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Contract: NAS 9 - 15388

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Part I. Studies to gain information on the molecular basis of atrophy by
antigravity muscle.

The philosophy of these studies has been to identify the time sequence of events in the soleus muscle of the rat following the immobilization of the hind limbs, so that the length of the soleus muscle within the fixed limb is less than its resting length. Information on potential causal factors of atrophy in antigravity muscles can be gained from observations on the time sequence of changes. In two separate studies, no significant decline in the weight of the soleus muscle could be detected during the first 72 hours of 100% immobilization.

Study #1.

Time after hind limb casted (hr)	Soleus weight (mg)	Initial Body weight (g)	N
0	248 ± 10*	343 ± 13	6
8	281 ± 16	344 ± 10	6
16	248 ± 10	343 ± 10	6
24	263 ± 10	342 ± 10	6
48	248 ± 8	344 ± 13	6
72	258 ± 10	346 ± 20	5

* mean $\pm$ SE

(NASA-CR-151822) BIOCHEMICAL ADAPTATIONS OF
ANTIGRAVITY MUSCLE FIBERS TO DISUSE ATROPHY
Progress Report, 1 Aug. 1977 - 31 Jul. 1978
(Texas Univ.) 28 p HC A03/MF A01 CSCI 06C

G3/51

Unclas
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Study #2

Time after hind limb casted (hr)	Soleus weight (mg)	Initial Body weight (g)	N
0	256 $\pm$ 14*	358 $\pm$ 1	6
24	258 $\pm$ 8	359 $\pm$ 2	6
48	257 $\pm$ 10	359 $\pm$ 2	6
72	240 $\pm$ 7	359 $\pm$ 1	6

* mean $\pm$ SE.

Previous studies by me indicated that the soleus muscle (of which approximately 85% of the soleus is composed of antigravity, i.e. slow-twitch or type I, fibers) began atrophying somewhere between the 2nd and the 4th day of limb immobilization (J. Appl. Physiol. 43:656-661, 1977). Thereafter, the time during which one-half of the final decrease in soleus weight occurred was 5 days. So, there is no question that this model produces atrophy of the soleus. The results of the above studies indicate that any changes in the chemical content of the soleus during the first 3 days of limb immobilization might be a causal factor in the resulting atrophy of the soleus.

It is well known that the rates of protein synthesis decline in atrophying muscle. However, there is very little known about the time sequence in the decline of protein synthesis in antigravity muscle within a limb that is fixed in a cast. We used continuous infusion techniques to measure the rates of protein synthesis within antigravity muscles. One group of rats had their hind limbs immobilized 12 hours prior to the start of the continuous infusion of ^{14}C -tyrosine, so that protein synthesis was measured between the 12th and 18th hour of immobilization. A second group served as a control. The control group received the anesthetic at the same time that rats in the immobilized group were anesthetized (sodium phenobarbital) so that their limbs could be cast. The dpm in protein from the washed TCA precipitate of the soleus and the dpm in L-tyramine were determined.

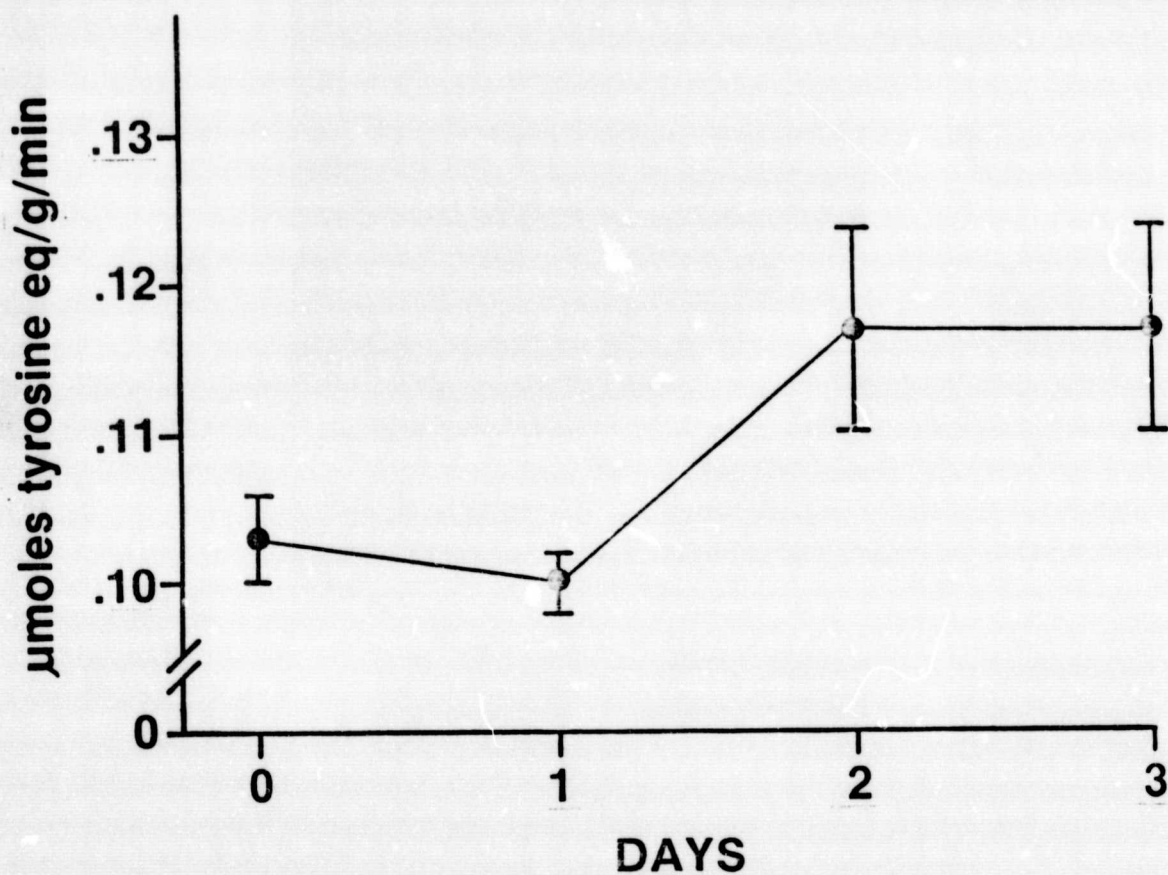
Group	N	$\frac{(S_B)}{\text{dpm}}$ mg protein	$\frac{(S_F)}{\text{dpm}}$ nmole tyrosine	S_B/S_F
Control	3	119.0 $\pm$ 4.3*	5.4 $\pm$ 1.5	25.1 $\pm$ 5.7
Hind-limb casted 12 hr prior to start of 6 hr of continuous infusion	4	31.4 $\pm$ 3.2***	6.9 $\pm$ 3.8	9.4 $\pm$ 3.4**

