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EXPERIMENTAL STUDIES ON THE CARBONIC ANHYDRASE ACTIVITY—XIV

EFFECT OF ALDOSTERONE AND ACTINOMYCIN D *IN VITRO* ON CARBONIC ANHYDRASE FROM LIVER AND KIDNEY OF MICE

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Abstract—After incubating kidney and liver slices in Krebs–Ringer phosphate solution with or without aldosterone, actinomycin D and cortisol, carbonic anhydrase activity in 77,000 *g* supernatant fraction was examined.

In the kidney, aldosterone increased and actinomycin D decreased the enzymic activity, while cortisol had no effect. In the liver, aldosterone and a smaller dose of actinomycin D increased, while cortisol decreased the enzymic activity. The elevation of renal carbonic anhydrase activity after aldosterone treatment was blocked by the concurrent addition of actinomycin D. The depression of renal carbonic anhydrase activity and the elevation of hepatic carbonic anhydrase activity after actinomycin D treatment were blocked by the concurrent addition of aldosterone.

Smaller doses of aldosterone and actinomycin D were added directly to kidney and liver homogenates and the changes in enzymic activity were examined. Kidney carbonic anhydrase activity was increased by aldosterone and decreased by actinomycin D. Liver enzymic activity was increased by aldosterone and a smaller dose of actinomycin D, although neither drug had a direct effect on the activities of renal and hepatic carbonic anhydrase in 700 *g* supernatant, 77,000 *g* precipitate and 77,000 *g* supernatant fractions.

IT IS now believed that steroid hormones act by stimulation of RNA synthesis followed by formation of specific enzyme protein in target organs.[1–4] Furthermore, the effect of aldosterone on the synthesis of RNA and protein has been reported in toad bladder and rat kidney.[5–8] However, the properties of more recently synthesized enzymes in the kidney after aldosterone treatment have not been fully investigated.

In the kidney, carbonic anhydrase is well known as an important enzyme regulating H^+–Na^+ and K^+–Na^+ exchange mechanism in the renal tubules.[9, 10] Any correlations between aldosterone and carbonic anhydrase may therefore be expected, but have not been established.

In previous reports,[11, 12] we observed that repeated, single administration of aldosterone *in vivo* into normal mice increased renal and hepatic carbonic anhydrase activity, and that concurrent administration of actinomycin D with aldosterone blocked the stimulatory effect of the hormone on enzymic activity. The present experiment was carried out *in vitro* to ascertain the mode of action of aldosterone and actinomycin D on renal and hepatic enzymic activity and to compare the results with those obtained *in vivo*.

MATERIALS AND METHODS

Animals

Adult male ddN strain mice (25–30 g) were used. The animals were fed commercial solid diet (Oriental Co.) and tap water *ad lib.* at room temperature (20°). They were fasted for 24 hr before sacrifice but allowed to drink water.

Drugs used

Actinomycin D (Merck, Sharp & Dohme) was dissolved in saline. D-aldosterone (Mann) was dissolved in 95% ethanol and diluted with saline to adequate concentration. Cortisol acetate (Merck) was suspended in saline. Various doses of these drugs were added to the incubation medium or enzyme preparation in 0·1 ml of each solvent. In the control experiment the solvent only was added.

Experimental procedure

After sacrifice by decapitation, livers and kidneys were removed, sliced and washed well with physiological saline. In each experiment, tissue slices from 40 to 50 animals were pooled and 1·5 g of each tissue was placed in 50-ml flasks containing 10 ml of Krebs–Ringer phosphate solution [KRP, 127 mM NaCl; 5·1 mM KCl; 2·7 mM $CaCl_2$; 1·2 mM KH_2PO_4; 1·2 mM $MgSO_4$ and 10 mM sodium phosphate buffer (pH 7·4)] with or without drugs and incubated under air at 37° with gentle shaking (50 times/min). After incubating for various lengths of time, the tissue slices were washed well with cold saline and 0·25 M sucrose and homogenized in glass-teflon homogenizer with 9 vol. of 0·25 M sucrose contained 0·1% sodium deoxycholate and 5 mM Na_2EDTA adjusted to pH 7·4 with 1 M Tris. The homogenate was centrifuged as described in the previous report[11] and 700 *g* supernatant, 77,000 *g* precipitate (microsomal fraction) and 77,000 *g* supernatant (supernatant fraction) were obtained.

