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THE COMBINED INFLUENCES OF STRETCH, MOBILITY AND ELECTRICAL STIMULATION IN THE
PREVENTION OF MUSCLE FIBER ATROPHY CAUSED BY HYPOKINESIS AND HYPODYNAMIA

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Department of Anatomy and Cellular Biology, Tufts University, Schools of
Medicine and Veterinary Medicine, 136 Harrison Ave., Boston, MA.

Dr. Geoffrey Goldspink (Principal Investigator)

Dr. David Goldspink, Dept. of Physiology, Queen's University, Belfast, Ireland

Dr. Paul Loughna

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Introduction

Disuse atrophy of muscle is currently being investigated using a wide variety of techniques including cage restraint, hind limb immobilization (plaster casting and joint pinning), denervation space flight and suspension systems (dorsal bonding, harness and tail fastened suspension). In this study we are employing two of these models, hind limb cast fixation and a suspension model. We have conducted a series of experiments to define the precise changes (morphological and biochemical) which occur in the hindlimb muscles of the rat in response to hypokinesia and hypodynamia (i.e. H + H). Although some earlier observations have been broadly reconfirmed, our use of more appropriate control animals and better methodology greatly improves the accuracy of, and provides more confidence in, these findings. In addition, some interesting new ideas and interpretations have arisen from this work (see below). Further, we have made good progress in establishing a solid base from which to devise suitable methods (i.e. exercises and patterns of electrical stimulation) for preventing, or retarding, the muscle atrophy associated with the weightless state.

Suspension experiments

In all suspension experiments carried out over this time period, male Sprague Dawley rats (initially 100 g) were suspended in a similar manner to that described by Mussacchia et al. (1) in a cloth harness so that only their forelimbs participated in weight-bearing and in the processes of feeding and drinking; both hindlimbs were totally free of all postural functions and were seldom moved after 1-2 hours in the harness. A 20 degree angle (head down tilt) was also imposed on the suspended animals in order to simulate the fluid shifts experienced during weightlessness (2). The resulting atrophy of

hindlimb muscles was defined and explained over a maximum period of 12 days of H + H. The food consumption of these suspended animals was monitored over the 12 days, and compared with ad libitum fed and fully mobile rats individually caged (Fig. 1). The suspended rats initially lost and then regained weight (Fig. 2), while their daily food intake was down by approximately 20% compared with the ad libitum fed controls. However, as the ad libitum fed mobile rats gained weight faster than the suspended rats, the food consumption per unit body weight was in fact consistently lower than that of the suspended rats. Changes in the nutritional state of an animal are known to influence muscle size and its rates of protein turnover, preferentially affecting fast twitch muscles relative to slow, oxidative muscles. Allowances must, therefore, be made for the important differences in the nutritional state of the suspended animals. Mobile control animals were therefore pair fed (twice daily to spread the time over which the reduced food ration was available) to the same level of intake as the suspended rats. Although similar differences in food consumption have previously been reported, they have never been controlled for. In fact, strange and often totally inappropriate controls have been used in the past, e.g. matched for size (weight) against the atrophic muscle, and, in so doing, completely disregarding inevitable differences in age and stages of muscle differentiation and metabolism.

After only 5 days of H + H appreciable muscular atrophy was apparent, this being preferentially expressed in certain muscles, i.e. soleus (41%) > plantaris (32%) > gastrocnemius (31%) > tibialis anterior (19%) > extensor digitorum longus (16%). These values were derived from the use of pair fed controls, but had ad libitum fed animals been used instead, these values would have increased by 3-10%. Despite allowing for the altered nutritional state, the suspended animals were still appreciably smaller than their pair fed

controls. When muscle size was normalized for such difference in animal size (i.e. expressed per 100 g of body wt.), the atrophy of the inactive muscles decreased, e.g. from 41% to 24% for the soleus. Nonetheless, the H + H induced atrophy was still more markedly expressed in the antigravity muscles (e.g. soleus) possessing a greater proportion of slow oxidative fibers. These results are similar to previous studies using this suspension model in the rat (3, 4). Protracted periods of H + H, e.g. up to 12 days, simply further exaggerated the degree of muscle wasting, while retaining the selective effects on the antigravity muscles. However, the fiber type composition of these muscles does not provide a complete explanation for their relatively far greater rate of atrophy. It was considered by the authors that the position of the foot in suspended rats which due to its weight hung in a plantar flexed position could have affected the rate of atrophy in those muscles examined.

Subsequent experiments were undertaken in which one limb subjected to H + H was simultaneously immobilized in a dorsi-flexed position while the contralateral remained freely suspended. This study was undertaken for two reasons: firstly, to establish if passive stretching of the antigravity muscles (e.g. soleus) would retard or prevent the atrophy induced by weightlessness, and secondly, to determine if the less pronounced atrophy of the fast twitch muscles (e.g. E.D.L.) was linked solely to the possession of less slow oxidative (S.O.) fibers, or whether the real atrophic responses to H + H were being masked, i.e. through the passive extension of the ankle (i.e. plantar flexing as occurs upon suspension) a stretch-related protection might be afforded these flexor muscles. In the latter situation the E.D.L. and tibialis anterior would be held at a longer, and hence stretched, length relative to the normal position when engaged in locomotory or postural functions. Two sets of control animals were used, each animal being caged

individually though allowed full mobility. One group of animals was fed ad libitum and the left foot of these animals was casted in a fully dorsiflexed position. A second group of animals was pair fed the same as the suspended animals.

Passive stretching of the soleus for 5 days simultaneous to H and H completely prevented wasting of this muscle when compared to pair fed controls (Fig. 3). Atrophy of the gastrocnemius muscle was also reduced to only 6% over a 5 day period by passive stretching (Fig. 4). The plantaris muscle, however, showed a less dramatic decrease in wasting from 32% to 19% (Fig. 5), which is probably due to the insertion of this muscle leading to only a slight stretch when the lower part of the limb is dorsi-flexed. When E.D.L. and tibialis anterior muscles were immobilized (fully shortened) to eliminate any possible influences of stretch which might arise from changes in limb position, an additional and very significant loss of muscle weight occurred (Figs. 6 & 7). For example, after 5 days the tibialis anterior was 14% smaller when this form of immobilization was coupled with H + H. So, although the cross-sectional areas of the S.O. fibers (established by histochemical staining for myosin ATPase activity in frozen muscle sections) were found to decrease more than the fast glycolytic (F.G.) and fast oxidative glycolytic (F.O.G.) fibers, the H + H selective effect on muscle types was not based purely on fiber compositions. The situation in this model is not, in fact, as simple as at first sight appears. Utmost care is clearly required in establishing appropriate controls and in employing reasoned caution in interpreting the data. Otherwise, a less accurate and oversimplified picture of events will inevitably emerge.

