Regionalized adaptations and muscle fiber proliferation in stretch-induced enlargement

S. E. ALWAY, P. K. WINCHESTER, M. E. DAVIS, AND W. J. GONYEA
Department of Cell Biology and Anatomy, University of Texas Southwestern Medical Center, Dallas, Texas 75235

ALWAY, S. E., P. K. WINCHESTER, M. E. DAVIS, AND W. J. GONYEA. *Regionalized adaptations and muscle fiber proliferation in stretch-induced enlargement.* J. Appl. Physiol. 66(2): 771–781, 1989.—The relative contribution of increases in fiber area to stretch-induced muscle enlargement was evaluated in the slow tonic fibers of the anterior latissimus dorsi of adult Japanese quails. A weight corresponding to 10% of the bird's body mass was attached to one wing. Thirty days of stretch in 34 birds averaged 171.8 ± 13.5% increase in muscle mass and 23.5 ± 0.8% increase in muscle fiber length. The volume density of noncontractile tissue increased in middle and distal regions of stretch-enlarged muscles. Mean fiber cross-sectional area increased 56.7 ± 12.3% in the midregion of stretched muscles. Further analysis indicated slow β-fiber hypertrophy occurred in proximal, middle, and distal regions; however, fast α-type fiber hypertrophy was limited to middle regions of stretched muscles. Stretched muscles had a significant increase in the frequency of slow β-fibers that were <500 μm^2 in all regions and fast α-type fibers in middle and distal regions. Total fiber number was determined after nitric acid digestion of connective tissue in 10 birds. Fiber number increased 51.8 ± 19.4% in stretched muscle. These results are the first to clearly show that muscle fiber proliferation contributes substantially to adult skeletal muscle stretch-induced enlargement, although we do not know whether the responses of the slow tonic anterior latissimus dorsi might be similar or different from mammalian twitch muscle.

muscular overload; anterior latissimus dorsi; fiber cross-sectional area; fiber length; noncontractile tissue

THE PERIOD of muscle fiber proliferation and total fiber number is considered to be determined either before or shortly after birth. This conclusion is based on studies from chick and rat muscles that have examined total fiber number in fetuses and mature animals (5, 6, 21). Thus any increase in muscle mass in adults is believed to be the result of fiber hypertrophy (10, 11).

One of the most widely used models for inducing hypertrophy in animals has been achieved by surgical ablation, tenotomy, or denervation of synergists. It has been shown that, in these models, the increase in muscle mass can be accounted for by hypertrophy of existing fibers in adult animals (8, 12, 32). However, an increase in split fibers has been observed in this model and may provide the impetus for an increase in fiber number (9, 26), although it is not clear if these fibers are in the process of splitting or fusing (12).

Another model for hypertrophy is weight-lifting exercise (9, 10, 14, 17–19). Most reports of weight-lifting-induced hyperplasia (15, 16, 19) have come from counts of histological sections using muscles with pinnate fiber arrangements. These results may not be representative of the entire muscle because it is known that not all fibers appear in cross section of a pinnately fibered muscle. However, recent evidence using direct counting after nitric acid digestion (26) as modified by Gollnick and co-workers (12) has shown a small but significant 9% increase in fiber number after weight-lifting exercise in the cat (18). In contrast, calculated mean fiber number in the biceps brachii of body builders was not different from untrained controls (23, 29). However, fiber area accounted for only 50% of the variation in muscle mass in these subjects.

The present experiments were conducted as an alternative method to surgical ablation to induce muscle hypertrophy. A stretch stimulus has been shown to induce extensive and rapid enlargement of the patagialis (7, 20) or anterior latissimus dorsi muscles (ALD) of the chicken (11, 31). In this model a weight is placed around the humerus thereby stretching the muscles of one wing (7, 11, 31). It is not clear whether fiber proliferation occurs in this model. Sola et al. (31) reported an increase in new fibers with the stretch model using counts from histological sections. In contrast, Gollnick and colleagues (11) did not find a change in fiber number. In their studies, total fiber number was determined by a technique which tweezed and counted muscle fibers under a dissection microscope after nitric acid digestion of connective tissue. They concluded that fiber area could independently account for the increase in muscle mass (11). Gollnick and associates (11), however, did document the occurrence of very small fibers in their cross sections which may have been new fibers that were too small to be seen by counting techniques employed under the low magnification of a stereomicroscope.

The purpose of these experiments was to determine if increases in fiber area could independently account for stretch-induced muscle enlargement. In the present studies the Japanese quail was used because it is a small bird and the ALD is more manageable for counting experiments. The second purpose was to determine if stretch generated uniform adaptations throughout the ALD.

METHODS

Adult Japanese quails (*Coturnix coturnix* Japonica) were obtained from a breeder (Truslow Farms, MA). The

birds were 6 wk old and weighed an average of 150 g. An accommodation period of 14 days was utilized to ensure stable body weights. The birds were housed in separate cages with 12 h of light. They received water and a diet of 18% protein (Purina turkey starter) ad libitum. The birds were weighed daily throughout accommodation and experimental periods. The bird's mass did not change over the duration of the study.

The anterior latissimus dorsi (ALD) functions to adduct and flex the humerus, so that it is brought upward and backward. The ALD of one wing was stretched for 30 days while the contralateral wing served as the intra-animal control. The stretching procedure was adapted from the model developed by Sola et al. (31). In this procedure a cardboard sleeve was placed around one wing so that it was maintained in a fully extended position (Fig. 1). The primary feathers were shortened to allow the tube to be placed over the wing to the shoulder. A gauze pad was placed between the tube and the birds' body to maintain the angle between the humerus and long axis of the thorax at 30° abduction. A lead weight was attached to the tube with tape. The weight of the tube, lead weight, and tape corresponded to 10% of the bird's body mass and this weight was not altered over the course of the experiment. This resulted in an obvious stretch to the muscles supporting the wing because the wing could not be returned to its normal resting position. The birds were able to function and eat normally and locomotion was not altered or impaired by the additional weight. Because the weighted wing was in a fully extended position, much of the stretch to the ALD would be relieved when the quail lay down. There were no apparent postural adjustments made by the unstretched contralateral wing.

The ALD arises from the supraspinous ligament of the first through fourth thoracic vertebra (Fig. 2). The posterior latissimus dorsi lies caudal to the ALD and is separated from the ALD by an aponeurosis. The ALD passes between the long and lateral heads of the triceps brachii to insert to the caudomedial aspect of the proximal portion of the humerus. After 30 days of stretch the animals were killed and the ALD muscles were removed. The birds were divided into three groups. Birds in *group A* were used for direct counts of total fiber number. The midbelly regions of ALD muscles in birds from *group B* were examined to determine fiber cross-sectional area and the volume density of noncontractile tissue. Regionalized adaptations of noncontractile tissue and fiber cross-sectional area were determined from birds in *group C*. The characteristics of the birds used in these studies are given in Table 1.

