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Effect of Aldosterone on Incorporation of Amino Acids into Renal Medullary Proteins

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Summary. Studies on the effects of pretreatment with aldosterone on the incorporation of ^{3}H leucine or ^{3}H methionine into proteins in renal slices were carried out in Joklik-modified minimal essential medium. Administration of aldosterone (2 μg/100 g body wt) to adrenalectomized rats increased ^{3}H leucine incorporation into trichloroacetic acid insoluble fractions of crude homogenates of cortical slices by 15.5 ± 0.4 % and of medullary slices by 53.5 ± 1.3 %. No increase in isotope incorporation was observed in slices of renal papilla or spleen prepared from the same rats. Aldosterone had no effect on the ^{3}H-leucine content of the trichloroacetic acid-soluble fractions of all three renal zones and the spleen. The dose of aldosterone that elicited a half-maximal increase in ^{3}H-methionine incorporation into proteins of renal medullary slices (0.45 μg of aldosterone/100 g body wt) was indistinguishable from that needed to elicit a half-maximal increase in the urinary K^+/Na^+ ratio (0.35 μg of aldosterone/100 g body wt). Dexamethasone, a potent glucocorticoid, at a dose of 0.8 μg/100 g body wt did not augment ^{3}H-leucine incorporation into renal medullary proteins but was effective at 8 μg/100 g body wt. Spirolactone (SC-26304), a potent anti-mineralocorticoid, abolished the effect of aldosterone on amino acid incorporation into medullary proteins when administered at a 100-fold higher dosage [i.e., 80 μg *vs.* 0.8 μg (per 100 g body wt)]. These results imply that the action of aldosterone on amino acid incorporation is mediated by the mineralocorticoid rather than the glucocorticoid pathway, presumably the mineralocorticoid receptors. Moreover, pretreatment of the rats with actinomycin D (70–80 μg/100 g body wt) erased the effect of aldosterone (0.8 μg/100 g body wt) on amino acid incorporation into medullary proteins.

In paired experiments with ^{3}H and ^{35}S methionine, aldosterone (0.8 μg/100 g body wt) increased methionine incorporation into trichloroacetic acid precipitable proteins of subcellular fractions of the renal medulla. The effect of aldosterone on incorporation of methionine into medullary cytosol proteins was analyzed further by polyacrylamide gel electrophoresis at pH 8.3 in tris-glycine buffer. The gel profiles indicate that aldosterone significantly increased methionine incorporation into at least one protein (independent of the isotope) with a molecular weight of ~31,000. This increase was inhibited by either pretreatment of the rat with actinomycin D (70–80 μg/100 g body wt or SC-26304 (80 μg/100 g body wt). Dexamethasone (0.8 μg/100 g body wt) did not increase incorpo-

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ration of methionine into the medullary cytosol proteins resolved by polyacrylamide gel electrophoresis.

The induction hypothesis posits that aldosterone augments the synthesis of specific proteins, rate-limiting for Na^+ transport, dictated by increased synthesis of mRNA [6, 20]. In accord with this hypothesis are the findings of high affinity receptors for aldosterone associated with the chromatin of various target tissues [1, 26] and augmentation of the incorporation of precursors into cytoplasmic polyadenylated RNA (putatively messenger RNA) [29, 39]. As expected, inhibitors of RNA and protein synthesis eliminate the mineralocorticoid response [6, 20]. Although some progress has been made in the characterization of the effects of aldosterone on protein synthesis, the primary mediators of the action on Na^+ transport have not been identified as yet.

In previous studies, aldosterone enhanced the incorporation of labeled amino acids into proteins (trichloroacetic acid precipitates) of the rat and rabbit kidney [10, 33]. In the isolated urinary bladder of the toad, aldosterone increased amino acid incorporation into discrete peptides with molecular weights that ranged from 12,000 to 38,000 [32]. Neither the subcellular site of origin nor the functions subserved by these proteins, however, have been defined.

The present report concerns a further analysis of aldosterone-dependent protein metabolism in the rat kidney based on administration of the hormone *in vivo* and labeling with radioactive amino acids, *in vitro*.

Materials and Methods

Physiological Preparations

Sprague-Dawley male rats were bilaterally adrenalectomized 7 days before use. The rats were given standard Purina chow pellets and 0.155 M NaCl (drinking water), *ad libitum*. On the day of the experiments, the rats weighed 180–200 g. Four series of experiments were completed: (i) Administration of various doses of aldosterone or the diluent, (ii) administration of various doses of dexamethasone or the diluent, (iii) administration of aldosterone, with or without prior treatment with actinomycin D, and (iv) administration of aldosterone with or without prior treatment with spirolactone (SC-26304)[1].

In the dose-response experiments, various amounts of aldosterone or dexamethasone were dissolved in 0.1 ml of absolute ethanol and diluted with 0.155M NaCl, such that a subcutaneous injection of 1 ml per 100 g body wt gave the desired dose. In the actinomycin D experiments, the drug was dissolved in 0.155 M NaCl (125 μg/ml) and injected

1 *Abbreviations:* SC-26304 = Spirolactone; Bis = N,N′-methylene-bis-acrylamide; TEMED = N,N′,N′-tetramethylethylenediamine.

intraperitoneally (70–85 μg/100 g body wt) 1 hr before the injection of aldosterone (0.8 μg/100 g body wt). In the spirolactone experiments, SC-26304 was dissolved in ethanol/propylene glycol/saline (2:5:5) to maximum solubility (12 mg/ml) and injected intraperitoneally 30 min before subcutaneous administration of aldosterone (0.8 μg/100 g body wt). The dose ratios of SC-26304/aldosterone were 25:1, 50:1, and 100:1.

Preparation of Kidney Slices

One hour after injection of the steroid (aldosterone or dexamethasone) or the diluent, the rats were killed by cervical dislocation and the kidneys were immediately perfused with 20 ml of ice-cold 0.155 M NaCl via the inferior vena cava. The perfused kidneys were transferred into ice-cold 0.25 M sucrose and dissected into 3 zones; cortex, medulla, and papilla. Slices, 300 μm thick, were prepared with a McIlwain tissue chopper (Brinkman Instruments) and collected in ice-cold 0.25 M sucrose. The excess sucrose solution was removed by pouring the collection through a single layer of Nytex (nylon cloth, 132 mesh). The slices were transferred directly into Joklik-modified minimal essential medium (MEM) (pH = 7.6) (3–20 mg wet wt of slices in 1 ml of medium) in a 10-ml Erlenmeyer flask. In the experiments with ^{3}H leucine, the medium was leucine-free, and similarly in the experiments with ^{3}H- or ^{35}S methionine, the medium was devoid of methionine. In the single isotope experiments, ^{3}H leucine or ^{3}H methionine was added to the medium to a final concentration of 2.5 μCi/ml. In the double isotope experiments, medullary slices from 6 rats, treated either with aldosterone (0.8 μg/100 g body wt) or the diluent were pooled (~0.7 to 1.0 g wet wt) and placed in 3 ml of methionine-free Joklik-modified MEM that contained either ^{3}H or ^{35}S methionine (final concentration = 100 μCi/ml), in a 25-ml Erlenmeyer flask. Sufficient nonradioactive methionine was added to the media to provide a constant specific activity (2 Ci/mmole) in all flasks.

All incubations were carried out at 20° for 2 hr, under an O_2/CO_2 (95:5) atmosphere. Under these conditions, the morphological and metabolic integrity of the slices are maximally preserved.[2] The incubations were usually terminated by the addition of cycloheximide (final concentration = 3×10^{-5} M). Ten min after the addition of cycloheximide, the slices were collected on a single layer of Nytex and were washed successively with 100 ml of ice-cold Joklik-modified MEM supplemented with 10 mM leucine or methionine (depending on the isotope used) and with 100 ml of ice-cold Joklik-modified MEM. In the experiments in which the time of incubation was limited to 1 hr or less, incorporation of the isotope was terminated with the washing procedure, without prior treatment with cycloheximide.

