Biochemical Pharmacology, Vol. 20, pp. 759–770. Pergamon Press, 1971. Printed in Great Britain

EXPERIMENTAL STUDIES ON THE CARBONIC ANHYDRASE ACTIVITY—XIII

EFFECT OF ALDOSTERONE AND ACTINOMYCIN D *IN VIVO* ON RENAL AND HEPATIC CARBONIC ANHYDRASE IN MICE

SHIRO SUZUKI and EIICHI OGAWA
Department of Pharmacology, School of Medicine, Gunma University, Maebashi, Japan

(*Received* 6 *April* 1970; *accepted* 7 *August* 1970)

Abstract—Time course of responses of renal and hepatic carbonic anhydrase activities in microsomal and supernatant fractions to aldosterone and actinomycin D were examined at intervals ranging from 3 to 24 hr after a single administration.

Aldosterone in doses of 1 μg/kg and 2 μg/kg injected i.p. temporarily increased renal and hepatic enzymic activities at 2–6 hr after treatment. A larger dose of aldosterone (30 μg/kg) injected s.c. continuously increased renal and hepatic carbonic anhydrase activity until 24 hr after injection.

After i.p. injection of actinomycin D (300 μg/kg), renal carbonic anhydrase activity was decreased gradually until 6 hr and thereafter returned to normal values, while hepatic carbonic anhydrase activity was increased gradually until 6 hr and thereafter returned to normal values.

The stimulatory effect of aldosterone on renal carbonic anhydrase activity was blocked by concurrent treatment with actinomycin D and the inhibitory effect of actinomycin D on this enzymic activity was blocked conversely by concurrent treatment with aldosterone.

ALDOSTERONE is known as a naturally occurring mineralocorticoid and plays an important role in the regulation of sodium and potassium metabolism in the kidney. Although the effect of aldosterone on sodium transport[1–3] or synthesis of m-RNA and protein[4–7] in toad bladder and mammals have been reported by many investigators, the final mechanism of its action is not clear. In particular the relation between aldosterone and kidney enzymes has not been fully investigated.

Of several enzymes in the kidney, carbonic anhydrase has been shown to play a role in the formation of H^+ and on H^+–Na^+ or K^+–Na^+ exchange mechanism in renal tubules.[8, 9] Therefore in the previous reports XI and XII, we examined the effect of repeated administration of aldosterone on renal carbonic anhydrase activity in normal and adrenalectomized mice and observed a significant increase of this enzymic activity after aldosterone treatment.[10, 11] In general however it has been reported by many workers that the effect of aldosterone on urinary electrolyte excretion appears in a shorter time after administration.[7, 12, 13] If carbonic anhydrase is a mediator of action of aldosterone in the kidney, any alterations of renal enzymic activity would be observed during the period of a maximal sodium retention by aldosterone. Therefore in this paper, the effect of aldosterone and actinomycin D on renal carbonic anhydrase and the presence of antagonism between the action of aldosterone and actinomycin D were examined.

Further this enzymic activity is observed in various organs and liver carbonic anhydrase is affected by adrenocorticoids,[14, 15] but the physiological function of this enzyme in the liver is not known. The alterations of liver carbonic anhydrase activity after aldosterone and actinomycin D treatment were also examined in this paper.

MATERIALS AND METHODS

Animals

Adult male ddN strain mice (25–30 g) were used. The animals were fed with commercial solid diet (Oriental Co.) and tap water *ad lib.* at room temperature of 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 Aqueous Vehicle No. 1 (Merck). Various doses of these drugs were administered s.c. or i.p. in 0·1 ml of each solvent. Control animals were administered the solvent only.

Separation of subcellular fractions

After sacrifice by decapitation, livers and kidneys were removed and minute incisions were made and washed well with cold distilled water which was then absorbed with blotting paper. After repeating this procedure several times and removing as much blood as possible, the livers and kidneys in a group of six to eight animals were pooled, weighed with a torsion balance and a 10% (w/v) homogenate was made in a Potter–Elvehjem type glass homogenizer fitted with a teflon pestle with 0·25 M sucrose containing 0·1% sodium deoxycholate and 5 mM Na_2EDTA adjusted to pH 7·4 with 1 M Tris.

Differential fractionation was carried out according to Schneider.[16] The homogenate was centrifuged in a refrigerated centrifuge at 2° for 10 min (9000 *g*) to sediment the nuclei and mitochondria. The supernatant was decanted and the sediment resuspended in the above solution and recentrifuged under the same conditions. The supernatant solutions from both centrifugations were then combined and centrifuged at 2° for 1 hr (77,000 *g*) using an ultracentrifuge to sediment the submicroscopic particles. The sediment was suspended in 0·25 M sucrose and used as the microsomal fraction. The supernatant obtained from 77,000 *g* centrifugation was used as the supernatant fraction.