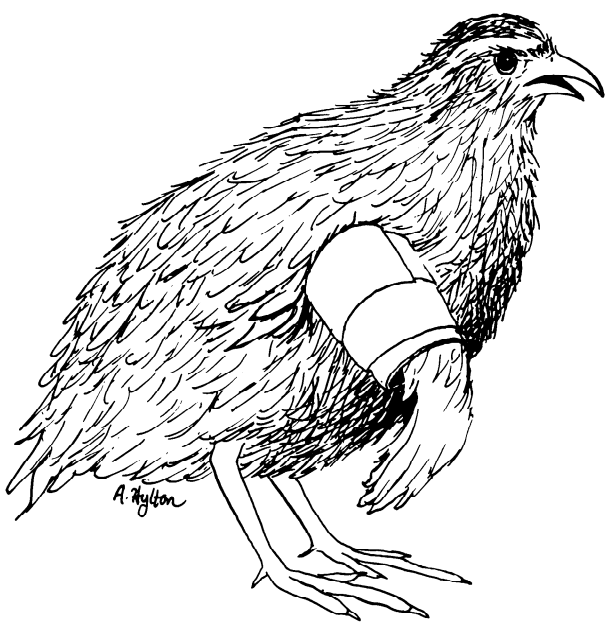

FIG. 1. Illustration of tube weight and tape attached to one wing of Japanese quail.

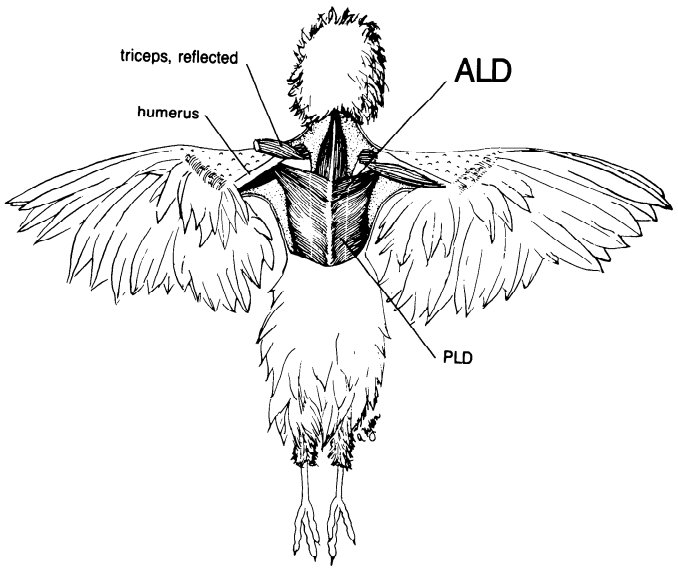

FIG. 2. Illustration of location and orientation of ALD of Japanese quail. PLD, posterior latissimus dorsi.

Experiments to Examine the Contribution of Fiber Cross-Sectional Area and Fiber Proliferation to Stretch-Induced Muscle Enlargement

Noncontractile and connective tissues. The changes in noncontractile and connective tissues were assessed from frozen histological sections stained for Gomori's trichrome (13). The tissue sections were obtained from the midbelly region of the ALD in birds from *group B*. The volume density of collagen and other noncontractile tissue was measured using an ocular square lattice grid containing lines having 121 intersection points. This was conducted under a light microscope and a minimum of 30 fields from each muscle was examined according to standard stereological techniques (33). Each grid covered an area of 65×10^3 μm^2. These results were used to correct for the noncontractile component which had accounted for part of the increase in stretch-induced muscle mass.

Fiber cross-sectional area. The ALD was removed from 12 birds from *group B* after 30 days of stretch. The muscles were cleaned of connective tissue and weighed. The midbelly of the muscle was sectioned for histochemistry. Control and stretched muscles were frozen in Freon or isopentane which was cooled to the temperature of liquid N_2. Before morphometric study, the tissue was

TABLE 1. *Characteristics of birds used for direct counts and morphometric studies*

	Birds for Direct Counts (*Group A; n* = 12)		Birds for Morphometrics (*Group B; n* = 12)		Birds for Regionalized Study (*Group C; n* = 10)	
	Before stretch	After stretch	Before stretch	After stretch	Before stretch	After stretch
Body mass, g	162.4±3.7	162.1±3.5	164.6±4.2	164.3±3.9	157.3±1.4	157.7±1.2
	C	S	C	S	C	S
Muscle mass, mg	54.5±4.3	152.4±16.2*	53.6±3.9	152.6±6.6*	54.7±3.2	143.9±11.7*

Values are means ± SE. C, control muscle; S, stretched muscle. * $P < 0.01$, control vs. stretch.

stored at −70°C in an ultrafreezer. Sections were cut at a thickness of 10 μm and stained for myosin adenosine-triphosphatase (ATPase) (3). Light micrographs were taken of the entire cross section in each of the muscle segments and a photographic montage was assembled from the micrographs.

Fiber areas were determined by planimetry from a minimum of 900 fibers from the ATPase reactions for birds in *group B*. This represents approximately 70% of the total number of fibers in the control ALD. The entire muscle was photographed at ×100 and a photographic montage assembled. The montage was composed of ~10–15 different fields which were numbered. The fields were randomly selected until areas had been determined on a minimum of 900 fibers. All the fibers encompassed in a field were traced. There was no attempt to differentiate between fiber types in these 12 birds which were sectioned only at the midbelly of the muscle.

Cross-sectional areas were determined from micrographs of ATPase reactions because it has been shown that this will not differ from areas obtained from reduced nicotinamide adenine dinucleotide tetrazolium reductase reactions (11).

Muscle fiber length. Muscle fiber length was measured in control and stretched tissue from 12 birds in *group A* that had undergone nitric acid digestion of connective tissue (12, 26). In this technique, the muscles were removed, frozen, and stored at −70°C until needed. The muscles were then placed in 20% nitric acid until the connective tissue was digested and the muscle fibers could be easily separated. The muscles were not fixed before nitric acid treatment because pilot studies indicated that fixation made the connective tissue much more resistant to digestion and the fibers were frail and fragmented upon dissection after nitric acid treatment. To account for shrinkage of the muscle fibers postexcision and due to treatment with nitric acid, muscle lengths were measured with a calibrated micrometer in situ after removal of the muscles and after digestion with nitric acid. Approximately 50 fibers from each muscle were tweezed and measured to the nearest 0.5 mm with a micrometer ocular under a dissection microscope. Muscle fiber length was corrected for shrinkage due to postexcision contraction and nitric acid digestion.

Total fiber number. Total fiber number was determined in 12 birds (*group A*) after nitric acid digestion of connective tissue (12, 26). Muscle fibers were carefully dissected from the ALD and counted under a dissection microscope. Three investigators participated in the direct counts. Fibers in the muscles of six birds were counted in a double-blind manner.

Experiments to Examine the Regionalized Adaptations of Fiber Cross-Sectional Area and Noncontractile Tissue to Stretch-Induced Muscle Enlargement

Control and stretch-enlarged ALD muscles were removed after 30 days of stretch in 10 birds from *group C*. The muscles were cleaned of excess connective tissue and weighed. The ALD was cut into three equal sections and labeled as proximal, middle, or distal segments. The segments were mounted in tragacanth gum and were frozen in Freon or isopentane which was cooled to the temperature of liquid N_2. Before morphometric study, the tissue was stored at −70°C in an ultrafreezer.

