Aldosterone Stimulation of Riboflavin Incorporation into Rat Renal Flavin Coenzymes and the Effect of Inhibition by Riboflavin Analogues on Sodium Reabsorption

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A B S T R A C T This study was designed to investigate a possible relationship between the effect of aldosterone upon urinary electrolytes and the incorporation of [^{14}C]riboflavin into renal [^{14}C]flavin mononucleotide (FMN) and [^{14}C]flavin adenine dinucleotide (FAD). Adrenalectomized Sprague-Dawley rats that weighed between 185 and 210 g were pretreated with 15 μg/100 g body wt dexamethasone intraperitoneally. 16 h later they were administered aldosterone (1.5 μg/100 g body wt) and [^{14}C]riboflavin (5.0 μCi/200 g body wt). The urethra of each rat was ligated, and the rats were sacrificed by decapitation 3 h later. The urine was aspirated from the bladders of each rat and analyzed for total Na$^+$ and K$^+$ excretion while the kidneys were removed and the formation of [^{14}C]FMN and [^{14}C]FAD was determined for each kidney. There was a significant increase in the formation of renal [^{14}C]FMN and [^{14}C]FAD (27.3 and 14.4%, respectively) after aldosterone treatment. Aldosterone significantly decreased the excretion of Na$^+$ by 50%, and increased that of K$^+$ by 55%.

To determine if the increased incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD was an important intermediary step in the aldosterone-induced alterations in urinary Na$^+$ and K$^+$, the riboflavin analogues 7,8-dimethyl-10-formylmethyl isoalloxazine or 7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine were given to the animals i.p. to diminish the conversion of riboflavin to FMN by competitively inhibiting the enzyme flavokinase (EC 2.7.1.26). These analogues were found to significantly counteract the decreased urinary excretion of Na$^+$ as a result of aldosterone from 26±9% to 124±58% (SEM) with a dose-related response when administered from 10 to 25 μg/100 g body wt. The same doses of the analogues that significantly increased the urinary output of Na$^+$ when administered simultaneously with aldosterone also significantly decreased the formation of renal [^{14}C]FMN from 15±4 to 38±3% when compared with the effects of aldosterone alone. The analogues exerted no significant effect on the increased urinary excretion of K$^+$ by aldosterone. The analogues alone had no influence on urinary Na$^+$ and K$^+$ output or the formation of renal [^{14}C]FMN and [^{14}C]FAD at the dose levels that we investigated.

These data strongly suggest that the enhanced synthesis of renal FMN and FAD may be a causative factor in the increased reabsorption of Na$^+$ as a result of aldosterone; and, consequently, riboflavin analogues may function as a novel class of antimineralocorticoids.

INTRODUCTION

The nature and physiologic roles of the aldosterone-induced protein have been a subject of disparate claims. Edelman and Fanestil (1) have inferred that the aldosterone-induced protein may stimulate Na$^+$ transport either by increasing the rates of NADH production by modifying the steps between citrate synthetase and α-ketoglutarate dehydrogenase in the citrate cycle and(or) by stimulating intramitochondrial oxidation of NADH at the flavoprotein-linked NADH dehydrogenase step. The increased rate of Na$^+$ transport would be a consequence of an increased rate of ATP production coupled to NADH oxidation.

Fazekas and Sandor (2) showed that ACTH increased the formation of [^{14}C]flavin mononucleotide

(FMN)[1] from [^{14}C]riboflavin in both the kidney and liver of the intact rat. They could not determine whether this observed effect was a result of ACTH per se or of an effect of the steroid hormones elaborated by the ACTH. Of considerable interest, therefore, is an antecedent report of Laszt and Verzár (3) in 1935 that asserts that the formation of FMN from riboflavin was promoted by the hormones of the adrenal cortex. The possibility that aldosterone increased the activity of the mitochondrial flavoprotein-linked NADH dehydrogenase (1) prompted Tan and Trachewsky (4) to investigate the influence of aldosterone on the formation of renal [^{14}C]flavin coenzymes from [^{14}C]riboflavin. We concluded from these latter studies (4) that mineralocorticoids specifically increased the formation of renal [^{14}C]flavin adenine dinucleotide (FAD) because spironolactone effectively blocked this aldosterone-induced increase. The dose level of spironolactone employed in these studies had already been shown to inhibit the reabsorption of Na$^+$ and secretion of K$^+$ as a result of the administration of aldosterone to bilaterally adrenalectomized rats.[2] This specific aldosterone antagonist had no effect by itself in the absence of aldosterone administration to the adrenalectomized rats.