Isotope Incorporation and Subcellular Fractionation

On completion of the incubations, as described above, the quantity of ^{3}H leucine or ^{3}H methionine into total protein (of the renal zones) was estimated as follows: All steps were carried out at 0–4°. The kidney slices were homogenized in 0.25 M sucrose (transferred directly from the ice-cold Joklik-modified MEM) at a 1:10 (wet wt/volume) ratio, with 10 strokes at full speed in a motor driven Teflon-glass Elvehjem-Potter homogenizer. The homogenates were divided into two equal parts and acidified with trichloroacetic acid (final concentration = 10%). DNA content of one portion was determined by the method of Burton [3]. The other portion was centrifuged at $10{,}000 \times g$ for 10 min in a Sorval centrifuge. The drained pellet was redissolved in 3 ml of 1 N NaOH at room

2 Law, P.Y., and Edelman, I.S. *(unpublished observation).*

temperature, and aliquots were used to determine protein content by the method of Lowry *et al.* [24], and ^{3}H or ^{35}S activity by liquid scintillation spectrometry in a Mark II Nuclear-Chicago instrument. For the latter assays, the aliquots were neutralized with 1 N HCl and added to Aquasol [in a ratio of 1:10(v/v)].

In the double isotope experiments, the medullary slices from both the aldosterone-treated and control rats were pooled before homogenization in 0.25 M sucrose (1:10, w/v). The subcellular fractions were obtained by modifications of the method of Lo *et al.* [23] as outlined in Fig. 1. All steps, including extraction of the ribosomal proteins, were carried out at 0–4°. The nuclei were purified by centrifugation in 2.2 M sucrose, 3 mM $CaCl_2$, as described by Marver *et al.* [26], and fractionated further into, (i) 0.15 M NaCl extract, (ii) histones and (iii) SDS-soluble chromosomal proteins by the method of Spelsberg *et al.* [34, 40]. The plasma membrane-rich fraction was obtained by distribution in an aqueous two-phase polymer (dextranpolyethylene glycol) system as described by Lesko *et al.* [21]. The plasma membrane, heavy mitochondria, and light mitochondria fractions were dissolved in 1.0 ml of 1 % SDS (2–6 mg protein/ml) at 24°. The microsomal pellet was suspended in 5 ml of 2 % Triton X-100 (1–2 mg protein/ml) to free the ribosomes from the membrane matrix. This suspension was centrifuged for 1 hr at 100,000 × g in a IEN model B-60 ultracentrifuge. The pellets were resuspended in 4.5 ml of 2 M LiCl-/2.4 M urea and extracted for 16 hr, to dissolve the ribosomal proteins. The cytoplasmic supernatants were processed by ultrafiltration in a Minicon (Amicon Co.), in order to remove free amino acids and molecules with mol wt ≦ 10,000. Routinely, the cytoplasmic supernatants were concentrated 10-fold and rediluted to the original volume with distilled water. This process was repeated twice in order to remove free amino acids and other solutes. The protein contents of the various fractions were determined by precipitation in 10 % trichloroacetic acid, dissolving the precipitate in 1 N NaOH, and analysis by the method of Lowry *et al.* [24].

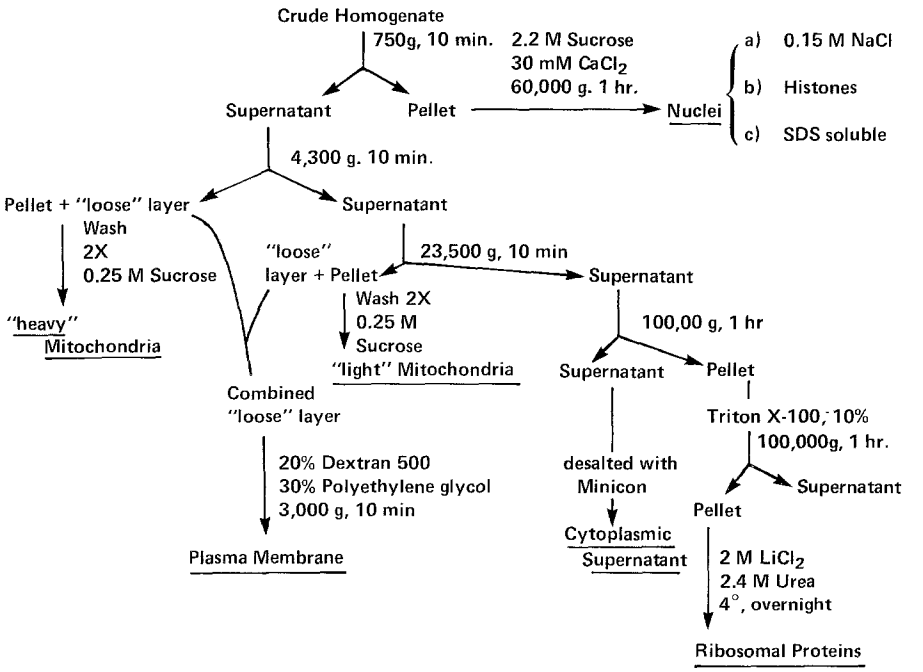


Fig. 1. Flow diagram of the method used to fractionate the kidney homogenates

Polyacrylamide Gel Electrophoresis

Renal medullary cytoplasmic supernatant proteins were analyzed by gel electrophoresis by the method of Davis [5]. The separation gels (0.7 cm × 10 cm) consisted of 7.5 % acrylamide and 0.25 % Bis in 0.75 M Tris-HCl and 5 mM TEMED at pH 8.8. The stacking gels (0.7 cm × 2.5 cm) made up of 2.5 % acrylamide and 0.6 % Bis in 0.062 M Tris-HCl and 5 mM TEMED at pH 6.7 were layered on the polymerized separating gels. The desalted, concentrated supernatants containing 20–30,000 dpm of isotope and 100 to 200 µg of protein in 0.1 ml of 10 % (w/v) sucrose were applied to the gels and electrophoresed at a constant current of 6 mA/gel, until the tracking dye (bromphenol blue) was 0.5 to 0.7 cm from the bottom of the separation gel. The computed pH in the separation gel was 8.3 during electrophoresis. The gels were fixed by immersion in 10 % trichloroacetic acid at 4° for 30 min, sliced (1.8 mm thick) with a razor blade assembly, and dissolved by the method of Tishler and Epstein [38]. Each slice was transferred into a counting vial and covered with 0.1 ml of 30 % H_2O_2. The capped vials were incubated overnight at 55° in a constant-temperature oven. After cooling, 10 ml of aquasol was added to each vial and the activity was assayed in a Mark II Nuclear-Chicago Liquid Scintillation Spectrometer. The dpm of each isotope and of the isotope ratios were calculated based on the external standard ratio. The isotope ratios were computed from the percent of the total ^3H and ^{35}S that was recovered in the slice.

Materials

d-Aldosterone was purchased from Vismara Terapeutic (Italy), dexamethasone from Sigma Chemical Co., and actinomycin D from Merck, Sharp and Dohme. Spirolactone (SC-26304) was provided, as a generous gift, by G.D. Searle Co. Leucine-free and methionine-free Joklik-modified MEM was obtained as a prepackaged powder from Gibco. ^3H leucine (50 Ci/mmole) was purchased from Schwarz and Mann, and 2-^3H methionine (2 Ci/mmole) from Amersham-Searle. ^{35}S methionine (200–400 Ci/mmole) and aquasol were obtained from New England Nuclear. The gel electrophoresis chemicals, acrylamide, N,N′-methylene-bis-acrylamide (Bis) and ammonium persulfate were purchased from Eastman Kodak Co. The acrylamide was recrystallized from acetone before use. Nytex was obtained from Swiss Silk Bolting Cloth Manufacturing Co., Ltd. All of the conventional chemicals were reagent grade or spectroquality.

Results

Effect of Aldosterone on ^3H Leucine Incoporation into Renal Proteins

To circumvent the problem of indirect effects on incorporation of amino acids into proteins *in vivo* (for example, on renal blood flow), we used *in vitro* incorporation of amino acids into kidney slices after injection of aldosterone, *in vivo*. The conditions of incubation of the slices included use of Joklik-modified MEM with an O_2/CO_2 (95:5) atmosphere at 20°. The incubation temperature (20°) was chosen on the

basis of three criteria[3]: (i) preservation of morphological integrity, (ii) maintenance of respiration throughout the period of incubation, and (iii) positive, linear dependence of amino acid incorporation on temperatures above and below 20°. At incubation temperatures between 30 and 37°, amino acid incorporation was a negative function of the temperature.

To characterize the determinants of amino acid incorporation into renal proteins, estimates were made of the time-course of uptake of ^{3}H leucine into the trichloroacetic acid-soluble fraction and of incorporation into trichloroacetic acid-precipitable peptides. Aldosterone (2 μg/100 g body wt) or the diluent were injected into adrenalectomized rats and the kidneys were removed 1 hr later. Uptake of ^{3}H leucine into the trichloroacetic acid-soluble pool of medullary homogenates attained steady-state levels (hyperbolic time-course) within 30 min of addition to the media.