*, mean $\pm$ SE. **, $P < 0.5$ and *** $P < 0.001$
from control.

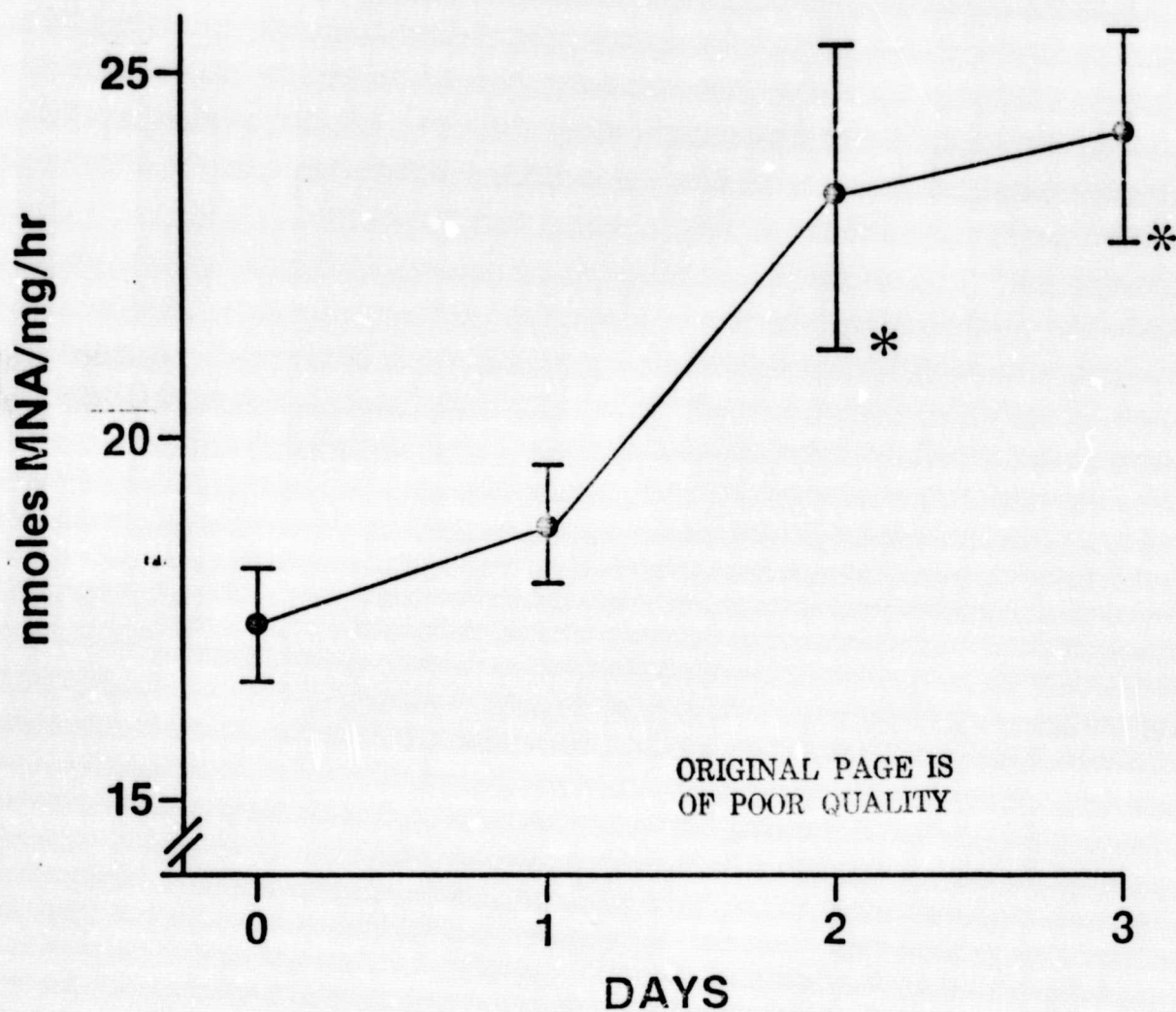
Thus, protein synthesis in the soleus declined by the 18th hour of disuse in a limb which had been immobilized. In about 2 weeks, we plan to undertake additional studies to determine which biochemical events related to protein synthesis in the soleus occur within the first 12 hours of limb immobilization.