Protein Turnover

Since intracellular protein constitutes approximately 20% of tissue mass,

the muscle wasting induced by H + H must result in a net loss of protein. This was indeed confirmed, and the changes in protein mass reaffirmed the atrophic trends within the different muscle types (see above). These changes in muscle protein must arise from alterations in the relative rates of protein synthesis and protein breakdown. To determine precisely what changes H + H had induced, we measured the protein synthetic and degradation rates (in vivo) using the most accurate technique currently available; i.e. after the injection (i.v.) of a large dose of ^3H -phenylalanine (5). Each rat was given a single injection administered into a lateral tail vein. The bolus injection dose contained 150 μmol of phenylalanine, including 65 μCi L-(4- ^3H) phenylalanine, in 1 ml of 0.9% saline per 100 g of body weight. All animals were decapitated 10 minutes after commencing the injection and the appropriate leg muscles rapidly dissected free under ice cold NaCl and frozen in liquid nitrogen. Muscles were subsequently homogenized in 0.2 N perchloric acid. Nucleic acids were extracted according to the modified method (6) of Schneider and Greco (7) and assayed spectrophotometrically as described by Goldspink and Goldberg (6). The specific radioactivity of the phenylalanine in the "flooded" free amino acid pool (SA) and that incorporated in protein (SB) were determined as described by Garlick et al. (8). This involved the prior hydrolysis of the washed protein pellet in 6M-HCl at 110 degrees C for 18 hours and the conversion of phenylalanine to B-phenylethylamine which was assayed fluorimetrically. All measurements of radioactivity were made in a LKB 1211 Rackbeta scintillation counter in a Triton X-100/xylene based scintillant. Fractional rates of synthesis K_s , as percent of the protein mass synthesized per day, were calculated as

$$K_s = \frac{SB}{SA} \times 100$$

where SA and SB are the specific radioactivities of phenylalanine in the tissue precursor pool and protein, respectively, and t is the time in days. Total protein synthesis (i.e. mg of protein synthesized per day) can be obtained as the product of the fractional rate (Ks) and the protein mass. Without altering the rate of synthesis (9) this "large dose" method (9, 10) effectively floods the tissue amino acid pool, thereby minimizing problems with location of the precursor pool for new proteins. For this reason, and the need to restrain animals for only 1 minute, this method now supercedes the constant infusion method, which involves several hours (usually 6) of restraint. Fractional rates of breakdown were calculated by subtracting the mean growth rate from the mean fractional rate of synthesis for any particular muscle. These calculated rates of breakdown have been shown to be in good agreement with induced changes in breakdown when measured "in vitro". Since such changes in protein turnover must precede observable changes in muscle growth (i.e. protein mass), turnover was measured in all five lower limb muscles after 0, 3 and 5 days of suspension, with or without passive stretching. For the sake of brevity, only the changes in two extreme muscle types, and at 3 days, will be described here.

In the soleus muscle, both the fractional (i.e. $KS = 15.4 \pm 1.7\%$ per day) and total (1.68 ± 1.5 mg of protein per day) rates of synthesis decreased by 30 and 47%, respectively, after 3 days of H + H. This decrease in the synthetic rate was found to correlate with a fall in the inactive muscle's ribosomal capacity (i.e. total RNA, or RNA/protein) rather than any change in its ribosomal activity (i.e. synthesis/unit RNA), with respect to the process of translation. In addition to these changes, the control rate of protein

degradation (i.e. $kb = 9.1\%$ per day) increased by 58% in the inactive soleus. Hence, the decrease in synthesis and the increase in breakdown together combine to explain the H + H induced atrophy. The additional imposition of stretch on this muscle retarded its atrophy (see above). This was explained by a 45% increase (ks now = 22% per day) rather than the former decrease (30%) in synthesis as registered with H + H alone. Although protein degradation was also increased by stretch, synthesis ($ks = 22\%/day$) exceeded breakdown (i.e. $kb = 17.3\%$ per day) such that the net effect was anabolic. This contrasts with the net catabolic effect in the H + H situation without stretch (i.e. $ks = 11.0-$ and $kb = 14.4\%$ per day). This anti-wasting action of stretch was therefore mainly manifested by its ability to stimulate protein synthesis. Fractional rates of protein synthesis in the gastrocnemius muscle, though of a very different fibre type composition, show a similar though less dramatic response to the soleus muscle under the same conditions (Table 1).

The less extensive atrophy of the E.D.L. muscle after 3 days of H + H alone was explained by a small fall in the total rate of synthesis (from 0.9 to 0.8 mg/day) and a small increase (5%) in its rate of degradation ($kb = 6.8\%$ per day). Removing the possibility of stretch-protection by preventing the foot from dropping, as normally occurs in the suspended animals, a further fall in the rate of synthesis (now only 0.5 mg/day) and an additional increase (10%) in the rate of protein breakdown occurred. This once again illustrates that the full atrophic effects will not be experienced in these flexor muscles unless such possible complications (i.e. stretch) are recognized and allowed for. These changes in protein turnover do, however, explain how H + H induces muscle atrophy, and how passive stretch can effectively modify these events, thereby reducing the extent of muscle wasting.

Having demonstrated the potential usefulness of passive stretch in

retarding muscle atrophy, a series of studies has been initiated in which the stretching of muscles is combined with direct electrical stimulation via the normal nerve supply. In the initial experiment we suspended two groups of rats for a period of 5 days. In one group both hindlimbs hung freely unhindered and in the second group both limbs were casted in a fully dorsiflexed position. In both groups the plantar flexor group of the lower limb was electrically stimulated (5 Hz) for one hour per day. The contralateral (non-stimulated) limb was used as a control for each animal. However, no difference was found in either group between stimulated and unstimulated limbs. We are at present proceeding with a further study in which one limb will be stimulated for considerably longer periods (12 hours each day and continuous stimulation). At present, however, we have not sufficiently processed these data to make comment.