Carbonic anhydrase assay

Carbonic anhydrase activity was measured manometrically according to Altschule and Levis.[13] The details of this procedure were reported in the previous paper.[11] The amount of protein in the enzyme preparation was determined by the Biuret reaction[14] with crystalline bovine serum albumin (Sigma Chem. Co.) used as protein standard.

RESULTS

Effect of actinomycin D (dose–response relation)

Figure 1 shows the effect of actinomycin D in doses ranging from 0·1 to 50 μg/ml incubation medium on supernatant carbonic anhydrase activity from liver and kidney. The incubation of liver and kidney slices without actinomycin D for 6 hr had no effect on enzymic activity compared with normal values. The addition of actinomycin D to incubation medium resulted in a decrease of enzymic activity in renal supernatant fraction and these decreases were almost parallel with increases of doses. However, the enzymic activity in hepatic supernatant fraction was elevated after actinomycin D treatment indicating a maximal increase at a dose of 10 μg/ml, while actinomycin D in a dose of 50 μg/ml failed to affect this enzymic activity.

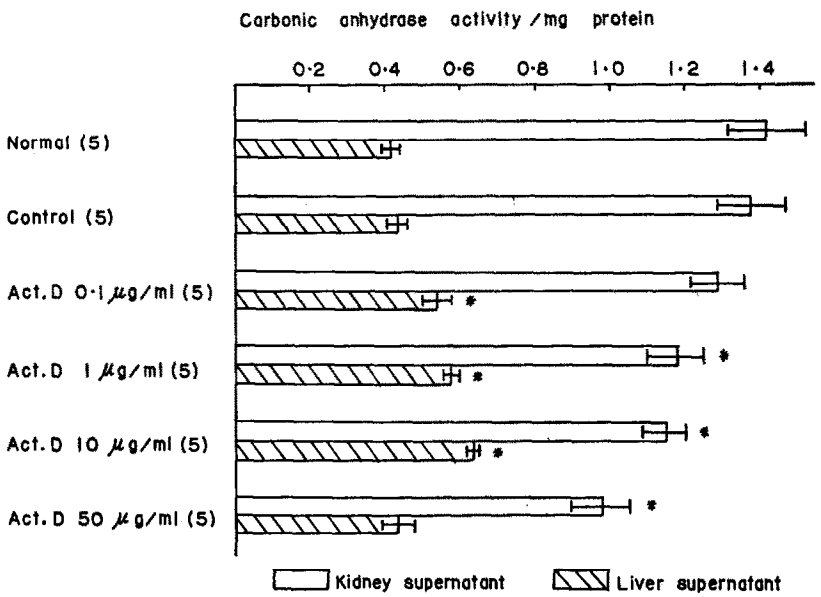

FIG. 1. Effect of various doses of actinomycin D on carbonic anhydrase activity in supernatant fractions from kidney and liver. Kidney and liver slices were incubated in Krebs–Ringer phosphate solution with or without actinomycin D at 37° for 6 hr. Numbers in parentheses represent the number of observations. Each bar represents the mean with standard deviation. * $P < 0{\cdot}05$ (when compared with control values).

Antagonism of actinomycin D to the action of aldosterone in the kidney

Figure 2(A) shows the changes of carbonic anhydrase activity from kidney slices at various times after treatment with aldosterone (1 μg/ml). In the control group, incubation of kidney slices without aldosterone produced no significant change on enzymic activity, while aldosterone in a dose of 1 μg/ml increased the enzymic activity in supernatant fraction gradually and significantly ($P < 0{\cdot}05$) over a 9-hr period. Figure 2(B) shows the concurrent effect of actinomycin D with aldosterone. After incubating kidney slices with aldosterone (1 μg/ml) for 0, 3 and 6 hr, actinomycin D was added to incubation medium. Actinomycin D (10 and 50 μg/ml incubation medium) treated with aldosterone from the beginning of incubation conversely inhibited the enzymic activity below control levels. The addition of actinomycin D (10 and 50 μg/ml) 3 and 6 hr after treatment with aldosterone also inhibited a stimulatory effect of aldosterone with a decrease of enzymic activity below initial levels.

