cific model for the complete trajectory for one molecule presents itself. If a molecule continued to deposit as a single row, R_g would have to increase significantly with M (linearly as recorded for oriented mat), which is not observed. Nevertheless, if the rows of stems did not increase in length but did increase in thickness, the observed close invariance of R_g with M would be accounted for. Such a situation would arise if, beyond a certain length, the rows of stems would fold up on themselves. Such a possibility is in fact plausible on a molecular basis. We could refer to the folding back of the ribbons near themselves as "superfolding," although this superfolding is not representatively "adjacent re-entrant." Figure 2 shows a schematic two-dimensional representation of the structure. It has always seemed unlikely that one molecule would fold into one uninterrupted ribbon along the growth face; at the very least, there will be competition between different chains for room on the growth face. Such effects are indeed inherent in theories of crystal growth [regime II in (*11*)]. In particular, when two growing ribbons meet, either one continues to deposit and the remaining parts of the other becomes excluded from the crystal (and may possibly become incorporated elsewhere later) or both double up on themselves. The doubling-up process would represent the least interference with crystallization and is strongly suggested by the present experimental results.

Such a possibility of multiple ribbons does not arise quite unexpectedly. In fact, the idea of the proximity of different rows of stems from the same molecule has already been derived from effects at much higher angles examined in earlier work; the intensities corresponded to multiple rather than single rows of stems for high molecular weight ["interference between ribbons" (*5*)]. Use of the mixed crystal infrared technique (*12*) has shown that, for very-high-molecular-weight DPE, stems of the same molecule are in close proximity. Our own infrared measurements, made on the same samples as used here, favor the formation of multiple ribbons (*13*).

If we suppose that one dimension in the x–y plane is substantially greater than the other, we can derive an estimate for the larger dimension [R_x, say; in reality this is likely to be along 110 (Table 1)]. From the derived R_x value an estimate of the longest likely sequence of folded stems is possible, which is 170 Å. With the knowledge of lamellar thickness and stem spacing, we can express this length as corresponding to 40 stems, or M in the neighborhood of 50,000. On this basis, the apparent R_g should decrease for M below a value in this range. Unfortunately, at low M the excess low-angle intensity is large compared with the signal from the individual solute molecules, and an R_g is not obtainable. The fact that R_x is close to R_z (Table 1) implies sheets that are about equidimensional.

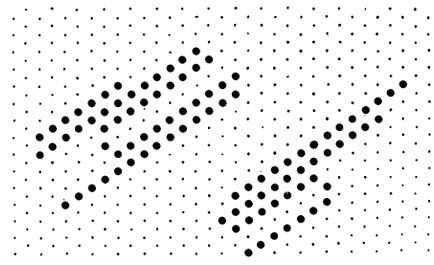

Fig. 2. Schematic representation of "stem" positions of two labeled molecules (large dots) as viewed in the chain direction (that is, 001). The 100 direction is shown horizontal, and the fold plane is (110) (*1*). Not to scale: the length of the rows is likely to be approximately 40 stems.

The estimates of R_z (even though imprecise since these are given by differences in R_g values) are larger than the 30 Å predicted from the known lamellar thickness of 105 Å. We can attribute this to the fact that some chains crystallize partly in neighboring lamellae; for example, a chain may divide itself approximately equally between two lamellae, or between three lamellae with the central lamella containing most of the chain. This is quite reasonable in view of the multilayered morphology of these crystals.

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In vitro Model for Stretch-Induced Hypertrophy of Skeletal Muscle

Abstract. *Mechanical stretch of embryonic chicken skeletal myotubes developed in vitro leads to many of the biochemical changes seen in skeletal muscle hypertrophy. These include increased amino acid accumulation, increased incorporation of amino acids into general cellular proteins and myosin heavy chains, and increased accumulation of total protein and myosin heavy chains. This model system should aid in understanding how the growth rate of skeletal muscle is regulated by its activity.*