Immobilization of hind limbs in casts.

The above studies demonstrate that passive stretch can counteract muscle disuse atrophy due to hypodynamia and hypokinesia. We have also started to investigate the effects of stimulation and immobilization on muscle. The anterior tibialis muscle of young male rabbits of the strain NZW weighing approximately 1.5 kg were stimulated at a frequency of 5 Hz for 4 or 7 days. The intensity of approximately 2 V was adjusted so that it did not cause any noticeable discomfort to the animal. In one group of animals the anterior tibialis muscle was stimulated only; in a second group stimulation was combined with stretching of the muscle by means of a plaster cast holding the foot in full plantar flexion; in a third group the muscle was stretched without stimulation. In each case the contralateral muscle served as control. The effects of immobilization and immobilization combined with stimulation

were investigated using histochemical techniques.

It was found that immobilization in the extended position resulted in an increase of the SO fibers (Fig. 8). "Slow" stimulation alone did not cause any significant increase in fiber size whereas a combination of immobilization in the extended position and stimulation resulted in a large increase in size of all three main fiber types. Along with increase in fiber area, sarcomeres were added onto the muscle fibers (Table 2). These sarcomeres, however, differed from those in the end regions of the control muscles and also from those in the middle regions of the experimental muscle: there was a greater proportion of slow oxidative and fast oxidative glycolytic fibers. Using a higher stimulation frequency however probably would not have produced this effect. It would, therefore, appear that this type of modification to the activity pattern can reprogram the synthesis of fiber specific proteins (via gene switching), in this case consisting of F.O.G. and S.O. typings. Neither stimulation nor stretch alone appears to produce these very rapid responses. The rates of protein synthesis under similar conditions are being measured in order to identify and localize any regionalized changes in response to stretch, continuous stimulation and a combination of both. Although the latter experiments have been initiated, the analytical work has not been sufficiently advanced to comment upon at this time. These, and other aspects of the proposed three year study are being pursued with the same interest and enthusiasm.

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Fig. 1. Food consumption in the suspended rats (●) and in ad libitum fed control rats (▲).

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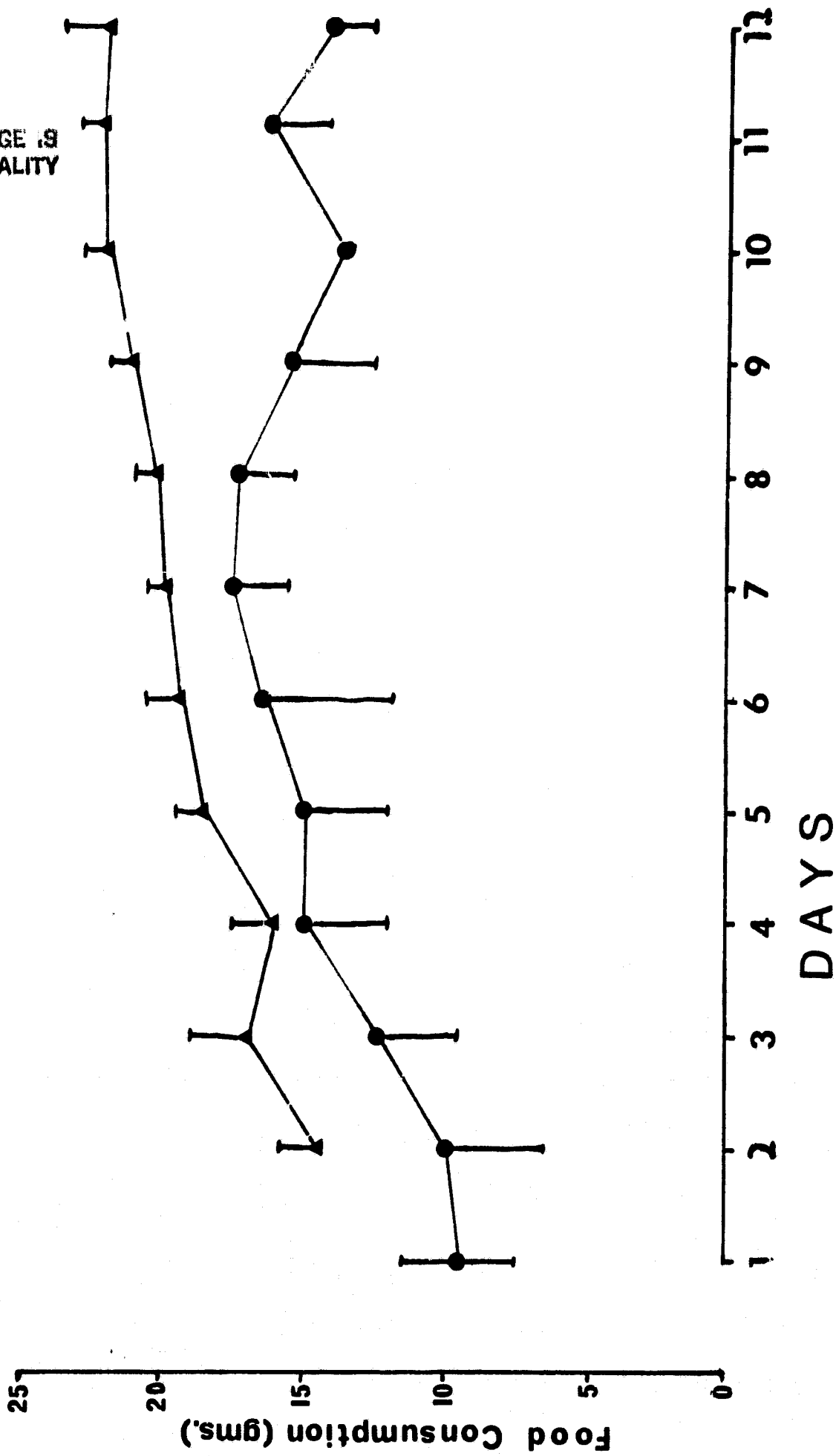


Fig. 2. Body weight changes for suspended rats (●), pair fed controls (■) and ad libitum fed controls (▲).