Fiber cross-sectional area. Sections were cut at a thickness of 10 μm and stained for myosin ATPase (3). Light micrographs were taken of the entire cross section in each of the muscle segments and a photographic montage was assembled. Fiber type was determined from the myosin-ATPase sections after acid preincubation at pH 4.35 and after alkaline preincubation at pH 10.45. Unlike most mammalian muscle fibers, but similar to the chicken (4, 11), the slow tonic fibers of the quail ALD do not reverse their staining pattern after acid and alkaline preincubations (Fig. 3). These fibers stain intensely for myosin-ATPase after both acid and alkaline pH preincubations. To avoid the confusion with mammalian fiber types, we have described all the fibers that had a strong ATPase reaction after both acid and alkaline preincubations as slow β-fibers (1, 27). Conversely, we have observed a second population of fibers in the ALD that appeared similar to mammalian type II fibers because they were acid-labile and alkaline-stable (see Fig. 3). This second population of fibers is referred to as fast α-type fibers. Based upon ATPase reactions, our experiments indicate that the ALD in the quail is composed of ~85% slow tonic β-fibers. Fiber cross-sectional areas for birds in *group C* were determined by planimetry from a minimum of 500 slow β-type fibers and all of the fast α-type fibers since this population usually had less than 500 fibers.

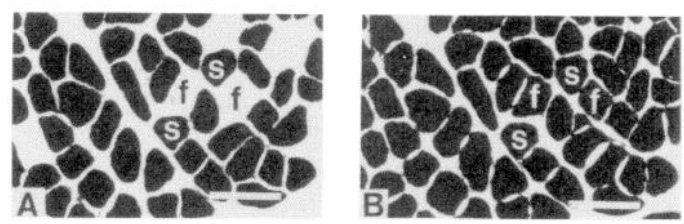

FIG. 3. Myosin-ATPase after pH preincubations of 4.35 (*A*) and 10.45 (*B*) in control ALD. f, fast α-fiber; s, slow β-fiber. Notice that slow β-fibers do not reverse staining intensity for myosin-ATPase after alkaline and acid pH preincubations. Bar, 100 μm.

Noncontractile tissue. The volume density of collagen and other noncontractile tissue was determined by stereological techniques as described above (33) for proximal, middle, and distal sections of the ALD on birds from *group C* ($n = 10$). Collagen and other noncontractile tissue were identified from frozen histological sections which were stained for Gomori's trichrome (13).

Fiber number from histological sections. Total fiber number and per cent fiber type were obtained from photographic montages of myosin ATPase reactions after acid preincubations from proximal, middle, and distal regions in 10 birds (*group C*). This data was obtained as a supplement to the direct fiber counts. Because fibers in the ALD are arranged in parallel and extend the entire length of the muscle (12), fiber numbers obtained from histological cross sections should agree with the results obtained from direct counts after nitric acid digestion.

Statistics

Descriptive statistics included means ± SE. The results of the total fiber number in control and stretched muscles that were obtained after nitric acid digestion of connective tissue were analyzed by a paired *t* test. Fiber numbers obtained from histological cross sections were compared in control and stretched muscles independently by a repeated-measures analysis of variance. Analysis of differences in fiber number among histological sections from control and stretched muscles were determined by a two-way analysis of variance (region × condition). The mean fiber areas obtained from the midbelly of control and stretched ALD muscles in birds from *group B* were compared by a paired *t* test. Comparisons of mean fiber area in the proximal, middle, and distal regions of control muscles and stretch-enlarged muscles were compared by a three-way analysis of variance (region × condition × fiber type). Regionalized adaptations of connective and noncontractile tissue were compared by a two-way analysis of variance (region × condition). The frequency of fibers <500 μm^2 were compared in control and stretched muscles by a one-group χ^2 analysis for each muscle region. $P < 0.05$ was selected to indicate statistical significance.

RESULTS

Stretch-Induced Muscle Enlargement

Thirty days of stretch in 34 birds resulted in an average 171.8 ± 13.5% (mean ± SE) (range of 92 to 286%, $P < 0.01$) increase in mass compared with the contralateral control muscles (54.1 ± 12.2 mg vs. 147.9 ± 16.8 mg). This is greater than the degree of enlargement reported in the chicken stretch model (11, 20) or exercise models (14, 15). The characteristics of the three groups of birds used in this study are given in Table 1.

The increase in ALD mass was not related to duration of stretch. Six birds were weighted for 40–53 days and the increase in ALD mass averaged 176.2 ± 4.6%. This did not differ from the remaining birds which were weighted for 30 days. Although the morphological data was obtained on these birds, their data were not included in any of the groups because the duration of stretch did not equal 30 days.

Analysis of three control birds which were not weighted has shown an average difference of 2.48 ± 0.4% in the number of fibers, 3.3 ± 0.2% in fiber area, 2.48 ± 0.1% in fiber length, and 0.8 ± 0.01% in collagen volume density between the midbelly regions of right and left ALD. There was a large interanimal variation in fiber number (14.3%) and length (10.7%).

Contribution of Fiber Cross-Sectional Area and Fiber Proliferation to Stretch-Induced Muscle Enlargement

Collagen and other noncontractile tissue. In experiments in *group B* birds ($n = 12$) that evaluated only the middle sections of the ALD, we observed a small but significant increase in collagen and noncontractile tissue. The noncontractile volume density averaged 24.2 ± 3.8% in control muscles and 32.9 ± 6.5% in stretch-overloaded muscles.

Muscle mass corrected for noncontractile tissue. The volume density of collagen and other noncontractile tissue was obtained from birds in *group B* and used for calculations of the contractile component of the ALD. When corrected for collagen the stretch-induced increase in muscle mass corresponded to 149.5 ± 3.7%. Estimates of the fiber component of the total muscle weight were obtained from the product of fiber number, average cross-sectional area, average fiber length, and percent collagen and other noncontractile tissue (Table 2).

Fiber cross-sectional area. Fiber cross-sectional area was determined in 12 birds in *group B* that had achieved a mean 184.7 ± 6.9% enlargement (uncorrected for connective tissue) in stretched compared with control muscles. Differentiation of fiber types was not obtained for these birds. Mean fiber area increased 56.7 ± 3.6%. This does not represent a simple shift to the right in the area-frequency curve indicating all fibers hypertrophied. Stretched muscles contained approximately 22% of the total fibers between 50 and 600 μm^2 compared with only 7.5% in their contralateral controls (Fig. 4*A*). This is further demonstrated when these same data are expressed as a cumulative frequency polygon (Fig. 4*B*). The origin of these small fibers is not known.

Fiber length. Muscle fiber length in the ALD were measured in tissue which had undergone nitric acid digestion. The effects of nitric acid on muscle length was determined in seven birds. The degree of shrinkage was 44.1 ± 1.8% and was similar for both control and stretched muscles. Because fibers appear to extend from origin to insertion in this muscle (11), the degree of muscle shrinkage was used to correct for fiber shrinkage. Fiber length corrected for shrinkage increased an average of 23.5 ± 0.8% (16.9 ± 0.7 vs. 20.8 ± 0.9 mm) after 30 days of stretch. These results suggest that muscle fiber length can explain part of the increase in muscle weight.

Observations of fibers during direct counts after nitric acid digestion of connective tissue indicated that most fibers in both control and stretched muscles extend the entire length of the ALD. This agrees with the observations in the chicken ALD (11).