Carbonic anhydrase assay

Carbonic anhydrase activity was measured according to Altschule and Levis.[17] The procedure was as follows; 1·0 ml of 0·2 M phosphate buffer (pH 6·8) and 0·5 ml of enzyme preparation was placed in one compartment of a boat-shaped glass vessel and in the other compartment 1·0 ml of 0·05 M sodium bicarbonate solution was added. The reaction vessel containing the reagents and enzyme was attached to its manometer and placed in a water bath for 8 min at 37°, then the vessel was shaken 120 times/min and the CO_2 produced was determined manometrically. Assays were made several times with the same enzyme preparation and mean values were calculated.

Protein assay

Protein amount of enzyme preparation was determined by Biuret reaction[18] with crystalline bovine serum albumin (Sigma Chem. Co.) used as protein standard.

RESULTS

Effect of aldosterone (time course experiment 1)

Six mice in each group were injected i.p. with 1 and 2 μg/kg of aldosterone and sacrificed at various times indicated in Fig. 1. In the 1 μg/kg dose injected group, the enzymic activity in kidney microsomes and supernatant was increased and its maximum was observed at 3 hr with a gradual return to normal values by 12 hr. In the 2 μg/kg dose injected group, a maximal increase of the enzymic activity in the kidney was observed at 4 hr later in microsomes and at 6 hr later in supernatant fraction.

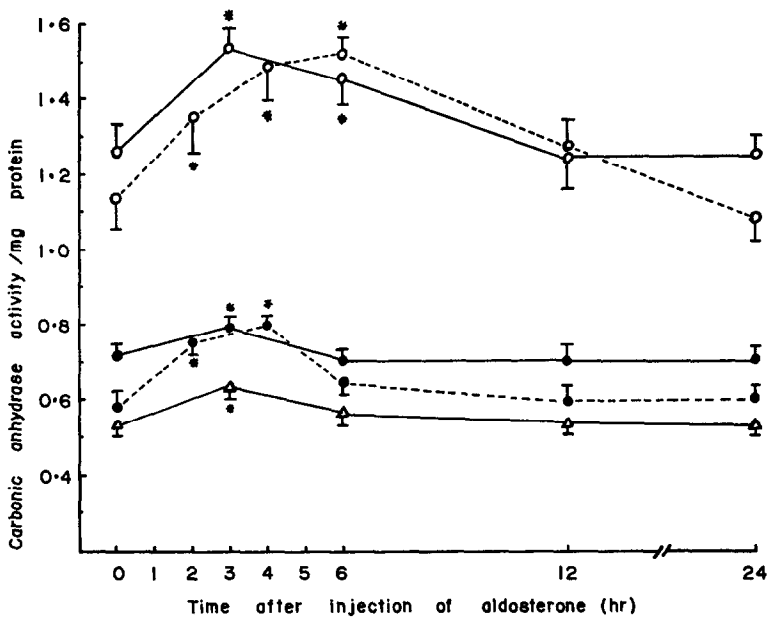

FIG. 1. Time course of response of carbonic anhydrase activity in supernatant fractions from kidney and liver to aldosterone. Each point with vertical line represents the mean with standard deviation from five observations. *P < 0·05 (when compared with 0 hr values). ○—○ kidney supernatant (1 μg/kg aldosterone); ○ - - - ○ kidney supernatant (2 μg/kg aldosterone); ●—● kidney microsomes (1 μg/kg aldosterone); ● - - - ● kidney microsomes (2 μg/kg aldosterone); △—△ liver supernatant (1 μg/kg aldosterone).

Between the two groups a slight difference was observed with maximal reaction times according to the injected doses. The enzymic activity in liver supernatant from the 1 μg/kg dose injected group was increased temporarily at 3 hr and a return to normal values was seen at 6 hr after treatment.

Effect of aldosterone (time course experiment 2)

Six mice in each group were injected s.c. with 30 μg/kg of aldosterone and sacrificed at various times indicated in the left half of Fig. 2. After the single injection of aldosterone, carbonic anhydrase activity in kidney microsomes, kidney supernatant and