Skeletal muscle is a highly adaptive tissue that must continuously respond to changes in the external environment. Thus, muscle activity leads to many biochemical changes, the type of activity determining the changes seen. For example, increased muscle work against high resistance leads to increased muscle size (hypertrophy) (*1*), whereas repetitive work against little resistance leads to increased oxidative capacity of the muscle (*2*). Although it has been known for more than 80 years that mechanical stretching of skeletal muscle increases its metabolic rate (*3*), the mechanism involved is still unknown. Because muscle is an elastic tissue that stretches and relaxes during activity, knowing the relationship of the mechanical movement of skeletal muscle to its metabolism is essential to understanding how muscle regulates its growth rate and the role of the nervous system in this regulation. Stretch has been implicated in such processes as early muscle growth and development (*4*), denervation hypertrophy (*5*), and compensatory hypertrophy (*6*). In this report we describe an in vitro model system in which it has been shown that mechanical stretch leads to skeletal

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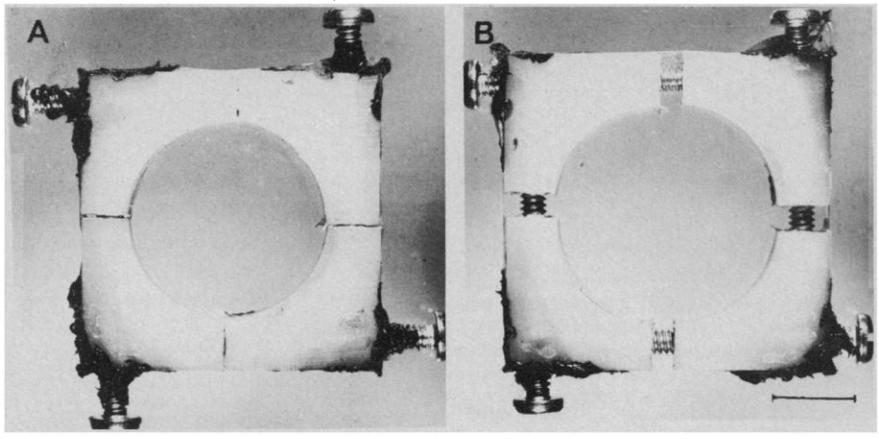

Fig. 1. Frames used to mechanically stretch skeletal muscle myotubes, with clear silicone membrane attached to base on which the cells were grown. (A) Frame before stretching. (B) Frame after a 10.8 percent stretch (scale bar, 1 cm).

muscle hypertrophy. This system should facilitate studies of the mechanism of stretch-induced hypertrophy.

Single-cell myoblasts were isolated from breast muscle of 12-day embryonic chicken (*7*) and plated into the well of a stretching frame (Fig. 1) with a total volume of 1.0 ml of growth medium (*8*). Myoblasts began fusion to form myotubes 24 to 48 hours after plating, and by 72 hours well-developed, contractile myotubes were present in the culture. To be certain that the myotubes were being stretched longitudinally when the elastic membrane on which they were grown was stretched, individual myotubes differing in orientation with respect to the frame were identified by use of an inverted phase-contrast microscope and the distance between identifiable myotube markers was measured with an eyepiece micrometer before and after the stretching procedure (Fig. 2). There was a linear relation between the increased diameter of the frame well and the distance between the myotube markers from 5 to 20 percent stretch (maximum distance measured); that is, a 10 percent increase in frame diameter led to a 10 percent increase in myotube longitudinal length, and so on.

Some of the characteristic changes seen in skeletal muscle hypertrophy in vivo were measured with the model system; these included (i) increased amino acid accumulation by the A transport system, as measured with the amino acid analog α-aminoisobutyric acid (AIB) (*9*); (ii) increased accumulation of amino acids into total and myofibrillar protein (*10*); and (iii) increased accumulation of total protein and myofibrillar protein (*11*). Tables 1 and 2 summarize the results of four experiments where myotubes were stretched 10.8 percent. The changes seen in vitro in the tissue culture model system are similar to those seen in vivo in the tenotomy model system, where mechanical stretch appears to be an important regulator of hypertrophy (*6*). Compensatory hypertrophy of a skeletal muscle occurs in the tenotomy model system when the tendons of synergistic muscles are cut and the remaining intact muscle is subjected to increased work (*12*). In hypertrophy in vivo, total muscle nitrogen and muscle-specific myofibrillar nitrogen increased 16 and 9.5 percent, respectively, 4 days after the tenotomy operation (*11*). The corresponding increases in the tissue culture system were 8.3 percent for total protein and 14.7 percent for muscle-specific myosin heavy chain after 18 hours of stretching. Accumulation of labeled amino acids into total protein and myofibrillar protein of the soleus in the in vivo hypertrophy model increased 80