Antagonism of actinomycin D to the action of aldosterone in the liver

Figure 3(A) shows the changes of carbonic anhydrase activity from liver slices at various times after treatment with aldosterone (1 μg/ml). The enzymic activity in supernatant fraction was increased gradually and significantly after aldosterone treatment. Figure 3(B) shows the concurrent effect of actinomycin D with aldosterone. In a group in which actinomycin D was added to incubation medium concurrently with aldosterone from the beginning of incubation, an elevation of enzymic activity by

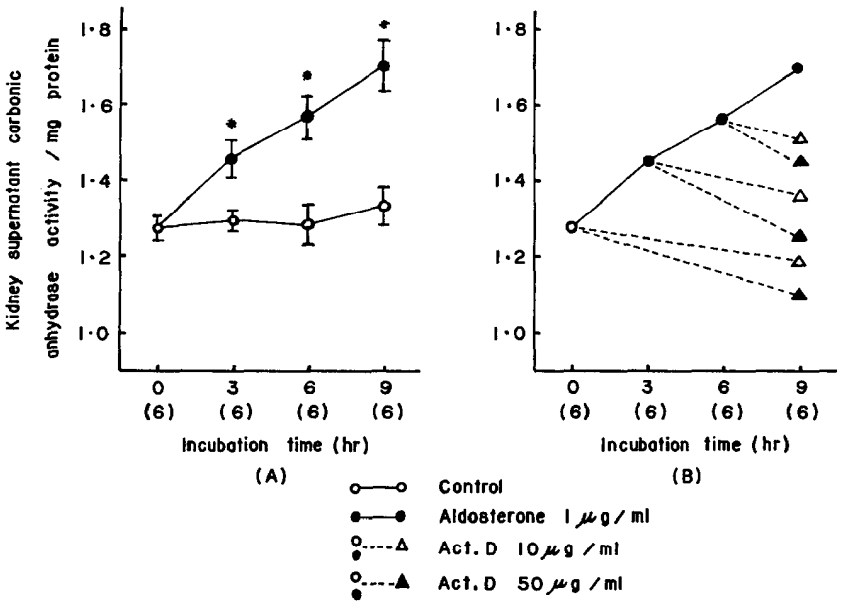

FIG. 2. Effect of aldosterone alone (A) and concurrently with actinomycin D (B) on carbonic anhydrase activity in kidney supernatant fraction. Numbers in parentheses represent the number of observations. In (A), each point with vertical lines represents the mean ± S.D. and in (B), each point represents the mean from six observations. * $P < 0.05$ (when compared with control values).

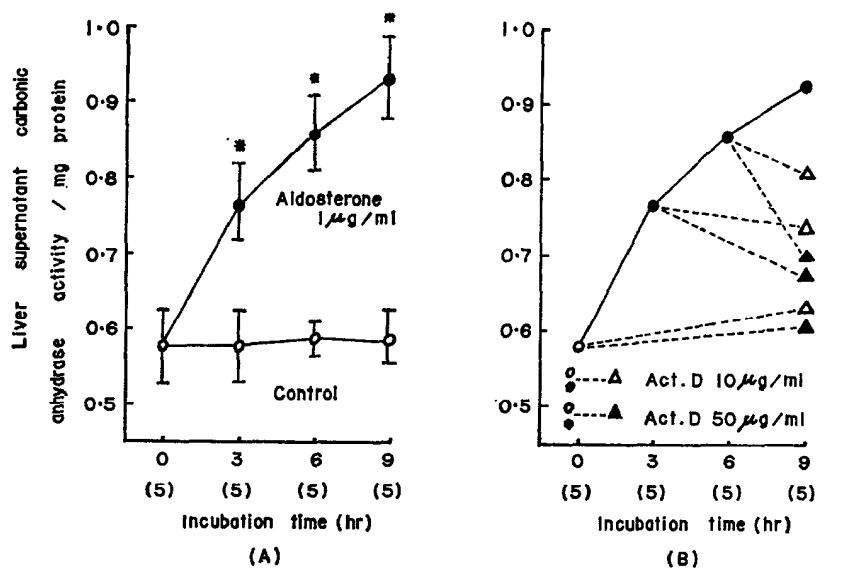

FIG. 3. Effect of aldosterone alone (A) and concurrently with actinomycin D (B) on carbonic anhydrase activity in liver supernatant fraction. Numbers in parentheses represent the number of observations. In (A), each point with vertical lines represents the mean ± S.D. and in (B) each point represents the mean from five observations. * $P < 0.05$ (when compared with control values).

aldosterone alone was inhibited by actinomycin D in normal levels. When actinomycin D (10 and 50 μg/ml) was added to incubation medium 3 and 6 hr after treatment with aldosterone, an elevation of enzymic activity was also inhibited and the decrease of enzymic activity was greater in 50 μg/ml dose treated group.

