AUTORADIOGRAPHIC LOCALIZATION
OF NEW RNA SYNTHESIS IN
HYPERTROPHYING SKELETAL MUSCLE

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ABSTRACT

Work-induced growth of rat soleus muscle is accompanied by an early increase in new RNA synthesis. To determine the cell type(s) responsible for the increased RNA synthesis, we compared light autoradiographs of control and hypertrophying muscles from rats injected with tritiated uridine 12, 24, and 48 h after inducing hypertrophy. There was an increased number of silver grains over autoradiographs of hypertrophied muscle. This increase occurred over connective tissue cells; there was no increase in the number of silver grains over the muscle fibers. Quantitative studies demonstrated that between 70 and 80% of the radioactivity in the muscle that survived fixation and washing was in RNA. Pretreatment of the animals with actinomycin D reduced in parallel both the radioactivity in RNA and the number of silver grains over autoradiographs. Proliferation of the connective tissue in hypertrophying muscle was evident in light micrographs, and electron micrographs identified the proliferating cells as enlarged fibroblasts and macrophages; the connective tissue cells remained after hypertrophy was completed. Thus, proliferating connective tissue cells are the major site of the increase in new RNA synthesis during acute work-induced growth of skeletal muscle. It is suggested that in the analysis of physiological adaptations of muscle, the connective tissue cells deserve consideration as a site of significant molecular activity.

INTRODUCTION

Goldberg (1971) recently reviewed the biochemical events during skeletal muscle hypertrophy and summarized the observations which suggest that both new and increased RNA syntheses are important prerequisites for work-induced growth of skeletal muscle fibers. That new RNA synthesis is essential is supported by the observation that treatment of animals with actinomycin D blocks work-induced growth of skeletal muscle (Goldberg and Goodman, 1969). Evidence that increased RNA synthesis is essential is indirect. During work-induced growth, there is a marked increase in the total RNA content and RNA concentration in muscle (Hamosh et al., 1967). Furthermore, an increased rate of incorporation of RNA precursors precedes the accumulation of RNA and declines before hypertrophy is complete (Sobel and Kaufman, 1970; Goldberg, 1971). Because the con-

centration and specific activity of the RNA precursor pool in homogenates of control and hypertrophying muscles is the same (Sobel and Kaufman, 1970), the increased incorporation strongly suggests increased RNA synthesis. Thus it has been proposed (Sobel and Kaufman, 1970) that muscular activity sufficient to produce hypertrophy leads to new and increased RNA synthesis that is essential to the growth of muscle fibers. This theory rests, however, on the assumption that the metabolic activity of the connective tissue in muscle has not contributed significantly to the documented increase in RNA synthesis. To examine the validity of that assumption, the present study was undertaken to localize by autoradiography the site(s) of the increase in new RNA synthesis. Initial experiments were performed to determine the optimal procedure for labeling RNA in vivo and preserving it for autoradiography. Thereafter, a portion of each muscle was fractionated to determine the molecular species that gave rise to the silver grains in the autoradiographs.

MATERIALS AND METHODS

Model of Skeletal Muscle Hypertrophy

Compensatory hypertrophy of the soleus muscle was induced by tenotomy of synergistic gastrocnemius and plantaris muscles in 250–300 g Sprague-Dawley rats as previously described (Hamosh et al., 1967). A sham operation was performed on the contralateral limbs.

In Vivo Labeling of RNA

To investigate skeletal muscle RNA synthesis, rats were injected with tritiated uridine or orotic acid (New England Nuclear, Boston, Mass.) subcutaneously while the animals were under light ether anesthesia. 4 h later, rats were sacrificed by cervical fracture and the soleus muscles rapidly excised, rinsed in 0.85% saline, chilled, and weighed on a Roller-Smith torsion balance (Roller-Smith Inc., Newark, N.J.). 50–60 mg of muscle were excised from the proximal and distal ends and weighed, and each sample was separately homogenized in 10 ml 0.2 M perchloric acid in an all-glass Duall grinder (Kontes Glass Co., Vineland, N.J.).

Fractionation of Fresh Muscle Homogenate

The homogenate was fractionated according to a modified Munro-Fleck (1966) procedure. After the initial centrifugation, the precipitate was washed three times with 0.2 M perchloric acid to make a total of 40 ml of supernatant fraction designated the "acid

soluble" fraction. The RNA was solubilized by incubation of the precipitate with 4 ml of 0.4 N NaOH for 1 h at 37°C. The digest was cooled and the non-RNA material precipitated with 2.5 ml of 1.2 M perchloric acid. After centrifugation, the pellet was washed twice with 0.2 M perchloric acid to make a total of 10 ml of supernatant fraction designated the "RNA" fraction. The RNA content of the muscle sample was determined by the absorbance at 260 nm of this fraction, using similarly digested yeast RNA (Type III, Sigma Chemical Co., St. Louis, Mo.) as a standard. The remaining acid precipitate was washed once with 10 ml of a 1:1 mixture of ether:ethanol, dried, and solubilized in 0.5 ml of 30% H_2O_2 by incubation at 50°C overnight. This fraction was designated the "remaining acid precipitate" and together with the RNA fraction constituted the "total acid precipitate."

Determination of Radioactivity in the Muscle Fractions

1 ml each of the acid soluble and RNA fractions, and the entire solubilized remaining acid precipitate were added separately to 10 ml of counting fluid containing Fluoralloy formula TLA and Bio-Solv Solubilizer BBS-3 (Beckman Instruments, Inc., Fullerton, Cal.) in toluene, in the ratio of 0.032:1:5 (w/v/v); radioactivity was determined in a Beckman scintillation counter. This method yielded a counting efficiency of between 35 and 38% for tritium. All counts were converted to disintegrations per minute per milligram of wet weight muscle. The percent of the total acid precipitate radioactivity incorporated into RNA was calculated from the ratio of the radioactivity in the RNA fraction to that in the total acid precipitate.

Fixation and Washing

Muscles for autoradiography were carefully dissected, mounted under slight tension, and immersed in 10 ml of 6% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 20 mM $CaCl_2$, for 18 h at 4°C, with constant shaking. Most of the radioactive RNA precrusors were removed by washing the muscle over a period of 5 days with 12 changes of the same sodium cacodylate buffer containing $CaCl_2$. The efficiency of the procedure is illustrated below. A few small blocks were trimmed from the fixed, washed muscle for OsO_4 fixation and autoradiography, and portions of the muscle adjacent to those selected for autoradiography were analyzed as follows.

Fractionation of Fixed Muscle Homogenate

To determine the molecular species that gave rise to the silver grains in the autoradiographs, 30–40 mg

portions of the fixed and washed muscles were homogenized and fractionated, and the radioactivity in the fractions was determined by the same method employed for fresh muscle, except for minor changes: (a) the muscle was homogenized and washed once with 5 ml portions of 0.2 M perchloric acid to make a total of 10 ml supernatant designated the acid soluble fraction; and (b) since both control and hypertrophied muscle lost about 15% of their wet weight during fixation and washing, the weights of fixed muscles were corrected by 15% to make the scintillation counting data obtained on the fixed muscle samples comparable with the data obtained on fresh muscle. Preliminary experiments (described below) showed that glutaraldehyde fixation did not affect the fractionation procedure.

Autoradiography

After additional fixation for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) with 20 mM $CaCl_2$, samples were rinsed several times in the same buffer, dehydrated in ethanol, and embedded in Epon 812 according to Luft (1961). Sections (0.5-1.0 μm thick) were mounted on slides and dipped in Kodak NTB2 emulsion that had been diluted 1:1 with water. The slides were stored for 10-20 wk in light-tight boxes containing anhydrous $CaSO_4$ at 4°C. They were developed for 2 min in Kodak D19 developer that had been diluted 1:1 with water, and examined by phase-contrast microscopy. The frequency of grains over muscle cells, fibroblasts, blood vessels (arterioles and venules), and capillaries (endothelial cells and pericytes), as well as the total number of grains over each cell type, was determined by examination of 10 high-power fields (magnification $\times$1,600) of each sample. The statistical significance of the difference between the number of grains over the different cell types in autoradiographs of the hypertrophied and control muscle was determined by the Student's t test. The fractionation of glutaraldehyde-fixed muscles was performed before fixation with OsO_4 because osmium interfered with scintillation counting. Autoradiographs of muscles before and after osmium fixation, however, were similar and this similarity suggests that large amounts of radioactivity are not lost during osmium fixation.

