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EFFECTS OF ALDOSTERONE, ACTINOMYCIN D, PUROMYCIN AND CYCLOHEXIMIDE ON RNA SYNTHESIS, CARBONIC ANHYDRASE AND ATPASE ACTIVITIES OF THE KIDNEY AND ON URINARY EXCRETION OF SODIUM IN ADRENALECTOMIZED MICE

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SUMMARY

Aldosterone (2 μg/kg) administered to adrenalectomized mice increased the synthesis of RNA from [6-^{14}C]-orotic acid in the kidney and increased the carbonic anhydrase activity but not the microsomal ATPase activity. However, urinary excretion of sodium was decreased 2–4 h after aldosterone administration. Aldosterone increased the specific radioactivity of RNA, but not the overall content of RNA in the kidney. The increase in specific radioactivity is primarily a nuclear RNA. The increase in carbonic anhydrase activity and decrease of urinary excretion of sodium produced by aldosterone were inhibited by actinomycin D (300 μg/kg), puromycin (60 mg/kg) and cycloheximide (1 mg/kg). These findings are consistent with the so called "induction hypothesis"—that is aldosterone would have its major effect in the nucleus, resulting in increased synthesis of RNA and ultimately in increased synthesis of specific enzymes. The results of this study indicate the possibility that kidney carbonic anhydrase may be a mediator of the action of aldosterone on tubular reabsorption of sodium in adrenalectomized mice.

INTRODUCTION

In 1948, Deane *et al.*[1] reported that rats maintained on a low-sodium diet developed hypertrophy of the glomerular zone of the adrenal glands; a sodium-rich diet resulted in atrophy of the glomerular zone. These observations suggested the existence of a mineralocorticoid. Simpson and Tait[2] in their quest for a substance which had mineralocorticoid activity in beef adrenal extracts, finally isolated aldosterone by paper chromatography[3, 4]. Several other studies[5] which preceded or were run simultaneously with those of Simpson *et al.*, led to the discovery of a substance with mineralocorticoid properties, isolated from hog adrenal extracts.

Many hormones are thought to exert their physiological actions by initiating the formation of new RNA followed by the synthesis of proteins (enzymes) which are assumed to mediate the action of hormones in the target tissue. This "induction hypothesis" was applied to the study of the mechanism of action of aldosterone and is currently recognized as a major concept for the action of aldosterone[6–9]. An important factor may be to define the enzymes responsible for the action of aldosterone on tubular sodium transport. Several effects of aldosterone on kidney or toad bladder enzyme systems, for example, (Na^+–K^+)-ATPase[10–16], cytochrome oxidase[17], succinic dehydrogenase[17, 18] and some tricarboxylic acid cycle enzymes[19, 20], have already been reported. Unlike the above enzymes, carbonic anhydrase is highly concentrated in the cortex of kidney[21], especially in its soluble fraction[22, 23], and has been shown to play an important role in the formation of H^+ and on Na^+–H^+ exchange mechanisms in the renal tubule[24]. Recently Mizuhira and Amakawa[25] suggested that carbonic anhydrase is a major factor in the transport of sodium from the tubular lumen into the tubular epithelium in rat kidney. Some relationship between antinatriuresis exerted by aldosterone and kidney carbonic anhydrase activity can therefore be expected. However, no systematic investigations of the early effect of aldosterone on mouse kidney carbonic anhydrase have been reported to date.

In the present experiment, changes of kidney RNA metabolism, kidney carbonic anhydrase and ATPase activities and urinary excretion of sodium after administration of aldosterone and/or inhibitors of RNA and protein synthesis were investigated to determine the physiological significance of carbonic anhydrase in the expression of the sodium retaining effect of aldosterone.

MATERIALS AND METHODS

Animals. Male ddN strain mice bred in our department and weighing 25–30g were used. Bilateral adrenalectomy was carried out under ether anesthesia through the dorsal route 3 days prior to the experiment. The adrenalectomized mice were maintained

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Trivial names: Carbonic anhydrase: carbonate hydrolyase (EC 4.2.1.1.), ATPase: Adenosine triphosphate-phosphohydrolase (EC 3.6.1.3.).

with commercial solid diet (Oriental Co., Japan) containing 104 meq/kg of Na and 107 meq/kg of K with 0.9% saline *ad lib.* as drinking water at a room temperature of about 25°C. Normal mice were maintained with solid diet and tap water *ad lib.* under the same conditions. On the day of experiment, the diet and drinking water were omitted in the early morning and animals were fasted for about 8 h until sacrificed (overnight fasting weakened the animals).

Drugs. Crystalline D-aldosterone (Mann) was dissolved in 95% ethanol and diluted with saline to the desired concentrations. Orotic acid-[6-^{14}C]-monohydrate (RCC, Amersham: specific radioactivity 61 mCi/mmol), actinomycin D (Kosmegen: Merck, Sharp & Dohme), puromycin dihydrochloride (Nutr. Biochem. Corp.) and cycloheximide (Reagent grade) were dissolved in saline. [^{22}Na] (RCC, Amersham: in NaCl form) was diluted with saline. Cortisol acetate (Merck) was suspended in aq. Vehicle No. 1 (Merck). All drugs were injected in 0.1 ml/20g body wt.

