(*25*). The second follows from nature of the active site as Zn (H_2O) $\rightleftharpoons$ Zn (OH^-) (*3, 5*) and applies to the very large number of organic reactions catalyzed by acid or bases, or involving water, such as hydrolysis, enol-keto rearrangement and aldol condensations, reactions that occur in intermediary metabolism and the biosynthesis of hormones.

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Vitamin A: Not Required for Adrenal Steroidogenesis in Rats

Abstract. *Previous work supporting the vitamin A dependency of adrenal function in rats neglected to take into account a secondary effect of the deficiency, a decrease in hepatic ascorbic acid biosynthesis. Vitamin A–depleted rats maintained on a diet free of ascorbate had a decrease in the activity of adrenal 3β-hydroxysteroid dehydrogenase, and extensive adrenocortical degeneration. The use of an ascorbate supplement prevented the symptoms. The results suggest that previous evidence for direct involvement of vitamin A in steroidogenesis may have been due to the production of a secondary deficiency, a chronic scorbutic condition.*

The vitamin A dependency of steroidogenic organ function in rats has remained a controversial subject. Both adrenal (*1, 2*) and gonadal (*3*) dysfunction have been reported in hypovitaminosis A. Attempts have been made to apply this theory as an explanation for the apparent anticarcinogenic effects of vitamin A. Light (*4*) has proposed the existence of subclinical vitamin A deficiencies which result in decreased adrenocortical output. The correction of such a condition in a patient with cancer would be the equivalent of steroid therapy. In addition, Van Thiel *et al.* (*5*) have shown that ethanol interferes with testicular vitamin A metabolism, and they theorized that this could account for the occurrence of sterility in alcoholics. However, no attempt has been made to reconcile these theories with recent work that does not support the involvement of vitamin A in steroidogenesis (*6, 7*). A review of this field revealed that none of the investigators who attempted to produce a hypovitaminosis A condition (*1–3, 6, 7*) noted an important effect of hypovitaminosis A in the rat, a reduction in the hepatic biosynthesis of ascorbic acid (*8*). An examination of the diets used indicated that in those experiments where significant steroidogenic dysfunction was noted both vitamins A and C were lacking in most of the reported diets (*1–3*). In one of the experiments that failed to support the aforementioned work (*7*), significant amounts of ascorbic acid were present in the diet (*9*). In another report (*6*) showing the absence of adrenal effects in vitamin A deficiency, no vitamin C was in the reported diet; but retinoic acid was used to deplete the vitamin A stores, and was then abruptly withdrawn. The effect of this regimen on the hepatic ascorbic acid synthesizing enzyme, gulonolactone oxidase, is not known.

With these facts in mind we decided to repeat the experiments that attempted to show vitamin A involvement in adrenal function. However, we maintained groups on vitamin A– and vitamin C–free diets.

We used 50 male, weanling Long-Evans rats (Charles River, Wilmington, Mass.). To eliminate the parameter of stress, the rats were handled every day for 3 weeks before and during the experiment. The animals were housed in wide mesh steel cages which allowed their feces and urine to drop through, leaving little residue and preventing ingestion of excreta. A light schedule of 10 hours of light (from 0800 to 1800) alternating with 14 hours of darkness was maintained. This regimen permitted stabilization of daily rhythms of enzyme and hormone activity so that normal fluctuations would occur at predictable time periods. At 6 weeks of age the rats were divided into groups and the experiment begun.

Custom-formulated diets were obtained from Nutritional Biochemicals Corp. Twenty animals were designated controls (group 1), and fed a nondeficient diet that contained 20 units of vitamin A and 1 mg of ascorbic acid per gram of feed. Groups 2, 3, and 4 (each containing ten animals) were maintained on diets free of vitamins A and C (*10*); this diet in every other aspect was identical to that given group 1. Vitamin supplements were provided as follows. Group 3 received an intraperitoneal injection of 10 mg of sodium ascorbate per 100 g of body weight per day; group 4 was given 20 international units of vitamin A palmitate per 100 g of body weight per day. Groups 1 and 2 were given equivalent injections of vehicle alone. Half of group 1 and all of groups 3 and 4 were pair-fed to group 2. The remaining animals of group 1 were given free access to their diet. However, in the results that follow the data from both control groups were pooled because there were no significant differences between them.