RESULTS

Isotopic Labeling of RNA In Vivo

Our earlier studies of RNA synthesis during skeletal muscle hypertrophy used tritiated orotic acid as the RNA precursor (Sobel and Kaufman, 1970). In similar experiments, however, we found that orotic acid is not particularly suitable for autoradiography because it does not yield muscle

RNA of high radioactivity: pulse labeling with 0.5 mCi [5-³H]orotic acid yields 40-50 dpm/mg wet weight muscle in the total acid precipitate; 75% of this radioactivity was in the RNA fraction. Therefore, we examined the extent and specificity of labeling RNA in vivo with tritiated uridine, the pyrimidine riboside that is unique to RNA. Comings (1966) had found that when [5-³H]-uridine was added to the media of cultured fibroblasts, the specificity of labeling RNA was improved when he provided an excess of DNA precursors at the time of labeling. We have observed a similar phenomenon during the in vivo incorporation into skeletal muscle RNA of uridine bearing tritium in different positions (Table I). The simultaneous injection of unlabeled thymidine (1 mg/g of body weight) increased the specificity of the labeling of RNA with no change in the ex-

TABLE I

Effect of Thymidine on the Incorporation of Various Tritiated Uridines into Rat Soleus Muscle RNA

Isotope and addition	Radioactivity in RNA fraction	Radioactivity in RNA*
	dpm/mg wet weight	%
0.5 mCi [³H]uridine (G) (8.8 Ci/mmol)		
No thymidine	28 ± 3	48 ± 2
Thymidine	33 ± 3	88 ± 1
1.0 mCi [5-³H]uridine (25 Ci/mmol)		
No thymidine	82 ± 10	51 ± 2
Thymidine	92 ± 9	69 ± 3
1.0 mCi [6-³H]uridine (25 Ci/mmol)		
No thymidine	150 ± 18	48 ± 2
Thymidine	154 ± 20	84 ± 3

Thymidine (1 mg/g body weight) was dissolved in distilled water (50 mg/ml) and, when given, injected subcutaneously immediately after the injection of the isotope. 4 h after injection of the isotope, unoperated animals were sacrificed and the proximal end of the soleus muscle was homogenized and fractionated as described in the Methods. Each value is the mean ± SEM of data from six animals. Note that thymidine injected with the various tritiated uridines significantly increased the specificity of labeling RNA in vivo without a change in the radioactivity of the RNA fraction. G refers to generally labeled.

* The ratio ($\times$ 100) of the radioactivity in the RNA fraction to that in the total acid precipitate.

tent of RNA labeling. Raising the thymidine dose to 4 mg/g or using cytidine (1 mg/g) did not further improve the specificity of uridine incorporation, nor did shortening the time of pulse labeling to 2 h or lengthening it to 6 h. We have not attempted to determine the extent to which the non-RNA radioactivity found in the acid precipitate fraction represents labeled uridine transformed into a DNA precursor and incorporated into DNA (Comings, 1966) and/or tight binding of labeled uridine to protein, as Morley and Kingdon (1972) demonstrated for thymidine. Interestingly, [6-³H]uridine was found to be slightly more specific than [5-³H]uridine for labeling RNA, with 84 vs. 69% of the incorporated radioactivity in RNA, and to make RNA more radioactive, with 154 vs. 92 dpm/mg wet weight muscle (both with $P < 0.10$). Thus, for the localization of new RNA synthesis by autoradiography, [6-³H]uridine with unlabeled thymidine (1 mg/g of body weight) was injected 4 h before sacrificing the animal. We verified that the greatest increase in net incorporation of [6-³H]uridine into RNA of hypertrophying muscles occurred 2 days after tenotomy; the time of this maximal increase is the same as that already documented with [5-³H]orotic acid (Sobel and Kaufman, 1970).

Effect of Fixation and Washing of Muscle on Free and Incorporated RNA Precursors

In a preliminary experiment, muscle RNA was labeled in vivo with either [6-³H]uridine or [5-³H]-orotic acid, the muscles were homogenized, and half of the homogenate was fixed with glutaraldehyde for comparison with the unfixed half. There was no difference in the radioactivity and ultraviolet absorbance at 260 nm of the RNA fractions from fixed and unfixed homogenates. These results indicated that glutaraldehyde preserved most of the RNA in the muscle homogenate and, moreover, that it did not interfere with the fractionation procedure or with the determination of the RNA content and radioactivity.

To determine the effect of fixation and washing of whole muscles on the distribution of free and incorporated [6-³H]uridine, four sets of paired soleus muscles were labeled in vivo, and one muscle of each pair was fixed with glutaraldehyde before fractionation (Table II). About 95% of the label present in the fractionated fresh muscles was in the acid soluble fraction and presumably would have interfered with the autoradiographic localization

TABLE II

Effect of Fixation and Washing on the Distribution of Radioactivity in the Various Fractions of Paired Soleus Muscles from Normal Rats Injected with Tritiated RNA Precursors

| Isotope injected | State of muscle fractionated | Radioactivity in fraction | | | Radioactivity in RNA* |
		Acid soluble	RNA	Remaining acid precipitate	
		dpm/mg wet weight muscle			%
Exp I					
1 mCi [6-³H]uridine	(a) fresh	2650 ± 73	155 ± 29	60 ± 5	72 ± 2
(25 Ci/mmol) with 250 mg thymidine	(b) fixed	30.4 ± 4.1	136 ± 30	64 ± 4	68 ± 2
Exp II					
0.5 mCi [5-³H]orotic	(a) fresh	969 ± 86	25.7 ± 1.5	7.4 ± 0.5	78 ± 1
acid (12.2 Ci/mmol)	(b) fixed	4.3 ± 0.3	20.9 ± 0.9	32.7 ± 2.1	38 ± 1

Unlabeled thymidine was injected with tritiated uridine as described in Table I, and the unoperated animals were sacrificed 4 h after injection of the isotope. One of each paired soleus muscles was removed, weighed, and the proximal half was fractionated immediately for comparison with the contralateral limb soleus muscle which was fixed and washed before fractionation. Details of the glutaraldehyde fixation and sodium cacodylate buffer (pH 7.4) washing procedure of the whole muscle, the fractionation method, and the determination of radioactivity in each fraction are given in the Methods. Each value is the mean ± SEM of values from four paired muscles. Note that fixation and washing preserved between 80 and 90% of the labeled RNA and removed about 99% of the unincorporated isotope.

* The ratio (× 100) of the radioactivity in the RNA fraction to that in the total acid precipitate.

of incorporated label if it were not removed. Repeated washes of the muscles in 0.1 M sodium cacodylate buffer (pH 7.4), however, removed most of this unincorporated label. After 12 washes in buffer over a period of 5 days, fixed muscles released only 1% as much radioactivity into the acid soluble fraction as did muscles fractionated before fixation. This method for elimination of the unincorporated isotope was considered adequate, since after the repeated washes with buffer only about 10% of the total radioactivity in the fixed muscle was released into the acid soluble fraction. About 80-90% of the radioactivity incorporated into RNA was preserved during fixation and repeated buffer washing. Since no loss was observed during fixation of muscle homogenates (described above), the slight loss probably reflects a difference in the accessibility to glutaraldehyde of RNA in whole muscles compared with that of RNA in a muscle homogenate. Finally, no free isotope in muscles labeled with [6-³H]uridine in vivo appeared to be deposited by the glutaraldehyde fixation into the remaining acid precipitate; at least fixed muscles had no more radioactivity in this fraction than unfixed muscles. It is apparent in this experiment that nearly 70% of the incorporated [6-³H]uridine retained by fixation was in RNA.

Similar results were obtained with muscles labeled in vivo with [5-³H]orotic acid, except that there was one important difference. The extent of nonspecific deposition of radioactivity in vivo into the remaining acid precipitate was significant and reduced the specificity of RNA labeling from 70% in unfixed muscles to 40% in fixed muscles (Table II). We have no explanation for the difference between the extent of nonspecific deposition of [5-³H]orotic acid into the remaining acid precipitate compared with that of [6-³H]uridine, particularly since this magnitude of nonspecific binding of [5-³H]orotic acid occurred only after in vivo labeling of muscle.

