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Induced Muscle Atrophy

Redacted for Privacy

Abstract approved: _____
Lavern J. Weber

Space flight results in loss of mass and function in skeletal muscle. Data from manned flights and animal biosatellites give evidence of possible changes in neuromuscular interactions. Components at the neuromuscular junction are a potential site of action for this space-induced muscle atrophy. We examined the nicotinic acetylcholine receptor population (B_{max}) and affinity (K_d), the activity of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), and the endogenous levels of acetylcholine (ACh) and choline (Ch). We assayed the hindlimb soleus, gastrocnemius and tibialis, and the forelimb triceps brachii muscles after 1, 3 or 8 weeks of supsension. B_{max} was greater than controls in the triceps brachii and in the gastrocnemius after 3 and 8 weeks of suspension respectively. Receptor K_d was decreased in the soleus muscles of rats suspended for 3 weeks. Enzyme activity decreased from control animals' levels in all 4 muscles, with the time to effect varying depending on the muscle

and the enzyme. No difference was found in endogenous ACh levels in the soleus, but levels were increased in the gastrocnemius (3 weeks), and decreased in the tibialis (3 weeks) and triceps brachii (1 week). Ch levels were decreased in the soleus (1 and 3 weeks) and gastrocnemius (3 weeks) with no change from control levels in the tibialis and triceps brachii.

Testosterone, as an anabolic hormone to skeletal muscle, was assayed in suspended and control rats. We found no difference from control animals in total testes tissue levels. Plasma levels were increased in suspended animals after 8 weeks.

Acetylcholine Synthesis, Storage, Degradation and Nicotinic Receptors in a Rat Model for Space-Induced Muscle Atrophy

by

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Chapter 1. Introduction

One of the first recognized and most consistent physiological consequences of space flight has been a loss of muscle mass and function, particularly in the postural antigravity muscles. Evolved to function under a constant gravitational load, muscle properties ranging from the tonic firing pattern of antigravity muscles to proprioreceptor feedback systems to contralateral and antagonistic muscle interactions are disrupted in weightlessness.

Muscle atony was reported in the literature as early as 1971 (Kakurin, et al. cited in Kozlovskaya, et al., 1981) and autopsy material after the tragic deaths of the Salyuz 11 crew (also in 1971) showed focal degeneration of muscle fibers in antigravity muscles after 23 days in space (Nicogossian and Parker, 1982). Losses in muscle mass and function have been attributed to the fluid shifts from the periphery to the head and torso that occur in microgravity and to the disuse of the weight-bearing and antigravity muscles. However, decreases continue to occur long after the fluid shifts have occurred (usually within the first 10 hrs inflight (Moore and Thornton, 1987)) and despite vigorous exercise routines. As little as one week of weightlessness on the space shuttle resulted in a 3.2% decrease in leg volume one week

postflight (i.e. after fluid redistribution) (Moore and Thornton, 1987). In the Skylab 2 crew, who performed half an hour of exercise each day, average leg volume losses after 28 days in space were about 10%. In the Skylab 4 crew, who exercised vigorously for 1.5 hours a day, leg volume losses after 84 days in space were limited to about 7%. However, even with vigorous exercise, arm extensor and leg flexor muscles showed about a 15% decrease in strength as measured by a dynamometer (Thornton and Rummel, 1977, cited in Nicogossian and Parker, 1982). Longer flights result in greater losses. Leg volume losses averaged 20% in the Soyuz 29 crew after 140 days in space. Vigorous exercise by the Soyuz 35 crew decreased losses to about 15% after 185 days weightlessness (Kozerenko, et al., 1981). Despite exercise it can be seen that losses are continuous throughout the duration of the flight. Analysis of astronaut and cosmonaut urinary excretion products has shown losses of nitrogen, potassium, phosphorus and amino acids that are indicative of muscle catabolism (Leach and Rambaut, 1977) (Leach, et al., 1979). The losses in nitrogen and phosphorus have not been corrected by diet or exercise (Thornton, 1981, cited in Nicogossian and Parker, 1982).

