Kinetics of RNA Synthesis in Toad Bladder Epithelium:

Action of Aldosterone during the Latent Period

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A B S T R A C T   Processing of RNA in the toad bladder
was analyzed by polyacrylamide-gel electrophoresis to
determine whether aldosterone causes any changes in the
1 hr before it potentiates transport of sodium ion. No
change was found in the quantity or in the specific ac-
tivity of bulk RNA labeled with uridine-5-^{3}H. In vivo
and in vitro with either uridine-5-^{3}H or with methio-
nine-(methyl)-^{3}H as precursors, processing of RNA was
extremely slow. Heterodisperse RNA was obvious af-
ter 30 min of continuous labeling, but labeling of the 40S
precursor of ribosomal RNA was not apparent for 60
min. Labeling of mature 28S and 18S RNA first became
apparent after 8 hr. ⌐7S RNA was the principal fast-
migrating species labeled at 30 min, and 4S RNA was
not heavily labeled until 1 hr. Aldosterone (5×10^{-7}
mole/liter) produced no changes. If care were not taken
to inhibit metabolism of native bacteria colonizing the
bladder, bacterial RNA of high specific activity predomi-
nated. We conclude that RNA metabolism in the toad
bladder is extraordinarily slow, that a major acceleration
of *de novo* synthesis in response to physiologic doses of
aldosterone was not demonstrable, and that some reports
to the contrary may have been influenced by artifacts
from bacterial RNA metabolism. Earlier evidence for
obligatory alterations in RNA metabolism during the
latent period is not strong.

INTRODUCTION

Application of aldosterone to the toad bladder or to the
mammalian nephron fosters subtle physiologic events
responsible for increased transport of Na⁺ in about an
hour (1–3). Although considerable support has arisen
for the proposal that synthesis of new RNA and protein
are obligatory during the latent period (4–8), much of
the evidence on which the contention is based is indirect.

That is, inferences have been drawn from experiments
such as those showing increases in the specific activity
of RNA or in radioautographic localization of acid-
precipitable material assumed to be RNA, and those
showing parallel depression in radioactive labeling and
in physiological response after treatment with actinomy-
cin. To our knowledge an increase in RNA synthesis
per se during the latent period or in labeling of a specific
class of RNA unequivocally derived from the host organ
has not been reported.

The studies to be described deal with three main
points: (*a*) the leisurely kinetics of RNA synthesis in
toad bladder as analyzed by gel electrophoresis; (*b*)
artifacts produced by metabolic products of bacteria con-
taminating preparations of toad bladder in vitro; and
(*c*) the lack of effect of aldosterone, even in somewhat
larger than physiologic doses, on RNA metabolism in
toad bladder mucosa during the latent period.

METHODS

Tissue and conditions of labeling. Hemibladders were
removed from specimens of *Bufo marinus* (National Re-
agents, Inc., Bridgeport, Conn.) that had been partially
immersed in saline solution for 48 hr and prepared for use
by pithing and exsanguination. For in vitro studies, unless
otherwise specified, one hemibladder and its paired control
were preincubated at room temperature for 4 hr with peni-
cillin (100 μg/ml) and streptomycin (50 μg/ml) in 5 ml
of frog sodium-Ringer solution (113.5 mM NaCl, 3.5 mM
KCl, 2.4 mM NaHCO₃, 0.9 mM CaCl₂, pH 7.9, 220 millios-
mols/kg). Subsequent labeling was with uridine-5-^{3}H (50
μCi/ml; specific activity 2 Ci/mmole, New England Nu-
clear Corp.) or with L-methionine-(methyl)-^{3}H (100 μCi/
ml), with penicillin and streptomycin. When appropriate,
D-aldosterone was added concurrently with the radioactive
label for an incubation period of 1 hr at a concentration
of 5×10^{-7} mole/liter.

For in vivo studies, hemibladders were removed at in-
tervals after injection into the ventral lymphatic sac of 500
μCi of uridine-5-^{3}H.

