Table 1. Effects of GTP, Mg, and EGTA on microtubule polymerization. Tubulin was partially purified by ammonium sulfate precipitation. All samples were in 0.1*M* MES buffer at *p*H 6.5. The final concentrations were: GTP, 1 m*M*; EGTA, 1 m*M*; $MgSO_4$, 0.5 m*M*; and $CaCl_2$, 1.5 m*M*. The protein concentration was about 2.5 mg/ml.

Additions	Polymerization
GTP	No
$MgSO_4$	No
EGTA	No
GTP + $MgSO_4$	Slight
GTP + EGTA	No
GTP + $MgSO_4$ + EGTA	Yes
ATP + $MgSO_4$ + EGTA	Yes
GTP + $MgSO_4$ + EGTA + $CaCl_2$	No

of ADA which was different from the other buffers tested was its ability to bind calcium and magnesium ions, an investigation was made of the effects of these ions on polymerization. The addition of 0.01*M* $MgCl_2$ to 0.1*M* ADA appeared to give slightly better polymerization, while the addition of 0.01*M* $CaCl_2$ nearly eliminated polymerization. The addition of Mg and Ca together also prevented polymerization. Since the estimated concentration of free Ca produced by 0.01*M* Ca in 0.1*M* ADA was only about 6×10^{-6} (based upon a stability constant of 10^4), a very low concentration of Ca was sufficient to block polymerization.

To investigate the role of Ca and Mg further the effects of the chelators ethylenediaminetetraacetate (EDTA) and ethylenebis(oxyethylenenitrilo) tetraacetate (EGTA) on microtubule polymerization were also determined. The EDTA alone, at 0.05*M*, did not allow polymerization to occur, but polymerization was observed if 0.01*M* $MgCl_2$ was added to the EDTA solution. Also EGTA alone, at 0.05*M*, allowed polymerization to occur to roughly the same extent as did ADA alone, or EDTA plus Mg.

On the basis of the results obtained with the crude, unfractionated homogenate, the ability of partially purified tubulin to polymerize in various mediums was determined. As shown in Table 1, microtubule formation could be obtained in a medium containing a nucleotide triphosphate [GTP and adenosine triphosphate (ATP) were equally effective], $MgSO_4$, and EGTA. None of these agents were effective alone, nor were any pair of them (the exception was GTP with $MgSO_4$ in which a few relatively short microtubules were observed). Polymerization could also be obtained with a more highly purified tubulin (Fig. 1), and again both GTP and ATP were effective in promoting polymerization.

Polymerization proceeds rapidly, appearing to be nearly complete within about 30 minutes at 35°C. Incubation at 0°C, after preincubation at 35°C for an hour, greatly decreases the amount of polymer, as does incubation in the presence of 0.1 m*M* colchicine. The ultrastructure of the repolymerized tubules appears normal, with the stain either penetrating the core or staining only the surface (Fig. 2). A noteworthy feature is the frequent presence of "decorated" tubules with large numbers of heterogeneous projections along the length of the tubule. The presence of these projections appears to depend upon the way the microtubule is stained and is probably a staining artifact, but could also represent a true property of the microtubule. It is also important that microtubule formation is spontaneous, and requires the addition of no nucleating centers. The possibility that some tubules survive the process of purification cannot be absolutely ruled out, but has to be considered extremely unlikely. No tubules are seen prior to the induction of polymerization.