Incorporation of ^{3}H leucine into total proteins of medullary homogenates was a linear function of time up to 120 min of incubation in slices prepared from aldosterone-treated (2 μg/100 g body wt) and control, adrenalectomized rats (Table 1). Similar studies were carried out on the time-dependence of incorporation of ^{3}H leucine into total trichloroacetic acid precipitable peptides of crude homogenates prepared from the renal cortex and papilla. Graphic analysis confirmed that incorporation was a linear function of time, in all three zones of the kidney, for at least 120 min. These results are also summarized in Table 1. The linear least squares regression had correlation coefficients of 0.987–0.999. Treatment with aldosterone augmented incorporation in that the slope of the regression relationship in the treated group was 14 %, 33 %, and 9 % greater in the cortex, medulla, and papilla, respectively. Only the effect on the medulla, however, was statistically significant (Table 1). The aldosterone-dependent increase in the rate of incorporation of ^{3}H leucine into medullary proteins was also evaluated at the end of the incubation (120 min); the specific activity of the trichloroacetic acid-insoluble fraction was 54 % greater ($P < 0.001$) in medullary homogenates prepared from aldosterone-treated rats than in those of diluent-injected controls. The linearity of incorporation of the precursor into proteins as a function of time, regardless of hormonal status (Table 1), eliminated the possibility that the response to the hormone was a consequence of modification of a nonlinear process.

To evaluate the possibility that the increase in the specific activity of the medullary proteins elicited by aldosterone may have resulted from

3 *Ibid.*

Table 1. Effect of aldosterone on the rate of incorporation of ^{3}H leucine into renal proteins[a]

Renal zone	Aldosterone (2 μg/100 g body wt)	Correlation coefficients (^{3}H activity *vs.* time)	Slope ($\times 10^3$ dpm/mg protein/hr)	Difference in slope (%)
Cortex	+	0.994	11.0 ± 0.5	+14
	−	0.996	9.6 ± 0.3	
Medulla	+	0.999	31.7 ± 0.2	+33[b]
	−	0.998	23.9 ± 0.7	
Papilla	+	0.987	44.2 ± 2.7	+9
	−	0.999	40.5 ± 1.4	

[a] Adrenalectomized rats were injected either with aldosterone or the diluent and the kidneys were removed 1 hr later. Slices of the zones were incubated with ^{3}H leucine at 20° for 15, 30, 60, 90, and 120 min. The trichloroacetic acid-precipitable fractions of the crude homogenates were assayed for ^{3}H activity and rates of incorporation determined by the linear regression method. $n=6$ pairs of rats at each incubation time. Results are given as mean ± SE.

[b] Statistically significant ($P<0.05$).

Table 2. Effect of aldosterone on the incorporation of ^{3}H leucine into renal proteins (based on DNA content)[a]

Tissue	Aldosterone (2 μg/100 g body wt)	^{3}H TCA-soluble (dpm/mg protein) $\times 10^{-4}$	^{3}H Protein (dpm/mg protein) $\times 10^{-4}$	Protein content (mg protein/ mg DNA)	^{3}H Protein/ DNA (dpm/mg DNA) $\times 10^{-4}$
Renal Cortex	+	2.53 ± 0.15	2.43 ± 0.08[b]	98.4 ± 2.7	227 ± 9
	−	2.33 ± 0.18	2.11 ± 0.13	91.4 ± 11.4	183 ± 26
Renal Medulla	+	2.79 ± 0.22	6.36 ± 0.60[b]	58.7 ± 6.8	354 ± 21[b]
	−	2.52 ± 0.17	4.55 ± 0.23	50.9 ± 6.8	240 ± 14
Renal Papilla	+	11.9 ± 1.15	8.53 ± 0.35	45.6 ± 5.1	297 ± 23
	−	12.4 ± 1.05	7.62 ± 0.58	45.8 ± 4.7	272 ± 21
Spleen	+	1.69 ± 0.15	2.62 ± 0.38	36.7 ± 4.3	121 ± 12
	−	1.96 ± 0.21	2.68 ± 0.25	35.6 ± 4.8	118 ± 14

[a] Adrenalectomized rats were injected with either aldosterone or the diluent and the kidneys were removed 1 hr later. Slices of the renal zones and the spleen were incubated with ^{3}H leucine for 2 hr at 20°. The trichloroacetic acid-soluble and precipitable fractions of the crude homogenates were analyzed for ^{3}H activity and protein content. The homogenates were also analyzed for DNA content. $n=10$ pairs of rats in kidney studies and 6 pairs of rats in spleen studies. Results are given as mean ± SE.

[b] Statistically significant $P<0.05$).

effects on the protein content of the slices, DNA was used as the reference mass in a second set of experiments. Adrenalectomized rats were injected with aldosterone (2 μg/100 g body wt) or the diluent. One hour later, slices were prepared from the three zones of the kidney, as well as the spleen, and incubated in [^{3}H]leucine for 2 hr at 20°. Incorporation into peptides was expressed in terms of dpm/μg of trichloroacetic acid-precipitable protein and dpm/μg of DNA in the crude homogenate (Table 2). Aldosterone augmented incorporation by 24 % in the cortex and 48 % in the medulla ($P < 0.01$), as compared to 8.5 % and 11 % increases in uptake of ^{3}H leucine into the trichloroacetic acid-soluble fractions of these zones. No significant effects were detected in the renal papilla or the spleen. The latter was used as an internal control; because of the absence of high affinity receptors for aldosterone, the rat spleen is considered to be a nontarget organ [26]. The results in Tables 1 & 2 also confirm the progressively higher rate of amino acid incorporation into proteins in the inner zones (i.e., cortex < medulla < papilla) noted by Kline and Liberti [17]. They attributed the higher specific activity in papilla to higher metabolic rates [17]. These differences, however, are attributable, in part, to differences in uptake of ^{3}H leucine into the trichloroacetic acid-soluble pool (Table 2). Thus, uptake of ^{3}H leucine into the soluble pool is ~fivefold greater in the papilla than in either cortex or medulla and would account for the entire enhancement in the labeling of the papillary proteins. This explanation, however, does not account for the lower rate of incorporation into cortical *vs.* medullary proteins, since the uptakes into the soluble pools were almost the same in these zones.

If the aldosterone-dependent increase in ^{3}H leucine incorporation into medullary proteins is related to mineralocorticoid action, there should be some correspondence between the doses of hormone required to elicit the effect on Na^+ transport and on amino acid incorporation. In a previous study, Kirsten and Kirsten [15] obtained a half-maximal increase in the urinary K^+/Na^+ ratio with 0.35 μg of aldosterone/100 g body wt, in adrenalectomized, Sprague-Dawley rats. To assess the dose-dependence of the effect on amino acid incorporation, pairs of adrenalectomized rats were injected with aldosterone or the diluent at various doses, up to 5 μg/100 g body wt. The kidneys were removed 1 hr later, and the medullary slices were incubated in ^{3}H methionine for 2 hr. Incorporation into total trichloroacetic acid-precipitable peptides of the crude homogenates increased to a maximal level at a dose of 1 μg/100 g body wt, and half-maximal increase was attained at 0.45 μg of

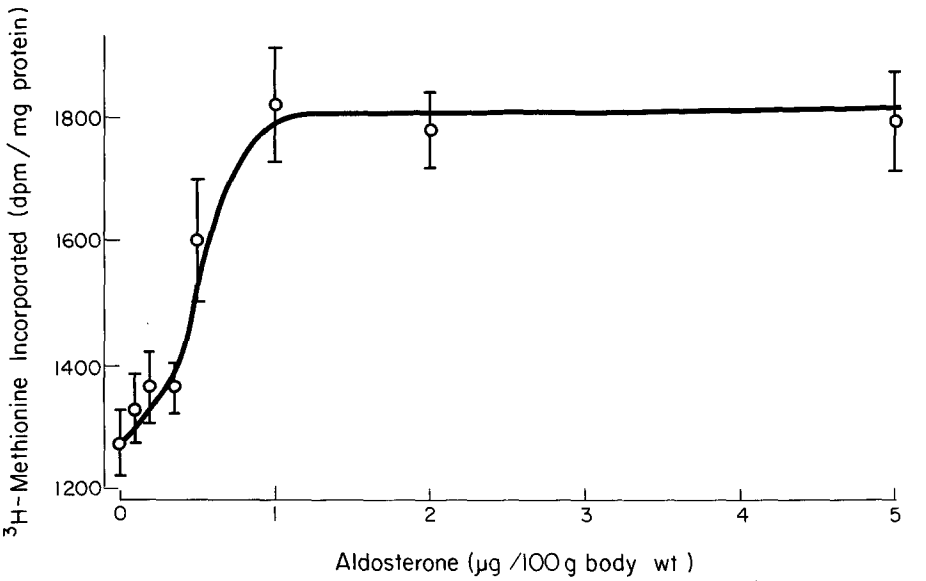


Fig. 2. Aldosterone-dependence of incorporation of ^{3}H methionine into renal medullary proteins. Adrenalectomized rats were injected with either the diluent or various doses of aldosterone and the kidneys were removed 1 hr thereafter. Medullary slices were incubated in ^{3}H methionine at 20° for 2 hr, and incorporation into proteins was assessed by precipitation with trichloroacetic acid. $n=6$ rats for each dose of aldosterone

aldosterone/100 g body wt (Fig. 2). At all doses of aldosterone, the specific activities of the trichloroacetic acid-soluble fractions did not differ significantly from that of the diluent-injected control. Moreover, the magnitude of the maximal aldosterone-dependent increase in methionine incorporation (+42 %) was the same as with leucine incorporation (+49 %) (*cf.* Table 2 and Fig. 4). The equivalence in the half-maximal dose for amino acid incorporation and for the change in urinary K^+/Na^+ ratio [15] suggests that these effects may be interrelated.