This present investigation was undertaken to examine the possible cause-effect relationship between the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD, and urinary excretion of Na$^+$ and K$^+$, after administration of aldosterone to bilaterally adrenalectomized rats. Just this year, Addison and McCormick (5) reported that there was a decrease in the biogenesis of flavoprotein components in hepatic mitochondria from riboflavin-deficient rats which could be reversed by an i.p. injection of riboflavin in 24 h. To determine if the increased formation of the renal [^{14}C]flavin coenzymes were causing the alterations in urinary Na$^+$ and K$^+$ output by aldosterone, we administered the riboflavin analogues 7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine and 7,8-dimethyl-10-formylmethyl isoalloxazine to the animals which would diminish the conversion of [^{14}C]riboflavin to [^{14}C]FMN by competitively inhibiting the enzyme flavokinase (EC 2.7.1.26) (6).

METHODS

Reagents. [D-2-^{14}C]Riboflavin (31 mCi/mmol) was purchased from the Amersham-Searle Corp., Arlington Heights, Ill. D-Riboflavin, FMN, FAD aldosterone (11,21-dihydroxy-18-oxo-5-pregnen-3,20-dione), dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione), and DEAE-Sephadex A-25 were obtained from the Sigma Chemical Co., St. Louis, Mo. The riboflavin analogues

7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine (U-2113) and 7,8-dimethyl-10-formylmethyl isoalloxazine (U-1003) were gifts from the Upjohn Co., Kalamazoo, Mich.

Experimental conditions. All experiments were performed with male Sprague-Dawley rats that weighed between 185 and 210 g. These animals were bilaterally adrenalectomized, fed, *ad libitum* on Purina rat chow (Ralston Purina Company Inc., St. Louis, Mo.), and given drinking water which contained 5% dextrose and 1% NaCl for 3 days. The night before the experiment on the 4th day, each rat received an i.p. injection of dexamethasone (15 μg/100 g body wt) and the food and water were removed. We have found that this dose of dexamethasone will suppress ACTH in our rats.[3] Dexamethasone was administered for the reasons already documented (4). On the morning of the 4th day, the animals were all weight-matched and divided into four equal and separate groups. At zero time, all rats were injected i.p. with a single dose of 5.0 μCi [2-^{14}C]riboflavin/200 g body wt, and various other solutions of aldosterone and(or) riboflavin analogue. The first group served as control, and received only the vehicles for all other injections. The second group was administered aldosterone (1.5 μg/100 g body wt). The third group received injections of aldosterone (1.5 μg/100 g body wt) and varying doses of one or the other of the two riboflavin analogues 7,8-dimethyl-10-(2'hydroxyethyl) isoalloxazine and 7,8-dimethyl-10-formylmethyl isoalloxazine. The fourth group was treated only with the same varying doses of one or the other of the two riboflavin analogues as the third group.

30 min after i.p. injections, the urethral outflow tracts of the rats were ligated under light ether-anesthesia and each animal was injected i.p. with 4 ml of 0.9% saline. 3 h later, the rats were sacrificed by decapitation, urine was aspirated from the bladder via an abdominal incision, and the kidneys were removed and quick-frozen for analysis of their radioactive flavin content the following day. The urinary excretion of Na$^+$ and K$^+$ in the adrenalectomized rats was used as an index of mineralocorticoid and antimineralocorticoid potency of aldosterone and the riboflavin analogues, respectively. The urinary Na$^+$ and K$^+$ concentrations were measured with an Instrumentation Laboratory model IL 443 flame photometer (Instrumentation Laboratory Inc., Lexington, Mass.).

Extraction, separation, and determination of radioactive flavins in tissue. Radioactive flavins were extracted from the tissue samples in the presence of nonradioactive carrier flavins. The separation and quantitation of radioactive flavins used newly developed techniques (7, 8) of isotope dilution and ion-exchange column chromatography with DEAE-Sephadex A-25. Each kidney to be analyzed was placed in a Potter-Elvehjem glass homogenizer equipped with a Teflon (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) pestle. To each kidney was added 7.0 ml methanol and 1.0 ml each of solutions of nonradioactive FAD, FMN, and riboflavin (100 μg/ml) prepared fresh daily. The kidneys were homogenized at 1,500 rpm for 5 min at 4°C followed by centrifugation at 100,000 g for 60 min at 4°C. This excessive homogenization in 70% methanol did result in grossly damaging the vast majority of the mitochondria, as shown by transmission electron microscopy, and thus insured a high yield of flavin nucleotides. The supernatant solutions were decanted into 15-ml conical centrifuge tubes and evaporated to dryness *in vacuo* at 40°C in a Vortex-Evaporator (Buchler Instruments, Div. Searle Diagnostics Inc., Fort Lee, N. J.).

The residues were dissolved in 1.0 ml water and transferred with a Pasteur pipette to a DEAE-Sephadex A-25 Bio-Rad

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[1] *Abbreviations used in this paper*: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

[2] Unpublished observation.

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[3] Unpublished observation.

