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Spironolactone Antagonism of Aldosterone Action on Na^+ Transport and RNA Metabolism in Toad Bladder Epithelium

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Summary. In earlier studies, aldosterone increased the incorporation of precursors into a class of cytoplasmic RNA with the characteristics of messenger RNA (mRNA), in toad bladder epithelium. In the present studies, this effect was analyzed further with a competitive antagonist, spironolactone (SC-9420). Paired hemibladders were labeled with ^{3}H-uridine (30 min pulse – 140 min chase), with or without aldosterone (3.5×10^{-8} M, 7×10^{-8} M) in the presence or absence of SC-9420 (7×10^{-6} M, 2.5×10^{-5} M) at molar ratios of 200:1 to 280:1. Cytoplasmic RNA, either the total phenol-SDS extract or polyadenylated-RNA (poly(A)(+)-RNA) obtained by oligo-deoxythymidylate-cellulose (oligo(dT)-cellulose) chromatography was analyzed in linear 5–20% sucrose gradients. Eight sets of experiments were completed in which the short-circuit current (*scc*) was monitored for 180 min and the incorporation of ^{3}H-uridine (30 min pulse – 150 min chase) was simultaneously determined on pools of epithelia from 5 to 10 hemibladders. The fractional change in *scc* correlated linearly with the fractional change in ^{3}H-uridine of 12S cytoplasmic RNA ($r = 0.95$, $p < 0.001$). The poly(A)(+)-RNA fraction had no detectable rRNA or tRNA and gave a heterogeneous pattern, typical of mRNA, in the sucrose gradients. In the presence of exogenous aldosterone, SC-9420 inhibited the incorporation of ^{3}H-uridine into poly(A)(+)-RNA (particularly 12S). These results support the inference that induction of mRNA mediates the action of aldosterone on Na^+ transport.

A variety of experiments indicate that the initial steps in the action of aldosterone on Na^+ transport involve binding of the steroid to stereospecific cytoplasmic receptors, transfer of this compler to chromatin acceptor sites, and induction of RNA synthesis [7]. The steps in this

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sequence of molecular events, however, have not been fully characterized. We recently noted that aldosterone increased the incorporation of ^{3}H-uridine into nonmethylated, 9S–12S cytoplasmic RNA prior to the onset of the effect on Na^+ transport, in the toad bladder [23]. Messenger RNA (mRNA) has been characterized by its minimal methyl content and sedimentation constants in the 5S to 30S range [20, 21]. Thus, our earlier findings suggested that aldosterone augments the synthesis of mRNA. Further evidence in support of this inference was obtained by Wilce *et al.* [27]. In the isolated toad bladder, aldosterone increased the incorporation of precursors into polyadenylated-RNA [poly(A)(+)-RNA]. Poly(A)(+)-RNA has been identified as the mRNA rich fraction [6]. This effect on poly(A)(+)-RNA was most marked during the latent period, namely within the first 30 min of the action of the hormone [27]. That the effect of aldosterone on nonmethylated 9S–12S cytoplasmic RNA was mineralocorticoid-specific was implied by the failure of cortisol (a potent glucocorticoid) to elicit these responses and by the effective inhibition by spironolactone (SC 9420) of both the biochemical and transport responses [23]. These findings prompted the use of spironolactone as a probe in a further analysis of the mediating role of induction of poly(A)(+)-RNA in the mineralocorticoid action of aldosterone.

The spirolactones compete with aldosterone for binding to the cytoplasmic receptor [11, 13]. The resultant spirolactone-receptor complex fails to bind to chromatin acceptor sites, presumably preventing initiation of induction of RNA synthesis [18]. An antagonist that acts at the first step in a cascade of molecular events should inhibit all subsequent steps. If augmented synthesis of mRNA mediates the action on Na^+ transport, there may be a quantitative relationship between the magnitudes of the effects of aldosterone on RNA metabolism (cytoplasmic 12S RNA and poly(A)(+)-RNA) and on Na^+ transport as modulated by spirolactone. This prediction was evaluated in the present studies.

Materials and Methods

Materials

The incubation medium (frog-Ringer's solution) contained (all in mM) 90 NaCl, 3 KCl, 25 $NaHCO_3$, 0.5 $MgSO_4$, 0.5 KH_2PO_4, 1 $CaCl_2$ and 6 glucose; pH = 7.6 (gassed with 3% CO_2); osmolality = 230 mOsm. Gentamycin sulfate was added to a final concentration of 10 μg/ml and the medium was filtered through a 0.45 μ Millipore filter. All glassware and other solutions were heat sterilized. D-Aldosterone was obtained from CalBioChem Corp., and oligo-deoxythymidylate-cellulose (T_2) (oligo(dT)-cellulose) from Collaborative Research. Spironolactone (SC 9420) was a generous gift from Searle Co. All of the conventional

reagents were either reagent grade or spectroquality. Aquasol(R) and 5-^{3}H-uridine (26–28 Ci/mmole) were purchased from New England Nuclear.

Transport and Labeling Procedures

Colombian female toads (*Bufo Marinus*) obtained from Tarpon Zoo (Florida) were partially immersed in saline (0.075 M) at room temperature for 48 to 72 hr before use. After double pithing and perfusion of the circulation with about 200 ml of oxygenated frog-Ringer's solution, hemibladders (mucosal side outside) were mounted as sacs on plastic cannulas. The sacs were filled with 5 ml and immersed in 90 ml of the frog-Ringer's solution and the temperature of the bath was maintained at 25 ($\pm$0.2)°C. The transepithelial potential difference (pd) and short-circuit current (*scc*) were measured at 30 min intervals by the method of Walser *et al.* [26]. Four hours after the sacs were mounted, three protocols were used: (1) spironolactone *vs.* control, (2) aldosterone *vs.* control, and (3) spironolactone + aldosterone *vs.* aldosterone. SC 9420 was dissolved in 100% ethanol; the final concentration of ethanol in frog-Ringer's solution did not exceed 0.2%. The antagonist or the diluent was always added to the serosal and mucosal media 20 min before addition of aldosterone or the diluent. At "t_o", aldosterone was added to the mucosal and serosal media. Ten minutes later, ^{3}H-uridine (10 μC/ml) was added to the serosal media of both the control and test preparations, and 30 min thereafter, a 100-fold excess of nonradioactive uridine was added to the same media (30 min pulse – 140 min chase). The incubation was terminated 180 min after addition of aldosterone. In one experiment a 30 min pulse format was used and the incubation was ended 40 min after addition of aldosterone.