Procedures for the assay of bulk RNA. After the administration of [^{14}C]-orotic acid (40 μCi/kg, ip), animals were sacrificed by decapitation and kidneys were removed, cut in pieces, washed well with cold saline and weighed with a torsion balance. Part of the tissue was homogenized in a Potter–Elvehjem type glass–Teflon homogenizer with distilled water. The rest of the tissue was homogenized in 0.25 M sucrose (adjusted to pH 7.4 with 1 M Tris buffer) and differentially fractionated at 2°C according to Schneider[26]; nuclear (700*g* for 15 min, twice), mitochondrial (9000*g* for 15 min, twice), microsomal (ppt. from 105,000*g* for 60 min) and soluble (supernatant from 105,000*g* for 60 min) fractions were obtained. Total RNA in homogenates and in each subcellular fraction was extracted by the modified Schmidt–Thannhauser procedure of Scott *et al.*[27] with the following two modifications: (1) Extraction of tissue lipids was carried out at room temperature to avoid the loss of RNA according to Munro and Fleck[28]. (2) Alkali digestion of sample was carried out with 1 N NaOH, shaking 120 rev./min for 1 h at 37°C in an incubator. The RNA concentration was determined by U.V. absorption at 260 nm. Radioactivity was counted in a liquid scintillation spectrometer (Packard, Tri-Carb, Model 2311) using the dioxane–naphthalene system (Dioxane 1000 ml; Naphthalene 100g; PPO 7g; Dimethyl POPOP 300 mg) according to Kragelund and Dyrbye[29]. Counting efficiencies were determined by external standardization and all results were corrected for background radiation and quenching.

Procedures for the preparation of carbonic anhydrase sample. Immediately after the sacrifice of the animals by decapitation, blood was taken by a micropipet and diluted with distilled water to the desired concentrations. The kidneys were removed, cut in pieces and washed well with cold isotonic sucrose which was then absorbed with blotting paper. After repeating this procedure several times, kidneys were homogenized with 9 vol. of 0.25 M sucrose containing 0.1% sodium deoxycholate and 5 mM Na_2EDTA adjusted to pH 7.4 with 1 M Tris buffer and differentially fractionated as described in the RNA study. The clear supernatant obtained from the 105,000*g* centrifugation for 60 min was used as the enzyme source. The greater part of the erythrocytes contained in kidney homogenate sediments with the nuclear fraction. To account for the erythrocytes destroyed by tissue homogenization, the hemoglobin released into the supernatant fraction was spectrophotometrically examined at 577 nm[30] as oxyhemoglobin (HbO_2). Enzyme activity due to the contaminating blood was calculated from HbO_2 concentration and subtracted from the kidney enzyme activity to obtain the true kidney enzyme activity. In some cases, animals under light ether anesthesia were perfused *via* the abdominal aorta with 0.25 M sucrose containing heparin. After perfusion, kidneys were removed, homogenized and fractionated as described.

Assay of carbonic anhydrase activity. The manometric method of Altschule and Levis[31] was modified to include a boat-shaped glass flask of about 50 ml capacity mentioned by Meldrum and Roughton[32] instead of the usual type flask. The reaction vessel attached to manometer and containing 1 ml of 0.2 M phosphate buffer (pH 6.8) and 0.5 ml of enzyme solution in one compartment and 1 ml of 0.05 M sodium bicarbonate in the other compartment as substrate was stood in an incubator for 8 min at 37°C to obtain temperature equilibration. After the mixing of enzyme with substrate, the vessel was shaken at 100 oscillations/min and the rate of evolution of carbon dioxide was estimated by manometer readings every 30 s for up to 7 min. The details of the assay method have been previously reported[23]. Carbonic anhydrase activity related to unimolecular velocity constants was calculated by the method of Mitchell *et al.*[33] originally described by Guggenheim[34] and was expressed by the difference (K) between the value related to the velocity constant for the reaction in the presence of the enzyme and, that related to the velocity constant of the control reaction. Enzyme activity was indicated as K (velocity constant)/mg protein in the kidney and K/μM HbO_2 in the blood.

Assay of ATPase activity. Kidney microsomal fraction was used as an enzyme source. (Na^+–K^+–Mg^{2+})-ATPase activity was assayed as follows: the enzyme sample was incubated at 37°C for 20 min with an incubation medium which consisted of 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 3 mM ATP (sodium free) and 25 mM Tris–HCl buffer (pH 7.4). Mg^{2+}-ATPase activity was assayed without Na^+ and K^+. Liberated Pi was determined according to the method of Allen[35] with a slight modification described by Nakamura[36]. Enzyme activity was expressed as μmol Pi/mg protein/20 min. The details of this procedure have been reported previously[23].

Protein assay. The amount of protein in enzyme preparations was determined by the Biuret reaction[37] with cryst. BSA (Sigma Chem. Co.) as a protein standard.

Estimation of urinary excretion of sodium. [^{22}Na] was used as tracer. One day prior to the experiment, animals were injected sc with [^{22}Na] (0.5 μCi/0.1 ml/20g body wt.) and individually housed in metabolic cages at room temperature (about 25°C). The next morning, the animals were deprived of diet and drinking water (0.9% saline) and transported to new metabolic cages under which was placed a pan with blotting paper to absorb the urine. Immediately after administration of aldosterone or drugs, 0.9% saline in a vol. of 15 ml/kg was subcutaneously administered. Radioactivity excreted into urine was measured by whole body γ scintillation counting at various times. Residual bladder urine was pushed out by gentle suprapubic pressure and was added to void urine before determination of [^{22}Na] radioactivity. Excretion rate of [^{22}Na] was expressed by count per min (c.p.m.).

RESULTS

Experiments on kidney RNA metabolism. Stimulatory effect of aldosterone on the incorporation of orotic acid into kidney RNA and its inhibition by actinomycin D. The effect of a single injection of aldosterone on the incorporation of [^{14}C]-orotic acid into RNA in kidney homogenates and in its subcellular fractions was examined. In addition, the antagonistic effect of actinomycin D with aldosterone was also studied. Table 1 summarizes the results obtained by homogenates. Specific radioactivity of RNA was significantly increased at 1.5 and 3 h after aldosterone administration without any alterations at 30 min and 5 h post injection. Pre-treatment with actinomycin D at 1 h prior to aldosterone significantly inhibited this effect on RNA. In neither case did aldosterone or actinomycin D affect the total RNA content in kidney homogenates.

