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Cordycepin and α-Amanitin: Inhibitors of Transcription as Probes of Aldosterone Action

Luke L. H. Chu* and Isidore S. Edelman

Department of Biochemistry and Biophysics, and Department of Medicine, and the Cardiovascular Research Institute, University of California School of Medicine, San Francisco, California 94122

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Summary. Evidence has been presented for the existence of distinct RNA polymerases (I and II) in the rat kidney similar to those described previously in rat liver and identified as nucleolar (I) and nucleoplasmic (II) in location. In adrenalectomized rats, administration of d-aldosterone (1 μg/100 g body wt) increased the ratio of polymerase I:II activity as defined by sensitivity to α-amanitin. The time-course of this shift correlated with the previously reported changes in urinary Na and K concentrations, in that a significant change in the I:II ratio was noted 1 to 2 hr before the usual peak change in urinary electrolyte concentrations. In the isolated urinary bladder of the toad, cordycepin inhibited incorporation of ^{3}H-uridine into epithelial RNA – with a half time of 4.3 min – and impaired the action of aldosterone on transepithelial Na transport. These findings as well as earlier studies in the literature imply that aldosterone augments ribosomal RNA synthesis, and that ribosomal or messenger RNA synthesis or both are required for the expression of mineralocorticoid activity.

Steroidal regulation of active Na transport in anuran and mammalian target tissues is apparently mediated by stimulation of DNA-dependent synthesis of RNA and the subsequent *de novo* synthesis of proteins [6, 7, 34]. This inference is based, in part, on the finding that actinomycin D impaired mineralocorticoid action [6, 10, 34]. In addition, aldosterone enhanced the incorporation of precursors into total or nuclear RNA of the urinary bladder of the toad, *in vitro* and of the rat kidney, both *in vivo* and in homogenates [5, 10, 12, 13, 22, 27].

Roeder and Rutter [25, 26] have recently separated and characterized two eukaryotic DNA-dependent RNA polymerases with distinct functions.

* *Present address:* Research Division, Veterans Administration Hospital, 4801 Linwood Blvd., Kansas City, Missouri 64128.

Polymerase I is localized in the nucleolus and transcribes nucleolar genes, and polymerase II is localized in the nucleoplasm and transcribes nucleoplasmic genes [1]. The RNA polymerases also differ in susceptibility to inhibitors of RNA synthesis. At low concentrations, actinomycin D predominantly impairs the synthesis of ribosomal RNA whereas high concentrations of this antibiotic inhibits virtually all DNA-dependent RNA synthesis [21, 30]. Recently, two inhibitors with somewhat greater specificity than actinomycin D have been described. α-Amanitin, a toxin isolated from the mushroom *Amanita phalloides*, inhibited RNA polymerase II selectively and had no effect on polymerase I [16]. In contrast, cordycepin (desoxyadenosine) blocked ribosomal RNA synthesis in mammalian tissue culture cells, which is an expression of polymerase I activity [29]. In addition, cordycepin suppressed the formation of polysomal messenger RNA (mRNA) (based on labeling of polyribosomes) but not of heterogeneous RNA (HnRNA) [20], although both mRNA and HnRNA are apparently nucleoplasmic in origin [4].

The availability of inhibitors of RNA synthesis that are somewhat more selective than actinomycin D prompted us to explore the role of specific classes of transcription in the mechanism of action of aldosterone. The results are consistent with the inference that aldosterone enhances renal polymerase I activity relative to polymerase II and that the products of these polymerases, either rRNA or mRNA, or both, are required for the mineralocorticoid response in the isolated urinary bladder of the toad. These results are in accord with the recent findings of Liew, Liu and Gornall [15] of enhancement of both Mg- and Mn-activated RNA polymerases in renal and cardiac nuclei by administration of aldosterone to adrenalectomized rats.

Materials and Methods

Materials

α-Amanitin was a generous gift from Professor Th. Wieland of the Max-Planck-Institute at Heidelberg. Cordycepin was purchased from Sigma Chemical Co., d-aldosterone (chromatographic grade) from Cal Biochem Co., and ^{3}H-uridine triphosphate, ^{3}H-uridine, and ^{3}H-leucine from New England Nuclear. The conventional reagents were of analytical grade quality.

Procedures

Rat Kidney Studies. Male, Sprague-Dawley rats (~120 g body wt) were adrenalectomized 3 to 7 days before use and maintained on 0.9% saline drinking water and Purina laboratory chow *ad libitum*. Sixteen hours before the study, low potassium diets

(1.2 μEquiv of K per gram dry wt, supplied by General Biochem) were substituted for the standard Purina chow. The rats were injected subcutaneously with either d-aldosterone (1 to 3 μg/100 g body wt) or the diluent (1 ml of 0.9% NaCl). At various times after injection, pairs of rats (control and steroid-injected) were anesthetized with ether and exsanguinated by cardiac puncture. The renal arteries were perfused with 20 ml of ice-cold 0.25 M sucrose, 3 mM $MgCl_2$, pH 8.0, titrated with Tris buffer (standard medium). Both kidneys were decapsulated, removed and put into the standard medium at 0 °C.

Purified renal nuclear fractions were prepared as described previously [31]. To assay for endogenous RNA polymerase activity, nuclear fractions obtained from a pair of kidneys were suspended in 3 ml of standard medium and 0.05-ml aliquots were added to equal volumes of stock solutions, such that the final concentrations in the incubation mixture were: sucrose = 125, $MgCl_2$ = 5, $MnCl_2$ = 1, $(NH_4)_2SO_4$ = 40 or KCl = 30 (in the low salt incubations), $(NH_4)_2SO_4$ = 120 (in the high salt incubations), Tris HCl = 100 (pH 8.0), mercaptoethanol = 20, ATP = 5, GTP = 5, CTP = 5 (all in mM) and ^{3}H-UTP = 20 μC/ml (24 C/mmole). In preliminary experiments, the concentrations of $MgCl_2$ and $MnCl_2$ were varied. The stock solutions were pre-warmed at 25 °C for 10 min, and the reaction was started by adding the nuclear suspension to the pre-warmed stock solutions in a shaking water bath and the mixtures were incubated for various times and at various temperatures as described under Results. The reaction was stopped by adding 1 ml of ice-cold 10% TCA containing 40 mM sodium pyrophosphate to each tube. The TCA-treated mixtures were immersed in an ice bath for 30 min and the precipitates were collected by filtration through glass fiber filter discs (Whatman, grade GF/c) under suction. Each disc was then washed 3 times with 5 ml of ice-cold 5% TCA, twice with 5 ml of ethanol/ether (3:1, v/v) and air dried. The filter discs were assayed for radioactivity in a dioxane-based scintillation solution [8] in a Mark II Liquid Scintillation Spectrometer (Nuclear Chicago). DNA content was determined by the method of Burton [2].

