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Induction of Citrate Synthase by Aldosterone in the Rat Kidney

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Summary. The possible induction of renal citrate synthase (E.C. 4.1.3.7) by aldosterone was evaluated in the adrenalectomized rat. Three hours after administration of aldosterone (0.8 μg/100 g body wt), renal cortical and medullary citrate synthase activity was significantly increased as reported previously by Kinne and Kirsten (Kinne, R., Kirsten, R. 1968. *Pfleugers Arch.* 300:244). In contrast, no change in this activity was detected in the renal papilla or the liver, under the same conditions. Kinetic analysis revealed that injection of aldosterone had no effect on the K_ms for acetyl-CoA and oxalacetate but augmented V_{max} of renal medullary citrate synthase activity by 40 %. The aldosterone-dependent increase in medullary citrate synthase activity was proportionate to the associated increase in the quantity of antiserum (specific for citrate synthase) required for half-maximal immuno-precipitation.

The possibility that aldosterone induced the synthesis of citrate synthase was evaluated in two sets of experiments. In the first set, adrenalectomized rats were injected intraperitoneally with either aldosterone (0.8 μg/100 g body wt) or the diluent, and simultaneously with ^3H or ^{35}S methionine (500 μCi/rat). The isotopes were reversed in about half of the experiments. Three hours after the injection, renal citrate synthase was isolated by ATP-sepharose column chromatography and immuno-precipitation with the specific antiserum. Aldosterone augmented methionine incorporation into renal citrate synthase by 55 % but had no effect on incorporation into the hepatic enzyme. In the second set, adrenalectomized rats were injected with either aldosterone (0.8 μg/100 g body wt) or the diluent, the kidneys were removed 1 hr later and medullary slices were incubated in either ^3H- or ^{35}S-methionine at 20° for 2 hr. Mitochondrial citrate synthase was isolated either by ATP-sepharose column chromatography and immuno-precipitation, or by polyacrylamide gel electrophoresis. Aldosterone increased methionine incorporation into the immuno-precipitates by 30 % and into the enzyme peak resolved by polyacrylamide gel electrophoresis by 43 %. The latter increase was eliminated by prior administration of either actinomycin D (70–80 μg/100 g body wt) or spirolactone (SC-26304) (80 μg/100 g body wt). An equimolar dose of dexamethasone (0.8 μg/100 g body wt) had no effect on the isotope ratio associated with citrate synthase activity in the polyacrylamide gels.

Although considerable evidence has been obtained indicating that induction of protein synthesis mediates the action of aldosterone on Na^+

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transport, relatively little information is available on the identity of the induced proteins or on the roles played in regulating Na^+ transport [4, 6, 17].

In the preceding paper [17], we reported that aldosterone augmented the incorporation of amino acids into renal medullary proteins; the effects were evident in the plasma membrane, mitochondrial, ribosomal, and cytosol fractions. One of the induced proteins with a molecular weight of 31,000 in the cytosol fraction was identified by polyacrylamide gel electrophoresis.

This paper addresses the question of induction of a specific mitochondrial enzyme (citrate synthase) by aldosterone, for a variety of reasons: Based on physiological experiments, it has been proposed that aldosterone regulates mitochondrial metabolism which in turn modulates active Na^+ transport [5, 9, 26, 27]. Aldosterone increased the activities of several mitochondrial enzymes, notably citrate synthase, in the rat kidney and urinary bladder of the toad [12–15]. The increase in citrate synthase activity correlated both with dose-response and time-course changes in Na^+ transport. The aldosterone-dependent increase in citrate synthase activity was accompanied by parallel increases in the mitochondrial NADH/-NAD^+ ratio, suggesting an influence on metabolic status. Moreover, spironolactone, an inhibitor of the mineralocorticoid receptor, and actinomycin D and puromycin, inhibitors of RNA and protein synthesis, blocked both the increases in citrate synthase activity and Na^+ transport in response to aldosterone [12, 15]. Our studies were designed to determine whether the aldosterone-dependent increase in renal medullary citrate synthase activity was a consequence of induction of the synthesis of this enzyme.

Materials and Methods

Physiological Preparations

Male, Sprague-Dawley rats were bilaterally adrenalectomized 7 days before use, and were maintained on standard Purina chow pellets and 0.155 M NaCl drinking water, *ad libitum*. On the day of the experiments, the rats weighed between 180–200 g. In the studies on amino acid incorporation into renal medullary proteins *in vivo*, the diluent (0.155 M NaCl) or aldosterone (0.8 μg/100 g body wt) was injected intraperitoneally 3 hr before removal of the kidneys. This dose is twice that required for a half-maximal increase in the urinary K^+/Na^+ ratio or in amino acid incorporation into medullary proteins [15, 17]. In the studies on amino acid incorporation *in vitro* (renal medullary slices), the diluent or aldosterone or dexamethasone (both at 0.8 μg/100 g body wt) were injected subcutaneously 1 hr before removal of the kidneys. The requirement for intact RNA synthesis in these experiments was tested by intraperitoneal injection of actinomycin D (70–80 μg/100 g body wt) 1 hr before

aldosterone or the diluent. This is twice the dose required for significant inhibition of the antinatriuretic response to aldosterone [8]. To assess the participation of the mineralocorticoid receptor system, spirolactone (SC-26304) was used as the antagonist with a well-established potency [10, 22]. SC-26304 dissolved in ethanol/propylene glycol/saline, 2:5:5 (80 μg/100 g body wt) was injected intraperitoneally 30 min before injection of the diluent or aldosterone. At this dose ratio (100:1), SC-26304 completely inhibits the aldosterone-dependent increase in the urinary K^+/Na^+ ratio [17].

Citrate Synthase Assays

Citrate synthase activity was determined by measuring the initial rate of generation of reduced coenzyme A (CoA-SH)[1] as described by Srere *et al.* [28]. For routine V_{max} assay, the reaction mixture contained, in 1 ml, 0.25 mM DTNB, 0.2 mM sodium oxalacetate, 0.1 mM acetyl-CoA, 100 mM Tris-Cl buffer, pH 8.0. The reaction was assessed at room temperature and initiated by the addition of the enzyme (0.006 to 0.04 units of activity in 10–50 μl). The enzyme was obtained by sonication of the mitochondrial factions of kidney and liver, suspended in 10 mM potassium phosphate buffer, pH = 7.4 (*see below* for details). The absorbance at 412 nm was measured continuously in a Gilford 2000 Recording Spectrophotometer. One unit of activity is defined as the liberation of 1 μmole of CoA-SH per min. To assess the K_m for oxalacetate, the reaction mixtures were adjusted to contain a fixed concentration of acetyl-CoA (either 5 or 10 μM) and variable concentrations of oxalacetate (10–40 μM). Similarly, the K_m for acetyl-CoA was determined at fixed concentrations of oxalacetate (either 5 or 10 μM) and variable concentrations of acetyl-CoA (10–40 μM). The protein content of the reaction mixtures was estimated by the method of Lowry *et al.* [21], using bovine serum albumin as the standard. Enzyme activity was expressed in units/mg of protein.

Antibody Titration of Citrate Synthase

Mitochondrial sonicates (particulate-free, prepared as described below) were assayed for citrate synthase content by neutralization with specific antibodies (rabbit anti-rat heart citrate synthase). In preliminary experiments, we found that the 0.1 ml of the antiserum precipitated 0.2 units of renal medullary citrate synthase when incubated for 24 hr at 4°, in pH 8 buffer. For the purposes of the present studies, the antiserum was diluted 1:50 with 10 mM potassium phosphate buffer, pH = 7.4. Mitochondrial sonicates in 0.145 M NaCl, 10 mM potassium phosphate buffer, pH = 7.4 (incubation medium) containing 0.05–0.09 units of citrate synthase activity were incubated with variable volumes of the 1:50 diluted antiserum for 24 hr at 4°. The incubation mixture was made up to a constant volume of 1 ml by addition of appropriate amounts of 10 mM potassium phosphate buffer, pH = 7.4. After 24 hr, the supernatants were collected by centrifugation at 8,000 × *g* for 10 min, and 0.1-ml aliquots were assayed for citrate synthase activity (*see below*). A plot of the log (activity with antiserum/activity with diluent) *vs.* amount of antiserum added yielded a straight line. The amount of antiserum (1:50 dilution) required to neutralize 50 % of the citrate synthase activity/mg protein present in the incubation mixture was then determined from the semi-log plot.