Table 1. Biochemical effects caused by stretching myotubes by 10.8 percent. Myotube cultures (72 hours) were preincubated at 37°C for 30 minutes with fresh medium [89 percent Eagle's basal medium, 5 percent horse serum, 5 percent chicken embryo extract, 1 percent penicillin-streptomycin (50 unit/ml)] containing 5 μCi of [^{3}H]AIB (New England Nuclear, 10.0 Ci/mmole) and 2 μCi of [^{14}C]leucine (New England Nuclear, 295 mCi/mmole) to allow reequilibration of the CO_2 content of the medium. Pairs of cultures were removed from the incubator and placed on a 37°C warming plate in a sterile laminar flow hood; one culture was stretched 10.8 percent and the stretched and control pair returned to the incubator. The stretching procedure took 2 to 3 minutes per pair. The cells remained stretched and in contact with the medium containing the radioactive substances for the total 18 hours of the experiment. After the experiment the cultures were removed in pairs, rinsed rapidly three times with 1 ml of ice-cold sodium phosphate-buffered saline (0.85 percent NaCl), *p*H 7.4, and drained well, and the cells were scraped into 1.5-ml Eppendorf test tubes. To each tube was added 500 μl of 2 percent sodium dodecyl sulfate and 20 percent glycerol in 10 m*M* tris-HCl, *p*H 8.5 (*18*), and the cells were frozen at −20°C until they were processed. On processing, the samples were warmed to room temperature and homogenized five times with a 22-gauge needle and five times with a 25-gauge needle attached to a 1-ml tuberculin syringe. Portions of the homogenized cells were taken and used to measure [^{3}H]AIB accumulation by liquid scintillation counting. To a 100-μl portion, 100 μl of ice-cold 20 percent trichloroacetic acid (TCA) was added and the mixture was centrifuged at 8000*g* for 2 minutes in a Brinkmann microcentrifuge. The supernatant fraction was aspirated, and the precipitate was washed twice with 100 μl of ice-cold 10 percent TCA. The supernatant fractions were combined and a portion was taken for scintillation counting (^{14}C radioactivity soluble in TCA). The TCA precipitate was dissolved by boiling for 5 minutes in 100 μl of 0.1*N* NaOH; radioactivity was determined in a portion. All liquid scintillation counting was done with a Beckman LS250 scintillation counter with an efficiency of 24 percent for ^{3}H and 55 percent for ^{14}C. Total protein was determined by the method of Lowry *et al.* (*21*) with bovine serum albumin as the standard. Values given below are means; *N* is the total number of cultures per experiment, and Δ is the difference between control and experimental values. Statistical analyses were by Student's *t*-test for paired values.

Experiment	*N*	[^{3}H]AIB accumulation			^{14}C radioactivity soluble in TCA			^{14}C radioactivity insoluble in TCA			Total protein		
		10^5 dis/min		Δ (%)	10^5 dis/min		Δ (%)	10^6 dis/min		Δ (%)	Control (mg)	Experimental (mg)	Δ (%)
		Control	Experimental		Control	Experimental		Control	Experimental				
1	20	8.7	10.49	21.0	11.02	12.10	8.0	0.89	1.16	30.0	0.594	0.650	9.4
2	20	7.62	10.20	34.0	8.26	8.92	8.0	1.67	1.81	8.4	0.567	0.621	9.5
3	18	12.83	15.68	21.5	10.30	10.80	4.9	1.30	1.43	10.0	0.618	0.655	6.0
4	19	7.26	10.46	44.0	7.53	7.70	2.3	1.43	1.55	8.4	0.745	0.817	9.7
Mean				30*			5.8†			14.2‡			8.7‡