Renal cytosol fractions were assayed for RNA polymerase activity by the method of Roeder and Rutter [26]. Renal homogenates were centrifuged for 10 min at 1,000 × *g* at 2 °C. The supernatants were made up to 0.01 M Tris HCl (pH 7.9) and 5 mM dithiothreitol, and centrifuged at 100,000 × *g* for 60 min at 2 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant fractions to yield a final concentration of 0.42 g/ml. These fractions were immersed in an ice bath for 30 min and recentrifuged at 100,000 × *g* for 60 min at 2 °C. The precipitates were resuspended in 2 ml of 0.05 M Tris HCl (pH 7.9), 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM dithiothreitol (T-G medium). To remove excess $(NH_4)_2SO_4$, these solutions were filtered through G-25 Sephadex columns (0.6 × 20 cm) previously equilibrated with the T-G medium. The void volume eluates were immediately applied to DEAE-Sephadex A-25 columns (0.6 × 10 cm) previously equilibrated with T-G medium. These columns were washed with 10 ml of T-G medium containing 0.1 M $(NH_4)_2SO_4$ and then with 5 ml of T-G medium containing 0.3 M $(NH_4)_2SO_4$. The fractions eluted with 0.3 M $(NH_4)_2SO_4$ were assayed for RNA polymerase activity as described previously [25]. In brief, 28 μmoles Tris HCl, pH 7.9, 0.8 μmole $MnCl_2$, 4 μmoles KCl, 3 μmoles NaF, 0.8 μmole mercaptoethanol, 10 μg pyruvate kinase (Cal Biochem), 2 μmoles phosphoenolpyruvate, 0.3 μmole each of GTP, CTP and ATP, 50 μg native calf thymus DNA (Cal Biochem) and 0.2 ml or 0.05 ml of the enzyme preparation in a final volume of 0.5 ml were incubated for 5 min at 30 °C. Incorporation was started by addition of 5 μC ^{3}H-UTP (20 C/mmole) and the mixtures were incubated for an additional 10 min at 30 °C. The reaction was terminated by the addition of equal volumes of ice-cold 10% TCA and 1 mg of bovine serum albumin. The precipitates were collected on glass fiber filter discs, washed, dried and assayed for radioactivity as described above for the nuclear RNA polymerase assays. The RNA polymerase assays were expressed in relative units (ratio of polymerase I:II activity).

Toad Bladder Studies. Active Na transport *in vitro* was estimated by the short-circuit current technique of Ussing and Zerahn [33]. Urinary hemibladders were excised after double pithing of toads (*Bufo marinus*) and each hemibladder was mounted in chambers filled with frog-Ringer's solution fortified with 10 mM glucose, 100 U/ml penicillin G and 0.1 mg/ml streptomycin as described previously [23]. The hemibladders were incubated at ~25 °C for 15 hr. All solutions were then replaced with fresh frog-Ringer's solution containing 5 mM glucose. "Time-zero" was designated as the time of addition of d-aldosterone (final concentration $= 7 \times 10^{-8}$ M) to the media. The details of the protocols are given in Results.

To assess the level of incorporation of precursors into RNA, hemibladders were pre-incubated for 15 hr as described above except that the tissue was suspended in 30 ml of frog-Ringer's solution in beakers. The hemibladders were then cut in half and incubated in fresh frog-Ringer's solution for an additional 60 min. The inhibitors, cordycepin or α-amanitin, were added to the media for varying periods of time before the addition of ^{3}H-uridine (1 μC/ml, 25 C/mmole). The tissue was incubated for 15 min at 25 °C and incorporation was terminated by transferring the hemibladders into 200 ml of ice-cold frog-Ringer's solution. The epithelial cells were collected by stretching the hemibladders over Lucite blocks and scraping the mucosal surface gently with the edge of a glass slide. These scrapings consist of sheets of epithelial cells with minimal adventitial elements [17]. The epithelial cells were suspended in ice-cold 10% TCA, homogenized in a Teflon-glass Potter-Elvehjem homogenizer and the homogenates centrifuged at $1{,}500 \times g$ for 10 min at 2 °C. These precipitates were washed successively as follows: twice with 5 ml of 5% TCA, twice with 5 ml of 95% ethanol/ether (3:1, v/v), and once with 5 ml of 5% TCA. All of the washings were carried out at 2 °C. The washed precipitates were extracted with 2 ml of 5% TCA at 90 °C for 15 min and the extracts were assayed for ^{3}H activity as described previously [8]. Aliquots of the extracts were analyzed for DNA [2].

To assess the effect of cordycepin on protein synthesis in the epithelium of the toad bladder, the same protocol was used as in the experiments on RNA synthesis with respect to pre-incubation in beakers. Cordycepin (final concentration = 30 μg/ml) was added to half of each hemibladder 30 or 60 min before the addition of ^{3}H-leucine (1 μC/ml, 35 C/mmole) to the media. The epithelial cell layers were harvested by scraping after 15 min of incubation at 25 °C. The epithelial cells were precipitated with cold TCA, and washed with cold TCA, ethanol and ethanol/ether as described above for the RNA experiments. After the extraction with hot 5% TCA, the precipitates were dissolved in 0.5 ml NCS (Searle-Amersham), mixed with toluene-based scintillation solution and assayed for ^{3}H-content by Liquid Scintillation spectrometry.

Results

Endogenous RNA Polymerase Activity in Rat Kidney Nuclei

To determine the optimum conditions for incorporation of ^{3}H-UTP into renal nuclear RNA, reaction mixtures were incubated for 5, 7, 10, 15 and 20 min, at 0, 10, 20, 25, 30 and 37 °C. Maximum incorporation was obtained when the nuclei were incubated for 20 min at 25 °C. At temperatures greater than 20 °C, incorporation was curvilinear with time. At 25°C, incorporation deviated from linearity only to a small degree in 10 min. Accordingly, all subsequent assays were carried out at 25 °C for 10 min.

