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CONTRACTILE PROTEIN ADAPTATIONS TO COMPENSATORY
HYPERTROPHY OF RAT PLANTARIS MUSCLE

by



Qian Tang

A Thesis
Submitted to the Faculty of Graduate Studies
through the Faculty of
Human Kinetics in Partial Fulfillment
of the Requirements for the Degree
of Master of Human Kinetics at
The University of Windsor

Windsor, Ontario, Canada

1982

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ABSTRACT

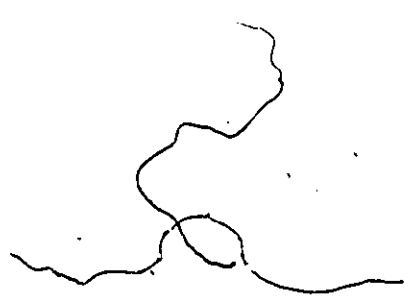
CONTRACTILE PROTEIN ADAPTATIONS TO COMPENSATORY HYPERTROPHY OF RAT PLANTARIS MUSCLE

by

Qian Tang

Protein synthesis, myosin ATPase activity and myosin light chain composition in rat plantaris muscle undergoing compensatory hypertrophy has been investigated. These parameters were correlated with the histochemical adaptation in the same muscle. At 2, 5, 15, 30 and 50 days post-surgery rat hindlimbs were perfused with buffer containing [^3H]phenylalanine. At these times, the weight of the hypertrophied plantaris muscle (HP) had increased by 11, 33, 33, 50 and 104%, respectively, compared to the contralateral control. Total muscle protein and myosin protein synthesis in the HP was found to be stimulated ($p < 0.05$) at 15 days post-surgery and returned to normal by 30 days. At the 30 day interval, the percent of histochemically determined alkaline-labile (SO) fibers had increased in the HP from 11% to 21% ($p < 0.05$) with little further change thereafter. The myosin slow light chain 1 component percent increased ($p < 0.05$) in the HP. Meanwhile, Ca^{++} -activated myosin ATPase activity (pH 9.8)

declined by 8.5% ($p < 0.05$). A transformation from fast to slow twitch muscle fibers was speculated. These observations suggest that the muscular adaptive remodelling process was stimulated between 15 and 30 days after the muscle was overloaded and was well established in less than 30 days. The early compensatory muscle growth could be due to decreased protein degradation.



To my wife Xiang, Mom and Dad

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INTRODUCTION

Skeletal muscle is composed of a mixture of muscle fibers each having different metabolic and contractile characteristics (Barnard et al., 1971; Baldwin et al., 1972; Peter et al., 1973). An increased workload can cause a muscle to undergo compensatory growth and alter its biochemical and histochemical properties (Goldberg et al., 1975; Holloszy, 1975; Baldwin et al., 1977). This adaptation ultimately leads to an increase in the muscle's capacity to generate tension.

In an effort to understand the relative plasticity of skeletal muscle, attention has focused on the underlying adaptive mechanisms for accomodating increased functional demand. Several animal models have been employed to overload skeletal muscle. They include treadmill running (Faulkner et al., 1971; Baldwin et al., 1972), weight training (Exner et al., 1973; Gonyea and Bonde-Peterson, 1978; Gonyea, 1980) and tentomy of synergists (Goldberg, 1969; Schiaffino, 1974). However, treadmill running and weight training have been found to induce only minimal growth while tenotomy results in a mild but transient muscular hypertrophy.

Recently, compensatory hypertrophy of the rat plantaris muscle induced by a total resectioning of the ipsilateral

gastrocnemius has been utilized by several investigators (Ianuzzo et al., 1976; Armstrong et al., 1979; Gollnick et al., 1981). With this model, hypertrophy presumably represents the muscular response to an elevated contractile demand placed on the muscle by removal of its synergists. This surgical model can induce a rapid, large and sustained muscular hypertrophy as well as a marked alteration in the muscle fiber type profile (review in Ianuzzo et al., 1981). It is a particularly useful and convenient model to examine muscle adaptation.

The process of muscular hypertrophy must be accomplished by a net synthesis of cellular materials, particularly the protein that constitutes the contractile elements. Previous studies have revealed that compensatory hypertrophy is accompanied by increased protein synthesis (Hamosh et al., 1967; Goldberg, 1968). Simultaneously protein catabolism was reported to be depressed (Goldberg, 1969). The increased protein synthesis together with decreased degradation are thought to account for the quantitative and qualitative changes in muscle protein found to occur during hypertrophy. However, these previous investigations most often concentrated on short-term (usually within 7 days post-surgery) muscle compensation. Since the composite half-life of tissue protein is considerably longer than 7 days (approximately 25 days,

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Gubjarnason et al., 1964), compensatory remodelling of the overloaded muscle would probably not be completed in such a short time. This being the case, a longer term study relating protein synthesis to the concomitant biochemical and histochemical changes would provide more insight into the mechanism(s) of muscle adaptation to enhanced functional demand.

A review of some contractile properties of hypertrophied muscle has shown that short-term surgically-induced hypertrophy is accompanied by a reduction of muscle contractility as reflected by reduced tension development per unit mass (Lesch et al., 1968; Gutman et al., 1969) and prolongation of contraction time (Vrbová, 1963; Lesch et al., 1968). This was suggested to be due to a reduced protein concentration caused by edema and/or a reduced specific activity of the enzymes, myofibrillar ATPase (Gutman et al., 1969) and myosin ATPase (Jaclecki and Kaufman, 1973). However, it has also been reported that muscle contractile properties are unaltered following chronic hypertrophy (Binkhorst and van't Hof, 1973). Therefore, the time course and extent to which contractile characteristics are altered in the muscle undergoing hypertrophy remains open to question.

Whereas there is confusion regarding the contractile character of hypertrophied muscle, there is no question that compensatory hypertrophy is accompanied by significant

histochemical alterations. A disproportional increase in cross-sectional area of the different fiber types has been observed. The hypertrophied muscle exhibits an increase in its percentage of histochemically determined alkaline-labile (SO) fibers as compared to its contralateral control (Guth and Yellin, 1971; Schiaffino and Bormioli, 1973; Ianuzzo et al., 1976). It has been speculated that this results from a transformation of fast-twitch to slow-twitch muscle fibers (Ianuzzo et al., 1976). The depressed myosin ATPase activity observed in hypertrophied muscle (Ianuzzo et al., 1981) is consistent with this histochemical observation since a low myosin ATPase activity has been found to be one of the characters of slow muscle (Bárány, 1967). This fiber transformation would represent a functional hypertrophic adaptation as slow-twitch fibers exhibit a greater energetic efficiency in maintaining isometric tension than do fast-twitch ones (Awan and Goldspink, 1970).

In an attempt to explain the significance of the histochemical adaptation in muscle, the composition of the myosin protein, one of the primary contractile elements, has been examined in the hypertrophied muscle. Ianuzzo et al. (1981) demonstrated a shift in the electrophoretic myosin light chain pattern towards that of a "slow" type myosin in the chronically hypertrophied rat plantaris muscle although they did not quantify this alteration. This indicated that a

substantial alteration may occur in the myosin molecule during the development of muscular hypertrophy. It was speculated that chronically altered ambulatory requirements of a muscle can alter myosin gene expression (Ianuzzo et al., 1981). Unfortunately, the synthesis process of this contractile protein has not been investigated. It is not adequate to generalize the finding in total muscle protein synthesis adaptation during muscular hypertrophy to the possible response of myosin protein synthesis.

No systematic information is presently available to indicate the relation between these histochemical, biochemical and electrophoretic alterations and their significances with regard to the hypertrophy process. Since such an examination would help to elucidate the factors responsible for altering genetic expression, the present study was undertaken to investigate the time course (2-50 days post-surgery) of alteration in total protein and myosin protein synthesis, histochemical properties, myosin structure and myosin catalytic capacity in the rat plantaris muscle undergoing surgically induced compensatory hypertrophy. It was anticipated that this investigation would offer some insight into the temporal relation among these parameters and allow some speculation with regard to the mechanisms of muscular adaptation to a functional overload.

MATERIALS AND METHODS

At various times post-surgery (2, 5, 15, 30 and 50 days), protein synthesis, myosin synthesis, myosin ATPase activity and light chain composition in the hypertrophied rat plantaris muscle were measured and related with the histochemically determined adaptation in the same muscle. Selected parameters were also examined at 150 days post-surgery.

Animals

Wistar rats (Woodlyn Farms, Guelph, Ontario, Canada) having initial body weights of 200-220 g were employed in this study. They were housed individually and were fed and watered ad libitum. All animals were maintained in a controlled environment with a temperature of 21°C and a 12 hr light/dark cycle.

Hypertrophy Model

Compensatory hypertrophy of rat plantaris muscle was induced by a total resectioning of the gastrocnemius muscle as described by Ianuzzo and Chen (1977). The contralateral leg served as a sham-operated control.

Determination of Protein Synthesis in Hypertrophying
Plantaris Muscle

Perfusion Procedures. To examine the protein synthetic process in muscle undergoing hypertrophy, animals were sacrificed at appropriate times post-surgery and a hindlimb preparation was perfused with a [^3H]phenylalanine buffer. The surgical procedures to prepare the hindlimb for perfusion were essentially those described by Jefferson (1975) as modified by Burton (1978). The hindlimb preparation was perfused with a Krebs-Henseleit bicarbonate buffer containing 15 mM glucose, bovine insulin (25 milliunits/ml), 0.4 mM phenylalanine (5 times normal plasma level) and the other 19 common amino acids (Tolman et al., 1973). [^3H]phenylalanine was added to the buffer to yield a final activity of 0.25 uCi/ml. The perfusate was gased with 95% O_2 : 5% CO_2 and maintained at 37°C. The hindlimb preparation was initially perfused with 50 ml of the perfusate which was collected and discarded. Thereafter 150 ml of the perfusate was recirculated at a flow rate of 10-12 ml/min for 60 min (Dohm et al., 1980), at which time the plantaris muscles of both the hypertrophied and control legs were excised, weighed and stored in 50% glycerol at -20°C for later use.

Myosin Extraction. Myosin extraction was carried out at 0-4°C by a modification of the procedure employed by Bhan

and Scheuer (1975). The muscle tissues was minced and homogenized with a Potter Elvehjem tissue homogenizer in 20 vol of 0.05 M KCl - 0.01 M KHPO_4 (pH 7.0). After centrifugation at 2,000 g for 10 min, the pellet was suspended in 20 vol of 0.05 M KCl - 0.01 M KHPO_4 , 1% Triton X-100 (pH 7.0) and stirred for 45 minutes. This fraction was then centrifuged at 2,000 g for 10 min, and the residue was washed twice with 0.05 M KCl - 0.01 M KHPO_4 (pH 7.0). The washed myofibrils were extracted with 30 vol of 0.47 M KCl - 0.01 M KHPO_4 and 0.02 M $\text{K}_4\text{P}_2\text{O}_7$ (pH 6.8) for 15 minutes. Following centrifugation for 30 min at 13,000 g, the supernatant was diluted 10-fold with cold double distilled water. The resulting myosin precipitate was dissolved in 18 ml of 0.47 M KCl - 0.01 M KHPO_4 , 0.02 M $\text{K}_4\text{P}_2\text{O}_7$ and 0.005 M MgCl_2 (pH 6.8) and centrifuged for 1.5 hr at 90,000 g. The supernatant was then fractioned with a saturated ammonium sulfate solution containing 0.01 M EDTA. The fraction precipitating from 35-45% saturation was collected by centrifugation at 13,000 g for 10 minutes. This fraction was resuspended in a solution of 0.6 M KCl in 0.05 M Tris and dialyzed against a buffer of 10 mM KCl, 1 mM EDTA, 1 mM Tris and 0.05 mM DTT (pH 7.0) for 14-18 hours. The myosin was then collected by a centrifugation at 30,000 g for 30 minutes. Employing this procedure, the myosin yield was on the order of 20 mg/g wet weight.