Effect of Actinomycin D on [6-³H]Uridine Incorporation into Muscles

The effect of actinomycin D on the in vivo incorporation of [6-³H]uridine provided independent evidence that most of the silver grains in autoradiographs of muscles from animals injected with [6-³H]uridine and thymidine represented radioactivity in RNA. 2 days after tenotomy,

actinomycin D in doses sufficient to block muscle RNA synthesis in vitro (Breuer and Florini, 1965) was administered 4 h before pulse labeling with [6-³H]uridine. The incorporation of radioactivity into the RNA fraction was reduced to about 30% of that found in the corresponding sham-operated and tenotomized limb soleus muscles of a control set of animals (Table III), whereas the incorporation of label into the remaining acid precipitate was unaffected. A parallel reduction in the numbers of silver grains in autoradiographs of sections of muscles from the animals injected with actino-

TABLE III

Effect of Actinomycin D on the Incorporation of [6-³H]Uridine into the RNA Fraction and the Grain Count over Corresponding Autoradiographs from Control and Hypertrophied Soleus Muscles

Soleus	Radioactivity in RNA fraction	Grains over autoradiographs
	dpm/mg wet weight muscle	*grains/hpf**
Saline-injected animals		
Control	99 ± 10	28 ± 6
Hypertrophied	164 ± 20	46 ± 5
Actinomycin D-treated animals		
Control	30 ± 8	21 ± 4
Hypertrophied	47 ± 8	23 ± 4

All animals were injected with 1 mCi [6-³H]uridine (25 Ci/mmol) with thymidine (1 mg/g body weight) 48 h after unilateral tenotomy. The actinomycin D-treated group of animals was injected intraperitoneally with actinomycin D (0.5 mg/kg body weight, Sigma Chemical Co., dissolved in 5 ml 0.85% saline) 4 h before injection of the tritiated uridine with unlabeled thymidine. Control animals were treated similarly with saline. All animals were sacrificed 4 h after injection of the isotope. The soleus muscles were removed, fixed, and washed and small portions were removed from the proximal end of the control and hypertrophied muscles for autoradiography and RNA fractionation as described in the Methods. Both control and hypertrophied soleus muscles in each group had about 25 dpm/mg wet weight as non-RNA acid-precipitate radioactivity. Note that actinomycin D pretreatment reduced the radioactivity in the RNA fraction of the control and hypertrophied muscles and the grain count over autoradiographs from the same muscles in a parallel fashion. Each value is the mean ± SEM of data from four animals.
* Corrected for background.

mycin D compared with the saline-injected control set of animals was also noted (Table III). The similar effect of actinomycin D on the incorporation of [6-³H]uridine into RNA and on the number of silver grains in corresponding autoradiographs strongly suggests that [6-³H]uridine incorporated into RNA accounts for the production of silver grains in the autoradiographs.

Autoradiography

Initially, we analyzed by autoradiography sections taken from the central portions of paired control and hypertrophied muscles 2 days after the tenotomy operation (Table IV), when the increase in the net rate of incorporation of RNA precursors is at a maximum (see above). As before, we fractionated 30–40 mg of the surrounding fixed muscle and found 85 and 81% of the incorporated [6-³H]-uridine in the RNA fraction of the hypertrophied and control muscle samples, respectively. By correction of the total number of grains per high-power field for background, we calculated that there were approximately 52 and 12 grains over comparable areas of sections of hypertrophied and

control muscles, respectively, due to incorporated [6-³H]uridine. The difference between these two figures, 40 grains/high power field (hpf), represented the number of grains over the section of the hypertrophied muscle that was not due to the control rate of incorporation of [6-³H]uridine and is, therefore, due to work-induced growth. The difference of 40 grains/hpf is about three times greater than the number of grains, 12 grains/hpf, calculated to represent the net rate of incorporation of [6-³H]uridine in the control muscle (see above). We also computed the difference between the number of grains over each cell type in paired autoradiographs of control and hypertrophied muscles. This calculation furnished the cellular distribution of the grains in the hypertrophied muscle autoradiographs due to hypertrophy. Surprisingly, more than 95% of the increase appeared over connective tissue cells (Figs. 1–4). The large blood vessels and muscle fibers showed no significant increase in labeling (Table IV). It was concluded that most of the increased [6-³H]uridine incorporation in hypertrophied muscle was due to increased RNA synthesis in connective tissue. The

TABLE IV

Mean Grain Counts over Different Cell Types in Paired Control and Hypertrophied Soleus Muscles of Rats Labeled In Vivo with [6-³H] Uridine 2 Days after Unilateral Tenotomy

	Soleus			Total difference
	Hypertrophied	Control	Difference	
	grains/hpf			%
Cell type				
Muscle	27.5 ± 1.3	26.5 ± 1.8	1.0 ± 1.6	---
Capillary	23.2 ± 6.1	8.2 ± 1.7	15.0 ± 4.8*	37
Fibroblast	26.5 ± 5.7	2.0 ± 1.0	24.5 ± 8.9*	61
Total	77.2 ± 10.2	36.7 ± 2.1	40.5 ± 14.6*	

Thymidine (1 mg/g body weight) was injected with 1.0 mCi [6-³H]uridine (25 Ci/mmol) 2 days after tenotomy. The animals were sacrificed 4 h later. After glutaraldehyde fixation and sodium cacodylate buffer (pH 7.4) washing, specimens for autoradiography and RNA fractionation were taken from the middle of control and hypertrophied muscles. Evidence of muscle cell hypertrophy was seen in the autoradiographs in that there were 13.1 ± 1.0 muscle cells/hpf for the hypertrophied muscles compared to 15.3 ± 1.1 muscle cells/hpf for the control muscles (Δ = 2.2 ± 0.6, $P < 0.02$). Each grain count is the mean ± SEM of the average number of silver grains per high power field over each cell type obtained by counting 10 hpf for each of six paired muscle samples and is uncorrected for background. The total background counts for the autoradiographs of the control and hypertrophied muscles were 24.6 ± 1.5 and 25.0 ± 2.0 grains/hpf, respectively. Since the background counts were not significantly different, the number of grains over each cell type due to hypertrophy was calculated as the difference between the hypertrophied and control grain counts (uncorrected for background) of each pair, and the difference is significant ($P < 0.05$) for the capillary cells and fibroblasts. No significant difference between the labeling of muscle cells in the control and hypertrophied muscles was observed.
* The difference is significant ($P < 0.05$).
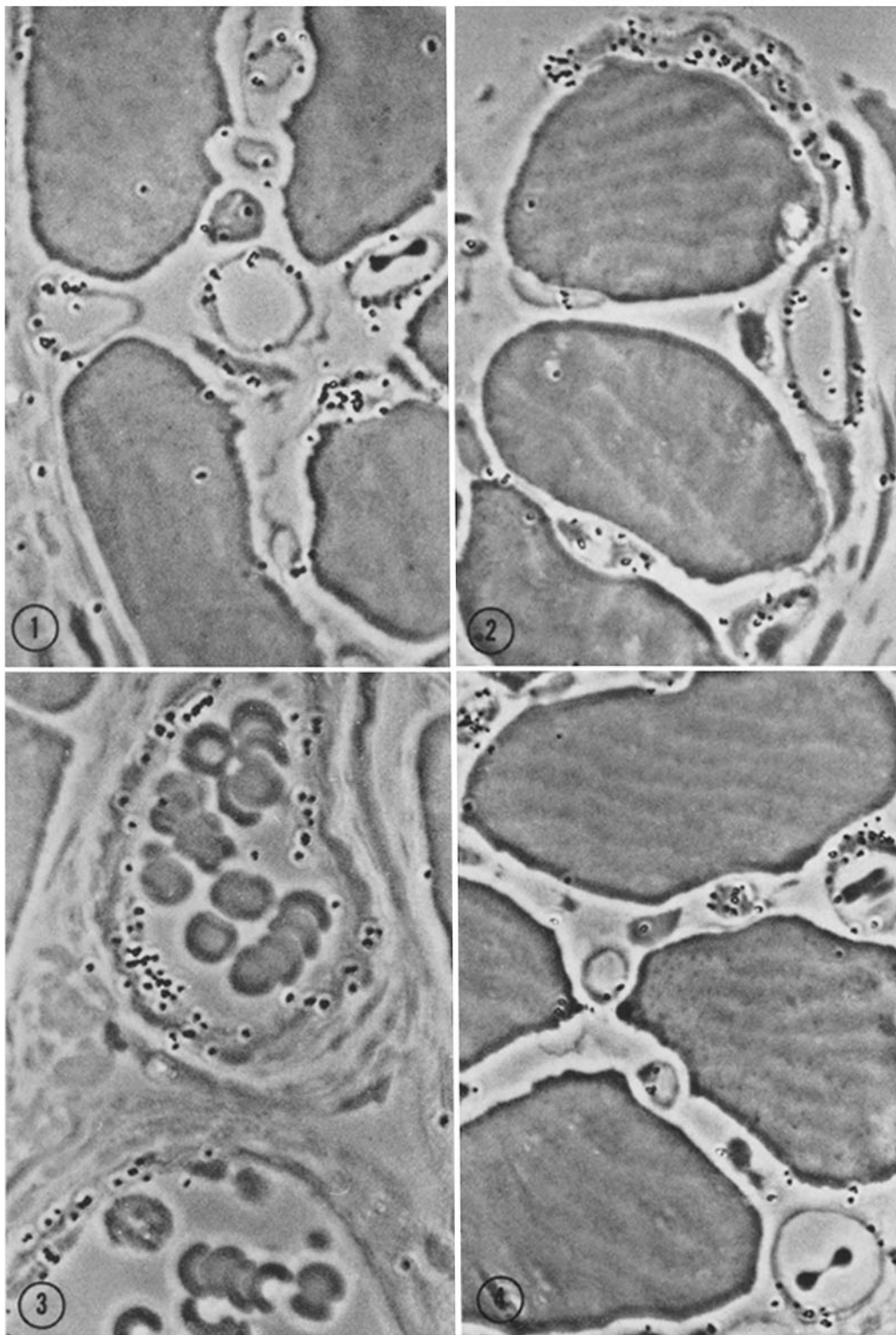
FIGURES 1-4 Phase-contrast autoradiographs of the hypertrophied soleus muscles labeled in vivo with [6-³H]uridine and analyzed in Table IV. The micrographs illustrate the localization of silver grains predominantly over capillaries, endothelia of larger blood vessels, and connective tissue cells. × 640.

