Cell-free and whole-tissue protein synthesis was studied in skeletal muscle of untrained male guinea pigs that had undergone a treadmill run to exhaustion. Experiments using explants from the gastrocnemius muscle maintained in organ culture demonstrated that the ability of the acutely exercised muscle to incorporate amino acids into protein had increased. Compared to polyribosomes prepared from several lower hind limb muscles of nonexercised guinea pigs, polyribosomes from the same muscle in exhausted guinea pigs had incorporated almost 50% more radioactive leucine into protein. However, the polysome profiles of control and exercised muscle were identical, and no difference in the total polysome RNA content could be detected. The efficiency of in-vitro protein synthesis using washed membrane-bound polyribosomes (microsomes) isolated from acutely exercised skeletal muscle was 50% greater than with microsomes from rested control muscle.

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PROTEIN SYNTHESIS IN SKELETAL MUSCLE FOLLOWING ACUTE EXHAUSTIVE EXERCISE

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The protein synthesis system in mature mammalian skeletal muscle demonstrates the capacity to respond and adapt to various pathologic states as well as to changes in functional demand. For example, various differences in the ribosomal capacity for protein synthesis have been observed in several types of muscular dystrophy,[6] and diabetes decreases the capacity of skeletal muscle to synthesize protein in vitro[9] and in vivo.[3] Microsomal and ribosomal protein synthesis is decreased in the skeletal muscles of rats after four to six days of protein starvation, and is enhanced after a single meal of protein.[14] The effects of changes in dietary regimen can be detected in vivo as well,[10] and these studies have correlated well with in-vitro results. An increase in the capacity for microsomal protein synthesis has been reported for skeletal muscle undergoing hypertrophy[2] and for cardiac muscle subjected to increased functional demand in vitro.[13]

Our experiments were designed to investigate the capacity for protein synthesis by tissue explants, microsomes, and polyribosomes isolated from the lower hind limb of guinea pigs 1 hr after a single bout of vigorous exercise (running on a motor-driven treadmill). The observations indicate that the exercised skeletal muscle, removed from in-vivo neural and hormonal influence, incorporates amino acids into protein more efficiently than similarly isolated, nonexercised muscle.

MATERIALS AND METHODS

Animals and Experimental Treatment. Mature, untrained male guinea pigs (Charles River), weighing 600–800 g, were used in all experiments. The experimental groups of animals were run to exhaustion on a motor-driven treadmill at a rate of 0.6 miles per hour on a 10% incline. Failure of the animals to respond to stimulation by low-voltage electrical shocks served as the criterion for exhaustion. The mean running time to exhaustion under these conditions was 58 min. One hour following exhaustion, the animals were sacrificed by cervical dislocation. Control animals were handled

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in an identical manner except that they were not run on the treadmill but did receive electrical stimulation comparable to exercised animals. Animals had free access to food and water at all times.

Preparation of Subcellular Fractions. After the animals were sacrificed, selected skeletal muscle (extensor digitorium longus, plantaris, soleus, and gastrocnemius) of the lower hind-limb was removed and dissected free of all obvious connective tissue and fat. The tissue (mean yield, 4.2 g per animal) was then placed in a chilled Petri dish containing 10 ml of medium A (0.25 mM sucrose, 10 mM $MgCl_2$, 250 mM KCl, 50 mM Tris-HCl buffer, pH 7.6). Unless otherwise specified, all subsequent procedures were performed at 4°C using ice-cold apparatus and solutions.

The isolated skeletal muscle (equal weights of control or experimental tissue) was minced as finely as possible with scissors in the Petri dish. The minced tissue was homogenized in two volumes of medium A for two separate periods of 15 sec each in a Polytron tissue homogenizer (Brinkman Instruments, Westburg, NY) with a setting of 3. The homogenates were centrifuged at 10,000 × g for 15 min in a Sorvall refrigerated centrifuge (Dupont Instruments, Newton, CT). The heavy fat layer which appeared was removed by filtration, and the resulting postmitochondrial supernatant was then centrifuged at 105,000 × g in a Beckman L2-50 ultracentrifuge (Beckman Instruments, Fullerton, CA) for 60 min. The resulting microsomal pellet was dispersed by stirring with a small magnetic stirring bar and resuspended in medium A. Aggregates in the final microsomal preparation were removed by low-speed centrifugation.