Specific activity of bulk RNA. Epithelium was scraped
from hemibladders washed in 0.25 M sucrose containing 5%

citric acid (w/v) and dispersed in sucrose-citrate buffer with a Potter-Elvehjem homogenizer. Total RNA was estimated by a modification of the methods of Scott, Fraccastoro, and Taft (9) and of Munro and Fleck (10) based on $A_{290nm}:A_{260nm}$ of a 1 N NaOH hydrolysate (1 hr at 37°C) of a washed cold 0.3 N perchloric acid precipitate. Recovery of RNA was linear at all concentrations.

Radioactivity was counted in 0.1 ml aliquots in 10 ml of Aquafluor (11) at an efficiency of 14%; no correction for quenching was made, as it was uniform in all samples.

Electrophoretic analysis of RNA. Epithelium derived from a single hemibladder was homogenized in 3 ml of buffer (0.01 M Tris, pH 7.4 at 20°C, 0.01 M NaCl, 0.0015 M MgCl₂) containing 20 μg/ml polyvinylsulfate (PVS) (Eastman Organic Chemicals, Rochester, N. Y.). Of the homogenate, 2 ml was extracted with phenol(Mallinckrodt 88%)–sodium dodecyl sulfate–chloroform–isoamyl alcohol in the presence of PVS (20 μg/ml) and 0.01 M EDTA by a modification of Penman's (12) method that has been demonstrated to preserve labile RNA precursors (13) even in an organ-like kidney, which is rich in nucleases. After extraction there was added to the aqueous phase 0.5 volume of a concentrated buffer containing 0.05 M Tris, pH 7.4 at 20°C, 2.5 M NaCl, and 0.25 M MgCl₂. RNA was precipitated with 2 volumes of ethanol at −20°C and resuspended in a 10-fold concentrate of homogenization buffer before digestion with electrophoretically pure DNase (200 μg, Worthington Biochemical Corp., Freehold, N. J.) for 2 min at 37°C. Precipitated RNA was dissolved in homogenization buffer and again extracted with phenol.

Methods of analysis of the purified RNA on 7-cm gels of 2.65% polyacrylamide (14) have been described (13, 15). Pertinent details are given in the legends to the figures.

RESULTS

Specific activity of bulk RNA. Table I shows that there was no difference between the amount of RNA extracted nor its specific activity in hemibladders treated with aldosterone compared with controls.

Kinetics of RNA processing in vitro. Under the conditions of electrophoresis shown in Fig. 1, the absorbance pattern shows major peaks of 28S and 18S RNA, so
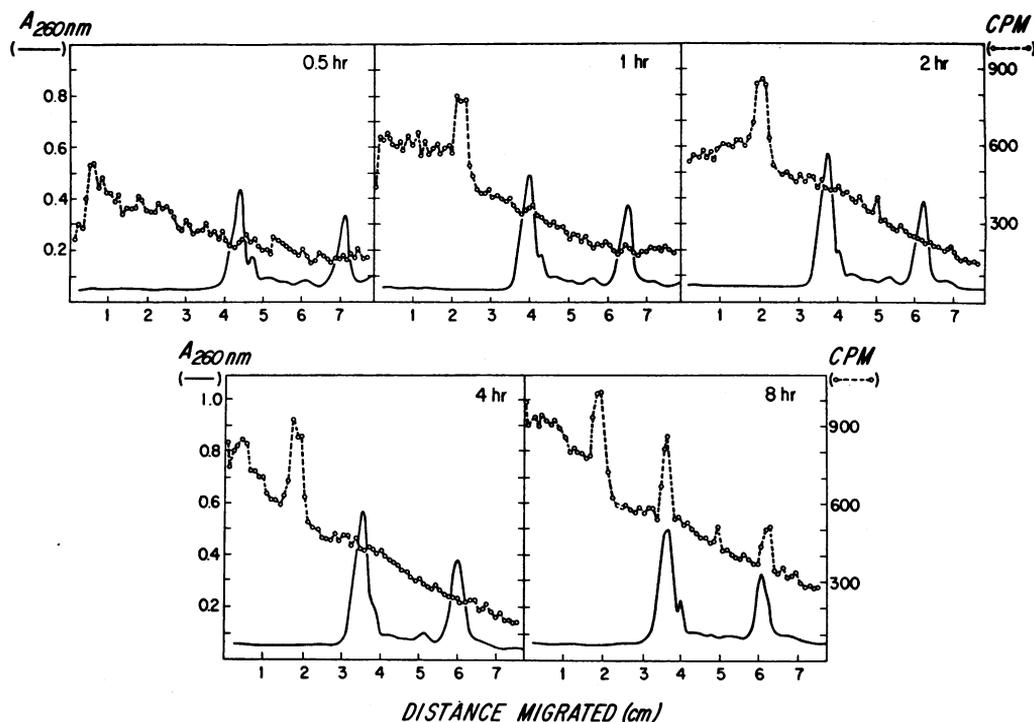