Approximately one half of the myosin pellet was dissolved in 0.6 M KCl and used for radioisotope scintillation counting and electrophoretic analysis. The remainder was stored in a solution of 0.6 M KCl, 20 mM EDTA, 2 mM DTT and 50% glycerol (pH 7.0 - 7.2) at -20°C for subsequent myosin ATPase determination. The concentration of myosin protein in both portions was quantified by the method of Lowry et al. (1951).

Total Protein Extraction. To isolate the total tissue protein, a 1 ml aliquot of the homogenate described in "Myosin Extraction" was precipitated overnight in an equal volume of 0.6 M TCA at 4°C. Following centrifugation at 2,000 g for 15 min, the protein precipitate was dissolved in 1N NaOH and was quantified according to Lowry et al. (1951).

Liquid Scintillation Counting. To determine the [^3H] phenylalanine incorporation into total muscle protein, aliquots (100 μl) of this fraction were suspended in a toluene based scintillation solution for counting in a Beckman LS-100 liquid scintillation counter. Similarly, aliquots of myosin (400 μl) were counted after being dissolved in a diluted PCS (Amersham) cocktail (PCS:Toluene, 2:1). The radioactivity was expressed as disintegrations per min (dpm) per mg protein.

Specific Radioactivity of Free Phenylalanine. After

the total tissue protein was precipitated with 0.6 M TCA (see above), the supernatant (TCA soluble fraction) was employed to determine the specific radioactivity of free phenylalanine in the muscle. TCA was removed from the aqueous phase according to the method of Warner and Finamore (1967). To the TCA soluble portion, a slight excess of 1N alamine in chloroform was added and the two phase system was mixed thoroughly and then centrifuged for 10 min at 2,000 g. The deacidified aqueous phase was removed carefully and dried in an oven at 50°C. The remaining phenylalanine was suspended in 400 ul of 0.3 M TCA and quantified by the method of Joyce et al. (1965). The radioactivity was determined as previously detailed for myosin.

Rate of Protein Synthesis. The protein synthetic rate (in both total and myosin fractions) was calculated by dividing the incorporation rate of [^3H] phenylalanine (dpm/mg protein/hr perfusion) by the specific radioactivity of free phenylalanine (dpm/nmole phenylalanine) in the muscle. This rate was expressed as nmole phenylalanine incorporated per mg protein per hr perfusion.

Histochemical Measurements

In order to estimate the percent alkali-labile (slow-twitch) and alkali-stabile (fast-twitch) fibers,

muscle cross-sections of 10 μ m thickness were stained for myofibrillar ATPase following an alkaline pre-incubation at pH 10.3 (Padykula and Herman, 1955; Guth and Samaha, 1969).

Fiber type populations were estimated by the classification of 25-30 fibers in each of eight regions evenly distributed throughout the muscle cross-sections. Those fibers which showed an intermediate staining intensity for myofibrillar ATPase were recorded as alkaline stable.

Fractionation of Myosin

Myosin samples (100 μ g) were separated on a sodium dodecyl sulfate (SDS) polyacrylamide disc gel system according to the procedure of Laemmli (1970). Separating gels (9 cm) of 10% acrylamide containing 0.375 M Tris-HCl (pH 8.8), 0.1% SDS and 1 cm stacking gels of 3% acrylamide containing 0.125 M Tris-HCl (pH 6.8), 0.1% SDS were prepared. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Electrophoresis was carried out with a current of 1.5 mA per gel. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 and the myosin light chains were identified by determination of their molecular weights using appropriate standards (Weber and Osborn, 1969).

The molar ratio of myosin light chains was determined by integration (Lowey and Risby, 1971) following scanning of

the SDS gels in a Gilford 2520 gel scanner.

Myosin ATPase Activity

The Ca^{++} -activated myosin ATPase activity at 25°C was assayed at both pH 7.0 and 9.8 using a modification of the method of Bárány and Close (1971). Myosin protein (0.15 - 0.2 mg/ml) was preincubated in a reaction mixture composed of 25 mM KCl, 10 mM CaCl_2 and either 60 mM histidine (pH 7.0) or 75 mM glycine (pH 9.8). The reaction was then initiated by the addition of Na_2ATP (3 mM). The method of Rockstein and Herron (1951) was used to measure inorganic phosphate liberation.

Statistical Analysis

The dependent t-test was employed for statistical comparison of data from hypertrophied muscle and its contralateral control. A p-value of 0.05 or less was considered significant.

RESULTS

Characteristics of the Hypertrophy Model

Data presented in Table 1 indicates that the rat plantaris muscle in the limb from which the gastrocnemius muscle had been removed demonstrated a large and sustained increase in wet weight when compared to its contralateral control. The increase ranged from 11% at 2 days to 104% at 50 days post-surgery with little additional change thereafter. This is similar to previous data employing the same surgical model (Ianuzzo and Chen, 1979).

In contrast to previous investigations, the water content (%) in the hypertrophied plantaris was not found to be higher than in the control muscle, even at the early times (2-5 days) post-surgery (Table 2). However, this does not eliminate the possibility of a surgically induced edema of the hypertrophied muscle since the perfusion procedure would tend to mask the difference in water content between hypertrophied and control muscle. Additionally, the sham operation performed on the contralateral limb was extensive enough to have possibly resulted in some edema in the control muscle.

There was no significant difference between the hypertrophied and control plantaris in terms of total muscle tissue protein or myosin yields in any group (Table 2).

Table 1. Time course for changes in muscle wet weight of control vs hypertrophied rat plantaris muscle (2-150 days postmyectomy).

Days Postsurgery	Body Wt. (g)	Control Plantaris Wt. (mg)	Hypertrophied Plantaris Wt. (mg)	Diff. (%)
2 (7)	241 ±4	242 ±5	*268 ±10	11 ±4
5 (4)	259 ±15	264 ±19	*346 ±13	33 ±10
15 (6)	308 ±24	330 ±30	**433 ±28	33 ±7
30 (6)	258 ±9	275 ±13	***411 ±15	50 ±3
50 (7)	398 ±10	381 ±21	***759 ±38	104 ±11
150 (9)	548 ±19	452 ±18	***942 ±53	111 ±13

Data are expressed as means ± SEM.

Numbers in parentheses are number of animals per group.

Control vs hypertrophied muscle are statistically significant at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2. Some characteristics of control and hypertrophied rat plantaris muscles (2-150 days postmyectomy).

Days Postsurgery		% Water	Total Tissue Protein (mg/g wet wt.)	Myosin (mg/g wet wt.)
2 (6)	CP	80.02 \pm 1.06	114.87 \pm 5.59	22.23 \pm 1.44
	HP	79.35 \pm 0.42	104.37 \pm 4.10	20.90 \pm 1.25
5 (4)	CP	82.25 \pm 1.27	110.03 \pm 4.09	16.46 \pm 0.75
	HP	83.23 \pm 1.25	97.74 \pm 3.90	14.12 \pm 0.58
15 (6)	CP	79.42 \pm 1.26	107.42 \pm 5.38	20.43 \pm 1.70
	HP	81.16 \pm 0.70	105.13 \pm 3.26	15.66 \pm 0.94
30 (6)	CP	77.22 \pm 1.33	172.15 \pm 9.83	28.24 \pm 1.69
	HP	76.80 \pm 1.43	172.30 \pm 10.04	23.01 \pm 1.84
50 (5)	CP	80.14 \pm 1.04	114.12 \pm 7.13	20.47 \pm 0.67
	HP	77.00 \pm 1.50	101.39 \pm 4.52	18.75 \pm 0.86
150 (4)	CP	74.17 \pm 1.92	128.08 \pm 7.20	20.91 \pm 1.65
	HP	76.48 \pm 1.16	124.95 \pm 1.00	20.63 \pm 1.19

Data are expressed as means \pm SEM.

Numbers in parentheses are number of animals per group.

CP, control plantaris; HP, hypertrophied plantaris.

Characterization of the Perfusion System

To establish the metabolic stability of the hindlimb perfusion system, a preliminary investigation was conducted. Our observations indicate that lactate produced by the hindlimb preparation decreased after the initial anoxia caused by the surgical procedure and remained at a relatively low level throughout the perfusion period (Fig. 1). This is consistent with previous experiments employing similar surgical and perfusion procedures (Burton, 1978). Further, the results in Table 3 demonstrate that the glycogen content and the percent water in the perfused muscle were quite similar to those found in normal controls. The findings suggest that the hindlimb preparation was metabolically stable. Incorporation of [^3H]phenylalanine into total muscle protein was found to increase proportionately to the perfusion time over a 60 min period (Fig. 2). As a sufficient amount of radioactivity in muscle protein could be detected at 60 min, this point was selected as the perfusion duration in subsequent experiments.

Finally, in order to estimate the intracellular availability of [^3H]phenylalanine in relation to the duration of the perfusion period, the radioactivity of the TCA soluble fraction of muscle tissue was examined at various times during perfusion. The results in Table 4 suggest that the

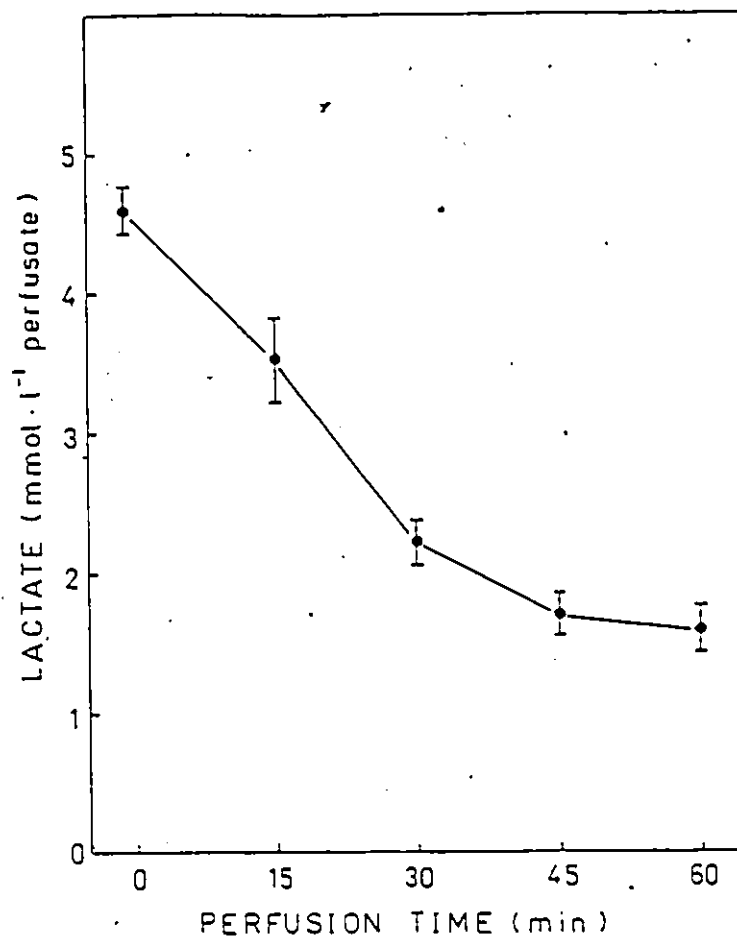


Fig. 1. Lactate production during perfusion.