Polyribosomes were prepared from microsomal fractions by extending the 105,000 × g centrifugation to 150 min. The microsomal pellet prepared in this manner was resuspended in medium B (3 ml of 2.5 M KCl in 10 mM $MgCl_2$ added to 9.75 ml of medium A). The suspension was treated with 10% deoxycholate (sodium salt, Sigma Chemical Co., St. Louis, MO) and 10% Lubrol WX to achieve final concentrations of 1% and 0.5%, respectively. The detergent-treated suspension was left on ice for 10 min and then layered over 10 ml of 1.0 M sucrose in medium A, henceforth referred to as the sucrose cushion. The suspension was centrifuged at 150,000 × g for 150 min to collect the washed polyribosomes. The resulting clear pellet had an absorption coefficient ($A_{260/280}$) of 1.7 (mean value for control and experimental preparations) and was resuspended in medium A by gentle stirring as above. Aggregates were removed by low-speed centrifugation.

The high-speed-supernatant fractions, S150, prepared by taking small aliquots (0.5–1 ml) of the 150,000 × g supernatant were chromatographed on a column (1 × 30 cm), Sephadex G25 (Pharmacia), previously equilibrated with medium A and eluted with the same medium. The material eluting in the void volume was collected and stored in aliquots of 100 μl.

Assay of Amino Acid Incorporation. Incubation mixtures were prepared containing either microsomes or polyribosomes (refer to figures 3 and 4 for exact quantities), 100 μl of the S150 fraction (5 mg protein/ml), and 50 μl of a master reaction mixture. The final concentrations of reagents in the 100-μl incubation mixtures were 45 mM Tris-HCl, pH 7.6; 8 mM $MgCl_2$; 10 mM β-mercaptoethanol; 130 mM KCl; 1.2 mM ATP; 0.1 mM guanosinetriphosphate, 20 mM creatine phosphate; 50 μg/ml creatine kinase; 100 μCi/ml [^{3}H]-leucine (New England Nuclear, Boston, MA, 47 Ci/mmole); and a mixture of the remaining 19 common amino acids, each at a concentration of 10 μM. The assay was initiated by the addition of either microsomes or polyribosomes, and the mixtures were incubated at 37°C for 30 min. The reaction was terminated by the addition of 4 ml of 10% trichloroacetic acid (TCA). The incubation mixtures were heated at 90°C for 10 min, and the precipitated protein was collected on glass fiber filters (Whatman) by filtration and washed with 10% TCA. The filters were dried and placed in vials with 7 ml of an Omnifluor-toluene (New England Nuclear Corp., Boston, MA) scintillation cocktail and counted in a Beckman LS-250 liquid scintillation counter.

Sucrose Density Gradient Analysis of Polyribosomes. A 10,000 × g postmitochondrial supernatant was treated as described above for the preparation of subcellular fractions. Aliquots of 0.4 ml were layered onto a 10%–40% linear sucrose density gradient prepared in homogenization medium. Gradients were centrifuged in a Beckman SW 50–1 at 189,000 × g and 4° C for 30 min. Fractions were collected from the bottom of the tubes, and the absorbance of each fraction at 260 nm was determined.

Preparation of Tissue Slices and Measurement of Amino Acid Incorporation into Protein. Slices (1 mm^3) were prepared from the gastrocnemius and soleus

muscles of control and experimental guinea pigs. The tissue was incubated in a modified Krebs-Ringer phosphate buffer (pH 7.4), containing 11 mM glucose, 0.05 mCi [^{3}H]-leucine, and a complete set of the remaining amino acids at the concentration found in rat plasma.[8] Tissue was incubated at 37°C with continuous gentle shaking and removed for the assay of protein synthesis at the time points indicated in the appropriate figure. The tissue was washed three times in standard Krebs-Ringer buffer containing 1 mM leucine. Trichloroacetic acid to a final concentration of 10% was added, and the mixture was heated to 90°C for 20 min. The protein was collected on glass fiber filters, washed with 10% trichloroacetic acid and ethanol-ether (1:1 v/v), and the filters were dried. Tissue samples were solubilized in 1 ml of 0.5 N NaOH by heating at 90°C for 30 min. Aliquots were then removed for liquid scintillation counting in a scintillant containing Triton X-100 and for the determination of RNA and protein. Microsomes, polyribosomes, and tissue slices were prepared and assayed for activity on the same day.