FIGURE 1 Kinetics of labeling in slower-migrating RNA. Hemibladders were labeled continuously with uridine-³H in vitro and processed as described in the text. RNA was run on 7-cm 2.65% polyacrylamide gels (5 ma/gel, 10 v/cm) for 5 hr, scanned at 260 nm, and analyzed for radioactivity.
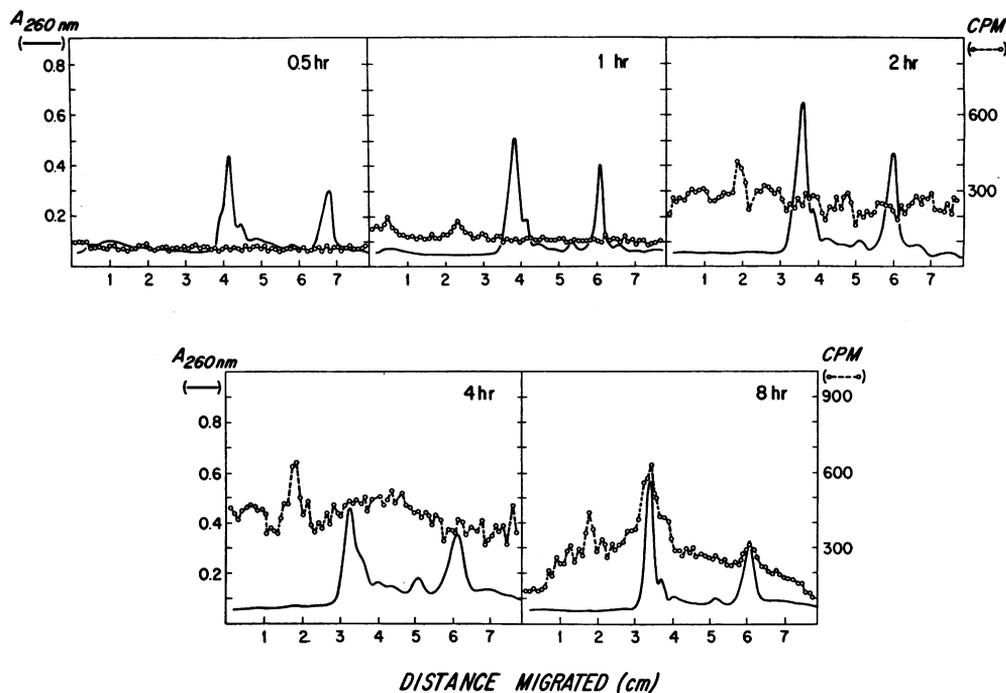
FIGURE 2 Kinetics of labeling of methylated RNA. Details were as in Fig. 1 except that the precursor was methionine-(methyl)-³H.

designated after coelectrophoresis with mouse kidney RNA (13). An inconstant minor peak was seen at the region corresponding to approximately 40S. On the faster-migrating side of the larger 28S peak there was a constant, much smaller "shoulder;" several minor areas of increased absorbance were consistently present between 28S and 18S. Identical characteristics were noted in heart and kidney from these animals.