Antagonism of aldosterone to the action of actinomycin D in the kidney and liver

Effect of actinomycin D treatment, either alone or concurrently with aldosterone, on supernatant carbonic anhydrase activity from kidney and liver slices were examined until 6 hr after incubation. As shown in Fig. 4(A), actinomycin D (20 μg/ml) alone decreased enzymic activity in renal supernatant fraction. Whereas 0·2 μg/ml of aldosterone added 3 hr after treatment with actinomycin D inhibited a decrease of enzymic activity induced by actinomycin D alone. Furthermore, aldosterone in a dose of 1 μg/ml conversely increased this enzymic activity ($P < 0.05$).

Figure 4(B) shows the effect of actinomycin D treatment, either alone or concurrently with aldosterone, on carbonic anhydrase activity in liver supernatant fraction. Actinomycin D (20 μg/ml) alone increased enzymic activity. However, addition of aldosterone (0·2 and 1 μg/ml) 3 hr after treatment with actinomycin D resulted in a decrease of enzymic activity below the initial levels.

Antagonism of actinomycin D to the action of cortisol

Effects of cortisol and actinomycin D treatment on supernatant carbonic anhydrase activity from liver and kidney slices were examined. Liver and kidney slices were

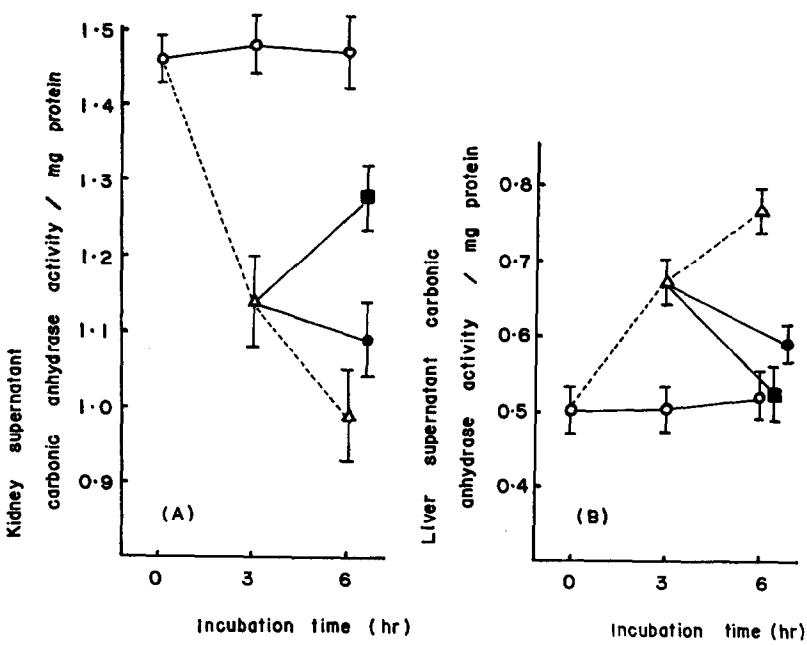

FIG. 4. Effect of actinomycin D treatment, alone or concurrently with aldosterone, on carbonic anhydrase activity in kidney (A) and liver (B) supernatant fractions. Kidney and liver slices were incubated in KRP. Each point with vertical lines represents the mean ± S.D. from five observations. ○ control; ● aldosterone 0·2 μg/ml; ■ aldosterone 1 μg/ml; △ act. D 20 μg/ml.