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1 *Abbreviations:* CoA = oxidized coenzyme A; CoA-SH = reduced coenzyme A; DTNB = 5,5′-Dithiobis-(2-Nitrobenzoic acid); SDS = sodium dodecyl sulfate; Bis = N,N′-methylene-bis-acrylamide; and TPN = Nicotinamide Adenine Dinucleotide Phosphate.

Isolation of Mitochondria

All procedures were carried out at 0–4° unless specified otherwise. The tissues (both kidney and liver) were suspended in 0.25 M sucrose, 5 mM EDTA and 10 mM potassium phosphate buffer, pH = 7.4 (1 g of tissue/5 ml of homogenizing solution) and homogenized with 10 strokes at full-speed in a motor driven Teflon-glass, Elvehjem-Potter homogenizer. Nuclei and cell debris were removed from these crude homogenates by centrifugation at 600 $\times g$ for 10 min. The supernatants were then centrifuged at 10,000 $\times g$ for 15 min, and the pellets were washed twice by resuspension in the homogenizing solution and recentrifuged at 10,000 $\times g$ for 15 min. The washed mitochondria were stored at −20° for from 1 to 7 days until use.

Partial Purification of Citrate Synthase

The frozen mitochondrial fractions were thawed slowly by immersion in an ice-water slurry. Two ml of 10 mM potassium phosphate buffer (pH = 7.4) were added to the mitochondrial fractions isolated from 1 g of tissue and sonicated 4 times in 2 min bursts, allowing 2 min for cooling between bursts, with a Micro-probe, Heat Systems-Ultrasonics Model W140D Sonifier. To limit heating of the suspensions, the tubes were immersed in a salt-ice bath (−20°) throughout sonication. The mitochondrial particulates were then removed by centrifugation for 90 min at 78,000 × g. These sonicate supernatants were used in the antiserum titrations (*see above*) or for purification of the enzyme. Protamine sulfate (final concentration = 0.1 %) was added to the supernatants and the mixtures were stirred for 30 min at 2–4°. The resultant precipitates were removed by centrifugation for 20 min at 20,000 $\times g$. The supernatants were made up to 50 % saturated ammonium sulfate with the solid salt and stirred for 30 min. These precipitates were removed by centrifugation at 20,000 $\times g$ for 20 min. The supernatants were adjusted to 80 % saturation with solid ammonium sulfate, stirred for 30 min and the precipitates were collected by centrifugation at 20,000 $\times g$ for 20 min. These precipitates were dissolved by the addition of 2 ml of potassium phosphate (10 mM) buffer, pH = 7.4. The ammonium sulfate was eluted by dialysis in cellulose dialysis tubing, against 2 liters of potassium phosphate (10 mM) buffer, pH = 7.4; the external solution was replaced twice over a 48-hr period. This partially purified preparation was then processed as described below.

Preparation of ATP-Sepharose

Sepharose 4B-adipic acid dihydrazide conjugate was prepared and coupled with periodate-oxidized ATP by the method of Lamed *et al.* [16], except for differences in the amounts of oxidized ATP used. Instead of adding 4–5 μmoles of oxidized ATP/ml of Sepharose hydrazide, we added twice this amount. The yield of ATP bound to Sepharose was determined by ashing 0.1 ml samples of the product in 1 ml of 10 % (w/v) $MgCl_2$ in ethanol and analyzing the ash for inorganic phosphate, as described previously [1]. The ATP-Sepharose used in this study contained 7.3 μmole of ATP/ml of packed column.

Further Purification of Citrate Synthase

The starting material for further purification was the partially purified preparation (dialyzed against excess 10 mM potassium phosphate buffer, pH = 7.4, *see above*). Routinely, partially purified citrate synthase (0.8 ml) was applied to 5 ml of ATP-Sepharose in a 0.7 × 13 cm column, and eluted with 5 mM potassium buffer, pH = 7.4; 2.5-ml fractions were

collected in 10-min intervals. Seventy percent of the applied protein was eluted in the first 15 fractions (total volume of the eluate = 38 ml). These eluates contained no detectable citrate synthase activity. The enzyme was then eluted from the column with 0.1 mM acetyl-CoA, 0.1 mM sodium oxalacetate, 5 mM potassium phosphate, pH = 7.4. Fractions 16–20 (total volume 12 ml; 2.5 ml/fraction) contained 100 % of the applied enzyme. To remove the remaining proteins before re-use, the columns were washed with 10 ml of 0.2 M KCl, 5 mM potassium phosphate buffer, pH = 7.4, and then equilibrated with 50 ml of 5 mM potassium phosphate buffer, pH = 7.4.

To complete the purification, fractions 16–20 were pooled and concentrated to 0.5 ml by ultrafiltration through a Minicon (mol wt exclusion limit of ~10,000) (Amicon Co.). An incubation mixture was then prepared, consisting of the concentrate, 0.4 units of citrate synthase activity in 0.15 ml of the eluting solution (0.1 mM acetyl-CoA, 0.1 mM sodium oxalacetate, 5 mM potassium phosphate, pH = 7.4), 0.1 ml of 0.145 M NaCl, 0.15 ml 0.04 M potassium phosphate buffer, pH = 7.4, and 0.2 ml of the 1:50 dilution of the antiserum (*see above*). These mixtures were incubated for 24 hr at 4° and the precipitates were collected by centrifugation at 10,000 × *g* for 15 min. The pellets were resuspended in 1 ml of 0.155 M NaCl and recentrifuged at 10,000 × *g* for 15 min, twice. The washed pellets (antibody-enzyme complex) were then dissolved in 1 ml of 4 % (w/v) SDS and assayed for ^{3}H and ^{35}S content (*see below*).

Incorporation of Methionine into Citrate Synthase

In the *in vivo* incorporation experiments, ^{3}H or ^{35}S methionine (500 μCi/rat) was injected intraperitoneally simultaneously with aldosterone or the diluent. Three hours later the rats were killed by cervical dislocation and the kidneys and liver were cleared of blood by retrograde perfusion through the inferior vena cava with 20 ml of ice-cold saline (0.155 M). The respective organs of the steroid-treated and control rats were pooled, homogenized and the mitochondrial fraction was collected by differential centrifugation (*see below*).

In the *in vivo* incorporation experiments, the rats were killed by cervical dislocation 1 hr after subcutaneous injection of aldosterone or the diluent, and the kidneys were perfused with 20 ml of ice-cold saline (0.155 M) via the inferior vena cava. The medullary slices were prepared as described previously [17] and suspended in Joklik-modified minimal essential medium (MEM), pH 7.6 (devoid of methionine) and gassed with 95 % O_2, 5 % CO_2. Medullary slices from 6 rats treated identically were combined and suspended in 3 ml of MEM in 25-ml Erlenmeyer flasks, and labeled with either ^{3}H- or ^{35}S-methionine (500 μCi/flasks) for 2 hr at 20°. To ensure equivalent specific activities (Ci/mmole), nonradioactive methionine was added to the ^{35}S methionine to yield a final specific activity of 0.5 Ci/mmole. After 2 hr of incubation, cycloheximide (final concentration = 3×10^{-5} M) was added to all flasks and the incubations were continued for an additional 10 min. The slices were then washed sequentially with 100 ml of ice-cold MEM containing 10 mM methionine and 100 ml of ice-cold MEM without added methionine. The experimental and control slices were then pooled and processed, by isolation of the mitochondrial fraction. The effects of aldosterone on incorporation of methionine into citrate synthase were evaluated by purification of the enzyme through ammonium sulfate precipitation, ATP-Sepharose chromatography and antibody precipitation or by polyacrylamide gel electrophoresis of the ammonium sulfate precipitates.