Comparison of the Effects of Aldosterone and Dexamethasone on Amino Acid Incorporation

Aldosterone binds to renal glucocorticoid receptors with an affinity equal to ~20 % that of dexamethasone [11]. The possibility that the effect of aldosterone on amino acid incorporation into medullary proteins was an expression of a glucocorticoid rather than a mineralocor-

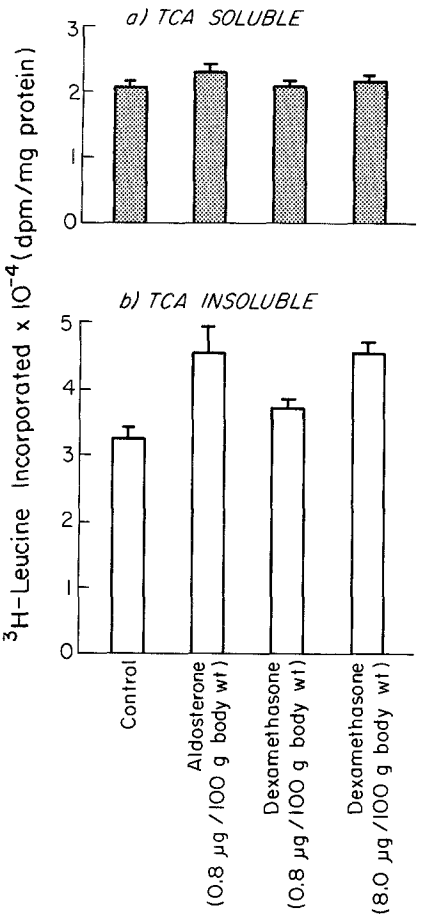


Fig. 3. Effects of aldosterone and dexamethasone on incorporation of ^{3}H leucine into renal medullary proteins. Pairs of adrenalectomized rats were injected with either the diluent or one of the steroids; 0.8 µg/100 g body wt of aldosterone, or 8 µg/100 g body wt of dexamethasone. The kidneys were removed 1 hr later, and medullary slices were incubated in ^{3}H leucine for 2 hr at 20°. (*a*): The effects of the steroids on ^{3}H leucine uptake into the trichloroacetic acid soluble fraction and (*b*) into the precipitates. $n=6$ rats for each point

ticoid pathway was evaluated by comparison with two dose levels of dexamethasone. Adrenalectomized rats were injected with either the diluent or aldosterone (0.8 µg/100 g body wt) or dexamethasone (0.8 µg or 8 µg/100 g body wt). One hour after injection, renal medullary slices were prepared and incubated with ^{3}H leucine for 2 hr at 20°. No significant change in the labeling of the trichloroacetic acid-soluble pool was noted with either steroid (Fig. 3*a*). Aldosterone elicited the usual increase in incorporation into proteins (+40 %) (Fig. 3*b*). In contrast, an equimolar

dose of dexamethasone had no significant effect on ^3H leucine incorporation. A 10-fold higher dose of dexamethasone, sufficient to produce glucocorticoid effects, however, elicited a comparable effect to that of aldosterone. Since the affinity of aldosterone for the renal mineralocorticoid receptor is 10-fold higher than the affinity of dexamethasone for the glucocorticoid receptor, and 50-fold higher than the affinity of dexamethasone for the mineralocorticoid receptor, these results imply that the effect of aldosterone on ^3H leucine incorporation into medullary proteins is an expression of a mineralocorticoid-specific pathway.

Effect of Actinomycin D on the Response to Aldosterone

To determine whether augmentation of incorporation of amino acids into medullary proteins by aldosterone required intact RNA synthesis,

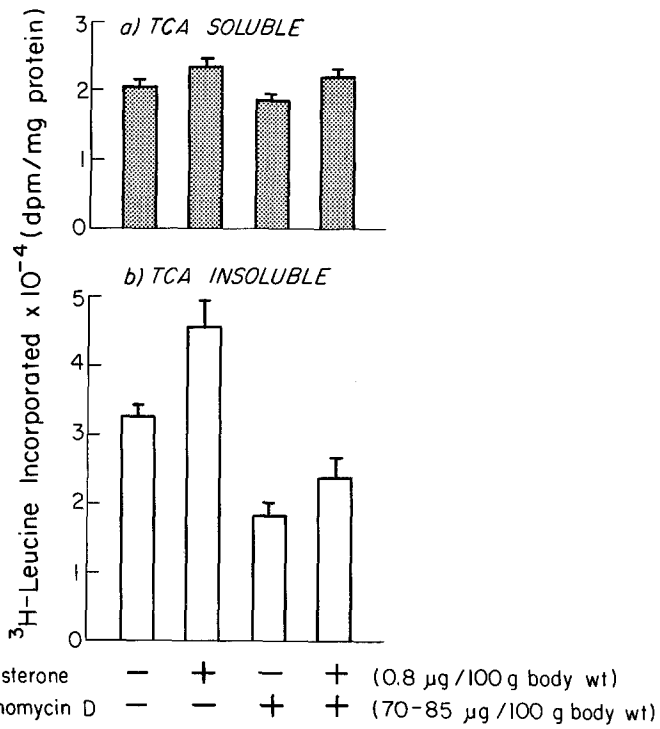


Fig. 4. Effect of actinomycin D on the aldosterone-dependent incorporation of ^3H leucine into renal medullary proteins. Adrenalectomized rats were injected with actinomycin D (70–80 µg/100 g body wt) or the diluent 1 hr before the administration of aldosterone (0.8 µg/100 g body wt). One hour after hormone was given, the kidneys were removed and medullary slices were incubated in ^3H leucine for 2 hr at 20°. Crude homogenates were extracted with trichloroacetic acid; (*a*): the acid-soluble ^3H leucine content and (*b*) that of the precipitates. $n=6$ rats for each point

actinomycin D (70–80 μg body wt), a well-characterized inhibitor of DNA-directed RNA synthesis, was administered 1 hr before aldosterone (0.8 μg/100 g body wt). Medullary slices were then incubated in ^{3}H leucine for 2 hr at 20°. As documented above (*cf.* Tables 1 and 2), aldosterone elicited a 40% increase ($P<0.05$) in incorporation of ^{3}H leucine into proteins of medullary crude homogenates (Fig. 4). These are the same results as those shown in Fig. 3. In the steroid-depleted rats, actinomycin D significantly depressed ^{3}H leucine incorporation (−45%, $P<0.01$) (Fig. 4). Pretreatment with actinomycin D of the rats given aldosterone reduced the steroid-dependent increment in the specific activity of the medullary proteins to the point of statistical insignificance (*cf.* last two bars in Fig. 4) and below that of the controls (*cf.* bars *1* and *4* in Fig. 4). None of these agents, either alone or in combination, had any effect on the uptake of ^{3}H leucine into the trichloroacetic acid-soluble pool (Fig. 4, upper panel).