The group in which vitamin A was replaced (group 4) was inserted primarily as a reference point for the interpretation of any morphological changes seen in the other deficient groups and as a check on the ability of the Long-Evans strain to synthesize ascorbic acid for functional needs. The dosage of vitamin A chosen for group 4 was that amount reported to produce maximum growth and longevity without appreciable liver storage (*11*). The ascorbate supplementation of group 3 was estimated from the work of Salomon and Stubbs (*12*), who found that on the average ascorbate synthesis was 6 mg per 100 g of body weight per day. However, recent work (*13*) has shown a sizable variation in the ascorbic acid requirements of a random population of guinea pigs, and since a similar variation might exist in the levels of synthesis in rats, a dose of ascorbate just in excess of the average determined rate of synthesis was used.

The body weight and the concentration of ascorbic acid in the blood (obtained from the tail vein) were determined week-

ly. A significant decrease (20 percent) in the blood ascorbic acid of the rats deficient in vitamins A and C (group 2), as compared to the controls or other experimental groups, was noted after 60 days on the deficient diet. An additional 30 days was allowed to elapse before the animals were killed. For this period groups 2 and 3 were in a condition of slow liver retinol depletion, whether with or without an induced chronic state of ascorbate deficiency. It has been shown (*8*) that there is an immediate decrease (up to 50 percent) in ascorbate synthesis which accompanies any significant depletion of liver vitamin A. Although in previous investigations (*1–3, 6, 7*) weight loss or the appearance of gross symptoms of vitamin A deficiency were criteria for the time of killing the animals, we used the fall in blood ascorbic acid as a criterion, since there were no significant alterations in the weight-growth curves of the four groups, nor were there any gross symptoms of vitamin A deficiency. The purpose of this was to demonstrate that functional and morphological changes occur in steroidogenic organs before any manifestations of peripheral vitamin A deficiency occur.

Animals were killed by rapid decapitation between 1100 to 1500 hours, after the animals were acclimated (about 1 hour) to the laboratory area. The time of killing, 3 to 7 hours after the onset of light, was chosen to correspond to periods of reported steady low levels of circulating corticosterone and ascorbic acid (*14*). Blood and adrenal ascorbic acid and corticosterone were determined by the dinitrophenylhydrazine method (*15*) and acid fluorescence (*16*), respectively. The activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), was measured by the rate of conversion of dehydroepiandrosterone to androstendione in adrenal homogenates (*17*). Adrenal protein was assayed by the technique of Lowry *et al.* (*18*). Liver vitamin A was measured by the Carr-Price method (*19*). Statistical significance of the data was determined by Student's *t*-test, at the $P < .01$ level. Some adrenals were sectioned by cryostat at 15 μm, and were stained with hematoxylin and eosin for general morphology, or were used for the Schultz modification (*20*) of the Liebermann-Burchardt reaction for the visualization of cholesterol and its esters.

Group 2 showed significant decreases in both blood and adrenal ascorbic acid, 18 and 33 percent, respectively, as compared to controls (Table 1). Group 3 showed a slightly higher concentration of ascorbic acid in the blood (18 percent) as compared to controls, as a result of administered excess replacement. However, the ascorbic acid concentration in the adrenals was identical to that of the control group.

Table 1. The effect of selected vitamin deficiencies on the adrenal weight and ascorbic acid in the rat. Group 1 rats received a normal diet containing 1 mg of ascorbic acid and 20 units of vitamin A palmitate per gram of feed. Groups 2, 3, and 4 were maintained on a diet deficient in vitamins A and C; group 3 received 10 mg of sodium ascorbate per 100 g of body weight; group 4 received 20 international units of vitamin A palmitate per 100 g of body weight per day. Numbers in each case represent the mean ± standard error of the mean.