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1 June 1972

RNA Metabolism in Tracheal Epithelium: Alteration in Hamsters Deficient in Vitamin A

Abstract. *The electrophoretic pattern of RNA molecules that are synthesized in vitro in tracheal epithelium from hamsters deficient in vitamin A differs from that of RNA synthesized in normal, pair-fed control hamsters. There is less RNA of low electrophoretic mobility in the epithelial cells deficient in vitamin A. This alteration is reversed after the deficient animals have been treated with vitamin A.*

The state of differentiation of the tracheal epithelium is determined by vitamin A (*1*, *2*). In vitamin A deficiency, the normal columnar tracheal epithelium, composed of ciliated or mucus-producing cells, which act as a barrier to infectious and other noxious agents (see cover), is replaced focally by squamous cells, which are neither ciliated nor mucus-producing. The extent of replacement by squamous cells (squamous metaplasia) generally parallels the severity and duration of vitamin A deficiency. After feeding vitamin A to previously deficient animals, new ciliated and mucus-producing cells repopulate the epithelium. We report here studies on the effect of vitamin A deficiency on RNA metabolism in this tissue. These studies show that the electrophoretic pattern of RNA molecules in tracheal epithelium from vitamin A–deficient hamsters differs from the pattern in normal, pair-fed controls. The percentage of newly synthesized, high-molecular-weight RNA species with low electrophoretic mobility is reduced in tracheal epithelium deficient in vitamin A.

The effect of vitamin A on RNA metabolism in the liver and intestine of the rat has been studied. Decreased isotopic labeling of RNA in the intestine of vitamin A–deficient rats has been shown to result from decreased synthesis, rather than from differences of entry of radioactively labeled uridine into intracellular uridine pools (*3*); a decrease in total cellular transfer RNA was also observed. Administration of vitamin A stimulated incorporation of [^{3}H]uridine (*4*) or [^{3}H]orotic acid (*5*) into RNA in the intestine of vitamin A–deficient rats. Studies of the incorporation of [^{3}H]uridine into nuclear and cytoplasmic fractions of the livers of vitamin A–deficient rats demonstrated that incorporation of [^{3}H]uridine into nuclear RNA was reduced by approximately 50 percent when compared to pair-fed controls (*6*). However, in a number of the above-mentioned experiments (*4–6*), the effects of vitamin A on the incorporation of uridine or

orotic acid into the RNA precursor pools were not measured.

In our studies, vitamin A–deficient and normal, pair-fed, littermate control hamsters were fed a vitamin A–deficient diet (*2*), modified for hamsters, starting at 12 days of age. Control hamsters received 200 μg of *all-trans*-retinyl acetate (Eastman) in 0.1 ml of cottonseed oil, intragastrically, each week. Deficient hamsters received only the cottonseed oil. When deficient hamsters had lost 4 to 8 g from their peak body weight (after 6 to 8 weeks), they were either killed or treated with 1 mg of retinyl acetate in 0.05 ml of propylene glycol, intraperitoneally, together with 1 mg of retinyl acetate in 0.2 ml of cottonseed oil, intragastrically. Hamsters that had been treated once with vitamin A were killed 1, 2, 7, and