Determination of Protein and RNA. Protein in all preparations was assayed by the method of Lowry et al,[7] and after the removal of protein, RNA was determined by the orcinol method.[9]

RESULTS

Analysis of Muscle Polyribosomes. By studying the sedimentation of polyribosomal material in sucrose density gradients, it was found that gentle homogenization using the Dounce apparatus (Kontes Glass Co., Vineland, NJ) decreased the yield of polyribosomes by 34% ± 6% when compared to the more vigorous Polytron homogenizer. No detectable increase in degradation of polyribosomes to ribosome monomers was found using Polytron homogenization.

Linear sucrose density gradient analysis of muscle cell lysates (fig. 1) from exercised and nonexercised muscle tissue revealed no significant differences with respect to yield or distribution of ribosomal or polyribosomal material.

Cell-free Protein Synthesis with Microsomes and Polysomes. Microsomes isolated from the lower hind limb of rested and exercised skeletal muscle were incubated in a protein synthesis system using optimal K^+, Mg^{2+}, ATP, and guanosine triphosphate concentrations. Final reaction mixtures and conditions for optimal protein synthesis were based on the kinetics and extent of the incorporation of [^{3}H]-leucine into polypeptide. Conditions for protein synthesis with experimental and control microsomes were optimized and identical, and the microsomes showed typical linear incorporation of labeled leucine for the initial 10 min of incubation, after which point a plateau was reached and maintained for at least 30 min (data not shown).

The results seen in figure 2 indicate that microsomes from control muscle incorporated 1,750 ± 230 counts per min (cpm), while microsomes prepared from exercised skeletal muscle tissue incorporated 3,350 ± 218 cpm. These values are significantly different ($p < 0.01$). No differences in protein or RNA content between exercised and control microsomes were detected (data not shown).

The in-vitro capacity for protein synthesis was further tested in washed membrane-free polyribosomes prepared as described above. Polyribosomes were initially centrifuged through a 2-M sucrose cushion. Although the activity of polyribosomes prepared in this manner was increased by 14 ± 3% when compared to the preparation using a 1-M sucrose cushion, the loss of RNA occasioned by the former procedure was significant, ranging from 20% to 50% as compared with the 1-M sucrose procedure. Sucrose density analysis of polyribosomes prepared using a 2-M sucrose cushion suggested either a loss or breakdown of light polyribosomal material during centrifugation; therefore, we have used centrifugation through 1-M sucrose in polysome resuspension buffer in all experiments.

The results presented in figure 3 indicate that polyribosomes prepared from exercised muscle tissue were significantly more active than corresponding control preparations. In-vitro protein synthesis was carried out under the same conditons used for skeletal muscle microsomes; this proved to be optimal for polyribosomes also. At a concentration of 5 μg/ml polyribosomal RNA, polysomes from exercised muscle tissue incorporated 9,850 ± 650 cpm, while control preparations incorporated 6,000 ± 540 cpm.

As shown in figures 1 and 2, increasing the concentrations of either microsomal protein or polyribosomal RNA inhibited protein synthesis in a cell-free system. We observed this routinely in all microsome and polyribosome preparations from skeletal muscle. Experiments using mixed sources of microsomes and polyribosomes and S150 fraction did not indicate the presence of any inhibitory substances, and the observed decrease in protein synthesis may be analogous to a substrate inhibition effect.