30 min of continuous labeling with uridine-³H resulted in a heterodisperse distribution of radioactivity with a tendency toward greater activity near the origin. Preponderance of radioactivity in the region migrating slower than 28S became more pronounced after 60 min, but in addition there then became apparent a solitary slowly migrating peak. By calculation from the logarithmic relation between molecular weight and electrophoretic mobility (13, 16, 17) as well as from comparison with studies of RNA from *Triturus* and from *Xenopus* (18–23), the peak is designated 40S; the major localized radioactivity remained in this species for at least 4 hr. Labeling of 28S and 18S RNA first became apparent at 8 hr. Increasingly obvious labeling of heterodisperse species migrating slower than 28S was noted in direct relation to time from 1 to 8 hr.

In order to distingush the methylated precursors of ribosomal RNA (rRNA) (24–26) from the heterodisperse background, labeling with methionine-(methyl)-³H was studied. Fig. 2 shows absence of labeling in the gel

after 30 min and a small peak near the origin and at the 40S region after 1 hr. Distinct concentration of radioactivity was present at 40S in 2 hr and 4 hr, with, in addition, a broad peak between about 36S and 18S at 4 hr. At 8 hr the major peak was at 28S, with secondary peaks at 40S and 18S; a shoulder in the region of 36S–32S was also noted. Although the heterodisperse background was high after 2 hr, the gradual predominance of slowly migrating heterodisperse species after labeling with uridine was not identified when methionine was the precursor.

Kinetics of RNA synthesis in vivo (*Fig. 3*). The pattern of labeling with uridine was much the same in vivo as in vitro, with the exception that the heterodisperse labeling was far less intense and showed far less propensity for the region migrating slower than 28S. A small peak of radioactivity in the 40S region at 1 hr was coincident with the increased absorbance there. The specific activity of this region increased for at least 4 hr. Labeling of 28S and 18S RNA began between 4 and 8 hr and appeared to increase slowly from the estimated time of onset until 12 hr.

Labeling of rapidly migrating RNA in vitro. Short periods of electrophoresis were used to display species of RNA migrating faster than 18S RNA. On the absorbance pattern the major peak at approximately 4S constantly had a shoulder on its trailing side, which we assumed represented 5S RNA (Fig. 4). After 30 min,

most radioactive label was heterodisperse in the slower-migrating part of the gel. The principal area of discrete incorporation corresponded to about 7S. After 1 hr the heavy heterodisperse label diminished considerably, and the discrete peaks were at ∼ 7S and 4S.

Effect of aldosterone. Since the latent period for the effect of aldosterone on Na$^+$ transport is about an hour, differences in labeling were sought only at this time and earlier. In six experiments no differences in the labeling characteristics of the larger RNA species were seen at 1 hr, as exemplified by Figs. 5 A and 5 B. There were likewise no qualitative differences in labeling of the smaller species of RNA (Figs. 5 C and 5 D).

Artifacts from bacterial contamination. Bacteriological examination of urine and of the bladder revealed *Klebsiella* species, *B. proteus,* and other Gram-negative bacilli, all of which were sensitive to streptomycin, and some of which were sensitive to penicillin.

In experiments in which hemibladders not preincubated and maintained in penicillin and streptomycin were labeled with uridine-³H for periods to 2 hr and compared with hemibladders treated with antibiotics as described above, the absorbance patterns of the species of stable RNA were identical. Fig. 6 A and B demonstrate, however, a major difference in characteristics of labeling. Although both hemibladders show active incorporation into 40S RNA, the hemibladder without antibiotics also has intense labeling at positions com-patible with 23S and 16S bacterial RNA. Fig. 6 C and D show an increase in labeling in the region around 4S RNA, likewise, when antibiotics are omitted. At the end of an experiment the colony count in the incubation medium without antibiotics was about 500,000/ml and in the media with antibiotics, 1000/ml. Culture of the antibiotic-treated bladder was sterile.