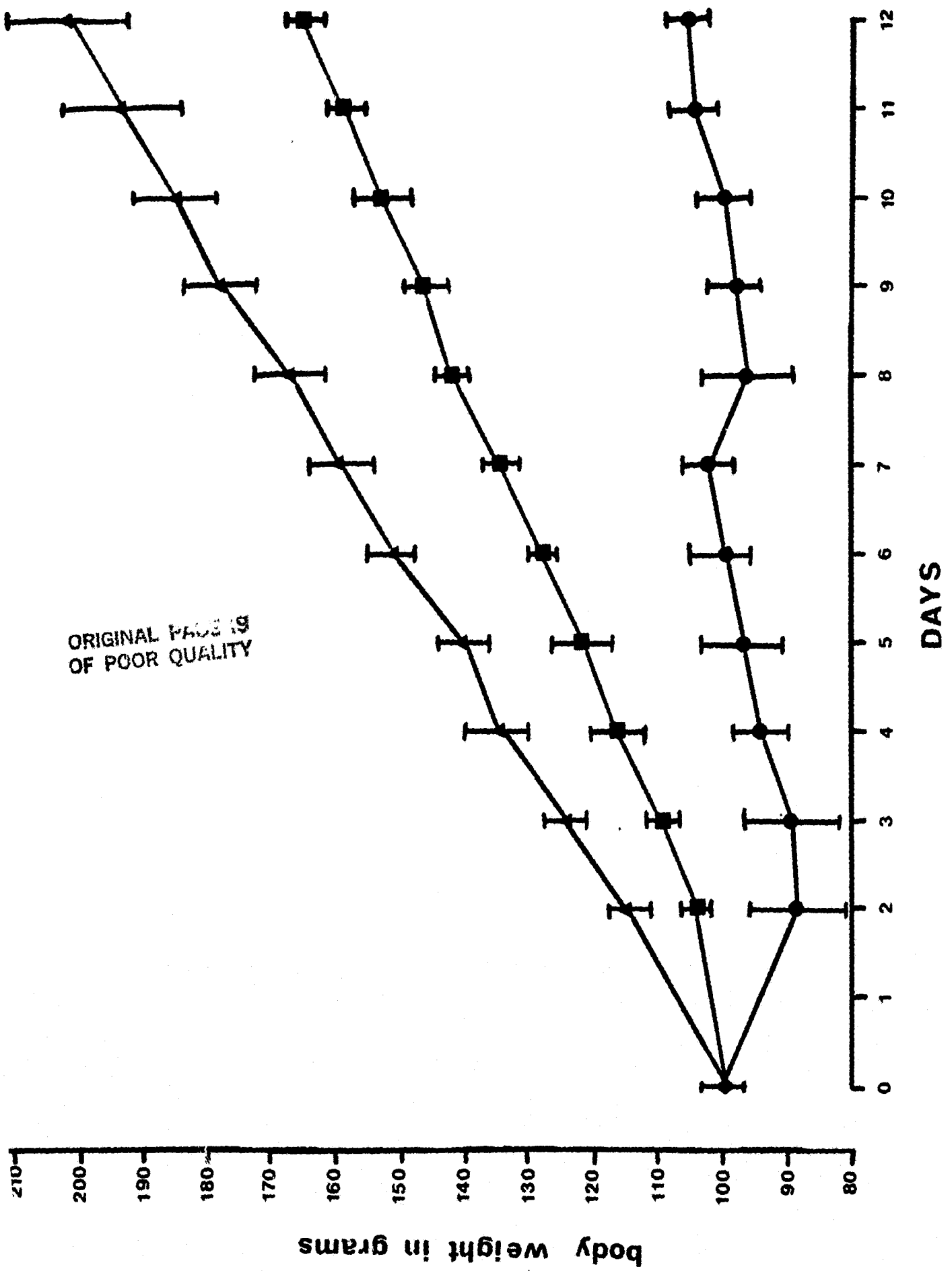


Fig. 3. Soleus muscles - weight changes over five day period

○ - suspended limb only.

● - suspended and immobilized in dorsiflexed position.

□ - pair fed.

▲ - ad libitum fed and immobilized in dorsiflexed position.

△ - ad libitum fed - contralateral limb.

(The same key is used in figures 3 - 7.)