validity of this conclusion rests on the assumption that the specific activities of the RNA precursor pools in control and hypertrophying muscle fibers are identical (see Discussion).

Examination of the percentage of each cell type that was labeled in the hypertrophied muscles compared with the controls suggested that more connective tissue cells were engaged in the synthesis of new RNA. Whereas about 20% of the sectioned capillaries and fibroblasts were labeled in the control soleus, over 50% were labeled in the hypertrophied soleus. Furthermore, there were almost twice as many fibroblasts in the sections of hypertrophied muscle compared with that of the controls. This figure was determined by comparison of the ratio of fibroblasts to muscle cells in the hypertrophied soleus (1.18 ± 0.13) with the ratio in the control muscle (0.59 ± 0.08); the difference between these two ratios (0.59 ± 0.13) was significant ($P < 0.01$). A similar comparison of the ratio of capillary profiles to muscle cells in the hypertrophied muscle (1.53 ± 0.10) with the ratio in the control muscle (1.37 ± 0.14) yielded an insignificant difference (0.16 ± 0.08; $P < 0.10$). Thus, during muscle hypertrophy there is connective tissue proliferation without a significant increase in the total number of capillaries.

The importance of connective tissue cells as a site of RNA synthesis in control as well as hypertrophying muscle is clear from the following calculation. We computed the number of grains over control muscle fibers due to incorporated [6-³H]uridine by correction of the grain count over muscle fibers (27 grains/hpf) for background, based on the determination that about 75% of the area of control muscle sections was occupied by muscle fibers (0.75×26 grains = 20 grains). This calculation yields 7 grains/hpf and comparison of this figure with the total number of grains over muscle sections (12 grains/hpf) suggests that about 40% of the [6-³H]uridine incorporation occurred in connective tissue cells in control muscle.

The unexpected absence of any autoradiographic evidence for increased RNA synthesis in the hypertrophied muscle fibers led us to consider whether the technique that we had used had failed to detect RNA synthesis in muscle, because new RNA synthesis in muscle fibers occurs much earlier than 2 days after the onset of hypertrophy or because we had used uridine as the RNA precursor whereas orotic acid had been used in the previous in vivo study of RNA synthesis during

muscle hypertrophy, or because we had examined the central portion of muscles.

We have confirmed the absence of radioactivity in the RNA of hypertrophy muscles with the autoradiographic analysis of the proximal portion
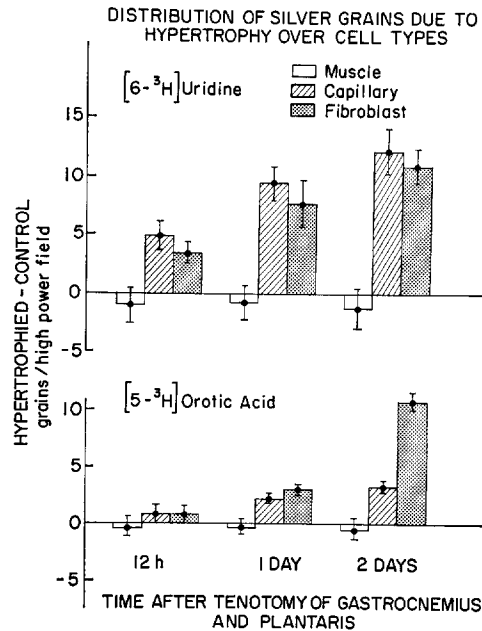