TABLE I

Effect of Aldosterone Administration to Bilaterally Adrenalectomized Rats on the Incorporation of [^{14}C]Riboflavin In Vivo into Renal [^{14}C]FMN and [^{14}C]FAD

Experimental group	Specific activity of flavins			Increase over control		
	Riboflavin	FMN	FAD	Riboflavin	FMN	FAD
	dpm/μg flavin			*%*		
Control (78)	151±18‡	322±33	878±32			
Aldosterone* (80)	180±12	410±37	1004±32	19.1	27.3§	14.4‖

* Aldosterone administered at 1.5 μg/100 g body wt. Numerals in parentheses refer to the number of rats in each group. Both kidneys were used from each rat and each kidney was processed separately.

‡ Mean±SEM calculated on the basis of the number of animals in each group.

§ $P < 0.05$ (significance determined by Student's t test for unpaired data).

‖ $P < 0.005$ (significance determined by Student's t test for unpaired data).

Econo column, 18 cm in length and 1 cm in internal diameter (Bio-Rad Laboratories, Richmond, Calif.). The tube was rinsed with an additional 1.0 ml water, which was also transferred to the column. When the extract had entered the gel bed, elution was started with 4.0 ml distilled water, and continued with 1% ammonium sulfate solution. Riboflavin was eluted first, followed by FMN and FAD. The flavin solutions were collected manually in separate polyethylene liquid scintillation vials. The volumes of the three flavin solutions were determined with 10-ml graduated cylinders, and each was thoroughly mixed. A 2.0-ml aliquot of each solution was transferred to a polyethylene liquid-scintillation vial and 17.0 ml Aquasol 2 (New England Nuclear, Boston, Mass.) was added. Radioactivity was determined in a Beckman liquid scintillation counter, model LS9000, which read disintegrations per minute directly (Beckman Instruments, Inc., Fullerton, Calif.). Another 4.0-ml aliquot of each solution was transferred to a quartz cuvette and absorbance determined at 450 nm in a Beckman dual beam spectrophotometer, model 25, with 1.0% ammonium sulfate as the blank. Calibration curves of riboflavin, FMN, and FAD from 0 to 50 μg/ml were determined with the original stock solutions prepared and utilized each day. The entire procedure yielded ≅100, 90, and 95% of the riboflavin, FMN, and FAD, respectively, which were added before extraction. The specific activity of each flavin in solution was calculated by dividing the radioactivity in disintegrations per minute by the flavin concentration in micrograms.

Statistical analysis. The results are expressed as mean±1 SEM. Statistical analysis was carried out with the Student's t test for unpaired data, and P values of <0.05 are considered significant.

RESULTS

Effect of aldosterone administration to bilaterally adrenalectomized rats on renal [^{14}C]FMN and [^{14}C]FAD formation, and on the urinary excretion of Na$^+$ and K$^+$. The formation of renal [^{14}C]FMN and [^{14}C]FAD from [^{14}C]riboflavin, (and radioactivity as a result of renal [^{14}C]riboflavin which was not incorporated into these flavin coenzymes) 3.5 h after i.p. injection in bilaterally adrenalectomized rats administered aldosterone (1.5 μg/100 g body wt) was compared to those

not given the hormone (Table I). Aldosterone administration significantly increased the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN by 27.3% ($P < 0.05$) and into renal [^{14}C]FAD by 14.4% ($P < 0.005$). The mean radioactivity as renal [^{14}C]riboflavin was greater in the aldosterone-treated than in control animals, but this difference was not statistically significant ($P > 0.05$).

In the same animals, as well as additional control and mineralocorticoid-treated ones, aldosterone administration significantly decreased the urinary output of Na$^+$ by 50% ($P < 0.0005$) and increased that of K$^+$ by 55% ($P < 0.0005$) 3.5 h after hormone treatment (Table II.)

Effect of riboflavin analogues on renal [^{14}C]FMN and [^{14}C]FAD formation, and on the urinary output of Na$^+$ and K$^+$ as a result of aldosterone administration. To determine if there was a causal relationship between the increased corporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD on the one hand, and the alterations in urinary Na$^+$ and K$^+$ excretion, on the other, after aldosterone treatment of the adrenalectomized animals, we administered the riboflavin

TABLE II

Effect of Administration of Aldosterone to Bilaterally Adrenalectomized Rats on Urinary Na$^+$ and K$^+$ Excretion

Experimental group	No. of rats	Na$^+$	P value	K$^+$	P value
		μeq/3h/rat		*μeq/3h/rat*	
Control	143	352±18‡		74±4	
Aldosterone*	133	176±15	<0.0005	115±5	<0.0005

* Aldosterone administered at 1.5 μg/100 g body wt.