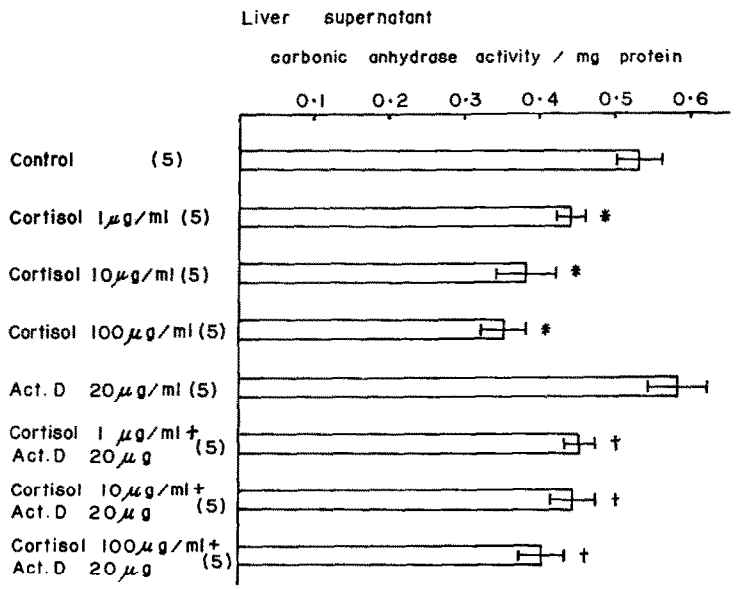

FIG. 5. Effect of cortisol treatment, alone or concurrently with actinomycin D, on carbonic anhydrase activity in liver supernatant fraction. Numbers in parentheses represent the number of observations. Each bar represents the mean with standard deviation. * $P < 0.05$ (when compared with control group). † $P < 0.05$ (when compared with actinomycin D treated group).

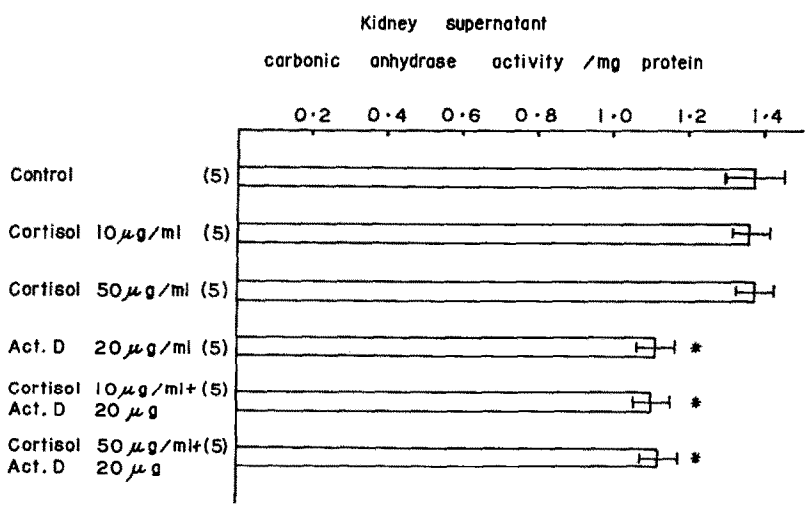

FIG. 6. Effect of cortisol treatment, alone or concurrently with actinomycin D, on carbonic anhydrase activity in kidney supernatant fraction. Numbers in parentheses represent the number of observations. Each bar represents the mean with standard deviation. * $P < 0.05$ (when compared with control group).

incubated in Krebs–Ringer phosphate solution with both drugs, either alone or concurrently for 6 hr, then slices were washed, homogenized, fractionated and the enzymic activity assayed. As shown in Fig. 5, after administration of the cortisol alone decrease in hepatic enzymic activity varied almost linearly with the dose. In groups in which cortisol was treated concurrently with actinomycin D, the enzymic activity was also decreased, although these decreases were slight compared with that of cortisol alone. Figure 6 shows the effect of cortisol and actinomycin D on renal carbonic anhydrase activity. Cortisol, treated either alone or concurrently with actinomycin D, had no effect on enzymic activity in supernatant fraction.

Direct effect of aldosterone and actinomycin D on homogenate carbonic anhydrase activity

In order to ascertain the direct effect of aldosterone and actinomycin D on the enzyme system, livers and kidneys were homogenized with 0·25 M sucrose containing Na_2EDTA and sodium deoxycholate. These homogenates were diluted with above solution in a concentration of 1 mg protein/0·5 ml and incubated in glass test tubes with or without aldosterone and actinomycin D under air at 37° with gentle shaking