Rats (n=6) were perfused with Krebs-Henseleit buffer for 60 min. At 0, 15, 30, 45, and 60 min, perfusate sample coming out of the hind-limb was collected. Perfusate lactate content was quantified by the method of Pryce (1969). Data are expressed as means \pm SEM.

Table 3. Glycogen and water content in normal and perfused rat plantaris muscle.

Condition	% Water	Glycogen	
		mg/g wet wt.	mg/g dry wt.
Control (N=6)	75.32 ± 0.78	4.04 ± 0.55	16.44 ± 2.60
Perfused (N=6)	77.52 ± 0.43	3.35 ± 0.16	14.97 ± 0.95

Data are expressed as means ± SEM.

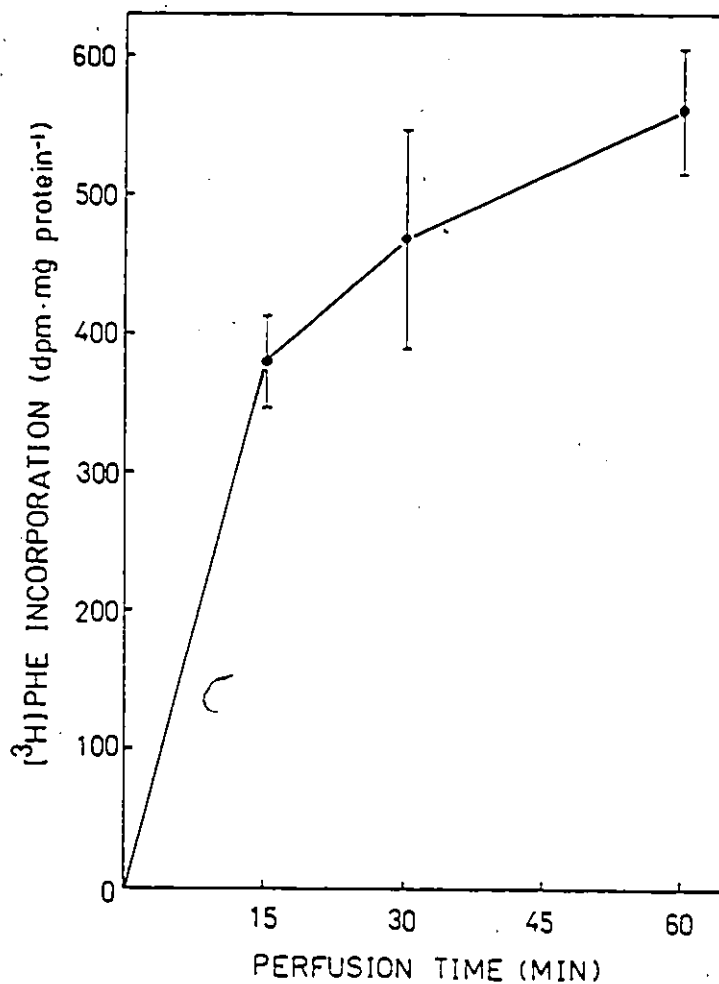


Fig. 2. Incorporation of [³H]phenylalanine into total muscle protein during perfusion.

Rats were perfused for 15, 30, and 60 min with [³H]phenylalanine buffer. Radioactivity was determined as described in "Materials and Methods". Each point represents the mean and SEM of 4-5 perfusions.

Table 4. The effect of perfusion duration on TCA soluble radioactivity.

Perfusion Time (min)	TCA Soluble Radioactivity (dpm/mg total muscle protein)
15 (N=3)	1811 ± 340
30 (N=4)	2519 ± 557
60 (N=3)	2380 ± 231

Data are expressed as means ± SEM. .

intracellular space for phenylalanine equilibrated early and remained stable after 30 min of perfusion.

[³H]phenylalanine Incorporation in Muscle Undergoing Compensatory Hypertrophy

The rate of [³H]phenylalanine incorporation into total muscle protein, when expressed as dpm/mg protein/hr perfusion was found to be elevated ($p < 0.05$) in the hypertrophied plantaris at 2, 5, and 15 days post-surgery (Fig. 3). The difference between hypertrophied and control muscles was no longer present by 30 days post-surgery and thereafter.

The incorporation of [³H]phenylalanine into myosin (dpm/mg myosin/hr perfusion) was not stimulated in the hypertrophied plantaris until 15 days post-ablation ($p < 0.05$) and then returned to control levels by 30 days post-surgery (Fig. 4).

Specific Radioactivity of Free Phenylalanine

To make a valid estimate of protein synthesis based on the incorporation of a radioactively labeled amino acid, the intracellular availability of the amino acid precursor should be known. In the present study, the specific radioactivity of [³H]phenylalanine in muscle was determined to estimate the specific activity of the phenylalanine precursor pool.

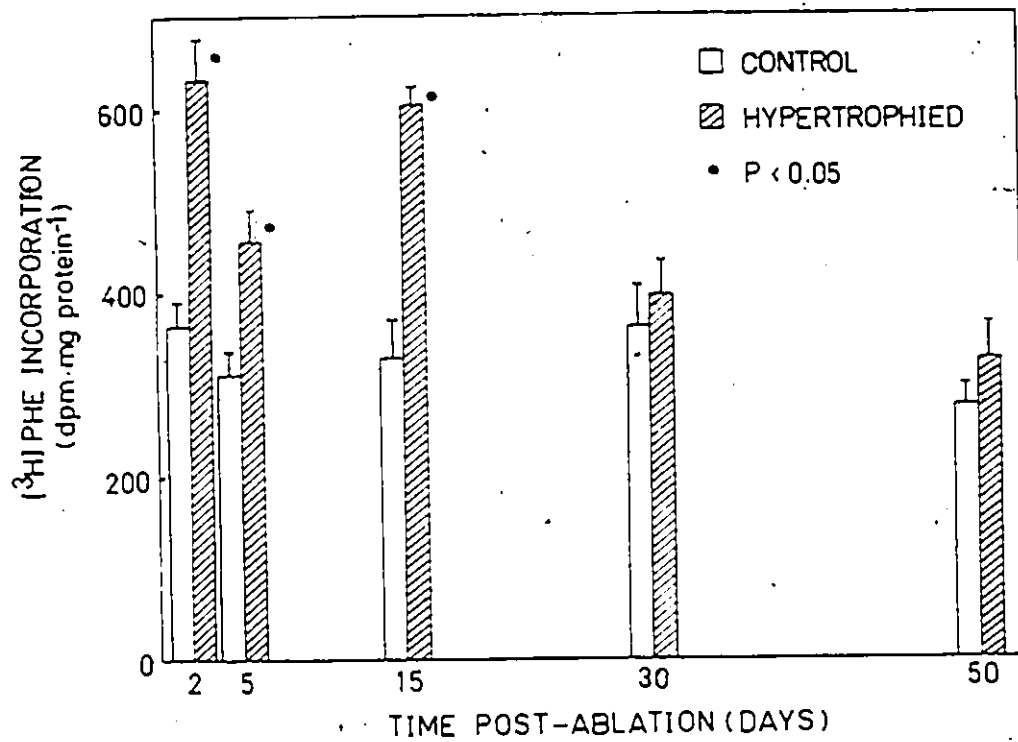


Fig. 3. Incorporation of [³H]phenylalanine into total tissue protein of control and hypertrophied rat plantaris muscle.

Values for each group represent means \pm SEM of 4-6 rats.

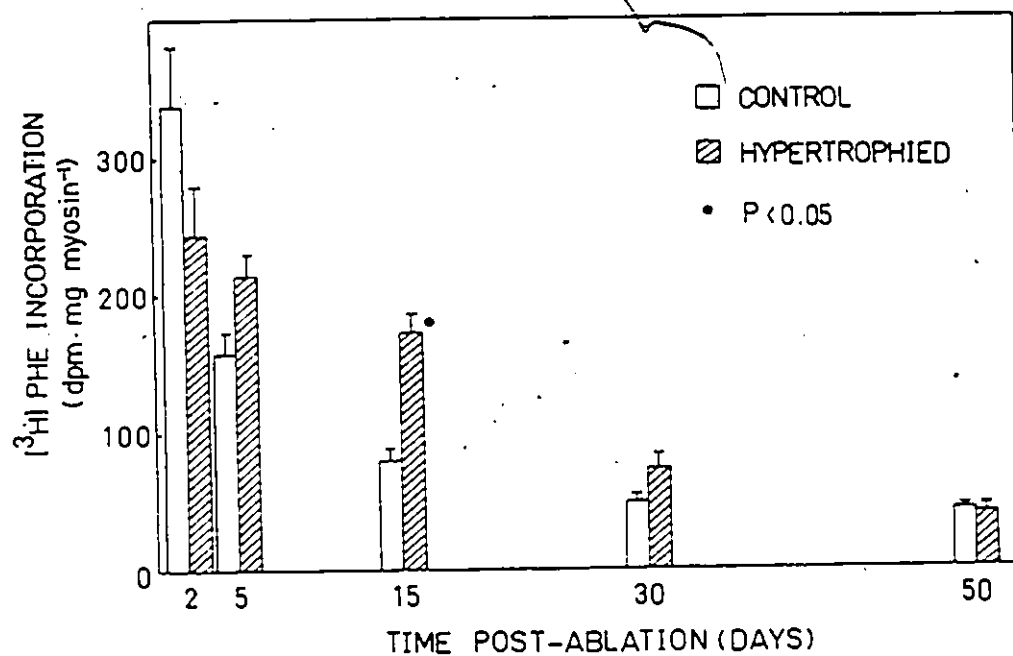


Fig. 4. Incorporation of [³H]phenylalanine into myosin protein of control and hypertrophied rat plantaris muscle.

Values for each group represent means \pm SEM of 4-6 rats.

Fig. 5 demonstrates that the free phenylalanine specific activity, as expressed in dpm/nmole phenylalanine was significantly higher ($p < 0.05$) in the hypertrophied than in the control plantaris at 2 days post-surgery, but lower by 50 days ($p < 0.05$). Further, a trend that the specific activity of free phenylalanine in both legs declined with time post-ablation was observed. The latter could be a result of an increased rate of protein degradation as muscle weight stabilized which resulted in a dilution of the labeled phenylalanine.