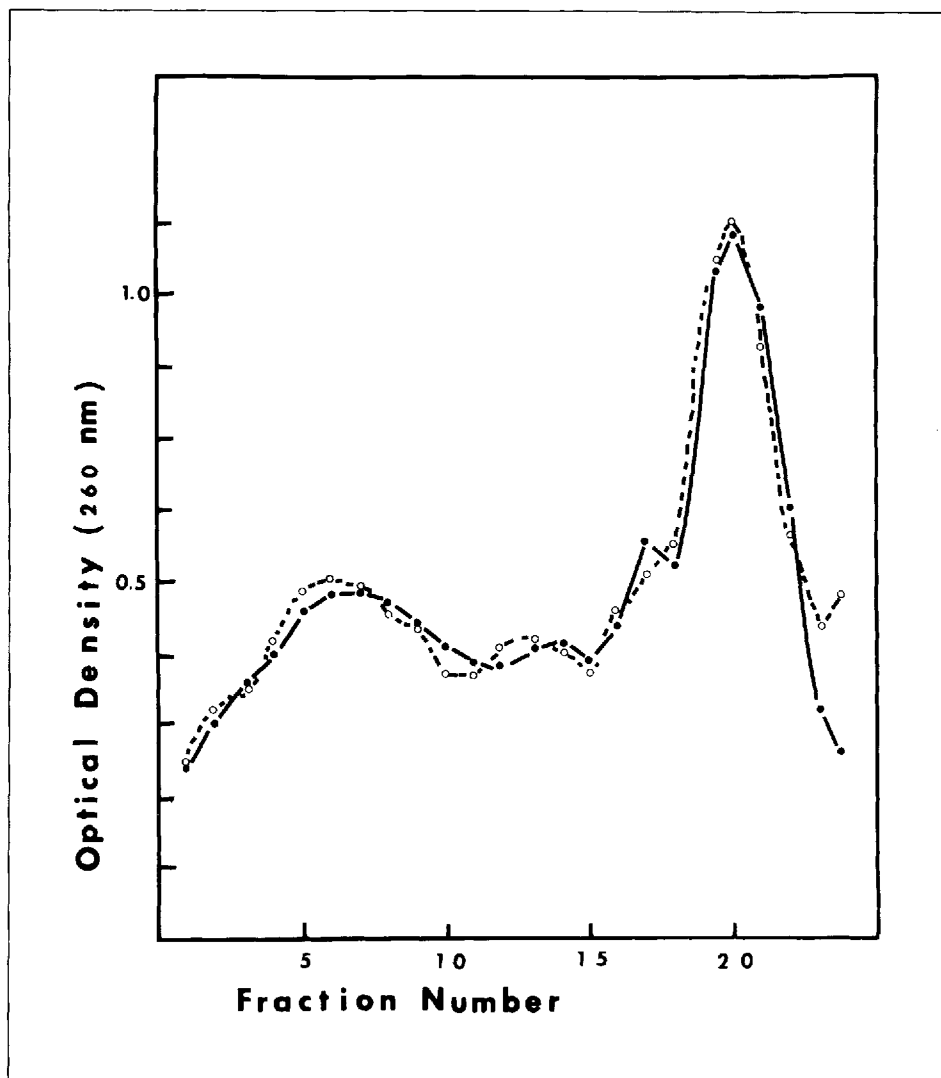

Figure 1. Distribution of polyribosomal and ribosomal material from skeletal muscle cell lysates on a 10%–40% linear sucrose density gradient. Each point represents the mean value of four different preparations. Control (---) and exercised (——) muscle polyribosomes.

Protein Synthesis by Intact Muscle. The gastrocnemius muscle and soleus muscle were used as sources of tissue to measure the protein synthetic activity of explants maintained in organ culture. The incorporation of labeled leucine into protein increased with time in both muscles, between 0.5 and 1.5 hr (fig. 4). The gastrocnemius explants (fig. 4A) from exercised animals incorporated 16,000 ± 970 cpm after 1.0 hr, while control nonexercised gastrocnemius preparations incorporated 12,500 ± 800 and 14,500 ± 840 cpm after 1.0 and 1.5 hr of tissue incubation. The soleus muscle explants (fig. 4B) from exercised animals were significantly more active in protein synthesis only after 1.5 hr of incubation, incorporating 19,500 ± 630 cpm, while control explants incorporated 16,300 ± 650 cpm into protein. When the protein synthesis capacity of the tissue explants is expressed relative to the amount of RNA present, the same relative differences persist.

DISCUSSION

A significant amount of information is currently available regarding the synthesis of protein during the development of cardiac hypertrophy, despite the fact that some investigations are not entirely in agreement with respect to the role of protein synthesis during adaptation to increased functional demand.[15] Little information is available concerning the role of the protein synthesis system during the adaptation of skeletal muscle to regularly performed, vigorous endurance training. The in-vitro techniques used in this investigation indicate that an increase in the efficiency of protein synthesis following a single bout of vigorous exercise may play a role in the development of increased capac-