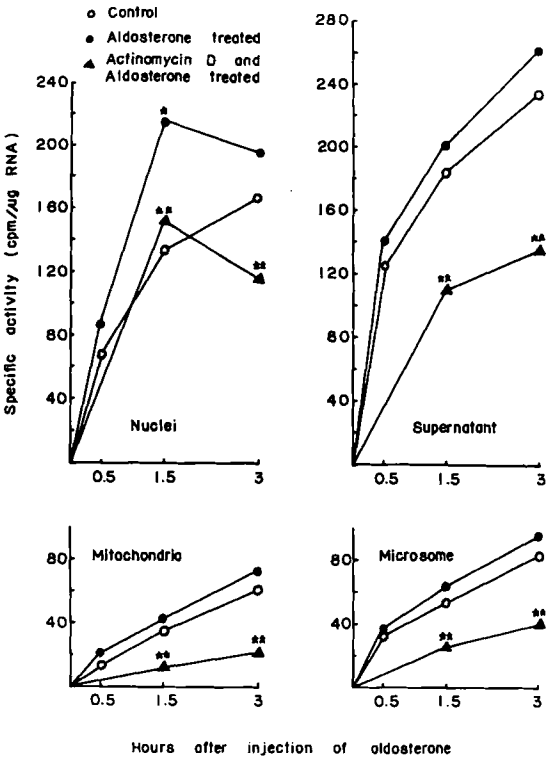

Fig. 1. Time-course of the effect of aldosterone (2 μg/kg, sc) and actinomycin D (300 μg/kg, ip) on RNA specific radioactivity in the kidney subcellular fractions from adrenalectomized mice. Each point represents the mean from several animals whose numbers are indicated in Table 1. *$P < 0.05$ when compared with control. **$P < 0.05$ when compared with aldosterone-treated groups.

The effect of aldosterone and actinomycin D on the incorporation of [^{14}C]-orotic acid into RNA in subcellular fractions from kidney is shown in Fig. 1.

Table 1. Effect of aldosterone and aldosterone + actinomycin D on the specific radioactivity of RNA and the total RNA content in kidney homogenates from adrenalectomized mice

Time (h) after injection	Group	No. of mice	RNA specific activity (c.p.m./μg RNA)	RNA amount (mg/g wet wt.)
0.5	Control	6	73 ± 7	5.20 ± 0.22
	Aldosterone	6	83 ± 7	5.09 ± 0.48
1.5	Control	10	107 ± 10	5.35 ± 0.10
	Aldosterone	10	127 ± 13*	5.41 ± 0.30
	Act. D + Ald.	8	52 ± 12†	5.32 ± 0.42
3	Control	10	141 ± 13	5.09 ± 0.36
	Aldosterone	10	168 ± 21*	5.06 ± 0.37
	Act. D + Ald.	8	77 ± 20†	5.08 ± 0.18
5	Control	8	148 ± 13	5.10 ± 0.16
	Aldosterone	8	165 ± 25	5.00 ± 0.25
	Act. D + Ald.	8	84 ± 13†	4.78 ± 0.25

Aldosterone (Ald. 2 μg/kg, sc) and [^{14}C]-orotic acid (40 μCi/kg, ip) were injected at the same time. Actinomycin D (Act. D 300 μg/kg, ip) was injected 1 h before aldosterone injection. Animals were sacrificed at various times after aldosterone injection. Each value represents the mean ± S.D.

* $P < 0.05$ when compared with controls.

† $P < 0.05$ when compared with the aldosterone-treated groups.

Table 2. Effect of the single administration of actinomycin D or cortisol on the incorporation of [^{14}C]-orotic acid into kidney RNA from adrenalectomized mice

Group	No. of mice	Homogenate RNA (mg/g wet wt.)	Specific radioactivity (c.p.m./μg RNA)				
			Homogenate	Nuclei	Mitochondria	Microsome	Supernatant
Control	7	5.29 ± 0.36	123 ± 21	132 ± 23	48 ± 12	61 ± 12	187 ± 39
Actinomycin D 300 μg/kg	7	4.94 ± 0.29	72 ± 6*	84 ± 10*	22 ± 10*	26 ± 5*	123 ± 21*
Control	6	5.32 ± 0.39	140 ± 25	196 ± 36	59 ± 10	79 ± 17	229 ± 55
Cortisol 1 mg/kg × 2	7	5.36 ± 0.36	150 ± 20	195 ± 48	76 ± 13	95 ± 15	225 ± 49

Actinomycin D was injected ip 1 h before orotic acid administration.
Cortisol was administered sc once daily for two days from the 2nd day after adrenalectomy.
All the animals were sacrificed 3 h after orotic acid administration.
Each value represents the mean ± S.D.
* $P < 0.05$ when compared with control.

A maximum increase of specific radioactivity of nuclear RNA by aldosterone was found at 1.5 h with a subsequent rapid decrease, whereas the specific radioactivity of RNA in the other fractions increased gradually until 3 h after aldosterone administration. Actinomycin D injected at 1 h prior to aldosterone administration inhibited the incorporation of [^{14}C]-orotic acid into RNA in all fractions. In another experiment (data not presented), 5 μg/kg of aldosterone also increased the [^{14}C]-orotic acid incorporation into kidney nuclear RNA at 1 h after injection without any effect in the homogenate or subcellular fractions.