Isolation of Cytoplasmic RNA and Poly(A)(+)-RNA

The hemibladders were washed twice with ice cold frog-Ringer's solution and all subsequent steps were carried out at 0–4 °C. The epithelial cells were harvested by scraping with a glass slide. Five to 10 paired hemibladders were pooled into test and control groups. The pooled cells were homogenized with 15 strokes at maximum speed in a motor driven Teflon-glass Potter-Elvehjem homogenizer. Cytoplasmic RNA was isolated by Penman's procedure [19], modified as previously described [23]. Poly(A)(+)-RNA of the cytoplasmic extracts was collected by a modification of the method of Aviv and Leder [1, 27].

Sucrose Gradient Analysis of RNA

Equal amounts, 1 to 2 A_{260} units, (1 A_{260} unit = 32 μg RNA, light path = 10 mm) of test and control cytoplasmic RNA or Poly(A)(+)-RNA (0.015–0.030 A_{260} units) were layered on 5 ml, 5–20% linear sucrose density gradients and centrifuged at 45,000 rpm in the SW 50.1 rotor of the L2-65B Beckman ultracentrifuge at 4 °C for 195 min. Thirty fractions were collected with an ISCO Model 183 gradient fractionator monitored at 254 nm (UA-5 Absorbance Monitor). Each fraction was added to 10 ml of Aquasol and analyzed for ^{3}H content in a Mark II Nuclear Chicago Liquid Scintillation Spectrometer. The recovery of radioactivity from the gradients varied from 90 to 95%.

Acid-Soluble Pool

Aliquots of the crude homogenates were acidified with ice-cold trichloroacetic acid (TCA) to a final concentration of 10%. The precipitates were collected by centrifugation

at $10{,}000 \times g$ for 10 min and then washed twice by the same procedure with ice-cold 5% TCA. The supernatants of all of these steps were pooled and assayed for ^3H content in Aquasol in a Mark II Nuclear Chicago Liquid Scintillation Spectrometer. The DNA content of the precipitates was determined by the technique of Burton [3].

Statistics

The significance of the difference in the mean values were estimated by the paired Student t test and the regression line by the method of least squares [25].

Results

Effects of Spironolactone on Na^+ Transport and RNA Metabolism

To assess the effects of SC 9420 in the absence of exogenous aldosterone, paired hemibladders were incubated with either the antagonist or the diluent for 180 min. At a concentration of 7×10^{-6} M, spironolactone had no significant effect on the *scc* at any time during the period of exposure. After 180 min, the scc_t/scc_0 ratios were 1.21 ± 0.14 and 1.30 ± 0.13 ($p > 0.4$) in the control and SC-9420-treated groups, respectively (Table 1). Similarly, except for a single fraction at about 4S, this concentration of spironolactone did not change the sucrose gradient sedimentation profile of cytoplasmic RNA (Fig. 1). At a higher concentration (2.5×10^{-5} M), however, spironolactone significantly depressed

Table 1. Effects of spironolactone and aldosterone on Na^+ transport

Pairs of hemibladders (No.)	SC-9420 (M)	Aldosterone (M)	scc_t/scc_0	p
9	0	0	1.21 ± 0.14	>0.4
	7×10^{-6}	0	1.30 ± 0.13	
8	0	0	0.90 ± 0.10	<0.01
	2.5×10^{-5}	0	0.65 ± 0.08	
8	7×10^{-6}	3.5×10^{-8}	1.02 ± 0.18	<0.01
	0	3.5×10^{-8}	1.52 ± 0.07	
8	2.5×10^{-5}	7×10^{-8}	0.98 ± 0.04	<0.01
	0	7×10^{-8}	1.35 ± 0.09	
8	0	0	0.92 ± 0.08	0.01
	0	7×10^{-8}	2.05 ± 0.25	

Paired hemibladders were incubated with or without SC-9420, aldosterone or a combination of these agents. scc_t denotes the short-circuit current at $t = 180$ min. and scc_0 at $t = 0$, where zero-time was the time of addition of SC-9420 (in the absence of aldosterone) or of the addition of aldosterone. In the combination experiments, the SC-9420 was always added 20 min before the aldosterone. The results are given as mean $\pm$ SEM. The p values were obtained by the paired student t test.

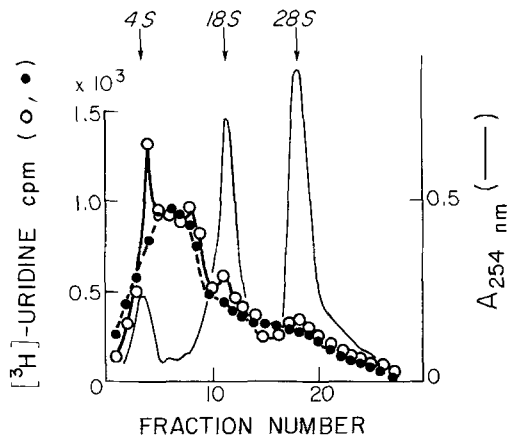

Fig. 1. Sucrose gradient analysis of the effects of spironolactone (7×10^{-6} M) on cytoplasmic RNA. Paired hemibladders were incubated in SC 9420 or the diluent and labeled with ^{3}H-uridine (30 min pulse—140 min chase). Epithelial cytoplasmic RNA was analyzed on a linear 5–20% sucrose gradient. The ^{3}H activity (cpm/fraction) of the hemibladders treated with SC 9420 (–•–) or with the diluent (–o–) is indicated by the ordinate on the left, and absorbance at 254 nm (A_{254}) is indicated by the continuous line and the ordinate on the right. The A_{254} peaks provided the calibrations at 4S, 18S and 28S. The ^{3}H activity has been normalized to the total RNA (i.e., the area under the absorbance curve)

the *scc*; the scc_t/scc_0 ratio was 28% lower than in the control hemibladders ($p < 0.01$) (Table 1). The incorporation of ^{3}H-uridine into 12S cytoplasmic RNA was impaired to some extent by spironolactone. The specific activity of the 12S RNA was 14% less in the spironolactone (2.5×10^{-5} M) group (Fig. 2). Since these hemibladders were pre-incubated in steroid-free media for only 4 hr, it is possible that at very high concentrations, spironolactone competed effectively with residual endogenous aldosterone.