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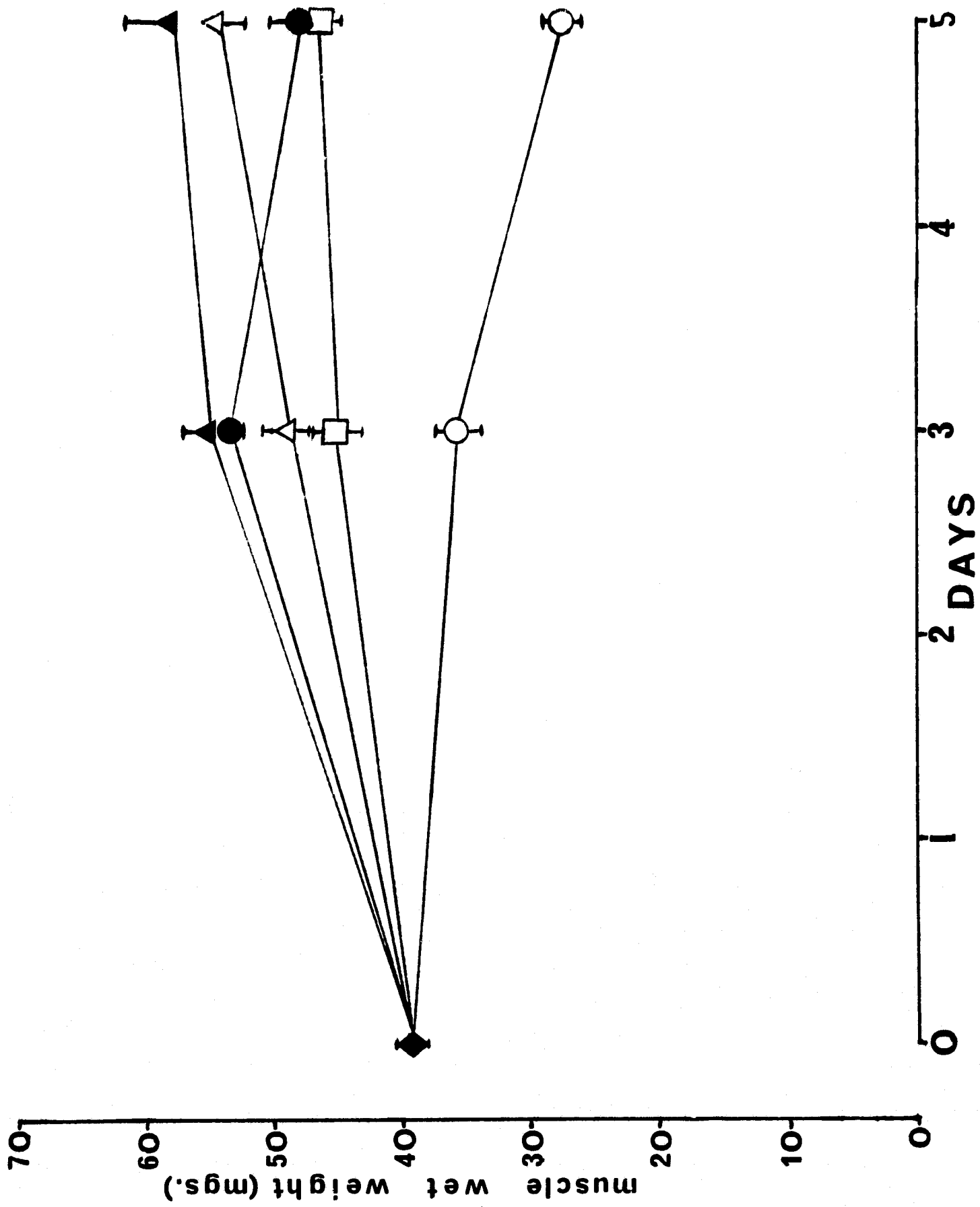


Fig. 4. Gastrocnemius muscle - weight changes over five day period

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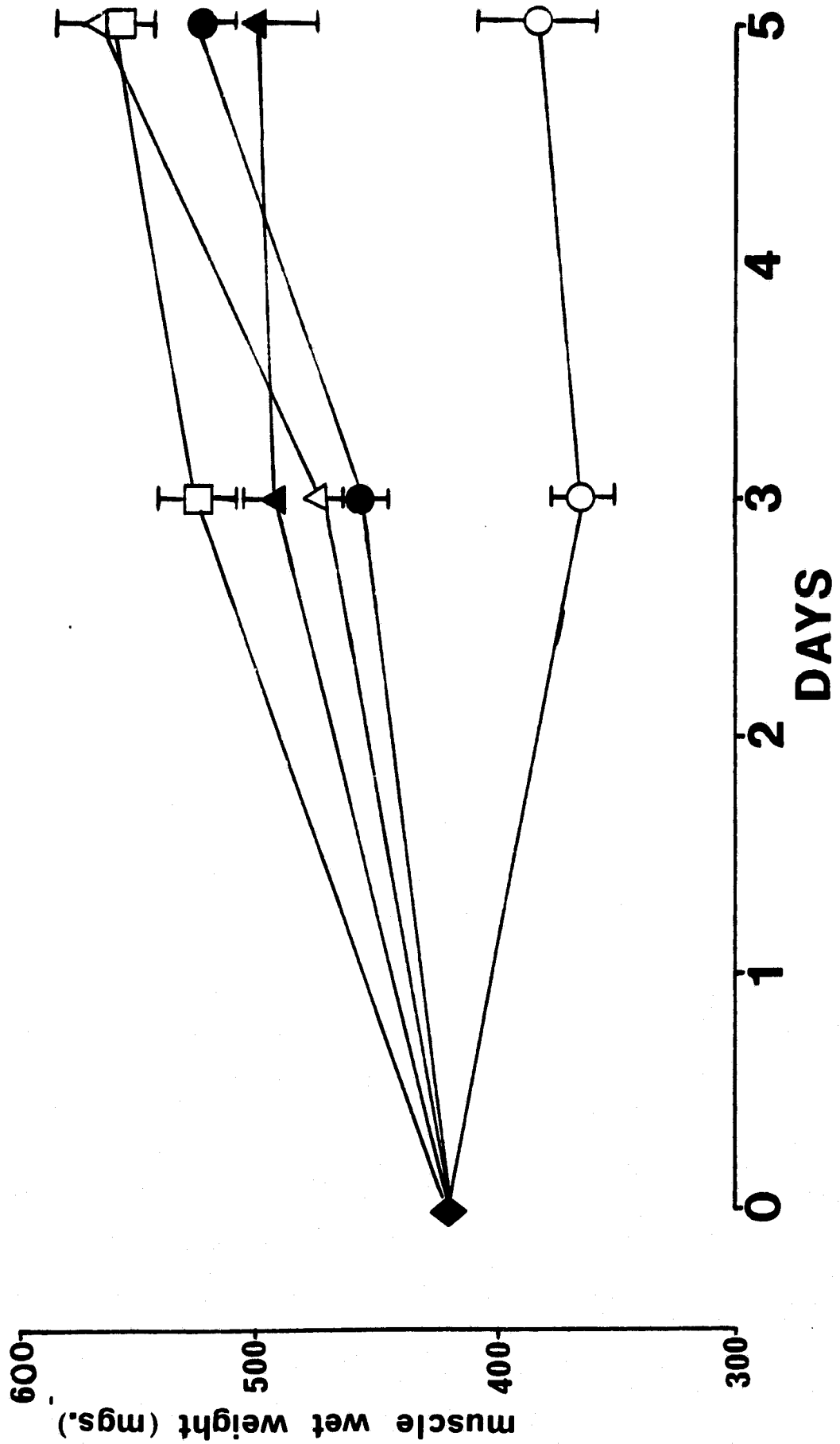