‡ Mean±1 SEM.

analogues 7,8-dimethyl-10-formylmethyl isoalloxazine and 7,8-dimethyl-10-(2'hydroxyethyl) isoalloxazine to the rats which would diminish the conversion of [^{14}C]-riboflavin to [^{14}C]FMN by competitively inhibiting the enzyme flavokinase (EC 2.7.1.26) (6).

The animals were divided into four equal and separate groups as described in Methods. A dose-response relationship was determined for each of the riboflavin analogues with regard to the urinary output of Na$^+$ and K$^+$, and the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD. The administration of 14–25 μg/100 g body wt of 7,8-dimethyl-10-formyl-methyl isoalloxazine simultaneously with aldosterone resulted in a significant 26±9 to 124±58% ($P < 0.05$) increase in the urinary excretion of Na$^+$ when compared to that as a result of aldosterone administration alone (Fig. 1). The optimal effect of the riboflavin analogue was found at 20 μg/100 g body wt where the increase in urinary output of Na$^+$ was 124±58% ($P < 0.05$) when compared to that as a result of aldosterone administration alone. At the lower-dose levels of 8.5 and 10 μg/ 100 g body wt and the higher ones of 30 and 35 μg/100 g body wt, this particular riboflavin analogue did not significantly alter the urinary output of Na$^+$ as a result of aldosterone treatment. At all dose levels investigated, this analogue exerted no significant effect on the increased excretion of K$^+$ by aldosterone.

The same dose levels of 7,8-dimethyl-10-formyl-methyl isoalloxazine that significantly increased the uri-nary output of Na$^+$ when administered simultaneously with aldosterone (14–25 μg/100 g body wt) also significantly decreased the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN from 34±8 ($P < 0.01$) to 38±3% ($P < 0.0005$) when compared to the urinary Na$^+$ excretion and incorporation of [^{14}C]riboflavin as a result of aldosterone alone (Fig. 2). At the lower-dose levels of 10 μg/100 g body wt and the higher levels of 30 and 35 μg/100 g body wt, this analogue did not significantly alter the formation of renal [^{14}C]FMN as a result of aldosterone administration alone. Thus, an inverse relationship was observed between the increase in urinary output of Na$^+$ and the decrease in the forma-tion of renal [^{14}C]FMN when the animals were treated with aldosterone plus 7,8-dimethyl-10-formylmethyl isoalloxazine compared with aldosterone alone (Figs. 1 and 2). At least eight rats were used for the aldo-sterone and control groups, respectively, at each dose level of analogue (plus aldosterone) studied. In addi-tion, 7,8-dimethyl-10-formylmethyl isoalloxazine by itself had no significant influence on urinary Na$^+$ and K$^+$ output, or the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD at the dose levels that we investigated compared with the control group of adrenalectomized rats given only the vehicles for injec-tion plus [^{14}C]riboflavin. At least eight animals were used for each dose level when this analogue was studied by itself. Interestingly, too, the mean radio-activity as renal [^{14}C]riboflavin was essentially the
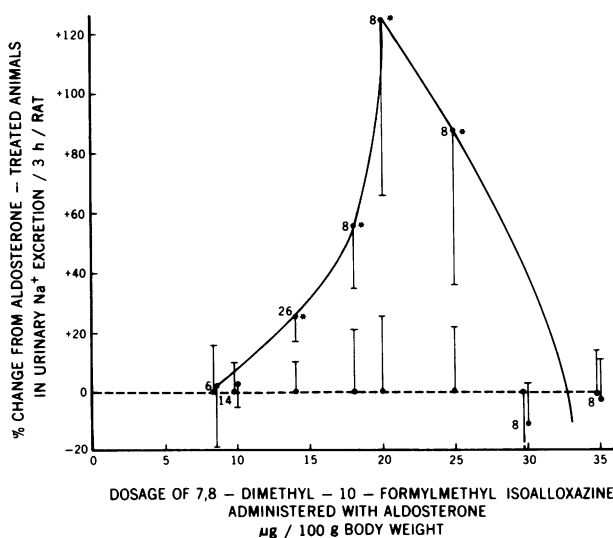

FIGURE 1 Effect of various dose levels of 7,8-dimethyl-10-formylmethyl isoalloxazine, administered simultaneously with aldosterone, on urinary Na$^+$ excretion compared with aldosterone administration alone. Asterisks (*) indicate values significantly different from zero aldosterone values ($P < 0.05$). Numerals on graph refer to number of rats treated with ribo-flavin analogue plus aldosterone; an equal number of rats was treated with aldosterone alone.
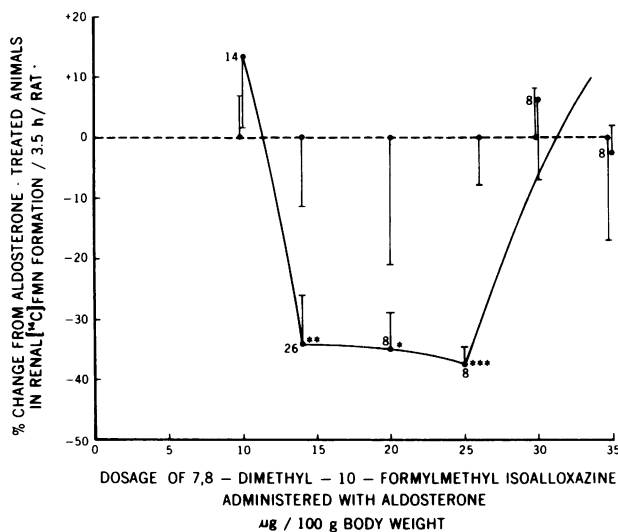