DISCUSSION

Processing of RNA. Although appreciable amounts of the 40S RNA precursor are processed to mature rRNA within an hour in the developing embryo of *Xenopus laevis* (21) and in cells cultured from *Xenopus* (22, 23) when maintained at 37°C, the kinetics of processing in an adult poikilotherm maintained at ambient temperatures might, predictably, be slower. For example, the rate of RNA synthesis in cultured chick-retinal cells is decreased by about 70–75% after a transition from 37° to 27°C and little processing of ribosomal-precursor RNA is found (27). Results from the present experiments indicate a processing time of at least 4 hr for label to appear in mature 28S rRNA in intact *Bufo marinus.* Part of the incorporation may actually be into the 32S immediate precursor of 28S RNA (28), which cannot be resolved under the conditions of these experiments. By comparison, 32S RNA becomes labeled within 8–10 min in nuclei of rat liver (29) and of mouse kidney (13). 18S RNA in liver and kidney becomes
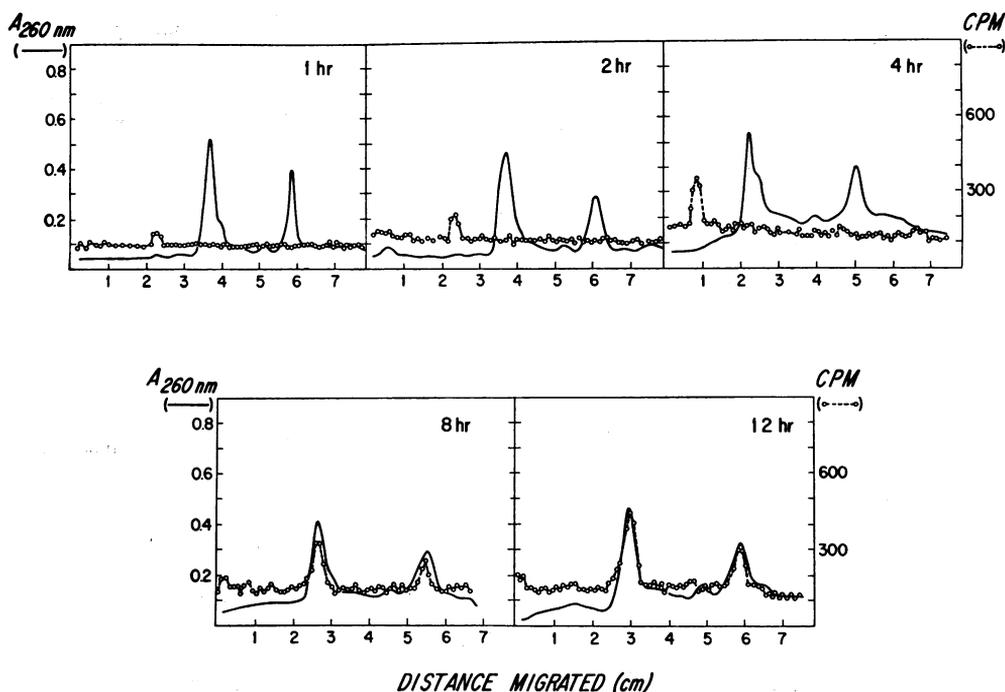

FIGURE 3 Kinetics of labeling in vivo. The technique was identical to that in Fig. 1 except that the uridine-³H was given parenterally.

labeled within 8–30 min, (13, 29, 30) and 28S RNA within 20–40 min. Processing time in vitro for labeling of tRNA is also prolonged in *Bufo* inasmuch as label does not appear at the 4S region until over 30 min, contrasted with less than 10 min in adult mouse kidney (30). Whether this label is in the terminal CCA triplet or in tRNA itself cannot be distinguished.

Since the rate of processing of toad bladder RNA is slow, conditions for the detection of rapidly labeled species of RNA newly synthesized in response to aldosterone should be optimal. No such species were found, although it is possible that even under these circumstances a quantity of RNA below the level of detection was made.

Methodology. The validity of the methods on which this conclusion is based may be inferred from their suitability when used to study RNA synthesis in *Xenopus* embryos (20) and in mouse kidney (13), an organ rich in nucleases (31, 32). The sensitivity of the methods permits detection of an estimated 200–600 molecules of 45S RNA turning over in 3–7 min per mouse kidney nucleus (13), but its sensitivity for the more slowly turning over RNA of toad bladder has not yet been estimated.