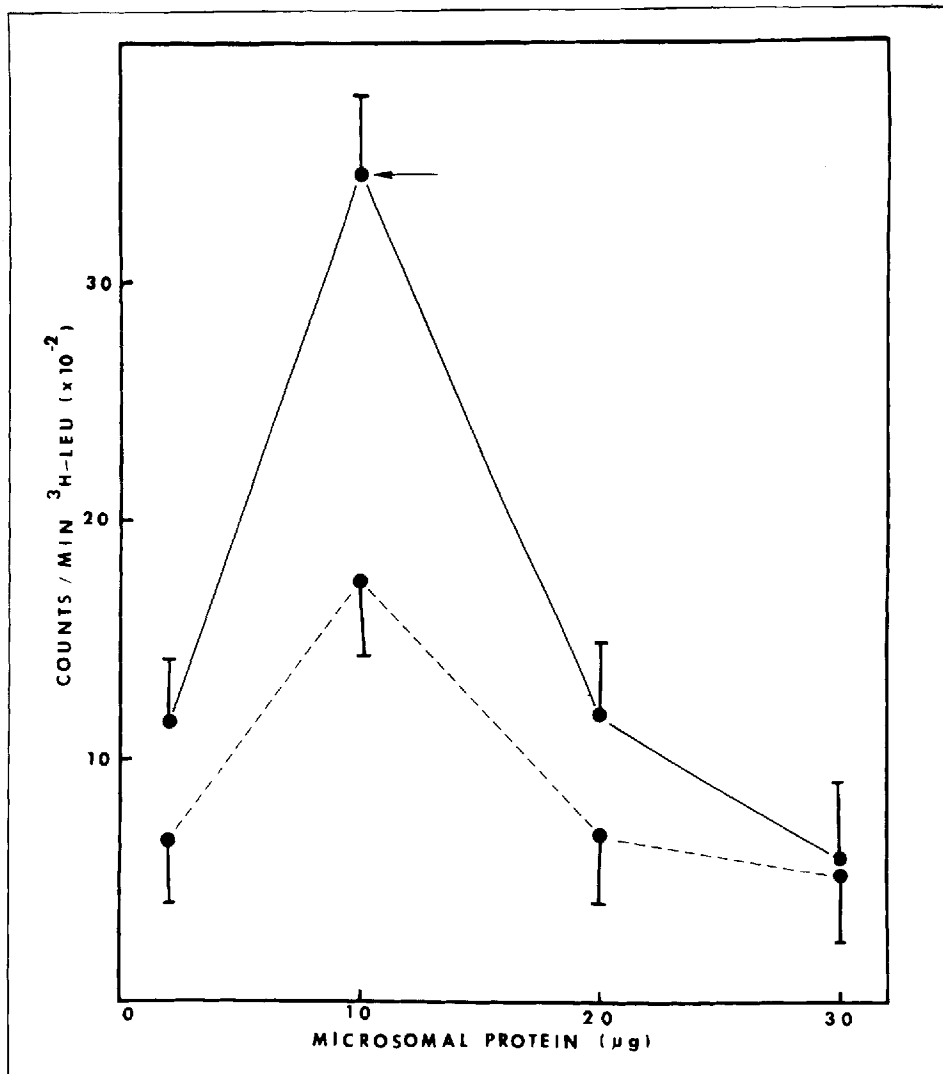

Figure 2. Incorporation of [^{3}H]-leucine into trichloroacetic acid-insoluble protein in a microsomal cell-free system derived from skeletal muscle cells. Values are mean ± SEM for five preparations. Arrow indicates point which is significantly different at $p < 0.01$. Control (---) and exercised (——) muscle microsomes.

ity for aerobic metabolism observed in endurance-trained skeletal muscle.

In-vitro polyribosomal and microsomal protein synthesis was enhanced in the skeletal muscle of the exercised animals. The results parallel the published finding that in-vitro microsomal protein synthesis is greater in hypertrophied skeletal muscle than in control muscle.[2] The enhanced protein synthesis capacity has been attributed to an increase in microsomal RNA. Microsomes isolated from left ventricular tissue subsequent to supravalvular constriction of the ascending aorta in rabbits are more active than those isolated from control animals. This enhanced activity is detected after 24 hr of overload stress and persists for five days. As in the case of hypertrophied skeletal muscle, the difference in activity was believed to be due to an increase in ribosomal RNA content of the microsome fraction of the stressed cardiac tissue.[11] Other experiments using isolated, overloaded perfused cardiac tissue suggested that an increase in cardiac messenger RNA synthesis may be responsible for activation of microsomal protein synthesis.[12]