FIGURE 2 Effect of various dose levels of 7,8-dimethyl-10-formylmethyl isoalloxazine administered in tandem with aldosterone, on the percent change in renal [^{14}C]FMN for-mation compared with aldosterone administration alone. As-terisks (*) indicate values significantly different from zero al-dosterone values ($P < 0.05$); **, $P < 0.01$; ***, $P < 0.0005$. Numerals on graph refer to number of rats treated with ribo-flavin analogue plus aldosterone; an equal number of rats was treated with aldosterone alone.

same for the control group of rats and the groups administered aldosterone and 7,8-dimethyl-10-formylmethyl isoalloxazine, on the one hand, and 7,8-dimethyl-10-formylmethyl isoalloxazine alone, on the other.

Essentially the same results were obtained when the riboflavin analogue 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine was investigated. The administration of 10–21 μg/100 g body wt of this analogue in tandem with aldosterone evoked a significant increase of 64 ±24 to 92±32% ($P < 0.025$) in the urinary excretion of Na$^+$ when compared with that as a result of aldosterone administration alone (Fig. 3). At the lower-dose levels of 4.5 and 7 μg/100 g body wt and the higher levels of 25 and 30 μg/100 g body wt, this particular riboflavin analogue did not significantly alter the urinary excretion of Na$^+$ as a result of aldosterone treatment. At all dose levels investigated, this analogue, too, had no significant effect on the increased excretion of K$^+$ by aldosterone.

The same dose levels of 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine that significantly increased the urinary excretion of Na$^+$ when administered in tandem with aldosterone (10–21 μg/100 g body wt) also significantly diminished the incorporation of [^{14}C]-riboflavin into renal [^{14}C]FMN from 15±4 ($P < 0.05$) to 31±5% ($P < 0.005$) when compared to the urinary Na$^+$

output and incorporation of [^{14}C]riboflavin as a result of aldosterone alone (Fig. 4). At the lower-dose levels of 4.5 μg/100 g body wt and the higher doses of 25 and 30 μg/100 g body wt this analogue did not significantly alter the formation of renal [^{14}C]FMN as a result of aldosterone administration alone. Again, as with the other riboflavin analogue, an inverse relationship was noted between the increase in urinary excretion of Na$^+$ and the decrease in the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN when the animals were treated with aldosterone plus 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine compared with aldosterone alone (Figs. 3 and 4). At least seven rats included in the aldosterone and control groups, respectively, at each dose level of analogue (plus aldosterone) studied.

7,8-Dimethyl-10-(2′-hydroxyethyl) isoalloxazine by itself also had no significant influence on the urinary excretion of N$^+$ and K$^+$ or the formation of renal [^{14}C]FMN and [^{14}C]FAD at the dose levels that we investigated when compared to the control group of adrenalectomized rats. At least seven animals were used for each dose level when this analogue was studied by itself. Again, the mean radioactivity as renal [^{14}C]-riboflavin was the same for the control group of rats and the groups administered aldosterone and 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine, on the one hand, and 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine alone, on the other.

DOSAGE OF 7,8 − DIMETHYL − 10 − (2′ − HYDROXYETHYL) ISOALLOXAZINE ADMINISTERED WITH ALDOSTERONE
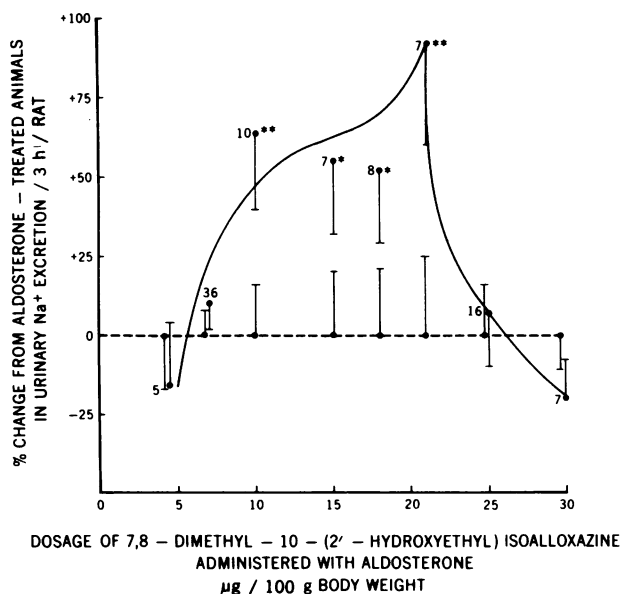μg / 100 g BODY WEIGHT