The Rate of Protein Synthesis in Muscle Undergoing Compensatory Hypertrophy

The rate of protein synthesis was estimated employing the specific activity of phenylalanine in the muscle. The total muscle tissue protein synthesis as expressed in nmole phenylalanine incorporated/mg protein/hr perfusion, was found to be enhanced ($p < 0.05$) in the hypertrophied plantaris at both the 15 and 50 day intervals (Fig. 6). Similarly, myosin protein synthesis (Fig. 7) was significantly elevated in the hypertrophied muscle at 15 days post-surgery ($p < 0.05$). When the rate of protein synthesis was corrected for the specific activity of the phenylalanine pool, the early hypertrophic response was no longer observed.

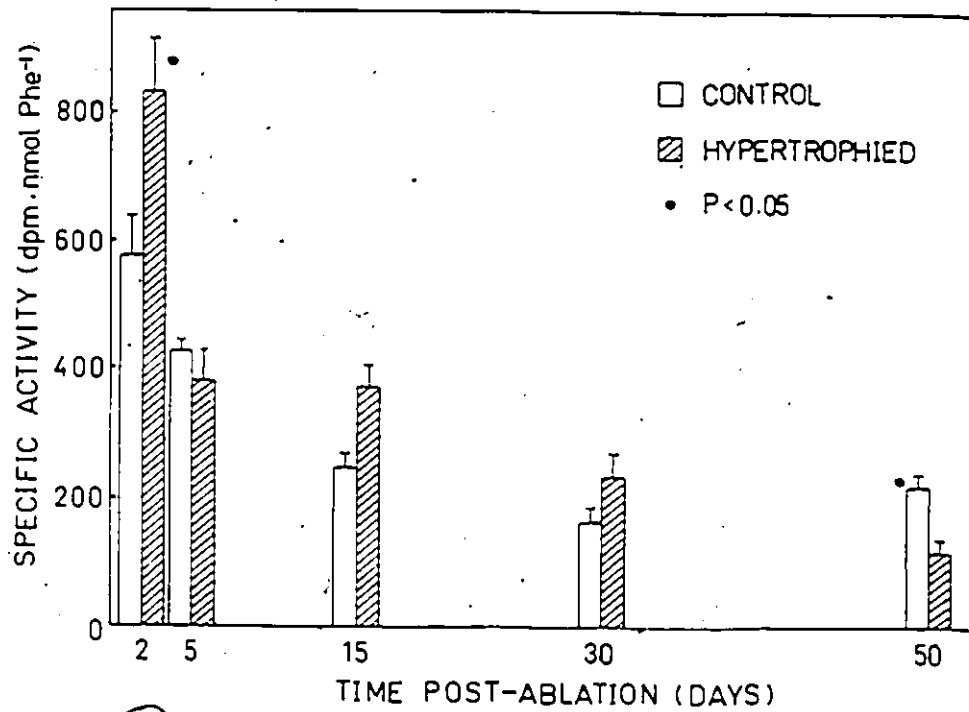


Fig. 5. Specific radioactivity of free phenylalanine in control and hypertrophied rat plantaris muscle.

Values for each group represent means \pm SEM of 4-6 rats.

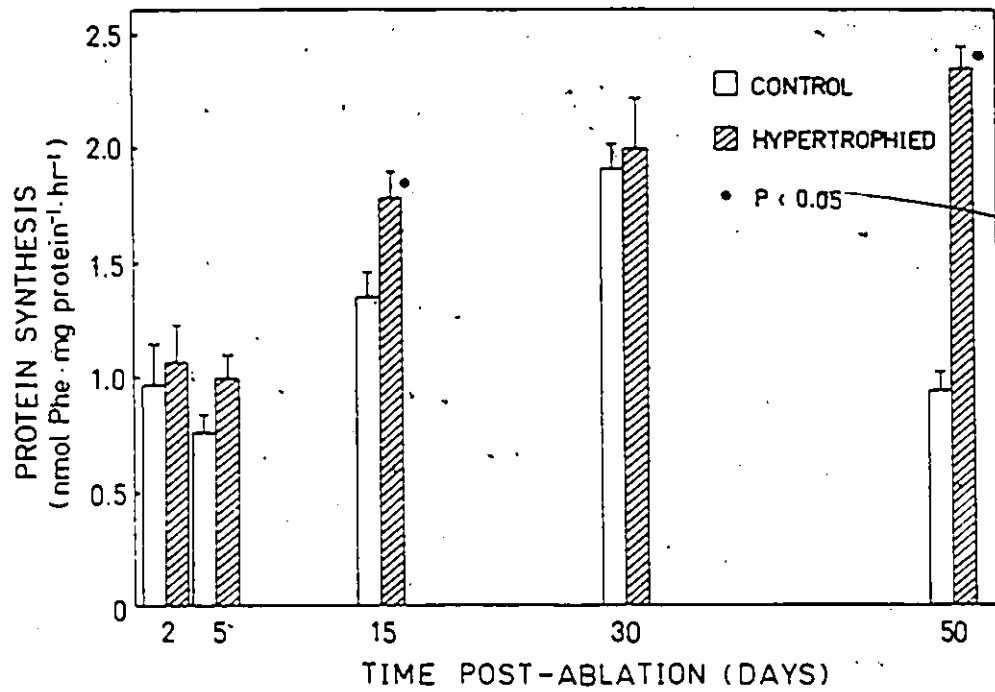


Fig. 6. Protein synthesis rate in control and hypertrophied rat plantaris muscle.

Values for each group represent means \pm SEM of 4-6 rats.

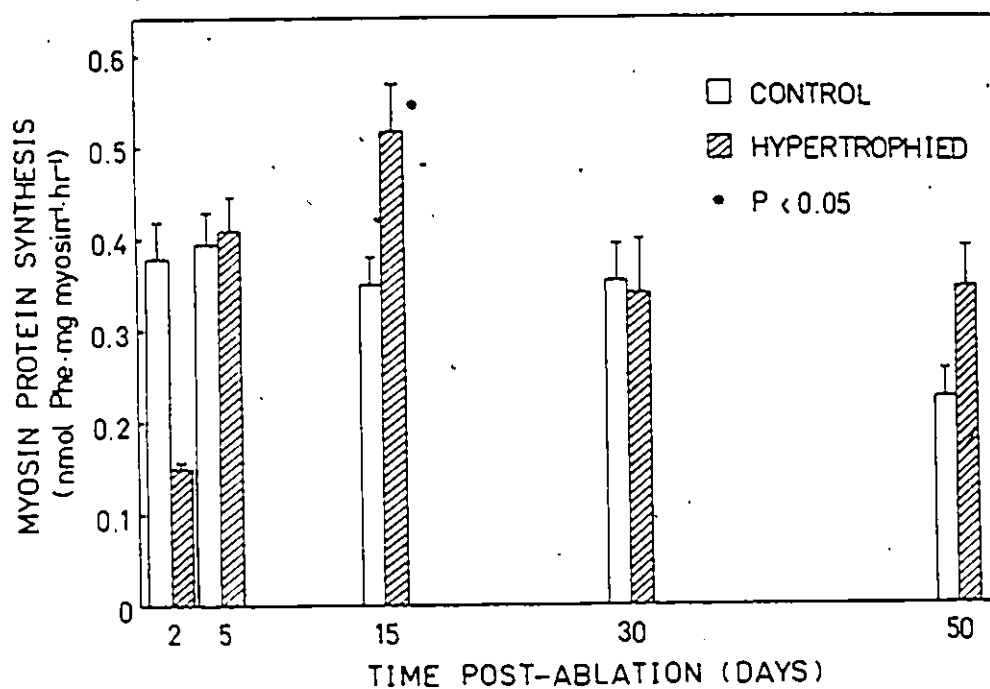


Fig. 7. Myosin protein synthesis rate in control and hypertrophied rat plantaris muscle.

Values for each group represent means \pm SEM of 3-6 rats.

Fiber Distribution in Muscle Undergoing Compensatory Hypertrophy

The hypertrophied plantaris muscle exhibited a large increase in its percentage of histochemically determined alkaline-labile (SO) fibers as compared to its contralateral control. As Table 5 indicates, the histochemical change was not evident until 30 days post-surgery. At this time, the percent of alkaline labile fibers increased from 11% of the total number in the control plantaris to 21% in the hypertrophied muscle ($p < 0.05$). The percentage of SO fibers in the hypertrophied plantaris stabilized thereafter at 25% ($p < 0.01$) and 24% ($p < 0.001$) by 50 days and 150 days post-surgery respectively.

Change in Myosin Light Chain Composition in Muscle Undergoing Compensatory Hypertrophy

Electrophoretic analysis of the myosin light chain composition of the hypertrophied plantaris muscle demonstrated an increase in the slow myosin light chain 1 (LCs1) component at 30 and 50 days post-surgery (Fig. 8). Slow myosin light chain 2 (LCs2) in the plantaris muscle was too weak to be detected in the SDS gel. Integrative analysis of SDS gels scans (Table 6) show that by 30 days post-surgery, the percentage of LCs1 increased ($p < 0.01$) from 5.0% in control to 9.7% in the hypertrophied plantaris

Table 5. Percent of alkaline-labile (SO) fibers in plantaris muscle undergoing compensatory hypertrophy.

Days Postsurgery	Alkaline Labile Fibers (%)	
	Control	Hypertrophied
2 (5)	10.45 \pm 0.63	12.07 \pm 1.12
5 (5)	8.43 \pm 1.53	9.83 \pm 1.08
15 (4)	10.16 \pm 0.62	15.36 \pm 2.92
30 (4)	11.41 \pm 1.97	*21.25 \pm 0.65
50 (5)	11.33 \pm 0.30	**25.29 \pm 1.33
150 (5)	10.53 \pm 0.63	***24.25 \pm 1.19

Values are means \pm SEM.

Numbers in parentheses are number of animals per group.