Fig. 5. Plantaris muscle - weight changes over five day period

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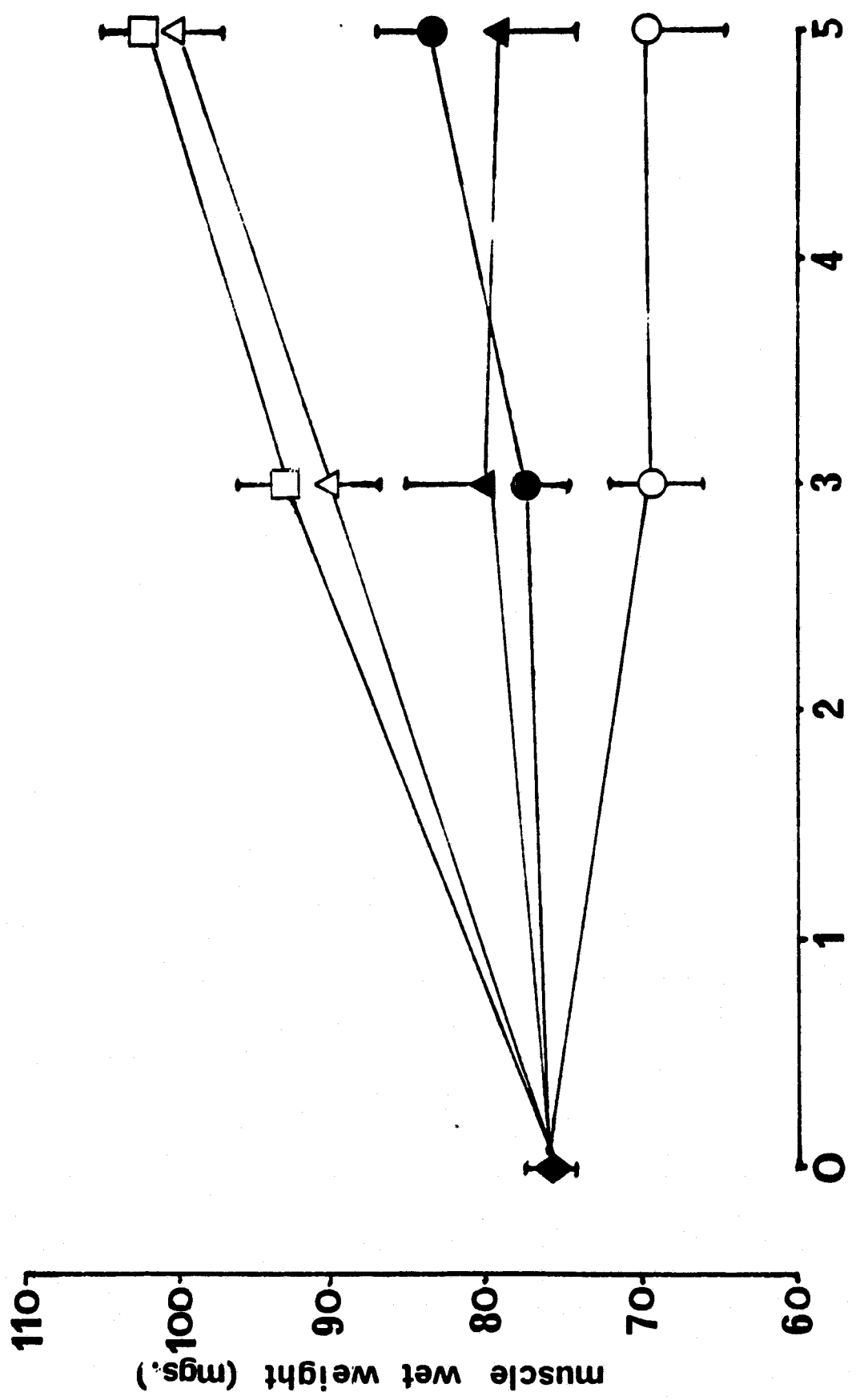


Fig. 6. Extensor digitorum longus muscle - weight changes over five day period

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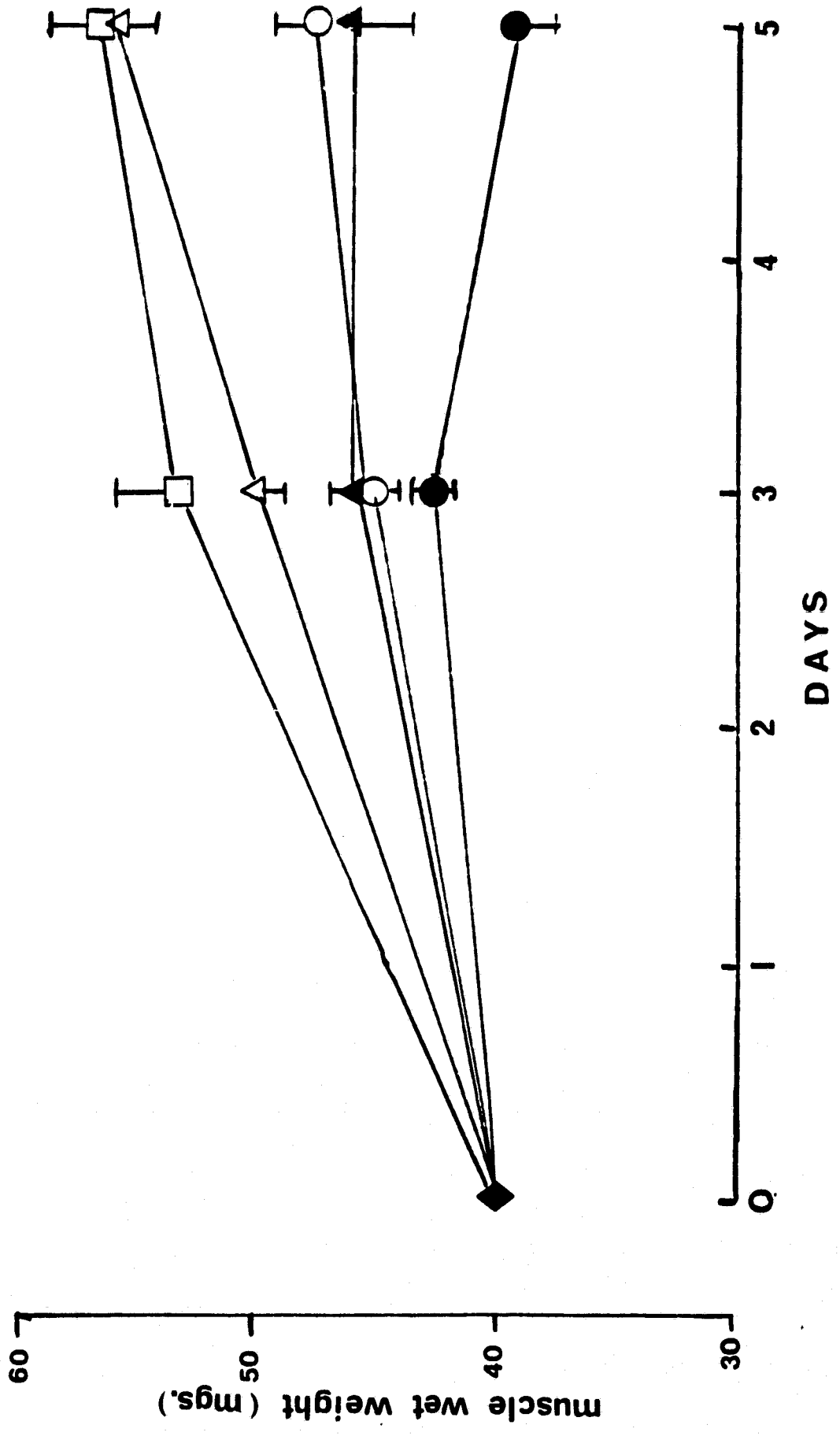