Cosmonauts and astronauts have exhibited postflight behavior suggesting neuromuscular dysfunction. Overcompensation in lateral movements, increases in reflex time, changes in postural control and coordination disturbances have all been noted (Kozlovskaya, et al., 1981) (Lestienne and Gurfinkel, 1988). Postflight shifts to higher frequency bands in

electromyograph (EMG) patterns similar to those seen in neuropathologic muscle were seen in the crew of Skylab 3 (LaFevers, et al., 1975). And after 175 days in space the crew of Soyuz 32, EMG analysis of muscle function showed the cost of a standard exertion by the posterior leg muscles to be increased more than two-fold. The magnitude of EMG effort decreased over time but had not returned to control levels by 35 days postflight (Kozlovskaya, et al., 1981). Proprioreceptor hyperactivity as evidenced by decreases in Achilles tendon reflex (Baker, et al., 1977) and vibrosensitivity thresholds of the foot (Kozlovskaya, et al., 1982) were also found. Inflight evidence of a possible neuromuscular junction transmission mediated effect was found by Baranski, et al. (1977) in their morphological study of Biosputnik 936 rat soleus neuromuscular junctions. They found synaptic vesicles to be decreased in both volume and number.

Our hypothesis is that neural input to the weight-bearing and antigravity muscles is decreased in weightlessness and that this decrease contributes to the losses seen in muscle mass and function. To investigate this hypothesis we looked for changes at the neuromuscular junction. In the first phase of our project we examined the nicotinic acetylcholine receptor population. Receptor population has been shown to be sensitive to changes in neural input in both denervation and nerve transmission blockade studies. In the second phase of our study we examined the activity of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT), the enzymes responsible for the

degradation and synthesis of the neuromuscular junction neurotransmitter, acetylcholine (ACh). Both enzymes have been shown to be decreased in denervation (Tucek, 1973) (Dettbarn, et al., 1987) and TTX nerve conduction blockade studies. The third phase of our study involved looking at endogenous storage levels of ACh and choline (Ch), a precursor to ACh synthesis. Choline active uptake by nerve terminals is tied to impulse activity (Tucek, 1985) and nerve activity affects the availability of ACh for release by affecting its exchange between intraterminal storage pools (Whittaker, 1986).

In our study we used the Morey-Holton rat-tail suspension model to simulate the effects of weightlessness. This model causes the fluid shifts and organ displacement common to microgravity and mechanically unloads (i.e., removes from weight-bearing) the hindlimbs. It has been shown to successfully mimic changes seen in flight animals in bone formation, electrolyte balance, and renal function (Morey, 1979) as well as changes in muscle mass and protein content (Morey-Holton and Wronski, 1981) (Musacchia, et al., 1987).

Whole body effects of the suspension model could have contributed to changes found at the neuromuscular junction. One such possible contribution would be by changes in hormone levels. Testosterone is known to be an anabolic hormone on skeletal muscle growth (Bergamini, 1975) (Breuer and Florini, 1966), to influence AChE and ChAT synthesis (Gutmann, 1976), and to show an effect on motorneuron axonal transport (Frolkis, et al., 1985)

and size (Kurz, et al., 1986). We found that the testes of suspended animals were smaller in size than those of the control animals. As the primary site of testosterone production in the male, the reduced testes size could result in hormonal changes of a consequence to our neuromuscular study. We therefore did a comparison study of the plasma and testes tissue levels of testosterone in our suspended and control rats. Our study showed that it was unlikely that there was an effect in the shorter suspension experiments, but at the longest suspension period (8 weeks), plasma testosterone levels were increased. These data are presented in Appendix A.

Chapter II. Nicotinic Acetylcholine Receptor Number (B_{max}) and Affinity (K_d) in Rat Skeletal Muscle in the Rat-Tail Suspension Model for Weightlessness

Introduction

Data from manned flights and biosatellites have shown skeletal muscle to be sensitive to the weightless condition. Losses in muscle mass (Moore and Thornton, 1987) and function (Oganov, et al., 1980) have been reported. The losses in mass have been attributed to the fluid shifts from the periphery to the head and torso that occur in weightlessness and to the unloading of the weight-bearing and antigravity muscles, resulting in a form of disuse atrophy. But there is a continued loss of mass after the fluid shifts have occurred and despite vigorous exercise regimens (Moore and Thornton, 1987) (Thornton and Rummel, 1977, cited in Nicogossian and Parker, 1982).