Control vs hypertrophied muscle are statistically significant at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

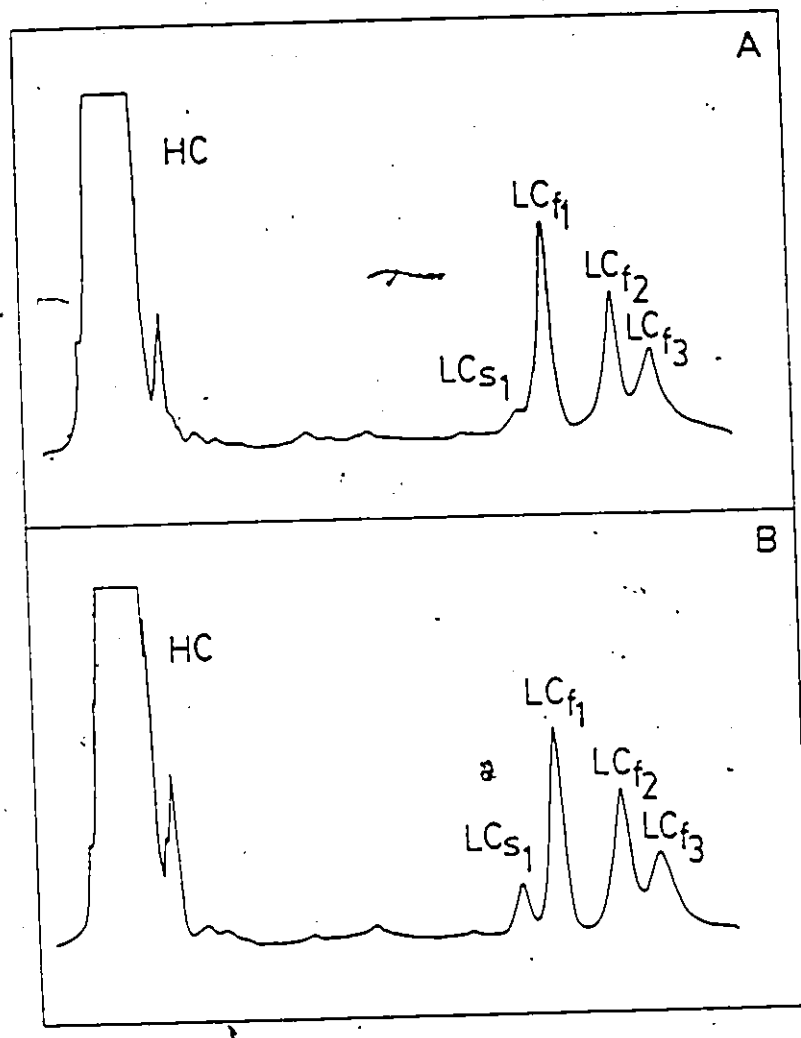


Fig. 8. Densitometric scans of SDS polyacrylamide gels from the electrophoresis of myosin protein from control and hypertrophied rat plantaris muscle.

Samples (80 μ l) containing 100 μ g of myosin were separated on a 3% stacking gel and 10% running gel using the method of Laemmli (1970). Densitometry of the Coomassie Brilliant Blue R-250-stained gels was performed on a Gilford 2520 gel scanner. Sources were: A, control plantaris; B, hypertrophied plantaris, 50 days postsurgery. Abbreviations used: - HC, heavy chain; LCs1, slow light chain 1; LCF1, fast light chain 1; LCF2, fast light chain 2; LCF3, fast light chain 3.

Table 6. Myosin light composition in plantaris muscle undergoing compensatory hypertrophy.

Days Postsurgery		Myosin Light Chain Components (%)			
		LCs1	LCf1	LCf2	LCf3
30	CP	5.0 \pm 1.4	42.9 \pm 1.6	26.6 \pm 1.8	25.6 \pm 1.4
(N=6)	HP	**9.7 \pm 1.4	**37.2 \pm 0.9	24.8 \pm 1.6	28.2 \pm 0.4
50	CP	3.3 \pm 0.6	43.1 \pm 1.9	24.2 \pm 3.3	29.7 \pm 1.3
(N=5)	HP	*8.6 \pm 1.7	39.8 \pm 2.0	20.9 \pm 3.3	30.8 \pm 1.0

Data are expressed as means \pm SEM.

CP, control plantaris; HP, hypertrophied plantaris.

Control vs hypertrophied muscle are statistically significant at: * $p < 0.05$; ** $p < 0.01$.

of the total light chain protein content. Similar differences ($p < 0.05$) were found at the 50 day interval. In addition, at 30 days post-surgery, the percentage of fast myosin light chain 1 (LCf1) was found to be significantly decreased ($p < 0.01$) in hypertrophied plantaris.

Myosin ATPase Activity in Muscle Undergoing Compensatory Hypertrophy

As presented in Table 7, the only significant difference ($p < 0.05$) in Ca^{++} -activated ATPase activity was observed at 30 days post-surgery (pH 9.8). There was an 8.5% decline in ATPase activity from 1.06 $\mu\text{mol}/\text{mg}/\text{min}$ in control muscle to 0.97 $\mu\text{mol}/\text{mg}/\text{min}$ in hypertrophied plantaris at this time.

Table 7. Myosin ATPase activity of plantaris muscle undergoing compensatory hypertrophy.

Days Postsurgery		Ca ⁺⁺ - Myosin ATPase Activity (umoles Pi/mg myosin/min at 25°C)	
		pH 7.0	pH 9.8
2 (11)	CP	0.22 ±0.01	1.25 ±0.06
	HP	0.23 ±0.02	1.21 ±0.07
5 (3)	CP	0.34 ±0.02	1.65 ±0.07
	HP	0.33 ±0.07	1.31 ±0.25
15 (10)	CP	0.21 ±0.01	1.16 ±0.07
	HP	0.22 ±0.02	1.17 ±0.04
30 (11)	CP	0.28 ±0.02	1.06 ±0.04
	HP	0.28 ±0.02	*0.97 ±0.04
50 (11)	CP	0.23 ±0.01	1.22 ±0.12
	HP	0.23 ±0.01	1.15 ±0.11
150 (4)	CP	0.30 ±0.01	0.77 ±0.06
	HP	0.30 ±0.02	0.78 ±0.13

Data are expressed as means ± SEM.

Number in parentheses are number of animals per group.

CP, control plantaris; HP, hypertrophied plantaris.

Control vs hypertrophied muscle are statistically significant at: * $p < 0.05$

DISCUSSION

The animal model employed in this study demonstrated a large and sustained muscular hypertrophy. The increase in muscle wet weight ranged from 11% at 2 days to 110% at 150 days post-surgery. A point of stable enlargement was reached between 30-50 days post-surgery with little additional change thereafter. This finding is consistent with previous investigations which have employed similar surgical procedures (Hubbard et al., 1975; Ianuzzo and Chen, 1979).

The increase in muscle mass during hypertrophy is thought to be related to an adaptive change in muscle protein turnover, since over 80% of the dry weight of muscle is protein. In the present study, a rat hindlimb preparation was perfused with radioactively labeled phenylalanine to investigate the time course and localization of protein synthesis in plantaris muscle undergoing compensatory hypertrophy. A preliminary experiment demonstrated the reliability of the perfusion system and the rat hindlimb preparation.

Employing a tenotomy model, Goldberg (1975) has shown that the hypertrophied plantaris and soleus demonstrated a more rapid protein incorporation of radioactive amino acid than did the control muscle as early as 8 hr after

tenotomy. This process continued for the next 6 days (Goldberg, 1969). Additionally, Hamosh et al. (1967) reported that cell-free extracts, taken from muscle 4 days after tenotomy of its synergist, showed an increased rate of amino acid incorporation. In the present study, the early phase (2, 5, and 15 days post-surgery) of compensatory hypertrophy was found to be accompanied by an increased incorporation of [^3H]phenylalanine into total muscle tissue protein when expressed as dpm per mg protein. This result seemed to be comparable to those of previous reports.

However, enhanced incorporation of labeled amino acid into protein does not necessarily indicate greater in vivo protein synthesis. Changes in muscle size, muscle perfusate flow rate and, particularly, the specific activity of intracellular amino acid pools can lead to a greater incorporation of radioactivity without any change in the rate of protein synthesis. Such complications are of concern in the present study since the size of hypertrophied muscle is different from the control, and the surgical procedure might affect blood or perfusate flow rate to the muscles. Therefore, to make a valid estimate of protein synthesis rate, the information of the amino acid precursor pool size is essential.

The specific activity of transfer RNA-bound amino acid has been used as representative of the immediate precursor

for protein synthesis (Henshaw et al., 1971; Martin et al., 1977). However, the procedure to isolate tRNA requires a tremendous amount of muscle tissue which is not practical in this experiment. Since the purpose of this experiment was to examine the relative change (compared with the contralateral control) in the rate of protein synthesis, it was not necessary to determine the absolute protein synthetic rate. Therefore, we employed the specific activity of [^3H]phenylalanine in muscle as an alternate estimate of the specific activity of the phenylalanine precursor pool. It is noteworthy that more recent data suggest that, for most amino acids, the precursor pool specific activity lies somewhere between the intra- and extracellular pool specific activities (Vidrich et al., 1977; Robertson and Wheatley, 1979; O'Hara et al., 1981), and thus our estimate (using the free phenylalanine specific activity) probably overestimates the true protein synthetic rate.

Using a perfused rat hindlimb preparation, Jefferson et al. (1977) and Dohm et al. (1980) reported protein synthesis rates of 84 and 72 nmol/hr per g of muscle respectively. If we express our results for control muscle in comparable units, they are similar (approximately 100 nmol/hr per g of muscle).

Although published data (Hamosh et al., 1967; Goldberg,

1969, 1975) suggest that protein synthesis is enhanced immediately after surgery, our corrected results for total muscle protein synthesis indicate that this is not the case. In the hypertrophied plantaris, the total muscle protein synthesis rate, expressed as nmol phenylalanine incorporated per mg protein per hr perfusion time, was not significantly higher than the control until 15 days post-surgery. However, the muscle mass started to increase immediately after the functional overload was induced by surgery. Therefore, the only speculation that can be made is that in the early phase, the muscular hypertrophy can be accounted for by a decreased rate of protein degradation. This hypothesis is made because any change in muscle mass is thought to be due to the imbalance of protein synthesis and protein degradation. Consistent with this speculation is the lower phenylalanine concentration in the hypertrophied plantaris muscle at the early intervals post-surgery. Further, as the rate of muscle growth declines at 30 days post-ablation and beyond, a gradual return to a normal rate of protein degradation may occur as suggested by the enhanced free phenylalanine concentration in the hypertrophied muscle during this period. Goldberg (1969) has reported the decline of protein catabolism in hypertrophied muscle 5 days after tenotomy.

Since the muscular compensatory adaptation would

ultimately lead to an increase in the muscle's capacity to generate tension, the adaptation occurring in the contractile proteins would be more meaningful than the general change in the total tissue protein. The present study showed that the synthesis rate of myosin, one of the primary contractile proteins in muscle, was stimulated at 15 days post-surgery and then returned to the control level by 30 days. These results could indicate that the total muscle tissue protein synthesis and synthesis of the contractile filament, myosin, respond to a functional overload with a similar time course.

To interpret the significance of the increase of contractile protein synthesis and to trace the fate of newly synthesized contractile elements, the possible histochemical adaptations in the muscle undergoing compensatory hypertrophy have to be considered. It has been documented that the histochemical profile of skeletal muscle can be changed by functional overload (Baldwin et al., 1972; Gonyea and Bonde-Peterson, 1978; Ianuzzo et al., 1981). In the present study, cross-sections of the rat plantaris, a muscle with more than 90% of its fiber population being fast-twitch (Ariano et al., 1973), were stained for myofibrillar ATPase following an alkaline pre-incubation at pH 10.3 to examine the histochemical adaptation and its time course during hypertrophy. At 30 days

post-surgery, the hypertrophied plantaris exhibited a significant increase in its percentage of histochemically determined alkaline-labile (SO) fibers (about 25%) as compared to its contralateral control (approximately 9-11%) and with little further change thereafter. This observation is consistent with previously reported investigations (Guth and Yellin, 1971; Ianuzzo et al., 1981).

In line with the histochemical observation, ~~electro~~phoretic analysis of the myosin light chain composition of the hypertrophying muscle demonstrated an increase in the myosin slow light chain component (LCs1) which followed a similar time course to the histochemical changes. Interestingly, at the 30 day time one of the myosin fast light chain component (LCf1) was found to be decreased when compared to the control muscle.