Fiber number. Direct fiber number counts were deter-

TABLE 2. *Muscle and fiber data from anterior latissimus dorsi before and after stretch*

	Control	Stretched	% Difference
Muscle mass, mg (n = 24)	54.1±3.1	152.5±4.1*	181.8±8.9
Corrected muscle mass, mg	41.0±1.4	102.3±2.1*	149.5±3.7
Area of fibers, μm^2 (n = 12)	1,397.8±39.9	2,190.9±104.6*	57.2±4.7
Fiber length, mm (n = 12)	16.9±0.7	20.8±0.9†	23.5±0.8
Total fiber number (n = 12)	1,281±83	1,945±121*	51.8±5.6
Calculated mass/fiber, μg	32.0±0.7	52.6±5.7†	64.4±4.4

Values are means ± SE. Corrected muscle weight is calculated from noncontractile collagen elements of 24.2 and 32.9% in control and stretched muscles, respectively. Percent collagen was calculated from data in 12 birds. Calculated mass/fiber was calculated from corrected muscle mass/total fiber number. * $P < 0.01$; † $P < 0.05$ control vs. stretch.

mined after nitric acid digestion of connective tissue in 12 birds from *group A*. The characteristics of these birds are given in Table 1. The mean number of fibers in control and stretched ALD were 1,281 ± 83 (mean ± SE) and 1,945 ± 121, respectively. This represents a mean increase in fiber number of 51.8 ± 5.6%. Although a transient increase in number cannot be totally discounted, data from three birds suggest that this is not the case. In these experiments total fiber number was determined by nitric acid digestion of connective tissue after 40–53 days of stretch. This stretch interval resulted in a 171.1± 10.2% increase in muscle mass and a 53.9 ± 12.5% increase in fiber number which is not different from birds stretched for 30 days.

Regionalized Adaptations of Fiber Cross-Sectional Area and Noncontractile Tissue to Stretch-Induced Muscle Enlargement

Regionalized adaptations of collagen and noncontractile tissue. Subsequent experiments conducted in birds of *group C* (n = 10) have shown that volume density of collagen and other noncontractile tissue was similar in all regions of the control ALD (Fig. 5). The volume density of collagen and other noncontractile tissue in the distal regions of stretched muscle was significantly greater than all regions in control muscle (Fig. 5). Within the stretched muscle, the distal region had a significantly greater volume density of noncontractile elements than the proximal region (9.6%). This demonstrates a preferential increase in connective tissue in the distal regions of the ALD in response to stretch.

Regionalized adaptations of fiber cross-sectional area. To determine whether fiber area increased uniformly in both fiber types throughout the ALD, slow β- and fast α-fiber cross-sectional areas were examined in 10 birds in *group C* with a mean uncorrected mass increase of 147.9 ± 6.8 (Table 1). Fast α-fibers had smaller areas than slow β-fibers in all regions of control and stretched ALD muscles (Fig. 6). Slow β-fiber mean area increased in all regions of the ALD relative to control, however, the distal region showed less hypertrophy than either proximal or middle regions. In contrast, fast α-fibers showed hypertrophy in only the middle region relative to the control muscles (Fig. 7).

Observation of only the means may be somewhat misleading because there is a shift of the area-frequency curve to both the right and left (Figs. 8 and 9). One-group χ^2 analysis completed on the area-frequency data indicated a significant increase in the per cent of small

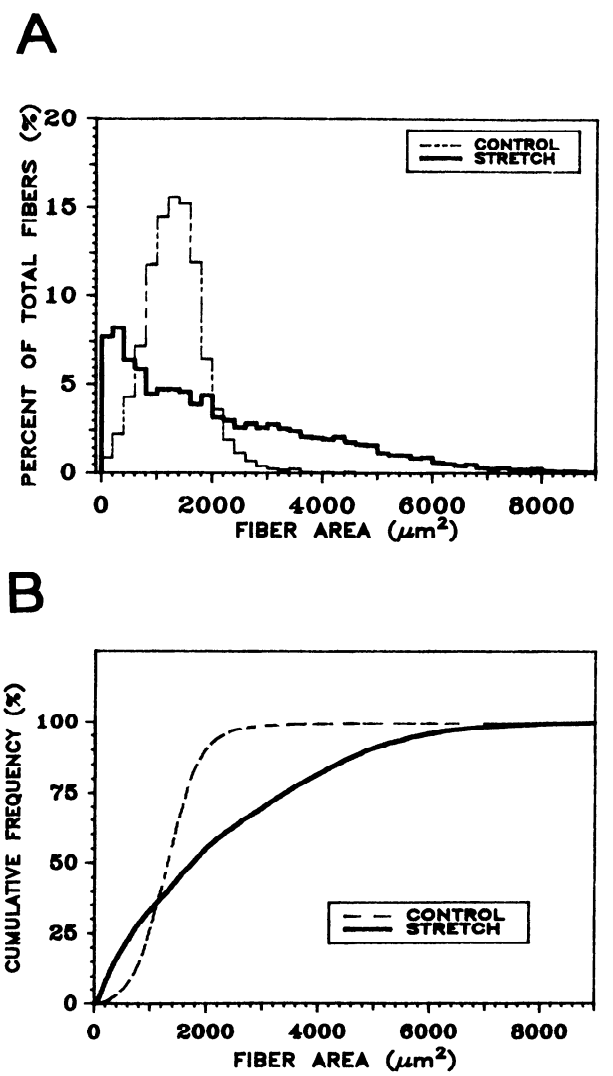

FIG. 4. Area-frequency distribution (*A*) and cumulative frequency polygon (*B*) after 30 days of stretch in control and stretch-enlarged ALD of 12 birds (from *group B*). Stretched muscles had significantly greater population of small fibers (50–600 μm^2) relative to control muscles ($P < 0.01$).

slow β-fibers in all regions of stretched muscles (Fig. 8). The frequency of slow β-fibers <500 μm^2 was ~3% in all regions of control muscles. The frequency of slow β-fibers <500 μm^2 was 11% and 13% in proximal and middle regions of stretched muscles, respectively. Of the slow β-fiber population in the distal segments, ~24% had fiber areas <500 μm^2. The frequency of fast α-fibers <500 μm^2 was significantly greater in stretched relative to control muscles in middle (37% vs. 16%) and distal

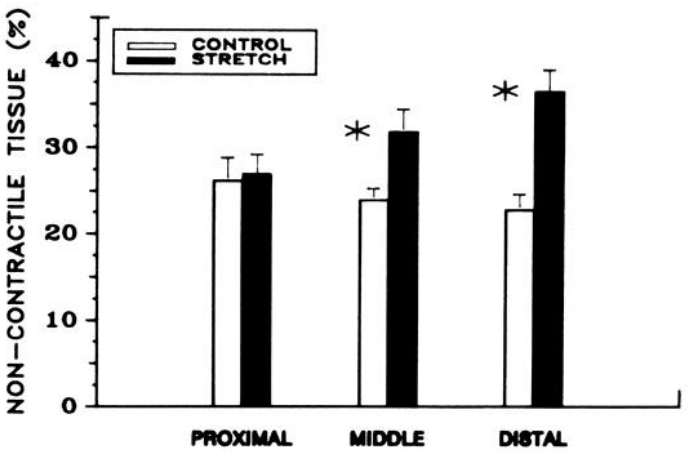

FIG. 5. Volume density of collagen and noncontractile tissue in proximal, middle, and distal regions of control and stretched ALD in 10 birds (*group C*) after 30 days of stretch. Noncontractile tissue in distal regions of stretched muscle is significantly greater than all regions in control and in proximal regions in stretched muscle, $P < 0.05$. * $P < 0.05$, control vs. stretch. Data are means ± SE.