Polyacrylamide Gel Electrophoresis

The starting material for analysis by polyacrylamide gel electrophoresis was the partially purified renal medullary citrate synthase preparation (mitochondrial sonicates

after successive treatment with protamine sulfate and ammonium sulfate) in 10 mM potassium phosphate buffer, pH = 7.4. The analysis was carried out as described by Davis [2]. Aliquots of approximately 200 µl containing 100–160 µg of protein were layered on the stacking gel (0.3 ml) made up of 1.25 % acrylamide (w/v) and 0.3 % Bis (w/v). The separating gel (0.7 × 10 cm) consisted of 5.0 % acrylamide (w/v) and 0.16 % Bis (w/v). Electrophoresis was performed in 0.01 M sodium citrate, 0.025 M Tris-HCl, 0.19 M glycine, pH = 8.3, at 4°. A constant current of 9 mA per cell was applied until the tracking dye, bromphenol blue, was 1 cm from the bottom of the gel. The gels were frozen in dry-ice and 1.8-mm slices were prepared with a fixed razor blade assembly. Citrate synthase activity was identified by adding 400 µl of the assay mixture (*see* enzyme assay above) to each of the thawed slices and incubating for 5 min at 22°. To each incubate, 400 µl of H_2O was then added, mixed, and the absorbance of the supernatants were measured at 412 nm. To assay for ^3H and ^{35}S contents, the slices were solubilized by addition of 300 µl of 30 % H_2O_2 (v/v) and incubation for 15 hr at 55°.

Radioassay

The ^3H and ^{35}S assays were obtained by addition of the samples to Aquasol (10 ml) and double-label counting at efficiencies of 51 % for ^{35}S and of 0 % for ^3H in the ^{35}S channel and of 10 % for ^{35}S and of 41 % for ^3H in the ^3H channel in a Mark II-Nuclear Chicago Liquid Scintillation Spectrometer. External standards were used for quench corrections in all of the assays.

Materials

Oxalacetate (sodium salt), acetyl-CoA, CoA, DTNB, ATP (sodium salt), sodium periodate, and dexamethasone were purchased from Sigma Chemical. Adipic acid dihydrazide, acrylamide, Bis and ammonium persulfate were supplied by Eastman Kodak Co. The acrylamide was recrystallized from 100 % acetone before use. Cyanogen bromide activated Sepharose 4B was obtained from Pharmacia (Sweden), *d*-aldosterone from Vismara Terapeutic (Italy), actinomycin D from Merck, Sharp & Dohme, and Joklik-MEM (methionine-free), as the prepackaged powder, from Gibco. [^3H]methionine (0.5 Ci/mmole) was purchased from Schwarz-Mann, and ^{35}S methionine (20–400 Ci/mmole) and Aquasol from New England Nuclear. Spirolactone (SC-26304) was a generous gift of G.D. Searle. The anti-citrate synthase was donated by Dr. P.A. Srere and prepared by immunizing rabbits with purified (homogeneous) rat heart citrate synthase, as described previously [23]. This preparation consisted of whole rabbit serum with a titer of 2 units of citrate synthase precipitated per ml of antiserum, and cross-reacts with this enzyme prepared from rat liver, spleen, kidney, and brain.

All of the conventional reagents were analytical or spectroquality grade.

Results

Effect of Aldosterone on Renal Medullary Citrate Synthase Activity

In previous studies, administration of aldosterone, at dosages down to 2 µg/100 g body wt, to adrenalectomized rats increased renal citrate synthase activity significantly [12, 15]. The results in the preceding paper

[17], however, indicate that a near-maximal increase in the urinary K^+/Na^+ ratio, and an incorporation of amino acids into medullary proteins is obtained at a dosage of 0.8 μg/100 g body wt (approximately twice the dose required for half-maximal responses). To insure that this lower dose is also effective in modulating renal citrate synthase activity, adrenalectomized rats were given 0.8 μg of aldosterone/100 g body weight and the kidneys were removed 3 hr after injection, at the peak of the response [15]. The kidneys were dissected into the three principal zones (cortex, medulla, and papilla) and assayed simultaneously with samples of liver. The concentration of the enzyme (units/mg protein) of the crude mitochondrial sonicates (particulate-free) was highest in the medulla and lowest in liver (Table 1). Aldosterone augmented citrate synthase activity in the medulla (+25 %) and cortex (+13 %) but had no significant effect in the papilla or liver. The observed aldosterone-dependent increase in citrate synthase activity could result either from changes in the kinetic properties or in the total activity (e.g., content) of the enzyme. The first possibility was evaluated by measuring K_ms for oxalacetate and acetyl CoA: The kidneys were removed 3 hr after injection of aldosterone (0.8 μg/100 g body wt) or the diluent, and mitochondrial sonicates (particulate-free) were prepared from the medulla. In the presence of fixed concentrations of acetyl-CoA (5 or 10 μM), the average K_ms for oxalacetate were 4.6 μM and 5.0 μM from the diluent and aldosterone treated medullas, respectively (Fig. 1*A* and Table 2). In the presence of fixed concentrations of oxalacetate (5 or 10 μM), the

Table 1. Effect of aldosterone on the activity of renal medullary and hepatic citrate synthase[a]

Tissue	Aldosterone	Citrate synthase[b]	Difference
Cortex	+	0.46±0.01	+12.9[c]
	−	0.40±0.01	
Medulla	+	0.86±0.02	+24.8[c]
	−	0.69±0.03	
Papilla	+	0.53±0.02	0
	−	0.53±0.02	
Liver	+	0.16±0.005	+7.3
	−	0.15±0.01	

[a] Adrenalectomized rats were injected either with aldosterone or the diluent, and the kidneys and liver were removed 3 hr later. The kidneys were dissected into the 3 major zones and particulate-free mitochondrial sonicates from these zones and the liver were assayed for citrate synthase activity. $n=9$ rats in each group (aldosterone *vs.* control). Results are given as mean ±SE.

[b] In units of enzyme activity/mg protein.

[c] Statistically significant ($P<0.005$).

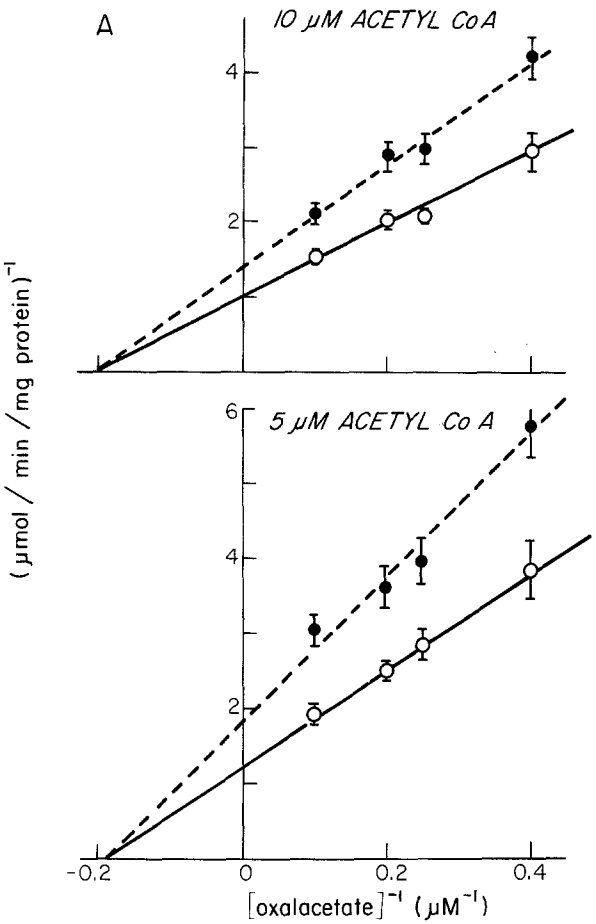

Fig. 1. Effect of aldosterone on the kinetic parameters of renal medullary citrate synthase. Adrenalectomized rats were injected with either diluent or aldosterone and the kidneys were removed 3 hr later. The renal medullas were assayed at fixed concentrations of acetyl CoA (5 or 10 μM) and variable concentrations of oxalacetate (*A*), or fixed concentrations of oxalacetate (5 or 10 μM) and variable concentrations of acetyl CoA (*B*). The results are analyzed by double reciprocal plots of the mean ±SE at each point (19). The medullas from the diluent-treated rats are denoted by (--●--) and from the aldosterone-treated rats by (—○—). $n=9$ rats in each group (aldosterone/diluent)

average K_m s for acetyl-CoA were the same (4.5 μM) for diluent and steroid-treated tissues, respectively (Fig. 1*B* and Table 2). These values are similar to those previously reported; namely 4.5 μM for oxalacetate and 5.0 μM for acetyl-CoA [23]. Clearly, aldosterone had no significant effect on either K_m. In accord with previous observations [12, 15], however, aldosterone significantly increased V_{max} for oxalacetate and acetyl-CoA to about the same extent, i.e., +36.6 % and +39.8 %, respectively (Table 2).