Effects of SC 9420 on the Response to Aldosterone

In previous studies, we found that at concentrations of 3.5×10^{-8} M and 7×10^{-8} M, aldosterone augmented the incorporation of ^{3}H-uridine into 9S–12S cytoplasmic RNA of toad bladder epithelium [23, 27]. At a molar ratio of 100:1, SC 9420 significantly inhibited this effect [23]. Similar experiments, at a molar ratio of SC 9420/aldosterone of 200:1 are summarized in Table 1. The scc_t/scc_0 ratios after 180 min of incubation were 50% greater with aldosterone (3.5×10^{-8} M) than with aldosterone (3.5×10^{-8} M) + spironolactone (7×10^{-6} M) ($p < 0.01$). Under these conditions, as shown in Fig. 3, spironolactone suppressed the incorpora-

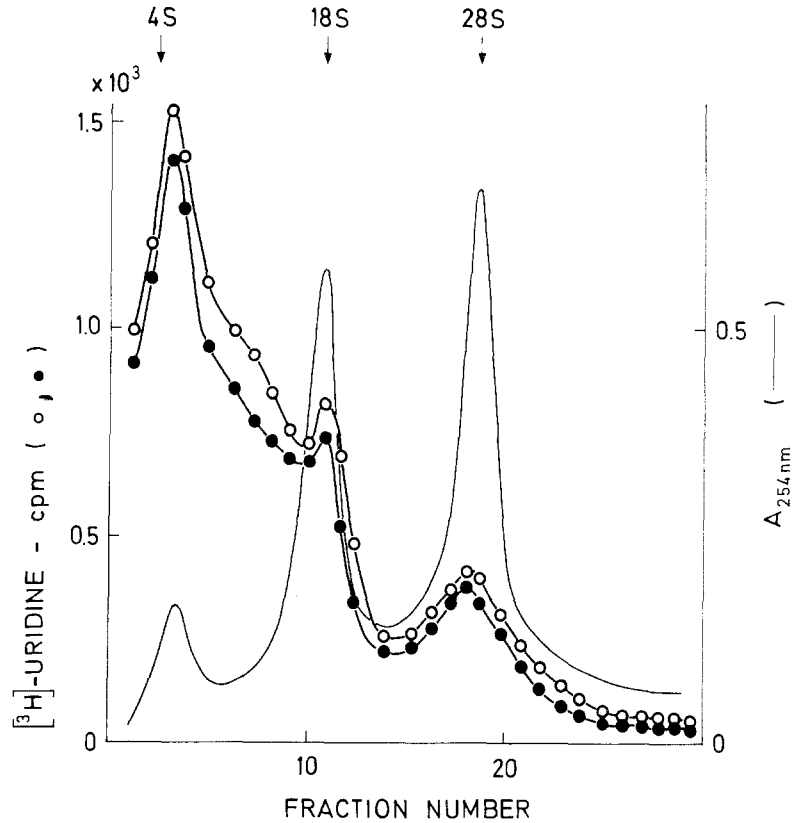

Fig. 2. Sucrose gradient analysis of the effects of spironolactone (2.5×10^{-5} M) on cytoplasmic RNA. Paired hemibladders were incubated in SC 9420 or the diluent, and labeled with ^{3}H-uridine (30 min pulse – 140 min chase). Epithelial cytoplasmic RNA was analyzed as described in the legend of Fig. 1

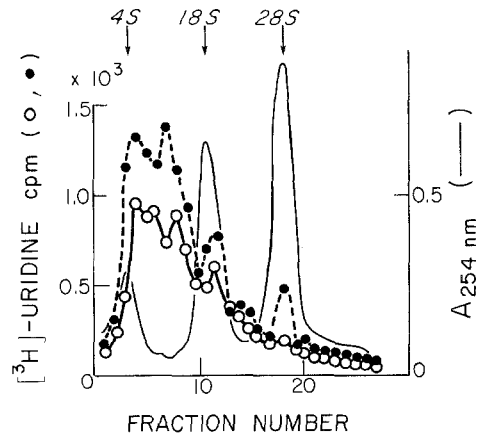

Fig. 3. Sucrose gradient analysis of the effects of spironolactone (7×10^{-6} M) and aldosterone (3.5×10^{-8} M) on cytoplasmic RNA. Paired hemibladders were incubated with SC 9420 or the diluent for 20 min. All hemibladders were then given aldosterone and labeled with ^{3}H-uridine (30 min pulse – 140 min chase). ^{3}H activity of the hemibladders incubated in aldosterone (–●–) or in aldosterone + SC 9420 (–○–) is indicated by the ordinate on the left. Epithelial cytoplasmic RNA was analyzed as described in the legend of Fig. 1

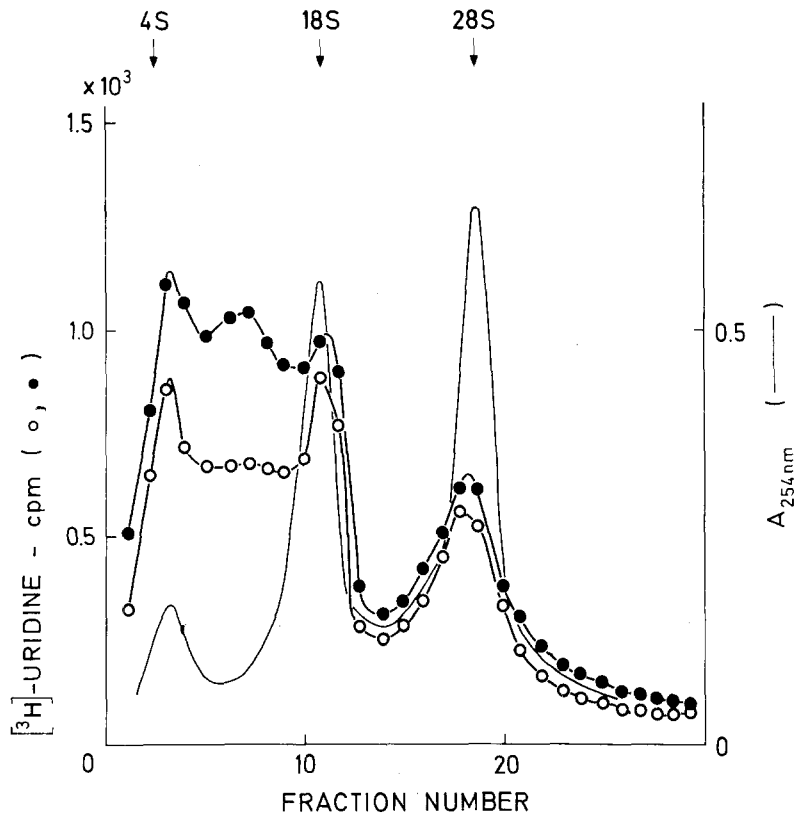

Fig. 4. Sucrose gradient analysis of the effects of spironolactone (2.5×10^{-5} M) and aldosterone (7×10^{-8} M) on cytoplasmic RNA. Paired hemibladders were incubated with SC 9420 or the diluent for 20 min. All hemibladders were then given aldosterone and labeled with ^{3}H-uridine (30 min pulse – 140 min chase). ^{3}H activity of the hemibladders incubated in aldosterone (–●–) or in aldosterone + SC 9420 (–○–) is indicated by the ordinate on the left. Epithelial cytoplasmic RNA was analyzed as described in the legend of Fig. 1

tion of ^{3}H-uridine into cytoplasmic RNA, particularly in the region between 4S and 18S. Inhibition of the small peak at 28S also was evident.