Table 2 shows the effect of a single injection of actinomycin D and cortisol on RNA specific radioactivity in kidney homogenate and the subcellular fractions at 3 h after orotic acid injection. Actinomycin D significantly inhibited the [^{14}C]-orotic acid incorporation into RNA in homogenates and in all of the subcellular fractions. However, RNA content in homogenates or in each subcellular fraction was not affected by actinomycin D. Pretreatment with cortisol

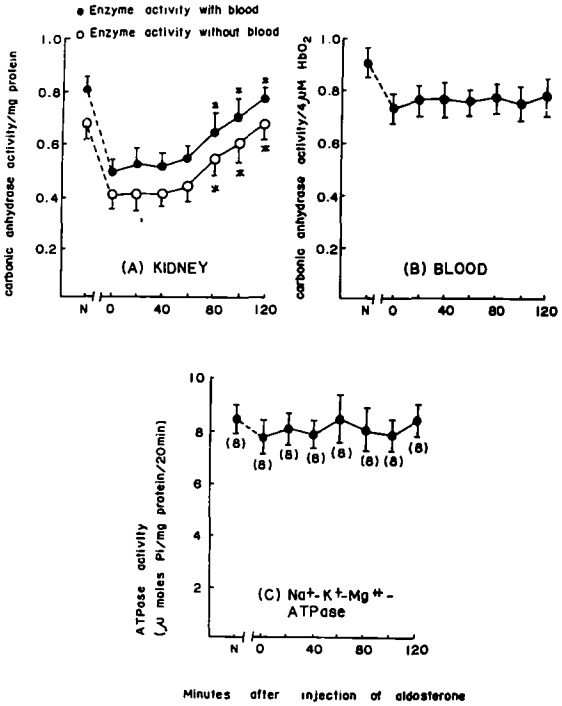

Fig. 2. Time-course of the effect of aldosterone on carbonic anhydrase and ATPase activities up to 2 h after injection. In Fig. (A), (●——●); kidney enzyme activity containing the enzyme activity due to contaminated blood. (○——○); true kidney enzyme activity (see text). Each point with vertical line or lines represents the mean ±S.D. Numbers in parentheses represent the number of animals. N indicates the normal value. *$P < 0.05$ when compared with 0 h value.

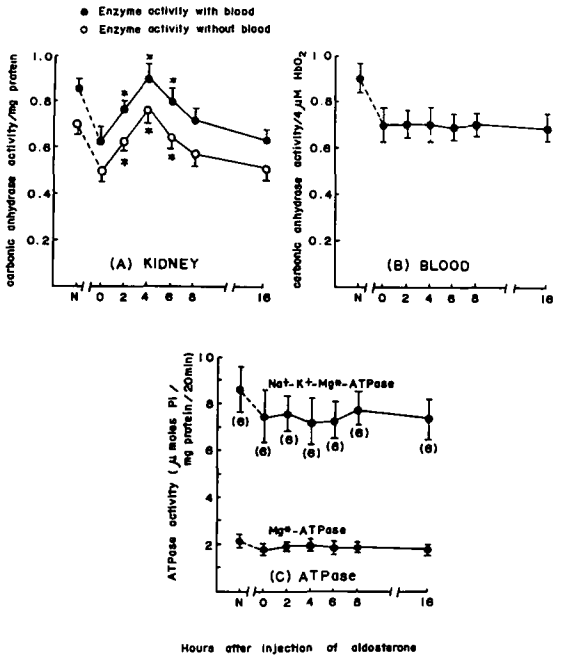

Fig. 3. Time-course of the effect of aldosterone on carbonic anhydrase and ATPase activities until 16 h after injection. In Fig. (A), (●——●); kidney enzyme activity containing the enzyme activity due to contaminated blood. (○——○); true kidney enzyme activity (see text). Each point with vertical line or lines represents the mean ±S.D. Numbers in parentheses represent the number of animals. N indicates the normal value. *$P < 0.05$ when compared with 0 h value.

did not show any effect on specific radioactivity of RNA in homogenates and subcellular fractions. Elevation of kidney nuclear RNA synthesis by aldosterone seems to be specific for its mineralocorticoid activity (Fig. 1).

Experiments on kidney enzyme systems. Effect of aldosterone on kidney carbonic anhydrase and ATPase activities. In Fig. 2, adrenalectomized animals were injected sc with 2 μg/kg of aldosterone and sacrificed at 20 min intervals up to 120 min. Kidney carbonic anhydrase activity which decreased with adrenalectomy was unaltered until 60 min after aldosterone treatment. Significant elevation of enzyme activity was initiated at 80 min after aldosterone and continued until 120 min. Blood carbonic anhydrase activity and kidney microsomal ATPase activity were not affected by aldosterone at any time. Figure 3 shows the changes of carbonic anhydrase and ATPase activities at longer time periods after aldosterone (2 μg/kg). The maximum increase of kidney carbonic anhydrase activity by aldosterone was observed at 4 h with a gradual return to control levels thereafter. Blood carbonic anhydrase and kidney microsomal ATPase activities were not altered after the treatment of aldosterone. As shown in Table 3, 0.5 μg/kg of aldosterone also increased kidney carbonic anhydrase activity, whereas kidney microsomal $(Na^+–K^+–Mg^{2+})$-ATPase activity had a tendency to decrease by a larger dose of aldosterone. Blood has the highest carbonic anhydrase activity and its contamination may have some influence on kidney carbonic anhydrase activity. From the above results, however, it may be postulated that blood carbonic anhydrase bears no relationship to the elevation of kidney carbonic anhydrase activity by aldosterone.

Effect of actinomycin D on aldosterone-induced elevation of kidney carbonic anhydrase activity. Adrenalectomized animals were injected with aldosterone (2 μg/kg, sc), either alone or in combination with actinomycin D (300 μg/kg, ip), and sacrificed at the various times indicated in Fig. 4. In Fig. 4A, aldosterone alone elevated kidney carbonic anhydrase activity at 1.5 and 3 h after injection. Actinomycin D itself did not affect the enzyme activity, but inhibited the stimulatory effect of aldosterone on kidney carbonic anhydrase activity when administered prior to aldosterone administration. Actinomycin D injected at 1.5 h after aldosterone also diminished the stimulatory effect of aldosterone on enzyme activity. In Figs. 4B and 4C, single or combined administration of aldosterone and actinomycin D had no significant effect on blood carbonic anhydrase and kidney ATPase activities. In other experiments (data not presented), a single injection of actinomycin D (300 μg/kg, ip) to adrenalectomized mice had no effect on kidney carbonic anhydrase or on kidney microsomal $(Na^+–K^+–Mg^{2+})$-ATPase and Mg^{2+}-ATPase activities at 3 h after injection.