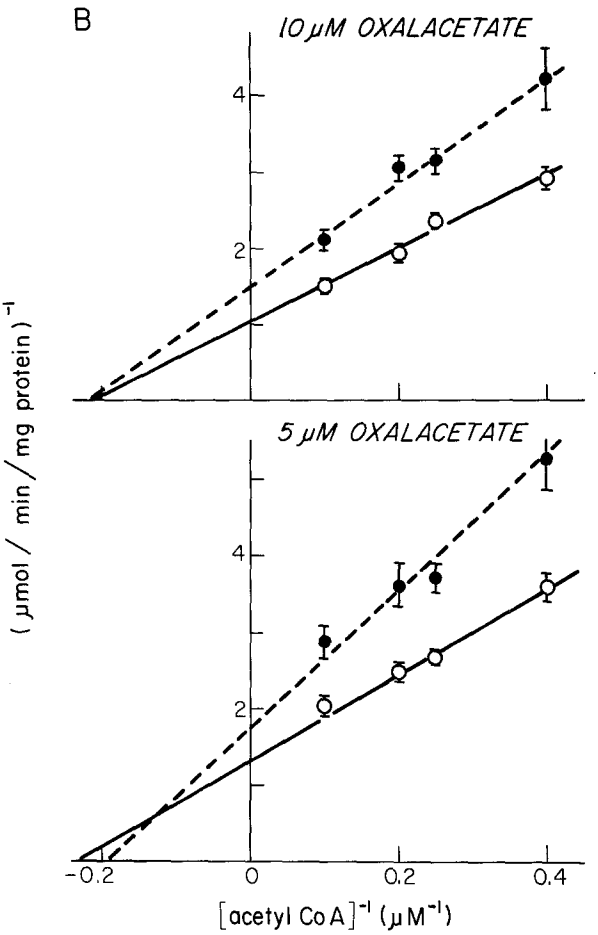

Table 2. Effects of aldosterone on kinetic parameters of renal medullary citrate synthase[a]

Aldosterone (0.8 μg/100 g body wt)	K_m (μM)				V_{max}(units/mg protein)			
	Oxalacetate		Acetyl CoA		Oxalacetate		Acetyl CoA	
	α	β	α	β	α	β	α	β
+	5.5±0.8	4.6±1.1	4.3±0.7	4.6±0.7	0.70±0.04	0.97±0.10	0.75±0.06	0.94±0.09
−	4.8±1.1	4.4±0.5	4.3±0.9	4.6±0.4	0.52±0.08	0.70±0.07	0.57±0.06	0.65±0.09
Difference (%)	+14.5	+4.5	0	0	+34.6[b]	+38.6[b]	+35.1[b]	+44.6[b]

[a] These results are computed from the Lineweaver-Burk plots shown in Fig. 1 ($A-B$). The experimental design is described in the legend to Fig. 1. The concentration of the cosubstrate, fixed at 5 μM is denoted by α and at 10 μM by β. Results are given as mean ±SE. $n=9$ rats in each group (steroid *vs.* control).

[b] Statistically significant ($P<0.005$).

Table 3. Titration of rat renal medullary citrate synthase with rabbit antiserum to the rat heart enzyme[a]

Status	Aldosterone (0.8 μg/ 100 g body wt)	Citrate synthase (units/ mg protein)	Difference (%)	Half-maximal titration (μl serum/ g protein)	Difference (%)
Normal	−	0.88±0.04	−22[b]	16.0±0.06	−29[b]
Adrenex	−	0.69±0.03	+25[b]	11.4±0.05	+39[b]
Adrenex	+	0.86±0.03		15.6±1.31	

[a] Assays were completed on normal (unoperated), and adrenalectomized rats injected either with the diluent or aldosterone. Three hours after injection, the kidneys were removed and particulate-free mitochondrial sonicates were prepared from the medulla. The sonicates, containing 0.05 to 0.09 units of citrate synthase were incubated with varying volumes of the diluted antiserum (1:50) for 24 hr at 4°. The precipitates were cleared from the solution by centrifugation and the supernatants were assayed for residual citrate synthase activity. The results are expressed in μl of antiserum required to remove half of the citrate synthase activity, normalized to the protein content of the sonicate. $n=12$ rats in each group (normal/adrenalectomized ± aldosterone). Results are given as mean ± SE.

[b] Statistically significant ($P<0.01$).

Antibody Titration

The proportionate increases in V_{max} for both substrates elicited by aldosterone could signify modulation of enzyme content. This alternative was explored by titration with anti-citrate synthase antibodies. Adrenalectomized rats were injected with diluent or aldosterone (0.8 μg/100 g body wt) and the kidneys were collected 3 hr later. The particulate-free mitochondrial sonicates from the renal medulla were assayed for citrate synthase activity and immuno-precipitated (Table 3). For purposes of comparison, kidneys were also obtained from normal, untreated rats and assayed simultaneously. The titration results are expressed as the volume of antiserum required to precipitate half of the citrate synthase activity in equal amounts of protein in the sonicates. Adrenalectomy depressed enzyme activity in the sonicates and half-maximum antibody titration by 28 % and 40 %, respectively. Administration of aldosterone restored both quantities to the normal values; citrate synthase activity increased 25 % and half-maximum titration, 39 % (Table 3). These results imply that the increase in enzyme activity (per mg of sonicate protein) is a consequence of an aldosterone-dependent increase in the quantity of enzyme extracted from the mitochondria.

Table 4. Partial purification of rat renal medullary and hepatic citrate synthase[a]

Tissue	Procedure	Citrate synthase (units/mg protein)	Cumulative yield (%)
Renal Medulla	Sonicate supernatant	0.42 ± 0.02	100
	Protamine sulfate	1.12 ± 0.04	107 ± 7
	Ammonium sulfate	2.38 ± 0.13	67 ± 11
	ATP-sepharose	30.0 ± 2.0	67[b]
Liver	Sonicate supernatant	0.10 ± 0.01	100
	Protamine sulfate	0.25 ± 0.02	96 ± 7
	Ammonium sulfate	0.62 ± 0.06	68 ± 14
	ATP-sepharose	20.0 ± 1.4	68[b]

[a] The particulate-free mitochondrial sonicates were treated sequentially with protamine sulfate and ammonium sulfate and chromatographed on ATP-sepharose, eluted with oxaloacetate and acetyl CoA. $n = 9$ separate preparations. Results are given as mean ± SE, except for the yield on ATP-sepharose chromatography which was determined in only two preparations.

[b] The yield in the two preparations in which it was measured was 100 % of that loaded on the ATP-sepharose column.