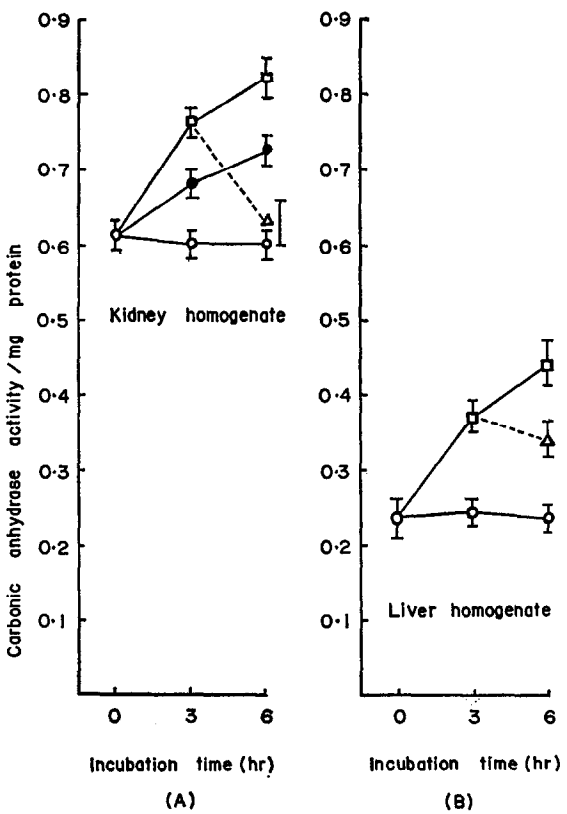

FIG. 7. Effect of aldosterone treatment, either alone or concurrently with actinomycin D, on carbonic anhydrase activity in kidney (A) and liver (B) homogenates. Each point with vertical lines represents the mean ± S.D. from five observations. ○ control; ● aldosterone 2 ng/mg protein; □ aldosterone 20 ng/mg protein; △ actinomycin D in 0·1 μg/mg protein.

(50 times/min) for 3 and 6 hr, then homogenates were cooled and immediately assayed for enzymic activity.

Figure 7(A) shows the changes of enzymic activity in kidney homogenate after aldosterone treatment either alone or concurrently with actinomycin D. Aldosterone (2 ng/mg protein and 20 ng/mg protein) induced increases of renal enzymic activity. When actinomycin D (0·1 μg/mg protein) was added to homogenate 3 hr after treatment with aldosterone (20 ng/mg protein), an increase of enzymic activity induced by aldosterone was inhibited and this enzymic activity was returned to normal levels. Figure 7(B) shows the changes of enzymic activity in liver homogenate after aldosterone treatment either alone or concurrently with actinomycin D. Carbonic anhydrase activity was increased after treatment with 20 ng/mg protein of aldosterone. When actinomycin D (0·1 μg/mg protein) was added to homogenate after treatment with aldosterone for 3 hr, an increase of enzymic activity induced by aldosterone alone was inhibited and this enzymic activity was decreased.

Figures 8(A) and (B) show the effect of actinomycin D treatment, either alone or concurrently with aldosterone, on carbonic anhydrase activity in kidney and liver homogenates. As shown in Fig. 8(A), actinomycin D (0·01 μg/mg protein and 0·1 μg/mg protein) decreased the enzymic activity in kidney homogenate and this decrease was greater in the 0·1 μg treated group. Addition of aldosterone (20 ng/mg protein) to kidney homogenate 3 hr after treatment with actinomycin D resulted in an increase of enzymic activity to normal levels. Figure 8(B) shows the effect of actino-

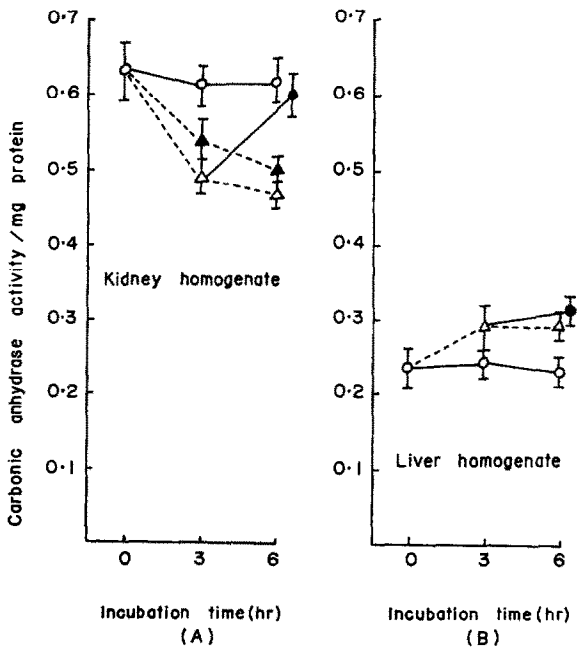

FIG. 8. Effect of actinomycin D treatment, either alone or concurrently with aldosterone, on carbonic anhydrase activity in kidney (A) and liver (B) homogenates. Each point with vertical lines represents the mean ± S.D. from five observations. ○ control; ▲ actinomycin D 0·01 μg/mg protein; △ actinomycin D 0·1 μg/mg protein; ● aldosterone 0·02 μg/mg protein.

mycin D treatment, either alone or concurrently with aldosterone, on carbonic anhydrase activity in liver homogenate. Actinomycin D in a dose of 0·1 μg/mg protein increased enzymic activity slightly but significantly ($P < 0·05$). Addition of aldosterone (20 ng/mg protein) to liver homogenate 3 hr after actinomycin D treatment had no significant effect on this enzymic activity.