Effect of aldosterone and puromycin, alone or in combination, on carbonic anhydrase and ATPase activities. Table 4 indicates the changes in kidney and blood carbonic anhydrase and kidney ATPase activities at 4 h after injection of aldosterone and puromycin. In the kidney, elevation of carbonic anhydrase activity by single or repeated administration of aldosterone was blocked by combined administration with puromycin. Puromycin alone had no effect on the enzyme activity. In blood, carbonic anhydrase activity was increased by repeated administration of aldosterone. This elevation may be due to an improvement of body conditions. Aldosterone and puromycin, alone or in combination, had no effect on the activities of $(Na^+–K^+–Mg^{2+})$-ATPase, $(Na^+–K^+)$-ATPase or Mg^{2+}-ATPase.

Effect of aldosterone and cycloheximide, alone or in combination, on carbonic anhydrase and ATPase activities. Table 5 indicates the changes of kidney carbonic anhydrase and ATPase activities at 4 h after administration of aldosterone and cycloheximide. Carbonic anhydrase activity was significantly elevated by aldosterone; this elevation was blocked by combined administration with cycloheximide, whereas, cyclo-

Table 3. Effect of the dose of aldosterone on kidney and blood carbonic anhydrase (CA) and kidney ATPase activities in adrenalectomized mice

Group	No. of mice	CA activity (K)		ATPase activity/mg protein	
		Kidney: K/ mg protein	Blood: K/ 2 μM HbO_2	$(Na^+–K^+–Mg^{2+})$-ATPase	Mg^{2+}-ATPase
Normal	7	0.65 ± 0.05	0.75 ± 0.04	8.24 ± 1.02	1.93 ± 0.31
Adrenalectomized	7	0.42 ± 0.07*	0.50 ± 0.07*	7.85 ± 0.68	2.01 ± 0.11
+ Aldosterone 0.5 μg/kg	6	0.60 ± 0.06†	0.50 ± 0.06	7.30 ± 1.15	1.99 ± 0.13
+ Aldosterone 20 μg/kg	7	0.65 ± 0.05†	0.50 ± 0.08	6.23 ± 0.90†	1.96 ± 0.20

Carbonic anhydrase activity (K) indicates the velocity constant obtained from the enzyme reaction (see text).

Kidney carbonic anhydrase activity represents the true enzyme activity obtained by the subtraction of contaminating blood carbonic anhydrase activity (see text).

Animals were injected sc with aldosterone and sacrificed 3 h later.

Each value represents the mean ± S.D.

* $P < 0.05$ when compared with normal.

† $P < 0.05$ when compared with adrenalectomized group.

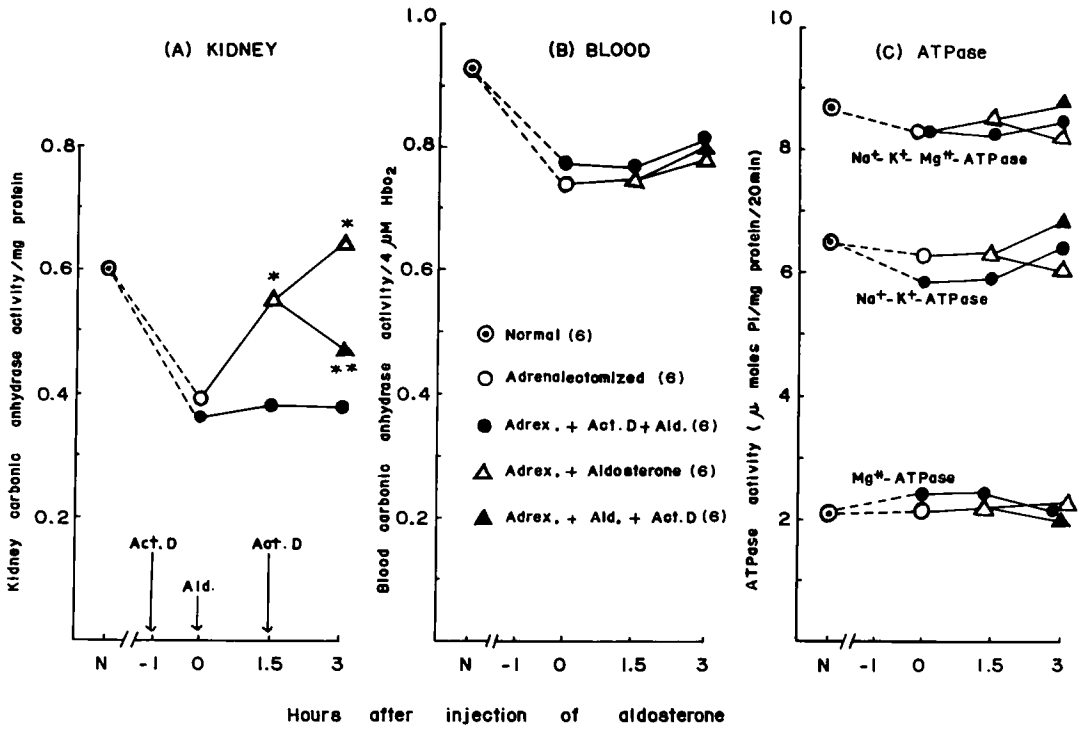

Fig. 4. Time-course of the effect of aldosterone and actinomycin D, alone or in combination, on carbonic anhydrase from kidney and blood and on kidney microsomal ATPase activities. Actinomycin D was injected ip 1 h prior to aldosterone administration. Kidneys were perfused with 0.25 M sucrose until red color disappeared. Each point represents the mean value. Numbers in parentheses represent the number of animals. N indicates the normal value. *$P < 0.05$ when compared with adrenalectomized group. **$P < 0.05$ when compared with adrenalectomized and aldosterone treated group.

heximide alone had no effect on the enzyme activity. Both aldosterone and cycloheximide had no significant effect on ATPase activities.