Methionine Incorporation into Citrate Synthase

Steroidal augmentation of extractable mitochondrial citrate synthase could result from a number of events, including: (i) induction of the synthesis of citrate synthase, (ii) a decrease in the rate of degradation of this enzyme, (iii) a differential increase in extractability of the enzyme *vs.* other mitochondrial proteins, (iv) a decrease in the synthesis of other mitochondrial proteins, or (v) an increase in the rate of degradation of other mitochondrial proteins. The first of these possibilities was assessed by a series of double-label experiments with ^{3}H- and ^{35}S methionine. In these experiments, the enzyme was isolated by two separate procedures: (i) Purification of the mitochondrial sonicates by treatment with protamine sulfate and ammonium sulfate, followed by ATP-Sepharose column chromatography and immunoprecipitation. (ii) Analysis of the ammonium sulfate precipitates of the mitochondrial sonicates by polyacrylamide gel electrophoresis.

The purification of renal and hepatic citrate synthase gave comparable results (Table 4). Precipitation of the bulk proteins with protamine sulfate resulted in a more than twofold increase in specific activity, and sequential precipitation with ammonium sulfate resulted in an overall increase in

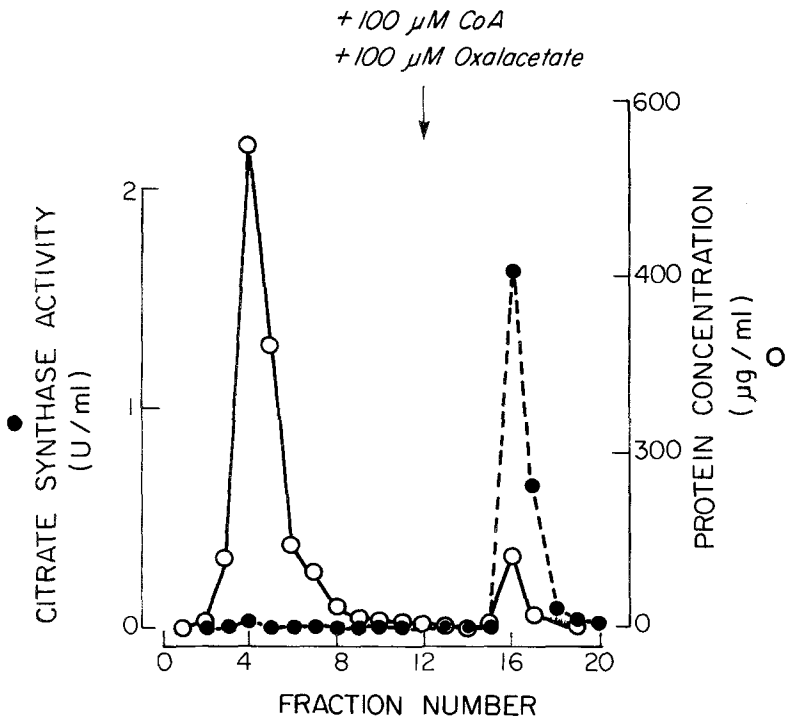

Fig. 2. ATP-sepharose chromatography of rat renal medullary partially purified citrate synthase. Medullary particulate-free mitochondrial sonicates were partially purified by treatment with protamine sulfate and ammonium sulfate (*see Methods*), applied to ATP-sepharose columns (0.7 × 13 cm) and sequentially eluted with 5 mM potassium phosphate buffer (pH = 7.4), and with 0.1 mM acetyl CoA + 0.1 mM oxalacetate in 5 mM potassium phosphate buffer (pH = 7.4). Each fraction was 2.5 ml. Protein concentration is denoted by (–o–) and citrate synthase activity by (--●--)

specific activities of about sixfold, with overall yields of about 68 %. The effectiveness of chromatography on ATP-Sepharose is indicated in Fig. 2. Initial elution with 5 mM potassium phosphate extracted 70 % of the applied protein but no detectable citrate synthase. Elution with 0.1 mM acetyl-CoA and 0.1 mM oxalacetate recovered 100 % of the applied enzymic activity in a single peak. Citrate synthase obtained from kidney mitochondria had a specific activity of 30 units/mg of protein in fractions 15–19 with a yield of 67 %, and from liver mitochondria the corresponding pool had a specific activity of 20 units/mg of protein with a similar yield (Table 4). Rat kidney citrate synthase, purified to homogeneity, has a reported activity of 100 units/mg of protein [23]. According to Srere[2], the anti-sera used in the

2 *Personal communication.*

present study precipitates only citrate synthase when partially purified by ATP-Sepharose chromatography.

A. *In vivo labeling.* Adrenalectomized rats were injected intraperitoneally with ^{3}H or ^{35}S methionine (500 μCi ea.) simultaneously with either aldosterone (0.8 μg/100 g body wt) or the diluent. In preliminary experiments, we found that the absolute specific activities (Ci/mg protein) of the purified citrate synthase obtained by intraperitoneal injection of labeled methionine was about the same as on injection into the lateral tail vein. In about half of the experiments, the isotopes ($^{3}H/^{35}S$) were reversed with respect to steroid administration (5 of the aldosterone-treated rats were given ^{3}H methionine and 4 were given ^{35}S methionine). The kidneys were removed 3 hr after injection and the appropriate pairs (from diluent- and aldosterone-treated rats) were pooled. The mitochondrial fractions were extracted by sonication, the enzyme was partially purified (including ammonium sulfate precipitation, *see* Table 4), and then chromatographed. The results of 9 experiments are summarized in Table 5. As in the earlier experiments, aldosterone elicited a significant increase in the activity (units/mg protein) of citrate synthase in the particulate-free mitochondrial sonicates. In the potassium phosphate eluates, the isotope ratio (aldosterone/control) were increased 20 % as compared to the isotope ratio in the sonicates prior to partial purification. The aldosterone/control isotope ratio (normalized to that of the crude sonicates) was also increased by 20 % in the citrate synthase enriched fraction. These results imply augmentation by aldosterone of the synthesis of some mitochondrial proteins, perhaps including citrate synthase. In contrast, aldosterone had no effect on the incorporation of methionine into hepatic mitochondrial proteins processed simultaneously (Table 5); although the absolute incorporation of radiomethionine (moles/mg protein) into the particulate-free mitochondrial sonicates was the same in liver and kidney. The possibility of enhanced synthesis of renal citrate synthase in response to aldosterone was tested by further separation of this enzyme from the other mitochondrial proteins in the acetyl-CoA-oxalacetate eluates by precipitation with the antiserum. As indicated in Table 5, aldosterone increased the isotope ratio of the immunoprecipitates by 44 %; this effect is in accord with the aldosterone-dependent increase in citrate synthase activity (+36 %). In contrast, aldosterone had no effect on the isotope ratio of the hepatic immunoprecipitate. These results are indicative of selective enhancement of the synthesis of renal citrate synthase.

Table 5. Effect of aldosterone on the incorporation of ^{3}H and ^{35}S methionine into rat hepatic and renal medullary citrate synthase, *in vivo*[a]

Tissue	Procedure	Normalized isotope ratios
Kidney[b]	Elution with potassium phosphate (fractions 3–7)	1.21 ± 0.09[e]
	Elution with oxaloacetate and acetyl CoA (fractions 16–20)	1.20 ± 0.07[e]
	Precipitation with antiserum[c]	1.44 ± 0.15[e]
Liver	Elution with potassium phosphate (fractions 3–7)	0.97 ± 0.06
	Elution with oxaloacetate and acetyl CoA (fractions 16–20)	0.91 ± 0.08
	Precipitation with antiserum[d]	1.03 ± 0.15

[a] Adrenalectomized rats were injected either with diluent or aldosterone (0.8 μg/100 g body wt) and 500 μCi of ^{3}H or ^{35}S methionine (and reversed in ~ half of the experiments). The kidneys and liver were removed 3 hr after injection, and the kidneys and livers were pooled, respectively, in pairs before homogenization. The particulate-free mitochondrial sonicates were partially purified by treatment with protamine sulfate and ammonium sulfate (cf. Table 4) and chromatographed on ATP-sepharose (cf. Fig. 2). The eluates obtained with the substrates, oxaloacetate and acetyl CoA were immuno-precipitated with rabbit antiserum, at maximal yield. The precipitates were analyzed for 3H and ^{35}S content after resolution in 4% SDS. The isotope ratios were normalized to that of the corresponding mitochondrial sonicate supernatants. The results obtained were indistinguishable regardless of the isotope assignments. Accordingly, all results are combined into single populations and expressed as mean ± SE. $n=9$ rats in each group (diluent *vs.* aldosterone).