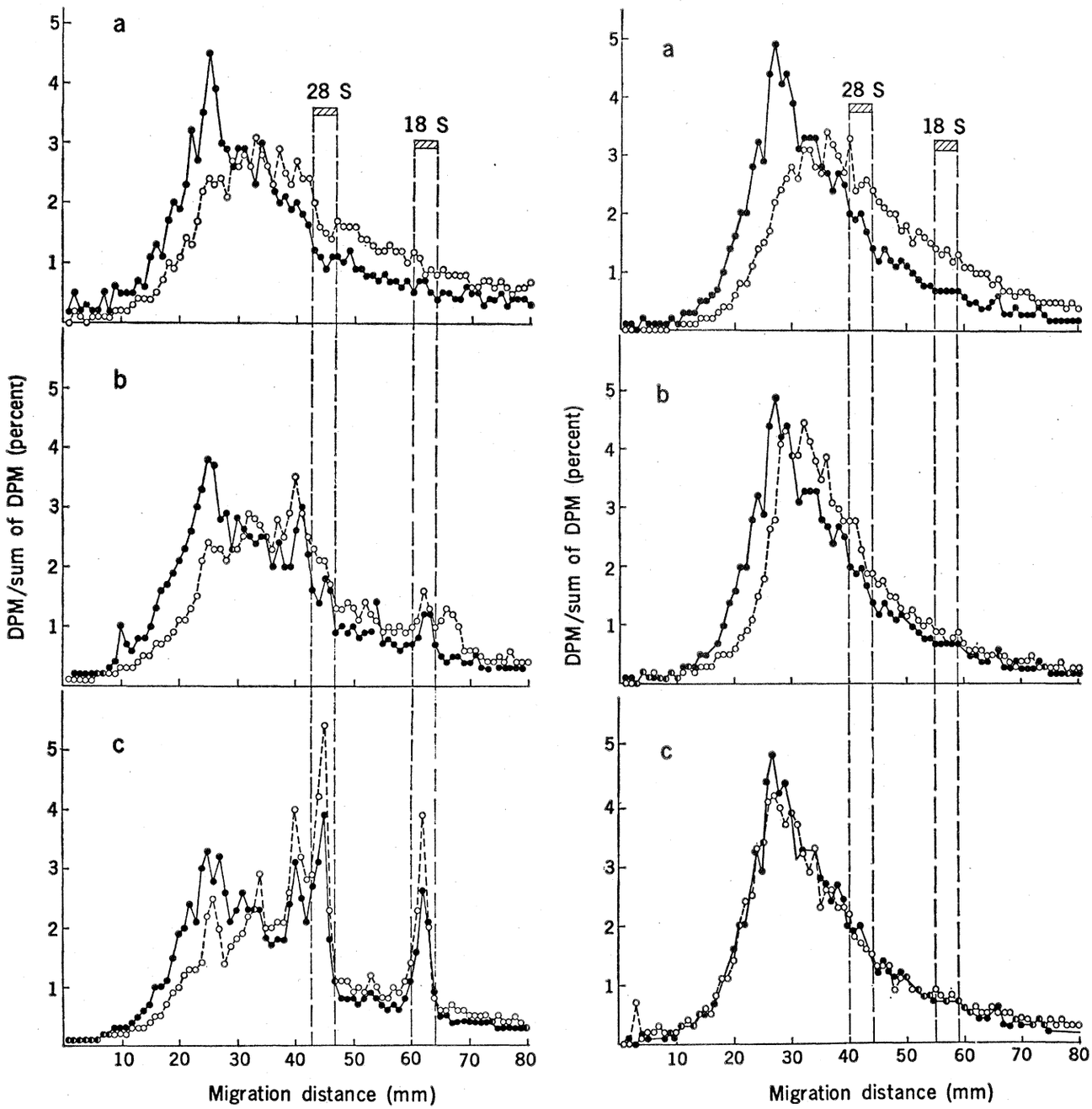

Fig. 1 (left). Electrophoretic pattern of RNA synthesized in vitro in tracheal epithelium from normal and vitamin A–deficient hamsters. ●—●, Preparations from normal animals; ○---○, preparations from vitamin A–deficient animals. (a) RNA prepared after 30-minute pulse with [5-^{3}H]uridine; (b) RNA prepared after 30-minute pulse with [5-^{3}H]uridine followed by 30-minute incubation in unlabeled medium; (c) RNA prepared after 30-minute pulse with [5-^{3}H]uridine followed by 90-minute incubation in unlabeled medium. The 28*S* marker in all the above experiments migrated 45 mm. Fig. 2 (right). Restoration of electrophoretic pattern of RNA synthesized in vitro in tracheal epithelium of vitamin A–deficient hamsters, after treatment with vitamin A. In all cases, RNA was prepared after incubation for 30 minutes with [5-^{3}H]uridine. (a) RNA from vitamin A–deficient hamsters before treatment with vitamin A; (b) RNA from vitamin A–deficient hamsters, 1 week after treatment with vitamin A; (c) RNA from vitamin A–deficient hamsters, 2 weeks after treatment with vitamin A. ●—●, Preparation from normal control animals; ○---○, preparations from vitamin A–deficient animals, either before or after treatment with vitamin A. The 28*S* marker in all of these experiments migrated 42 mm.