Effect of aldosterone and actinomycin D on subcellular fractions

In order to examine the direct effect of aldosterone and actinomycin D on enzymic activities in subcellular fractions from liver and kidney, 700 *g* supernatant, 77,000 *g* precipitate and 77,000 *g* supernatant were added aldosterone and actinomycin D and incubated in glass test tubes under air at 37° with gentle shaking (50 times/min) for 3 and 6 hr. After incubation, solutions were cooled and assayed for enzymic activity. The results are listed in Table 1 and indicated that aldosterone and actinomycin D had no significant effects on carbonic anhydrase activities in any fractions.

TABLE 1. EFFECT OF ALDOSTERONE* AND ACTINOMYCIN D* ON LIVER AND KIDNEY CARBONIC ANHYDRASE ACTIVITY IN SUBCELLULAR FRACTIONS

Liver carbonic anhydrase: Mean ± S.D.† (Enzyme unit/mg protein)			Incubation time (hr)	Kidney carbonic anhydrase: Mean ± S.D.† Enzyme unit/mg protein)		
0	3	6		0	3	6
			700 *g* supernatant			
0·40±0·03	0·40±0·03	0·41±0·03	Control	0·59±0·05	0·59±0·04	0·58±0·06
	0·38±0·05	0·39±0·04	Aldo. 0·02 μg		0·60±0·03	0·59±0·04
	0·40±0·04	0·38±0·04	Act. D 0·1 μg		0·59±0·04	0·58±0·05
			77,000 *g* precipitate			
0·35±0·04	0·34±0·03	0·35±0·03	Control	0·75±0·05	0·73±0·04	0·75±0·06
	0·33±0·04	0·36±0·03	Aldo. 0·02 μg		0·74±0·05	0·77±0·04
	0·35±0·05	0·34±0·03	Act. D 0·1 μg		0·73±0·04	0·75±0·03
			77,000 *g* supernatant			
0·48±0·04	0·48±0·05	0·49±0·03	Control	1·28±0·06	1·26±0·05	1·29±0·06
	0·49±0·04	0·47±0·03	Aldo. 0·02 μg		1·30±0·08	1·26±0·05
0·55±0·05	0·53±0·04	0·54±0·05	Control	1·32±0·09	1·30±0·09	1·31±0·08
	0·55±0·03	0·56±0·02	Act. D 0·1 μg		1·32±0·05	1·30±0·06
	0·53±0·05	0·55±0·04	Act. D 5 μg		1·32±0·07	1·30±0·05

* Aldosterone and actinomycin D indicate a dose per mg protein.
† Each value ± S.D. was obtained from four observations.

DISCUSSION

In the report XIII[12] in this series we examined the effect of aldosterone and actinomycin D *in vivo* on renal and hepatic carbonic anhydrase activity in mice and observed that aldosterone (1–30 μg/kg) increased renal and hepatic enzymic activity. Whereas actinomycin D (50–1000 μg/kg) decreased renal enzymic activity and there is a straight-line relationship between the depression of renal enzymic activity and log-dose of actinomycin D. On the liver carbonic anhydrase, smaller dose of actinomycin

D increased the enzymic activity, while larger dose of it decreased it. Cortisol had an inhibitory effect on the enzymic activity in the liver without any effect in the kidney. Further, the increases of renal and hepatic enzymic activity induced by aldosterone were blocked by the concurrent administration of actinomycin D. The decrease of renal enzymic activity and increase of hepatic enzymic activity induced by a smaller dose of actinomycin D were blocked conversely by the concurrent administration of aldosterone.

In the present experiment, aldosterone increased renal and hepatic carbonic anhydrase activity, while actinomycin D decreased renal enzymic activity. Changes of carbonic anhydrase activity in the liver after actinomycin D treatment are complex and smaller doses of actinomycin D increased the enzymic activity, while larger doses decreased it. Cortisol decreased the enzymic activity in the liver with no effect in the kidney. These results obtained *in vitro* were similar to those obtained *in vivo*.