Max and coworkers (Arch. Biochem. Biophys. 146:227-232, 1971) followed the time course of the change in the activities of lysosomal enzymes in fast-twitch muscle within limbs that were fixed with pins. However, none of the lysosomal enzymes studied by these workers were proteolytic. Moreover, they only found 1 of the 5, non-proteolytic lysosomal enzymes changing in fast-twitch muscle in the first 7 days of limb fixation. We chose to follow the time course of the change in enzyme activity of two proteolytic enzymes in the soleus muscle of limb immobilized in rats. The results are given next.

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There is no significant change in the activity of cathepsin D (μmoles tyrosine equivalents/g/min) in the soleus during the first 72 hours that the soleus is within an immobilized limb.



Cathepsin B activity (nmoles 4 - methoxy - 2 - naphthalamine) was significantly increased (*, $P < 0.05$ from control) by the 2nd day of limb fixation in the soleus.

These results suggest that changes in the rates of protein synthesis precede changes in the rates of protein degradation in the soleus of limbs that are immobilized. To collect more information on this matter, we are in the middle of performing an experiment which is relating the time sequence of changes in protein degradation and cathepsin B activity within the same soleus. Rats are casted 0,1,2 or 3 days. Soleus muscles are incubated in vitro in the presence of Krebs-Ringer bicarbonate buffer, cycloheximide, insulin, glucose, penicillin, and 95% O₂ - 5% CO₂. Tyrosine released into the media is an index of the rate of protein degradation. Cathepsin B is measured in the same muscles. Results will be reported in the next monthly report.

Part II. Studies on the work capacity of antigravity muscle during atrophy and during recovery from atrophy.

A copy of the manuscript that is to be submitted for scientific review to determine whether it merits publication in the Journal of Applied Physiology is attached.

RECOVERY OF SKELETAL MUSCLE AFTER
3 MONTHS OF HIND-LIMB IMMOBILIZATION IN RATS

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Running head: Skeletal muscle recovery from prolonged limb fixation.

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ABSTRACT

Adult, female rats had their hind limbs immobilized with plaster casts for 90 days and groups of these animals were then sacrificed at 0, 14, 28, 60, 97, and 120 days after the casts were removed. The recovery of maximal isometric tension was slower than the recovery of protein content in the soleus since it took 120 days of recovery from limb immobilization for maximum isometric strength of the soleus to increase to a level which was not significantly different than control values. Glycogen and ATP levels per mg of wet weight in the soleus, which were significantly depressed after 90 days of hind-limb fixation, had recovered by 60 days after cast removal. However, there was no significant depression in ATP levels per mg of protein in the soleus during recovery from 90 days of immobilization. The gastrocnemius muscle, which also showed significant decreases in wet weight and protein content after 90 days of immobilization, had increased sufficiently by the 120th day of recovery to become not significantly different in wet weight and protein content from control. Calcium concentration, which in the gastrocnemius increased significantly after 90 days of limb immobilization, returned to control levels during the period after cast removal. Thus, skeletal muscle can recover from 90 days of inactivity.

Index terms: skeletal muscle, recovery from atrophy, rehabilitation

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It is well known that skeletal muscle atrophies when limbs are immobilized so that these muscles are at lengths less than their resting length (4,5,7-9,12,14,16,18). Moreover, decreases in muscular strength in animals (7,8,16) and in humans (14) after limb immobilization have previously been reported. There is little known about whether skeletal muscles do return to their preatrophy level of strength following a lengthy period of disuse produced by limb fixation. It was our hypothesis that slow-twitch muscles would not return to their preatrophy strength level after disuse atrophy caused by limb immobilization. Under normal conditions, slow-twitch fibers are recruited for tonic activities, such as supporting the weight of the body against gravity (6). The removal of this function by either limb immobilization or weightlessness should result in their preferential atrophy. Indeed, others have previously reported a preferential degeneration of slow-twitch muscle fibers, as compared to fast-twitch muscle fibers, during limb immobilization (3,8,9,12,13,15,17,18). The soleus muscle of the rat is composed mainly of slow-twitch muscle fibers (1). We hypothesized that these degenerated fibers would not leave sufficient muscular tissue to permit regrowth of the soleus following removal of limb immobilization. We decided to immobilize limbs for 90 days to permit long-term atrophy and to use maximal isometric tension as a functional index of whether the soleus could return to a control level after 90 days of limb immobilization. In addition, other information on the time course of muscular regrowth was obtained so to gain additional information on the capacity of skeletal muscle to regrow after undergoing atrophy for 90 days.

MATERIALS AND METHODS

Animal care: Retired, female breeder rats of a Wistar strain (specific pathogen-free, HLA - W rats) were obtained from Hilltop Labs (Scottsdale, PA) and were provided Wayne lab chow and water ad libitum.

Experimental procedures: Hind limbs of rats were immobilized for 90 days with plaster of Paris using procedures described previously by us (4,5). Rats were anesthetized with sodium pentobarbital (4 mg/100 g BW) during initial fixation of limbs, during replacement of plaster casts, and at sacrifice. In all experiments, limbs were fixed so that the soleus and gastrocnemius muscles were at a length less than their resting lengths. Casts were removed and replaced at 2-4 week intervals so as to minimize movement within the cast by atrophying limbs.