Group	Adrenal weight (mg)	Protein (mg)/ adrenal (mg)	Ascorbic acid (mg)/ adrenal protein (mg)	Ascorbic acid (μg)/ 100 ml of blood
1	24.0 ± 1.0	0.261 ± .008	0.0195 ± .0007	808.3 ± 38.7
2	31.7 ± 1.6	0.289 ± .023*	0.0161 ± .0011*	541.6 ± 10.7*
3	40.6 ± 4.3	0.268 ± .014	0.0198 ± .0015	979.15 ± 85.6
4	28.4 ± 2.2	0.284 ± .010*	0.0183 ± .0011	627.5 ± 41.68†

*Significant compared to normal group, with $P < .01$. †Significant compared to normal group, with $P < .05$.

Group 4 showed only slight decreases in both blood and adrenal ascorbic acid, and this decrease represents the basal levels produced by the liver with minimal vitamin A replacement. The ratio of protein (milligrams) to adrenal weight (milligrams) (Table 1) indicates whether an increase in weight is due to an increase in the protein content of the gland. The increased weight in group 3 was not due to an increase in protein content, but the slight increase in weights in groups 2 and 4 were accompanied by a significant increase in protein.

The adrenal 3β-HSD activity and corticosterone levels, as well as the blood corticosterone levels when the animals were killed are shown in Table 2. The synthesis of adrenal steroids requires 3β-HSD, since the conversion of a 3β-hydroxy to a 3-keto group is necessary for the biological activity of most steroid hormones. There are conflicting reports concerning this enzyme's activity: some suggest that it is dependent on vitamin A (*1, 5, 6*), and others suggest vitamin C (*21*). The enzyme levels of group 2 were significantly lowered as compared to those of the other groups, whose values did not differ. This relation held whether the activities were expressed per two adrenal glands or per milligram of protein. The adrenal stores of corticosterone of group 3 were 36 percent lower ($P < .05$), while the blood levels were 34 percent higher. There were no differences in the blood and adrenal corticosterones of group 2 compared to the control group. Liver vitamin A of the two groups deficient in vitamin A (groups 2 and 3) showed a depletion of 50 and 70 percent, respectively, when compared to the control group (*22*).

The morphological appearance of the gland from an animal supplemented with vitamin C and deficient in vitamin A (Fig. 1D) was indistinguishable from that of the control group (Fig. 1A). The animals deficient in vitamins A and C (group 2) and the group given minimal vitamin A replacement (group 4) had hyperplasia of the glomerulosa with infiltration of the zona fasciculata and a lack of a distinct boundary between the zones (Fig. 1, B and E). In group 2 there were areas of multiple cell cytolysis forming "lumina" in which could sometimes be seen necrotic nuclei from the disintegrated cells (Fig. 1C). Similar hyperplasia and tissue destruction found in rabbits and guinea pigs on a scorbutic diet (*23*) were indistinguishable from that produced by prolonged stimulation of adrenocorticotrophic hormone (ACTH) in stress (*24*). Selye and Stone suggested that this syndrome was caused by extensive demands on the adrenal cortex as a result of long-term or overstimulation by ACTH (*25*). Staining for adrenal cholesterol showed undetectable amounts in group 2 (Fig. 1G), implying that less than 3 to 6 percent 7-hydroxycholesterol was present (*26*); the group deficient in vitamin A but with vitamin C replaced (Fig. 1H) and the control (Fig. 1F) group showed varied de-

Table 2. Effect of selected vitamin deficiencies on blood, adrenal, and liver. Group 1 rats received a normal diet containing 1 mg of ascorbic acid and 20 units of vitamin A palmitate per gram of feed. Groups 2 and 3 were maintained on a diet deficient in vitamins A and C, in addition to which group 3 received 10 mg of sodium ascorbate per 100 g of body weight per day. Numbers in each case represent the mean ± standard error of the mean.