Fig. 7. Anterior tibialis muscle - weight changes over five day period

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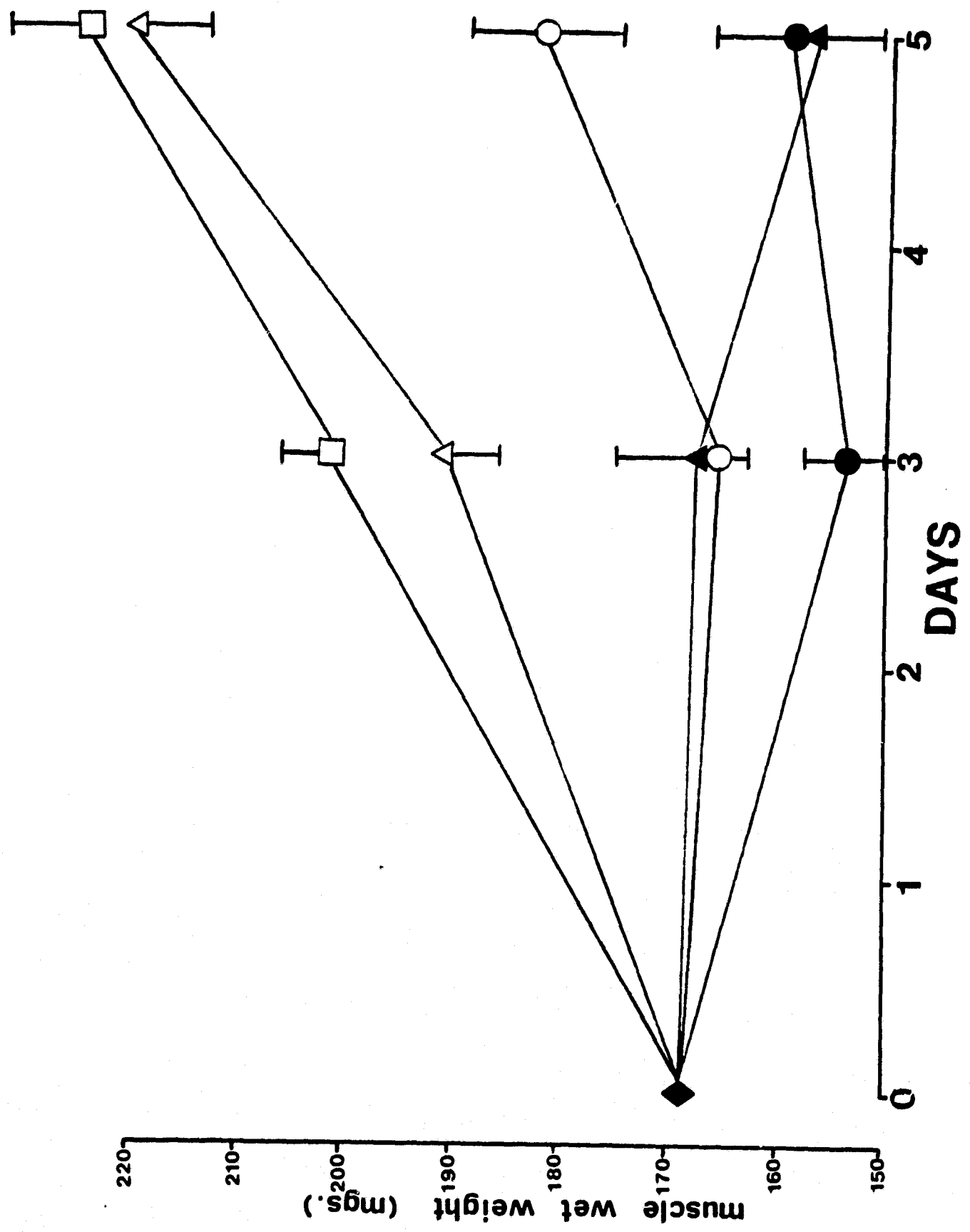
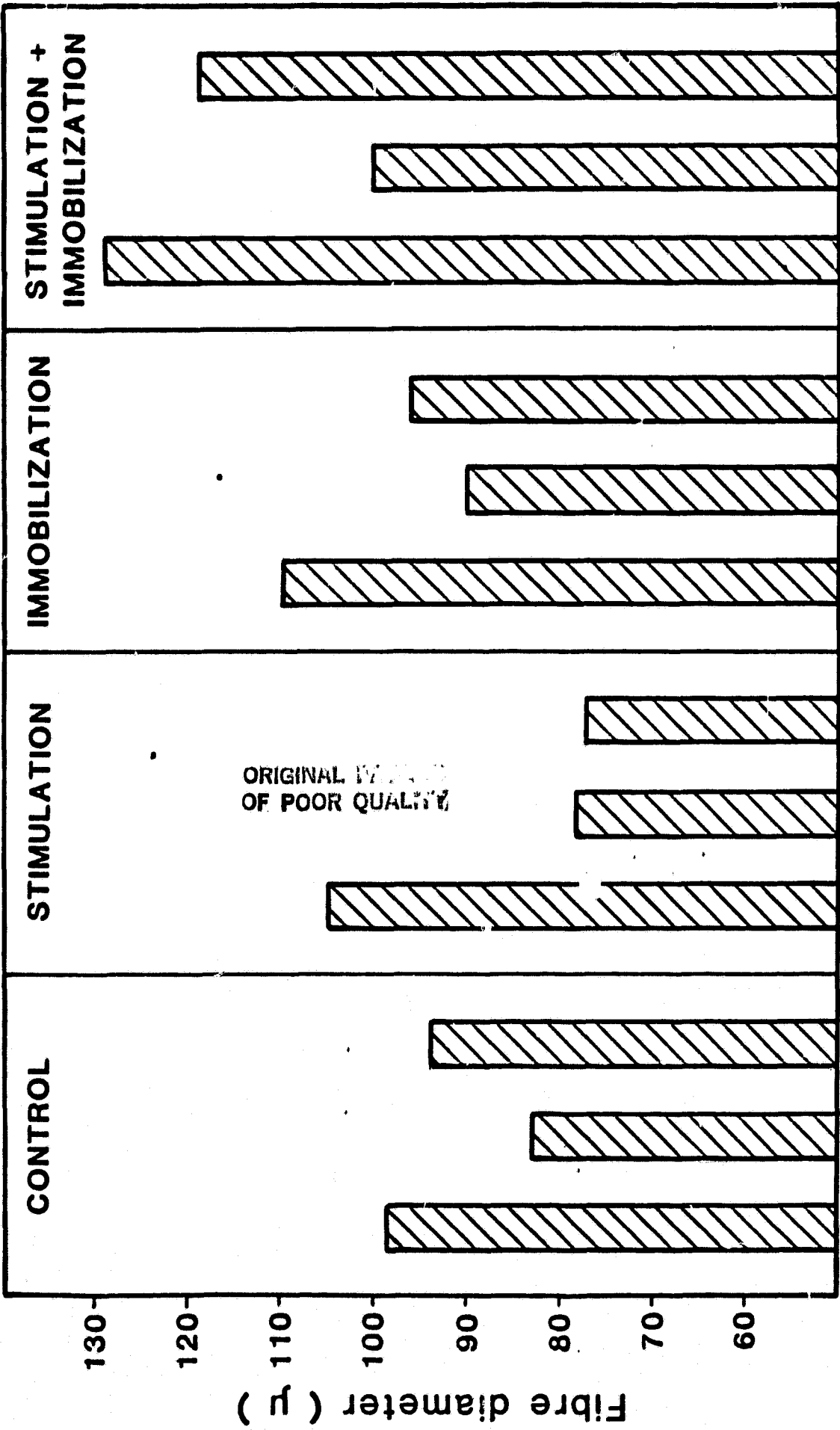