14 days after treatment. In these experiments, normal, pair-fed hamsters were treated in the same way as the deficient animals were. Tracheas were excised from hamsters overdosed with sodium methohexital; the isolated tracheas were placed in 5 ml of Leibovitz L-15 medium (*7*) containing [5-^{3}H]uridine (20 μc/ml; 26 c/mmole) and incubated, with shaking, in a water bath at 37°C.

Tracheal epithelium maintained its normal ultrastructure and incorporated uridine into RNA linearly for as long as 4 hours (*8*). After incubation, the tracheal epithelium was scraped from supporting tissues, and whole-cell RNA was prepared with sodium dodecyl sulfate and phenol (*8*). Samples of approximately 2 μg of tracheal RNA were applied to a polyacrylamide-agarose composite gel, and were fractionated by electrophoresis in the apparatus described by Raymond (*9*). Gels contained 0.5 percent agarose and 1.7 percent acrylamide (*N,N'*-methylenebisacrylamide and acrylamide, 1 : 19) (*10*), and 0.031 percent *N,N,N',N'*-tetramethylenediamine was used as the catalyst (*11*). A buffer of tris(hydroxymethyl)aminomethane (tris), ethylenediaminetetraacetic acid (EDTA), and borate, *p*H 8.3 (*10*), was used for electrophoresis, which was performed at 5° to 10°C for 2½ hours at 200 volts and approximately 45 ma. Cytoplasmic RNA (5 μg, from hamster liver) labeled with [2-^{14}C]uridine for 20 hours in vivo was subjected to electrophoresis with each experimental sample to identify the 18*S* and 28*S* species of RNA. Since the aim of our studies was to obtain the best electrophoretic resolution of the newly synthesized, high-molecular-weight RNA, we used relatively porous gels, and did not examine RNA fractions with lower molecular weight than that of the 18*S* marker. The gels were divided into 1-mm slices and each slice was treated with 0.1 ml of H_2O and 1.0 ml of NCS (Amersham/Searle Corp.) solubilizer. Radioactivity in each sample was measured after addition of toluene, 2,5-diphenyloxazole (PPO), and para-bis-2-(5-phenyloxazolyl)-benzene (POPOP) scintillator. The counts per minute were corrected for crossover between channels, for efficiency of counting, and for background counts to give the number of disintegrations per minute (dpm). The sum of the disintegrations per minute recovered from all slices of a gel was determined, and the percentage of this total in each slice was calculated. These percentages were plotted as a function of the gel slice number, which is equal to the migration distance in millimeters.

In Fig. 1, the electrophoretic patterns of tracheal RNA isolated from either vitamin A–deficient or normal, pair-fed control hamsters are compared after three intervals of incubation. Whole-cell RNA was prepared from samples of tracheal epithelium immediately after a 30-minute pulse with [5-^{3}H]uridine (Fig. 1a), and again 30 and 90 minutes after the tracheas were transferred to unlabeled medium (Fig. 1, b and c). High-molecular-weight species represented a progressively smaller proportion of total radioactive RNA with increasing time of incubation, but the proportion of these high-molecular-weight species was consistently smaller in the RNA from vitamin A–deficient animals. At all three incubation times, there was a decrease of RNA species migrating 30 mm or less in vitamin A–deficient animals as compared with the controls. In order to analyze the above data numerically, we have chosen a reference point of 30-mm migration, which has no inherent significance, but which operationally allows comparison between normal and vitamin A–deficient animals. Analysis of the data shows that only 28.1 percent of the total radioactive RNA from the vitamin A–deficient animals migrated 30 mm or less (Fig. 1a), as compared to 46.3 percent of the total radioactive RNA from the normal animals. In Fig. 1b, 27.5 percent of the radioactive RNA from the vitamin A–deficient animals migrated 30 mm or less, as compared to 43.3 percent from the normal animals; and in Fig. 1c, the percentages were 23.3 percent and 36.4 percent, respectively, for the vitamin A–deficient, as compared to the normal, animals.