Rats were grouped and sacrificed at the following times; 0, 14, 28, 60, 97, or 120 days after immobilization. On the day of sacrifice, rats were anesthetized with sodium pentobarbital (4 mg/g body wt., IP.), and the soleus muscle of the right leg was exposed and dissected free of surrounding tissue, leaving the nerve and blood supply intact. Additional anesthetic was supplied as needed. After dissecting down to the femur at its distal end, the fibula was removed from its insertion on the tibia, small holes were drilled in both the femur and tibia and the entire leg was immobilized by means of a needle passed through these holes and fixed at either end. The exposed muscles were kept moistened throughout the procedure with 0.9% NaCl solution maintained at 37°C by means of a water bath. A 4-0 suture silk was tied around the distal tendon of the soleus, passed over a small pulley and attached to a Grass FT03C force-displacement transducer which allowed the muscle to be loaded during

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stimulation. The muscle was stimulated directly by placing stainless steel electrodes at the proximal and distal ends of the muscle. Muscle length was then adjusted to a length at which active twitch tension was maximal. After loading with 5 g., the soleus was stimulated for one minute with repetitive pulses of 15 ms duration and 15 ms delay between pulses. The highest tension produced during the minute of work was designated the maximal isometric tension. After these procedures, the contralateral, resting soleus was removed and immediately frozen by liquid-nitrogen cooled Wollenburger tongs for the purposes of metabolite determinations. Gastrocnemius muscles were also dissected and frozen.

Assay procedures: Muscles which had been stored at -80°C were prechilled in liquid N_2 , rapidly weighed to the nearest 0.1 mg, and placed in a homogenizer cooled to -40°C in a methanol-dry ice bath. A 0.1N HCl-methanol solution (0.1 ml/50 mg tissue weight) was placed on the muscle in the homogenizer and allowed to penetrate the muscle for approximately 30 minutes at -40°C . Following this, 0.02 N HCl solution (1 ml/50 mg tissue weight) was added and the sample brought to 0°C in an ice bath. The muscles were homogenized completely and an aliquot was removed for glycogen and protein determination. Then 0.125 ml of a 3M perchloric acid solution containing 10 mM EDTA was then added for each ml of remaining homogenate. The precipitated protein was removed by centrifugation at 3,000 rpm in a IEC (PR - 6000) centrifuge for 10 minutes at 4°C . The supernatant was neutralized by adding 0.133 ml of a 2M KOH, 0.4 M imidazole, and 0.4 M KCl solution for each ml of supernatant and the precipitate was removed by centrifugation. The concentrations of ATP, CP and glycogen were determined using enzymatic fluorometric techniques as described by Lowry (11). Protein was determined by the Lowry procedure (10).

Calcium was measured by an atomic absorption spectrophotometer after ashing the muscles in an oven for 8 hours at 550°C (2).

The unpaired two-tail student t test was employed to test for significant differences and a probability level of 0.05 was designated as significant.

RESULTS

The wet weight of the soleus was significantly lower ($P < 0.01$) on the 90th day of limb immobilization as compared to control values (Fig. 1). However, by the 14th day of recovery from limb fixation, soleus wet-weight was not significantly different from control values. Likewise, the protein content of the soleus (mg protein/soleus) was significantly less ($P < 0.01$) after 90 days of limb fixation, but was not significantly different from control values from the 14th to 120th day of recovery. The protein concentration of the soleus (mg protein/g wet soleus wt) was significantly less than control at 90 days of limb fixation ($P < 0.05$) and after 14 and 28 days of recovery ($P < 0.02$) from the 90-day limb fixation (Fig. 1).

The maximum isometric tension produced during a minute of repetitive, direct stimulation was significantly lower in soleus muscles for the first 97 days following limb immobilization (Fig. 2). By the 120th day of recovery the tension per soleus was not significantly different than control (Fig. 2). The correlation between protein content and maximal isometric tension in the soleus during recovery was 0.95. When the maximum tension was expressed per mg of protein in the soleus, recovery values were consistently 20-30% less than control, which further shows that muscle tension increased more slowly than protein content during recovery. When maximum tension of the soleus was expressed as a function of its wet weight, these values were significantly

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lower than control for the following recovery days: 0 ($P < 0.001$), 14 ($P < 0.01$), 28 ($P < 0.01$) and 97 ($P < 0.05$). There was no significant difference between controls and groups at 60 and 120 days of recovery for the expression of maximum tension per unit of soleus wet weight (Fig. 2).

Both the wet weight and the protein content of the gastrocnemius were significantly lower than control values at 0, 14, 28, 60, and 97 days of recovery (Fig. 3). The concentration of calcium in the gastrocnemius was significantly increased ($P < 0.01$) after 90 days of immobilization (Fig. 4). However, there was no significant difference for the calcium concentration in the gastrocnemius between control values and 120-day recovery from a previous 90-day limb fixation.

Selected metabolites were studied to determine their time course of recovery after the 90 days of limb fixation. When glycogen and ATP values are expressed per unit of wet weight of the soleus, they were found to be significantly lower than control at 14, 28 and 97 days of recovery (Table 1). However, when ATP levels were expressed per unit of protein, ATP per mg of soleus protein did not significantly differ from control for any recovery group (Table 2). When glycogen was expressed per mg of protein in the soleus, then only the recovery groups at 14 and 97 days were significantly less than control.