FIGURE 3   Effect of various dose levels of 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine, administered in tandem with aldosterone, on urinary Na$^+$ excretion compared with aldosterone administration alone. Asterisks (*) indicate values significantly different from zero aldosterone values ($P < 0.05$); **, $P < 0.025$. Numerals on graph refer to number of rats treated with riboflavin analogue plus aldosterone; an equal number of rats was treated with aldosterone alone.

DOSAGE OF 7,8 − DIMETHYL − 10 − (2′ − HYDROXYETHYL) ISOALLOXAZINE ADMINISTERED WITH ALDOSTERONE
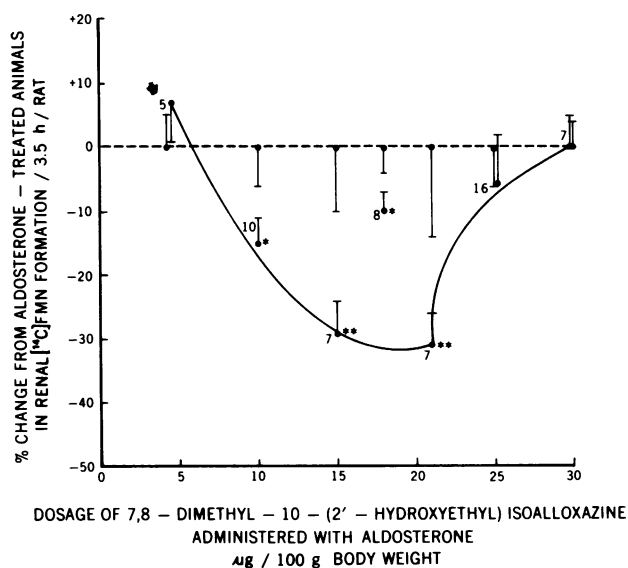μg / 100 g BODY WEIGHT

FIGURE 4   Effect of various dose levels of 7,8-dimethyl-10-(2′-hydroxyethyl) isoalloxazine, administered simultaneously with aldosterone, on the percent change in renal [^{14}C]FMN formation compared with aldosterone administration alone. Asterisks (*) indicate values significantly different from zero aldosterone values ($P < 0.05$); **, $P < 0.005$. Numerals on graph refer to number of rats treated with riboflavin analogue plus aldosterone; an equal number of rats was treated with aldosterone alone.

DISCUSSION

These investigations confirm and extend our previous studies (4) which showed that 1 h after the administration of aldosterone to adrenalectomized rats there was a significant increase of 16–19% in the incorporation of [^{14}C]riboflavin into renal [^{14}C]FAD. The recent development of techniques which permit simultaneous measurements to be made of radioactive riboflavin, FMN, and FAD in tissues (7,8) has greatly expanded the potential for investigating hormonal control of riboflavin metabolism. The present results clearly show that 3.5 h after the administration of aldosterone to adrenalectomized rats there was a significant increase in the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD by 27.3 and 14.4%, respectively (Table I). The results were found to be essentially the same as we have for all four groups of animals if the labeling were expressed per milligram of homogenate protein. Protein content of the homogenates was determined by the method of Ehresmann et al. (9). This finding of a 27.3 and 14.4% increase in [^{14}C]FMN and [^{14}C]FAD, respectively, is compatible with the idea that after 3.5 h aldosterone administration to adrenalectomized rats increased renal flavokinase (EC 2.7.1.26) (6) and FAD pyrophosphorylase (EC 2.7.7.2) activities (10). Rivlin (11) has also demonstrated that in adult rats, thyroxine increased hepatic flavokinase activity more than FAD pyrophosphorylase activity. More recently, Fazekas et al. (12) have shown that in adult rats, thyroxine produced a greater quantitative increase in hepatic [^{14}C]FMN than in [^{14}C]FAD from [^{14}C]riboflavin.

It is worth noting that in no group of animals in the present investigation was there a significant effect of aldosterone administration upon the radioactive riboflavin concentration in the kidney. The data found are compatible with the view that vitamin transport into kidney may not be a critical locus of hormonal control, and that regulation of enzyme activities may be the main control point.