Extractions at ambient temperatures avoid artifacts of dimerization produced by extractions above 55°C (33, 34). That the techniques separated the RNA in essentially undegraded form may be concluded from the clean display of large amounts of the labile precursors of rRNA and of the heterogenous RNA, both of which are especially sensitive to degradation.

These arguments for the integrity of the RNA extracted from the toad bladder thus suggest that both the shoulder detectable by absorption measurements on the

leading side of the 28S RNA peak and the rapidly labeled peak at about the 7S position are valid representations of species of RNA actually existing in vivo. The identity of the peak on the leading side of 28S is obscure. The rapidly labeled peak at ⁓7S is likely similar to one of the low molecular weight monodisperse varieties of RNA described in HeLa cells (35) and ascites tumors (36), and the slow processing in toad bladder may now enable its precursor-product relations to be assessed more easily.

Evaluation of other results. Reconciliation of these data with conclusions of an enhancement of RNA synthesis in either toad bladder or in rat kidney as a consequence of treatment with aldosterone may be considered under three headings: (a) experiments in which nonphysiological doses of aldosterone were used; (b) experiments in which bacterial metabolism was not controlled; and (c) experiments in which evidence of RNA synthesis was indirect. Comparisons will be limited for the most part to events during the latent period of approximately 1 hr between the application of aldosterone to the bladder and the demonstration of increased Na^+ transport. The reason for this restriction is primarily that stimulation of sodium transport is associated with secondary changes, such as an increase in oxidative metabolism, which may themselves produce changes in RNA metabolism (1). In addition, a stimulation of nucleic acid and protein metabolism by cations of the alkali metals has been described (37–39).

Thus, the demonstration of increased incorporation of uridine-³H into toad bladder RNA at various times following doses of aldosterone 10 times (5), 50 times (8), and 150 times (7) the dose (7×10^{-8} mole/liter) producing a maximal physiologic response in Na^+ transport
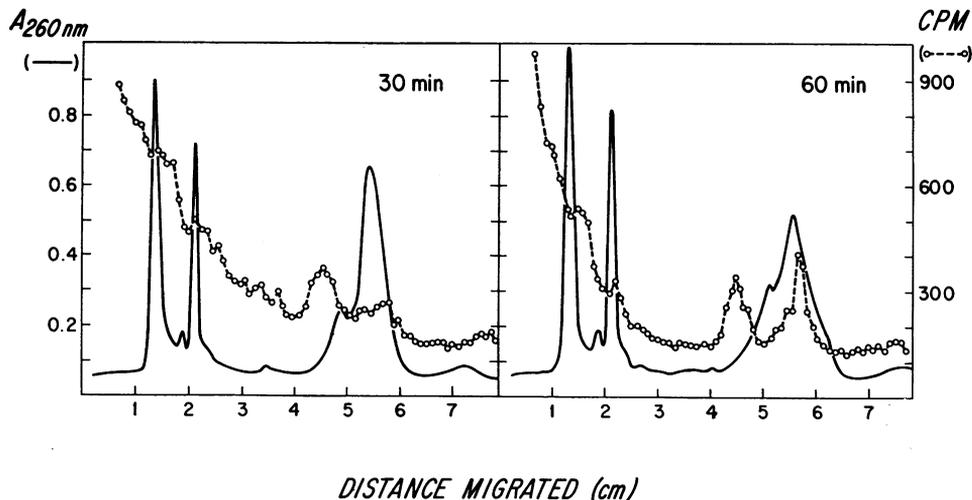

FIGURE 4 Labeling in rapidly migrating RNA. The precursor was uridine-³H in vitro and electrophoresis was for 1.5 hr.

(40) is not necessarily applicable to the natural situation. A similar reservation concerns the increased labeling of renal RNA with orotate-^{14}C reported 30 min after injection of aldosterone in amounts 10 or 20 times the dose that produces a maximal response, especially as the subjects were adrenalectomized rats not maintained on glucocorticoid replacement (41). In rats given more nearly physiologic doses of aldosterone, increased incorporation of orotate-^{14}C was not significant until more than 60 or 90 min later (6, 42).