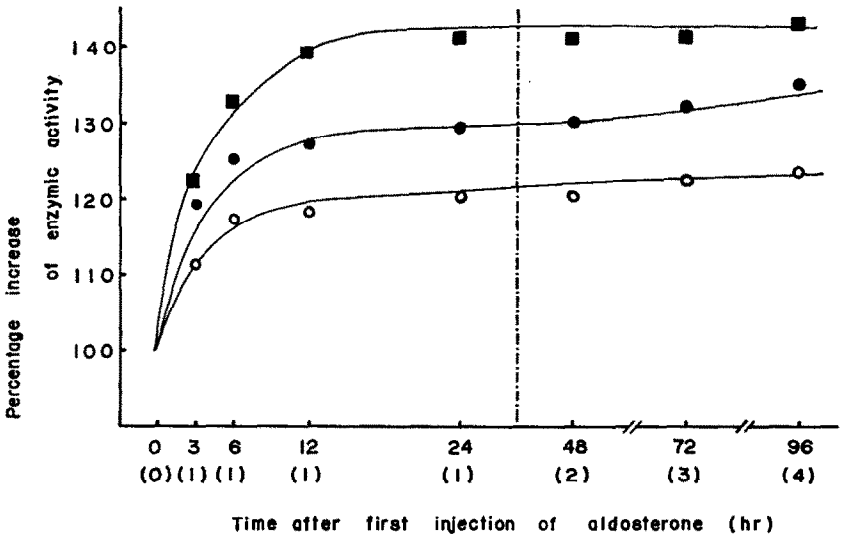

FIG. 2. Time course of changes of carbonic anhydrase activities in three fractions after single (left half) and repeated (right half) administration of aldosterone (30 μg/kg). Numbers in parentheses represent the frequency of aldosterone administration. Each point represents the mean from five observations. ■—■ kidney microsomes; ●—● liver supernatant; ○—○ kidney supernatant.

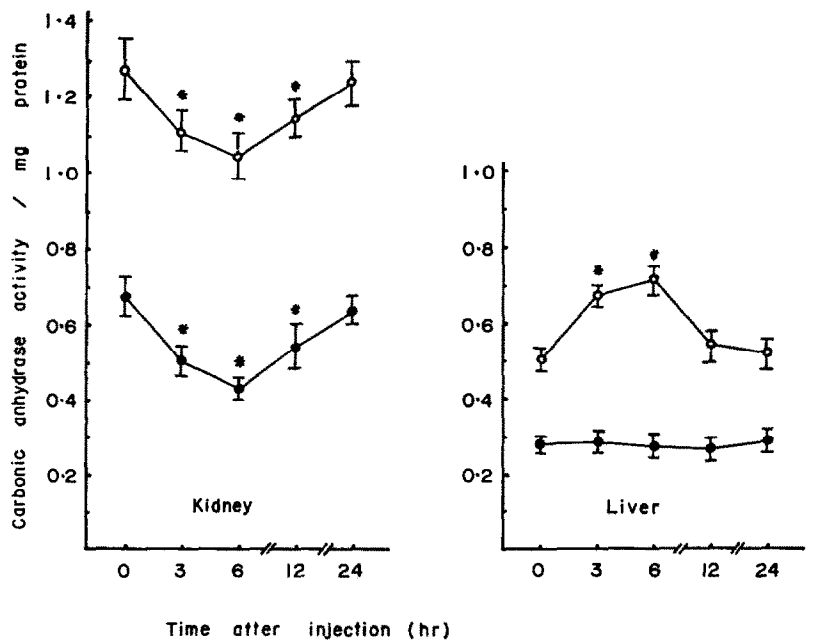

FIG. 3. Time course of response of carbonic anhydrase activity in microsomal and supernatant fractions from kidney and liver to actinomycin D (300 μg/kg). Each point with vertical lines represents the mean $\pm$ S.D. from five observations. * $P < 0{\cdot}05$ (when compared with 0 hr values). ○—○ supernatant; ●—● microsomes.

liver supernatant were all increased gradually until 24 hr. The right half of Fig. 2 indicates the effect of repeated administration of aldosterone. In each group six animals were used. Aldosterone (30 μg/kg) was injected s.c. once daily several times and animals were sacrificed 24 hr after the last injection. Carbonic anhydrase activities in three fractions were also increased, and its rate of increase was similar to that observed at 24 hr after the single injection. Among these three fractions, increased rate of enzymic activity ranged in magnitude in the order of kidney microsomes greater than liver supernatant greater than kidney supernatant.

Effect of actinomycin D (time course experiment)

Figure 3 shows the changes of carbonic anhydrase activities from liver and kidney at intervals from 3 to 24 hr after the single i.p. injection of 300 μg/kg of actinomycin D. In each group, eight animals were used respectively. Carbonic anhydrase activities in kidney microsomes and supernatant were decreased gradually after the administration of actinomycin D and a maximal decrease was observed at 6 hr. Thereafter the enzymic activity was recovered gradually and returned to the normal values at 24 hr. In the liver supernatant, enzymic activity was increased by actinomycin D and its maximal increase was observed 6 hr after the injection. Thereafter enzymic activity was decreased gradually and returned to the normal values 24 hr later. Enzymic activity in liver microsomes was not affected by actinomycin D in any times.