Results of another experiment are shown in Fig. 2. Here, tracheas were obtained from vitamin A–deficient animals before treatment with vitamin A (Fig. 2a), and 1 or 2 weeks after treatment with vitamin A (Fig. 2, b and c, respectively), as described previously. The RNA was prepared from tracheas that had been incubated with [5-^{3}H]uridine for 30 minutes. The electrophoretic patterns of RNA molecules from vitamin A–deficient hamsters, before and after vitamin A treatment, are compared with the patterns from a normal, pair-fed control hamster in Fig. 2. As in Fig. 1, before treatment with vitamin A (Fig. 2a) there was a decrease in the proportion of slowly migrating RNA species. After 1 week of treatment with vitamin A, there was an observable increase in the proportion of slowly migrating RNA species (Fig. 2b), and after 2 weeks of treatment (Fig. 2c), the RNA pattern of the previously deficient animals was indistinguishable from that of the control. Numerical analysis of the experiments in Fig. 2 shows that in Fig. 2a, 43.1 percent of the total radioactive RNA from the normal animals migrated 30 mm or less, while only 23.8 percent of the total radioactive RNA from the vitamin A–deficient animals migrated 30 mm or less. After 1 week of treatment of the deficient animals with vitaman A (Fig. 2b), the percentage had risen to 28.5 percent; after 2 weeks of treatment (Fig. 2c), 42.5 percent of the total radioactive RNA migrated 30 mm or less, as compared to 43.1 percent in the control animals. Electrophoretic patterns of newly synthesized tracheal RNA were also examined 1 and 2 days after administration of vitamin A to previously deficient animals; these electrophoretic patterns were indistinguishable from those of untreated, vitamin A–deficient animals.

The observed decrease in the proportion of slowly migrating RNA synthesized in vitamin A–deficient tracheas has also been detected in tracheal epithelium of hamsters in the "plateau phase" of vitamin A deficiency, that is, after animals cease gaining weight, but before they lose weight. No changes in the electrophoretic pattern of tracheal RNA molecules have been observed in RNA prepared from normal animals treated (as previously described) with a single 2-mg dose of retinyl acetate. Essentially the same amount of RNA from either vitamin A–deficient or normal tracheas was applied to the gels, although the specific activity of the RNA from vitamin A–deficient tracheas was approximately 30 percent lower than that of RNA from control animals. Because it was essential to make comparisons between gels containing different levels of radioactivity, the analysis of electrophoretic patterns was performed on a percentage basis. Analysis based on percentage distribution is independent of precursor pool effects, since the total amount of RNA synthesized in each case is an internal control against which to compare individual RNA species.

These results indicate that there is an alteration in the electrophoretic pattern of RNA of tracheal epithelium in vita-

min A deficiency; a smaller proportion of RNA species with low electrophoretic mobility is found in vitamin A–deficient animals than is found in normal controls. This alteration is reversed after vitamin A treatment, with 2 weeks necessary for complete restoration of the normal electrophoretic pattern. Although migration of RNA molecules in gel electrophoresis generally bears an inverse reaction to molecular weight (*10*), variations in secondary structure can alter mobilities (*11*, *12*). The change observed in the vitamin A–deficient animals is a relative lack of RNA molecules with low electrophoretic mobility. It is not known at present whether this represents a deficiency of RNA molecules with specific nucleotide sequences or whether these sequences are present, but with mobilities somehow changed. Alterations in rates of synthesis, processing, or degradation of RNA molecules might account for the observed changes in electrophoretic pattern. Parallel morphologic studies indicate that these changes persist despite the disappearance of squamous metaplasia and restoration of normal cellular populations within 1 week after vitamin A treatment (*13*). Thus, the changes in electrophoretic pattern cannot be completely explained as the result of an altered cellular population. Analogous to this observed effect of vitamin A on the electrophoretic pattern of RNA is the fact that (*14*) there is a relative deficiency of RNA molecules of low electrophoretic mobility in mammary gland organ cultures maintained on medium without hydrocortisone, and an increase in such molecules upon addition of hydrocortisone to the cultures.