If the observed hormonal effect was secondary to an increase of the intracellular-riboflavin pool, then when the animals were administered aldosterone in tandem with one or the other of the two riboflavin analogues, we should have definitely observed an increase in the amount of renal [^{14}C]riboflavin because our data show that under these conditions the analogues diminish the conversion of riboflavin to FMN when used at the appropriate dose levels. The radioactivity as renal [^{14}C]riboflavin, however, was essentially similar for the control group of rats and those administered aldosterone in tandem with one or the other of the analogues. By the very same token, if the observed hormonal effect (i.e., an increase in the incorporation of [^{14}C]riboflavin

into renal [^{14}C]FMN and [^{14}C]FAD when aldosterone was administered) was secondary to a decreased rate of degradation of riboflavin (13, 14), then when the animals were administered aldosterone simultaneously with one or the other of the two riboflavin analogues, we should have also noted an increase in the amount of renal [^{14}C]riboflavin because the analogues diminished the conversion of riboflavin to FMN when used at the appropriate dose levels; this, as already seen, was not the case. If the observed hormonal effect was secondary to a decreased rate of degradation of FMN, then the rats that were treated with aldosterone and the analogue should have had as much renal [^{14}C]FMN as the group given aldosterone alone; again, this was not found to be so. The observed lower content of renal [^{14}C]FMN in the former group could not have been a result of the effect of the analogues on the conversion of riboflavin to FMN because the analogues by themselves did not lower the amount of renal [^{14}C]FMN below that of the control group. Lastly, if the effect of aldosterone on the increased labeling of renal [^{14}C]FMN was secondary to an increased rate of degradation of FAD to FMN (15), then the animals administered aldosterone in tandem with the analogues should have had as high a level of renal [^{14}C]FMN as the group given aldosterone alone. As already noted, this was not the case. The lower amount of renal [^{14}C]FMN in the group administered aldosterone and analogue compared to aldosterone alone could not have been a result of the effect of the analogues on the conversion of riboflavin to FMN because the analogues by themselves did not lower the amount of renal [^{14}C]FMN below that of the control group. Thus it would appear that the effect of aldosterone was to directly increase the conversion of renal [^{14}C]riboflavin to [^{14}C]FMN and [^{14}C]FAD. It was this increment in renal [^{14}C]FMN formation that was subject to inhibition by the appropriate dose levels of the riboflavin analogues.

The present investigation was undertaken to examine the possible cause-effect relationship between the incorporation of [^{14}C]riboflavin into renal [^{14}C]FMN and [^{14}C]FAD, and the urinary excretion of Na^+ and K^+ after the administration of aldosterone to bilaterally adrenalectomized rats. Edelman and Fanestil (1) have inferred that the effect of aldosterone might be to increase the activities of some enzymes of the citric-acid cycle and the mitochondrial flavoprotein-linked NADH dehydrogenase. Several reports on the enzymic stimulating property of aldosterone have appeared (16) and it was concluded that the effect of aldosterone was dependent upon the increased enzymic activities of the citrate cycle. As well, oxythiamine, an inhibitor of the conversion of pyruvate to acetyl CoA, blocked the pyruvate-dependent effect of aldosterone (1). A number of enzymes require FMN or FAD as a coenzyme, includ-

ing mitochondrial NADH dehydrogenase (17), lipoamide dehydrogenase (EC 1.6.4.3) (18) from pyruvate dehydrogenase (EC 1.2.4.1) (19) and α-ketoglutarate dehydrogenase (EC 1.2.4.2) (19, 20), and α-glycerophosphate dehydrogenase (EC 1.1.99.5) (21, 22). Because flavoprotein enzymes are generally stabilized against denaturation by their coenzymes (11), the lack of adequate coenzyme formation in adrenalectomized animals would be expected to limit the accumulation of these flavoprotein holoenzymes as a group. Thus, among the various mechanisms by means of which aldosterone enhances the active reabsorption of Na^+ in the kidney, one should also consider an increased formation of flavin coenzymes and the consequent effects upon the activities of flavin-dependent enzymes.

That a causal relationship appeared to exist between the increased incorporation of [14C]riboflavin into renal [14C]FMN and [14C]FAD, and the reduction in the urinary excretion of Na^+ after aldosterone administration to the adrenalectomized rats, was shown by the inverse relationship between the increase in urinary output of Na^+ and the decrease in the formation of renal [14C]FMN when the animals were treated with aldosterone plus riboflavin analogue compared with aldosterone alone (Figs. 1–4). That the riboflavin analogues were counteracting the effect of aldosterone on the decreased urinary output of Na^+ and the increased incorporation of [14C]riboflavin into renal [14C]FMN and were not evoking an effect by themselves, was demonstrated by the fact that the analogues alone had no significant influence on urinary Na^+ and K^+ output, or on the formation of renal [14C]FMN and [14C]FAD, at the dose levels that we investigated, when compared with the control group of adrenalectomized animals. Consequently, it would appear that the increased incorporation of [14C]riboflavin into renal [14C]FMN and [14C]FAD, observed after aldosterone administration to adrenalectomized rats, was a causal factor in the active reabsorption of Na^+ in the kidney as a result of aldosterone.