Group	Liver vitamin A (unit/g)	Corticosterone (μg)/adrenal protein (mg)	Corticosterone (μg)/ 100 ml of blood	3β-HSD activity per minute	
				Androstendione (μg)/two adrenals	μg/mg of adrenal protein
1	1165 ± 6.1	0.0704 ± .0055	18.76 ± 6.5	7.65 ± 0.23	0.306 ± .04
2	580 ± 16.3*	0.0603 ± .0049	19.70 ± 0.5	2.65 ± 0.17*	0.136 ± .03*
3	345 ± 6.1*	0.0473 ± .0059†	28.38 ± 7.0*	7.48 ± 0.31	0.407 ± .08

*Significant compared to normal group, with $P < .01$. †Significant compared to normal group, with $P < .05$.

grees of staining, an indication that 12 to 25 percent hydroxycholesterol and 40 to 50 percent total lipids were present. ACTH lowers adrenal cholesterol, and depletion occurs in long-term stimulation (*27*). Also, the decrease in adrenal cholesterol in scurvy is attributed to diminished acetylation in the body (*28*) with decreased acetate uptake and utilization by the adrenal (*29*). Vitamin A is nonessential for cholesterol biosynthesis (*2, 30*).

In the control group, the levels of 3β-HSD, blood and adrenal ascorbic acid, and of corticosterone and a positive Schultz reaction indicated that the glands actively produced and stored corticosterone and maintained blood levels. In the group in which vitamin C was replaced, the 3β-HSD activity, blood and adrenal ascorbic acid were the same as that in the controls; the corticosterone was slightly lower in the gland and higher in the blood, and the intracellular cholesterol was slightly increased with a resulting increase in adrenal weight. This indicated that the average gland at the time the animals were killed produced and released corticosterone. In contrast, the group deficient in vitamins A and C (group 2) had low gland and blood ascorbic acid, no detectable cholesterol, very low 3β-HSD, and normal corticosterone levels. This indicated that the glands produced adequate corticosterone, but at the peak of glandular capability (that is, under high ACTH stimulation). That this stimulation had been long-term can be seen by the hyperplasia of zona glomerulosa and cytolysis, and by hypertrophy as evidenced by an increase in the gland weight and a larger ratio of protein weight to adrenal weight. The group in which vitamin A was replaced (group 4) showed a small decrease in ascorbic acid in the gland and blood. The increase in the ratio of gland weight to protein weight was due to the hyperplasia of zona glomerulosa seen in the stained sections. These adrenals, containing a low level of ascorbic acid, present a similar histological picture to that of the animals of the vitamin C–deficient group. The lower level of tissue