Table 1. Renal nuclear incorporation of ^{3}H-UTP[a]

	Condition	Rel. activity (%)
Part I	Control	100.0
	+Act. D, 3.5 μg/ml	25.7
	+EDTA, 10 mM	7.0
	−ATP	23.8
	−GTP	12.9
	−CTP	0.0
	+RNase (250 μg/ml)	13.0
Part II	RNase treatment of ppt.	2.0
	DNase treatment of ppt.	100.0

[a] 0.2 ml aliquots of a purified nuclear suspension were added to 0.8 ml of incubation mixture as described in Materials and Methods, with the addition or deletion of the reagents specified in the table. In Part I, the result is expressed as a percentage of the control sample, which incorporated 10,280 cpm per filter disc. In Part II, filter discs containing the precipitate were carefully immersed in 5 ml of 0.1 M Tris HCl, pH 8.0, containing 250 μg/ml RNase or DNase plus 0.4 mM Ca and 3 mM Mg. The vials were incubated at 22 °C for 15 hr. These filter discs were then carefully washed on the filter holder and the remaining radioactivity determined. The result is expressed as a percentage of the similarly treated control sample, which incorporated 9,800 cpm per filter disc.

The validity of the assay for RNA polymerase activity was determined in a series of control studies (Table 1). Addition of either actinomycin D (3.5 μg/ml) or EDTA (10 mM) or RNase (250 μg/ml), or omission of either ATP or GTP or CTP reduced incorporation of ^{3}H-UMP into renal nuclear RNA to 26% or less of the control value. Treatment of the TCA precipitates on the filter discs (after incorporation of ^{3}H-UTP under optimal conditions) with RNase (250 μg/ml) at 22 °C for 15 hr reduced recovery of labeled RNA to 2.0% of the control value. In contrast, DNase (250 μg/ml) under the same conditions gave a recovery of 100%.

The contributions of nucleolar (I) and nucleoplasmic (II) RNA polymerases to total polymerase activity was evaluated by altering the Mg and Mn concentrations, and the ionic strength of the assay media. As shown in Fig. 1, optimum activity was obtained at a Mn concentration of 1.5 to 2.0 mM in both low and high salt concentrations, and at a Mg concentration of 7 to 9 mM in low salt and 3 to 5 mM in high salt. The profiles of activity as a function of divalent metal ion concentration and ionic strength bear a close resemblance to those of purified rat liver RNA polymerases I and II [25]. These results imply the existence of similar nucleolar and nucleoplasmic RNA polymerases in rat liver and kidney. The relative contributions of

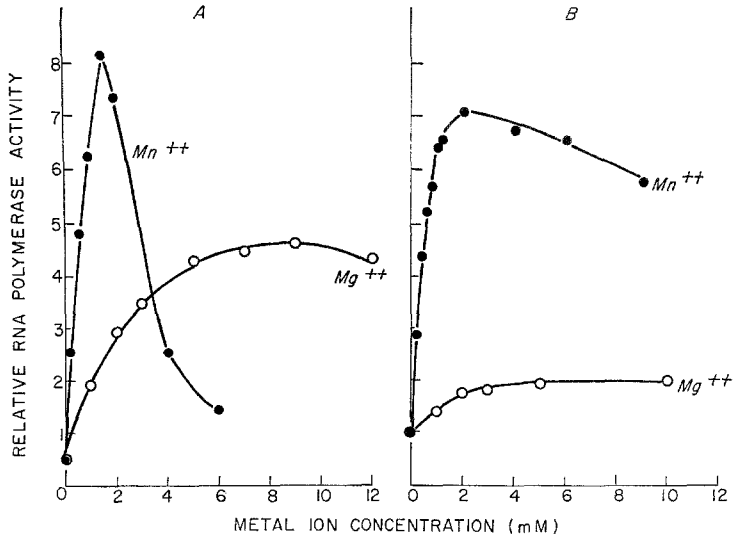

Fig. 1. Effects of ionic strength and divalent metal ion concentrations on renal nuclear RNA polymerase activity. Renal nuclear fractions were incubated in either 0.03 M KCl, (low salt media, panel *A*) or in 0.12 M $(NH_4)_2SO_4$ (high salt media, panel *B*). All incubation tubes contained 0.1 M Tris HCl, pH 8.0, 0.02 M mercaptoethanol, 5 mM ATP, GTP and CTP, and 10 μC/ml of ^{3}H-UTP (24 C/mM). Either $MgCl_2$ or $MnCl_2$ was added at the concentrations indicated in the figure. Each point is the average of two experiments expressed as a fraction of the activity relative to that obtained in the absence of divalent metal ions in high salt media which averaged 250 cpm per filter disc

polymerase I and polymerase II activities to total polymerase activity can be assessed with the toxin, α-amanitin. Rat liver RNA polymerase II was shown previously to be selectively inhibited by this toxin [16]. The effects of varying concentrations of α-amanitin on renal nuclear RNA polymerase activity are shown in Fig. 2. α-Amanitin inhibited polymerase activity by 85% in high salt media and by 50% in low salt media. Since the activity of polymerase II is enhanced in media of high ionic strength, these results are in accord with the inference that α-amanitin inhibits renal nucleoplasmic RNA polymerase II selectively [16].

To assess the relative contributions of polymerases I and II to total polymerase activities, we assumed that α-amanitin at 1 μg/ml completely inhibited polymerase II activity. Thus, renal nuclear RNA polymerase activity expressed in terms of the ratios of I:II was calculated by measuring total activity in the presence and absence of α-amanitin since other polymerases, e.g., polymerase III, usually contribute very little to total activity [16, 25].

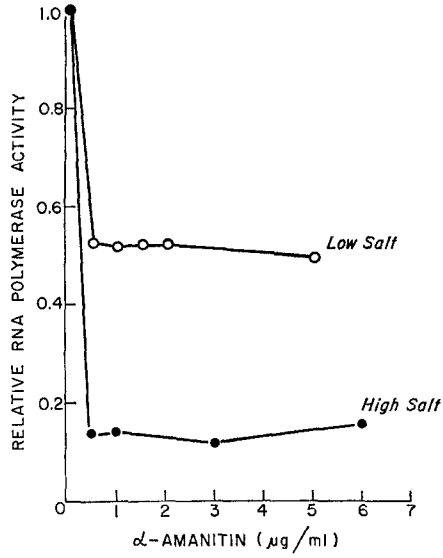

Fig. 2. Effect of α-amanitin on nuclear RNA synthesis. Purified nuclear suspensions were added to preincubated mixtures in either 0.04 M $(NH_4)_2SO_4$ (low salt ∘—∘—∘) or 0.12 M $(NH_4)_2SO_4$ (high salt •—•—•) with various amounts of α-amanitin. The mixtures contained 5 mM $MgCl_2$ and 1 mM $MnCl_2$. ^{3}H-UTP (20 μC/ml) was added after 5 min of pre-incubation with α-amanitin. Each point represents the average of two experiments expressed as a fraction of the activity of the uninhibited control samples which had 1,900 cpm per filter disc in low salt medium and 4,200 cpm per filter disc in high salt medium