Effect of actinomycin D (dose–response relation)

Eight mice in each group respectively were injected i.p. with actinomycin D in doses ranging from 50 to 1000 μg/kg. They were sacrificed 6 hr after treatment with actinomycin D and carbonic anhydrase activity was examined. The results are presented in Fig. 4 and Table 1. In the kidney, actinomycin D decreased the enzymic activities in microsomal and supernatant fractions and these data using a semi-log plot (Fig. 4) show that a straight-line relationship exists between the depression of enzymic activity and the dose of actinomycin D. In the liver, 50 and 150 μg/kg doses elevated the enzymic activity in microsomal and supernatant fractions, while 500 μg/kg dose had no

TABLE 1. EFFECT OF ACTINOMYCIN D ON BLOOD CARBONIC ANHYDRASE ACTIVITY

Actinomycin D dose (μg/kg)	No. of mice	Carbonic anhydrase activity: Mean ± S.D. Enzyme unit/0·5 ml of 1:100
0	8	1·10±0·08
50	8	1·11±0·07
150	8	1·11±0·09
500	8	1·12±0·10
1000	8	0·87±0·09*

Animals were sacrificed 6 hr after the i.p. injection with actinomycin D and blood was diluted with distilled water to 100-fold. Enzymic activity was assayed with 0·5 ml of diluted blood.
* $P < 0·05$.

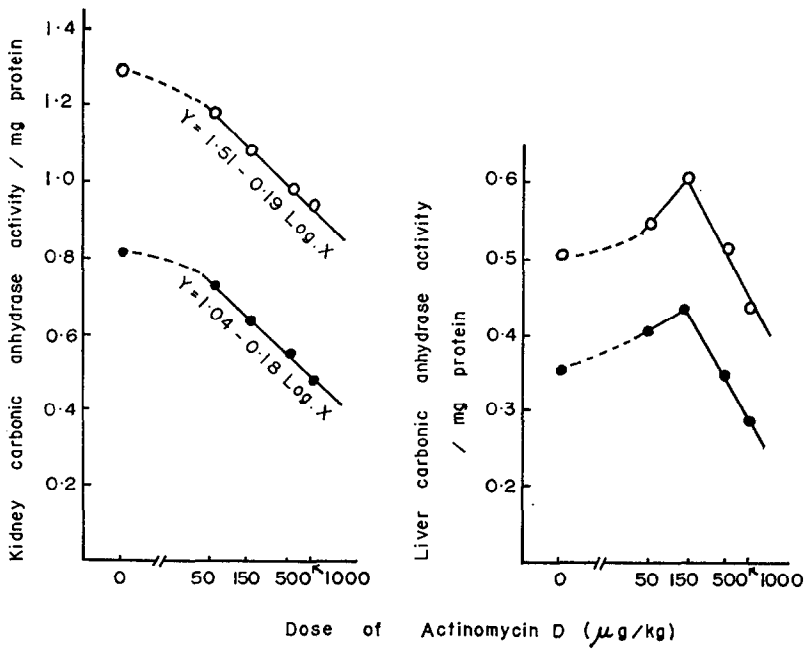

FIG. 4. Effects of various doses of actinomycin D on renal (left half) and hepatic (right half) carbonic anhydrase activity. Each point represents the mean from five observations. ○—○ supernatant; ●—● microsomes.

effect and 1000 μg/kg dose decreased the enzymic activities in both fractions. Actinomycin D in doses from 50 to 500 μg/kg had no effect on blood enzymic activity, while 1000 μg/kg dose decreased it (Table 1).

Antagonism of actinomycin D to the action of aldosterone (1)

In this case, six animals in each group were injected with 30 μg/kg of aldosterone s.c. or 300 μg/kg of actinomycin D i.p., either alone or concurrently with each other, and sacrificed 3 and 6 hr after treatment. As shown in Fig. 5(A), kidney carbonic anhydrase activities in microsomal and supernatant fractions were increased after aldosterone treatment. When 300 μg/kg of actinomycin D was injected 3 hr after aldosterone treatment, the effect of aldosterone was blocked and enzymic activity was decreased to the normal values. Figure 5 (B) shows the concurrent effect of aldosterone on the action of actinomycin D. Single injection of 300 μg/kg of actinomycin D decreased renal enzymic activities in microsomal and supernatant fractions. This decrease was prevented by the concurrent administration of aldosterone and the enzymic activity restored to the normal values.