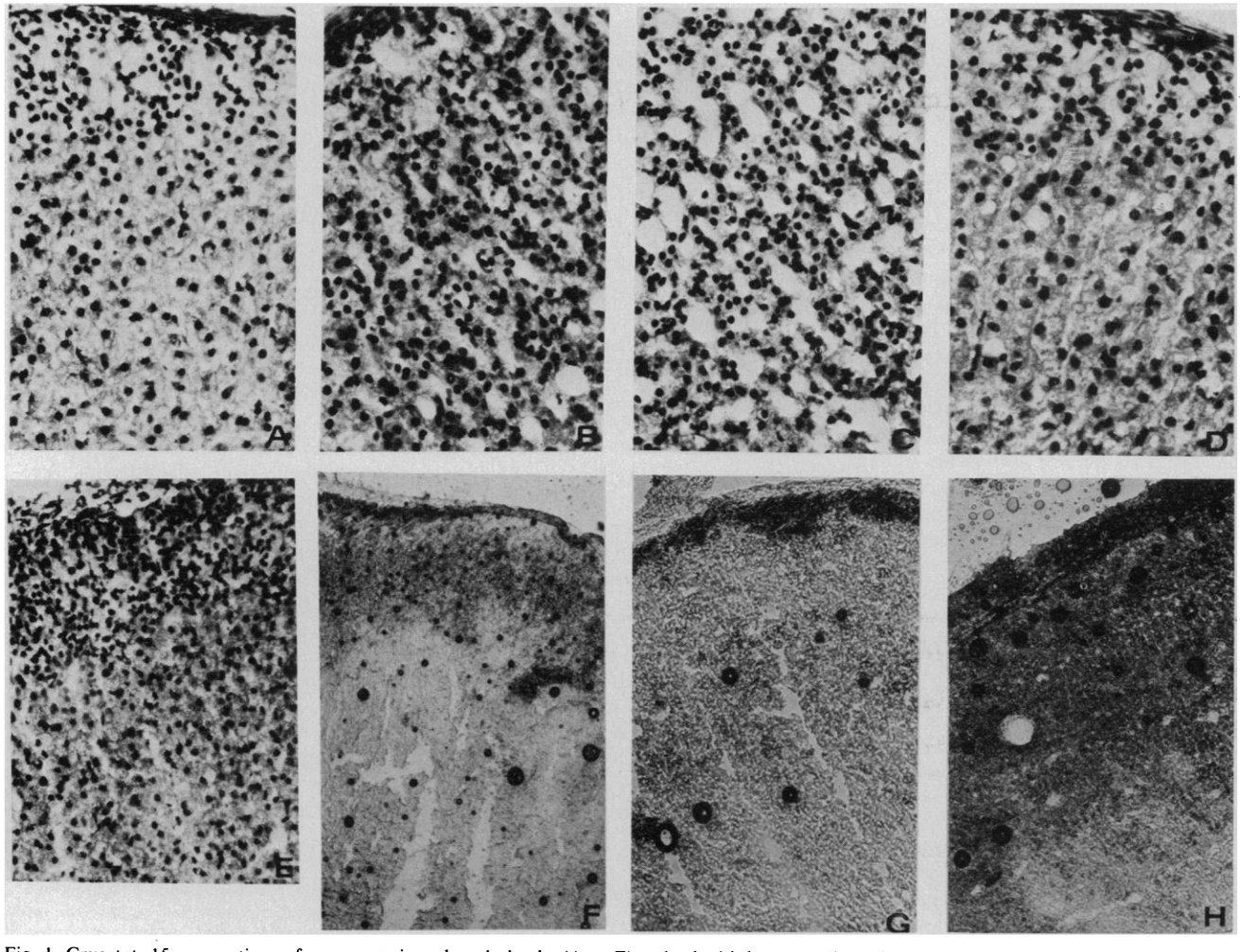

Fig. 1. Cryostat, 15-μm sections of representative adrenal glands; (A to E) stained with hematoxylin and eosin; (F to H) stained for cholesterol by the Schultz technique (*20*). (A) Normal rat adrenal from group 1 ($\times$ 125). (B) Rats deficient in vitamins A and C (group 2). Vitamin C deficiency causes hyperplasia of zona glomerulosa, which infiltrates the fasciculata, resulting in an indistinct boundary between the zones ($\times$125). (C) From rats deficient in vitamins A and C (group 2). The zona fasciculata of the vitamin C–deficient adrenal has areas of focal necrosis or cytolysis. These form lumina, into which protrude nuclei of the disintegrated cells ($\times$ 125). (D) Glands of rats deficient in vitamins A and C, but supplemented with ascorbic acid (group 3) appear normal ($\times$125). (E) Glands of rats deficient in vitamins A and C but with minimal vitamin A supplement (low level liver synthesis of ascorbic acid) (group 4) show only hyperplasia of zona glomerulosa ($\times$100). (F) In the adrenal gland from the normal rats (group 1), the dark staining reaction product outlines the zona glomerulosa with moderate precipitation in zona fasciculata. It indicates the presence of cholesterol and its esters ($\times$10). (G) Glands from rats deficient in vitamins A and C (group 2). The glands deficient in vitamin C show very slight reaction in the zona glomerulosa and none in the zona fasciculata. It indicates a depletion of 7-hydroxycholesterol to less than 3 to 6 percent, which is not detectable by the Schultz reaction ($\times$25). (H) In glands from rats deficient in vitamins A and C, but with vitamin C replaced (group 3), intense staining in the zona glomerulosa and throughout the zona fasciculata indicates increased cholesterol deposits. Other glands tested showed moderate amounts of reaction product similar to the control group ($\times$ 25).

and blood ascorbic acid in this group represents hepatic synthesis on a low intake of vitamin A, with no liver storage.