The increased percentage of alkaline-labile fibers and the change in the myosin light chain composition suggest that the contractile proteins in the plantaris muscle can adapt to chronic stimulation and become more like slow-twitch muscle fibers. The significance of this adaptation is, as suggested by Awan and Goldspink (1970), the greater energetic efficiency of slow-twitch fibers in maintaining isometric tension.

With this in mind, the ultimate effect of a change in

myosin phenotype should be an alteration in the myosin ATPase activity of the muscle, as this parameter is intimately related to the contractile characteristics of a muscle. In fact, in the present study, the Ca^{+} -activated myosin ATPase activity was found to decline (at pH 9.8) in the hypertrophied plantaris at 30 days post-surgery.

Collectively, the biochemical and ~~histochemical~~ observations from this study suggest that the muscular adaptive remodelling process was stimulated between 15 and 30 days after the muscle was overloaded. By 15 days, contractile protein synthesis was enhanced and histochemical staining of the muscle with myofibrillar ATPase showed a slight increase in the percent of slow fibers. This remodelling process appeared stable prior to 30 days post-surgery when the myosin protein synthesis rate returned to normal. At this point, the percentage of histochemically determined slow fibers was significantly higher than in the control muscle and had reached a plateau. Concomitantly, the ATPase activity of isolated myosin was depressed and the compositional analysis of myosin showed a shift from a predominate "fast" light chain type to a "slow" one.

Together with the histochemical and enzymatic observations, these data suggest that a transformation from fast to slow muscle fibers may occur during compensatory hyper-

trophy though this is not conclusive. Our observations strongly suggest that this remodelling process was well established in less than 30 days. This coincides well with the postulated half-life of tissue protein (25 days).

Although the observed decrease in myosin ATPase activity was consistent with the change in the histochemical observation and expected electrophoretic profile of the hypertrophied muscle, this enzymatic activity was not depressed to the extent that would have been anticipated on the basis of the apparent myosin compositional change. Recently, several investigators (Wagner and Weeds, 1977; Wagner and Giniger, 1981) have suggested that Ca^{++} -activated myosin ATPase activity may primarily reflect the phenotype of the myosin heavy chain. Therefore, we can only speculate, that the lack of coincidence between the apparent compositional changes and the Ca^{++} -activated myosin ATPase activity indicates that the histochemical adaptation in muscle induced by compensatory overload mainly reflects the change in myosin light chain components and this change is sufficient to allow the muscle to meet the increased functional demand. The myosin heavy chain component may (not be altered seriously as indicated by the slight depression in myosin ATPase activity. This speculation is also supported by the lack of agreement concerning the alteration in contractile properties of hypertrophied

muscle (Jablecki and Kaufman, 1973; Binkhorst and van't Hof, 1973). Hence, as noted earlier, since myosin ATPase activity and muscle contractile characteristics are closely correlated, the inability to clearly observe a depressed contractile activity in hypertrophied muscle reinforces our observation of minimal change in myosin ATPase activity.

The observations mentioned above and the fact that myosin synthesis was enhanced at 15 days post-surgery with similar myosin yields in control and hypertrophied muscles coupled with a compositional change in LCs1 strongly suggest a selective synthesis in the myosin molecule during the adaptative growth. This finding is interesting because most previous models employed to alter myosin profile in skeletal muscle such as cross-inervation (Bárány and Close, 1971; Hoh et al., 1980) and electrical stimulation (Salmons and Sréter, 1976) have resulted in a co-ordinate expression of the total myosin molecule. Our observation suggests that possibly a complete alteration of myosin molecule is not necessary for adaptation to a functional overload. Further, as our model involves no direct manipulation of motor neuron, it may represent a more physiologic assessment of the muscle's adaptive process.

In summary, this study demonstrates that in response to functional overload, rat plantaris muscle responds by both an increase in muscle size and a change in fiber

composition. The early hypertrophic response of muscle may be due to a decreased protein degradation. By 15 days post-surgery, increased total protein and myosin protein synthesis are detectable. This muscle remodelling process is well established by 30 days. At this point, an increased percentage of alkaline-labile fibers, a reduced myosin ATPase activity and a shift towards a slow myosin light chain pattern are observed. These observations collectively suggest a transformation of a fast muscle fiber to slow fiber possibly in an effort to achieve energetic efficiency. The findings from the present study could indicate that myosin gene expression can be altered by a chronic functional overload favoring synthesis of the slow myosin type. In light of the minor adjustment in Ca^{++} -activated myosin ATPase activity however, some reservations regarding the completeness of the transformation must be retained.

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APPENDICES

Perfusion Buffer

Buffer:

Salt	% (g/100 ml)	Molarity	mls used
NaCl*	0.9	0.154	1000
KCl	1.15	0.154	40
CaCl ₂ ·2H ₂ O	1.22	0.110	36
KH ₂ PO ₄	2.11	0.154	10
MgSO ₄ ·7H ₂ O	3.82	0.154	10
Na ₂ .EDTA 2H ₂ O	3.72	0.100 pH 7.4	6
NaHCO ₃ *	1.30	0.154	210

* Made fresh.

1. To 1000 ml NaCl add KCl, CaCl₂, KH₂PO₄, MgSO₄ and EDTA.
2. Add the following to the buffer, dissolve and gas with 95% O₂:5% CO₂
 - a. 3.94 g Glucose for 300 mg/100 ml (15 mM in perfusate).
 - b. One times normal plasma levels of amino acids.
9.84 ml of 200x amino acids without tyrosine (see below), 29.7 mg tyrosine. For 5x phenylalanine add 69 mg.
3. Add NaHCO₃.
4. Add insulin 25 mU/ml. (0.328 cc for 1 stock volume).
5. Gas buffer for 10 min with 95% O₂:5% CO₂.

Amino acid mixture (200 x):

Amino Acid	Plasma Level (mM)	MW	mg/200 ml
Asp	.0380	133.10	202
Thr	.2697	119.12	1286
Ser	.2434	105.09	1024
Asn.H ₂ O	.0626	150.10	376
Gln	.6666	146.15	3897
Pro	.1858	115.13	856
Glu	.0757	147.13	445
Gly	.4080	75.07	1226
Ala	.4705	89.09	1676
Val	.1732	117.15	812
Cys	.0380	121.16	184
Met	.0465	149.21	278
Ile	.0905	131.17	474
Leu	.1607	131.17	844
Phe	.0538	165.19	356

Lys HCl	.4181	182.69	3056
His	.0633	155.16	392
Arg	.1322	174.20	922
Trp	.0690	204.22	564
Tyr	.0833	181.19	-

1. Weigh out amino acids into a beaker.
2. Dissolve in approximately 80% of the total volume of 0.9% NaCl.
3. Adjust pH to 7.4 with 1N NaOH.
4. Make the final volume 200 ml with 0.9% NaCl.
5. Freeze convenient size aliquots.
6. Batches of amino acids may be weighed out and stored dry in the beaker.

Perfusate Lactate Determination

Stock solutions

- A. Precipitating reagent. Dissolve 5 g sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in 400 ml H_2O . Add 11 ml 90% analytical-reagent grade orthophosphoric acid (H_3PO_4), followed by 2.18 g copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). Make up to 500 ml with H_2O . (This solution is stable for a few months in a brown bottle at room temperature.)
- B. Colour reagent. Dissolve 1.5 g p-hydroxy-biphenyl in 100 ml H_2O . (Store in a brown bottle at room temperature.)
- C. Sulfuric acid. Analytical-reagent grade.

Standard

Dissolve 42.6 mg of lithium lactic acid (Sigma) in 5 ml H_2O , followed by 0.1 ml sulfuric acid. Bring to 100 ml with H_2O . (Brown bottle, 0-4°C.)

Procedure

- 1) To make lactate standard:

	Blank	20 mg%	40 mg%	60 mg%	80 mg%
LA standard (ul)	0	25	50	75	100
H_2O (ul)	100	75	50	25	0
Solution A (ml)	3.9	3.9	3.9	3.9	3.9

- 2) Remove 50 ul from perfusate sample, add 50 ul H_2O and 3.9 ml solution A.
- 3) Mix thoroughly.
- 4) Centrifuge at 2,000 rpm for 5 min.
- 5) Transfer 1 ml of the sample supernate to another tube.
- 6) Add 6 ml H_2SO_4 RAPIDLY. Allow to stand for 1-2 min and then mix with a vortex mixer.
- 7) Cool in cold tap water.
- 8) Add 100 ul colour reagent (solution B) and vortex immediately. Allow to stand for 10 min.

- 9) Boil the tubes for 90 sec and cool in cold tap water.
- 10) Read O.D. at 565 nm.

Muscle Glycogen Determination

Stock solutions

- A. 30% KOH saturated with Na_2SO_4
Dissolve 30 g KOH in 100 ml H_2O . Add Na_2SO_4 to saturate point.
- B. 95% Ethanol
Use from stock.
- C. 5% Phenol
Dissolve 5 g phenol in 100 ml H_2O .
- D. 95-98% Sulfuric acid
Use from stock.

Standard

Glycogen 1 mg/1 ml H_2O .

Glycogen extraction procedure

- 1) Put a piece of muscle tissue (approximately 100 mg) in 1 ml solution A.
- 2) Stopper each tube and boil the tubes in a water bath for 30 min.
- 3) Cool in ice or cold tap water.
- 4) Add 1.2 ml 95% ethanol and mix with a vortex mixer.
- 5) Store the tubes on ice for 60 min or in refrigerator overnight.
- 6) Centrifuge 20 min at 3,000-5,000 rpm and aspirate the supernatant.
- 7) Dissolve sediment with 5 ml H_2O (Vortex).

Glycogen quantification procedure

- 1) Make standard by using samples of 20, 40, 60, and 80 μg of glycogen standard.
- 2) Remove 0.25 ml from muscle glycogen sample.
- 3) Make each sample up to 1.0 ml volume with H_2O .

- 4) Make blank with 1.0 ml H₂O.
- 5) Add 1.0 ml 5% phenol (solution C).
- 6) Add 5 ml 95-98% sulfuric acid RAPIDLY directing the stream against the surface of the solution.
- 7) Allow to stand 10 min.
- 8) Shake with a vortex mixer and put the tubes in a 25-30° C water bath for 10-20 min.
- 9) Read O.D. at 490 nm.