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Inhibition of Angiotensin Conversion in Experimental Renovascular Hypertension

Abstract. *Constriction of the renal artery and controlled reduction of renal perfusion pressure is followed by a prompt increase in systemic renin activity and a concomitant rise in blood pressure in trained, unanesthetized dogs. The elevated blood pressure induced by the renal artery stenosis can be prevented by prior treatment with the nonapeptide Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, which blocks conversion of angiotensin I to angiotensin II. Further, the nonapeptide can restore systemic pressure to normal in the early phase of renovascular hypertension. These results offer strong evidence that the renin-angiotensin system is responsible for the initiation of hypertension in the unilaterally nephrectomized dog with renal artery constriction.*

Although some evidence suggests that the renin-angiotensin system may become relatively unimportant in the maintenance of blood pressure in chronic renovascular hypertension in animals with one kidney removed (*1*), data are not available to define the role of this system in the initiation of the elevated blood pressure. Gutmann and co-workers (*2*) demonstrated in unanesthetized dogs previously subjected to unilateral nephrectomy that both systemic renin activity and blood pressure rise within minutes after renal artery constriction and controlled reduction of renal perfusion pressure. To determine whether the rise in renin activity and blood pressure are causally related, similar experiments were performed in which animals were given the nonapeptide Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (*3*), a potent inhibitor of the angiotensin-converting enzyme (angiotensin I to angiotensin II).

Under sterile conditions, polyvinyl catheters were inserted into the aorta, renal artery, and inferior vena cava of each of ten anesthetized male mongrel dogs (26 to 34 kg), and an externally inflatable cuff was placed about

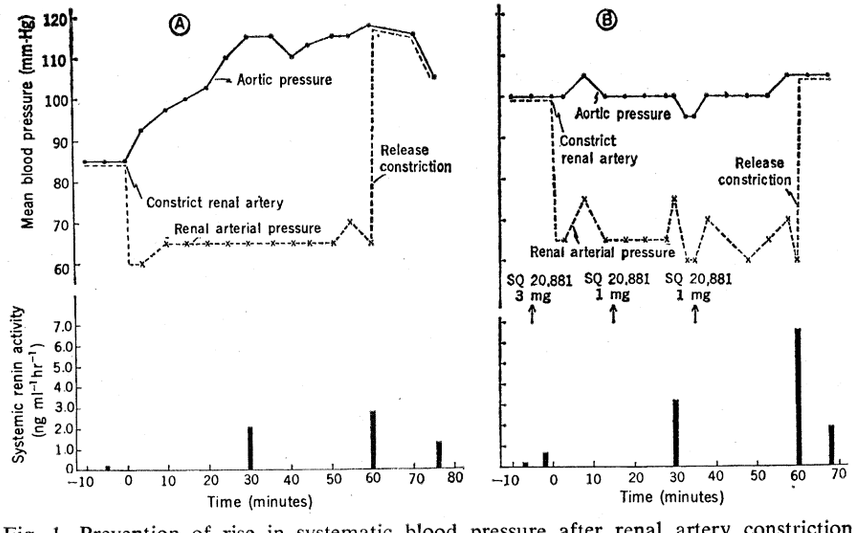

Fig. 1. Prevention of rise in systematic blood pressure after renal artery constriction by blockade of conversion of angiotensin I to angiotensin II by the nonapeptide. Data are for (A) an untreated dog and for (B) the same dog after intravenous administration of the drug.