As shown in Fig. 6(A), liver supernatant carbonic anhydrase activity which was increased by the single injection of 30 μg/kg of aldosterone was decreased by the concurrent administration of actinomycin D below normal values. Liver supernatant enzymic activity which was increased by the treatment with 300 μg/kg of actinomycin D was decreased by the concurrent administration of aldosterone [Fig. 6(B)]. In the liver, similar results were observed after actinomycin D and aldosterone treatment.

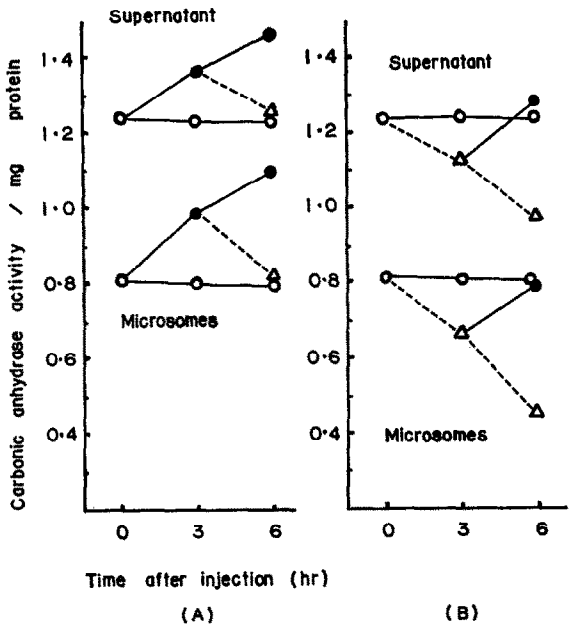

FIG. 5. Effects of aldosterone (A) and actinomycin D (B) administration, either alone or concurrently with each other, on renal carbonic anhydrase activity. Each point represents the mean from five observations. —○— control; —●—aldosterone 30 μg/kg s.c.; - - △ - - actinomycin D 300 μg/kg i.p.

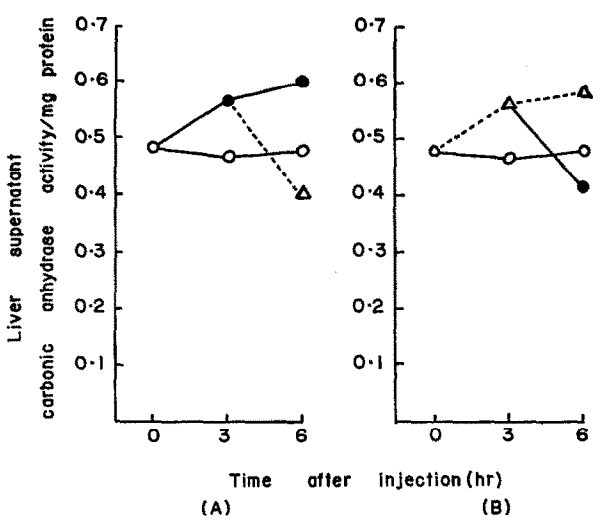

FIG. 6. Effects of aldosterone (A) and actinomycin D (B) administration, either alone or concurrently with each other, on hepatic supernatant carbonic anhydrase activity. Each point represents the mean from five observations. —○— control; —●— aldosterone treated; - - △ - - actinomycin D treated.

Antagonism of actinomycin D to the action of aldosterone (2)

In Table 2 is presented the effect of actinomycin D on aldosterone pretreated mice. Aldosterone was administered s.c. for 3 days in a dose of 15 μg/kg/day and on the fourth day, animals received i.p. injection of actinomycin D 6 hr before sacrifice. In normal mice, hepatic carbonic anhydrase activities in microsomal and supernatant fractions were increased by 150 μg/kg of actinomycin D, but unaltered by 500 μg/kg dose. Renal carbonic anhydrase activities in microsomal and supernatant fractions were decreased by 150 and 500 μg/kg of actinomycin D. In aldosterone pretreated mice, hepatic carbonic anhydrase activities in microsomal and supernatant fractions were decreased and approached the normal values after treatment of actinomycin D. Renal carbonic anhydrase activities in both fractions were decreased after actinomycin D treatment with no differences between 150 and 500 μg/kg doses.