Experiments on urinary excretion of sodium. Urinary excretion of [^{22}Na] in normal, adrenalectomized and aldosterone-treated mice. Figure 5A indicates the cumulative excretion of [^{22}Na] in urine in normal and adrenalectomized mice at various times after NaCl loading. Urinary [^{22}Na] excretion rates in adrenalectomized mice were significantly higher than that in normal mice. Figure 5B shows the excretion of [^{22}Na] in urine at various times after single injections of aldosterone in doses of 0.5 and 2 μg/kg. At 1 h after aldosterone administration, urinary excretion of [^{22}Na] showed no difference between control and aldosterone treated groups. At 2 h after aldosterone

Table 4. Effect of aldosterone and puromycin, alone or in combination, on kidney and blood carbonic anhydrase (CA) and ATPase activities in adrenalectomized mice

Group	No. of mice	CA activity (K)		ATPase activity/mg protein	
		Kidney: K/ mg protein	Blood: K/ 2 μM HbO_2	$(Na^+-K^+-Mg^{2+})$-ATPase	Mg^{2+}-ATPase
1. Normal	5	0.69 ± 0.05	0.70 ± 0.03	8.91 ± 1.16	2.04 ± 0.18
2. Adrenalectomized	6	0.45 ± 0.04*	0.48 ± 0.02*	8.26 ± 1.14	1.97 ± 0.16
3. + Puromycin 60 mg/kg, ip	5	0.45 ± 0.06	0.48 ± 0.06	8.02 ± 1.21	2.10 ± 0.09
4. + Ald. 2 μg/kg × 1, sc	5	0.65 ± 0.03†	0.47 ± 0.03	8.15 ± 1.30	2.10 ± 0.12
5. + Ald. 2 μg/kg × 1 + Puromycin 60 mg/kg	5	0.50 ± 0.06‡	0.45 ± 0.02	8.04 ± 0.88	1.89 ± 0.16
6. + Ald. 10 μg/kg × 3, sc	5	0.73 ± 0.08†	0.62 ± 0.05†	7.97 ± 1.25	1.81 ± 0.05
7. + Ald. 10 μg/kg × 3 + Puromycin 60 mg/kg	6	0.48 ± 0.05§	0.61 ± 0.04	8.70 ± 0.59	2.04 ± 0.16

In groups 5 and 7, animals were injected with aldosterone and puromycin at the same time and were sacrificed 4 h later. In groups 6 and 7, aldosterone was injected once daily for 3 days and animals were sacrificed 4 h after the least injection. Kidneys were perfused with 0.25 M sucrose containing heparin. Each value represents the mean ± S.D.

* $P < 0.05$ when compared with normal group.
† $P < 0.05$ when compared with adrenalectomized group.
‡ $P < 0.05$ when compared with aldosterone (2 μg/kg) treated group.
§ $P < 0.05$ when compared with aldosterone (10 μg/kg × 3) treated group.

Table 5. Effect of aldosterone and cycloheximide, alone or in combination, on kidney carbonic anhydrase and ATPase activities in adrenalectomized mice

Group	No. of mice	Carbonic anhydrase activity/mg protein	ATPase activity/mg protein	
			$(Na^+-K^+-Mg^{2+})$-ATPase	Mg^{2+}-ATPase
Adrenalectomized	5	0.56 ± 0.02	8.51 ± 1.43	2.05 ± 0.23
+ Ald. 2 μg/kg, sc	6	0.74 ± 0.03*	8.29 ± 1.17	1.96 ± 0.23
+ Cyclo. 1 mg/kg, ip	6	0.55 ± 0.05	8.51 ± 1.67	2.10 ± 0.32
+ Ald. 2 μg/kg + Cyclo. 1 mg/kg	5	0.51 ± 0.04†	8.12 ± 0.77	2.14 ± 0.25

Animals were injected with aldosterone (Ald.) and cycloheximide (Cyclo.), alone or in combination, at the same time and were sacrificed 4 h later.

Kidneys were perfused with 0.25 M sucrose containing heparin. Each value represents the mean ± S.D.

* $P < 0.05$ when compared with adrenelectomized group.

† $P < 0.05$ when compared with aldosterone-treated group.

administration, however, [^{22}Na] excretion was decreased. The maximum inhibitory effect of aldosterone on [^{22}Na] excretion was observed from 2 to 4 h after administration. [^{22}Na] excretion between 4–6 h after aldosterone showed no difference compared with that in controls.

Antagonistic effect of actinomycin D on aldosterone-induced inhibition of urinary excretion of [^{22}*Na*]. As shown in Fig. 6, excretion of [^{22}Na] into urine was significantly increased from an early stage after combined administration of actinomycin D and aldosterone. The most significant effect of actinomycin D was observed between 1 to 2 h after aldosterone (between 2 to 3 h after actinomycin D treatment). According to another experiment (data not presented), actinomycin D alone had no significant effect on urinary excretion of [^{22}Na] in adrenalectomized mice until 4 h later.