Concerning the mechanism of action of aldosterone and actinomycin D, an increased incorporation of [^{3}H]-uridine[6, 15] and [^{14}C]-orotic acid[16] into RNA fraction under the influence of aldosterone have been reported with toad bladder and adrenalectomized rat kidney. Further Porter *et al.*[6] reported that [^{3}H]-aldosterone was selectively associated with the nuclei of toad bladder epithelial cells in contrast to the inactive steroid [^{3}H]-progesterone which was randomly distributed between the nuclei and cytoplasmic region. Ausiello and Sharp[17] have reported that the physiological receptor sites responsible for the stimulation of sodium transport by aldosterone are located in the nuclei of mucosal epithelial cells of the toad bladder. Merkle and Brussalis[18] found histologically that the nuclear size of epithelium in the distal segment of nephron enlarged after treatment of aldosterone to guinea pig. Dingman and Sporn[19] have reported that the major binding site of [^{3}H]-actinomycin D in the rat liver was the nuclear fraction, while [^{3}H]-cortisol mainly bound with the supernatant fraction. Williams and Baba[20] observed by electron microscope autoradiography that the [^{3}H]-aldosterone injected into rat aorta mainly bound with mitochondria and nucleus, but major binding site of [^{3}H]-cortisol is the mitochondria or plasma membrane of renal tubular cells in a shorter time after injection.

Further it is noteworthy that the carbonic anhydrase activity of mouse kidney is increased by the *in vivo* administration of aldosterone and DOCA,[21] decreased by adrenalectomy[22] and unaffected by other steroid hormones. The carbonic anhydrase activity of mouse liver, however, is increased by aldosterone,[12] DOCA,[21] testosterone[23] and progesterone[24] and decreased by cortisol[21] and oestradiol.[24]

From these observations, we could offer the following hypothesis. Kidney cells may have one receptor which is sensitive to mineralocorticoids, especially aldosterone; liver cells may have several receptors. One is sensitive to aldosterone and related to activation of carbonic anhydrase and the others are sensitive to steroid hormones contained cortisol and related to the elevation or depression of the enzymic activity. The stimulating effect of a smaller dose of actinomycin D on liver carbonic anhydrase activity may be due to the blocking effect of these antibiotics on the liver receptor which is sensitive to cortisol and a larger dose of actinomycin D may block all metabolic processes. We previously reported the elevation of liver carbonic anhydrase activity after adrenalectomy in mice.[22] This phenomenon seems to indicate the disappearance of the control by glucocorticoid which may be more predominant than aldosterone concerning the regulation of liver carbonic anhydrase activity. Further as

indicated in Figs. 7 and 8 and Table 1, carbonic anhydrase activity in liver and kidney homogenates was affected by aldosterone and actinomycin D. However, the enzymic activity in 700 *g* supernatant, 77,000 *g* precipitate and 77,000 *g* supernatant were not affected by aldosterone and actinomycin D *in vitro*. Therefore aldosterone-sensitive receptors may exist in the nuclei.

According to our unpublished data with adrenalectomized rats, aldosterone affects the carbonic anhydrase activities of liver and kidney, while actinomycin D had no effect on this enzymic activity *in vivo* and *in vitro*. However, the effect of actinomycin D on carbonic anhydrase activities of liver and kidney appears again 2 hr after pretreatment of adrenalectomized rats with aldosterone *in vivo*. Therefore aldosterone also has a trigger-like property to the action of actinomycin D.

On the effect of aldosterone and actinomycin D *in vitro* on any other enzymes than carbonic anhydrase in the liver and kidney, Feldman *et al.*[25] have reported that the addition of aldosterone to rat kidney homogenate and mitochondrial fraction did not affect succinic dehydrogenase and cytochrome oxidase activities. Chignell *et al.*[26] found that kidney slices and microsomes showed no change in Na^+–K^+-ATPase levels after incubation with aldosterone. Bonting *et al.*[27] stated that the stimulatory effect of aldosterone on sodium transport in toad bladder is not due to direct stimulation of the Na^+–K^+-ATPase system. Weber *et al.*[28] have reported that the addition of actinomycin D to liver homogenate or supernatant failed to affect the activities of gluconeogenic enzymes. From the results of our previous and present studies, it could be concluded that the kidney carbonic anhydrase is sensitive to actinomycin D and mineralocorticoids, especially aldosterone, since the dose of aldosterone necessary to induce a significant alteration of kidney carbonic anhydrase activity *in vivo* is less than that of DOCA.

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