FIGURE 5 The difference between the number of grains over each cell type in paired autoradiographs of control and hypertrophied muscles at various times after tenotomy. Animals were injected with 0.5 mCi [5-³H]orotic acid (12.2 Ci/mmol) or 1.0 mCi [6-³H]uridine (25 Ci/mmol) with thymidine (1 mg/g body weight) at various times after tenotomy. 4 h later, the soleus muscles were removed, fixed, and washed, and small portions were removed from the proximal end of the control and hypertrophied muscles for autoradiography and RNA fractionation as described in the Methods. The mean grain counts over each cell type in the autoradiographs and the difference between the mean grain counts due to hypertrophy was determined as in Table IV. In rats injected with [6-³H]uridine with thymidine, 70 ±2% and 75 ±2% of the radioactivity of the fixed control and hypertrophied muscle samples, respectively, were recovered in the RNA fraction; whereas, in rats injected with [5-³H]orotic, only 40 ±3% and 42 ±2% of the incorporated radioactivity were recovered in the RNA fraction of the corresponding muscles. Note that when a significant change in the number of silver grains occurs, it appears over only the capillary and fibroblast cells; no significant increase in labeling of muscle cells in hypertrophying muscle was observed. Each value is the mean ± SEM of data from four animals.
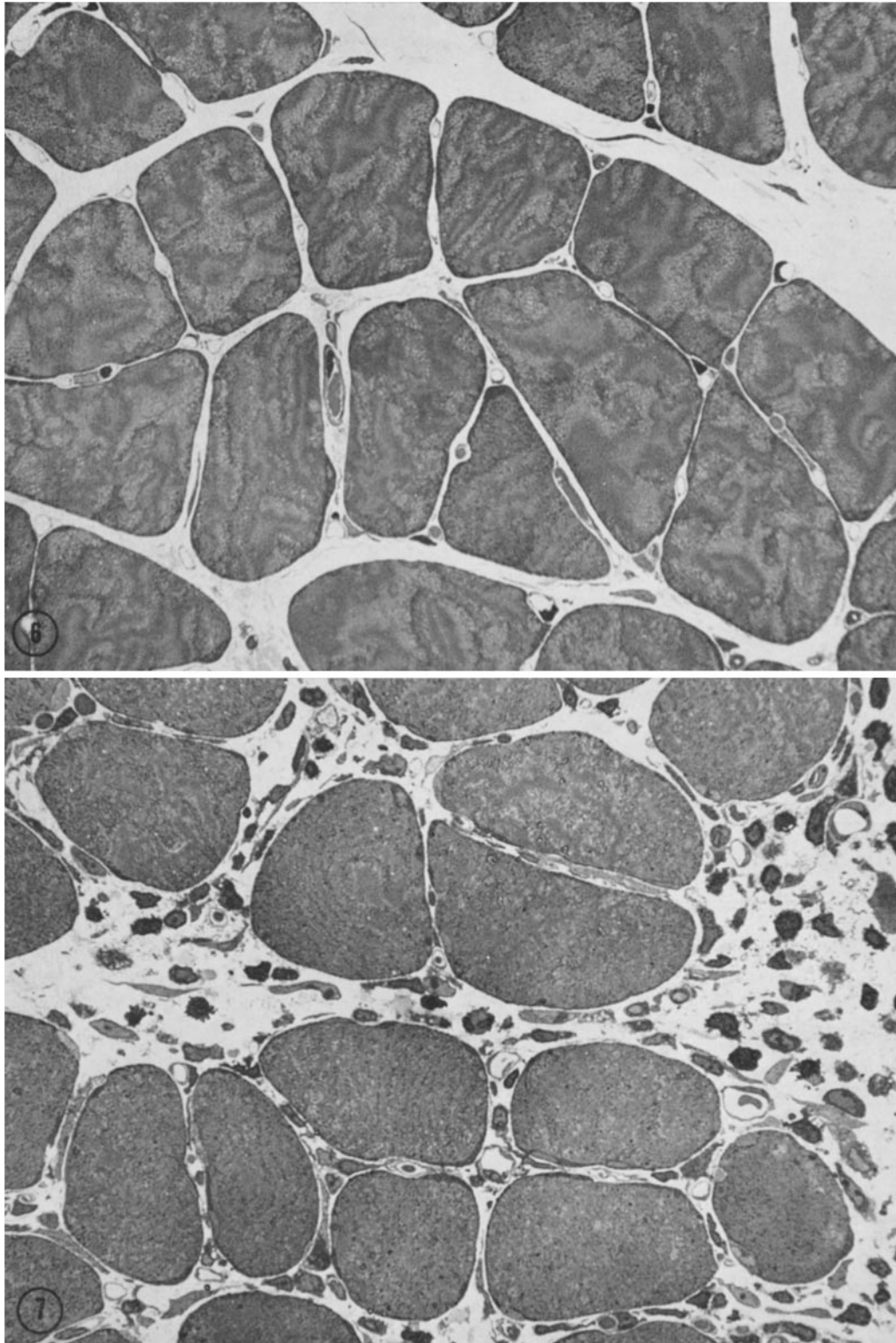
FIGURES 6 and 7   Light micrographs of the distal portions of rat soleus muscles, illustrating the normal distribution of capillaries and connective tissue cells between muscle fibers in the control muscle (Fig. 6) and the capillary pleomorphism and cellular infiltration that occurs during hypertrophy (Fig. 7). These fields are from slightly different portions of the control and hypertrophying muscles, and therefore histological evidence of muscle cell enlargement, such as that documented in the text, is not readily apparent. × 1,880.

of muscles allowed to hypertrophy for 12, 24, and 48 h, and labeled in vivo with either [5-^{3}H]orotic acid or [6-^{3}H]uridine (Fig. 5). Autoradiographs from muscles labeled with [5-^{3}H]orotic acid compared with those of muscles labeled with [6-^{3}H]uridine had lower grain counts and did not show an increase in RNA labeling until 24 h after tenotomy. These differences between the autoradiographs are probably because muscles labeled with [5-^{3}H]-orotic acid were much less radioactive (30–40 dpm/mg) than muscles labeled with [6-^{3}H]uridine (130-200 dpm/mg). Nevertheless, in both cases, the increase in labeling of the hypertrophied muscles compared with the controls appeared over fibroblasts and capillaries; at no time did an increase appear over the muscle fibers themselves.

Difference between the Proximal and Distal Portions of Hypertrophied Muscles

Hamosh et al. (1967) and Sobel and Kaufman (1970) noted that the distal half of hypertrophied soleus muscles were infiltrated with connective tissue, and they did not use this portion of the hypertrophied muscle in their studies. Since the autoradiographs in this study of the proximal and central portions of the hypertrophied soleus showed that connective tissue cells are the major site of new RNA synthesis, it seemed likely that the rate of RNA synthesis of these infiltrated, distal regions might be increased over that in the proximal portion of hypertrophying muscles (see Figs. 6 and 7). Therefore, we separated hypertrophying and control muscles into proximal and distal portions and fractionated them separately (Table V). In control muscles, there was no difference in either the RNA concentration or the net rate of [5-^{3}H]-crotic incorporation between the two portions. In hypertrophied muscles, the RNA concentration and the net rate of orotic acid incorporation in the proximal portion were only 20 and 30% above the respective levels in the corresponding control muscles, whereas in the distal portions, the same indices of RNA synthesis were 50 and 160%, respectively, above control levels. Furthermore, the magnitude of the difference between the extent of incorporation of isotope into RNA in the two portions of the hypertrophying soleus was even more dramatic when [6-^{3}H]uridine was used as the RNA precursor. 2 days after tenotomy, the distal portion incorporated 320% more radioactivity into RNA than the control, compared with a 60% increase above the control level in the proximal portion.

TABLE V

Comparison of RNA Concentration and Incorporation of [5-^{3}H]Orotic Acid into RNA in Proximal and Distal Halves of Paired Soleus Muscles 2 Days after Unilateral Tenotomy Operation

Soleus	Radioactivity in fraction		RNA concentration
	Acid soluble	RNA	
	dpm/mg wet weight		µg/mg wet weight
Control limb			
Proximal half	785 ± 23	30.8 ± 1.7	3.00 ± 0.11
Distal half	790 ± 28	28.0 ± 2.0	2.97 ± 0.10
Tenotomized limb			
Proximal half	820 ± 21	39.5 ± 5.1	3.55 ± 0.10
Distal half	910 ± 23	72.4 ± 4.0	4.41 ± 0.11

2 days after unilateral tenotomy of the gastrocnemius and plantaris muscles, rats were injected with 0.5 mCi [5-^{3}H]orotic acid (12.2 Ci/mmol) and sacrificed 4 h later. The wet weight of the hypertrophied soleus muscle (158 ± 7 mg) was greater than that of the control (117 ± 5 mg), and the difference, 41 ± 4 mg, was highly significant ($P < 0.001$). The control and hypertrophied soleus muscles were bisected and each portion was weighed and fractionated as described in the Methods. 75 ± 2% and 74 ± 2% of the total incorporated radioactivity was recovered in the RNA fraction of the control and hypertrophied soleus muscles, respectively. Values are the means ± SEM of data from six different animals. Note that there is no difference in the data from the proximal and distal halves of the control muscles, whereas both the radioactivity of the RNA fraction and the RNA concentration of the proximal half of the hypertrophied muscle are moderately increased, and the same measurements in the distal half of the hypertrophied muscle are greatly increased compared with the control muscle data.

This large difference in the labeling of RNA of the distal portion of the hypertrophied muscle with [6-³H]uridine is consistent with the autoradiographic data. In contrast to a 50% increase in the number of silver grains due to incorporated [6-³H]uridine over autoradiographs of sections from the proximal portion of hypertrophied soleus muscles 48 h after tenotomy (see Fig. 5), there was a 300% increase in the number of silver grains over autoradiographs of sections from the middle portion (see Table IV). The correlation between the magnitude of the increase in [6-³H]uridine incorporation into RNA and the number of silver grains above background occurring over autoradiographs from the corresponding regions of the hypertrophied muscles further strengthened our conclusion that most of the silver grains above background in the autoradiographs of sections of muscles labeled in vivo with [6-³H]uridine represented radioactivity incorporated into RNA.