DISCUSSION

After 90 days of limb fixation, the protein content of the soleus was 67% less than control values with maximum isometric tension being 75% less than control. During recovery, the correlation between the absolute values for protein content and tension in the soleus was 0.95, which suggests a connection between tension development and protein content in the soleus

during recovery. Since the ratio of tension to protein content was consistently 20-30% less than control during recovery, this suggests that during recovery, tension tended to return to control levels more slowly than protein content. This suggestion is supported by the observation that although protein content in the soleus was not significantly different by the 14th day of recovery, it took 120 days for tension to become not significantly different from control.

The hypothesis in our present study was based upon previous investigations that reported slow-twitch muscle exhibited a greater degree of atrophy than fast-twitch muscle when limbs were immobilized (8,9,12,18) and that slow-twitch fibers had a preferential degeneration during limb fixation (3,15,17). Since slow-twitch fibers had degenerated, we hypothesized that these changes would be irreversible, i.e. the remaining muscle fibers in the soleus after 90 days of disuse would be unable to regenerate sufficient muscular mass to permit the soleus to regain its pre-atrophy level of maximum isometric tension. The return of soleus tension during recovery disproved our hypothesis. Because of our hypothesis, no functional measurements such as isometric tension, were made on fast-twitch muscle during its recovery from atrophy. Although fast-twitch muscle did have a smaller amount of atrophy than slow-twitch muscle after 90 days of disuse, fast-twitch muscle regrew more slowly than slow-twitch muscle after immobilization had ended. It is thus possible that recovery of maximal isometric tension by a fast-twitch muscle might occur even more slowly than in a slow-twitch muscle.

Our present data provides a time course for recovery, and therefore extends the information provided by a recent report in which it was shown that ATP, CP and glycogen levels per unit of muscle weight were significantly lower in human skeletal muscle after 5 weeks of limb immobilization and that these

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metabolites returned to control levels when biopsies were first made 150 days after casts were removed (14). Our data indicates that it takes at least 28 days for ATP and glycogen levels per unit of wet weight in the soleus to return to control values after the 90-day immobilization period. Moreover, it also indicates that there is no significant change in ATP after immobilization when these levels are expressed per mg of protein in the soleus. This is due to the quantity of protein per unit of wet weight in the soleus being reduced to 68% of control concentration after 90 days of limb fixation. Glycogen per mg of soleus protein was only significantly lower in the 14 and 97-day recovery groups.

The means for soleus tension and metabolites in the groups at 0, 14, 28, and 97 days of recovery were significantly less than control. On the other hand, the means of similar measurements in the group at 60 days of recovery were often not significantly different from control. Since the numbers of animals in the 60-day recovery group were higher than in the 97-day group (6 vs 4 animals respectively), the smaller sample size in the 97-day group might be responsible for this group having lower tensions and metabolites in the soleus than the 60-day recovery group. Two animals in the 97-day group were accidentally eliminated from the experiment: one by overdose of anesthetic, and the other by a broken leg.

Calcium concentration is increased in atrophied muscles of limbs fixed by casts in animals (2) and in human skeletal muscle during bed rest of healthy humans (unpublished observations, Giannetta, Narahara, and Booth). Data in the present study show that this change is reversible.

Since the immobilization of human limbs is often occurring for 3 months and since humans have experienced up to 3 months of weightlessness in the Skylab program, a 3-month time period was chosen for the immobilization of the hind

limbs of rats. Results of the present study indicate that slow-twitch muscles in adult rats having free cage activity after 90 days of hind-limb immobilization do regain maximal isometric tension and metabolite levels after removal of the casts. However, the recovery of maximal isometric tension by the soleus takes months. These data suggest that the atrophy of slow-twitch muscles is reversible following 3-month periods of weightlessness, limb immobilization, or bed rest is reversible.

ACKNOWLEDGMENTS

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TABLE 1. Glycogen, ATP, and CP per mg of wet weight in soleus during recovery from 90 days of hind-limb fixation.

Group		ATP	CP	Glycogen
		(nmoles/mg wet wt)		
Control	(5)	3.4 ± .2	7.3 ± .7	10.2 ± .8
90-D immobilized				
plus:				
0-D recovery	(5)	2.4 ± .2***	5.0 ± .8	4.3 ± .8****
14-D recovery	(5)	2.5 ± .2**	4.8 ± .8	4.3 ± .3****
28-D recovery	(5)	2.2 ± .4**	4.8 ± .8	7.3 ± 1.0*
60-D recovery	(6)	2.9 ± .2	7.2 ± 1.2	9.2 ± .9
97-D recovery	(4)	2.3 ± .3**	4.9 ± .7	5.9 ± .9***
120-D recovery	(4)	2.9 ± .1	5.1 ± .9	9.0 ± .8

Values are means ± SE. Number of animals in each group is given in parentheses.

Data were compared using unpaired 2-tail student t-test.

**** P < .0001

*** P < .01

** P < .02

* P < .05

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TABLE 2. Glycogen, ATP, and CP per mg of protein in soleus during recovery from 90 days of hind-limb fixation.