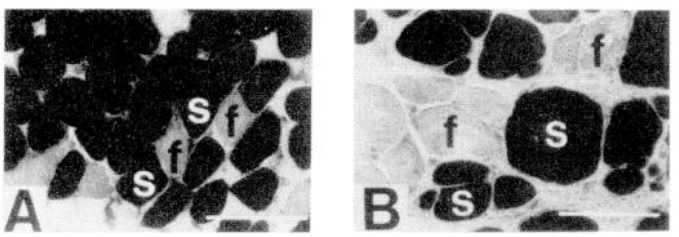

FIG. 6. Myosin-ATPase after acid preincubation in middle regions of control (*A*) and stretched (*B*) ALD. f, fast α-fiber; s, slow β-fiber. Note uniform fiber sizes in control tissue whereas stretched muscle has large ranges in fiber size. Bar, 100 μm.

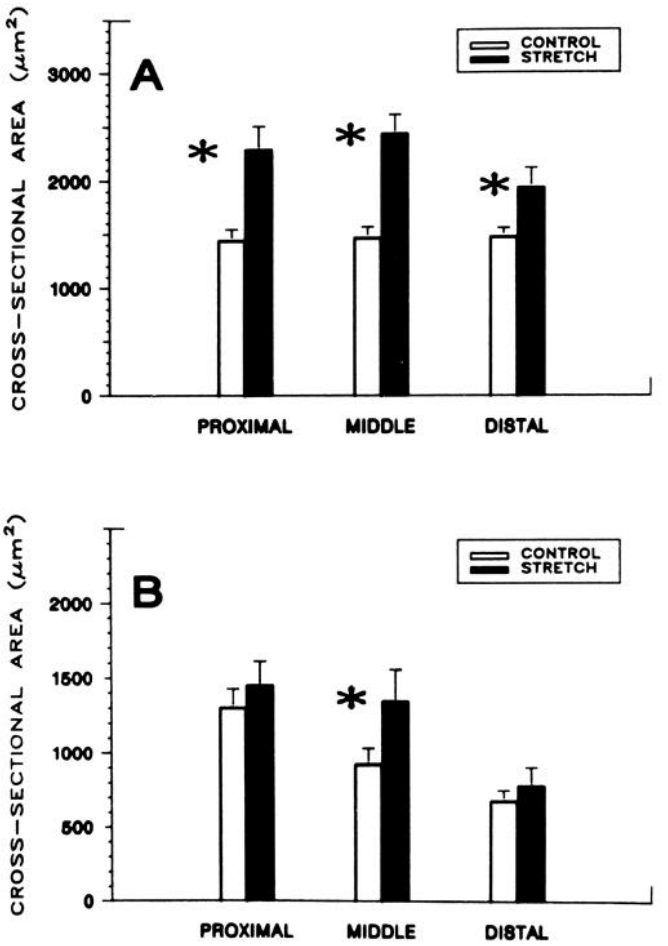

FIG. 7. Mean fiber cross-sectional area in proximal, middle, and distal regions of control and stretched ALD after 30 days of stretch. Data are means ± SE for a minimum of 500 slow β-fibers (*A*) and all of fast α-fibers (*B*) in cross section. * $P < 0.05$, control vs. stretch.

regions (57% vs. 33%). The proximal regions had ~16% of the population of fast α-fibers from control muscles with areas <500 μm^2 but this did not differ from the frequency observed in stretched muscles (12%).

Adaptations of fiber number and fiber type by regions. The percent increase in fiber number obtained from histological cross sections taken at 90° to the long axis of the muscle in control or stretched muscles was not different from the percent increase in total fiber number achieved by direct counts after nitric acid digestion. Counts from photographic montages of middle regions of the ALD indicated a 74.4 ± 18.7% increase in fiber number in stretched muscles. Analysis by repeated measures ANOVA indicated that fiber number obtained from histological cross sections was not different in proximal, middle, or distal regions of their respective control or stretched muscle (Table 3). The percent distribution of slow β-fiber types in the middle region of the ALD was 92.3 ± 2.2% in control and 82.8 ± 3.1% in stretched muscles. This represents a significant increase in the per cent of fast α-fibers in stretched muscles (Table 3).

DISCUSSION

Fiber proliferation has been reported after resistance exercise in cats (15, 18) but has led to conflicting results in the chicken stretch model (11, 31). The present experiments examined stretch-induced muscle enlargement in the ALD of the adult Japanese quail. Because the electromyographic activity does not increase as a result of stretch, enlargement of the ALD is thought to occur as a result of a stretch stimulus (20). We cannot, however, rule out some voluntary contraction of the muscles of study. Irrespective of the mechanism, the results demonstrate that increases in fiber cross-sectional area, fiber length, and noncontractile tissue do not totally account for the stretch-induced increase in muscle mass. Furthermore, the data provide evidence for proliferation of fibers in adult muscle. These results are the first to demonstrate that fiber proliferation can make major contributions to overall muscle enlargement, although we do not know if the magnitude of fiber proliferation observed in the slow tonic ALD might be similar or different from mammalian twitch muscle or hyperplasia induced by exercise. The exercise-induced increases in fiber number observed in cats (18) and the positive correlation of fiber number to muscle mass observed in elite body builders (S. E. Alway, W. H. Grumbt, W. J. Gonyea, and J. Stray-Gundersen, unpublished observations) suggest a similar hyperplastic response.

Fiber numbers obtained by histological counts were 10% and 30% greater in control and stretched muscles, respectively, than those obtained by direct counts after