The preferential localization of connective tissue infiltration and RNA synthesis in the distal portion of the soleus muscle raised the question of whether these changes were related to skeletal muscle hypertrophy or whether they represented an inflammatory response to the cut tendons lying immediately adjacent to the muscle. In an attempt to distinguish between these two alterna-

tives, we took advantage of the observation (Goldberg and Goodman, 1969) that spinal cord section to prevent movement of the lower limbs also prevents work-induced hypertrophy of the tenotomized limb muscle. In these experiments the cut tendon in the tenotomized limb was left near the distal soleus as usual. 2 days later, the animals were pulse labeled with [5-³H]orotic acid and the proximal and distal segments of the soleus muscles from the sham-operated and tenotomized limbs (Table VI) were fractionated separately. In these animals there was no difference in the appearance of, the concentration of RNA in, and the net rate of incorporation of [5-³H]orotic acid into RNA in proximal and distal segments of the soleus muscles between the sham-operated and tenotomized limbs. Thus, muscle activity appears to be necessary to develop the connective tissue infiltration and increased RNA synthesis.

It is noteworthy that whereas the increased activity during compensatory hypertrophy is accompanied by an increase in RNA synthesis, decreased activity of muscles produced by spinal section may be accompanied by a decrease in RNA synthesis. This conclusion is suggested by the observed decrease in the net rate of incorporation of [5-³H]orotic acid into RNA in the paralyzed muscles (50% control levels) with no change in the

TABLE VI

Effect of Spinal Section on RNA Concentration and Incorporation of [5-³H]Orotic Acid into RNA in Proximal and Distal Halves of Paired Soleus Muscles 2 Days after Unilateral Tenotomy Operation

Soleus	Radioactivity in fraction		RNA concentration
	Acid soluble	RNA	
	dpm/mg wet weight		*μg/mg wet weight*
Control limb			
Proximal half	752 ± 25	15.0 ± 1.2	2.77 ± 0.10
Distal half	741 ± 21	17.7 ± 1.7	3.01 ± 0.10
Tenotomized limb			
Proximal half	746 ± 23	16.1 ± 1.4	2.75 ± 0.05
Distal half	760 ± 22	18.6 ± 1.2	3.12 ± 0.10

The experiment was carried out exactly as described in Table V except that spinal section was done immediately after tenotomy of the gastrocnemius and plantaris. There was no difference in the wet weight of the control (121 ± 4 mg) and tenotomized limb soleus muscles (122 ± 3 mg). 80 ± 2% and 77 ± 2% of the radioactivity in the control and tenotomized limb soleus were recovered in the RNA fraction. Values are the means ± SEM of data from six different animals. Note that there is no significant difference in the RNA radioactivity and concentration of the corresponding portions (proximal and distal) of the control and hypertrophied muscle. Also, compared with the data in Table V, the control muscle after spinal section incorporated one-half as much radioactivity into RNA as the control muscle from animals with intact spinal cords, with no change in the acid soluble radioactivity.

radioactivity of the acid soluble fractions (cf., Tables V and VI). It remains to be determined whether this represents a genuine decrease in RNA synthesis and, if so, whether this decrease in RNA synthesis with muscle inactivity occurs predominantly in muscle fibers or in connective tissue cells.

DISCUSSION

Autoradiography

The autoradiographic studies reported here required the development of a method of labeling of RNA in vivo that met the following criteria: (a) specific labeling of RNA, (b) high specific radioactivity of the RNA, and (c) a method of tissue preparation that led to maximum retention of radioactivity in the RNA with maximum removal of the unincorporated label. The specific labeling of RNA was achieved by the simultaneous injection of unlabeled thymidine with [6-^{3}H]uridine. Glutaraldehyde fixation and subsequent repeated washes in sodium cacodylate buffer (pH 7.4) effectively preserved the radioactivity in the RNA and eliminated the unincorporated label. The fractionation method of glutaraldehyde-fixed tissue allowed the determination of the molecular species responsible for the production of silver grains in the autoradiographs. Independent evidence that the silver grains in the autoradiographs were predominantly due to label incorporated into RNA was provided by comparison of the effect of actinomycin D on the incorporation of label into RNA and the number of silver grains over autoradiographs from corresponding muscle samples.

The remarkable finding of this study is that at all the time points that were investigated, the increase in the net incorporation of RNA precursors after the onset of hypertrophy was localized by autoradiographs to proliferating connective tissue within the muscle; there was no significant increase in the number of silver grains over muscle fibers in hypertrophied muscles compared with the control limb muscles. It is important to consider whether a significant increase in RNA synthesis occurs in muscle fibers during work-induced growth, but that our methods failed to demonstrate it. The fact that the grain counts over both control and hypertrophied muscle fibers were significantly above background suggests that [6-^{3}H]uridine enters muscle fibers and is incorporated into RNA. However, if the specific activity of the RNA precursor pool in the hypertrophied muscle fiber decreased, for example, because of an increase in the size of the pool, the rate of incorporation of label into RNA could have remained the same as that in the control despite an increased rate of RNA synthesis in hypertrophying muscle fibers. We have previously noted no difference in either the concentration or the specific activity of the UTP pool between control and hypertrophied muscles labeled in vivo with [5-^{3}H]orotic acid 1 day after the tenotomy operation, when there was a 50–60% increase in the net rate of incorporation of radioactivity into hypertrophying muscle RNA (Sobel and Kaufman, 1970). That measurement, however, was made on whole muscle homogenates, and the possibility of a difference existing between specific radioactivity of the RNA precursor pool of control and hypertrophied muscle fibers cannot be entirely excluded until one is able to separate the muscle fibers from the connective tissue cells before the determination of the UTP precursor pool size and radioactivity.

RNA and Protein Metabolism and Muscle Hypertrophy

After tenotomy of the gastrocnemius and plantaris muscles, there is a rapid accumulation of myofibrillar proteins in the hypertrophying soleus. The maximum dry weight gain occurs 4 days after the operation (Lesch et al., 1968), but the maximum increase in myofibrillar protein occurs about 2 days after tenotomy (Table VII). Although some of the accumulation of myofibrillar protein that occurs during hypertrophy is due to a decrease in the rate of protein degradation (Goldberg, 1969), Goldberg (1968) has also shown that there is an early increase in the rate of synthesis of myofibrillar proteins.

An essential role for RNA in the accumulation of the myofibrillar protein in muscle during hypertrophy, and presumably in its synthesis, was postulated from the following observations: (a) the total RNA content of muscles increased during hypertrophy (Hamosh et al., 1967); (b) the increased rate of incorporation of RNA precursors into RNA occurs in vivo within 1 day of the onset of hypertrophy (Sobel and Kaufman, 1970); and (c) actinomycin D inhibits the development of hypertrophy (Goldberg and Goodman, 1969). Goldberg (1971) recently confirmed the findings that hypertrophied skeletal muscle has both a

TABLE VII

Fractionation of Tissue Nitrogen in Control and Hypertrophied Soleus Muscles

Soleus muscle	Protein		
	Myofibrillar	Sarcoplasmic	Stromal
	mg nitrogen per muscle		
2 days postoperative			
Control	1.88 ± 0.04	0.51 ± 0.03	0.89 ± 0.08
Hypertrophied	2.15 ± 0.09*	0.69 ± 0.04*	1.07 ± 0.11
4 days postoperative			
Control	2.01 ± 0.09	0.56 ± 0.03	0.82 ± 0.07
Hypertrophied	2.21 ± 0.08*	0.77 ± 0.04*	1.01 ± 0.10
6 days postoperative			
Control	2.05 ± 0.04	0.56 ± 0.05	0.67 ± 0.13
Hypertrophied	2.20 ± 0.05*	0.71 ± 0.05*	1.01 ± 0.18*

The time-course of the increase in the various muscle protein fractions during hypertrophy was examined as previously described (Hamosh et al., 1967). All values represent the mean of five separate determinations and are reported as the mean ± SEM. P value determined by Student's t test for paired variables.
* $P < 0.01$

higher RNA content and an increased rate of incorporation of RNA precursors into RNA. These data suggested that the increased synthesis of RNA in muscle cells was responsible for the increased synthesis of muscle proteins.

The present finding, however, that essentially all of the increased de novo RNA synthesis that occurs during skeletal muscle hypertrophy takes place in connective tissue, indicates that it would be prudent to reexamine the above interpretation of the relationship between RNA and protein synthesis during skeletal muscle hypertrophy.