Group	ATP	CP	Glycogen
		(nmoles/mg wet wt)	
Control	18.5 ± 1.7	40.6 ± 8.6	53.4 ± 3.0
90-D immobilized			
plus:			
0-D recovery	19.5 ± 1.4	35.3 ± 0.9	44.0 ± 24.3
14-D recovery	17.6 ± 1.2	32.5 ± 3.5	39.3 ± 1.8**
28-D recovery	15.9 ± 2.5	34.5 ± 5.6	52.8 ± 7.5
60-D recovery	15.5 ± 0.9	37.0 ± 4.1	48.8 ± 3.3
97-D recovery	13.4 ± 1.2	29.2 ± 3.6	35.2 ± 5.8*
120-D recovery	15.8 ± 0.6	28.1 ± 5.1	49.6 ± 4.1

Values are means ± SE with statistical analysis identical to Table 1.

** P < 0.01

* P < 0.05

FIGURE LEGENDS

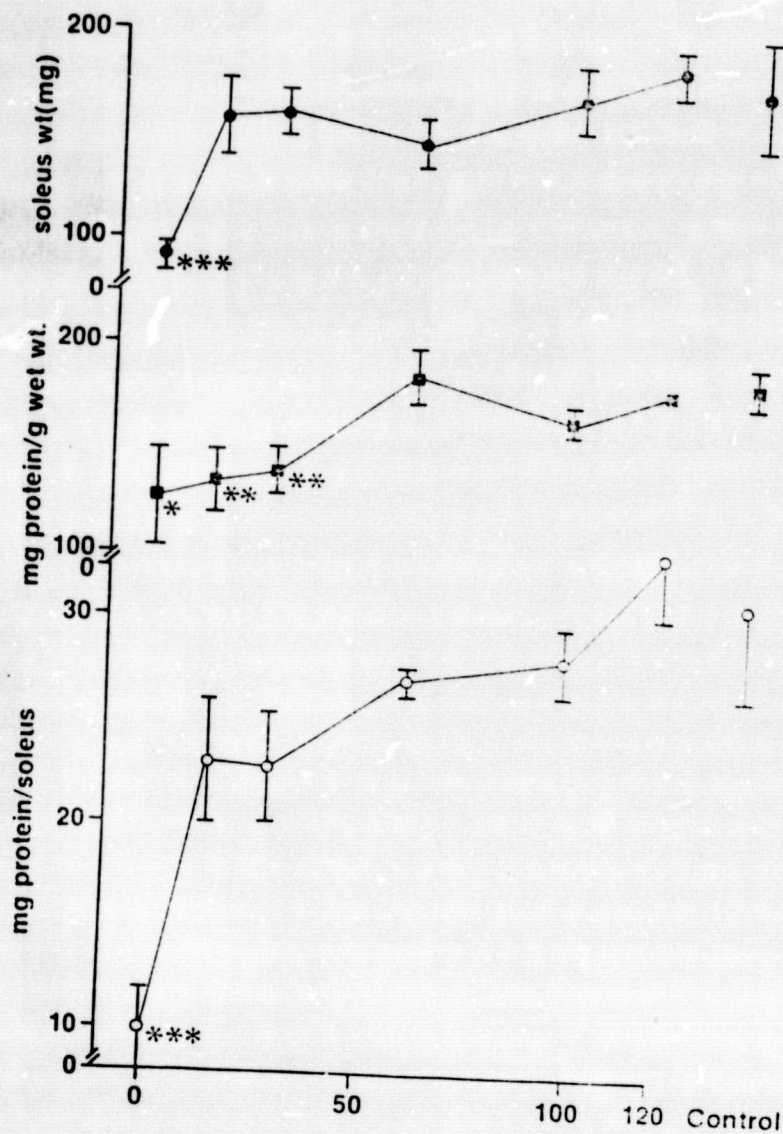
Figure 1. Recovery of wet weight, protein concentration, and protein content in soleus muscle after 90 days of limb fixation. The abscissa is labelled 0 to 120 days of recovery from the limb fixation with non-casted control values given at the extreme right-hand side of the figure. The upper third of the ordinate gives soleus wet weight (mg), the middle third gives protein concentration in the soleus (mg protein/g wet wt) and the lower third gives protein content of the soleus (mg protein/muscle). Values are means $\pm$ SE. Observation per time point are from 4 to 6 animals. The mean of each recovery time point was compared to the control value with an unpaired 2-tail student t test. * $P < 0.05$; ** $P < 0.02$; and *** $P < 0.01$.

Figure 2. Recovery of maximum isometric tension in soleus from 90 days of limb fixation. The x-axis is 0 to 120 days of recovery from the limb fixation with control values given at the far right-hand side of the figure. Recovery days are 0, 14, 28, 60, 97 and 120. The upper third of this figure gives maximum isometric tension produced by the soleus (g). The middle third gives maximum isometric tension of the soleus expressed per unit of protein in soleus (g/mg protein). The lower third of the figure gives maximum isometric tension of the soleus expressed per unit of wet soleus weight (g/mg wet weight). Values are means $\pm$ SE. Observations per time point are from 3 to 6 animals. The mean of each recovery time point was compared to the control value with a 2-tail student t-test. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