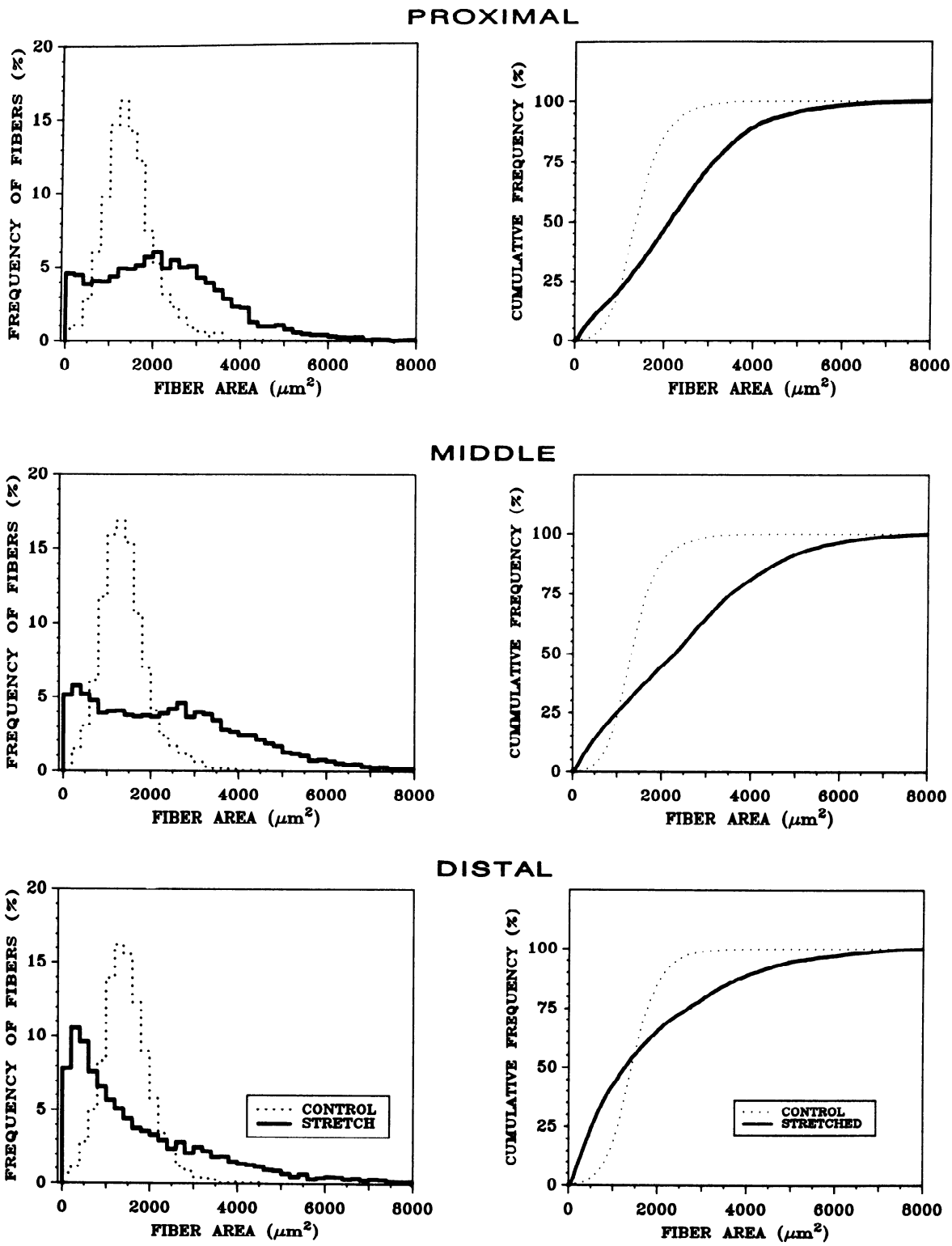

FIG. 8. Fiber area-fiber frequency histograms and cumulative frequency polygons for slow β-fibers in proximal, middle, and distal regions of control and stretched ALD in 10 birds (*group C*) after 30 days of stretch.

nitric acid digestion of connective tissue. This discrepancy is likely the result of missing many of the small fibers during direct counts under the stereomicroscope but identifying them with higher magnification in the photomicrographs. If we assumed that all slow β- and fast α-fibers <500 μm^2 were missed with direct counting techniques, this would account for 4.9% and 20.8% of all fibers (calculations from the middle region) of control and stretched ALD. The remainder of the discrepancy in histological and direct counting techniques may have been the result of missing additional small fibers that exceeded 500 μm^2. Although direct counting after nitric acid digestion is the most widely accepted technique (12, 32), this study suggests that the nitric acid technique

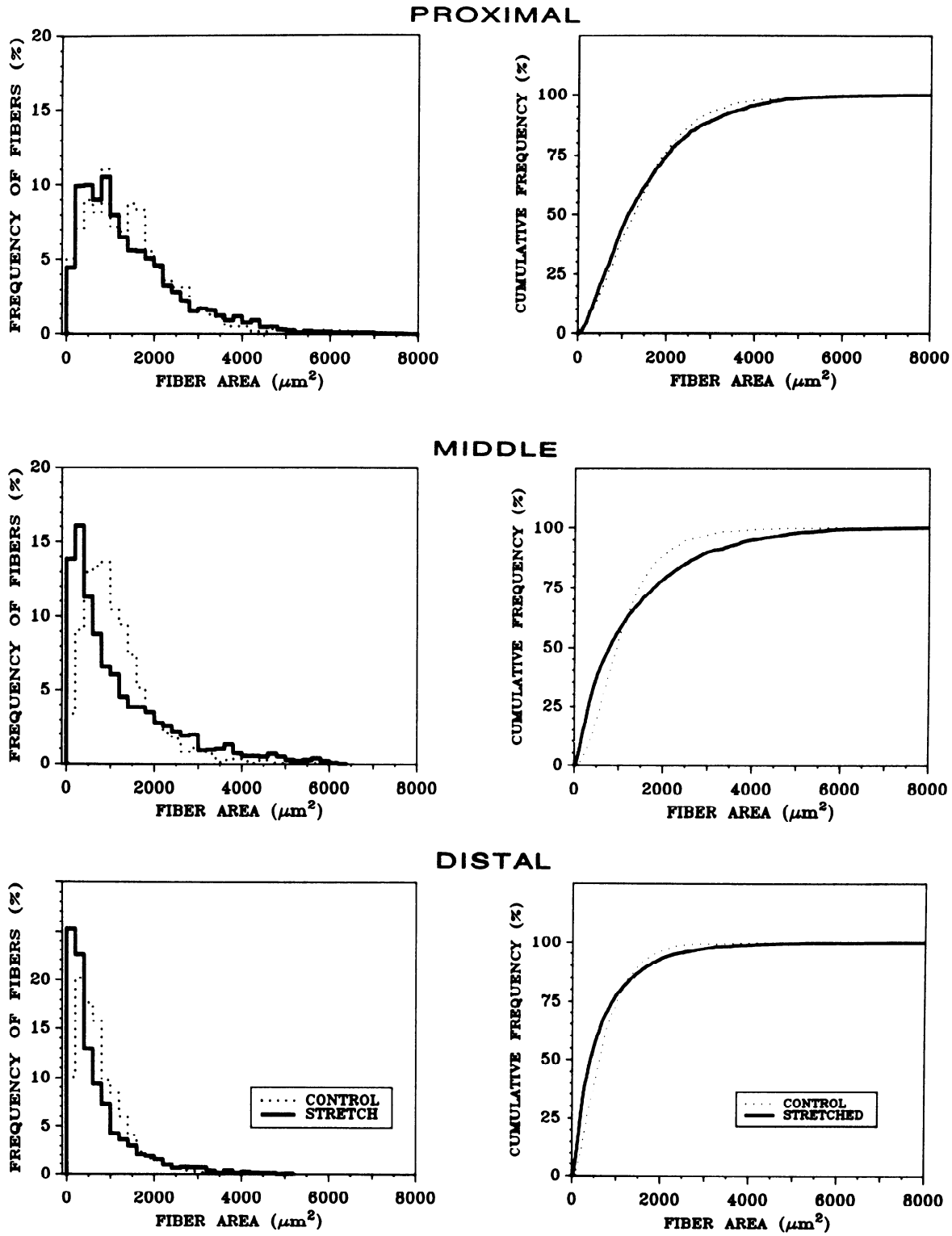

FIG. 9. Fiber area-fiber frequency histograms and cumulative frequency polygons for fast α-fibers in proximal, middle, and distal regions of control and stretched ALD in 10 birds (*group C*) after 30 days of stretch.

may miss many of the small fibers in stretch-overloaded muscles and may explain the difference in results between Gollnick and co-workers (11) and Sola and associates (31). Furthermore, the histological data of Gollnick et al. (11) demonstrated an increase of 2.5-fold in the standard error of the mean in stretched vs. control fibers. It is not clear if this increase in variance indicates that there were large as well as small fibers in the stretched muscles or if the increased variance occurred simply as a result of larger fibers. We do, however, suggest that determination of fiber cross-sectional area from as few as 50 fibers (12) may be misleading because a large sample of fibers is needed to adequately reflect the distribution and frequency of fiber areas. Important shifts in the frequency-area distribution are missed if only a mean fiber area is obtained. Possibly because the stretch

TABLE 3. *Fiber number and type from histological sections of myosin ATPase*

	Control						Stretched					
	Proximal		Middle		Distal		Proximal		Middle		Distal	
Percent slow β-fibers	93.4±2.2		92.3±2.1		93.9±2.1		83.8±3.1*		82.8±2.9*		85.1±3.1*	
Fiber type	β	α	β	α	β	α	β	α	β	α	β	α
Fiber number	1,328 ±103	94 ±37	1,338 ±105	112 ±42	1,314 ±102	86 ±34	1,980* ±294	383* ±100	2,093* ±255	436* ±100	2,083* ±301	362* ±94

Values are means ± SE. Fiber type was based upon myosin ATPase reactions at acid and alkaline pH preincubations. * $P < 0.05$, control vs. stretch in the same segment and same fiber type.

model produces greater degrees of muscle enlargement in the quail than the chicken (7, 11, 20, 31) the muscle proliferation may also be greater.

