin system in *Rana catesbeiana* was obtained by Johnston, Davis, Wright, and Howards (10) who found that frog renin increased the concentration of aldosterone in postcaval plasma, although its steroidogenic effect was less than that of ACTH. The negative results with valine-5-angiotensin amide in the present study suggest that the homologous angiotensin peptide may be required. The steroidogenic potency of synthetic angiotensin is variable in different mammalian species. Its stimulatory effect is short-lived in the sheep (20) and variable in the rat (21, 26), where dose (27) and mode of administration (28) may determine its effectiveness.

The present study demonstrates secretion of aldosterone in the bullfrog and reciprocal changes in that secretion in response to changes in the salinity of the environment. Aldosterone secretion was strongly stimulated by mammalian ACTH and suppressed by dexamethasone. The method described makes the bullfrog a suitable experimental subject for controlled physiologic studies of the regulation of the adrenocortical secretion. Several questions not answered by the available data require further investigation. Although both pituitary and extrapituitary trophic mechanisms exist in the bullfrog, their integration in vivo and

relative participation as mediators of the sodium or volume depletion stimulus is not known. Another question is whether anuran interrenal, although without zonation histologically, exhibits the characteristics of function zonation of the mammalian adrenal cortex, namely, the independent regulation of the secretion of electrolyte-active and of carbohydrate-active steroids. Corticosterone is secreted by the bullfrog (10), but it is not clear whether the steroid functions as a glucocorticoid in this species or is released from the interrenal as an aldosterone biogenetic precursor. Frog renin increased the plasma level of both steroids (10) in *Rana catesbeiana*, but a saline environment decreased the peripheral plasma level of aldosterone but not that of corticosterone in *Bufo marinus* (24).

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