The results of our experiments with in-vitro protein synthesis by microsomes and polyribosomes from acutely exercised skeletal muscle indicate that exercise stress induces positive changes in the efficiency of protein synthesis at the level of translation, and that these changes in efficiency cannot be accounted for by quantitative differences in protein or RNA present in the in-vitro systems. The RNA contents of control and exercised microsomes and polysomes were equivalent, and the polyribosome profiles for control and exercised tissue were indistinguishable. The presence

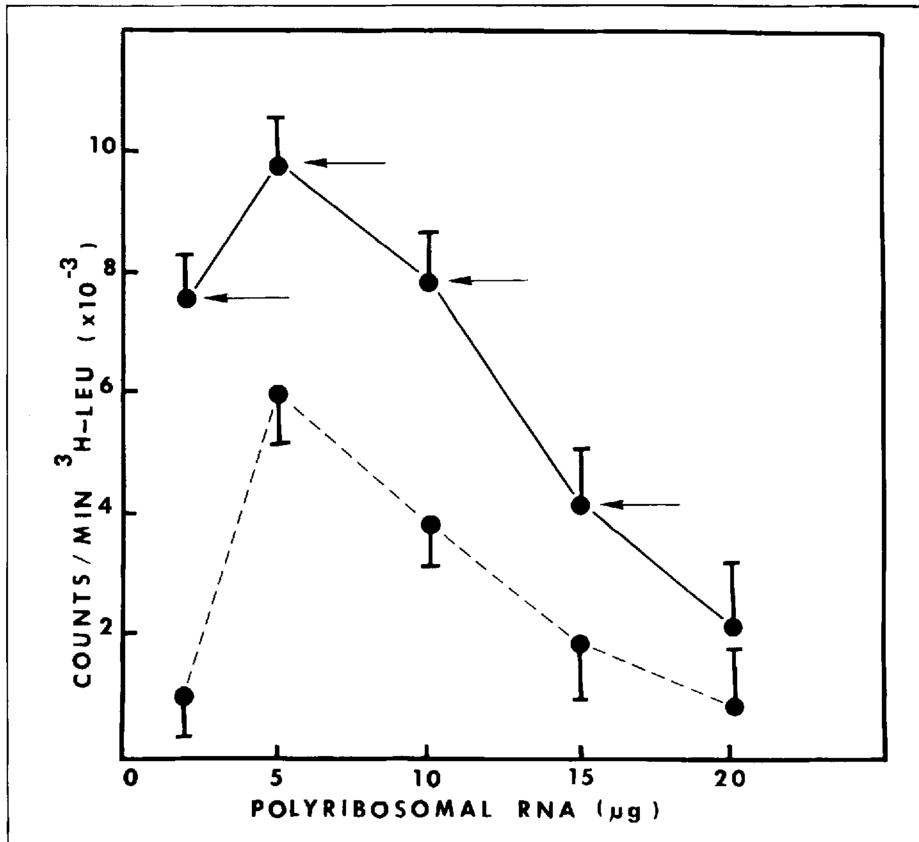

Figure 3. Incorporation of [^{3}H]-leucine into trichloroacetic acid–insoluble protein in a polyribosomal cell-free system derived from skeletal muscle cells. Values are mean ± SEM for five preparations. Arrows indicate points which are significantly different at $p < 0.01$. Control (---) and exercised (——) muscle polyribosomes.