Myosin Isolation

Stock solutions

- A. 40 mM KCl in 50 mM Tris, pH 7.0
3.06 g Tham brought to 1000 ml with H₂O. Adjust pH to 7.0. 2.98 g KCl brought to 1000 ml with Tris solution. Check pH.
- B. 0.05 M KCl
0.01 M KHPO₄
pH 7.0
Dissolve 1.36 g KH₂PO₄ into 1 l H₂O. Dissolve 1.74 g K₂HPO₄ into 1 l H₂O. Add one solution to another while checking pH to get pH = 7.0. 3.73 g KCl brought to 1 l with KHPO₄ solution. Recheck pH.
- C. 0.05 M KCl
0.01 M KHPO₄
1% (v/v) Triton X-100
pH 7.0
Make KHPO₄ solution as for sol. B. Dissolve 3.73 g KCl into 800 ml KHPO₄ solution. Add 10 ml Triton X-100. Bring to 1 l with KHPO₄ solution. Recheck pH.
- D. 0.47 M KCl
0.01 M KHPO₄
0.02 M K₄P₂O₇
pH 6.8
Make 1 l KH₂PO₄ and 1 l K₂HPO₄ as for sol. B. Mix up to get KHPO₄ solution, pH 6.8. 35.04 g KCl and 6.6 g K₄P₂O₇ brought to 1 l with KHPO₄ solution. Recheck pH.
- E. 0.47 M KCl
0.01 M KHPO₄
0.02 M K₄P₂O₇
0.05 M MgCl₂
pH 6.8
204 mg MgCl₂ brought to 200 ml with sol. D. Make fresh daily.
- F. 10 mM EDTA
Saturated (NH₄)₂SO₄
0.39 g EDTA and more than 51.84 g (NH₄)₂SO₄ (3.9 M saturated) brought to 100 ml with H₂O in a ice bath.
- G. 0.6 M KCl in 0.05 M Tris
3.03 g Tham brought to 500 ml with H₂O. 22.36 g KCl brought to 500 ml with Tris buffer.

H. Dialysis buffer

1 mM EDTA
10 mM KCl
1 mM Tris
0.05 mM DTT
pH 7.2

Dissolve 1.11 g EDTA, 2.25 g KCl, 0.36 g Tham and 22.1 mg dl-dithiothreitol into 800 ml SINGLE distilled H₂O. Adjust pH to 7.2. Bring to 3 l with single distilled H₂O.

I. 0.6 M KCl

20 mM EDTA
2 mM DTT
50% (v/v) glycerol
pH 7.0 - 7.2

4.47 g KCl, 0.74 g EDTA, 31 mg DTT and 50 ml glycerol brought to 90 ml with H₂O. Adjust pH to 7.0-7.2. Bring to 100 ml with H₂O.

Procedure

- 1) Remove frozen muscle tissue (200-400 mg) from glycerol and wash in a small beaker with sol. A by stirring vigorously with a stirring rod.
- 2) Dry a piece (20-30 mg) of tissue in oven to determine the water content of the muscle. Weigh the remainder.
- 3) Mince tissue, homogenize in 10 ml sol. B.
- 4) Take 1 ml mixture and add 1 ml 0.6 M TCA. Precipitate total tissue protein overnight (0-4°C).
- 5) Centrifuge the remaining mixture at 2,000 g for 10 min.
- 6) Suspend pellet in 15 ml sol. C, homogenize and wait for 45 min with occasionally stirring.
- 7) Centrifuge at 2,000 g for 10 min.
- 8) Wash pellet with 15 ml sol. B and centrifuge at 2,000 g for 10 min.
- 9) Repeat 8).
- 10) Homogenize pellet in 20 ml sol. D and stir for 15 min.

- 11) Centrifuge at 13,000 g for 30 min.
- 12) Pour supernatant into 200 ml ice-cold double distilled H_2O and precipitate overnight.
- 13) Aspirate clear supernatant and pellet cloudy precipitate by spinning at 13,000 g for 15 min.
- 14) Dissolve pellet in 18 ml sol. E and centrifuge in a ultra-centrifuge at 90,000 g for 1.5 hr.
- 15) After centrifugation decant off the supernatant fluid into a centrifuge tube.
- 16) For the 34% $(NH_4)_2SO_4$ add 9.27 ml sol. F at a rate of approximately 2 drops/sec. The solution is constantly stirred with a mini-magnetic stirring bar and kept in an ice bath.
- 17) Centrifuge at 13,000 g for 10 min.
- 18) The supernatant is decanted into a centrifuge tube and 5.45 ml sol. F is added to precipitated the 45% fraction. Stir for 20 min in an ice bath.
- 19) Centrifuge at 40,000 g for 30 min.
- 20) Resuspend myosin pellet with 5 ml sol. G.
- 21) The myosin solution is dialyzed against 3 l sol. H for 14-18 hr in a cold room.
- 22) Collect pellet by spinning at 40,000 g for 30 min.
- 23) Suspend 1/3 of myosin pellet in 1 ml sol. I and stored at $-20^\circ C$ for myosin ATPase assay. The remaining pellet is dissolved in 3-5 ml sol. G for electrophoresis.
- 24) All steps are at $0-4^\circ C$.

SDS Polyacrylamide Gel Electrophoresis

Stock solutions

- A. 38.94% (w/v) acrylamide
1.06% (w/v) bis-acrylamide
Bring 38.94 g acrylamide and 1.06 g N,N'-methylene-bis-acrylamide to 100 ml with H₂O.
- B. 1.5 M Tris-HCl
pH 8.8
Dissolve 18.17 g Tham into 80 ml H₂O. Adjust pH to 8.8 with conc. HCl. Bring to 100 ml with H₂O.
- C. 0.5 M Tris-HCl
pH 6.8
Dissolve 6.06 g Tham into 80 ml H₂O. Adjust pH to 6.8 with conc. HCl. Bring to 100 ml with H₂O.
- D. 1% (w/v) SDS
Dissolve 1 g SDS into 100 ml H₂O.
- E. 0.08% ammonium persulfate
Dissolve 20 mg ammonium persulfate into 25 ml H₂O.
Make fresh shortly before use.
- F. 2% (w/v) TEMED
Dissolve 0.5 ml TEMED into 25 ml H₂O.
- G. Dialysis buffer
0.0625 M Tris-HCl pH 6.8
0.1% SDS
0.1% 2-mercaptoethanol
Dissolve 7.58 g Tham into 800 ml H₂O. Adjust pH to 6.8 with HCl. Add 1 g SDS and 1 ml 2-mercaptoethanol. Bring to 1000 ml with H₂O.
- H. Running buffer
0.192 M glycine
0.025 M Tris
0.1% SDS
pH 8.3
To make 2x concentrated stock solution, dissolve 3.03 g Tris and 14.41 g glycine into 400 ml H₂O. Adjust pH to 8.3 with HCl. Add 1 g SDS and bring to 500 ml with H₂O. Dilute 1:1 with H₂O immediately use.

I. Staining solution

0.25% coomassie brilliant blue R-250

45.4% methanol

9.2% acetic acid

Mix 90.8 ml methanol with 90.8 ml H₂O. Add 18.4 ml acetic acid. Dissolve 0.5 g coomassie brilliant blue R-250 into this solution. Filter through Whatman #1 filter paper.

J. Destaining solution A

25% (v/v) isopropyl alcohol

10% (v/v) acetic acid

250 ml isopropyl alcohol and 100 ml acetic acid brought to 1000 ml with H₂O.

K. Destaining solution B

10% (v/v) isopropyl alcohol

10% (v/v) acetic acid

100 ml isopropyl alcohol and 100 ml acetic acid brought to 1000 ml with H₂O.

L. Gel storing solution

7.5% acetic acid

7.5 ml acetic acid brought to 100 ml with H₂O.

Preparation of gels

10% running gel (Sufficient to make 12 10 cm gels)

1) Combine the following:

Sol. A 12 ml

Sol. B 12 ml

Sol. D 4.8 ml

Sol. F 1.2 ml

2) Deaerate for 4 hr.

3) Add 18 ml sol. E while gently shaking the bottle.

4) Pipet gel solution into each gel tube to the 9 cm mark.

5) Layer H₂O on the top of each gel and cap.

6) Allow to polymerize for 1 hr.

3% stacking gel (Sufficient to make 12 1 cm gels)

1) Combine the following:

Sol. A 0.9 ml
H₂O 0.9 ml
Sol. C 2.0 ml
Sol. D 0.8 ml
Sol. F 0.4 ml.

2) Deaerate for 4 hr.

3) Add 3 ml sol. E while shaking the bottle gently.

4) Remove H₂O on the top of 10% gel.

5) Layer 3% gel solution on the top of 10% gel.

6) Layer H₂O on the top of each gel and cap.

7) Allow to polymerize overnight.

Sample preparation

1) Dialyse protein samples against dialysis buffer overnight.

2) Place 100 ug protein in a small test tube.

3) Add the following:

5 ul 2-mercaptoethanol
2 ul tracking dye
30 ul 50% glycerol.

4) Vortex tubes to get homogeneous solution.

Running eletrophoresis

1) Dilute running buffer 1:1 with H₂O.

2) Put gel tubes in chamber. Remove upper and lower caps. Remove water from the top of each gel.

3) Fill lower and upper compartments of chamber with running buffer.

4) Insert electrodes from power supply (positive at bottom end).

5) To equilibrate, pre-run at 1.5 mA per gel for 1 hr.

7) Layer sample containing 100 ug of protein on top of gel with syringe.

- 8) Electrophoresis (1.5 mA per gel) until the tracking dye reaches the bottom of the gels (Approximately 4-5 hr).

Staining and destaining

- 1) Remove gels from tubes and put them in staining solution overnight.
- 2) Rinse gels with H_2O and place them in destaining solution A for 8-10 hr.
- 3) Transfer gels to destaining solution B until the gel background is clear.
- 4) Store gels in 7.5% acetic acid solution.

Protein Determination

Stock solutions 2% Na_2CO_3 in 0.1N NaOH
 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 2% NaK Tartrate (cold)

Solution "C" (Make fresh daily)
 0.5 ml 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 0.5 ml 2% NaK Tartrate
 50 ml 2% Na_2CO_3

Standard Bovine Serum Albumin 1 mg/ml H_2O

Procedure

- 1) Make standard by using samples of 20, 40, 60, and 80 ug of BSA standard.
- 2) Bring 20-40 ul sample of protein to be determined up to 0.4 ml volume with 0.1N NaOH.
- 4) Make a blank with 0.4 ml 0.1N NaOH.
- 5) Add 2 ml solution C and let stand 10 min at room temp.
- 6) Add 0.2 ml of Folin-phenol reagent (Fisher) (dilute 1:1 with H_2O), agitate and wait 30 min at room temp.
- 7) Read O.D. at 750 nm.

Deacidification of the TCA Soluble Fraction

Stock solutions

1N alamine in chloroform

- 1) Bring 57.5 ml alamine to 100 ml with chloroform.
- 2) Add an equal volume of 0.01 N NaOH to alamine in a separatory-funnel and mix by inversion.
- 3) Remove washed alamine (lower phase) and discard NaOH.
- 4) Repeat 2) and 3).
- 5) Wash alamine with 0.01 N NaOH employing 10-20% more by volume and discard NaOH.
- 6) Repeat 5).
- 7) Repeat 5).
- 8) Collect washed alamine and filter it through Whatman #1 filter paper. Store washed alamine in a brown bottle at room temperature.

Procedure

- 1) Add washed alamine employing 10% more by volume to the TCA soluble fraction and mix with a vortex mixer.
- 2) Check pH of the mixture with pH test paper (should be 6.5 - 7.0).
- 3) Centrifuge at 3,000-5,000 rpm for 10 min.
- 4) Remove the aqueous phase to a glass vial and dry in an oven or in a water bath at 50°C.
- 5) Suspend dried portion in 400 μ l 0.3 M TCA. Determine phenylalanine concentration and radioactivity.