At the outset, it should be emphasized that it is not known whether the hypertrophied muscle cell has a higher RNA content than that of the control muscle cell. If future work should prove that there is an increased RNA content in the hypertrophied muscle cells, our present results would indicate that this increase could not be due to an increase in the rate of synthesis of the major type of RNA (i.e., ribosomal RNA) in the muscle cell. If indeed there is an increase in the content of total and ribosomal RNA in the muscle cell per se, it is therefore probably due primarily to a decrease in the rate of degradation of ribosomal RNA.

Since there is evidence that RNA synthesis is essential for hypertrophy, i.e. the process is inhibited in vivo by actinomycin D, the restrictions imposed by the present results still allow for the following possibilities: (a) increased synthesis of

RNA is essential for hypertrophy, but it takes place in cells other than the muscle, e.g., fibroblasts. (The type of RNA in fibroblasts whose synthesis is stimulated has not been identified.) In relation to this point, it should be noted that electron micrographs (see below) indicate that the connective tissue cells of the hypertrophied muscle have a greater abundance of rough endoplasmic reticulum, a site of ribosomal RNA, than do the fibroblasts in the control muscle. (b) Since the autoradiography technique may not have detected the increased synthesis of a minor species of RNA in the muscle (such as mRNA), it is still possible that hypertrophy is dependent on the enhanced synthesis in muscle of this RNA type.

It is also conceivable, however, that continuing RNA synthesis is essential, but an increased rate of synthesis is not. In that case, the simplest hypothesis that is consistent with our present data is that the control of a posttranscriptional event, such as an increase in the half-life of mRNA or an increase in the rate of translation of mRNA, is responsible for the early increase in the rate of synthesis of myofibrillar protein. Since our investigation was limited to the first 2 days after the induction of hypertrophy, it remains to be determined if a significant increase in RNA synthesis occurs in muscle fibers during long-term adaptation of muscle to work-induced growth.

Connective Tissue Metabolism and Muscle Hypertrophy

It is difficult to evaluate our finding that almost all of the enhanced RNA synthesis that accompanies muscle hypertrophy occurs in proliferating connective tissue, and that there is a preferential increase in the number of connective tissue cells and the rate of RNA synthesis in the distal portion of the hypertrophied soleus muscle. It is possible that these changes are unrelated to physiological hypertrophy and are, instead, an artifact of the tenotomy procedure that we have used to induce hypertrophy. Against this possibility is our finding that the changes in connective tissue are abolished by spinal cord section; the changes therefore are dependent on muscle activity. Also, in electron micrographs of the hypertrophied muscle, the infiltrate did not appear to be inflammatory; at least it included no leukocytes, damaged cells, or fibrin exudates (Florey, 1970). Instead, it was composed of larger and more numerous examples of the two types of cells usually present in the endomysium of muscle: (a) smooth, flattened cells filled with ribosome-studded endoplasmic reticulum (which we identified as fibroblasts after Porter (1966); and (b) villous, rounded cells filled with phagocytic vacuoles and dense granules (which we identified as macrophages after Ross (1968) (Figs. 8–10). Whether these different cell types represent different cell lines or simply different states of differentiation of a single cell line remains to be determined (Follett and Goldman, 1970; Comings and Okada, 1970). These cells did not penetrate the muscle fiber's basal lamina or form close contacts with them. Both cell types appeared in regions where collagen bundles were disorganized or replaced by a fine, amorphous, dense material, as if the cells were reorganizing the collagenous matrix of the muscle. Furthermore, the connective tissue infiltrate persisted; it was present in hypertrophied muscle examined by light microscopy 6 days after the tenotomy operation. In spite of these considerations, we have no way of convincingly ruling out the possibility that the proliferation of connective tissue cells with their increased RNA synthesis is not an artifact of the tenotomy technique.

The presence of the proliferating interstitial cells may be responsible, in part, for the previously noted marked increase in soluble proteins in hypertrophying muscles (Hamosh et al., 1967).

Our present results, however, emphasize the caution one must exercise in attributing a biochemical change to a portion of a mixed cell population (muscle cells and interstitial cells) when both kinds of cells have the potential to cause the change. In this regard, it remains to be determined whether the increased RNA polymerase activity that appears 3 days after the tenotomy operation (Sobel and Kaufman, 1970) reflects activity in muscle fibers or connective tissue cells.

While it is most likely that the connective tissue proliferation is a secondary response to metabolic products of, or structural changes in, muscle fibers undergoing hypertrophy, it is also possible that there is a more dynamic relationship between connective tissue and muscle cells. In this regard it may be significant that in both hypertrophied skeletal (Hamosh et al., 1967) and cardiac muscle (see below) there is an increase in collagen in the hypertrophied tissue. Also suggestive of an intimate relationship between fibroblasts and muscle cells is the finding of Hauschka and Konigsberg (1966) that muscle cell differentiation in vitro is dependent upon the presence of a macromolecule produced by fibroblasts and that the necessary macromolecule is probably collagen. We hope to determine if such a relationship exists during skeletal muscle hypertrophy, i.e., if the proliferating connective tissue directly influences the accumulation of the muscle cell proteins.