Effect of Aldosterone on Renal Nuclear RNA Polymerase Activities

Adrenalectomized rats were injected either with d-aldosterone (1 μg/100 g body wt) or an equal volume of saline, and the kidneys were removed either 1, 3 or 6 hr after injection. Previous studies showed that the effect of aldosterone on urinary Na and K excretion is maximum at 3 to 4 hr after injection [10]. The results in Table 2 indicate that there was a significant increase in the I:II ratio at 1 hr, a peak effect at 3 hr and a decline towards the ratio found in the steroid-deficient state in 6 hr. The time-course of the change in the I:II ratio is similar to that of the changes in urinary electrolyte concentrations but appears sooner. Similar results were obtained in the corresponding assays in media of high ionic strength. Two additional control studies were carried out to test the possibility that the changes in nuclear RNA polymerase activities were artifactual: (1) It has been observed that RNA polymerases may be released into the supernatant fraction during preparation of nuclear fractions [14]. We therefore assessed the possibility that aldosterone simply shifted the relative quantities of polymerases I and

Table 2. Ratio of nucleolar (I)/nucleoplasmic (II) RNA polymerase activity of kidney nuclei of adrenalectomized rats [a]

No. of rats	Time of injection [b]	Ratio (I:II) (Mean ± SE)	p [c]
24	Control	0.47 ± 0.02	—
11	1	0.59 ± 0.04	<0.01
11	3	0.65 ± 0.05	<0.01
12	6	0.53 ± 0.04	n.s.

[a] Aldosterone was injected subcutaneously at a dose of 1 μg/100 g body wt at the indicated times before removal of the kidneys for assay. These assays were carried out in low ionic strength media with and without α-amanitin (1 μg/ml) as described in Materials and Methods.

[b] The absolute rate of incorporation of ^{3}H-UTP in the absence of α-amanitin averaged 2,000 ± 40 cpm per filter disc which is equivalent to 5 μμmoles UMP/mg DNA.

[c] The p values are based on a comparison of the ratios obtained after injection of aldosterone to that of the saline-injected control group and calculated by the Student's t test (unpaired).

II leaked into the cytosol fraction. (2) The presence of aldosterone in the nuclear fraction may nonspecifically stabilize polymerase I to a greater extent than polymerase II.

The former possibility was tested by isolating RNA polymerases I and II from renal cytosol fractions by the method of Roeder and Rutter [26]. In the final stage of isolation, total polymerase recovery was obtained by a one-step elution of the DEAE-Sephadex A-25 column with 0.3 M $(NH_4)_2SO_4$. Linearity of the assay system with time was tested over a 20-min interval at 30 °C. The time-course of incorporation of ^{3}H-UTP (20 C/mmole) into RNA with calf thymus DNA as template, was almost linear for the entire 20-min period of incubation. The validity of the assay system was evaluated by conventional control studies. The results are given in Table 3 and indicate the requirements for nucleoside triphosphates and template DNA. Moreover, incorporation was sensitive to actinomycin D but not rifampicin, an inhibitor of mitochondrial RNA polymerase [28]. The effect of aldosterone on the ratio of polymerase I:II activities in renal cytosol fractions was examined with α-amanitin as described above in the experiments on renal nuclear fractions. The results in Table 4 indicate that administration of aldosterone (3 μg/100 g body wt) to adrenalectomized rats had no effect on the polymerase I:II ratio of the cytosol fraction in either high salt or low salt media. The aldosterone-dependent rise in the I:II ratio of the nuclear fraction, therefore, could not be attributed to differential leakage of the two polymerases into the cytosol fraction which would have been reflected in a reciprocal fall in the I:II ratio.

Table 3. Assay for RNA polymerase activity in renal cytosol

Condition	% [a]
Complete system [b]	100
−ATP, CTP, GTP	2
−DNA	2
−Enzyme [c]	10
+Actinomycin D (5 μg/ml)	30
+Rifampicin (30 μg/ml)	110

[a] The absolute rate of incorporation of ^{3}H-UTP into RNA in the complete system was 2,500 cpm per filter disc. Each value is the average of 4 samples.
[b] The complete system is described in Materials and Methods. The mixtures were incubated at 30 °C for 10 min.
[c] 0.5 mg bovine serum albumin was used to substitute for the cytosol preparation in the (-enzyme) condition.

Table 4. Ratio of RNA polymerase I:II in renal cytosol [a]

No. of rats	Condition [b]	Ratio I:II [c] (Mean ± SE)	p
	A. High Salt		
12	Control	0.32 ± 0.05	n.s.
12	Aldosterone	0.31 ± 0.04	n.s.
	B. Low Salt		
4	Control	0.52 ± 0.03	n.s.
4	Aldosterone	0.55 ± 0.11	n.s.

[a] Aldosterone (3 μg/100 g body wt) or diluent was injected into adrenalectomized rats 1 hr before removal of the kidneys.
[b] Condition A: 0.2 ml cytosol preparation in 0.5 ml final volume. Condition B: 0.05 ml cytosol preparation in 0.5 ml final volume.
[c] Absolute incorporation (in the absence of α-amanitin) ranged from 1,500 cpm to 4,000 cpm per disc in (A) and from 300 to 1,300 in (B) in the various preparations. α-Amanitin (2.5 μg/ml) was used to inhibit polymerase II activity.

The possibility that the presence of aldosterone in the nuclear fractions during assay for RNA polymerase activity had a differential effect on the stability of polymerases I and II was assessed by the addition of aldosterone (10^{-6} M) to incubation mixtures that contained purified renal nuclei obtained from untreated adrenalectomized rats. The nuclear fractions were pre-incubated with or without added aldosterone for 5 min at 25 °C. Tracer incorporation was initiated by the addition of 5 μC/ml of ^{3}H-UTP (20 C/mmole) and the mixture was incubated at 25 °C for 10 min. In three

separate preparations, no significant difference was noted in the presence or absence of aldosterone in the incubation mixture.