Effect of SC-26304 on the Response to Aldosterone

The antagonist, SC-26304, was used as a further index of the role of the mineralocorticoid receptor system in the aldosterone-induced increase in incorporation of amino acids into medullary proteins. Spirolactones competitively inhibit binding of aldosterone to the putative receptors and thereby block attachment of the steroid-receptor complex to the chromatin of target cell nuclei [27]. SC-26304 was selected because of its high affinity for the aldosterone receptor and effective inhibition of the mineralocorticoid response in the adrenalectomized rat [12, 27]. The limited solubility of SC-26304 in water dictated the use of a nontoxic solvent; we chose ethanol/propylene glycol/saline (2:5:5). Various doses of SC-26304 were injected intraperitoneally 30 min before administration of aldosterone (0.8 μg/100 g body wt). In three hours, the urinary K^+/Na^+ ratio increased more than 12-fold in response to aldosterone, and a 25:1, spirolactone to aldosterone dose ratio inhibited the steroid-dependent rise by 55% (Fig. 5). At a dose ratio of 100:1, inhibition was complete. Administration of SC-26304 alone, even at a super-maximal dose (200 μg/100 g body wt), had no significant effect on the urinary K^+/Na^+ ratio.

The effect of SC-26304 on the aldosterone-dependent increase in amino acid incorporation into medullary proteins was studied at a dose

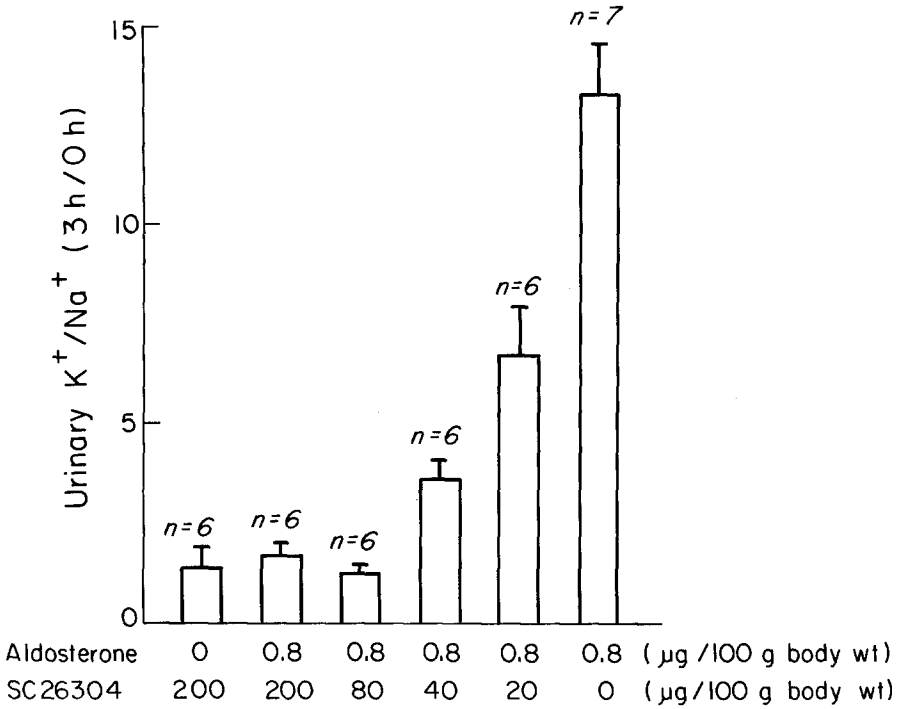


Fig. 5. Effect of spirolactone (SC 26304) on the antinatriuretic response to aldosterone. Adrenalectomized rats were injected intraperitoneally with either the diluent [ethanol p-propylene glycol/saline (2:5:5)] or various doses of SC 26304 (suspended in this vehicle), 30 min before administering aldosterone (0.8 μg/100 g body wt) or an equal volume of saline. The rats were anesthetized with Inactin (6 mg/100 g body wt) and urines were collected 3 hr after hormone by catheterization. The K^+/Na^+ concentrations were measured by flame photometery. *n* denotes the number of rats at each dose of SC 26304

ratio of 100:1. Adrenalectomized rats were injected with either the diluent or aldosterone alone (0.8 μg/100 g body wt) or SC-26304 alone (80 μg/100 g body wt) or SC-26304 (80 μg/100 g body wt) injected intraperitoneally 30 min before subcutaneous injection of aldosterone (0.8 μg/100 g body wt). Medullary slices were prepared 1 hr after injection and incubated in ^3H leucine for 2 hr at 20°. The results obtained with the diluent and with aldosterone are taken from Fig. 3, for comparison. SC-26304 alone depressed incorporation by 26% (not statistically significant) (Fig. 6). Aldosterone had no significant effect on incorporation of ^3H leucine into medullary proteins when administered to rats pretreated with SC-26304. None of these agents had significant effects on the ^3H leucine content of the trichloroacetic acid-soluble fraction.

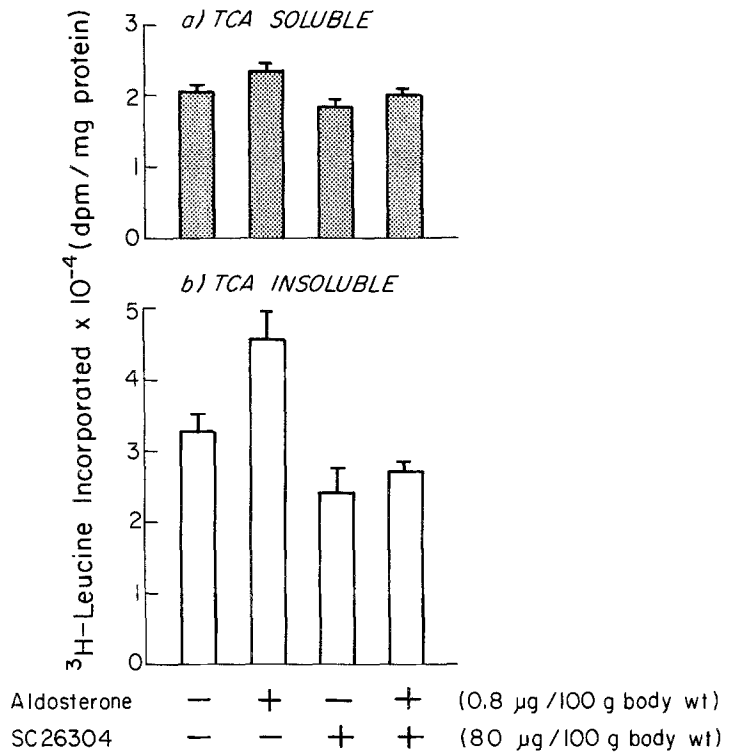


Fig. 6. Effect of spirolactone (SC 26304) on the aldosterone-dependent incorporation of ^{3}H leucine into renal medullary proteins. Adrenalectomized rats were injected with either the vehicle (*see* legend Fig. 5) or SC 26304 (80 µg/100 g body wt) 30 min prior to the administration of aldosterone (0.8 µg/100 g body wt). The kidneys were removed 1 hr after the steroid was given and medullary slices were incubated in ^{3}H leucine for 2 hr at 20°. Crude homogenates were then extracted with trichloroacetic acid; (*a*): the acid-soluble ^{3}H leucine content and (*b*) that of the precipitates. $n=6$ rats for each point

Subcellular Loci of Action of Aldosterone

To define the possible roles of augmented incorporation of amino acids into proteins in the action of aldosterone requires knowledge of the subcellular distribution of the labeled proteins. Adrenalectomized rats were injected subcutaneously with either the diluent or aldosterone (0.8 µg/100 g body wt) or dexamethasone (0.8 µg/100 g body wt). In addition, groups of rats were injected intraperitoneally with SC-26304 (80 µg/100 g body wt), 30 min before injection of the same dose of aldosterone. One hour after injection with aldosterone (or diluent) medullary slices from 6 rats treated comparably were pooled and incubated with either ^{3}H- or ^{35}S methionine for 2 hr at 20°. In three incubations of these pools, the

Table 3. Isotope ratio of various subcellular fractions of rat kidney medulla[a]

Subcellular fractions	Aldosterone/ control ($n=6$)	SC-26304 + Aldosterone/ SC-26304 ($n=4$)	Dexamethasone/ control ($n=4$)
Nucleus			
0.15 M NaCl	1.23 ± 0.20	1.13 ± 0.08	1.07 ± 0.35
histones	1.15 ± 0.11	1.18 ± 0.28	2.09 ± 0.99
nonhistones	1.12 ± 0.13	1.15 ± 0.14	0.93 ± 0.15
Plasma membrane	1.39 ± 0.22	1.01 ± 0.08	1.06 ± 0.05
"Heavy" mitochondria	1.45 ± 0.23	1.12 ± 0.17	1.06 ± 0.11
"Light" mitochondria	1.63 ± 0.19[b]	1.05 ± 0.12	1.07 ± 0.03
Ribosomal proteins	1.48 ± 0.19[b]	1.17 ± 0.15	1.12 ± 0.03
Cytosol	1.33 ± 0.21	1.06 ± 0.08	1.21 ± 0.30

[a] Adrenalectomized rats were treated with aldosterone and other agents as described in the text. Medulla of the kidneys were incubated in modified Joklik MEM as described in the method. Routinely, for every incubation, slices from 6 animals were pooled and placed in 3 ml of media containing 300 μCi of either ^{3}H- or ^{35}S methionine. For every incubation which used ^{3}H methionine to label the experimental tissues, the same number of incubations were carried out using ^{35}S methionine to label the experimental tissues. The crude homogenates were subfractionated as outlined in Fig. 1. The isotope ratios of each subcellular fraction was normalized to that of the trichloroacetic acid-soluble fraction of the 100,000 × g supernatant. n = number of incubations carried out.