TABLE 2. EFFECT OF ACTINOMYCIN D ON CARBONIC ANHYDRASE AND ITS INTERACTION WITH ALDOSTERONE

Group	No. of animals	Carbonic anhydrase activity: Mean ± S.D. Enzyme unit/mg protein			
		Liver		Kidney	
		Microsomes (5)	Supernatant (5)	Microsomes (5)	Supernatant (5)
Normal	8	0·35 ± 0·02	0·51 ± 0·03	0·81 ± 0·07	1·29 ± 0·06
Actinomycin D 150 μg/kg	8	0·44 ± 0·03*	0·61 ± 0·03*	0·64 ± 0·06*	1·08 ± 0·08*
Actinomycin D 500 μg/kg	8	0·35 ± 0·03	0·52 ± 002	0·55 ± 0·05*	0·97 ± 0·09*
Aldosterone 15 μg/kg × 3	8	0·51 ± 0·04*	0·65 ± 0·01*	1·11 ± 0·05*	1·54 ± 0·07*
Aldosterone + actinomycin D 150 μg/kg	8	0·32 ± 0·05†	0·55 ± 0·04†	0·92 ± 0·04†	1·14 ± 0·06†
Aldosterone + actinomycin D 500 μg/kg	8	0·30 ± 0·03†	0·54 ± 0·01†	0·91 ± 0·06†	1·15 ± 0·06†

Numbers in parentheses represent the number of observations.
* $P < 0.05$ (when compared with normal group).
† $P < 0.05$ (when compared with aldosterone treated group).

Antagonism of actinomycin D to the action of cortisol

Table 3 shows the effect of actinomycin D on cortisol pretreated mice. Cortisol was administered s.c. for 4 days in a dose of 3 mg/kg/day and on the 5th day, animals received i.p. injection of actinomycin D 6 hr before sacrifice.

In normal mice, hepatic carbonic anhydrase activities in microsomal and supernatant fractions were increased by 150 μg/kg of actinomycin D, but unaltered after 500 μg/kg dose. Renal supernatant carbonic anhydrase activity was decreased by 150

and 500 μg/kg doses. In cortisol pretreated mice, hepatic carbonic anhydrase activities in both fractions which were decreased by cortisol were increased and returned to the normal values after actinomycin D treatment. Renal supernatant carbonic anhydrase activity which was unaltered by cortisol was decreased after actinomycin D treatment similar in normal mice.

TABLE 3. EFFECT OF ACTINOMYCIN D ON CARBONIC ANHYDRASE AND ITS INTERACTION WITH CORTISOL

Group	No. of animals	Carbonic anhydrase activity: Mean ± S.D. Enzyme unit/mg protein		
		Liver		Kidney
		Microsomes (5)	Supernatant (5)	Supernatant (5)
Normal	6	0·39 ± 0·03	0·49 ± 0·05	1·32 ± 0·09
Actinomycin D 150 μg/kg	6	0·53 ± 0·05*	0·61 ± 0·04*	1·05 ± 0·10*
Actinomycin D 500 μg/kg	6	0·38 ± 0·03	0·48 ± 0·04	0·99 ± 0·07*
Cortisol 3 mg/kg × 4	6	0·23 ± 0·02*	0·37 ± 0·02*	1·32 ± 0·06
Cortisol + Actinomycin D 150 μg/kg	6	0·43 ± 0·03†	0·46 ± 0·03†	1·10 ± 0·08†
Cortisol + Actinomycin D 500 μg/kg	6	0·46 ± 0·04†	0·52 ± 0·03†	1·05 ± 0·08†

Numbers in parentheses represent the number of observations.
* $P < 0·05$ (when compared with normal group).
† $P < 0·05$ (when compared with cortisol treated group).

DISCUSSION

The authors assume that some enzyme systems may play a role in the regulation of sodium metabolism in the kidney as a mediator of the action of aldosterone. As shown in Fig. 1, maximal stimulatory effect of smaller doses of aldosterone (1 and 2 μg/kg) on renal carbonic anhydrase activity appeared 3 and 6 hr after administration and thereafter this enzymic activity returned to normal levels. Fimognari *et al.*[7] reported with adrenalectomized rats that subcutaneous administration of aldosterone in a dose of 2 μg/rat caused a maximal sodium retention and potassium excretion at 3–4 hr after treatment. Sonnenblick *et al.*,[13] reported that the aldosterone injected intravenously into man produced a marked reduction in sodium excretion and the peak of this effect occurred in 2–4 hr after treatment. According to our unpublished data, maximal replacement effect of physiological doses of aldosterone (0·5–2 μg/kg) on the alterations of renal carbonic anhydrase activity caused by adrenalectomy was observed 4 hr after administration in mice and rats. A correlation seems to exist between the maximal effect of aldosterone on urinary solute excretion and changes of renal carbonic anhydrase activity.