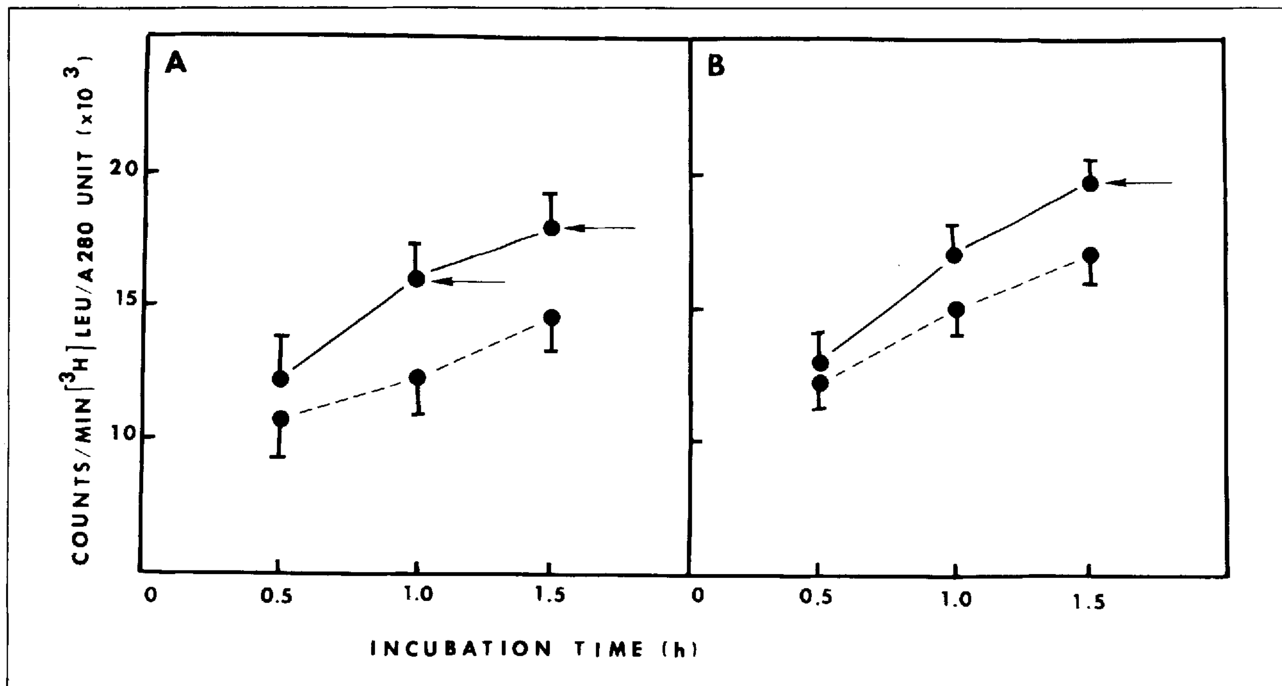

Figure 4. Time course of [^{3}H]-leucine incorporation into trichloroacetic acid–insoluble protein by gastrocnemius muscle explants (A) and soleus muscle explants (B) from guinea pigs. Values are mean ± SEM for six preparations. Arrows indicate points which are significantly different at $p < 0.01$. Control (---) and exercised (——) muscle explants.

of small quantities of regulatory RNA or protein molecules cannot be excluded as a possible explanation of the results presented here, especially in view of the purification of a species of RNA that has been implicated in regulation of the translation of myosin messenger RNA,[4] but it is clear that gross changes in the synthesis of stable RNA species cannot explain the results presented here.

Tissue explants from both the gastrocnemius and soleus muscles of exercised animals incorporated significantly more amino acids into protein than explants from control muscles after 1.0 hr and 1.5 hr of incubation in organ culture medium containing [^{3}H]-leucine. The results agree with the increased translational efficiency of polyribosomes and microsomes isolated from exercised skeletal muscle. The higher net amino acid incorporation by control and exercised soleus muscle explants, as compared with gastrocnemius muscle explants, is consistent with the finding that skeletal muscles containing a higher proportion of slow-twitch fibers are more active in protein synthesis and have a higher RNA concentration than do muscles with fewer slow-twitch fibers.[1] The protein synthesis of the exercised gastrocnemius muscle explants is significantly greater than control values after 1.0 hr of incubation, while explants of the exercised soleus muscle do not show any significant difference in protein synthesis until 1.5 hr of incubation. These results may be explained by the difference in oxidative capacity between the two muscles. Compared to the gastrocnemius, the soleus muscle has a higher relative capacity for aerobic metabolism and demonstrates no significant adaptation to endurance training.[5] Therefore, the high aerobic demand of the treadmill running is better met by the higher capacity for aerobic metabolism of the soleus muscle than by the gastrocnemius muscle.

Our results are in general agreement with the hypothesis that protein synthesis in skeletal muscle is correlated with the functional demands placed on that tissue. Extensions of these experiments, combined with efforts to isolate and translate messenger RNA from developmentally stable skeletal muscle, should enhance our understanding of the molecular mechanism(s) by which mature mammalian skeletal muscle is able to maintain its functional state as well as to adapt specifically to reduce relatively severe demands on its metabolic and contractile processes.

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