In view of the evidence that aldosterone evokes an increase in total RNA synthesis [10] and in both Mg- and Mn-dependent RNA polymerase activity [15], our results may imply that aldosterone stimulates both polymerase I and II *in vivo* and that the effect on I is greater than on II.

Effect of α-Amanitin and Cordycepin on the Response of the Urinary Bladder of the Toad to Aldosterone

To assess the possible relevance of the effect of aldosterone on nuclear RNA synthesis to mineralocorticoid action, we exploited the isolated toad bladder preparation. In these studies we made use of cordycepin as an inhibitor of transcription and explored the possible use of α-amanitin for this purpose. The concentrations necessary to inhibit RNA synthesis in intact toad bladder epithelial cells was determined in preliminary studies by effects on ^{3}H-uridine incorporation.

Paired halves of hemibladders were incubated at room temperature without or with varying concentrations of α-amanitin in beakers for 30 min, at which time ^{3}H-uridine (1 μC/ml) was added to the media. The epithelial cells were harvested by scraping after exposure to ^{3}H-uridine for 1 hr, and assayed for incorporation into total cellular RNA. No significant decrease in RNA synthesis was obtained at concentrations of α-amanitin up to 10 μg/ml. In view of the failure of α-amanitin to inhibit uridine incorporation in toad bladder epithelium, the action of aldosterone on transepithelial Na transport should be unimpaired. Paired hemibladders were preincubated in steroid-free media for 15 hr and d-aldosterone (7×10^{-8} M) was then added to the serosal and mucosal media of both hemibladders. α-Amanitin (10 μg/ml) was added to one of each pair at zero time. In five pairs of hemibladders, this toxin had no significant effect on the latent period or the magnitude of the aldosterone-induced increase in short-circuit current (SCC).

Cordycepin was used as an alternative to α-amanitin. The efficacy of cordycepin inhibition of RNA synthesis in intact toad bladder epithelium was tested with ^{3}H-uridine. Cordycepin (30 μg/ml) was added to the media of one of each pair of quarter-bladders either simultaneously with or 15 to 60 min before the addition of ^{3}H-uridine (1 μC/ml) to the media. Incorporation was allowed to proceed for 15 min at 25 °C. Two sets of experiments were carried out: (1) hemibladders continuously supplemented with glucose (10 mM) for the 15 hr of pre-incubation in steroid-free media, and (2)

Table 5. Cordycepin inhibition of RNA synthesis in toad bladder epithelium[a]

Condition of the system	Time of administration (min before ^{3}H-uridine)	Incorporation of ^{3}H-uridine (% of control)	No. of pairs
A. Substrate-supplemented system	0	73 ± 9[b]	10
	15	63 ± 7	10
	30	56 ± 10	10
	60	56 ± 3	10
B. Substrate-depleted system	30	66 ± 10	7
	60	40 ± 5	9

[a] One of each pair of hemibladders was exposed to cordycepin (30 µg/ml) for the indicated periods of time prior to the addition of ^{3}H-uridine (1 µC/ml) to the serosal and mucosal media of both hemibladders. All hemibladders were incubated in ^{3}H-uridine for 15 min at 25 °C and the ^{3}H-uridine incorporated into acid-precipitable material was measured in terms of cpm/mg of DNA in the sample. The results are expressed as per cent of incorporation of the paired uninhibited control hemibladder. The substrate-rich hemibladders were incubated in 10 mM glucose-standard Ringer's solution (plus streptomycin, 100 µg/ml, and penicillin, 100 U/ml) for 15 hr before measurement of RNA synthesis and in 5 mM glucose-standard Ringer's during the period of ^{3}H-uridine incorporation. Substrate-free standard Ringer's solution was used both for the 15 hr of pre-incubation and as the replacement solution during the period of ^{3}H-uridine incorporation in the substrate-depleted pairs. The absolute rates of incorporation for the control samples were 11,700 ± 920 cpm/mg DNA in (A) and 17,750 ± 2,270 cpm/mg DNA in (B).

[b] Mean ± SE.

hemibladders depleted of substrate by pre-incubation for 15 hr in substrate-free media. In substrate-repleted bladders, cordycepin inhibited uridine incorporation into RNA by 44% after exposure to the inhibitor for 30 min or longer (Table 5). In substrate-depleted bladders, pre-treatment with cordycepin for 30 min inhibited incorporation by 34% and pre-treatment for 60 min by 60%. These results indicate that the time needed to reach the steady-state level of inhibition may be prolonged in the substrate-depleted state but the magnitude of inhibition is as great or greater than in substrate-repleted bladders. On the assumption that the rate of inhibition is first-order, the half times of inhibition were 4.3 min in the substrate-repleted system and 19.1 min in the substrate-depleted system but the latter value is less reliable since it is based on only two points. These findings indicate that cordycepin is an effective inhibitor of RNA synthesis in the intact toad bladder epithelium.

Inasmuch as protein synthesis is regulated by the supply of nuclear RNA's (rRNA, mRNA and tRNA), control studies were needed to evaluate the specificity of inhibition of RNA synthesis during the period of initiation

Table 6. Effect of cordycepin on ^{3}H-leucine incorporation in toad bladder epithelium[a]

Conditions	Time of administration (min before ^{3}H-leucine)	Incorporation of ^{3}H-leucine[b] (% of control)	No. of pairs
A. Substrate-repleted	30	97 ± 15[c]	9
	60	105 ± 14	13
B. Substrate-depleted	30	93 ± 11	10
	60	99 ± 9	12

[a] The conditions for pre-incubation and of exposure to cordycepin (30 μg/ml) are described in footnote a of Table 5.
[b] Absolute rates of incorporation (during 15-min pulse period with 1 μC/ml ^{3}H-leucine, 35 C/mmole) in the control incubations were: 551,500 cpm/mg DNA, 30-min pretreatment (A); 671,400 cpm/mg DNA, 60-min pretreatment (A); 546,500 cpm/mg DNA 30-min pretreatment (B); 672,400 cpm/mg DNA 60-min pretreatment (B).
[c] Mean ± SE.

of mineralocorticoid action. The results summarized in Table 6 indicate that cordycepin had no significant effect on the incorporation of ^{3}H-leucine into epithelial protein for exposure times of 75 min (60 min of pretreatment and 15 min pulse of ^{3}H-leucine), regardless of the supply of exogenous substrate. The initial inhibitory action of cordycepin, therefore, was limited to that on transcription.