[b] Statistically significant ($P<0.05$).

control slices were labeled with ^{3}H methionine and the aldosterone slices with ^{35}S methionine. In three additional incubations the isotopes were reversed. The effect of aldosterone on the isotope ratios of the trichloroacetic acid-precipitable proteins were independent of the isotope used to label the experimental or control slices. Thus the results given in Table 3 combine the findings simply in terms of experimental/control ratios. Aldosterone tended to increase incorporation of methionine into the trichloroacetic acid-precipitable proteins of all of the subcellular fractions, except for those in the nuclear fraction. This effect was greatest in the mitochondrial and ribosomal fractions, but was statistically significant only in the light mitochondrial and ribosomal fractions. That these increases were mediated via the mineralocorticoid receptor pathway was indicated by two sets of findings: (i) An equal dose of dexamethasone (0.8 μg/100 g body wt) failed to elicit a statistically significant increase in methionine incorporation into proteins of any of the cell fractions, with the possible exception of nuclear histones; (ii) pretreatment with SC-26304 (30 min before aldosterone) reduced the isotope ratios to close to unity in all cell fractions.

The results in Table 3 suggest that aldosterone may regulate the synthesis of many proteins. To elucidate the nature of the regulatory process, however, requires an analysis of the effects of the hormone on metabolism of discrete proteins. For this purpose, we elected to obtain more detailed information on the effects of aldosterone, first on cytoplasmic proteins, identified by electrophoretic separation, and on mitochondrial proteins, particularly citrate synthase. The latter results are reported in the following paper. In addition, studies are now underway on the influence of aldosterone on the synthesis of plasma membrane proteins.

Effect of Aldosterone on Cytosol Proteins

The cytosol fraction was chosen for detailed study for two reasons, the absolute specific activities (dpm/mg protein) of the cytosol proteins was higher than in any other fraction, and these soluble proteins could be resolved, under nondenaturing conditions, by polyacrylamide gel electrophoresis.

Adrenalectomized rats were injected subcutaneously with either the diluent or aldosterone (0.5 or 0.8 μg/100 g body wt) and the kidneys were removed 1 hr later. Medullary slices were incubated in either ^{3}H methionine or ^{35}S methionine for 1 hr at 20°. The cytosol fractions were then analyzed by polyacrylamide gel electrophoresis. Parallel gels were stained with 1% amido black or sliced and analyzed for ^{3}H or ^{35}S content. Figure 7 displays typical patterns obtained by staining and radio-assay for ^{3}H methionine. Components that failed to migrate into the gel were detected both by staining and radio-assay. A large number of bands and a hetero-disperse labeling pattern were routinely obtained in the separation gel, representing most of the cytosol proteins applied to the gel in that 75% of the radioactivity applied to the gel was routinely recovered from the slices of the separation gel after H_2O_2 oxidation. In all of these analyses, a low molecular weight peptide, identified both by staining and radioassay, migrated very close to the dye front. This provided a convenient reference for the migrational front of each gel. The radioactivity maximum of this band was defined as the electrophoretic front.

To assess the effects of aldosterone on the synthesis of renal cytosol proteins, medullary slices of aldosterone (0.8 μg/100 g body wt) or diluent-treated rats, were pooled (6 rats from each group) and incubated

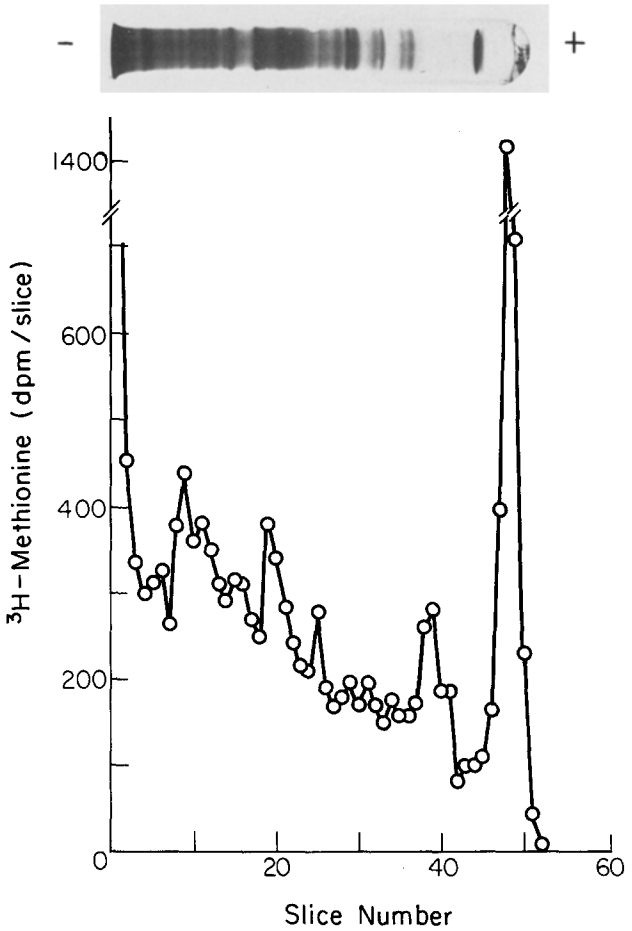


Fig. 7. Polyacrylamide gel electrophoresis of renal medullary cytosol. Adrenalectomized rats were injected with either the diluent or aldosterone (0.5 or 0.8 μg/100 g body wt) and after 1 hr the medullary slices were incubated in ^{3}H- or ^{35}S-methionine for 1 hr at 20°. The cytosol proteins were analyzed in 7.5% acrylamide gels (0.7 × 10 cm) in tris-glycine buffer (running pH = 8.3). The proteins were stained with amido black and gel slices were assayed for ^{3}H or ^{35}S content by liquid scintillation spectrometry. A representative gel, staining pattern (above) and assayed for ^{3}H-content (below) is shown

in either ^{35}S or ^{3}H methionine. Six incubations were completed; in three, ^{3}H methionine was used to label the experimental (aldosterone) pool, and in the other three, ^{35}S methionine was used to label the experimental pool. Since the results were indistinguishable, regardless of the isotope assignments, the data were averaged as experimental/control isotope ratios normalized to the total ^{3}H and ^{35}S recovered from each gel (Fig. 8). Significant augmentation in methionine incorporation in re-