To confirm the correlations between the effects of spironolactone on the Na^+ transport and RNA responses to aldosterone, similar experiments were completed at a higher concentration of the hormone. At 2.5×10^{-5} M spironolactone and 7×10^{-8} M aldosterone (molar ratio = 280:1), the scc_t/scc_0 ratio was inhibited by 37% (Table 1). This effect was accompanied by a marked inhibition of labeling of cytoplasmic RNA in the 4S to 18S region (Fig. 4).

We reported previously on the effects of aldosterone, at a concentration of 3.5×10^{-8} M, on the *scc* and on the incorporation of ^{3}H-uridine

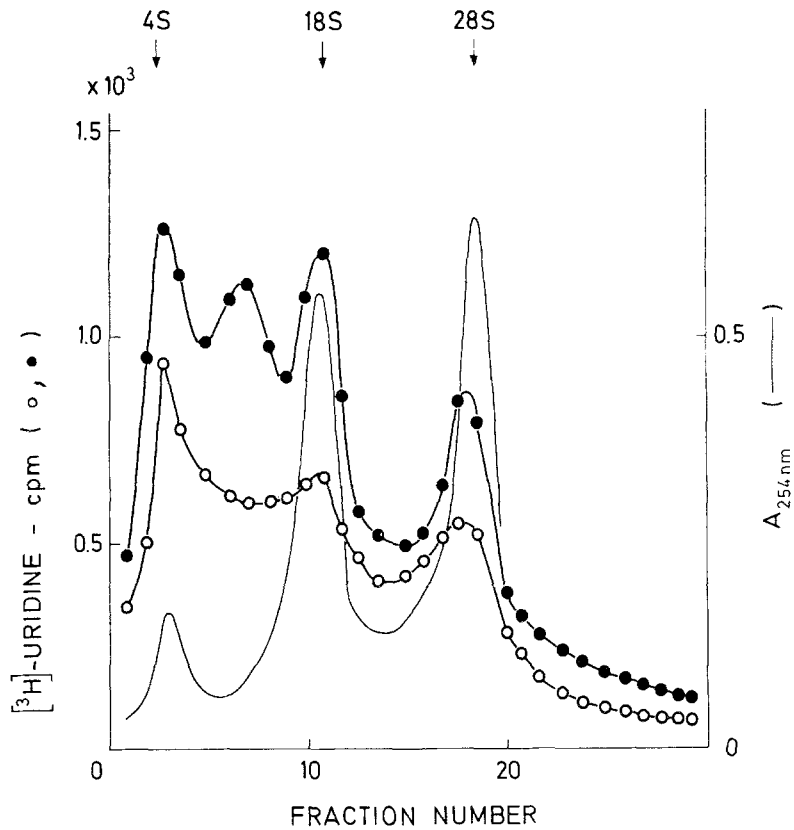

Fig. 5. Sucrose gradient analysis of the effects of aldosterone (7×10^{-8} M) on cytoplasmic RNA. Paired hemibladders were incubated in aldosterone (–●–) or the diluent (–o–) and labeled with ^{3}H-uridine (30 min pulse – 140 min chase). Epithelial cytoplasmic RNA was analyzed as described in the legend of Fig. 1

into cytoplasmic RNA of the toad bladder [23]. To provide a basis of comparison for the results obtained with spironolactone (2.5×10^{-5} M) and aldosterone (7×10^{-8} M) (*see* above), the response to aldosterone alone at 7×10^{-8} M, was documented. As shown in Table 1 and in Fig. 5, at this concentration aldosterone augmented the *scc* (+123%, $p < 0.01$) and the labeling of cytoplasmic RNA throughout the gradient, especially in the 4S to 18S region.

Quantitative Relationship Between Na^+ Transport and RNA Metabolism

To assess the quantitative relationships between Na^+ transport and RNA metabolism under the influence of the agonist and antagonist,

Table 2. Summary of the effects of spironolactone and aldosterone on Na^+ transport and 12S cytoplasmic RNA

SC-9420 (M)	Aldosterone (M)	*scc* fractional change	12S RNA fractional change	Source
3.5×10^{-6}	0	1.03	1.08	[23]
0	0			
7×10^{-6}	0	1.07	1.03	Table 1
0	0			Fig. 1
2.5×10^{-5}	0	0.72	0.86	Table 1
0	0			Fig. 2
0	3.5×10^{-8}	1.19	1.22	[23]
3.5×10^{-6}	3.5×10^{-8}			
0	3.5×10^{-8}	1.41	1.53	Table 1
7×10^{-6}	3.5×10^{-8}			Fig. 3
0	7×10^{-8}	1.37	1.46	Table 1
2.5×10^{-5}	7×10^{-8}			Fig. 4
0	3.5×10^{-8}	1.97	1.75	[23]
0	0			
0	7×10^{-8}	2.23	1.76	Table 1
0	0			Fig. 5

Paired hemibladders were labeled with ^{3}H-uridine for 30 min followed by a 140 min chase, and *scc*'s were measured for 180 min (*see* legends of Table 1, Figs. 1–5). The same format was used in the experiments of Rossier *et al.* [23]. The fractional change in *scc* was calculated as: $(scc_t/scc_0)_e/(scc_t/scc_0)_c$, where the subscript "*e*" (experimental) denotes spironolactone (alone) or aldosterone (alone) and the subscript "*c*" (control) either the diluent or spironolactone+ aldosterone in combination. The fractional change in labeling of 12S cytoplasmic RNA in the sucrose gradients was calculated as: (^{3}H activity of 12S fractions)$_e$/(^{3}H activity of 12S fractions)$_c$, where "e" and "*c*" refer to the additions described above. The 12S point was determined by interpolation of a line joining the 4S, 18S and 28S peaks. ^{3}H activity represents the sum of the three fractions that form the 12S region of the gradient.

the 12S class of cytoplasmic RNA was chosen for measurement. The 12S point in the sucrose gradients was identified by reference to the 4S, 18S and 28S absorbance peaks at 254 nm. The three fractions that formed the 12S peak were pooled and the specific activities used to compute the fractional changes summarized in Table 2. It is apparent by inspection that the effects of the agonist and antagonist on the *scc*'s correlates with the effects on labeling of 12S cytoplasmic RNA with ^{3}H-uridine. The quantitative assessment of the correlation between these parameters is shown in Fig. 6 (data points from Table 2). The dependence of the fractional change in *scc* on the fractional change in the specific activity of 12S RNA fits a least squares linear regression with a correlation coefficient of 0.95 ($p < 0.001$).