Antagonistic effect of puromycin and cycloheximide on aldosterone-induced inhibition of urinary excretion of [^{22}*Na*]. Urinary excretion of [^{22}Na] after combined administration of aldosterone (2 μg/kg, sc) with puromycin (60 mg/kg, ip) and cycloheximide (1 mg/kg, ip) was examined. Puromycin and cycloheximide were injected at the same time with aldosterone. In Fig. 7A, urinary excretion of [^{22}Na] was significantly increased by combined administration of puromycin and hormone. The most pronounced changes were observed between 2–4 h after puromycin. Fig. 7B shows the effect of cycloheximide. A significant increase in urinary excretion of [^{22}Na] was observed in the 2–4 h period after cycloheximide. According to another experiment (data not presented), puromycin and cycloheximide alone had no significant effects on urinary excretion of [^{22}Na] in adrenalectomized mice.

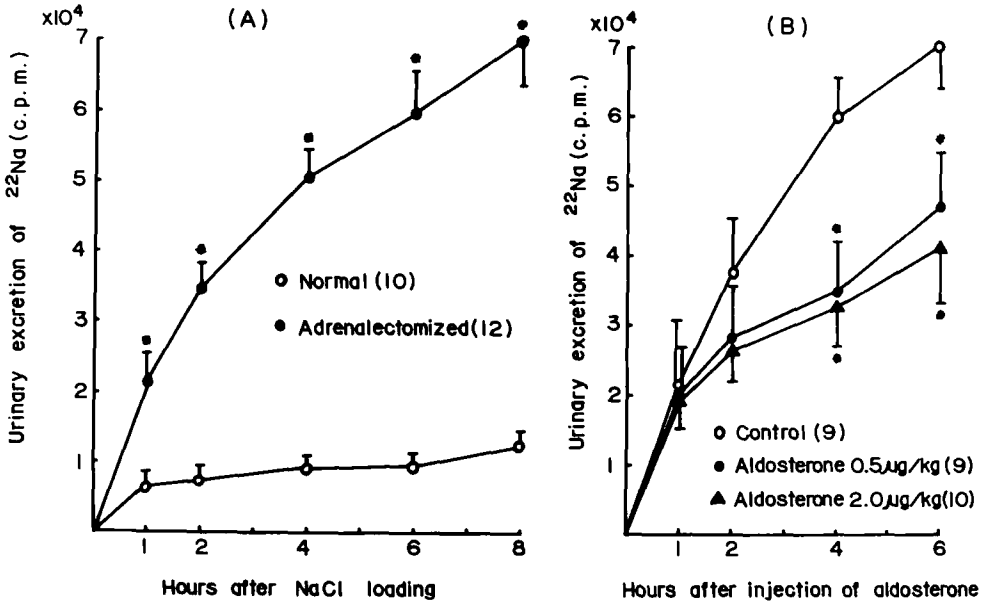

Fig. 5. Time-course of excretion of [^{22}Na] in urine. (A) Urinary excretion of [^{22}Na] in normal and adrenalectomized mice at various times after NaCl loading. (B) Urinary excretion of [^{22}Na] in adrenalectomized mice at various times after aldosterone treatment. Each point with vertical line represents the mean ±S.E.M. Numbers in parentheses indicate the number of animals. *$P < 0.05$ when compared with control.

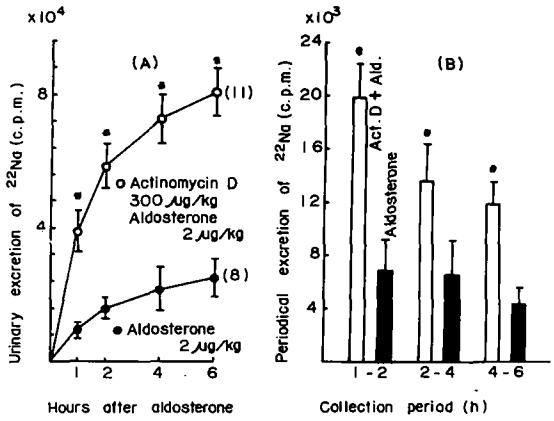

Fig. 6. Effect of the combined administration of actinomycin D with aldosterone on urinary excretion of [^{22}Na] at various times (A) and in each collection period (B). Actinomycin D was administered 1 h prior to aldosterone administration. The height of the point or bar with vertical line or lines represents the mean ±S.E.M. Numbers in parentheses represent the number of animals. $*P < 0.05$ when compared with aldosterone alone treated group.

DISCUSSION

According to the general "induction hypothesis" applied to aldosterone, a 1–2 h latent period is seen prior to the onset of the effect of this hormone on urinary excretion of sodium in mammals *in vivo* and on membrane transport of sodium in toad bladder *in vitro*. This time seems to be necessary for the synthesis or activation of intermediates involved in active sodium transport[8]. In the present experiment, a stimulatory effect of aldosterone on nuclear RNA synthesis and carbonic anhydrase activity of the kidney appeared at about 1 and 1.5 h, respectively, after treatment. Inhibition of urinary excretion of sodium by aldosterone administration appeared about 2 h later. The relationship between the above three parameters seems to agree quite well with the "induction hypothesis" concept.

In adrenalectomized animals, increased excretion of sodium and a decrease of potassium excretion in urine are commonly observed. Aldosterone replacement increases urinary loss of potassium whereas sodium excretion is reduced, so that 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. The tubular Na^+–H^+ exchange mechanism remains unclear; the following mechanism is presented as an explanation for the Na^+–H^+ exchange phenomenon[24, 38]. In the proximal and distal tubule, carbon dioxide is hydrated by carbonic anhydrase to form H_2CO_3 which dissociates to H^+ and HCO_3; H^+ moves from the interior of the cells to the tubular lumen in exchange for Na^+ derived from filtered $NaHCO_3$ in the proximal tubule and from Na_2HPO_4 in the distal tubule. In the present experiment, aldosterone activates kidney carbonic anhydrase activity *via* RNA synthesis. According to our inference, activation of kidney carbonic anhydrase may increase the supply of H^+ to the hydrogen secretory pump followed by the enhancement of Na^+–H^+ exchange in renal tubule. Kidney glutaminase is known to be an enzyme associated with H^+ excretion into urine. However, the enzyme activity is unaltered after adrenalectomy and mineralocorticoid replacement[39–41]. Glutaminase may have no relationship with the exchange mechanism induced by aldosterone.