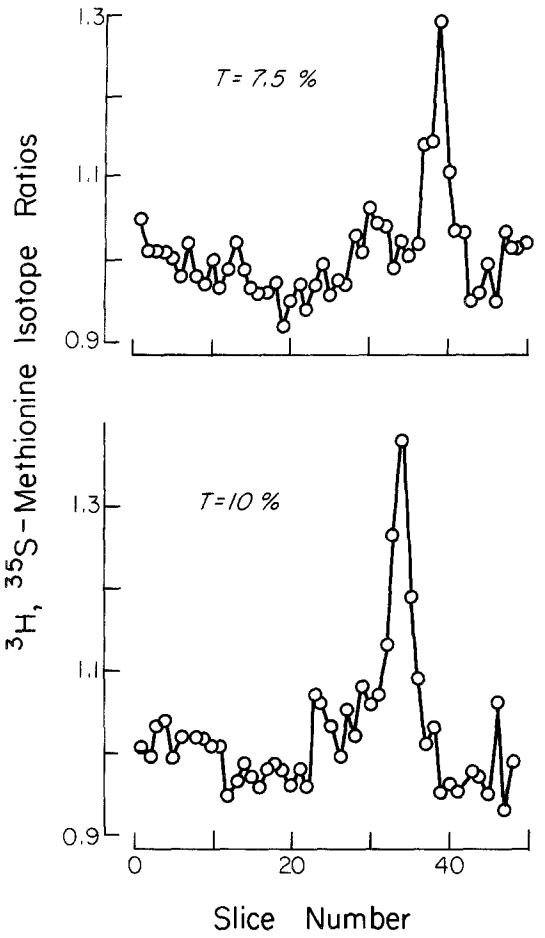


Fig. 8. Effect of aldosterone on incorporation of radio-methionine into medullary cytosol proteins analyzed by polyacrylamide gel electrophoresis. Adrenalectomized rats were injected with aldosterone or diluent and medullary slices were labeled with ^3H- or ^{35}S-methionine as described in the legend of Fig. 7. The cytosol proteins were analyzed on 7.5% and 10% total acrylamide gels. The isotope ratios were normalized to the total ^3H and ^{35}S content recovered from all of the slices, i.e., in each slice the ratio was $\%^3H/\%^{35}S$ or the reverse. In all experiments, 8 gels were run simultaneously; 4 gels of cytosol labeled with ^3H methionine in slices from the aldosterone-treated rats and 4 gels of cytosol labeled with ^{35}S methionine in slices from the diluent-treated rats, and in the other 4 gels the isotopes were reversed. The results are an average of 6 incubations. In 3 incubations, ^3H methionine was used in slices from aldosterone-treated rats and ^{35}S methionine in slices from diluent-treated rats. In the other 3 incubations the isotopes were reversed. In each incubation 6 aldosterone-treated and 6 diluent-treated rats were used to provide the medullary slices. Thus, each cytosol pool used to determine the isotope ratios was provided by a total of 12 rats. The results were indistinguishable regardless of which isotope was used to label the hormone-treated slices and are summarized as the mean of all experiments

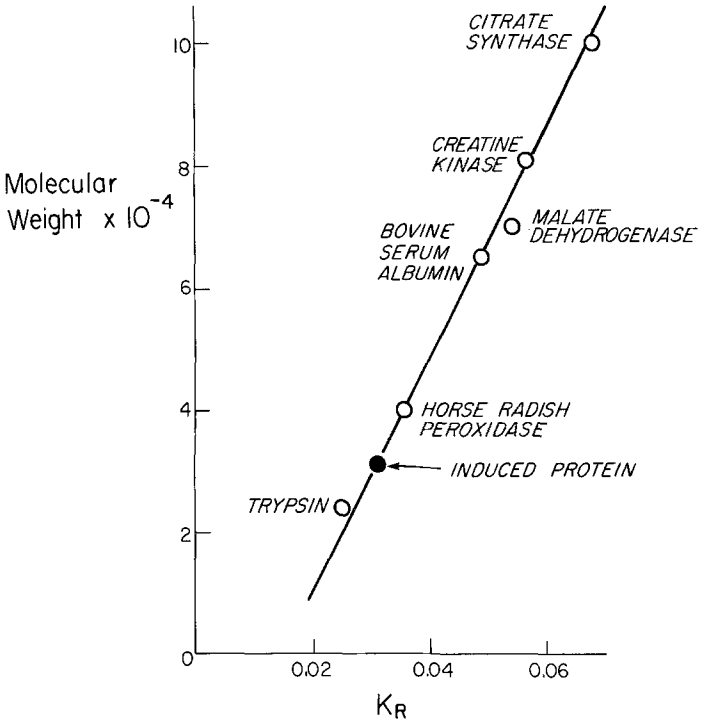


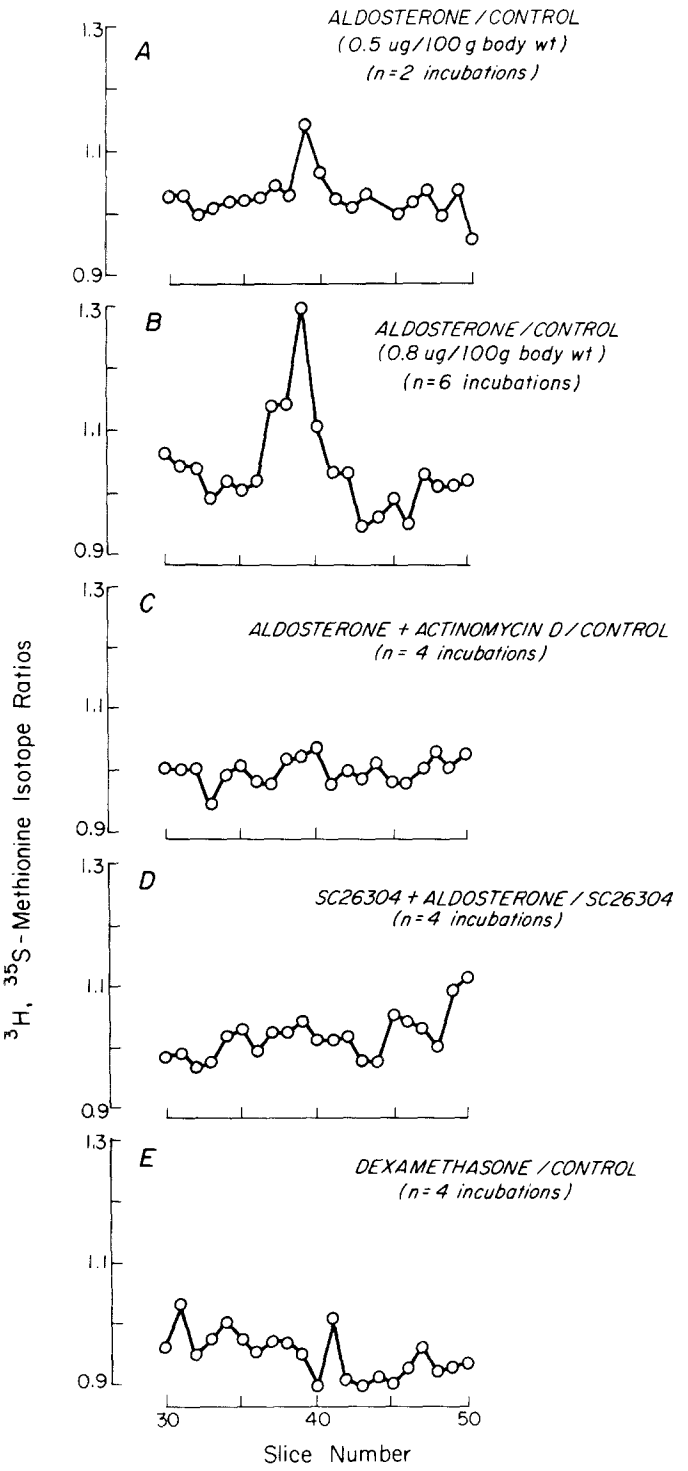
Fig. 9. Estimation of the molecular weight of the aldosterone-induced protein. The peak of the increase in isotope ratio (*see* Fig. 8) elicited by aldosterone was used to identify the aldosterone-induced protein. The slope of the relative mobility of this protein as a function of total acrylamide concentration (Ferguson plot) was used to estimate the retardation coefficient (K_R) of the induced protein. The molecular weights of the standard proteins were obtained from the following references: trypsin [14], creatine kinase [19], horseradish peroxidase [25], citrate synthase [28], bovine serum albumin [36], malate dehydrogenase [37]

sponse to aldosterone was obtained in a protein fraction with an R_f of 0.78 in 7.5% acrylamide gels and of 0.68 in 10% gels (Fig. 8). Since the assignments of the ^3H and ^{35}S methionine were reversed in half of the experiments ($n=8$, 7.5% gels and $n=6$, 10% gels), these findings are not attributable to an isotope effect.

To estimate the molecular weight of the induced protein, the retardation coefficient (K_R) was calculated by the Ferguson equation (9):

$$\text{Log}(R_f) = \text{Log}(Y_0) - K_R \cdot T$$

where T is the percent of total acrylamide and Y_0 is the R_f extrapolated to $T=0$. This method was also used to determine the retardation coefficients of a series of standard proteins (Fig. 9)—analyzed under the


A
ALDOSTERONE / CONTROL
(0.5 ug/100 g body wt)
(n=2 incubations)
B
ALDOSTERONE / CONTROL
(0.8 ug/100g body wt)
(n=6 incubations)
C
ALDOSTERONE + ACTINOMYCIN D / CONTROL
(n = 4 incubations)
D
SC26304 + ALDOSTERONE / SC26304
(n=4 incubations)
E
DEXAMETHASONE / CONTROL
(n=4 incubations)
1.3
1.1
0.9
30
40
50
Slice Number
^{3}H, ^{35}S-Methionine Isotope Ratios

same electrophoretic conditions at $T=3.5\%$, 5%, 7.5%, and 10%. Based on the linear relationship of the molecular weights to the K_R of the standards, the aldosterone induced protein(s) (at the maximum increase in the isotope ratio) has a mol wt of 31,000 (Fig. 9).