RNA Synthesis during Cardiac Hypertrophy

Fanburg (1970) recently reviewed the changes in the isotopic labeling and content of nucleic acids during cardiac hypertrophy after coarctation of the aorta in rats. During work-induced cardiac hypertrophy, there is a transient increase in the labeling of RNA by isotopic precursors and a subsequent increase in cardiac RNA content that is similar to that observed during work-induced skeletal muscle hypertrophy. It is not known whether the changes in RNA synthesis in hypertrophying cardiac muscle occur in muscle or connective tissue cells, but it has been shown that during hypertrophy of cardiac muscle, connective tissue cells proliferate (Morkin and Ashford, 1968). In addition, it has been shown that during cardiac hypertrophy after coarctation of the aorta a parallel increase in collagen occurs with the increase in myofibrillar protein (Bartošová et al., 1969; Buccino et al., 1969). It is possible that in
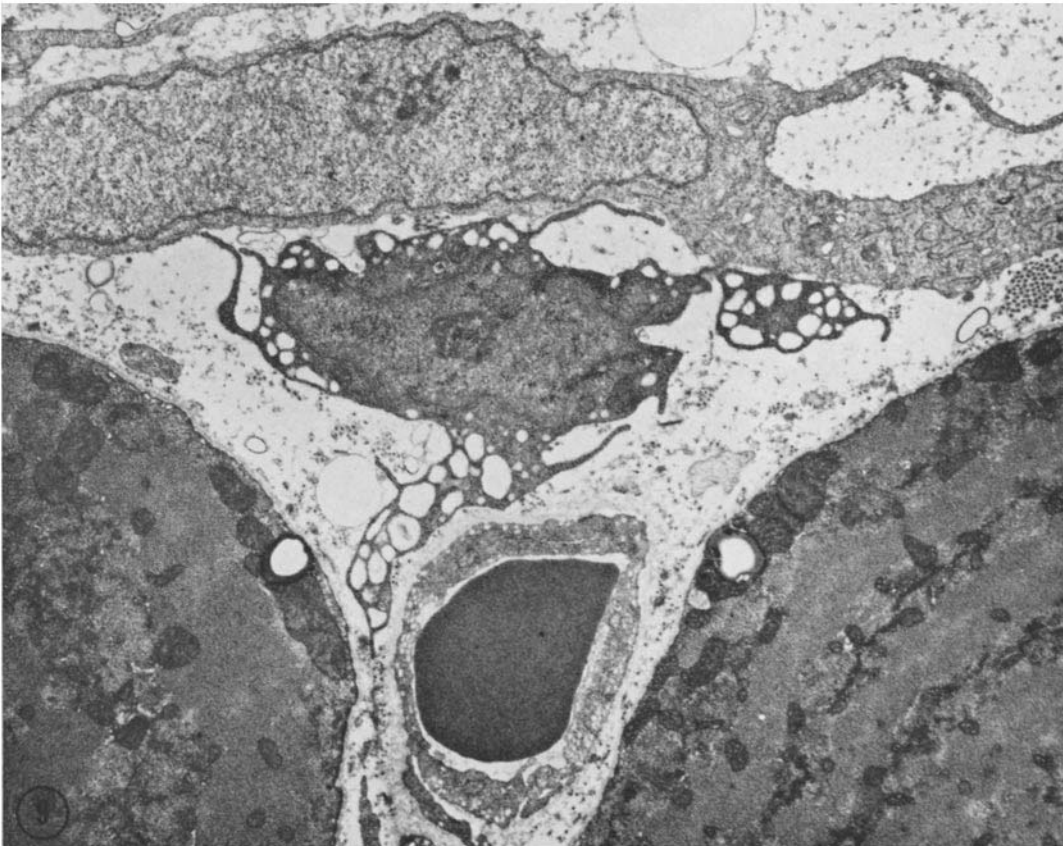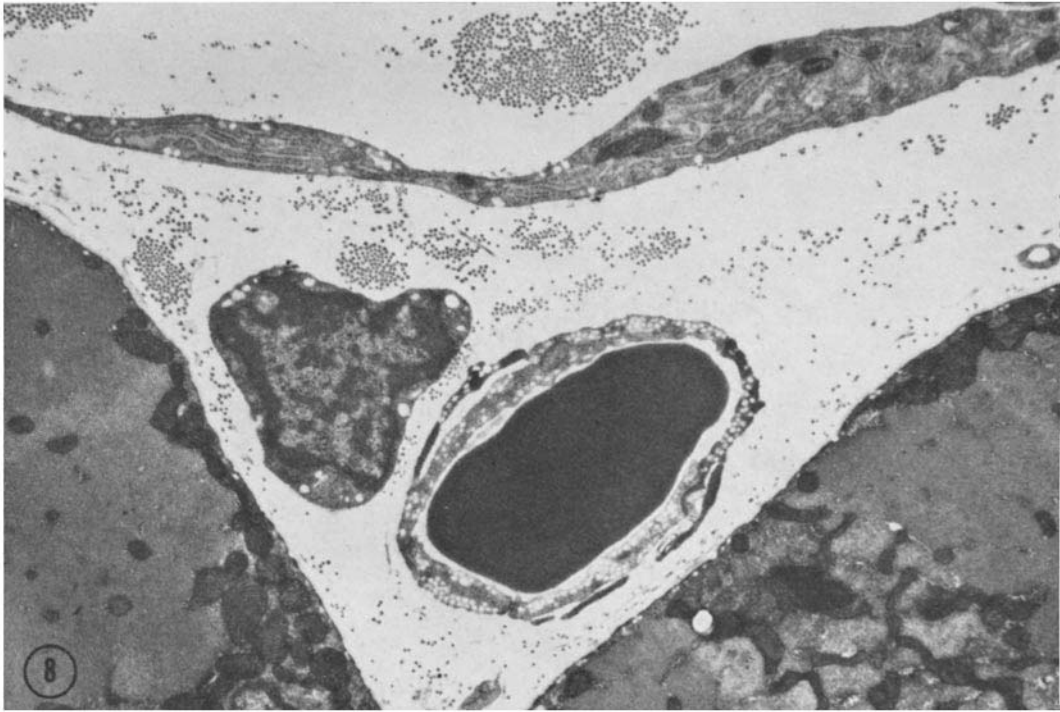
FIGURES 8 and 9   In the interstices between muscle fibers in the rat soleus are normally found capillaries surrounded by pale endothelia and darker pericyte processes (adjacent pericyte perikaryon is not connected in this plane of section); the processes of the adjacent fibroblasts are thin (Fig. 8). In muscles allowed to hypertrophy for 2 days, the pericytes are enlarged, villous, and vacuolated; the fibroblasts are more numerous and enlarged (Fig. 9). × 12,500.

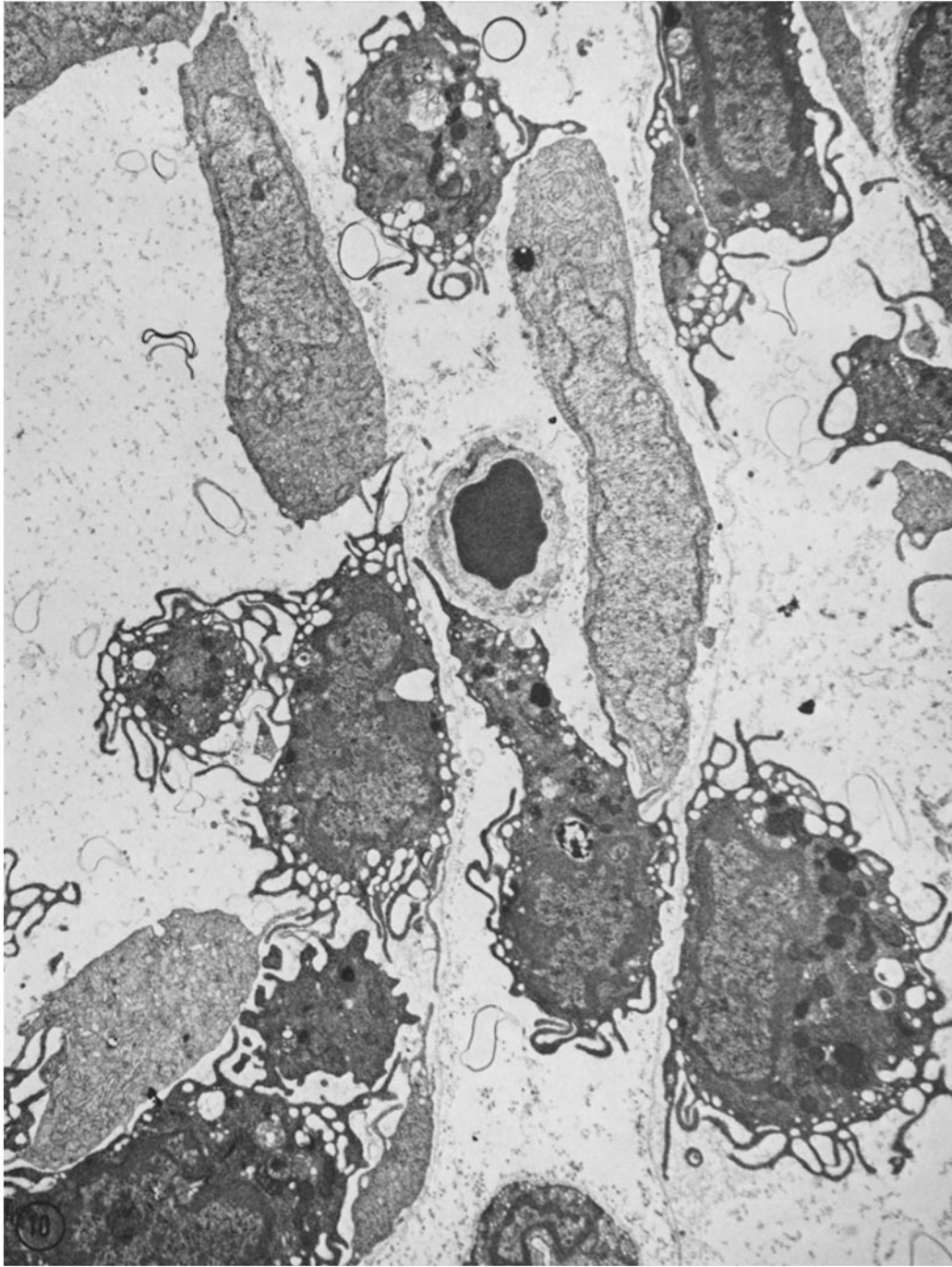FIGURE 10 In regions of hypertrophying muscles infiltrated with connective tissue, two cell types predominate: (a) pale, extended, smooth cells filled with swollen cisternae of rough endoplasmic reticulum, identified as fibroblasts; and (b) darker, rounded, villous cells filled with vacuoles containing variable amounts of debris, identified as macrophages. × 10,000.

cardiac muscle, as we have shown in skeletal muscle, the site of most new RNA synthesis during hypertrophy may be the connective tissue cells. Though there are major differences between cardiac and skeletal muscle, it is tempting to think that the elucidation of the mechanism of hypertrophy in skeletal muscle will shed light on the mechanism of cardiac hypertrophy, and in this instance, that the findings of this study may stimulate a similar investigation into the anatomical localization of in vivo RNA changes during cardiac hypertrophy.

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