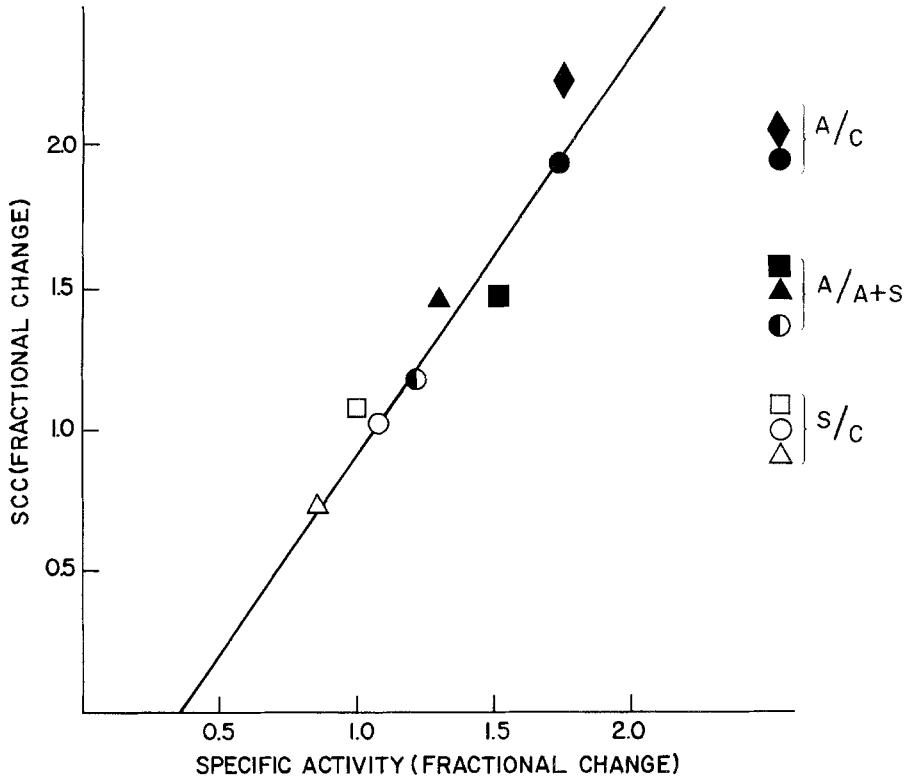

Fig. 6. Relationship between Na^+ transport and 12S cytoplasmic RNA. The fractional changes in *scc* on the ordinate and the fractional changes in specific activity of 12S cytoplasmic RNA on the abscissa were computed as the ratio of the respective values of the paired hemibladders, and are listed in Table 2. The points correspond to: aldosterone (7×10^{-8} M)/diluent (◆); aldosterone (3.5×10^{-8} M)/diluent (●); aldosterone (3.5×10^{-8} M)/aldosterone (3.5×10^{-8} M)+SC9420 (7×10^{-6} M) (■); aldosterone (7×10^{-8} M)/aldosterone (7×10^{-8} M)+SC9420 (2.5×10^{-5} M) (▲); aldosterone (3.5×10^{-8} M)/aldosterone (3.5×10^{-8} M)+SC 9420 (3.5×10^{-6} M) (◐); SC 9420 (7×10^{-6} M)/diluent (□); SC 9420 (3.5×10^{-6} M)/diluent (○); SC 9420 (2.5×10^{-5} M)/diluent (△)

Effects of Spironolactone and Aldosterone on Cytoplasmic Poly(A)(+)-RNA

The results in Fig. 6 may reflect activation by aldosterone of parallel pathways regulating Na^+ transport and RNA metabolism independently, or sequential activation of RNA synthesis and Na^+ transport. If the latter is the case, a reasonable candidate for the mediator is mRNA. This possibility was explored further by studies on the relationships between the effects of the modulators on *scc* and cytoplasmic poly(A)(+)-RNA.

Table 3. The effects of spironolactone and aldosterone on the incorporation of ^{3}H-uridine into the acid soluble pool and RNA fractionated by oligo (dT) cellulose chromatography

Pairs of hemi-bladders (No.)	SC-9420 (M)	Aldo-sterone (M)	Acid-soluble pool (cpm/mg DNA)	Cytoplasmic RNA (cpm/mg RNA)	High-salt eluates (cpm/mg RNA)	Low-salt eluates (cpm/mg RNA)
9	7×10^{-6}	0	520	570	465	8150
	0	0	675	740	570	8830
Fractional change:			0.77	0.77	0.82	0.92
8	0	3.5×10^{-8}	795	710	745	7820
	7×10^{-6}	3.5×10^{-8}	875	535	560	6360
Fractional change:			0.91	1.33	1.33	1.23

Paired hemibladders were incubated in SC-9420 or the diluent (upper half), or in aldosterone with or without spironolactone (lower half). The protocols are described in the legends of Table 1 and Figs. 1 and 3. The acid-soluble pools, cytoplasmic RNA extracts and oligo (dT)-cellulose chromatography (high-salt and low-salt eluates) were obtained as described in the text. The sources of the RNA, i.e., the toad bladder epithelia, were from the corresponding pairs listed in Table 1 and Figs. 1 and 3. The high salt eluates were obtained with 0.5 M KCl−0.01 M Tris HCl (pH=7.4), and the low-salt eluates (poly(A)(+)-RNA) with 0.01 M Tris HCl (pH=7.4). RNA was measured by absorbance at 260 nm (1-A_{260} unit=32 μg RNA; light path=10 mm).