To determine whether all the components of muscle mass were accounted for, muscle mass and fiber lengths were estimated (Table 4) from the measured means and standard deviations of data presented in Table 2. Because standard deviations are normally used to describe variance in data within an animal and standard errors are used for variances of the mean within a group of animals, the standard deviation of the measured data allowed a better comparison than the standard error of the mean for estimating the expected range for a given value. Data were calculated according to the formula: muscle mass = fiber CSA × fiber number × fiber length × muscle density. The high and low ranges for length or mass were obtained by adding or subtracting, respectively, the standard deviation to the mean of each component of the above formula. Previous experiments in our laboratory have shown that the relative amount of total protein and the relative wet weight of the ALD were not different in control and stretch-enlarged muscles (24). This suggests that muscle density did not change in stretch-enlarged muscles. Thus, muscle density was assumed to be 1.06 g/cm^3 (25) for calculations of muscle mass and fiber length in both control and stretched muscles. It can be seen that the calculated fiber lengths and muscle weights (Table 4) are not statistically different from their respective measured components (Table 2) and fall within the ranges of the samples. Thus, when fiber number is considered along with fiber area, fiber length, and connective tissue, all of the stretch-induced muscle enlargement can be explained.

TABLE 4. *Calculated muscle length and mass from ranges in data*

	Control	Stretched
Calculated corrected mean muscle mass, mg	32.1	94.0*
Range in calculated corrected muscle mass, mg	21.5–49.4	52.3–153.1
Calculated mean fiber length, mm	21.6	22.6*
Range in calculated fiber length, mm	17.9–27.3	17.1–32.1

Muscle mass corrected for connective tissue was calculated from fiber number × fiber length × fiber cross-sectional area × muscle density. Fiber length was calculated from muscle mass corrected for connective tissue ÷ (fiber number × fiber cross-sectional area × muscle density). Ranges are calculated from the standard deviations given for each variable used to determine muscle weight or fiber length. * $P < 0.01$, control vs. stretch.

TABLE 5. *Determination of fiber length in stretch-enlarged ALD if fiber number did not change*

Measured and uncorrected fiber length, mm	11.7
Calculated mean fiber length, mm	34.4
Measured shrinkage in stretched muscles, %	44.1
Percent shrinkage in fiber length if fiber number did not change	66.0

Fiber length was calculated from muscle mass corrected for connective tissue ÷ (fiber number obtained after direct counts in control muscle × fiber cross-sectional area × muscle density).

It is possible that the corrections made for the degree of shrinkage after nitric acid digestion may have led to erroneous fiber lengths. We have assumed that the degree of muscle shrinkage would be the same as fiber shrinkage because all fibers appear to run the entire length of the muscle. If we presumed that fiber number did not change in the enlarged ALD poststretch, we could then estimate the fiber lengths that would be necessary to explain the results. These calculations (Table 5) indicate that if fiber number did not change, fiber length would have to be 34.4 mm. If this were true, fiber length must have shrunk by 66% from nitric acid digestion, which would represent a 22% greater shrinkage than seen in control muscles. These calculations demonstrate that it is very unlikely that an error in determining fiber length could account for all of the increases in muscle mass that have been attributed to the increases in fiber number.

The increase in small fibers seen poststretch enlargement in the ALD of the quail and also the chicken (22) are not seen in the patagialis of the chicken (7). This apparent discrepancy can be explained by the different fiber distributions in these muscles. The control ALD has more than 90% slow β-fibers whereas the patagialis has only fast α-fibers (7). Our data have shown that although there is an increase in the small fiber population of fast α-fibers, the greatest increase in small fibers is seen in slow β-fibers. This discrepancy between muscles could also be explained if we speculated that fiber hypertrophy might precede fiber proliferation during adaptation to stretch, then a patagialis enlargement of only 70% with 6 wk of stretch (7) could be largely explained by increases in fiber area. If this were true, our data would agree with Frankeny and co-workers (7), because we found almost a 60% increase in mean fiber CSA. In addition, these muscles are functionally dissimilar because the patagialis is an elbow flexor, although the ALD adducts the wing. This resulted in differences in the

manner a stretch was applied, namely, the patagialis was stretched because the elbow was straightened (7, 20); however, the ALD received its stimulus from a gravitational pull of a weight added to one wing. Thus, although the mass of both the patagialis and the ALD are thought to increase largely as the result of a passive stretch because EMG activity is not increased during these experiments (20), it is conceivable that part of the stimulus to hypertrophy could be a voluntary isometric contraction of the ALD against the resistance. Finally, an increase in fiber length may be linked to increases in fiber number, although it is not clear if fiber length could be the stimulus initiating fiber proliferation or if longer fibers are the result of a greater relative load. Studies which have demonstrated an increase in the number of small fibers and in total fiber number after stretch (2, 22, 31) have found similar increases in fiber length to that observed in the present study. Conversely, where stretch-induced enlargement has not shown fiber proliferation (7, 12) fiber length remained unchanged. Goldspink and Howells (10) and Williams and Goldspink (34) have shown that stretch is an important stimulus to increase muscle fiber length and fiber CSA in the rat, although they did not examine fiber number. Models which have induced enlargement by surgical ablation have not demonstrated changes in fiber length or fiber proliferation (12, 28, 30), although, surgical ablation models may not be comparable to the present stretch models.

Some of the small fibers in the quail may contain embryologically immature myosin similar to those observed after stretch in the chicken (22), although we did not use immunocytochemical markers necessary to make this determination. If this was the case, these new fibers must have expressed myosin ATPase characteristics exclusive to either slow β- or fast α-fibers. The rate of new fast α-fibers formation appeared to exceed the rate of slow β-fibers proliferation and supported findings of an increase in the fast myosin expression of the ALD after 30 days of stretch (24). It is not known if this increase in fast myosin corresponds to an embryonic isoform because adult and embryonic myosin isoforms comigrate. An alternate explanation is that fiber proliferation occurred as a result of both an increase in new embryonic-like fibers (22) and by fiber splitting (10) which would result in daughter cells with identical myosin expressions to the parent fiber. The present study does not allow us to differentiate between different mechanisms for fiber proliferation.

These results are the first demonstration that fiber proliferation can make major contributions to overall muscle enlargement. Fiber proliferation does not appear transient at least up to 50 days of stretch. It is important to note that the quail model of stretch-induced enlargement involves greater levels of muscular enlargement than have previously been associated with hypertrophic stimuli (in some cases 300% enlargement was achieved). We believe that this model has good potential for clarifying the mechanisms involved in postnatal fiber proliferation during muscle enlargement.