In summary, depletion of liver vitamin A will cause a decrease in the hepatic synthesis of ascorbic acid, confirming previously reported work (*8*). Rogers (*31*) has suggested that this effect is due more to inanition than the vitamin A deficiency. However, the fact that our vitamin A–depleted groups did not show any significant differences in their weight-growth curves from the controls tends to rule out this possibility. In addition, if inanition was the cause of the decrease in ascorbic acid synthesis, then depletion studies with other vitamins should have shown adrenocortical effects. To our knowledge this is not the case.

The result of the reduction in ascorbic acid biosynthesis is a decrease in the activity of adrenal 3β-HSD, and a series of histological changes in the adrenal which resemble scurvy in the guinea pig (*23, 24*). These changes have been attributed to the vitamin A dependency of adrenocortical function (*1, 2*). The use of an ascorbate supplement in vitamin A–depleted rats prevented all of the aforementioned symptoms.

The above data provide a possible explanation for the conflicting reports concerning the effects of hypovitaminosis A on the adrenal cortex of the rat; that is, a lack of vitamin C in their diet. Further work is needed to ascertain whether the "protective" effect of ascorbic acid is still present in more extreme cases of vitamin A–deficiency. However, our work shows the importance of ascertaining that only one deficiency exists when performing vitamin depletion studies.

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Cyclic Stretching Stimulates Synthesis of Matrix Components by Arterial Smooth Muscle Cells in vitro

Abstract. *Rabbit aortic medial cells were grown on purified elastin membranes, which were then subjected to repeated elongation and relaxation or to agitation without stretching. Cells remained attached to the membranes, and cyclic stretching resulted in a two- to fourfold increase in rates of collagen, hyaluronate, and chondroitin 6-sulfate synthesis over those in agitated or stationary preparations. Synthesis of types I and III collagen was increased to the same degree. Stretching did not increase rates of chondroitin 4-sulfate or dermatan sulfate synthesis. Differences were not attributable to differences in cell number, for DNA synthetic rates were not increased by stretching. The model system devised to demonstrate these effects provides a means for relating various modes of mechanical stimulation to cell metabolism.*

Recent investigations have revealed consistent qualitative and quantitative relationships between the composition of arterial walls and estimates of medial stress (*1, 2*). These findings suggest that physical forces related to pressure and flow direct medial cell biosynthesis, thereby modulating structural adaptations to hemodynamic changes. Differential synthetic response to mechanical stimuli would be expected to play a major role in the differentiation, growth, and maintenance of cardiovascular and other connective tissues (*3*). The mechanisms regulating tissue adaptations to physical stresses are poorly understood, and quantitative data relating mechanical forces directly to cellular biosynthesis are not available. Since information of this kind could provide a basis for understanding the limitations of blood vessel wall adaptation in normal and diseased states, we devised a model system in which cultured aortic medial cells could be subjected to a variety of mechanical stimuli similar to those that prevail in the arterial wall. The system consists of a purified elastin membrane suspended in cell culture medium and coupled to an apparatus designed to stretch and relax the membranes at various desired frequencies and amplitudes. We report here that aortic medial cells attached to such elastin membranes and subjected to cyclic stretching consistently synthesized collagen and certain acid mucopolysaccharides much more rapidly than did cells growing on stationary membranes or on membranes subjected to agitation without stretching. To our knowledge this is the first demonstration of a direct relationship between mechanical forces and the synthesis of connective tissue components by smooth muscle cells.

The elastin membranes were prepared by slicing blocks of fresh bovine aortic me-