If precautions are not taken to eliminate or to identify species of nascent RNA produced by bacteria (7, 8) the results may be confused by incorporation of precursors into bacterial RNA. Copious Gram-negative bacteria colonizing the toad bladder were responsible for the vast majority of the rapidly labeled RNA shown in Fig. 6, as in the perfused rat heart (43). In our experiments only when the bacteria were inhibited by appropriate antibiotics was the processing of RNA in the bladder itself identifiable. Similar processing in vivo without antibiotics proves that the observations were not a result of damage to the bladder from penicillin or streptomycin.

Barring a decrease in RNA catabolism, the only direct way of ascertaining an increase in RNA synthesis is to determine that more RNA is present. Although labeling experiments substitute for direct quantitative estimation in most quickly changing situations of physiologic interest, appreciation should be clear that increased labeling is not the same as increased synthesis. The rate of labeling is determined by the specific activity of the immediate precursor pools and by the activities of the enzymes involved in synthesis. Thus, the increased specific activity of the uridine triphosphate (UTP) pool is probably responsible for part of the enhanced labeling of RNA in regenerating liver immediately after partial hepatectomy (44) and for the labeling of RNA in the kidney of the castrate mouse (45); in phytohemagglutinin-stimulated lymphocytes (46, 47) and in isoproterenol-stimulated mouse salivary glands (48) newly induced uridine-kinase activity selectively increases the specific activities of the uridine nucleotide pools. The specific activities of UTP pools were not measured in the experiments reported in this paper; if, however, aldosterone had influenced the pool, like cortisone (49) it
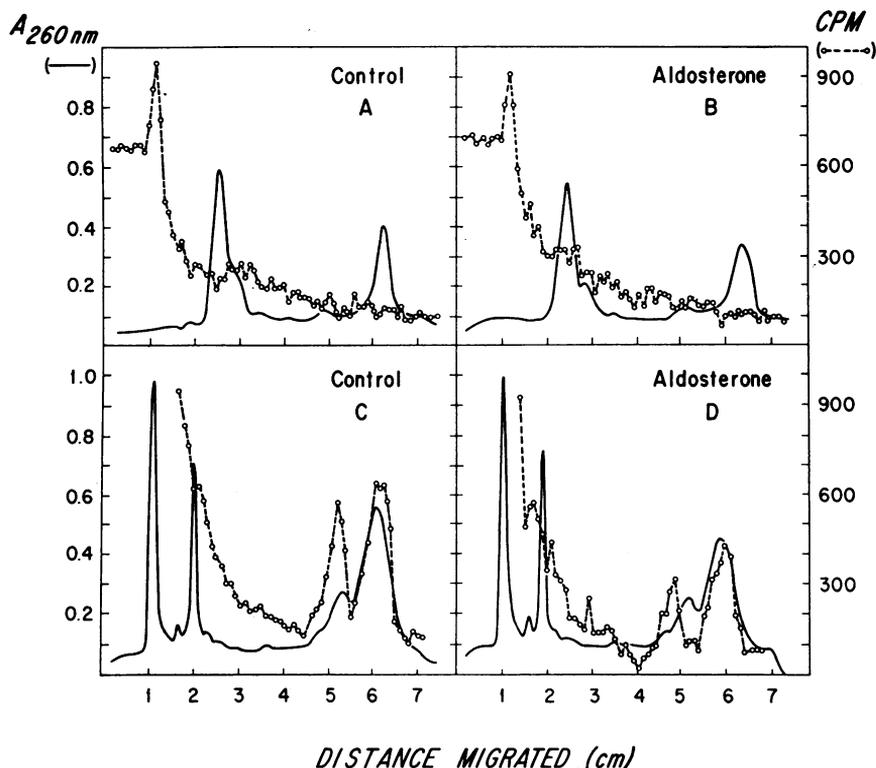