The physiological consequences of inhibition of RNA synthesis by cordycepin was assessed by the SCC technique of Ussing and Zerahn [33]. As shown in Fig. 3*A*, exposure of one of each pair of hemibladders to cordycepin depressed the basal rate of Na transport only to a small degree. The SCC_t/SCC_0 ratios at 3 hr were 0.88 ± 0.05 in the control and 0.72 ± 0.05 in the cordycepin-treated hemibladders. Moreover, the time-course and the magnitude of the response to vasopressin were identical in the control and cordycepin-treated hemibladders. Nonspecific toxicity with respect to Na transport, therefore, appears to be negligible. In contrast, simultaneous addition of cordycepin and aldosterone inhibited the response to the steroid almost completely (Fig. 3*B*). These results support earlier findings of the need for unimpaired RNA synthesis for the expression of the mineralocorticoid action of aldosterone [5–7, 10, 22, 34].

Earlier studies showed that in *Bufo marinus* obtained from Columbia, S.A., aldosterone elicited the typical increase in Na transport only in substrate-repleted bladders [6, 9, 11, 23]. In substrate-depleted bladders, aldosterone failed to evoke an increase in Na transport but the response was elicited without a significant latent period by the addition of substrates to the media three or more hours after aldosterone. This finding implied that

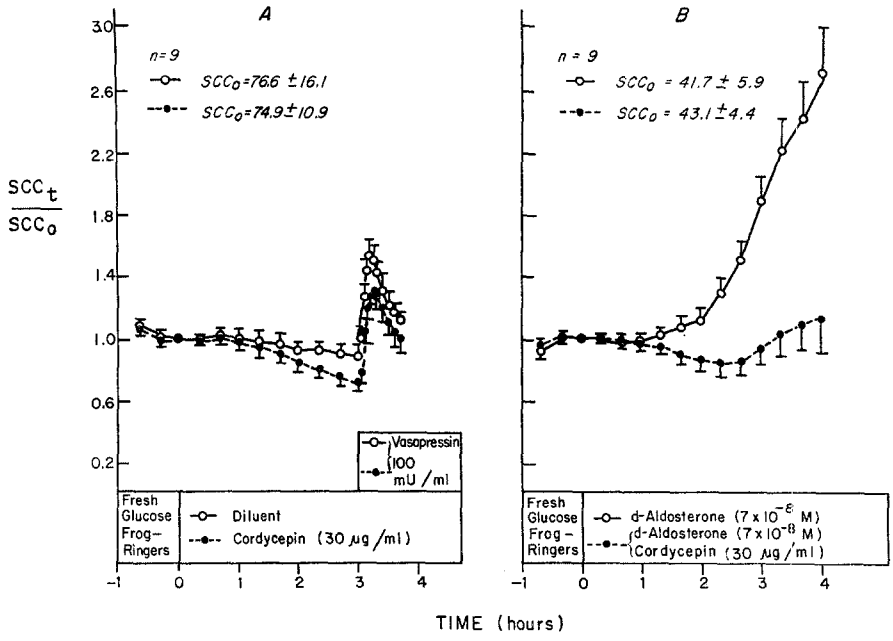

Fig. 3. Effect of cordycepin on aldosterone stimulation of Na transport in the toad bladder. Pairs of hemibladders were pre-incubated in steroid-free glucose (10 mM)-frog Ringer's for 15 hr. The media were then exchanged for fresh glucose (5 mM)-frog Ringer's solution. In the experiments shown in panel *A*, cordycepin (30 µg/ml) was added to the serosal and mucosal media of one of each pair (---•---) and vasopressin (100 mU/ml) was added to the serosal media of both hemibladders at 3 hr. In the experiments shown in panel *B*, aldosterone (7×10^{-8} M) was added to the serosal and mucosal media of both hemibladders and cordycepin (30 µg/ml) was added to the serosal and mucosal media of one of each pair (---•---). SCC_t/SCC_0 denotes the short-circuit current at time "*t*" divided by that at time zero. Each point and vertical line represents the mean ± SEM. *n* denotes the number of pairs of hemibladders. SCC_0 denotes the absolute short-circuit current at time zero and is given as the mean ± SEM

the proteins induced by aldosterone had been synthesized and their effects on Na transport were expressed promptly, when sufficient substrate was made available. Support for this inference was obtained with cycloheximide, an inhibitor of protein synthesis at the ribosomal level [9]. Aldosterone-induced proteins apparently accumulated silently in the substrate-depleted cells and exerted their effects on Na transport on repletion with substrate despite post-induction blockade of protein synthesis. These results suggest that post-induction inhibition of RNA synthesis with cordycepin should not prevent the immediate mineralocorticoid response to substrate in aldosterone-pretreated substrate-depleted bladders. Two sets of experiments were completed to test this inference. In the first set, pairs of hemibladders were incubated in substrate-free media for 15 hr and aldosterone (7×10^{-8} M)