A correct interaction between neural input and muscle activity is known to be required to maintain both nerve and muscle function and integrity. Studies that inhibit neural input such as denervation (Cangiano, 1984), tetrodotoxin blockade of nerve impulse (Spector, 1985), or botulinum toxin blockade of the neurotransmitter, acetylcholine (ACh), release (Thesleff,1960) lead to changes in muscle contraction properties, fiber diameter, and receptor population. Studies on cross-reinnervation (Foehring, et al., 1987a) and on frequency, duration and pattern of nerve stimulation (Westgaard and Lomo, 1988) have shown muscle fiber

type (e.g. fast glycolytic or slow oxidative) to be sensitive to the type of innervation. The converse has also been shown. Muscle activity is required for correct interaction of the nerve at the neuromuscular junction. Muscle activity participates in initiation of neural contact (Smith, et al., 1986) and endplate formation (Peng, 1986), nerve sprouting (Barker and Ip, 1965), and prevention of extrajunctional receptors (Jones and Vrbova, 1970). Studies that block axonal transport with colchicine (Hoffman and Thesleff, 1972) or utilize direct muscle stimulation (Hill and Bennett, 1986) prove that trophic substances are transported both directions between nerve and muscle.

Data from American astronauts and Russian cosmonauts give evidence of impaired neuromuscular function as a result of space travel. Postflight electromyographical (EMG) data show changes in spectral patterns similar to those seen in neuropathologic muscle (La Fevers, et al., 1975) (Kenyon and Young, 1986). Kozlovskaya, et al., (1981) in their analysis of motor function after long term flights (140 and 175 days) report severe atonia, a loss of strength and an increase in the EMG cost of contraction in leg and trunk muscles. They also report decreases in reflex action and contralateral movement and disturbances in central mechanisms of postural and locomotor activities. Baker, et al., (1977) report postflight changes in Achilles tendon reflex which they attribute to changes in the muscle servofeedback system. In addition, Baranski, et al., (1979) in their morphological study of Biosputnik

936 rat soleus muscles found decreases in both size and number of synaptic vesicles.

We investigated the possibility of changes at the neuromuscular junction correlating with the changes reported in space-induced muscle atrophy. We used the Morey-Holton rat-tail suspension model as a model for weightlessness. This model mimics the fluid shifts seen in space and mechanically unloads the hindlimbs. It also results in changes in electrolyte balance, bone resorption, renal function, and muscle mass and protein similar to those seen in flight animals (Morey, 1979) (Morey-Holton and Wronski, 1981) (Musacchia, et al.,1987). As the nicotinic acetylcholine receptor at the neuromuscular junction has been shown to be sensitive to neural input, we investigated it as a possible site of modulation and as a possible mechanism of action of space-induced muscle atrophy.

Materials and Methods

Supplies: Male Sprague-Dawley rats were from Simonsen Labs, Gilroy, CA. Supplies for suspension were as follows: Compound benzion tincture, Whiteworth, Inc., Gardena, CA; Fas-Trac adhesive strip, used in the tail suspension, was from DePuy, Inc., Warsaw, IN; and 1/2 inch waterproof medical tape, Johnson and Johnson Products, Inc., New Brunswick, NJ. ^{125}I-α-bungarotoxin (BuTX) was from Du Pont, NEN Research Prods., Boston, MA. Unlabeled bungarotoxin, bovine serum albumin (BSA), ethylenediaminetetraacetatic acid (EDTA), phenylmethyl sulfonyl fluoride (PMSF), and polyethylenimine (PEI) were from Sigma Chemical Co., St. Louis, MO. The Whatman GF/B glass filters were from VWR Scientific, Portland, Or. All other chemicals were reagent grade.

Suspension: Animals (mean wt 150.1 gm, n = 100) were weight matched at the beginning of the experimental protocol and pair-fed throughout. Experimental animals were suspended by the tail at a 30° head down tilt as previously described (Royland, et al., 1989). Unanesthesized rats' tails were cleaned, treated with tincture of benzoin to toughen the skin, and a strip of Fas-Trac orthopedic tape, starting at the tail base, ran 2/3 of the way up one lateral side of the tail, a loop was made in the tape, and the tape ran back down the opposite of the tail Waterproof adhesive tape was spiraled around the Fas-Trac tape to help hold it in place. Constant watch for loss of circulation was kept on the tail tips. A clip in the loop was attached to a rotating, swivel device (built in

this lab) which elevated the hindlimbs and allowed the animal free access to all of the cage. Suspension height was adjusted as the animals grew to maintain the correct 30° head down tilt. Control animals were housed singly, unrestrained in identical cages. Records of daily food consumption and weekly weight gains were kept. Animals were suspended for 1, 3 or 8 wks.

