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.

KENNETH A. GRUBER*
LAURA V. O'BRIEN†
ROBERT GERSTNER

Department of Anatomy,
New York University,
342 East 26 Street, New York 10010

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* Present address: Roche Institute of Molecular Biology, Nutley, N.J. 07110.
† Present address: Department of Anatomy, University of Rochester School of Medicine, Rochester, N.Y.

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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-

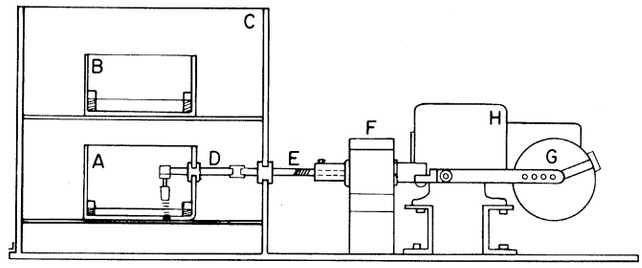

Fig. 1. Diagrammatic representation of the apparatus used for cyclic stretching or agitation of cells cultured on elastin membranes. Culture dishes containing the stretched or agitated membranes (*A*) and the paired stationary controls (*B*) are maintained in an airtight chamber (*C*). The dish containing a stretched or agitated membrane is equipped with a plunger (*D*) passing through an airtight seal in the side of the dish. The plunger is connected to a disk (*G*) mounted on the shaft of a variable speed motor (*H*) by means of an adjustable rod (*E*) passing through an airtight seal in the wall of the airtight chamber, and a guide support (*F*). Further details may be found in the text.

diums on a rotary meat slicer. Sections 400 μm thick were autoclaved and subjected to extraction with hot alkali to remove nonelastin components (*4*). Amino acid analysis as well as inspection by transmission and scanning electron microscopy indicated that such treatment yielded a sheet composed entirely of a network of elastin plates and interconnecting elastin fibers. The smooth muscle cells were prepared by culturing cells grown out of explants of rabbit thoracic aorta in the manner described by Ross (*5*). Cells were subcultured once, plated onto the elastin membranes at confluency, and allowed to grow for 3 days before the start of each experiment.

In the series of experiments that forms the basis of this report, rates of synthesis of protein, collagen, mucopolysaccharide, and DNA were determined under three conditions: (i) when the cells were grown on membranes that were stretched cyclically, (ii) when the cells were grown on fixed elastin membranes that were displaced linearly without stretching (that is, agitated), and (iii) when the cells were grown on stationary elastin membranes. For each of the stretching or agitation experiments, the biosynthetic activity of cells derived from the same explant and grown under the same conditions but attached to a stationary membrane was measured at the same time to serve as a paired control. Elastin membranes destined for cyclic stretching were mounted on a frame, one end of which could be displaced on tracks. The frame was then fixed in a specially designed rectangular culture dish equipped with a plunger passing through one of its sides by way of a watertight seal. The end of the plunger within the dish was then attached to the movable edge of the membrane mounting frame, while the end of the plunger extending outside the flask was connected by means of an extensible rod to a disk mounted on the shaft of a variable speed motor. The entire assembly could be adjusted to subject the elastin membranes to different initial tensions and to stretching through different amplitudes over a range of rates. Elastin membranes destined to be agitated without stretching were mounted on similar frames. The movable edge was fixed with a setscrew after the desired initial tension was established and then attached to the plunger. The entire frame could then be displaced linearly to and fro within the flask at the desired frequency and amplitude. During the experiments, the cultures were maintained in an airtight chamber gassed with a mixture of 10 percent CO_2 and 90 percent air at 37°C. The rod connecting the culture dish plunger to the motor passed through an airtight seal in a wall of the chamber. A diagram of the apparatus is furnished in Fig. 1; detailed specifications for its construction will be provided elsewhere (*6*).