Accordingly, the cytoplasmic extracts (shown in Figs. 1 and 3) were subjected to oligo(dT)-chromatography. In the absence of exogenous aldosterone, SC 9420 (7×10^{-6} M) depressed the incorporation of ^{3}H-uridine into total cytoplasmic RNA by 23%, but this effect was the same as the decrease in the ^{3}H content of the TCA-soluble extract (acid-soluble pool) (Table 3). As expected, the specific activity of the poly(A)(+)-RNA (low-salt eluates) was 12 to 15-fold higher than the total cytoplasmic RNA or the RNA in the high-salt eluates. SC 9420 had a minimal effect on the specific activity of the poly(A)(+)-RNA pool (Table 3). The low-salt eluates were also analyzed by sucrose gradient centrifugation. The specific activity of poly(A)(+)-RNA sedimenting at <9S was unaffected by spironolactone, but was lowered by 20 to 40% in the 10S to 28S region (Fig. 7).

In the presence of exogenous aldosterone, SC 9420 diminished the specific activity of total cytoplasmic RNA, the RNA in the high-salt eluates and the poly(A)(+)-RNA despite a small rise in ^{3}H content of the acid-soluble pool (Table 3). The sucrose gradient analysis of these poly(A)(+)-RNA fractions revealed a prominent peak in the 9S to 18S region, centered at 12S, that was completely erased by spironolactone (Fig. 8). These results are in accord with an aldosterone-dependent induc-

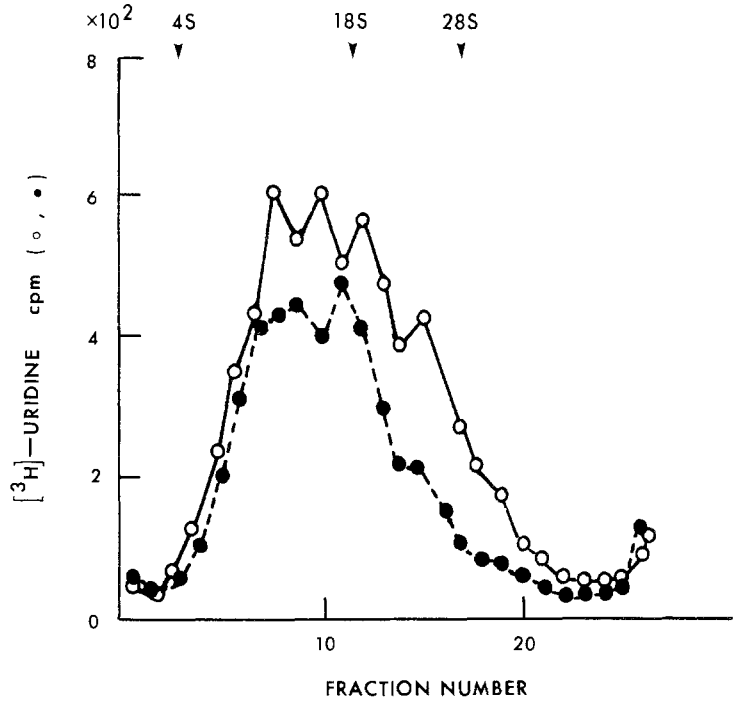

Fig. 7. Sucrose gradient analysis of the effects of SC 9420 (7×10^{-6} M) on Cytoplasmic Poly(A)(+)-RNA. Paired hemibladders were incubated in SC 9420 or the diluent and labeled with ^{3}H-uridine (30 min pulse − 140 min chase) as described in legend of Fig. 1. Epithelial cytoplasmic RNA extracts were fractionated by oligo(dT)-cellulose chromatography. The poly(A)(+)-RNA pool was analyzed on linear 5–20% sucrose gradients. The standard S values were determined from the A_{254} peaks (4S, 18S, 28S) of total cytoplasmic RNA centrifuged in identical gradients (parallel tubes) at the same time. The ^{3}H activity of the hemibladders treated with SC 9420 (-●-) or with the diluent (-o-) is indicated by the ordinate

tion of 12S poly(A)(+)-RNA. In these experiments, a pulse-chase format was used; specifically a 30 min pulse of ^{3}H uridine coincident with the first 30 min of exposure to aldosterone followed by a 150 min chase. Since spironolactone apparently inhibits the first step in the mineralocorticoid pathway, the binding of aldosterone to the receptor [18], the inhibitory effect on poly(A)(+)-RNA metabolism should have been exerted very early in the course of action of adosterone.

To insure that the inhibitory effect of SC 9420 was imposed during the latent period (i.e., first 60 min), paired hemibladders were preincubated for 20 min with either SC 9420 (7×10^{-6} M) or the diluent and

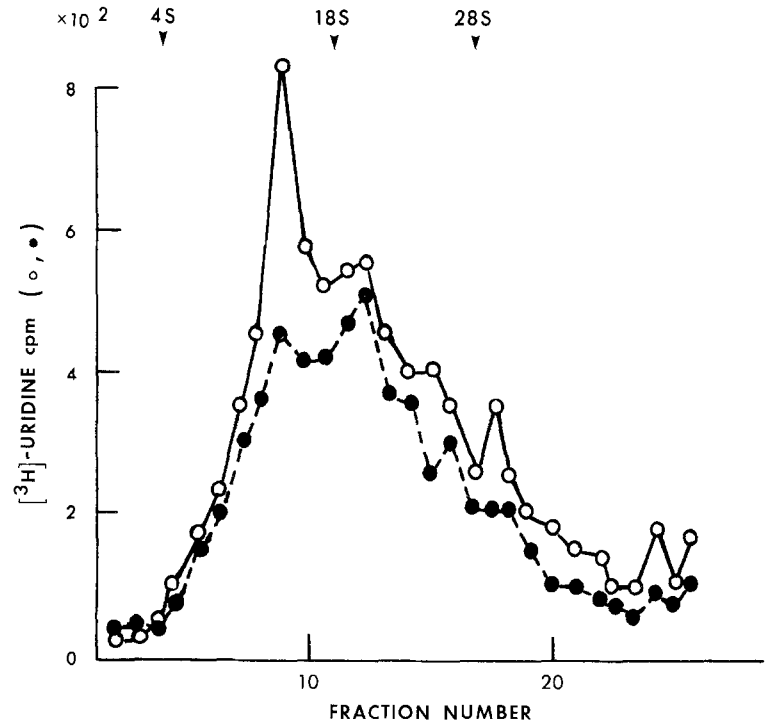

Fig. 8. Sucrose gradient analysis of the effects of SC 9420 (7×10^{-6} M) and aldosterone (3.5×10^{-8} M) on cytoplasmic poly(A)(+)-RNA. The incubation and labeling conditions are described in the legend of Fig. 3. The poly(A)(+)-RNA (oligo(dT)- cellulose chromatography) was analyzed as described in the legend of Fig. 7. Aldosterone is denoted by –o– and aldosterone+SC 9420 by –●–