Solutions: Stock solutions consisted of 10% PEI and 20 and 50 mM monobasic potassium phosphate (KH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4). Stock PEI was diluted to 0.3% the day of use. Buffer recipes follow: Buffer A -- 20 mM Na_2HPO_4 plus 5 mM EDTA, 0.1 mM PMSF, 0.1 M NaCl, pH 7.5; buffer B -- 20 mM Na_2HPO_4:KH_2PO_4, 4:1 v:v, plus 0.1 mM EDTA, 0.1 mM PMSF, 0.4 M NaCl, pH 7.5; buffer C -- same as buffer B except 0.1 M NaCl; buffer D -- 50 mM Na_2HPO_4:KH_2PO_4, 5:1, v:v, plus 100 mg BSA/liter; buffer wash -- same as buffer B except no EDTA added and plus 100 mg BSA/liter.

Tissue Preparation: Animals were killed by decapitation and the body chilled on ice during muscle dissection. The muscles dissected out were the unloaded tonic soleus, phasic gastrocnemius and tibialis and the loaded phasic triceps brachii. The thymus and adrenals were dissected out at this time and weighed. Muscles were weighed and quick frozen with acetone and dry ice and held at -70 ° C until analyzed. A 1:20 homogenate of the muscle, tissue wt:vol, in buffer A with two, 15 sec bursts at power level 6 on a Kinematica polytron and the homogenate filtered through two layers of gauze. The homogenate was then centrifuged at 35k x g

for 30 min at 4° C in a Beckman J2-21 centrifuge. The supernatent was discarded and the pellet resuspended 1:10, tissue wt:vol, in buffer B with one, 5 sec burst on the polytron. This homogenate was centrifuged at 10k x g for 10 min at 4° C, the supernatent saved and centrifuged at 35K x g for 30 min at 4° C, and the pellet resuspended with a 5 sec burst on the polytron in buffer C to give 1 gm original tissue/ml. Solutions and homogenates were kept on ice throughout. At the 1 and 3 wk time periods muscles from 4 animals were combined for each binding experiment for the gastrocnemius, tibialis and triceps brachii, and muscles from 8 animals for the soleus. At the 8 wk time period muscles from 3 animals were combined for the soleus and muscles from 2 animals for each of the other muscles. Protein determination was by the method of Lowery, et al. (1951) using BSA as a protein standard.

Receptor Binding: Receptor affinity (K_d) and number (B_{max}) were determined using the specific nicotinic radioligand, ^{125}I-α-BuTX (17.0 μci/μgm), in either inhibition or saturation assays. For the inhibition assays, 100 μl of homogenate were preincubated for 30 min at room temperature with 25 μl 0.1 μM unlabeled BuTX (nonspecific) or buffer C (total), 25 μl hot spike (0.35 nM ^{125}I-α-BuTX plus increasing concentrations of unlabeled BuTX to make up the desired concentrations) was added, and the samples incubated for 2.5 hrs at 30° C. The reaction was stopped by dilution with 4 ml of the buffer wash and filtration at 15 psi through a GF/B glass fiber filter presoaked in PEI to decrease nonspecific filter binding. The filters were washed with two, 4 ml volumes of the wash and

counted in a Beckman G-5500 gamma counter. Saturation assays were the same except the hot spike consisted of increasing concentrations of labeled BuTX. Binding was linear over the protein concentrations used and at equilibrium under the conditions described. Specific binding was defined as total - nonspecific binding and averaged approximately 60%.

Data Analysis: Raw data was analysed with Elsevier-BIOSOFT, a ligand-binding analysis program that uses a weighted least-squares curve-fitting algorithm with objective measurement of goodness of fit (Munson and Rodbard, 1980) (McPherson, 1985). The Student's paired t-test was used to test differences between control and experimental animals. Analysis of variance (ANOVA) was used to test for differences between time groups and Fisher's LSD test to see which groups differed.

Results

There was no difference between control and experimental animals normalized thymic weights. Normalized adrenal weight was different only at the 8 wk time period where it was increased (Table II.1).

Growth data showed that except at the 4 wk measurement, suspended animals gained less weight than their pair-fed controls (Fig. II.1). Analysis of weight gained per gm of food consumed showed that, except during the third week, suspended animals exhibited a significant decrease in growth efficiency (Table II.2). Muscle weights normalized to body weight were reduced in the soleus and gastrocnemius at all times measured and in the tibialis after 8 wks of suspension. The triceps brachii showed no difference between control and experimental animals in normalized muscle weight (Table II.3). Table II.4 shows mg protein/gm tissue in these purified membrane preparations. The only differences found were increases in the soleus and triceps brachii after 3 weeks suspension.