On the relation between aldosterone and any other enzymes than carbonic anhydrase in the kidney, Feldman *et al.*[19] reported an increase in the activities of succinic dehydrogenase and cytochrome oxidase in homogenate of rat kidney 3 hr after injection of aldosterone (100 μg/rat) *in vivo* without a correspondent change *in vitro*. However, the dose of 100 μg/rat of aldosterone seems to be too enormous as a physiological dose. Domján and Fazekas[20] found no rise in rat kidney succinic dehydrogenase after 4 days of administration of aldosterone (1 μg/kg) to adrenalectomized rats. Further, no significant effect of adrenalectomy or DOCA and cortisol administration on kidney glutaminase I activity have been reported.[21, 22] Since recently Na^+–K^+-ATPase is considered to be an important enzyme in Na^+ transport mechanism in the kidney,[23] several investigations as to the effect of aldosterone on this enzymic activity have been reported. Katz and Epstein[24] reported that there is a close correlation between microsomal Na^+–K^+-ATPase and sodium reabsorption in rat kidney and then adrenalectomy decreased this enzymic activity and reabsorption of sodium in renal tubules. However it has been reported that the administration of aldosterone to adrenalectomized rats produced no detectable changes in kidney Na^+–K^+-ATPase levels after 3 hr, even though maximal sodium retention may be observed at that time.[25, 26] 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. Jørgensen[28, 29] have considered with adrenalectomized rats that an increase of kidney Na^+–K^+-ATPase activity after aldosterone treatment is not due to a primary action of this hormone but a secondary effect by other events, e.g. alterations of Na^+ and K^+ concentration in plasma.

In view of these observations it is concluded that carbonic anhydrase is more sensitive to and more closely associated with aldosterone action than other enzymes in the kidney. As previously reported,[10, 11] the effects of aldosterone on kidney carbonic anhydrase activity differ in mice and rats, being conducive in the former and inhibitory in the latter. Therefore it is difficult to conclude the mechanism of action of aldosterone on renal carbonic anhydrase only with the alterations of the enzymic activity.

Recently it has become established that steroid hormones cause a stimulation of RNA synthesis followed by formation of specific protein in target organs.[30–33] Edelman and his co-workers have also reported that the aldosterone acts on toad bladder and rat kidney by increasing DNA-dependent RNA synthesis giving an increased rate of protein synthesis.[4, 5, 7] Actinomycin D blocks the action of aldosterone in sodium transport correlating closely with the degree of inhibition of RNA and protein synthesis.[34] Similar experimental results have been obtained by other investigators with rat kidney[6, 12] and toad bladder.[35, 36] In the present experiment, a single injection of aldosterone increased renal carbonic anhydrase activity, while actinomycin D decreased this enzymic activity. Further, an increase of enzymic activity induced by aldosterone alone was blocked by the concurrent administration of actinomycin D. Therefore, some correlation may exist between aldosterone and actinomycin D on the mechanism or site of action. Presumably, aldosterone may cause a synthesis of carbonic anhydrase in mouse kidney, however, no definite conclusions can be drawn from the present study. This problem must be investigated in future studies.

Liver also indicated the carbonic anhydrase activity although in less degree com-

pared with that in the kidney and this enzymic activity was stimulated by aldosterone and inhibited by cortisol. Drews and Bondy[37] reported that the pretreatment of rats with cortisol *in vivo* results in a marked increase in incorporation of [^{3}H]-cytidine triphosphate into rat liver nuclei *in vitro*. However, the incorporation of ^{32}P into DNA of regenerating rat liver is depressed under the influence of cortisone without similar effects in normal rat liver.[38] Stevens *et al.*[39] have reported that cortisol inhibited the synthesis of DNA of mouse lymphatic tissue as measured by the incorporation of [^{14}C]-thymidine, whereas Weber *et al.*[40] observed that cortisone induces *de novo* synthesis of liver glucose-6-phosphatase, fluctose-1, 6-diphosphatase, aldolase and lactic dehydrogenase and this synthesis is blocked by injection of actinomycin D in rats. Further it must be noted that the liver carbonic anhydrase activity is affected by not only aldosterone and cortisol but also oestradiol,[41] progesterone[41] and testosterone.[42] Effect of glucocorticoids on nucleic acid metabolism or enzyme protein synthesis is complicated and its mode of action seems to be different in different organs. The inhibitory effect of glucocorticoid on hepatic carbonic anhydrase has not been investigated by others and precise mechanism of this action is not clear, however, an increase of hepatic enzymic activity by smaller dose of actinomycin D may be derived from an antagonism of this drug to the action of cortisol.

It is well known that blood has a high carbonic anhydrase activity. In the present experiment, a smaller dose of actinomycin D had no effect on blood enzymic activity (Table 1). Therefore, the elevation of hepatic enzymic activity by actinomycin D may be independent of enzymic activity of blood contained in the liver.

It is interesting that aldosterone affects both renal and hepatic carbonic anhydrase activity, whereas cortisol affects hepatic carbonic anhydrase only. Presumably the organ specificity may exist between the action of aldosterone and cortisol.