Phenylalanine Determination

Stock solutions

- A. 0.6 M succinic acid, pH 5.88
70.9 g succinic acid
800 ml H₂O
Adjust pH to 5.88 with 5 N NaOH and dilute to 1 l with H₂O. Check pH at least once a week.
- B. 0.03 M ninhydrin
Dissolve 1.069 g ninhydrin in 200 ml H₂O.
- C. 0.005 M l-leucylalanine
Dissolve 202.33 mg leucylalanine in 200 ml H₂O.
- D. Buffered ninhydrin peptide solution
Mix solution A, B, and C by the volume ratio 5:2:1. Prepare fresh daily.
- E. Buffered ninhydrin solution
Mix solution A, B, and H₂O by the volume ratio 5:2:1. Prepare fresh daily.
- F. Na₂CO₃
NaKC₄H₄O₆·4H₂O
1.325 g Na₂CO₃
55.0 mg NaKC₄H₄O₆·4H₂O
Bring to 500 ml with H₂O.
- G. 0.6 mM CuSO₄·5H₂O
Dissolve 149.8 mg CuSO₄·5H₂O in 1 l H₂O.
- H. Copper, carbonate, tartrate solution
Mix solution F and G by the volume ratio 3:2. Prepare fresh daily.
- I. 0.6 M TCA
Dissolve 98.04 g of trichloroacetic acid in 1 l H₂O.

Standard

10 mg% phenylalanine. Dissolve 4.0 mg l-phenylalanine in 40 ml of 7.5% bovine albumin solution.

Reference solution

20 ppb quinine sulfate dihydrate in 0.1N H₂SO₄

Dissolve 5 mg quinine sulfate in 500 ml 0.1 N H_2SO_4 to make 10 ppm stock. Dilute 10 ul 10 ppm solution to 5 ml 0.1N H_2SO_4 before use.

Procedure

- 1) To 100 ul TCA solution (solution I) in a small tapered test tube, add 100 ul 7.5% bovine albumin (blank); 50 ul 7.5% BSA plus 50 ul phenylalanine standard (5 mg%); 100 ul phenylalanine standard (10 mg%); and 100 ul tested sample.
- 2) Mix thoroughly and allow to stand for 10 min with occasional mixing.
- 3) Stopper each tube and centrifuge at 2000 rpm for 10 min.
- 4) Transfer 20 ul of the sample supernate to each of two small test tubes and add 300 ul solution D. The two tubes provide for duplicate determination.
- 5) Transfer 20 ul of the sample supernate to a third test tube and add 300 ul solution E. (This is a check for fluorescence due to substances other than phenylalanine and may be omitted if interferences are known to be absent). In the same way, set up the blank and standard tubes using the appropriate supernate.
- 6) Stopper the tubes and incubate for 2 hr in a 60°C water bath.
- 7) Cool in cold tap water.
- 8) Add 5 ml of solution H to each tube and mix.
- 9) Read fluorescence in Beckman 772 Ratio Fluorometer. Set the phosphor sleeve to 360 nm.

Calculation

Fs = fluorescence of sample
 Fb = fluorescence of blank
 Fstd = fluorescence of standard

$$\frac{F_s - F_b}{F_{std} - F_b} \times 5 \text{ (or 10)*} = \text{mg Phe per 100 ml of sample.}$$

* For use of 10 mg% standard.

Radioactivity Determination

For total protein:

Scintillation cocktail

- A. Add 42 ml PPO-POPOP (Amersham) to 1 l Toluene to yield a final concentration of 4 g PPO and 50 mg POPOP per liter.
- B. Mix 1 l above solution with 600 ml Ethylene Glycole Monomethyl Ether.

Procedure

- 1) Add 100 ul sample to 3 ml cocktail in a mini-vial, agitate.
- 2) Make blank with 100 ul 1N NaOH.
- 3) Count 10 min at 1% error in Beckman LS 100 liquid scintillation spectrometer.

For TCA soluble fraction and myosin protein:

Scintillation cocktail

- A. Dilute PCS (Amersham) with Toluene (PCS:Toluene, 2:1 v/v).

Procedure

- 1) Add 200-400 ul sample to 10 ml cocktail, agitate.
- 2) Make blank with 200-400 ul 0.3 M TCA (for TCA soluble fraction) or 0.6 M KCl (for myosin).
- 3) Count 10 min at 1% error in Beckman LS 100 liquid scintillation spectrometer.

Myofibrillar ATPase Histochemistry

Stock solutions

- A. 2% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
10 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ brought to 500 ml with H_2O .
- B. 1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ brought to 500 ml with H_2O .
- C. 0.18 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
13.23 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ brought to 500 ml with H_2O .
- D. 1.0 M 2-amino-2-methyl-1-propanol (AMPL)
8.914 g AMPL brought to 100 ml with H_2O .
- E. Pre-incubation medium for demonstration of acid-labile, alkali-stable (FT) M-ATPase:
 - 100 mM AMPL
 - 18 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 - pH = 10.3
 - 10 ml 1.0 M AMPL
 - 10 ml 18 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 - 70 ml H_2O
 - Adjust pH to 10.3 with conc. HCl and bring to 100 ml with H_2O . (This solution is stable for at least 1 week).
- F. Incubation medium for demonstration of M-ATPase:
 - 100 mM AMPL
 - 18 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 - 50 mM KCl
 - 3.08 mM ATP
 - 1) 10 ml 1.0 M AMPL
10 ml 18 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0.3728 g KCl
70 ml H_2O
Adjust pH to 9.4 with conc. HCl and bring volume to 100 ml with H_2O .
(This solution is stable for at least 1 week.)
 - 2) Add 0.017 g ATP per 10 ml of solution F (1) and READJUST pH to 9.4 with dilute acid or base.
(This solution should be prepared shortly before use.)

Staining*Procedure

- 1) Tissue sections can be left for at least 1 day at room temperature, and have been left for up to 2 weeks, on

occasions, without having any observable effects on M-ATPase histochemistry. Tissue sections need not be left sitting for any time, before commencing staining procedure.

- 2) Pre-incubate in solution E for 10 min at 37°C.
- 3) Rinse with H₂O 3 times
Soak in H₂O for 1 min
Rinse with H₂O 3 times.
- 4) Incubate in solution F (2) for 30 min at 37°C.
- 5) Repeat 3).
- 6) Incubate in 1% CaCl₂·2H₂O for 3 min at room temperature.
- 7) Repeat 3).
- 8) Incubate in 2% CoCl₂·6H₂O for 3 min at room temperature.
- 9) Repeat 3).
- 10) Incubate in 1% (NH₄)₂S for 1 min at room temperature.
- 11) Repeat 3).
- 12) Soak in 80% EtOH for 2 min.
- 13) Soak in 90% EtOH for 2 min.
- 14) Soak in 100% EtOH for 2 min, twice.
- 15) Soak in xylene for 2 min.
- 16) Mount in permount.

Ca⁺⁺-activated Myosin ATPase Assay

Stock solutions

- A. pH 7.0
- | Stock | Final conc. | To make 50 ml |
|---|-------------|---------------|
| 29 mM KCl | 25 mM | .1080 |
| 70.59 mM histidine | 60 mM | .5840 |
| 11.77 mM CaCl ₂ ·2H ₂ O | 10 mM | .0865g |
- B. pH 9.8
- | Stock | Final conc. | To make 50 ml |
|---|-------------|---------------|
| 29 mM KCl | 25 mM | .1080 |
| 88.23 mM glycine | 75 mM | .2650 |
| 11.77 mM CaCl ₂ ·2H ₂ O | 10 mM | .0865g |
- C. ATP solution
30 mM Na₂ATP (99%)
pH 7.0
- D. 11.74 g (NH₄)₆Mo₇O₂₄·4H₂O
38 ml conc. H₂SO₄
Dilute to 1000 ml with H₂O.
- E. 10 g FeSO₄·7H₂O
15 ml 1N H₂SO₄
Dilute to 100 ml.
- F. Mix 500 ml sol. D with 100 ml sol. E.
- G. 2% SDS in 50 mM Tris, pH 7.2.

Procedure

- 1) Place 0.85 ml sol. A (for pH 7.0) or sol. B (for pH 9.8) in 13 x 100 mm tubes.
- 2) Add 50 ul myosin solution.
- 3) Pre-incubate for 5 min (for pH 7.0) or 7 min (for pH 9.8) at 25°C in a shaking water bath.
- 4) Add 100 ul ATP solution (sol. C) to initiate the reaction.
- 5) Run the reaction for 5 min (for pH 7.0) or 3 min (for pH 9.8) while in a 25°C shaking water bath.

- 6) Terminate the reaction with SDS solution (sol. G).
- 7) Prepare blank for each reaction by adding sol. A or sol. B and ATP solution, incubate 5 min then add SDS solution followed by myosin solution.
- 8) Add 3 ml sol. F using an Oxford pipettor.
- 9) Vortex and read O.D. immediately at 720 nm.

Data of Protein Synthesis

[³H] phenylalanine Incorporation into Total Tissue Protein
(dpm/mg protein)

Days	Control	Hypertrophied
2 (6)	363 ±54	632 ±85 *
5 (4)	310 ±55	456 ±74 *
15 (6)	329 ±81	603 ±41 *
30 (6)	361 ±89	396 ±74
50 (6)	274 ±48	324 ±77

[³H] phenylalanine Incorporation into Myosin Protein
(dpm/mg myosin)

Days	Control	Hypertrophied
2 (5)	338 ±86	244 ±71
5 (4)	158 ±33	214 ±32
15 (6)	80 ±17	173 ±26 *
30 (6)	49 ± 9	74 ±23
50 (6)	44 ± 5	44 ±12

Specific Activity of Free Phenylalanine on Muscle
(dpm/ nmol Phe-)

Days	Control	Hypertrophied
2 (5)	579 ±122	830 ±169 *
5 (4)	428 ± 32	384 ± 94
15 (6)	248 ± 49	372 ± 63
30 (6)	166 ± 44	238 ± 66
50 (6)	220 ± 37	117 ± 33 *

Protein Synthesis Rate
(nmol Phe. /mg protein/hr perfusion)

Days	Control	Hypertrophied
2 (5)	0.965 \pm 0.34	1.057 \pm 0.32
5 (3)	0.760 \pm 0.15	0.988 \pm 0.19
15 (6)	1.350 \pm 0.22	1.775 \pm 0.22 *
30 (5)	1.904 \pm 0.21	1.997 \pm 0.44
50 (4)	0.935 \pm 0.16	2.339 \pm 0.19 **

Myosin Protein Synthesis Rate
(nmol Phe. /mg myosin/hr perfusion)

Days	Control	Hypertrophied
2 (3)	0.378 \pm 0.08	0.150 \pm 0.01
5 (3)	0.393 \pm 0.07	0.409 \pm 0.07
15 (6)	0.350 \pm 0.06	0.518 \pm 0.10 *
30 (6)	0.355 \pm 0.08	0.341 \pm 0.10
50 (5)	0.227 \pm 0.06	0.345 \pm 0.09

Data are expressed as means \pm SEM.

Number in parentheses are number of animals per group.

Control vs hypertrophied muscle are statistically significant at: * $p < 0.05$; ** $p < 0.01$.

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