The effects of cyclic stretching or agitation on protein, collagen, mucopolysaccharide, and DNA synthesis were determined by measuring the incorporation of radioactive precursors into the respective macromolecules. After 2 days of cyclic stretching or agitation, radioactive precursors were added and stretching or agitation was continued for 8 hours. Protein and collagen synthesis was measured by [^{14}C]proline incorporation into nondialyzable proline and hydroxyproline, respectively. Separation of hydroxyproline and proline was carried out by the method of Lukens (*7*). Mucopolysaccharide synthesis was measured by incubating the cells in the presence of [^{3}H]acetate. Analysis consisted of 0.5*N* NaOH treatment, trichloroacetic acid precipitation to remove contaminating protein, dialysis, and enzymic degradation by chondroitin ABC lyase (E.C. 4.2.2.4) and chondroitin AC lyase (E.C. 4.2.2.5). The mucopolysaccha-

Table 1. Effects of cyclic stretching or agitation on protein, collagen, DNA, and mucopolysaccharide synthesis. Data are from five typical experiments. For each experiment, cells from the same explant were grown on a pair of identical unstretched elastin membranes for 3 days. One membrane of each pair was then stretched cyclically or agitated for 2 days, while the other served as a simultaneous stationary control. Eight hours before harvest, cells were incubated in the presence of labeled precursors. Results for each experiment are expressed as counts per minute in cells and culture medium per microgram of DNA. To study the effect of cyclic stretching or agitation on protein and DNA synthesis, cells were incubated in the presence of [^{14}C]proline (0.2 μc/ml) and [^{3}H]thymidine (1 μc/ml). Stretching was associated with a 3-fold increase in [^{14}C]proline uptake into proline and a 3.7-fold increase of [^{14}C]proline uptake into hydroxyproline, while thymidine incorporation was unchanged compared to the stationary paired membrane. Agitation without stretching was associated with a 1.7-fold increase in [^{14}C]proline uptake into proline and a 1.25-fold increase in [^{14}C]proline uptake into hydroxyproline, while [^{3}H] thymidine incorporation was increased 1.8-fold. To study the effect of cyclic stretching on collagen-type synthesis, cells were incubated in the presence of [^{14}C]proline (0.2 c/ml), ascorbate (50 μg/ml), and β-aminoproprionitrile (50 μg/ml). Type I and type III collagen showed comparable increases in synthesis in stretched compared to stationary membranes, 2.75- and 3.45-fold, respectively. To study the effect of cyclic stretching or agitation on mucopolysaccharide synthesis, cells were incubated in the presence of [^{3}H]acetate (2 μc/ml). Incorporation of [^{3}H]acetate into hyaluronate, chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate was determined as described in the text. Stretching was associated with a 3.8-fold increase in hyaluronate and a 2.9-fold increase in chondroitin 6-sulfate synthesis, compared to stationary paired controls. Dermatan sulfate synthesis showed only a 1.6-fold increase in the agitated preparation.

Precursor incorporation	Counts per minute per microgram of DNA			
	Paired membranes		Paired membranes	
	Stretched	Stationary	Agitated	Stationary
Effect of cyclic stretching or agitation on protein and DNA synthesis				
[^{14}C]Proline into proline	49,600	16,300	17,400	10,600
[^{14}C]Proline into hydroxyproline	7,100	1,900	2,000	1,600
[^{3}H]Thymidine into DNA	25,000	23,600	12,500	7,000
Effect of cyclic stretching on collagen-type synthesis				
[^{14}C]Proline into type I collagen	20,600	7,500		
[^{14}C]Proline into type III collagen	10,820	3,110		
Effect of cyclic stretching or agitation on mucopolysaccharide synthesis				
[^{3}H]Acetate into hyaluronate	15,730	4,800	9,300	10,170
[^{3}H]Acetate into chondroitin 4-sulfate	140	140	620	475
[^{3}H]Acetate into chondroitin 6-sulfate	1,540	530	1,500	1,500
[^{3}H]Acetate into dermatan sulfate	830	670	1,230	780

ride disaccharide units were separated according to previously described methods (*8*). For the study of DNA synthesis, [^{3}H]thymidine incorporation into material precipitable with trichloroacetic acid was determined. Total DNA was measured by the method of Burton (*9*). The collagens were isolated and characterized by labeling the cells with [^{14}C]proline in the presence of vitamin C and β-aminoproprionitrile, extraction from cells or culture medium with dilute acetic acid, and precipitation with 5 percent NaCl in the presence of carrier collagen. This precipitate was dissolved and treated with pepsin, reprecipitated with 15 percent KCl, and passed through a Bio-Gel A-5m column. After reduction and alkylation of disulfide groups, the collagen type was identified by carboxymethyl cellulose chromatography (*10*).