*Significant at $P < .01$. †Significant at $P < .05$. ‡Significant at $P < .02$.

and 84 percent, respectively, during 6 days of labeled amino acid injections (*10*), whereas the increases seen in the tissue culture system were 14.2 and 16.3 percent, respectively, after 18 hours of stretching. Accumulation of AIB over a 15-hour period increased approximately 80 percent 1 day after tenotomy in vivo (*9*) and 30 percent after 18 hours in vitro. To be sure that the increased accumulation of [^{3}H]AIB was not due to volume changes of the cells on stretching, the cellular volume was measured with [^{14}C]urea (*13*). It was found to be unchanged by 18 hours of mechanical stretch. The finding that some of the changes measured in vitro were smaller than those seen in vivo may be due to the fact that in the model system stretch of the myotubes occurred only once, whereas in vivo, the stimulus involves repeated stretch and relaxation. In organ-cultured rat diaphragm muscle the increased accumulation of AIB and decreased protein degradation are greater with both muscle stretch and electrical stimulation than with either alone (*14*).

A number of variables were tested to optimize conditions for the in vitro model system. Stretching from 7.5 to 13 percent gave results similar to stretching by 10.8 percent, but when the amount of stretch was increased to 20.8 percent all of the biochemical changes observed decreased to or became lower than the control values (data not shown). Since 5 percent CO_2 inhibits the increased metabolic rate in stretched muscle (*15*), the stretch experiments were repeated in medium buffered with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (*p*H 7.4) instead of the normal 5 percent CO_2-HCO_3 buffer system. Identical results were obtained (data not shown).

All of the experiments reported here were done with cultures containing both myotubes and fibroblasts. It is known that fibroblasts proliferate in the tenotomy model system and that some of the changes seen in skeletal muscle hypertrophy in vivo occur in the fibroblasts and not in the muscle cells [for instance increased RNA content (*16*)], whereas others occur in both muscle cells and fibroblasts [increased phosphtidylinositol turnover (*17*)]. To see whether mechanical stretch induced fibroblast proliferation, the DNA content of the cultures was measured (*18*) 18 hours after stretching. A 6.7 percent increase of the total DNA content of the culture was observed after stretch, compared to a 30 percent increase in the in vivo tenotomy model system 4 days after tenotomy (*11*). It will be important to determine which of the changes seen on mechanical stretch occur in muscle cells and which occur in fibroblasts. This distinction can be made readily with the in vitro tissue culture model system since fibroblast-free myotube cultures can be grown (*7*).

Two other tissue culture model systems for skeletal muscle hypertrophy have been developed. One involves growth in media supplemented with high levels of creatine (*19*), and the other involves long-term electrical stimulation of embryonic myotubes (*20*). It is difficult to compare all our results with those obtained in these systems, since our experiments were designed to be compared with the in vivo tenotomy model system (long-term accumulations rather than rates of incorporation). Nonetheless, some differences can be noted. In the electrical stimulation and creatine model systems, myosin heavy chain synthesis or accumulation, or both, were stimulat-