FIGURE 5 Labeling after treatment with aldosterone (5×10^{-7} mole/liter). Continuous labeling was with uridine-^{3}H in vitro for 1 hr. Slower-migrating species (electrophoresis for 5 hr) are shown without (A) and with (B) aldosterone added. Faster-migrating species (electrophoresis for 1.5 hr) are shown without (C) and with (D) aldosterone.
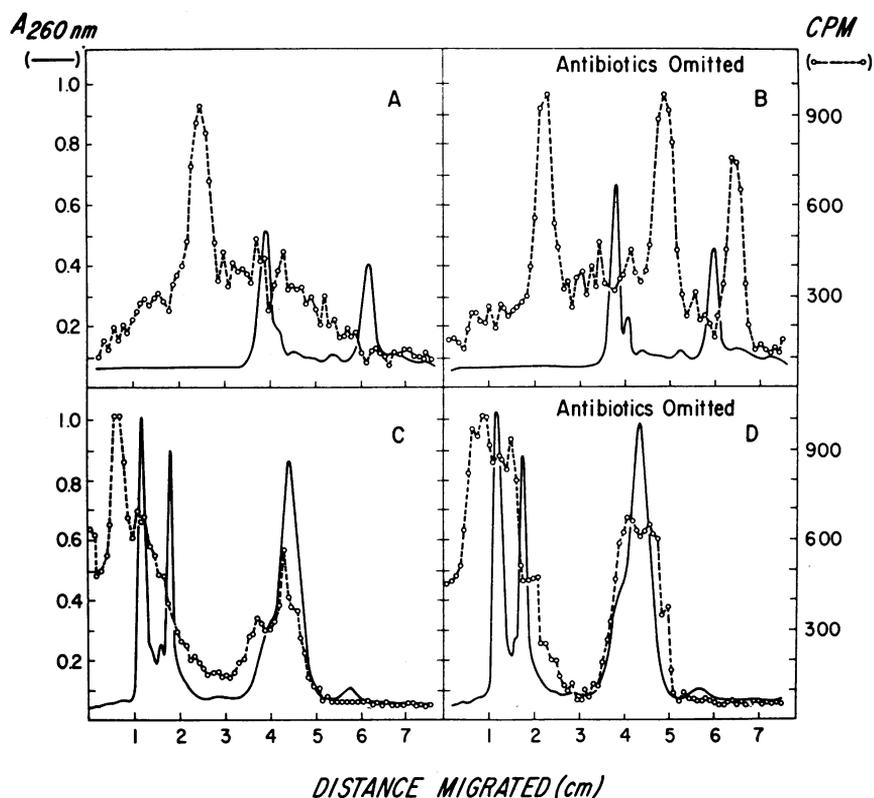
FIGURE 6 Effect of resident bacteria on labeling patterns. Labeling was for 2 hr in vitro with uridine-^{3}H. Slower-migrating species (electrophoresis for 5 hr) in bladders treated with penicillin and streptomycin (A) are compared with species from bladders not so treated (B). Faster-migrating species (electrophoresis for 1.5 hr) are also shown with (C) and without (D) penicillin and streptomycin.

would seem more likely to have increased the specific activity of the pool and hence to have produced an effect contrary to the results reported, rather than to have caused them.

Inferences about RNA synthesis from radioautographic studies are subject not only to these hazards, but, unless carefully controlled, also to the possibility that the localized radioactive label may not be in RNA (50). Deductions about RNA and protein metabolism from kinetics after administration of actinomycin and other inhibitors (1, 51) may be confounded by other effects of these drugs unrelated to the central problem (52–54). For these reasons, experiments that rely heavily on such indirect methods have not been regarded as final proof.

We conclude that direct proof of an action of physiologic concentrations of aldosterone in causing increased RNA synthesis during the latent period has not yet been published. Nonetheless, despite the difficulty in making deductions about RNA and protein metabolism from kinetics after administration of inhibitors, since experiments with actinomycin D suggest that an effect of aldosterone may be manifested in RNA synthesis, it is not

impossible that aldosterone could have an action on RNA synthesis that is small and below the sensitivity of current techniques of measurement.

ACKNOWLEDGMENTS

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