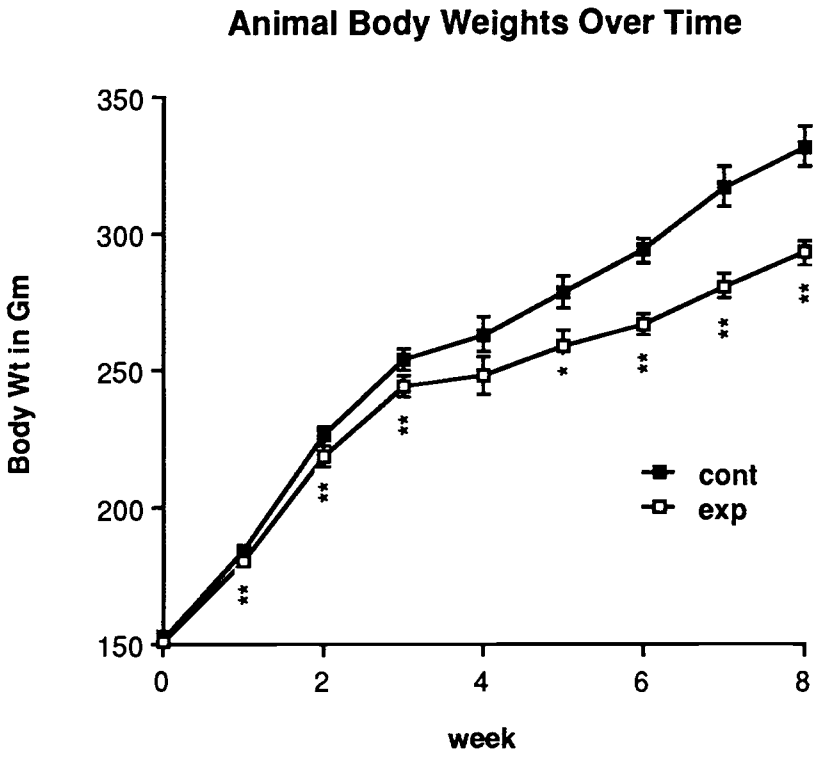
Receptor number (B_{max}) expressed as fmol bound/mg protein was different only in the gastrocnemius 8 wks into the experimental protocol where it was increased in suspended animals (Table II.5). In expressing B_{max} in terms of tissue wet wt (fmol bound/gm tissue), receptor number was increased over controls in the 3 wk soleus and triceps brachii and in the 8 wk gastrocnemius. Whole muscle receptor population (fmol

Table II.1. Thymus and adrenal weights normalized to body weight

		% body wt x 10^{-1} ($\bar{X}$± SE)	
		Thymus	
Wk	N	Cont	Exp
1	15	2.78 ± .09	2.67 ± .09
3	8	1.97 ± .08	2.13 ± .11
8	6	1.16 ± .04	1.17 ± .12
		Adrenal	
1	16	0.19 ± .008	0.19 ± .004
3	7	0.16 ± .004	0.16 ± .007
8	5	0.13 ± .005	0.14 ± .004*

Note: Comparisons done with Student's paired t-test, *$P < .05$.

Figure II.1.



Control and suspended animal body weights in gm $\bar{X} \pm SE$) over time. At t_0 and wk 1, n = 42; at wk 2 and 3, n = 26; and at wks 4 thru 8, n = 6, for each treatment. Comparisons done with a paired Student's t-test; * P <.05, ** P < .01.

Table II.2. Growth efficiency over time expressed as weight gained in gm per gram of food consumed.

week	1	2	3	4	5	6	7	8
cont	.22	.24	.16	.11	.07	.09	.12	.08
exp	.19**	.19**	.14	.08**	.05*	.04*	.07*	.05*
% cont	86.4	79.2	87.5	72.7	71.4	71.4	44.4	58.3

Note: N = 42 for wks 0 and 1, 26 for wks 2 and 3, and 6 for wks 4 through 8, for each treatment. Comparisons done with Student's paired t-test; *P < .05, **P < .01.

Table II.3. Normalized muscle weights expressed as percent of control.

wk	Sol	Gast	Tib	T. brac
1	67.1**	88.0**	102.9	104.4
3	58.0**	77.4**	100.0	99.5
8	69.0**	79.8**	97.5*	103.3

Note: Weights were normalized as % of body wt. Percents are calculated from means of normalized weights. For each treatment, at 1 wk, n = 12 except for the soleus where n = 18; at 3 wks, n = 12 except for the soleus where n = 23; and at 8 wks, n = 6 for all muscles. Comparisons done with a Student's paired t-test on the normalized muscle weights; *P < .05, **P < .001.