The possible relevance of the induction of the 31,000 mol wt cytosol protein(s) to mineralocorticoid action was evaluated by varying the dose of aldosterone (submaximal with respect to Na^+ and K^+ excretion) [15]. The results in Fig. 10 (*cf.* panels *A* and *B*) indicate that incorporation of radio-methionine into the 31,000 peak was increased 29.6 and 14% by injection of 0.8 and 0.5 μg/100 body wt, respectively. The dependence of the magnitude of the increase in the isotope ratio of the 31,000 protein(s) is within the range of doses that modulate Na^+ and K^+ excretion [15] and is similar to the dependence on aldosterone dosage of incorporation of ^3H methionine into total medullary proteins (*cf.* Figs. 2 and 10).

Three additional criteria were applied to evaluate the possible relevance of the induction of 31,000 mol wt cytosol protein(s) to mineralocorticoid action; the inhibitory effects of actinomycin D and SC-26304, and the ability of an equimolar dose of dexamethasone (0.8 μg/100 g body wt) to elicit the effect. Pairs of adrenalectomized rats were treated as described in the legend of Fig. 10. In half of each set of incubations of medullary slices, the isotopes ^3H and ^{35}S methionine were reversed and the cytosol proteins were resolved in 7.5% total acrylamide gels. Actinomycin D (80 μg/100 g body wt) administered 1 hr before aldosterone (0.8 μg/100 g body wt) completely eliminated the increase in labeling of the 31,000 mol wt protein(s) (Fig. 10*C*), implying a dependence on DNA-directed RNA synthesis. Similarly, SC-26304 (80 μg/100 g body wt) given 30 min before aldosterone (0.8 μg/100 g body wt) prevented augmentation of labeling of these components (Fig. 10*D*), implying that this

Fig. 10. Dose-dependence of aldosterone action on incorporation of labeled methionine into cytosol proteins, and the effects of actinomycin D, SC 26304, and dexamethasone. Adrenalectomized rats were given either, (*A*) aldosterone (0.5 μg/100 g body wt) or the diluent, (*B*) aldosterone (0.8 μg/100 g body wt) or the diluent, (*C*) aldosterone (0.8 μg/100 g body wt) and actinomycin D (70–85 μg/100 g body wt), or the diluent, (*D*) SC 26304 (80 μg/100 g body wt) ± aldosterone (0.8 μg/100 g body wt), or (*E*) dexamethasone (0.8 μg/100 g body wt) or the diluent. The kidneys were removed 1 hr after administration of the steroid and medullary slices were incubated in ^3H or ^{35}S methionine and processed as described in the legends of Fig. 7 and 8. Each panel represents the average isotope ratios of 8 gels (with 4 by 4 isotope reversal). The isotope ratios of the components with R_f's of 0.6 to 1.0 are shown; the peaks in *A* and *B* represent the 31,000-Dalton component. For each incubation 6 rats comprised the experimental group and 6 rats the control group

effect is mediated by the high affinity aldosterone (mineralocorticoid) receptors. This inference was reinforced by the finding that dexamethasone (0.8 μg/100 g body wt), a potent glucocorticoid, failed to elicit an increase in labeling of the cytosol protein(s) (Fig. 10*E*).

Discussion

The hypothesis that induction of RNA and protein synthesis mediates the action of aldosterone on Na^+ transport is based on four lines of evidence; the characteristic latent period, nuclear localization of the aldosterone-receptor complex, inhibition of the ion transport effects by inhibitors of RNA and protein synthesis, and augmentation of incorporation of precursors into RNA and proteins of target tissues [8]. The relevance of enhanced synthesis of RNA to mineralocorticoid action has been supported by a series of studies on the isolated toad bladder: Aldosterone at low concentrations ($<10^{-7}$ M) increased the incorporation of ^3H uridine into cytoplasmic polyadenylated 9-12 S rapidly labeled RNA (putatively mRNA) during the latent period (with respect to Na^+ transport) [29]. This effect was not obtained with either the inactive stereo-isomer, 17α iso-aldosterone, or with cortisol and was antagonized by the antimineralocorticoid (SC-9420) [29]. Aldosterone also augmented the amount of cytoplasmic polyadenylated RNA that hybridized to ^3H poly-uridine. Moreover, the increase in Na^+ transport correlated linearly with the increase in labeling of the 12 S species, under a wide variety of conditions [39]. These results implicate induction of RNA, more specifically mRNA, in steroidal regulation of Na^+ transport. The presumed protein mediators, however, are as yet unidentified.

Fimognari *et al.* [10] and Simone and Solomon [33] detected aldosterone-dependent increased incorporation of ^3H leucine into rat and rabbit kidney proteins, *in vivo*. Aldosterone also augmented amino acid incorporation into a discrete protein (mol wt = 12,000) in the isolated toad bladder [2]. These studies, however, did not define the subcellular loci of action of the mineralocorticoid.

The present studies explore the relevance of enhanced protein synthesis in a mammalian target tissue, the rat kidney, to mineralocorticoid action. At doses of only two to three times that required to elicit a half-maximal effect on Na^+/K^+ concentrations in the urine (0.8 μg/100 g body wt), aldosterone increased incorporation of labeled amino acids (^3H leucine or ^3H methionine) into proteins of the medulla and to a lesser

extent in the cortex. Sensitivity of this effect to actinomycin D implies a dependence on DNA-directed RNA synthesis. The possibility that aldosterone acted via illicit occupancy of the glucocorticoid receptor system [11] was contradicted by the failure of equimolar doses of dexamethasone, a potent glucocorticoid [30], to elicit the effect on amino acid incorporation into medullary proteins. A further assessment was made with a potent anti-mineralocorticoid, SC-26304 [12]. In doses sufficient to inhibit virtually all of the aldosterone-dependent changes in urinary Na^+/K^+ ratios, this spirolactone eliminated the enhanced amino acid incorporation in the medulla.

Although the results obtained on incorporation of labeled amino acids into total protein were encouraging, ultimately identification of the induced proteins is essential to an understanding of the mechanism of action on ion transport. As a preliminary step in this direction, the effect of aldosterone on cytosol proteins was assessed by labeling with ^3H- and ^{35}S methionine and resolving the products by polyacrylamide gel electrophoresis. At a modest dose (0.8 μg/100 g body wt), aldosterone augmented methionine incorporation into at least one cytosol protein, with an apparent mol wt of 31,000. Reversal of the isotopes did not modify the effect. The increase in isotope ratio implies an increase in the synthesis of the 31,000 component. Moreover, induction of this protein(s) was inhibited by actinomycin D and spirolactone (SC-26304). Dexamethasone, at an equimolar dose, did not mimic this action of aldosterone. Similar results were obtained in the isolated toad bladder; aldosterone stimulated the incorporation of ^{35}S methionine into three cytosol proteins with apparent mol wt in the 17,000–38,000 range [32]. In contrast, corticosterone did not induce the synthesis of this group of proteins. These results, as well as ours, are consistent with induction of cytoplasmic protein(s) via the mineralocorticoid receptor system.

The possible role, if any, of the induced cytosol proteins in steroidal modulation of Na^+ transport requires an understanding of the rate-limiting steps in transepithelial ion transport. In the past, the analyses of the nature and site of mineralocorticoid action of aldosterone-induced protein have been based on the Ussing-Skou model [7]. The primary events, according to this model, consist of entry of Na^+ across an apical plasma membrane diffusion barrier, driven by the trans-apical electrochemical gradient, and extrusion of Na^+ across the basal-lateral membrane by an ATP-dependent Na^+ pump. There are now a large number of candidates for the role of a mediator(s) in aldosterone regulation of Na^+ transport, including an amiloride-sensitive apical

"Na^+ permease" [4], modulators of phospholipid metabolism [13], a membrane protein phosphatase [22] carbonic anhydrase [35], the Na^+ pump (or the enzymatic equivalent, $(Na^+ + K^+)$-dependent adenosine triphosphatase) [18, 31] and mitochondrial enzymes regulating oxidative phosphorylation (e.g., citrate synthase) [15, 16]. In no case, however, has convincing evidence been obtained that one of these candidates plays a unique role in determining the magnitude and the time-course of the change in Na^+ transport. In the following paper, we describe experiments evaluating induction of one of these candidates, citrate synthase, by aldosterone.

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