Let us consider the possibility that the observed effect on FMN- and FAD-labeling is a consequence of increased oxidative metabolism secondary to the stimulation of Na^+ transport by aldosterone, rather than a primary hormonal effect. If this were so, then when the animals were administered aldosterone in tandem with the appropriate dose levels of one or the other of the two riboflavin analogues which abolished the increment in renal [14C]FMN formation observed as a result of aldosterone treatment, the amount of urinary Na^+ excreted should have been the same as found in the group administered aldosterone alone because the primary effect of the hormone would have been to stimulate Na^+ transport. This, however, was never observed; the urinary Na^+ output was identical to that of the con-

trol group. Consequently, it would appear that the primary effect of aldosterone is to increase the formation of renal [14C]FMN and [14C]FAD which then leads to an increase in Na^+ reabsorption.

It is intriguing that at the higher-dose levels of 7,8-dimethyl-10-formylmethyl isoalloxazine (30 and 35 μg/100 g body wt) and 7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine (25 and 30 μg/100 g body wt), these analogues given in tandem with aldosterone did not significantly alter the urinary output of Na^+ nor the formation of renal [14C]FMN as a result of aldosterone administration alone. This phenomenon may be explained by the findings of Ogunmodede and McCormick (23), who showed that analogues of riboflavin which cannot function in place of the natural vitamin may enhance utilization of riboflavin by decreasing the excretory loss and (or) metabolic destruction of the vitamin itself. Consequently, these higher-dose levels of our two analogues may, in effect, allow more [14C]riboflavin to be available for the enzyme flavokinase and thus reverse the competitive inhibition at this enzymic point by these two analogues. Each rat received ≅60 μg of riboflavin in the 5.0 μCi of [14C]riboflavin/200 g body wt. This amount of riboflavin is approximately twice the minimum daily requirement of a rat corresponding to our experimental animal (24, 25). Both of our riboflavin analogues had their peak effect against the aldosterone-induced increase in the formation of renal [14C]FMN (and decrease in urinary Na^+ excretion) at around 20 μg/100 g body wt. It is worth noting in this regard that the Michaelis constant value for riboflavin is 12.0 μM and the Inhibitor constant values for 7,8-dimethyl-10-formylmethyl isoalloxazine and 7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine are 9.7 and 6.8 μM, respectively (6); this is to say that there are similar affinities of all three compounds for the flavokinase.

The dose levels at which we have used our two riboflavin analogues should not present any problem of acute or chronic toxicity. In studies at the Upjohn Company the estimated mean lethal dose of 7,8-dimethyl-10-(2'-hydroxyethyl) isoalloxazine, when administered i.p. to mice, was in excess of 1,000 mg/kg body wt.[4] In rats, doses of 20 mg/kg body wt gave a response in decreased weight gain but no other gross evidence of riboflavin deficiency was noted. Observations with 7,8-dimethyl-10-formylmethyl isoalloxazine were essentially equivalent to those with the hydroxyethyl derivative.[5]

An interesting point worth mentioning is that although the analogues were supplied as chemically prepared

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[4] Riboflavin Analog U-2113. 1953. The Upjohn Company. In House Report.
[5] Riboflavin Analog U-1003. 1953. The Upjohn Company. In House Report.

derivatives of riboflavin, both occur as natural catabolites of the vitamin, and are excreted into urine and milk. Presumably, they arise as a result of bacterial degradation, especially in rumen where microfloral activity is high (26, 27).

In adrenalectomized animals, increased excretion of Na^+ and a decreased K^+ excretion in urine are commonly observed. Aldosterone replacement increases urinary loss of K^+, whereas Na^+ excretion is reduced (Table II), so that the urinary Na^+-K^+ ratio returns to a normal level. Therefore, the concept of an aldosterone-dependent Na^+-K^+ exchange in the distal tubule has been widely accepted. However, the physiological response to aldosterone in the adrenalectomized animal can be divided into two separable responses; namely, the antinatriuretic and the kaliuretic response, and this has been reported for the rat (1, 28) and the dog (29, 30). We found that the two riboflavin analogues at all dose levels investigated exerted no significant effect on the increased urinary excretion of K^+ by aldosterone, whereas they did (at certain dose levels) counteract the effect of aldosterone on the decreased urinary output of Na^+. These observations are certainly compatible with the view that the antinatriuretic and kaliuretic responses to aldosterone can be separated.

The results of this present investigation strongly suggest that the enhanced biosynthesis of renal FMN and FAD may be a causative factor in the increased reabsorption of Na^+ after aldosterone replacement; and, consequently, riboflavin analogues may function as a novel class of anti-mineralocorticoids.

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