REFERENCES

1. J. Crabbé, *Endocrinology* **69**, 673 (1961).
2. J. Crabbé, *J. clin. Invest.* **40**, 2103 (1961).
3. G. W. G. Sharp and A. Leaf, *Nature, Lond.* **202**, 1185 (1964).
4. I. S. Edelman, R. Bogoroch and G. A. Porter, *Proc. natn. Acad. Sci. U.S.A.* **50**, 1169 (1963).
5. G. A. Porter, R. Bogoroch and I. S. Edelman, *Proc. natn. Acad. Sci. U.S.A.* **52**, 1326 (1964).
6. T. R. Castles and H. E. Williamson, *Proc. Soc. exp. Biol. Med.* **119**, 308 (1965).
7. G. M. Fimognari, D. D. Fanestil and I. S. Edelman, *Am. J. Physiol.* **213**, 954 (1967).
8. R. F. Pitts, *Am. J. Med.* **9**, 356 (1950).
9. R. W. Berliner, *Fedn Proc.* **11**, 695 (1952).
10. S. Suzuki and E. Ogawa, *Biochem. Pharmac.* **17**, 1855 (1968).
11. S. Suzuki and E. Ogawa, *Biochem. Pharmac.* **18**, 993 (1969).
12. H. E. Williamson, *Biochem. Pharmac.* **12**, 1449 (1963).
13. E. H. Sonnenblick, P. J. Cannon and J. H. Laragh, *J. clin. Invest.* **40**, 903 (1961).
14. S. Suzuki and E. Ogawa, *Gunma J. Med. Sci.* **11**, 265 (1962).
15. S. Suzuki and E. Ogawa, *Gunma J. Med. Sci.* **12**, 239 (1963).
16. W. C. Schneider, in *Biochemist's Handbook* (Ed. C. Long), p. 810, E. & F. N. Spon, London (1961).
17. M. D. Altschule and H. D. Levis, *J. biol. Chem.* **180**, 557 (1949).
18. E. Layne, in *Methods in Enzymology* (Eds. S. P. Colowick and N. Kaplan), Vol. 3, p. 450, Academic Press, New York (1957).
19. D. Feldman, C. V. Wende and E. Kessler, *Biochim. biophys. Acta* **51**, 401 (1961).
20. G. Domján and A. G. Fazekas, *Enzymologia* **23**, 281 (1961).
21. L. Goldstein and C. J. Kensler, *J. biol. Chem.* **235**, 1086 (1960).
22. L. Goldstein, *Nature, Lond.* **205**, 1330 (1965).
23. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
24. A. I. Katz and F. H. Epstein, *J. clin. Invest.* **46**, 1999 (1967).

25. C. F. CHIGNELL and E. TITUS, *J. biol. Chem.* **241,** 5083 (1966).
26. E. J. LANDON, N. JAZAB and L. FORTE, *Am. J. Physiol.* **211,** 1050 (1966).
27. S. L. BONTING and M. R. CANADY, *Am. J. Physiol.* **207,** 1005 (1964).
28. P. L. JØRGENSEN, *Biochim. biophys. Acta* **151,** 212 (1968).
29. P. L. JØRGENSEN, *Biochim. biophys. Acta* **192,** 326 (1969).
30. P. KARLSON, *Deutsch. Med. Wsch.* **86,** 668 (1961).
31. P. KARLSON and C. E. SEKERIS, *Acta Endocr.* **53,** 505 (1966).
32. W. D. NOTEBOOM and J. GORSKI, *Proc. natn. Acad. Sci., U.S.A.* **50,** 250 (1963).
33. T. H. HAMILTON, C. C. WIDNELL and J. R. TATA, *Biochim. biophys. Acta* **108,** 168 (1965).
34. D. D. FANESTIL, and I. S. EDELMAN, *Fedn Proc.* **25,** 912 (1966).
35. J. CRABBÉ and P. DE WEER, *Nature, Lond.* **202,** 298 (1964).
36. G. ROUSSEAU and J. CRABBÉ, *Biochim. biophys. Acta* **157,** 25 (1968).
37. J. DREWS and P. K. BONDY, *Proc. Soc. exp. Biol. Med.* **122,** 847 (1966).
38. J. W. GUZEK, *Acta Endocr.* **59,** 10 (1968).
39. W. STEVENS, C. COLESSIDES and T. F. DOUGHERTY, *Endocrinology* **78,** 600 (1966).
40. G. WEBER, R. L. SINGHAL and N. B. STAMM, *Science* **142,** 390 (1963).
41. S. SUZUKI, E. OGAWA and K. SHIBATA, *Gunma J. Med. Sci.* **10,** 237 (1961).
42. S. SUZUKI, E. OGAWA and K. SHIBATA, *Gunma J. Med. Sci.* **10,** 228 (1961).