We thank Anita Hylton for illustrations in Figs. 1 and 2.

This work was supported by Biomedical Research Support Grant 2-S07-RR-07175-11 awarded to S. E. Alway by the National Institutes of Health.

Received 2 May 1988; accepted in final form 8 September 1988.

REFERENCES

1. ASHMORE, C. R., T. KIKUCHI, AND L. DOERR. Some observations on the innervation patterns of different fiber types of chick muscle. *Exp. Neurol.* 58: 272–284, 1978.
2. BARNETT, J. G., R. G. HOLLY, AND C. R. ASHMORE. Stretch-induced growth in chicken wing muscles: biochemical and morphological characterization. *Am. J. Physiol.* 239 (*Cell Physiol.* 8): C39–C46, 1980.
3. BROOKE, M. H., AND K. K. KAISER. Muscle fiber types: how many and what kind? *Arch. Neurol.* 23: 369–379, 1970.
4. BUTLER, J., E. COSMOS, AND J. BRIERLY. Differentiation of muscle fiber types in aneurogenic brachial muscle of the chick. *J. Exp. Zool.* 224: 65–80, 1982.
5. CHIAKULAS, J. J., AND J. E. PAULY. A study of postnatal growth of skeletal muscle in the rat. *Anat. Rec.* 152: 55–62, 1965.
6. ENESCO, M., AND C. P. LEBLOND. Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. *J. Embryol. Exp. Morphol.* 10: 530–562, 1962.
7. FRANKENY, J. R., R. G. HOLLY, AND C. R. ASHMORE. The effects of graded duration of stretch on normal and dystrophic skeletal muscle. *Muscle Nerve* 6: 269–277, 1983.
8. GOLDBERG, A. L. Work-induced growth of skeletal muscle in normal and hypophysectomized rats. *Am. J. Physiol.* 213: 1193–1198, 1967.
9. GOLDSPINK, G. Malleability of the motor system: a comparative approach. *J. Exp. Biol.* 115: 375–391, 1985.
10. GOLDSPINK, G., AND K. F. HOWELLS. Work-induced hypertrophy in exercised normal muscles of different ages and the reversibility of hypertrophy after the cessation of exercise. *J. Physiol. Lond.* 239: 179–193, 1974.
11. GOLLNICK, P. D., D. PARSONS, M. REIDY, AND R. L. MOORE. Fiber number and size in overloaded chicken anterior latissimus dorsi muscle. *J. Appl. Physiol.* 54: 1292–1297, 1983.
12. GOLLNICK, P. D., B. F. TIMSON, R. L. MOORE, AND M. REIDY. Muscular enlargement and number of fibers in skeletal muscles of rats. *J. Appl. Physiol.* 50: 936–943, 1981.
13. GOMORI, G. A rapid one-step trichrome stain. *Am. J. Clin. Pathol.* 20: 661–664, 1950.
14. GONYEA, W. J. Muscle fiber splitting in trained and untrained animals. *Exercise Sports Sci. Rev.* 8: 19–39, 1980.
15. GONYEA, W. J. The role of exercise in inducing skeletal muscle fiber splitting. *J. Appl. Physiol.* 48: 421–426, 1980.
16. GONYEA, W. J., G. C. ERICSON, AND F. BONDE-PETERSEN. Skeletal muscle fiber splitting induced by weight-lifting exercise in cats. *Acta Physiol. Scand.* 99: 105–109, 1977.
17. GONYEA, W. J., D. SALE, AND J. A. DIXON. Increases in muscle fiber number in response to weight-lifting exercise. *Med. Sci. Sports Exercise* 14: 111–113, 1982.
18. GONYEA, W. J., D. SALE, F. GONYEA, AND A. MIKESKY. Exercise induced increases in muscle fiber number. *Eur. J. Appl. Physiol. Occup. Physiol.* 55: 137–141, 1986.
19. HO, K. W., R. ROY, C. D. TWEEDLE, W. W. HEUSNER, W. D. VAN HUSS, AND R. E. CARROW. Skeletal muscle splitting with weight-lifting exercise in rats. *Am. J. Anat.* 157: 433–440, 1980.
20. HOLLY, R. G., J. G. BARNETT, C. R. ASHMORE, R. G. TAYLOR, AND P. A. MOLÉ. Stretch-induced growth in chicken wing muscles: a new model of stretch hypertrophy. *Am. J. Physiol.* 238 (*Cell Physiol.* 7): C62–C71, 1980.
21. KATSUTA, S. Light and electron microscopic studies in the postnatal development of skeletal muscle fibers in rats. *Jpn. J. Physiol.* 24: 201–208, 1979.
22. KENNEDY, J. M., B. R. EISENBERG, S. KAMEL, L. J. SWEENY, AND R. ZAK. Nascent muscle fibers appearance in overloaded chicken slow-tonic muscle. *Am. J. Anat.* 181: 203–215, 1988.
23. MACDOUGALL, J. D., D. G. SALE, S. E. ALWAY, AND J. R. SUTTON. Muscle fiber number in biceps in bodybuilders and control subjects. *J. Appl. Physiol.* 57: 1399–1403, 1984.
24. MATTHEWS, W., R. J. JENKINS, AND W. J. GONYEA. Myosin isozyme expression in response to stretch-induced hypertrophy in

the Japanese quail. *Anat. Rec.* In press.
25. MENDEZ, J., AND A. KEYS. Density and composition of mammalian muscle. *Metabolism* 9: 184–188, 1960.
26. REITSMA, W. Skeletal muscle hypertrophy after heavy exercise in rats with surgically reduced muscle function. *Am. J. Phys. Med.* 48: 237–257, 1969.
27. ROUAND, T., AND J. P. TOUTANT. Histochemical properties and innervation pattern of fast and slow-tonic fibre types of the anterior latissimus dorsi muscle of the chick. *Histochem. J.* 14: 415–428, 1982.
28. ROY, R. R., I. D. MEADOWNS, K. M. BALDWIN, AND V. R. EDGERTON. Functional significance of compensatory overloaded rat fast muscle. *J. Appl. Physiol.* 52: 473–478, 1982.
29. SALE, D. G., J. D. MACDOUGALL, S. E. ALWAY, AND J. R. SUTTON. Voluntary strength and muscle characteristics in untrained men and women and male bodybuilders. *J. Appl. Physiol.* 62: 1786–1793, 1987.
30. SNOW, M. H., AND B. S. CHORTKOFF. Frequency of bifurcated muscle fibers in hypertrophic rat soleus muscle. *Muscle Nerve* 10: 312–317, 1987.
31. SOLA, O. M., D. L. CHRISTENSEN, AND A. W. MARTIN. Hypertrophy and hyperplasia of adult chicken anterior latissimus dorsi muscles following stretch with and without denervation. *Exp. Neurol.* 41: 76–100, 1973.
32. TIMSON, B. F., B. K. BOWLIN, G. A. DUDENHOEFFER, AND J. B. GEORG. Fiber number, area and composition of mouse soleus muscle following enlargement. *J. Appl. Physiol.* 58: 619–624, 1985.
33. WEIBLE, E. R. *Stereological Methods.* New York: Academic, 1979, vol. 1.
34. WILLIAMS, P., AND G. GOLDSPINK. Connective tissue changes in immobilized muscle. *J. Anat.* 138: 343–350, 1984.