In the series of experiments reported here, the cyclically stretched membranes were stretched 10 percent beyond their resting length and returned to this position 52 times per minute; the agitated membranes were displaced without stretching for a similar distance and at the same frequency. Transmission and scanning electron micrographs of cultures grown on these elastin membranes showed that the cells formed numerous attachments to the elastin plates as well as to each other; such attachments persisted after prolonged stretching or agitation (*11*). We have therefore presumed that when the elastin membranes were stretched, cells anchored to the membranes were likewise stretched or deformed. The data reported here are drawn from a total of 75 experiments performed with the apparatus described above. Each stretching or agitation experiment was repeated at least three times.

Cyclically stretched preparations incorporated [^{14}C]proline into protein 3 to 5 times more rapidly and into hydroxyproline 4 to 5 times more rapidly than did stationary controls. There was, however, no difference in [^{3}H]thymidine incorporation between stretched or stationary cultures. By contrast, cells subjected to agitation without stretching incorporated [^{14}C]proline into protein only 1.2 to 2 times more rapidly and into hydroxyproline only 1.2 to 1.5 times more rapidly than did stationary cultures, but the stimulation was accompanied by a nearly twofold increase in rate of [^{3}H]thymidine incorporation. Protein, collagen, and DNA synthesis are compared from two typical experiments for cyclic stretching and for agitation without stretching in Table 1. Characterization of the collagens synthesized by cyclically stretched cells indicated that stretching increased the rate of synthesis of both type I and type III collagen to the same degree (Table 1). The effects of cyclic stretching and agitation on mucopolysaccharide synthesis in typical experiments are also compared in Table 1. After cells were stretched for 2 days there was a threefold stimulation of [^{3}H]acetate incorporation into hyaluronate and chondroitin 6-sulfate but no significant increase in incorporation into chondroitin 4-sulfate or dermatan sulfate over that in stationary cultures. In contrast, agitation led to no significant changes in mucopolysaccharide synthesis over that in stationary cultures. Thus, stretching and agitation had different effects on cell synthesis. Stretching resulted in marked stimulation of peptide proline (protein) and peptide hydroxyproline (collagen) synthesis without stimulation of DNA synthesis. Agitation resulted in parallel stimulation of protein, collagen, and DNA synthesis, but the stimulation of synthesis of both protein and collagen was much lower than that induced by stretching. The effect of stretching on collagen synthesis was consistently greater than the effect of stretching on overall protein synthesis. Stretching resulted in a differential increase in rates of hyaluronate and chondroitin 6-sulfate synthesis, while agitation had no effect on rates of mucopolysaccharide synthesis.

It is theoretically possible that the mechanically induced differences in radioactive precursor incorporation into protein were due in part to alterations in size of intracellular precursor pools or to alterations in rates of reutilization of radioactive amino acid (*12*) rather than to increased rates of synthesis. Although rates of equilibration between exogenous amino acids and the several possible intracellular pools are unknown, studies utilizing HeLa cells (*13*) and skeletal muscle cells (*14*) indicate that [^{3}H]leucine in the medium equilibrates with the intracellular free pool within 15 minutes. It is therefore probable that our data, based on findings after a relatively long labeling time of 8 hours, reflect synthetic rate. Such considerations are not applicable to the data on mucopolysaccharide precursor incorporation; in these experiments, the differential incorporation of a single precursor into different mucopolysaccharides should reflect differences in synthetic rate.

Stimulation of macromolecular synthesis by cyclic stretching of smooth muscle cells in our in vitro model parallels some of the changes in composition of artery walls that have been associated with presumed increases in medial tensile stress. For example, collagen accumulation during growth is much more rapid in the ascending aorta than in the pulmonary trunk, which is at lower pressure (*15*); aortic collagen production is greatly accelerated by experimental hypertension (*2*); and sulfated mucopolysaccharides are selectively increased in both aortas and pulmonary arteries that have been subjected to hypertension (*16*). The model system described in this report should permit detailed characterization of the relative effects of variations in frequency, amplitude, or pattern of stress on cell proliferation and on the elaboration of specific matrix components. Since in vivo findings are apparently reproduced in this system, such studies may also be expected to contribute insights into the mechanisms by which tissues normally subjected to mechanical stress differentiate, grow, adapt, and involute.

DONALD Y. M. LEUNG
Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

SEYMOUR GLAGOV
Department of Pathology, University of Chicago

MARTIN B. MATHEWS
Departments of Pediatrics and *Biochemistry, University of Chicago*

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