all hemibladders were then challenged with aldosterone (3.5×10^{-8} M). Ten min after the addition of aldosterone (t_0). ^{3}H-uridine was added to all of the media, for 30 min. At the end of this period of incubation ($t = 40$ min), the scc_t/scc_0 ratios were 1.24 ± 0.10 and 1.06 ± 0.12 (n=9 pairs, $p > 0.13$) in the aldosterone, and aldosterone+spironolactone groups, respectively. The cytoplasmic RNA extracts of the pooled epithelia were fractionated by oligo(dT)-cellulose chromatography and the poly(A)(+)-RNA pools were then analyzed by sucrose gradient centrifugation (Fig. 9). As noted previously, aldosterone promoted incorporation of ^{3}H uridine into poly(A)(+)-RNA in the 11S–13S region of the gradient and SC 9420 eliminated the 12S peak. These results confirm that spironolactone inhibition of incorporation of ^{3}H uridine into poly(A)(+)-RNA (particularly the 12S class) precedes the antagonism of the effect of aldosterone on Na^+ transport.

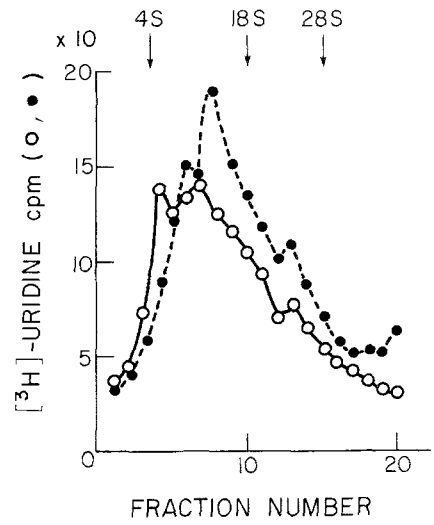

Fig. 9. Sucrose gradient analysis of the effects of SC 9420 (7×10^{-6} M) and aldosterone (3.5×10^{-8} M) on cytoplasmic poly(A)(+)-RNA labeled with ^{3}H-uridine for 30 min. The experimental conditions were as described in the legend of Fig. 8, except that labeling with ^{3}H-uridine was limited to a 30 min pulse introduced 10 min after addition of aldosterone. Aldosterone is denoted –●– and aldosterone + SC 9420 by –o–

Discussion

In the isolated toad bladder, the spirolactones are competitive inhibitors of the aldosterone-dependent increase in *scc* [22]. In the former experiments, the spirolactones did not impair either the *scc* response to vasopressin or glucose uptake from the medium. In the rat kidney, the spirolactones impaired the binding of ^{3}H aldosterone to cytoplasmic receptors; the pattern conformed to that of competitive inhibition [9, 11]. In the adrenalectomized rat (*in vivo*), a spirolactone (SC 26304) inhibited the binding of ^{3}H aldosterone to renal cytoplasmic and nuclear receptors, and the aldosterone-dependent rise in the urinary K^+/Na^+ ratio, proportionately [18]. Additional findings indicated that ^{3}H-SC-26304 occupied the same cytoplasmic receptor sites as ^{3}H aldosterone but the spirolactone-receptor complex failed to translocate to the nucleus and bind to the chromatin acceptor sites [18]. At very high concentrations, however, spirolactones may have a direct toxic effect. Thus, at concentrations greater than 7×10^{-5} M, spirolactone depressed the *scc* across the isolated toad bladder abruptly [5, 22]. For this reason, the maximum concentration of SC 9420 used in the present study was 2.5×10^{-5} M. In aldoste-

rone-free media, 2.5×10^{-5} M SC 9420 inhibited the *scc* and incorporation of ^{3}H uridine into 12S cytoplasmic RNA, proportionately. The effect on the *scc* was maximal after 180 min of incubation (Tables 1 and 2). In view of the relatively brief period of pre-incubation of the hemibladders in steroid-free media, i.e., 4 to 5 hr, these results may reflect displacement of endogenously bound aldosterone by spironolactone, or mild toxicity.

Our results confirm the findings of Hutchinson and Porter [14] of inhibition by spirolactones of the incorporation of precursors into unfractionated cytoplasmic and nuclear RNAs. Moreover, aldosterone-dependent augmentation of incorporation of ^{3}H-uridine into 12S cytoplasmic RNA and 9S–18S poly(A)(+)-RNA is markedly impaired by spironolactone (Tables 2 and 3 and Figs. 4, 8 and 9). These results are also in accord with our earlier findings of aldosterone-dependent increases in incorporation of ^{3}H-uridine into 9S–12S cytoplasmic RNA (spironolactone-sensitive) and into poly(A)(+)-RNA [23, 27].

The implication of induction of mRNA, inferred from the effects of aldosterone on (nonmethylated) 12S cytoplasmic RNA and poly(A)(+)-RNA, in mineralocorticoid action raises two further questions: How many distinct mRNA's are induced? For which proteins are these mRNA's coded? Although satisfactory answers to these questions are not yet available, recent studies imply that aldosterone induces a number of proteins of varying properties. In the isolated toad bladder, aldosterone enhanced the incorporation of amino-acid precursors into electrophoretically separated peptides with molecular weights of 12,000 [2], and of 17,000 to 38,000 [24]. Neither the subcellular distribution nor the functions subserved by these proteins have been identified as yet. Similar studies, in the adrenalectomized rat, identified induction of a cytosol protein (of unknown function) with a molecular weight of 31,000[1]. Augmentation of the activities of a variety of enzymes by aldosterone has also been described in both toad bladder and rat kidney. In the toad bladder, aldosterone increased the activity of a phosphatase that acted on a membrane-bound phospho-protein [17]. In both the toad bladder and the rat kidney, aldosterone increased the activities of several mitochondrial enzymes, including glutamate-oxalacetate transaminase, isocitrate dehydrogenase (TPN^{+}) and citrate synthase [15, 16]. Recently, it was found that aldosterone stimulated the incorporation of amino acids into renal citrate synthase and that this effect was inhibited

[1] P.Y. Law and I.S. Edelman. Effect of aldosterone on incorporation of amino acids into renal medullary proteins (*in preparation*).

by actinomycin D and spirolactone (SC-26304)[2]. It is probable, therefore, that aldosterone induces the synthesis of a variety of proteins with many functions. The roles played by these proteins in enhanced transepithelial Na^+ transport remains to be elucidated.