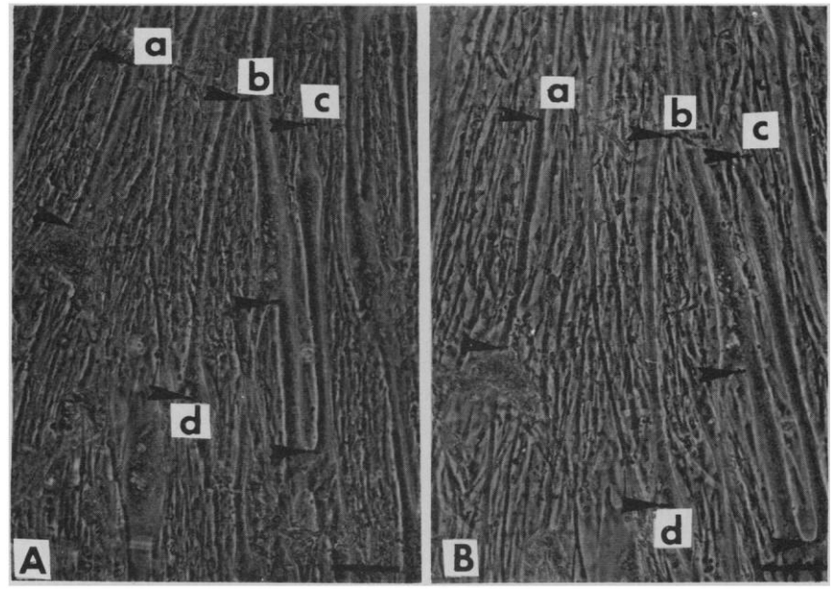

Fig. 2. Myotube cultures (72 hours) before and after mechanical stretch. Arrows labeled *a* to *d* show four myotubes with endogenous markers (for instance, myotube branch points) before (A) and after (B) a 20.8 percent stretch (scale bars, 100 μm).

Table 2. Effect of a 10.8 percent myotube stretch on myosin heavy chain labeling and accumulation. Cultures were labeled and stretched as described in the legend of Table 1. To a 50-μl portion of the homogenate, 50 μl of 16*M* urea, 10 m*M* dithiothreitol, 15 percent glycerol, and bromphenyl blue was added and the mixture was boiled for 5 minutes (*18*). A portion was placed on 5 percent polyacrylamide gels and electrophoresed with the tris-borate system of Paterson and Strohman (*22*), using a Bio-Rad model 220 slab gel electrophoresis apparatus (1.5-mm-thick gels). The gels were stained for 1 hour in 0.013 percent (weight to volume) Coomassie brilliant blue R-250, 50 percent (by volume) methanol, and 11 percent (by volume) acetic acid, and were destained in 7.5 percent acetic acid and 5 percent methanol (by volume) for 18 to 20 hours with a Bio-Rad model 222 diffusion destainer. The slabs were dried on filter paper (Bio-Rad model 224 slab drier). The myosin heavy chain band was cut out and scanned at 620 nm with a Joyce-Loebl Chromoscan in the reflectance mode. Purified myosin heavy chain from adult chicken breast muscle (*23*) was used as a standard and at least three concentrations of protein (linear range, 0.3 to 0.8 μg; correlation coefficient > .99) were used on each slab. After quantitation, the band was placed in a scintillation vial and dissolved overnight at 40°C with 30 percent H_2O_2; the ^{14}C radioactivity was then counted. Values given below are means; *N* is the total number of cultures per experiment, and Δ is the difference between control and experimental values. Statistical analyses were by Student's *t*-test for paired values.

Experiment	*N*	^{14}C radioactivity in myosin heavy chains			Myosin heavy chain		
		10^5 dis/min					
		Control	Experimental	Δ (%)	Control (μg)	Experimental (μg)	Δ (%)
1	19	0.90	1.09	21.5	33.6	41.4	23.0
2	20	1.31	1.52	16.0	72.1	80.1	11.2
3	20	1.42	1.47	3.5	53.9	59.0	9.5
4	18	0.66	0.81	24.0	75.4	88.7	15.0
Mean				16.3*			14.7†

*Significant at $P < .05$. †Significant at $P < .02$.

ed preferentially when compared to total protein (creatine system, 120 versus 0 percent for incorporation of label; electrical stimulation system, 39 versus 29 percent for incorporation of label and 29 versus 7 percent for accumulation). The stretch model system did not preferentially stimulate accumulation of labeled amino acids into myosin, but did cause a greater accumulation of myosin compared to total protein (14.7 versus 8.7 percent, respectively). Thus, the mechanism for these changes in the stretch model system may be different from the mechanism in either the elevated creatine or electrical stimulation systems. Use of a combination of experimental model systems for studying growth regulation in skeletal muscle should help to elucidate the biochemical mechanisms involved. Once they are understood, the regulatory role of the nervous system in skeletal muscle growth may be better understood.