[b] Separate renal homogenates were prepared and assayed for citrate synthase activity. The mitochondrial sonicates gave the following values (units/mg protein): 0.67 ± 0.05 (aldosterone-treated) and 0.51 ± 0.04 (diluent-treated), the difference (+36%) was statistically significant, $P < 0.02$).

[c] $n=8$ eluates precipitated with antiserum.

[d] $n=7$ eluates precipitated with antiserum.

[e] Statistically significant ($P < 0.01$).

B. *In vitro labeling.* The inference of induction of renal medullary citrate synthase by aldosterone was explored further in a series of *in vitro* labeling experiments. Aldosterone (0.8 μg/100 g body wt) or the diluent was injected into adrenalectomized rats and the kidneys were removed 1 hr later. Renal medullary slices were incubated for 2 hr at 20°, in either ^{3}H or ^{35}S methionine; the isotopes were reversed with respect to the aldosterone *vs.* diluent-treated slices in half the experiments. The slices from each pair of rats (steroid- and diluent-treated) were combined at the end of the period of incubation, homogenized and processed as indicated under *Methods.* In the *in vivo* experiments, the isotope contents (^{3}H and ^{35}S) of the entire

Table 6. Effect of aldosterone on the incorporation of [^{3}H]- and [^{35}S]-methionine into rat renal medullary citrate synthase, *in vitro*[a]

Procedure	Normalized isotope ratios
Elution with potassium phosphate (fractions 3–7)	1.10 ± 0.05
Elution with oxaloacetate and acetyl CoA (fractions 16–20)	1.34 ± 0.27
Precipitation with antiserum	1.30

[a] Adrenalectomized rats were injected with either the diluent or aldosterone (0.8 µg/100 g body wt). The kidneys were removed 1 hr later, and medullary slices were incubated in either ^{3}H- or ^{35}S-methionine (500 µCi/flask) for 2 hr at 20°. In half the experiments the isotopes were reversed with respect to the steroid/diluent pretreatments. The respective paired slices were pooled, homogenized, processed (partial purification of the mitochondrial sonicates as in *Methods*), and chromatographed on ATP-sepharose (cf. Fig. 2). The isotope ratios were normalized to that of the corresponding TCA-soluble fractions of the particulate-free mitochondrial sonicates. The results were combined into single populations, expressed as mean $\pm$SE, since the isotope assignments had no effect on the findings. To obtain adequate material, the medullas from 6 rats in each group (aldosterone *vs.* diluent) were combined in a particular incubation. $n = 4$ incubations, 2 of which were treated with antiserum.

particulate-free mitochondrial sonicates were used as the reference base for the isotope ratios on ATP-Sepharose chromatography and immuno-precipitation (Table 5). In the *in vitro* experiments, we elected to avoid contributions of the labeled proteins in these sonicates to the computation of the normalized isotope ratios by precipitating the proteins with trichloroacetic acid and using the ^{3}H and ^{35}S content of the acid-soluble pool as the reference base (Table 6). The results in Table 6 indicate that aldosterone had a minor effect (+10 %) on the incorporation of methionine into mitochondrial proteins other than citrate synthase and a more pronounced effect on incorporation into citrate synthase, as judged by the isotope ratio of the immuno-precipitates (+30 %). The fact that there was no change in the isotope ratio on immuno-precipitation of the acetyl CoA-oxaloacetate eluates (fractions 16–20, Fig. 2) suggests that some of the other mitochondrial proteins present in these fractions were also induced, since citrate synthase constitutes about 40 % of the proteins in these fractions. The aldosterone-dependent increase in incorporation of methionine into medullary citrate synthase presumably represents induction of the synthesis of this enzyme for two reasons: (i) The isotope ratio method (with isotope reversal) should yield equivalent increases in labeling of all proteins if the effect were on the radio-specific activity of the free pool of methionine. (ii)

Aldosterone had no effect on the trichloroacetic acid soluble radiomethionine content of renal medullary cytosol [17].

The effect of aldosterone on *in vitro* incorporation of methionine into renal medullary citrate synthase was also evaluated by polyacrylamide gel electrophoresis. The particulate-free mitochondrial sonicates were applied to the gels and analyzed under nondenaturing conditions which enabled the identification of the distribution of citrate synthase by enzymatic assays of the gel slices. These assays revealed that citrate synthase migrated as a single sharp peak with an $R_f = 0.4$, centered at slice 22 (Fig. 3). Since methionine incorporation was highest in the proteins with R_fs of 0.2 to 0.6 (slices 11–30, inclusive) and the protein bands, stained with amido black, were well-resolved in this region (Fig. 3), the analysis was restricted to this region of the gel. To normalize the results, the isotope content of a particular slice was divided by the total amount of the same isotope recovered in all of the slices in this region (11–30). To avoid isotope effects, the isotopes were reversed, with respect to prior treatment with aldosterone, in half of the incubations. The patterns in Fig. 4 indicate enhanced methionine incorporation into at least two proteins in the particulate-free mitochondrial sonicates, one of which ($R_f = 0.4$) co-migrates with citrate synthase. The increases in isotope ratio of this component were +40 % (aldosterone-pretreated labeled with ^3H methionine) and +60 % (aldosterone-pretreated labeled with ^{35}S methionine). These values are similar to the aldosterone-dependent increases in methionine incorporation into citrate synthase isolated by ATP-Sepharose chromatography and immuno-precipitation, and in citrate synthase activity (*cf.* Tables 5 and 6).

The available evidence implies that the action of aldosterone on Na^+ transport is mediated by induction of DNA-dependent RNA synthesis [6, 27]. If the increase in methionine incorporation into citrate synthase is a consequence of or dependent on hormonal augmentation of the products of transcription, this effect should be eliminated by inhibitors of DNA-dependent RNA synthesis, such as actinomycin D. The kidneys were removed from aldosterone (0.8 μg/100 g body wt) and diluent-treated rats, 1 hr after injection and medullary slices were incubated in ^3H- or ^{35}S methionine for 2 hr at 20°. The same procedure was used in pairs of rats pretreated with actinomycin D (70–80 μg/100 g body wt), one of which received aldosterone (0.8 μg/100 g body wt). Particulate-free mitochondrial sonicates were partially purified with protamine sulfate and ammonium sulfate, and then analyzed by polyacrylamide gel electrophoresis, as shown in Fig. 4. The normalized isotope ratios of the putative citrate synthase ($R_f = 0.4$) and a well-labeled reference protein ($R_f = 0.34$) are given in Table 7.