Fig. 8. Effects of immobilization, stimulation and immobilization and stimulation on changes in diameters of the three different fibre types in the anterior tibialis muscle of the rabbit.



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Fibre diameter (µ)

130
120
110
100
90
80
70
60

CONTROL

STIMULATION

IMMOBILIZATION

STIMULATION +
IMMOBILIZATION

SO FOG FG SO FOG FG SO FOG FG SO FOG FG

Table 1. Fractional rates of synthesis (% per day) for three muscles in suspended rats and ad libitum fed and pair fed controls.

	<u>Soleus</u>	<u>Extensor digitorum longus</u>	<u>Gastrocnemius</u>
Zero time	15.4 + 1.65	11.25 + 1.65	13.30 + 0.57
3 day suspended	10.96 + 1.09	14.02 + .0038	7.59 + 1.03
3 day suspended & immobilized	21.89 + 2.06	8.03 + 1.14	13.80 + 1.00
3 day pair fed	17.53 + 2.4	13.04 + 1.11	11.20 + 0.48
3 day ad lib fed	13.91 + 0.46	13.99 + 1.28	12.25 + 0.50
3 day ad lib fed & immobilized	14.09 + 1.13	8.46 + 0.95	12.50 + 0.71
5 day suspended	11.91 + 1.20	10.28 + 1.67	7.46 + 1.53
5 day suspended & immobilized	18.91 + 1.40	6.28 + 1.13	9.84 + 1.02
5 day pair fed	13.53 + 0.67	10.12 + 1.17	11.17 + 0.53
5 day ad lib fed	16.74 + 1.07	14.97 + 1.32	13.14 + 0.79
5 day ad lib fed & immobilized	14.31 + 0.89	9.93 + 1.07	12.78 + 0.72

Table 2. Muscle weight, length and serial sarcomere number in stretched stimulated muscles and contralateral controls.

	EXPERIMENTAL	CONTROL
	mean + S.D.	mean + S.D.
Muscle weight (g)	3.5 + 0.5*	2.6 + 0.3
Muscle length (cm)	6.0 + 0.2*	5.2 0.1
Sarcomere number	20469 + 738*	18337 + 437

*p < 0.05

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Submitted for publication

1. Williams, P.E., Watt, P.W., Bicik, V. & Goldspink, G. Muscle fibres can produce different end regions when stimulation is combined with stretch. Muscle and Nerve.
2. Goldspink, D.F., Loughna, P. & Goldspink, G. Changes in muscle size nucleic acids and protein turnover in response to hypokinesia/hypodynamia and passive stretch. J. Appl. Physiol. Respirat. Environ. Exercise Physiol.

In preparation

1. Histological changes in skeletal muscles subjected to hypokinesia/hypodynamia and passive stretch.

Geoffrey Goldspink, PhD, DSc