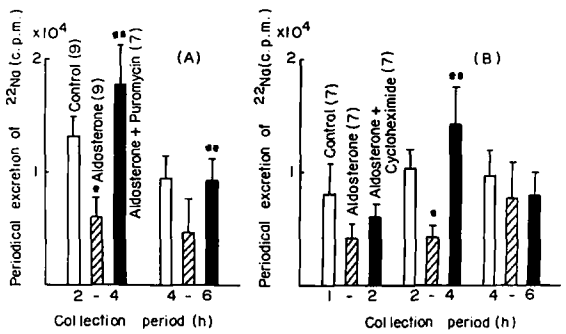

Fig. 7. Effect of the combined administration of puromycin (A) and cycloheximide (B) with aldosterone on urinary excretion of [^{22}Na] at each collection period. The height of the bar with vertical line represents the mean ±S.E.M. Numbers in parentheses represent the number of animals. $*P < 0.05$ when compared with control. $**P < 0.05$ when compared with aldosterone alone treated group.

Traditionally, natural adrenocorticoids have been classified as mineralocorticoids (aldosterone and DOC) and glucocorticoids (cortisol, cortisone and corticosterone). A mineralocorticoid has its main effect on sodium retention, whereas glucocorticoids influence carbohydrate, protein and fat metabolism. However, cortisol and corticosterone have weak and modest but significant activities on sodium retention. Therefore, to examine the effect of glucocorticoids on kidney RNA metabolism and carbonic anhydrase activity may be necessary to postulate the specific role of aldosterone on them. Cortisol alone had no effect on kidney RNA synthesis (Table 2). Cortisol and corticosterone have no relationship with mouse kidney carbonic anhydrase activity as previously reported[23, 42]. Therefore, increases of both RNA synthesis and carbonic anhydrase activity in the kidney and the antinatriuresis induced by aldosterone may be due to its mineralocorticoid activity and the glucocorticoid activity may have no connection with them at all.

According to the current concept, actinomycin D is considered to bind with template DNA and interfere with the synthesis of RNA by DNA-dependent RNA polymerase followed by the inhibition of protein synthesis[43, 44]. Puromycin inhibits the amino acid transfer from transfer RNA into ribosome protein followed by the depression of protein synthesis[45, 46]. Cycloheximide is considered to inhibit the protein synthesis by several mechanisms[47–49]. Antagonism of actinomycin D of the aldosterone-stimulated tubular sodium reabsorption in adrenalectomized rats was demonstrated by Castles and Williamson[50]. Wiederholt[51] and Fimognari *et al.*[52]

observed similar results without any effect in potassium excretion. Elevation of membrane transport of sodium by aldosterone and its inhibition by actinomycin D, puromycin and cycloheximide were also reported with toad bladder *in vitro*[53–55]. These results may show that antinatriuresis by aldosterone is dependent on the synthesis of DNA-dependent RNA and kaliuresis by aldosterone is independent of RNA synthesis, though Rodriguez *et al.*[56] recently reported that aldosterone stimulated [^{42}K] uptake in toad bladder *in vitro* and actinomycin D completely abolished this increased uptake produced by aldosterone, which suggested the stimulatory effect of aldosterone on potassium transport in toad bladder *via* DNA-dependent RNA synthesis. Recently Lifschitz *et al.*[57] examined the effect of aldosterone and actinomycin D on urinary excretion of sodium and hydrogen in adrenalectomized dogs and concluded that antinatriuresis by aldosterone is dependent on DNA-dependent RNA synthesis, while increased hydrogen ion excretion by aldosterone may be independent of the synthesis of RNA. Our Na^+–H^+ exchange hypothesis is at variance with the above statement.

In the present experiment, about 40% of the carbonic anhydrase activity in normal mouse kidney disappeared after adrenalectomy. Aldosterone administration returned the enzyme activity to a normal level. This fraction is also influenced by actinomycin D, puromycin and cycloheximide. The residual carbonic anhydrase activity after adrenalectomy is independent of actinomycin D, puromycin and cycloheximide as shown in Fig. 4 and Tables 4, 5. According to our unpublished data, puromycin and cycloheximide inhibit kidney carbonic anhydrase activity in normal mice. These results show the importance of endogenous aldosterone in the maintenance of the kidney carbonic anhydrase system. A similar relationship was also observed in urinary excretion of [^{22}Na] after actinomycin D, puromycin and cycloheximide administration. These phenomena seem to indicate the significance of kidney carbonic anhydrase as a mediator in the action of aldosterone in the "induction hypothesis". Of course data on the actual elevation of carbonic anhydrase synthesis by aldosterone together with the activation of its enzyme activity may be needed to emphasize the role of aldosterone in the "induction hypothesis". These studies are now in progress in our laboratory.

The occurrence of (Na^+–K^+)-ATPase in the kidney has been previously studied[14, 58–61]. Further, inhibition of this enzyme activity and of renal tubular Na reabsorption by cardiac glycosides are parallel[62, 63]. These findings offer clear evidence for the role of the ATPase system in tubular Na^+ reabsorption. However, a single injection of aldosterone to adrenalectomized rats in physiological doses did not show a direct effect on this enzyme system[11, 13] except for the reports by Knox and Sen[15] and Schmidt *et al.*[16] who presented the possibility that (Na^+–K^+)-ATPase might be a target enzyme of the action of aldosterone in adrenalectomized rat kidney. In the present experiment, aldosterone, actinomycin D, puromycin and cycloheximide did not affect (Na^+–K^+)-ATPase activity. Aldosterone may have no relationship with ATPase in mouse kidney.

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