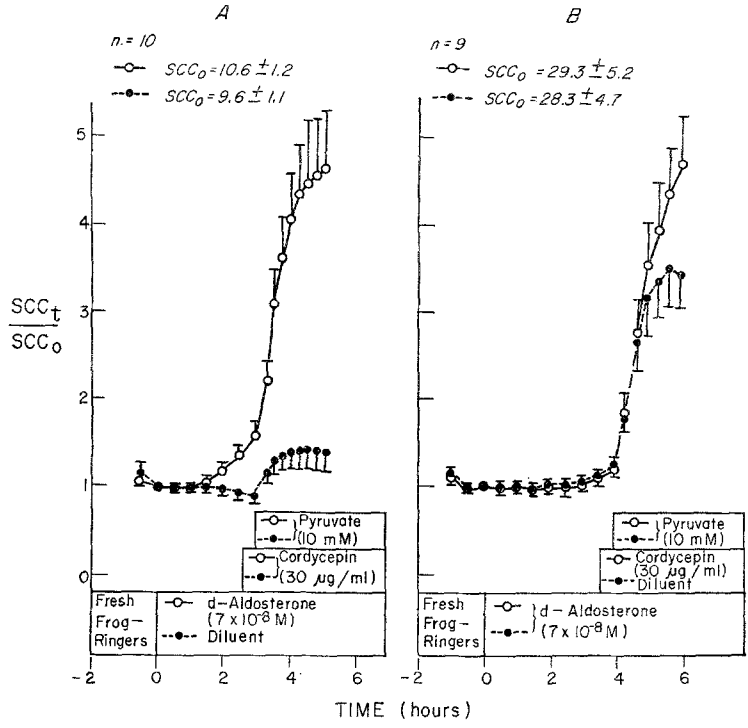

Fig. 4. Effect of cordycepin on the response to substrate in substrate-depleted, aldosterone-pretreated hemibladders. Pairs of hemibladders were pre-incubated in substrate-free frog-Ringer's solution for 15 hr and the media were then exchanged for fresh substrate-free frog-Ringer's solution. In the experiments shown in panel *A*, d-aldosterone (7×10^{-8} M) was added to the serosal and mucosal media of one hemibladder (—∘—) and at $t = 2$ hr 40 min cordycepin (30 µg/ml) was added to the serosal and mucosal media of both hemibladders. At $t = 3$ hr, Na pyruvate (10 mM) was added to the serosal and mucosal media of both hemibladders. In the experiments shown in panel *B*, d-aldosterone (7×10^{-8} M) was added to the media of both hemibladders at $t = 0$ and cordycepin (30 µg/ml) to the media of one hemibladder (---•---) at $t = 3$ hr 40 min. At $t = 4$ hr, Na pyruvate (10 mM) was added to the media of both hemibladders. The conventions used in this figure are defined in the legend of Fig. 3

was added to the serosal and mucosal media of one of each pair. Both hemibladders were treated with cordycepin (30 µg/ml) 2 hr and 40 min after addition of aldosterone (i.e., $t = 2$ hr 40 min), and with pyruvate (10 mM) at $t = 3$ hr. As shown in Fig. 4*A*, there was a striking enhancement of the response to pyruvate in the aldosterone-pretreated hemibladders with no appreciable latency, despite the presence of cordycepin in the medium. Thus, steroid-substrate synergism was preserved. As a further test of the validity of this conclusion, both members of pairs of hemibladders were given aldosterone and one of each pair was treated with cordycepin 3 hr later. The immediate response to pyruvate was prompt in both members

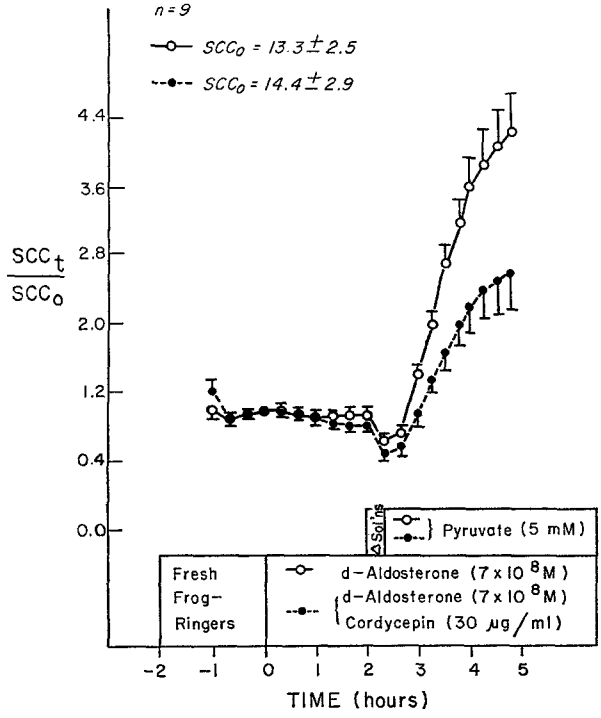

Fig. 5. Effect of cordycepin on aldosterone stimulation of Na transport in substrate-depleted bladders. Pairs of hemibladders were pre-incubated in substrate-free frog-Ringer's solution for 15 hr and the media were then exchanged for fresh substrate-free frog-Ringer's solution. At time zero, d-aldosterone (7×10^{-8} M) was added to the serosal and mucosal media of both hemibladders and cordycepin (30 µg/ml) to the media of one of each pair (---•---). At $t = 2$ hr, all the media were exchanged four times with fresh steroid-free and inhibitor-free frog-Ringer's solution. At $t = 2$ hr 40 min, Na pyruvate (5 mM) was added to the media of both hemibladders. The conventions used in this figure are defined in the legend of Fig. 3

of the pair and no significant difference was seen during the first hour of the substrate-mediated increase in SCC (Fig. 4*B*). The divergence in the response after the first hour presumably represents the decay of the pre-induced RNA and the aldosterone-induced protein.

To assess the specificity of the inhibitory action of cordycepin, additional sets of experiments were carried out in substrate-depleted hemibladders in which cordycepin was added simultaneously with aldosterone. The washout was introduced to avoid any short-lived toxic effects of cordycepin. The results in Fig. 5 indicate that steroid-substrate synergism was considerably reduced when the inhibitor was added at the same time as the steroid. The two curves differed significantly by 20 min after the addition of pyruvate to the media, suggesting that steroid-substrate synergism requires intact machinery of transcriptive processes. That this effect of cordycepin is not a

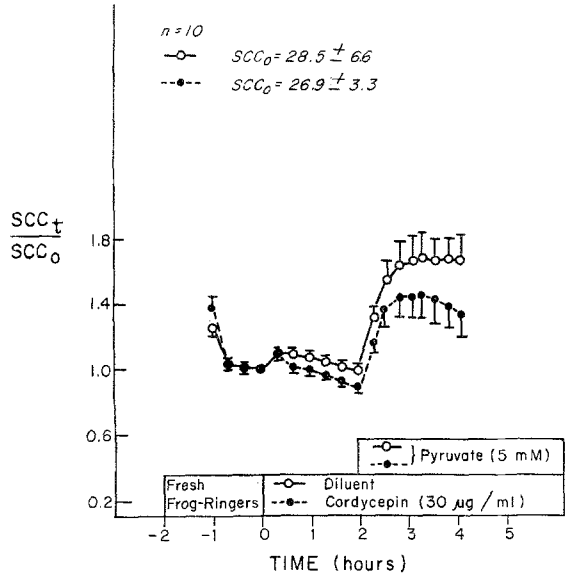

Fig. 6. Effect of cordycepin on pyruvate stimulation of Na transport in steroid-depleted bladders. Pairs of hemibladders were pre-incubated in substrate-free frog-Ringer's solution for 15 hr and the media were then exchanged for fresh substrate-free frog-Ringer's solution. At time zero, cordycepin (30 µg/ml) was added to the serosal and mucosal media of one of each pair (---•---). At $t=2$ hr, Na pyruvate (5 mM) was added to the media of both hemibladders. The conventions used in this figure are defined in the legend of Fig. 3

consequence of impaired energy metabolism is indicated in the experiments shown in Fig. 6. In steroid-depleted hemibladders exposed to cordycepin for 2 hr, the response to pyruvate is unimpaired for 2 hr after the addition of the substrate.