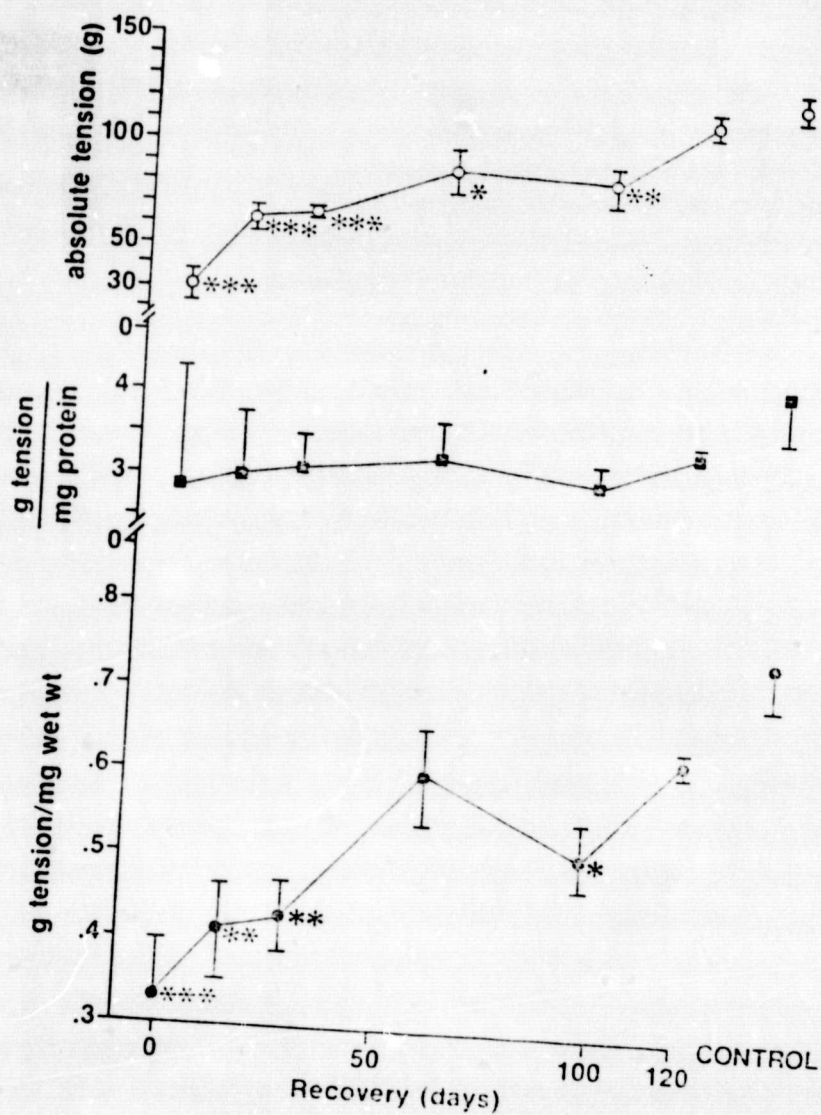
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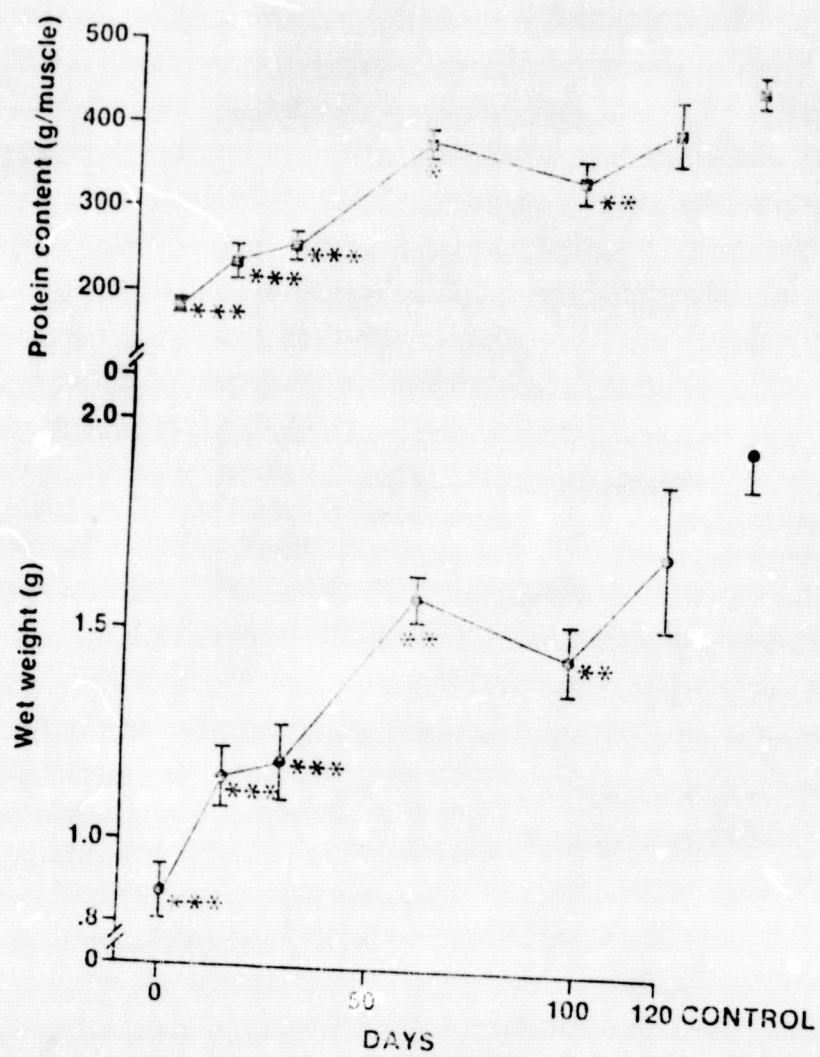
Figure 3. Recovery of wet weight and protein content of gastrocnemius from 90 days of limb fixation. The x-axis is 0 to 120 days of recovery from limb fixation with control values are given on the right-hand side of the figure. Recovery days are 0, 14, 28, 60, 97 and 120. The upper part of the figure is protein content of gastrocnemius. The lower part of the figure is the wet weight of the gastrocnemius. Values are means $\pm$ SE. Observations per time point are from 4 to 6 animals. The mean of each recovery time point was compared to the control mean with an unpaired 2-tail student t test. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