In the present study, a remarkable correlation ($r=0.95$) between the augmentation of Na^+ transport and labeling of 12S cytoplasmic RNA was found (Fig. 6). Although this correlation (even if the 12S RNA represents mRNA) does not establish a causal relationship, these findings certainly are in accord with the inference that induction of mRNA's mediate mineralocorticoid action.

The linear relationship (rather than a saturation function) between the fractional change in Na^+ transport and the fractional change in the specific activity of 12S cytoplasmic RNA may indicate that the induced proteins are rate-limiting with respect to the transport response; implying that under the conditions of these experiments the Na^+ pump operates below the maximal capacity provided, of course, that the Na^+ pump itself is not induced. It is of interest that a linear relationship has also been described for the aldosterone-dependent fractional increases in *scc* and citrate synthase activity in the toad bladder [15, 16]. That the Na^+ pump operates at less than maximal capacity under maximal aldosterone stimulation in the isolated toad bladder is also implied by the finding that vasopressin increases the *scc* to a considerable further extent even in the presence of excess steroid [10, 12]. This argument assumes that vasopressin and aldosterone act on the same cells in the epithelium.

The implication of induction of mRNA synthesis in the mechanism of action of aldosterone is consonant with steroid-dependent accumulation of specific mRNA's in a number of other systems (e.g., responses to progestins, estrogens, androgens and glucocorticoids [7]). With respect to aldosterone, the present results are also in accord with the sensitivity of the mineralocorticoid response to actinomycin D and cordycepin, well-characterized inhibitors of RNA synthesis [4, 8].

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[2] P.Y. Law and I.S. Edelman. Induction of renal citrate synthase by aldosterone (*in preparation*).

References

1. Aviv, H., Leder, P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Nat. Acad. Sci. USA* **69**:1408
2. Benjamin, W.B., Singer, I. 1974. Aldosterone-induced protein in toad urinary bladder. *Science* **186**:269
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the calorimetric estimation of DNA. *Biochem. J.* **62**:315
4. Chu, L.L.H., Edelman, I.S. 1972. Cordycepin and α-amanitin: Inhibitors of transcription as probes of aldosterone action. *J. Membrane Biol.* **10**:291
5. Crabbé, J. 1963. The Sodium Retaining Action of Aldosterone. Presses Acad. Europeene, Edit. Arscia S.A., Brussels
6. Darnell, J.E., Philipson, L., Wall, L., Adesnik, M. 1971. Polyadenylic acid sequences: Role in conversion of nuclear RNA into messenger RNA. *Science* **174**:507
7. Edelman, I.S. 1975. Mechanism of action of steroid hormones. *J. Steroid Biochem.* **6**:147
8. Edelman, I.S., Bogoroch, R., Porter, G.A. 1963. On the mechanism of action of aldosterone on sodium transport: The role of protein synthesis. *Proc. Nat. Acad. Sci. USA* **50**:1169
9. Fanestil, D.D. 1968. Mode of spirolactone action: Competitive inhibition of aldosterone binding to kidney mineralocorticoid receptors. *Biochem. Pharmacol.* **17**:2240
10. Fanestil, D.D., Porter, G.A., Edelman, I.S. 1967. Aldosterone stimulation of sodium transport. *Biochim. Biophys. Acta* **135**:74
11. Funder, J., Feldman, D., Highland, E., Edelman, I.S. 1974. Molecular modifications of anti-aldosterone compounds: Effects on affinity of spirolactones for renal aldosterone receptors. *Biochem. Pharmacol.* **23**:1493
12. Handler, J.S., Preston, A.S., Orloff, J. 1969. Effect of adrenal steroid hormones on the response of the toad's urinary bladder to vasopressin. *J. Clin. Invest.* **48**:823
13. Herman, T.S., Fimognari, G.M., Edelman, I.S. 1968. Studies on renal aldosterone binding proteins. *J. Biol. Chem.* **243**:3849
14. Hutchinson, J.H., Porter, G.A. 1970. Spirolactone inhibition of aldosterone induced nuclear RNA synthesis in toad urinary bladder. *Res. Commun. Chem. Path. Pharmacol.* **1**:363
15. Kirsten, R., Kirsten, E. 1972. Redox state of pyridine nucleotides in renal response to aldosterone. *Am. J. Physiol.* **223**:229
16. Kirsten, E., Kirsten, R., Sharp, G.W.G. 1970. Effects of sodium transport stimulating substances on enzyme activities in the toad bladder. *Pfluegers Arch.* **316**:26
17. Liu, A.Y.-C., Greegard, P. 1974. Aldosterone-induced increase in protein phosphatase activity of toad bladder. *Proc. Nat. Acad. Sci. USA* **71**:3869
18. Marver, D., Stewart, J., Funder, J.W., Feldman, D., Edelman, I.S. 1974. Renal aldosterone receptors: Studies with ^{3}H-aldosterone and the anti-mineralocorticoid ^{3}H-spirolactone (SC-26304). *Proc. Nat. Acad. Sci. USA* **71**:1431
19. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. *J. Mol. Biol.* **17**:117
20. Perry, R.P., Greenberg, J.R., Kelley, D.E., Latorre, J., Schochetman, G. 1973. Messenger RNA: Its origin and fate in mammalian cells. *In:* Gene Expression and Its Regulation. F.T. Kenney, B.A. Hamkalo, G. Favelukes, J.T. August, editors. p. 149. Plenum Publishing Company, New York
21. Perry, R.P., Kelley, D.E. 1974. Existence of methylated mRNA in mouse L cells. *Cell* **1**:37
22. Porter, G.A. 1968. *In vitro* inhibition of aldosterone-stimulated Na^+ transport by steroidal spirolactones. *Mol. Pharmacol.* **4**:224

23. Rossier, B.C., Wilce, P.A., Edelman, I.S. 1974. Kinetics of RNA labeling in toad bladder epithelium: Effects of aldosterone and related steroids. *Proc. Nat. Acad. Sci. USA* **71**:3101
24. Scott, W.N., Sapirstein, V.S. 1975. Identification of aldosterone-induced proteins in the toad's urinary bladder. *Proc. Nat. Acad. Sci. USA* **72**:4056
25. Snedecor, G.W., Cochran, W.G. 1967. Statistical Methods. (6th ed.) Iowa State University Press, Ames
26. Walser, M. 1969. Reversible stimulation of sodium transport in the toad bladder by stretch. *J. Clin. Invest.* **48**:1714
27. Wilce, P.A., Rossier, B.C., Edelman, I.S. 1977. Action of aldosterone on Poly(A)(+)-RNA and Na^+ transport in the toad bladder. *Biochemistry* **15**:4279