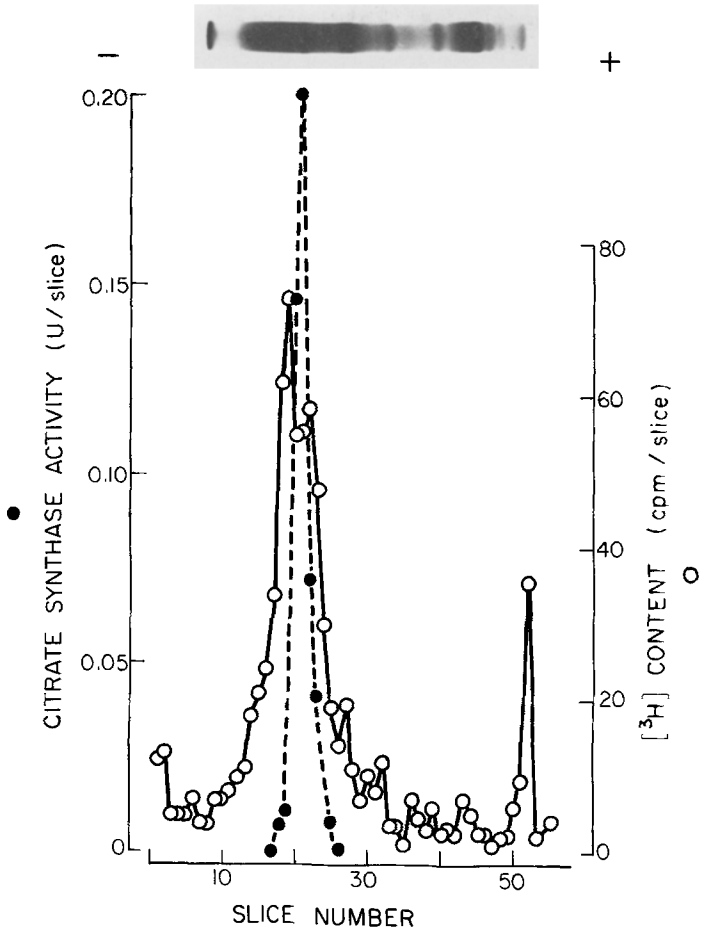

Fig. 3. Polyacrylamide gel electrophoresis of partially purified rat renal medullary citrate synthase. Renal medullary slices, prepared from adrenalectomized rats were incubated in ^{3}H methionine for 2 hr at 20°. Citrate synthase was partially purified by treating the particulate-free mitochondrial sonicates with protamine sulfate and ammonium sulfate. The gels (0.7 × 10 cm) contained 5% total acrylamide, 0.16% Bis in Tris-glycine buffer, pH = 8.3. The current, 9 mA/gel was applied for 1½–2 hr at 4°. The gels were stained for protein with 1% amido black in 7% acetic acid; parallel gels (slices) were analyzed for citrate synthase activity (denoted by --●--) or for ^{3}H content (denoted by −○−)

Aldosterone augmented methionine incorporation at the citrate synthase peak (+43 %) but had no effect on incorporation into the reference protein, although the absolute amounts of methionine incorporated into these two proteins was about the same. Actinomycin D had no effect on methionine incorporation into the reference protein but eliminated the response to aldosterone (Table 7).

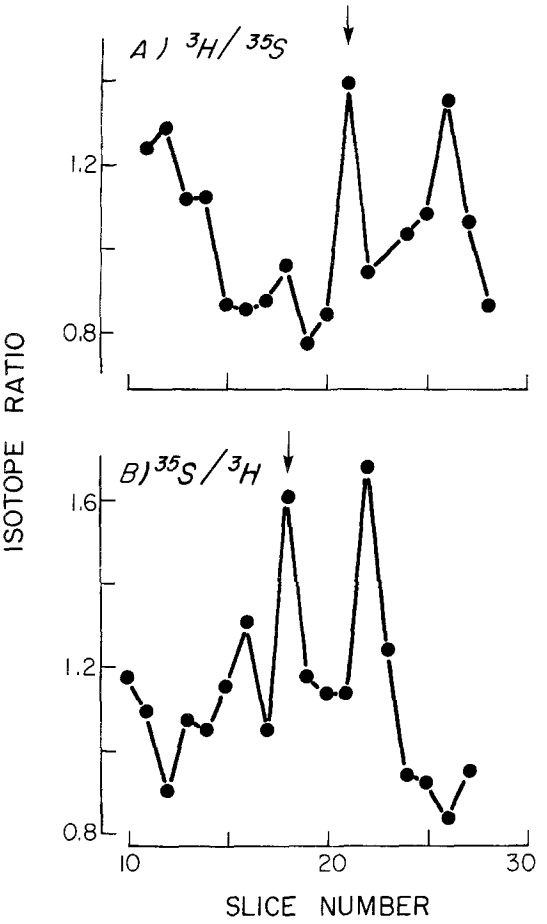

Fig. 4. Effect of aldosterone on the incorporation of ^{3}H or ^{35}S methionine into rat renal medullary mitochondrial proteins, analyzed by polyacrylamide gel electrophoresis. Adrenalectomized rats were injected with either the diluent or aldosterone (0.8 μg/100 g body wt); the kidneys were removed 1 hr later and medullary slices were incubated in ^{3}H- or ^{35}S-methionine (500 μCi/flask) for 2 hr at 20°. The particulate-free mitochondrial sonicates were partially purified by treatment with protamine sulfate and ammonium sulfate, and analyzed by polyacrylamide gel electrophoresis (*see* legend Fig. 3). The isotope ratios were normalized to that of the samples loaded on the gels. (*A*): Results obtained on labeling the renal medullas from the aldosterone-treated rats with ^{3}H methionine and the diluent-treated rats with ^{35}S methionine. (*B*): Results of incubations with the isotopes reversed. The arrows (↓) indicate the peak of the citrate synthase activity ($R_f = 0.4$). To obtain adequate yields, the medullary slices from 6 rats, treated identically were pooled and incubated with the appropriate isotope. $n = 2$ incubations for each assignment of isotopes. The results are the mean values of 6 parallel gels of each incubation

Table 7. Effects of actinomycin D and spirolactone (SC-26304) on the aldosterone-dependent increase in methionine incorporation into renal medullary citrate synthase and a comparison of the response to dexamethasone[a]

Experimental/control	Normalized isotope ratios	
	Citrate synthase maximum ($R_f = 0.4$)	Reference protein ($R_f = 0.34$)
Aldosterone/diluent	1.43 ± 0.10[b]	0.95 ± 0.08
Aldosterone + actinomycin D/actinomycin D	1.04 ± 0.06	0.92 ± 0.07
Aldosterone + SC-26304/SC-26304	1.07 ± 0.08	1.03 ± 0.05
Dexamethasone/diluent	0.94 ± 0.07	0.95 ± 0.11

[a] Adrenalectomized rats were injected with either aldosterone or dexamethasone (0.8 μg/100 g body wt) or diluent; pairs of rats were also pretreated with either actinomycin D (70–80 μg/100 g body wt) or SC-26304 (80 μg/100 g body wt). The kidneys were removed 1 hr later, medullary slices were incubated in ^{3}H- or ^{35}S-methionine, the particulate-free mitochondrial sonicates were partially purified and analyzed by polyacrylamide gel electrophoresis (cf. Figs. 3 and 4). Citrate synthase was located by enzyme assay. In half the experiments, the isotopes were reversed; the results of all incubations are combined, expressed as mean ±SE, as no isotope effect was apparent. The isotope ratios were normalized to that of the partially purified preparation loaded on the gel. To obtain adequate yields, the medullary slices from 6 rats, treated identically, were pooled and incubated with the appropriate isotope. $n = 4$ incubations (with isotope reversal) for the aldosterone *vs.* diluent experiments, and $n = 2$ incubations (with isotope reversal) for all of the other experiments. The incubations were analyzed on multiple gels; $n = 12$ gels for the aldosterone *vs.* diluent experiments, and $n = 8$ gels for all of the others.

[b] Statistically significant ($P < 0.005$).

Modulation of RNA metabolism and of active Na^+ transport by aldosterone appears to be mediated by binding of the steroid to high affinity (mineralocorticoid) receptors [6, 22, 24]. The participation of this receptor system in the aldosterone-dependent increase in incorporation of methionine into citrate synthase was evaluated with two agents, spirolactone (SC-26304), a competitive inhibitor of binding of aldosterone to the mineralocorticoid receptor [10, 22], and dexamethasone, a potent glucocorticoid with a relatively low affinity (1/50th that of aldosterone) for the mineralocorticoid receptor [7]. The procedure used in these experiments was as described above in those on the effects of actinomycin D. Pretreatment with SC-26304 (80 μg/100 g body wt) erased the aldosterone-dependent increase in methionine incorporation at the peak of citrate synthase activity and had no effect on the isotope ratio of the reference protein (Table 7). In addition, administration of dexamethasone (0.8 μg/100 g body wt) failed to elicit augmentation of methionine incorporation at the peak of citrate synthase activity or into the reference

protein. The findings summarized in Table 7 imply that the increase in methionine incorporation into citrate synthase (or a protein that co-migrates with citrate synthase) is mediated by the mineralocorticoid receptors and modulation of DNA-dependent RNA synthesis.