Figure 4. Calcium concentration in the gastrocnemius from control, immobilized and post-immobilized groups. Calcium concentration (y-axis) was determined in the gastrocnemius of the control group, the group immobilized 90 days, and the group which had casts removed 120 days earlier following 90 days of limb fixation. Values are means $\pm$ SE. Observations are 4 to 6 animals per group. The mean of recovery time points was compared to control mean with an unpaired 2-tail student t-test. * $P < 0.01$.

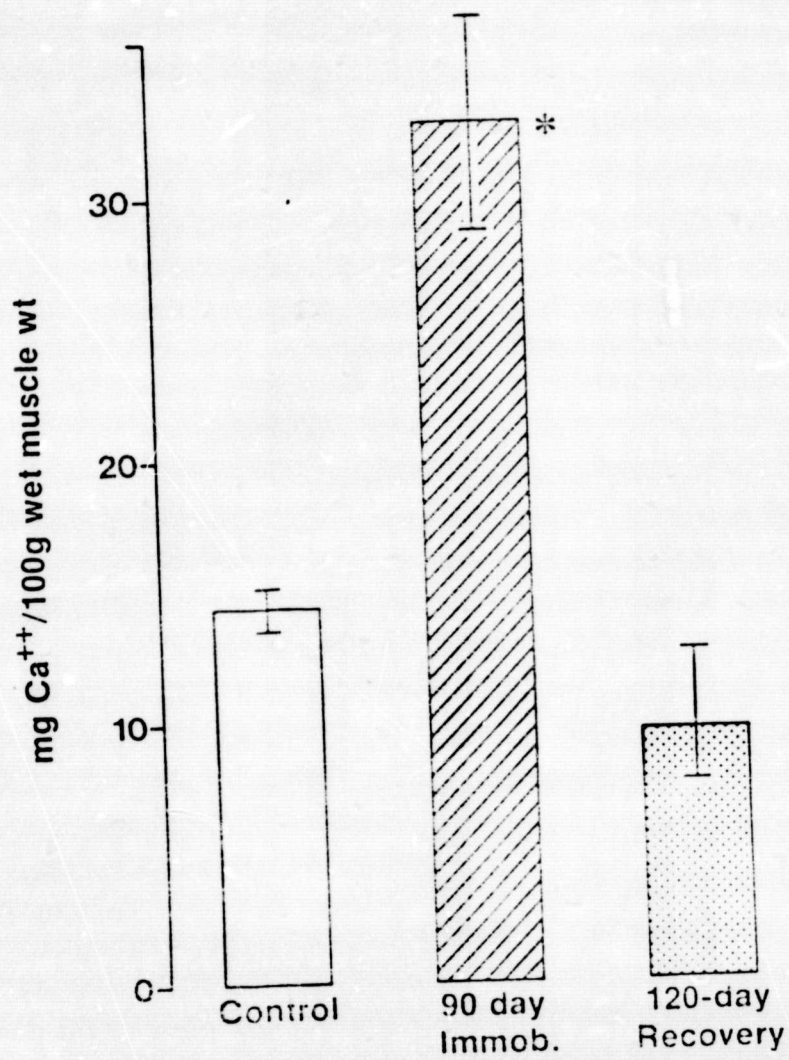


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Part III. Studies on the recovery of degenerated antigravity fibers after removal of hind-limb casts.

Rats had their hind limbs immobilized for 28 days and soleus muscles were stained for myosin ATPase activity. Cross-sections of these fibers are now at photography for enlargement so that fibers can be typed (i.e. antigravity or not) and then counted. We are trying a new procedure for stretching the soleus and comparing this procedure to our previous procedure (Pflügers Arch. 342:231-238, 1973). These data should be analyzed in the next monthly report.

Part IV. Studies on the atrophy and recovery of bone. I am just finishing these analyses. The data collected to date follows:

EXPERIMENTAL GROUP	N	BODY WEIGHT (g)	TIBIA WET WEIGHT (g)	ASH WEIGHT (g)	GRAMS CALCIUM PER TIBIA
Control	2	362*	.67 ± .05	.333 ± .018	.132 ± .001
90-Day Immobil	2	306*	.63 ± .00	.313 ± .001	.130 ± .006
60-Day Recov	4	364 ± 10	.73 ± .02	.346 ± .001	.139 ± .002
120-Day Recov	2	456 ± 36	.80 ± .03	.385 ± .010	.150 ± .001

* only one body weight

Evidently, the model employed by me does not produce much bone atrophy while the soleus muscle in the same rats atrophies to 33% of its control weight. I have no explanation for these observations. I will have the remaining tibia in this experiment analyzed within the next 10 days and the final data for this experiment will be reported in next month's report.