Discussion

A series of physiological and biochemical studies on anuran bladder and skin, and on the mammalian kidney suggest that stimulation of DNA-dependent RNA synthesis mediates the action of aldosterone on transepithelial Na transport. Incorporation of ^{3}H-uridine into total RNA has been found to be enhanced by aldosterone in the isolated urinary bladder of the toad, and in rat kidney *in vivo* and rat kidney homogenates *in vitro* [5, 10, 12, 22]. In these studies, actinomycin D inhibited both the physiological effect on Na transport and incorporation of uridine into RNA. If the basic premise is correct, two questions that remain to be answered are the nature of the increase in RNA synthesis, and the relationship between enhanced synthesis of specific classes of RNA and the increase in Na transport.

The present report provides evidence of the existence of distinct RNA polymerases in the rat kidney with the properties described previously for rat liver [1, 25, 26]. Polymerase I mediates nucleolar RNA synthesis and polymerase II regulates nucleoplasmic RNA synthesis and is inhibited by α-amanitin. To provide additional information on steroid induction, we made use of this toxin in studies on the rat kidney. In renal nuclear fractions, α-amanitin-insensitive RNA polymerase activity was increased by pretreatment with aldosterone, implying a shift in favor of nucleolar RNA polymerase activity. The time of appearance of this effect (1 hr) preceded and, therefore, correlated with the known changes in urinary Na and K excretion [10].

The possibility that the steroid-induced rise in the I:II ratio was a consequence of a selective release of polymerase II from the nucleus during the preparative procedure was negated by assay of the cytosol fraction (Table 4). In contrast to the results obtained with the nuclear fraction, administration of aldosterone had no effect on the I:II ratio of the cytosol fraction (*cf.* Tables 2 and 4). We have no explanation of why the ratio of polymerases in the cytosol fraction (presumably of nuclear origin) did not reflect the changes noted in the nuclear fraction. In any case, no evidence was obtained of a differential loss of polymerases to the cytosol sufficient to explain the shift in the activities in the nuclear fraction.

The finding of a significant increase in the nuclear RNA polymerase I:II ratio could be a result of either (1) an increase in nucleolar RNA synthesis alone, or (2) a selective decrease in nucleoplasmic RNA synthesis, or (3) a decline in both but less of a decline in nucleolar RNA synthesis, or (4) a rise in both with a proportionally greater increase in nucleolar RNA synthesis.

Earlier studies indicate that aldosterone-receptor complexes formed in the cytoplasm bind to renal chromatin with a high degree of specificity [19, 31] which is consistent with a nuclear site of action. In adrenalectomized rats, aldosterone increased incorporation of precursors into RNA [10, 12] and formation of RNA hybridizable to DNA [3] but did not increase template activity of isolated renal chromatin [32]. These results as well as the recent findings of Liew *et al.* [15] indicating that aldosterone enhances the activities of both Mg- and Mn-activated renal RNA polymerase support the interpretation of an increase in the activities of both I and II, with a proportionally greater increase in the activity of polymerase I. Aldosterone could enhance the activity of RNA polymerase in several ways, e.g., an increase in *de novo* synthesis, a decrease in the rate of degradation or direct activation. We failed to detect a shift in the I:II ratio on addition of aldosterone to the assay medium nor did aldosterone enhance Mg- or Mn-

activated RNA polymerase activity in renal nuclei treated with the steroid *in vitro* [15]. Thus, direct activation of the enzyme by the steroid seems to be an unlikely possibility.

The inhibitors α-amanitin and cordycepin were used in an attempt to assess the possible roles of nucleolar and nucleoplasm gene expression in the action of aldosterone on Na transport. The isolated toad bladder system was used for this purpose since it is a convenient model system. α-Amanitin proved to be inactive in the isolated toad bladder both with respect to effects on incorporation of ^{3}H-uridine into RNA and on basal or aldosterone-stimulated active Na transport. Similar negative results have been obtained in hepatoma cell (HTC) cultures (G. M. Tomkins, *personal communication*) and in intact muscle (J. Florini, *personal communication*), probably because of a failure of the toxin to penetrate the intact plasma membrane in these tissues. Cordycepin, however, inhibited both ^{3}H-uridine incorporation and the aldosterone-dependent increase in Na transport (Table 5 and Fig. 3). The specificity of cordycepin as an anti-mineralocorticoid is indicated by a number of findings: In substrate-enriched media, base-line Na transport was only minimally inhibited and the response to vasopressin was unimpaired (Fig. 3*A*). These results imply that the intrinsic components of the Na transport apparatus (e.g., Na+K-activated ATPase) and the increase in energy supply necessary to sustain an increase in Na transport are unimpaired by this toxin. This inference is reinforced by the lack of inhibition of either basal Na transport in substrate-deprived bladders or of the response to the addition of substrate (Figs. 3*A* and 6). Moreover, in substrate-depleted bladders pre-induced with aldosterone, substrate-steroid synergism was not depressed by addition of cordycepin 20 min before the substrate (Fig. 4). It appears, therefore, that cordycepin does not interfere with activation of Na transport by aldosterone-induced protein. In addition, the finding that cordycepin did not impair incorporation of ^{3}H-leucine into epithelial protein for more than an hour reinforces the inference of an action at steps preceding translation.

It has recently been observed in HeLa cell cultures that cordycepin blocked both ribosomal and polysomal messenger RNA synthesis but not HnRNA production [20, 29]. Darnell, Philipson, Wall and Adesnik [4] postulated that HnRNA is processed to mRNA by linkage with polyadenylic acid and subsequent transport to the cytoplasm. Cordycepin inhibition of mRNA formation was ascribed to interference with processing of HnRNA. Thus, the results we obtained with cordycepin in the toad bladder system could mean that either ribosomal RNA or mRNA synthesis is required for mineralocorticoid action. Obviously, both processes might act in concert.

In view of the findings of enhancement of nucleolar RNA polymerase activity by estradiol-17β in uterus [1, 24], testosterone in prostate [18], and cortisone in liver [1, 35], the aldosterone-dependent shift in renal polymerase I:II activity may represent an effect common to most if not all steroids. The increase in polymerase I activity could provide a means of increasing the capacity for protein synthesis but not involve the directed synthesis of specific proteins. The unique physiological properties of the steroids in particular target tissues, such as the action of aldosterone on active Na transport, would then be a consequence of induction of specific classes of mRNA.

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