Discussion

Aldosterone augments the activities of several mitochondrial enzymes, including citrate synthase, isocitrate dehydrogenase (TPN^+), and glutamate-oxaloacetate transaminase in the rat kidney and the urinary bladder of the toad [12–15]. Our findings confirm the effect of aldosterone on renal citrate synthase activity. The zonal distribution pattern of this effect, greatest in the medulla (Table 1), is consistent with a mineralocorticoid response since the medulla is richer in distal and collecting tubules than either the cortex or papilla, and the action of aldosterone on ion transport has been localized to these segments of the nephron [11]. The lack of an effect of aldosterone on hepatic citrate synthase activity is in accord with this interpretation in that mineralocorticoid receptors were not detectable in the rat liver [3].

The aldosterone-dependent increase in renal medullary citrate synthase activity could reflect activation of a fixed number of enzyme units or recruitment of additional enzyme molecules. The potential for activation (or the reverse) is indicated by the finding that a variety of substances modify the activity of citrate synthase: $NADH/NAD^+$, $NADPH/NADP^+$ (oxidation-reduction potential), ATP/ADP (phosphorylation potential), ionic strength, acetyl CoA, oxaloacetate and ketone bodies inhibit the activity [29]. Even in the crude preparations (mitochondrial sonicates), however, pretreatment with aldosterone had no effect on the K_ms for acetyl CoA or oxalacetate but significantly augmented V_{max} for both substrates (Table 2). The changes in V_{max} can be attributed either to changes in the rate constants (for each of the substrates) or to changes in the initial enzyme concentration. Aldosterone enhanced V_{max} to about the same extent when acetyl CoA concentration was varied. Since citrate synthase has been characterized as accepting the substrates randomly [28], an activation mechanism seems less likely in that the individual substrate rate constants would have to be increased to the same extent. Moreover, the proportionate increases in enzyme activity and in the quantity of antiserum required to titrate the enzyme (per unit of protein in the particulate-free mitochondrial sonicates) suggests accumulation of additional enzyme molecules. The alternative explanation would be an equivalent decrease in the bulk of the proteins, other than citrate synthase, in the sonicates.

The possibility of induction of the synthesis of citrate synthase by aldosterone, as the basis for the increase in enzyme activity, was explored in studies on methionine incorporation. Two techniques were used to isolate citrate synthase after partial purification by sequential treatment of the mitochondrial sonicates with protamine sulfate and ammonium sulfate; (i) ATP-Sepharose affinity-elution chromatography, followed by immunoprecipitation with rabbit antirat citrate synthase, and (ii) polyacrylamide gel electrophoresis under nondenaturing conditions. With respect to the former method, the findings of Srere[2], indicate that the antiserum to citrate synthase does not co-precipitate significant quantities of nonspecific proteins in the substrate (acetyl CoA and oxalacetate) eluates of the ATP-Sepharose column.

The effect of aldosterone on methionine incorporation into immunoprecipitated citrate synthase was evaluated by labeling with either ^{3}H- or ^{35}S methionine *in vivo* or in renal medullary slices, *in vitro*. In the *in vivo* labeling experiments, aldosterone augmented methionine incorporation into immuno-precipitated renal citrate synthase by 44 % and citrate synthase activity by 36 % (Table 5). In contrast, no effect was found in incorporation of radiomethionine into immuno-precipitated hepatic citrate synthase. In these experiments the kidneys were processed 3 hr after simultaneous injection of the amino acid and aldosterone, at the time of the peak effect on urinary Na^{+}/K^{+} concentrations [15]. Reversal of the assignment of the isotopes to the control and steroid-treated rats essentially insured that the effect was not a consequence of an isotope effect, and implies induction of the synthesis of the enzyme.

This inference was also evaluated in *in vitro* labeling of renal medullary slices for 2 hr beginning 1 hr after administration of the steroid. Aldosterone enhanced methionine incorporation into the immuno-precipitated enzyme by 30 % and into the electrophoretic band associated with the peak of enzyme activity ($R_f = 0.4$) by 43 % (Tables 6 and 7). The use of the isotope ratio method and the selectivity of the effect by comparison with a neighboring well-labeled reference protein, $R_f = 0.34$, supports the inference of induction of the synthesis of the enzyme. In view of the evidence that aldosterone acts at the transcriptional (or post-transcriptional processing of RNA) level [5, 6, 24], the obliteration of the steroid-dependent increase in methionine incorporation by actinomycin D is also in accord with this inference.

In earlier studies, based to a considerable extent on the substrate-dependence of the action of aldosterone on Na^{+} transport across the toad

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bladder, it was proposed that modulation of mitochondrial metabolism may mediate the physiological response [5, 6, 9]. This proposal recently received some support in studies on the effect of aldosterone on the affinity of the metabolic reactions that drive transepithelial Na^+ transport [25]. In any case, the process appears to be initiated by binding of aldosterone to high-affinity mineralocorticoid receptors. To evaluate the potential relationship of the induction of citrate synthase to the mineralocorticoid response, three elements were introduced into the experiments: (i) A submaximal dose of aldosterone was used (0.8 μg/100 g body wt) sufficient to elicit 80 % or more of the effect on Na^+ transport (or urinary Na^+/K^+ ratios) [15] but below the level needed for cross-over into the glucocorticoid domain [7]; (ii) an equimolar dose of dexamethasone, a potent glucocorticoid, was tested for its effect on methionine incorporation into the electrophoretic band at the citrate synthase peak; (iii) SC-26304 is a potent competitive inhibitor of binding of aldosterone to the mineralocorticoid receptor as well as of the effects on RNA synthesis and Na^+ transport [22, 24] and this inhibitor was also tested for its effects on methionine incorporation into the electrophoretically defined enzyme.

The failure of a low dose of dexamethasone to elicit the increase in methionine incorporation into citrate synthase, as well as the effectiveness of a limited dose [only $2 \times K_{1/2}$] of aldosterone implies that the mineralocorticoid receptors (Type *I*, in the classification of Feldman *et al.* [7]) rather than the glucocorticoid receptors (Type *II*), mediate the apparent increase in the synthesis of citrate synthase (*see below* for discussion on the use of "apparent" in this context). The implication of the Type *I* receptors in this response was reinforced by the effective blockade provided by SC-26304.

The evidence summarized in the preceding paper [17] and the results described in this communication provide a reasonable basis for concluding that aldosterone induces the synthesis of a variety of renal medullary proteins, including citrate synthase, initiated by binding to the mineralocorticoid receptors. The conclusion that aldosterone induces the synthesis of citrate synthase is based on both immuno-precipitation and electrophoresis. Both methods, however, carry some degree of uncertainty: In the former case, the selectivity of precipitation is based on published results. To be certain that selective precipitation of the enzyme was achieved in the present studies would require a separate analysis of the composition of the product. In the latter case, more detailed analysis, e.g., variations in pH of the running gels and in the percent acrylamide, is required to assert with complete confidence that the radioisotope content of the band coincident with the peak of citrate synthase activity is, in fact, incorporated into citrate synthase. Nevertheless, the similarity of the findings with the two methods

lends considerable credence to the conclusion. There remains, however, an additional question concerning possible effects of aldosterone on the degradation of citrate synthase. The correspondence between the magnitude of the increases in citrate synthase activity and in incorporation of methionine into the enzyme (as defined by immuno-precipitation and electrophoresis), however, suggests that aldosterone has little effect on the degradation of citrate synthase (*cf.* Tables 2, 5, 6, and 7). Nevertheless, a definitive evaluation of the possibility of steroidal regulation of degradation of the enzyme requires a new set of experiments that address this question directly.

The observed increase in citrate synthase activity may relate to the well-defined action of aldosterone on Na^+ transport in a variety of ways. If citrate synthase is rate-limiting for ATP synthesis at the site of mineralocorticoid action (distal segment of the nephron), modulation of the available ATP/ADP could alter both Na^+ conductance and active Na^+ transport [6, 20]. Alternatively, the effects of aldosterone on citrate synthase and other mitochondrial proteins could modify lipid metabolism and various transport functions as proposed by Lien *et al.* [18].

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