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8. Cells were plated at a density of 4.25×10^3 per square millimeter (2×10^6 per frame) in 1 ml of 84 percent basal medium (Eagle's), 10 percent pretested horse serum, 5 percent chicken embryo extract, and 1 percent penicillin-streptomycin (50 unit/ml). Frames were machined from nylon (3.8 cm square by 0.65 cm high) and held together with stainless steel screws. One screw rotation equaled a 3.6 percent increase in the frame well diameter (initial diameter, 2.54 cm). An elastic silicone membrane (General Electric, MEM 213) was attached to the bottom and sides of the frame with RTV 102 cement (General Electric). The frames were washed with distilled water, coated with collagen (*7*), autoclaved, and preincubated for at least 4 hours with 1 ml of Earle's balanced salt solution in a 100-mm Falcon plastic petri dish.
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Phycomyces: Modification of Spiral Growth after Mechanical Conditioning of the Cell Wall

Abstract. *Mature stage IVb* Phycomyces *sporangiophores show left-handed spiral growth; that is, viewed from above, the sporangium rotates clockwise. Mechanical conditioning of the cell wall by the Instron technique increases the ratio of the rotational to the elongational growth rate. This result is in agreement with the fibril reorientation model of spiral growth, which suggests that cell wall microfibrils, initially oriented in a nearly transverse right-handed direction in the upper region of the growing zone, are displaced during growth toward the longitudinal axis, causing the observed left-handed spiral growth.*

Although many plants show some spiral growth during development, few examples are as striking as the early developmental stages of *Phycomyces* (*1–3*). The young stage I sporangiophore shows left-handed spiral growth (clockwise rotation of the sporangium, viewed from above). In stages II and III, elongational and rotational growth cease as the sporangium enlarges and matures. After the formation of the mature sporangium spiral growth is again initiated, but this time in the right-handed direction; this stage is called IVa and lasts about 90 minutes.

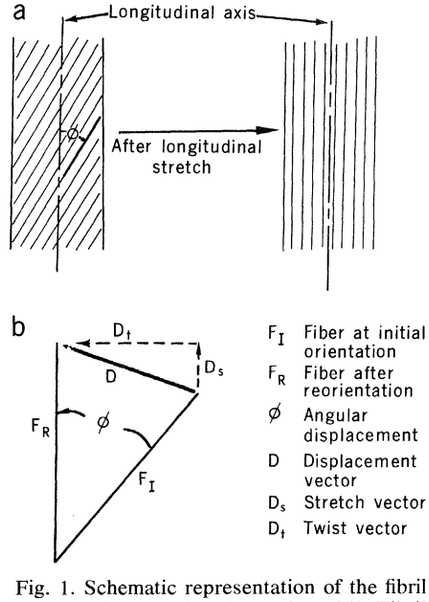

Fig. 1. Schematic representation of the fibril reorientation model [redrawn from (*4*)]. Fibril reorientation results from longitudinal stretch (a). This orientation is represented by a displacement vector **D** with components $\mathbf{D}_t$ and $\mathbf{D}_s$ (b).

During the end of this stage the rotation rate slowly decreases to zero, only to start again in the clockwise direction, which marks the last and final stage of development, called stage IVb. This entire reversal or rotation occurs during continued stalk elongation.

To our knowledge *Phycomyces* is unique in this reversal. A model to explain the mechanism of stage IVb cell wall growth has recently been proposed (*4*), based on the following two experimental findings. First, Ahlquist and Gamow (*5*) measured an abrupt change in the mechanical extensibility of the chitinous cell wall during the transition from stage II to stage IVb. During this period the cell wall changes from an essentially elastic body in stage II to a viscoelastic body in stage IVb. Second, Ortega *et al.* (*6*) found that the ratio of the rotational to the elongational growth rate was significantly larger in the lower regions of the growing zone than in the upper regions. These two experimental findings support the following model: the left-handed spiral growth of stage IVb occurs because the obliquely situated microfibrils in the primary cell wall are reorientated toward the longitudinal axis of the cell under internal turgor pressure.

Results of electron and polarizing microscopy suggest that the fibrils in the inner wall of the growing zone are either in the direction of a right-handed spiral or flatly transverse (*7–9*); in this respect the primary cell wall of *Phycomyces* is similar to the primary cell wall of most higher plants (*10*). Apparently, in the growing sporangiophore the fibrils lie in a nearly flat right-handed spiral; the measured ex-

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