Table II.4. Milligrams of protein in a purified membrane preparation per gram wet weight of tissue, expressed as percent of control.

wk	Sol	Gast	Tib	T. brac
1	150.9	103.9	100.6	113.4
3	212.0**	96.8	94.1	125.4*
8	122.0	93.5	107.2	85.0

Note: Tissues from 4 (1 and 3 wks) or 2 (8 wks) animals were combined for each control and experimental soleus preparation. N = 3 preparations for all cases calculated from means of preparation values. Comparisons done with a Student's paired t-test; *P < .05, **P < .01.

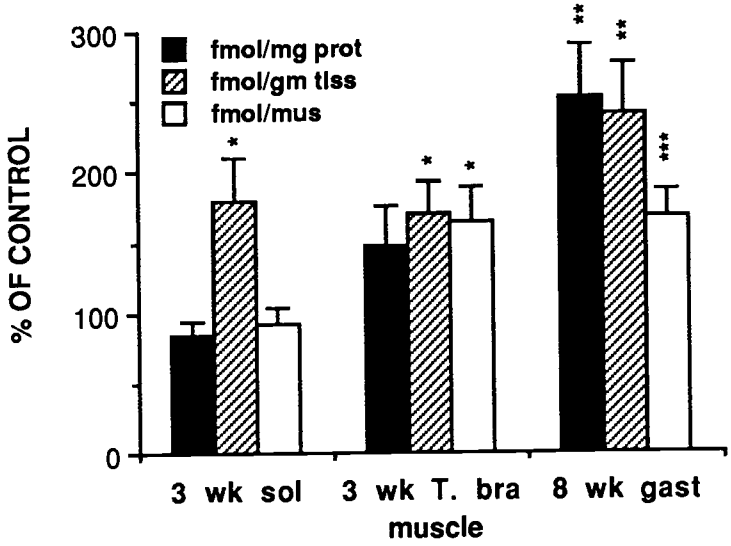
Table II.5. Receptor population (B_{max}) after 1, 3 or 8 weeks of 1, 3 or 8 weeks of suspension.

		fmol/mg protein (X ± SE)			
wk		Sol	Gast	Tib	T. brac
1	c	16.5 ± 2.1	12.8 ± 0.4	21.8 ± 2.3	36.5 ± 5.8
	e	18.0 ± 0.1	19.7 ± 4.1	20.7 ± 2.5	37.4 ± 4.0
3	c	20.6 ± 2.7	15.1 ± 2.7	8.1 ± 1.5	16.0 ± 5.1
	e	16.8 ± 1.8	18.9 ± 4.8	10.3 ± 3.1	20.4 ± 3.3
8	c	10.7 ± 0.1	2.7 ± 0.5	5.9 ± 0.6	7.8 ± 1.2
	e	9.8 ± 1.1	6.5 ± 0.6**	8.1 ± 0.3	8.5 ± 1.5

Note: Binding done as described in methods, n as described in table II. 4 note. Comparisons done with Student's paired t-test; *P < .05, **P < .01. c = control, e = experimental.

muscle receptor population (fmol bound/muscle) was increased in the 3 wk triceps brachii and 8 wk gastrocnemius (Fig. II.2). No other differences between control and experimental animals' B_{max}'s were found regardless of the way receptor number was expressed. In the soleus, analysis of fmol bound/mg protein between time groups in both control and experimental animals showed 1 wk values equal to 3 wk values which were greater than 8 wk values. Tissue wet weight values (fmol/mg tissue) increased between 1 and 3 wk and decreased between 3 and 8 wk in both control and experimental muscles. Whole muscle values (fmol/muscle) increased between 1 and 3 wks in control and experimental muscles, but decreased between 3 and 8 wk only in experimental animals. Control animals' gastrocnemius receptor number was not significantly different between 1 and 3 wk and increased between 3 and 8 wks whether expressed as fmol/mg protein or fmol/gm tissue. Fmol bound/muscle increased between 1 and 3 wk and then decreased between 3 and 8 wk. Experimental animals' B_{max} expressed as fmol/mg protein increased only between 1 and 8 wk and was unchanged when expressed as fmol/gm tissue or fmol/muscle. In the tibialis, control and experimental animals' receptor numbers followed the same pattern. Fmol bound/mg protein or /gm tissue were increased only between 1 and 3 wk and unchanged in the whole muscle. In the triceps brachii receptor number/mg protein increased in both control and experimental animals between 1 and 3 wk and again between 3 and 8 wk. Fmol bound/gm tissue

Figure II.2.



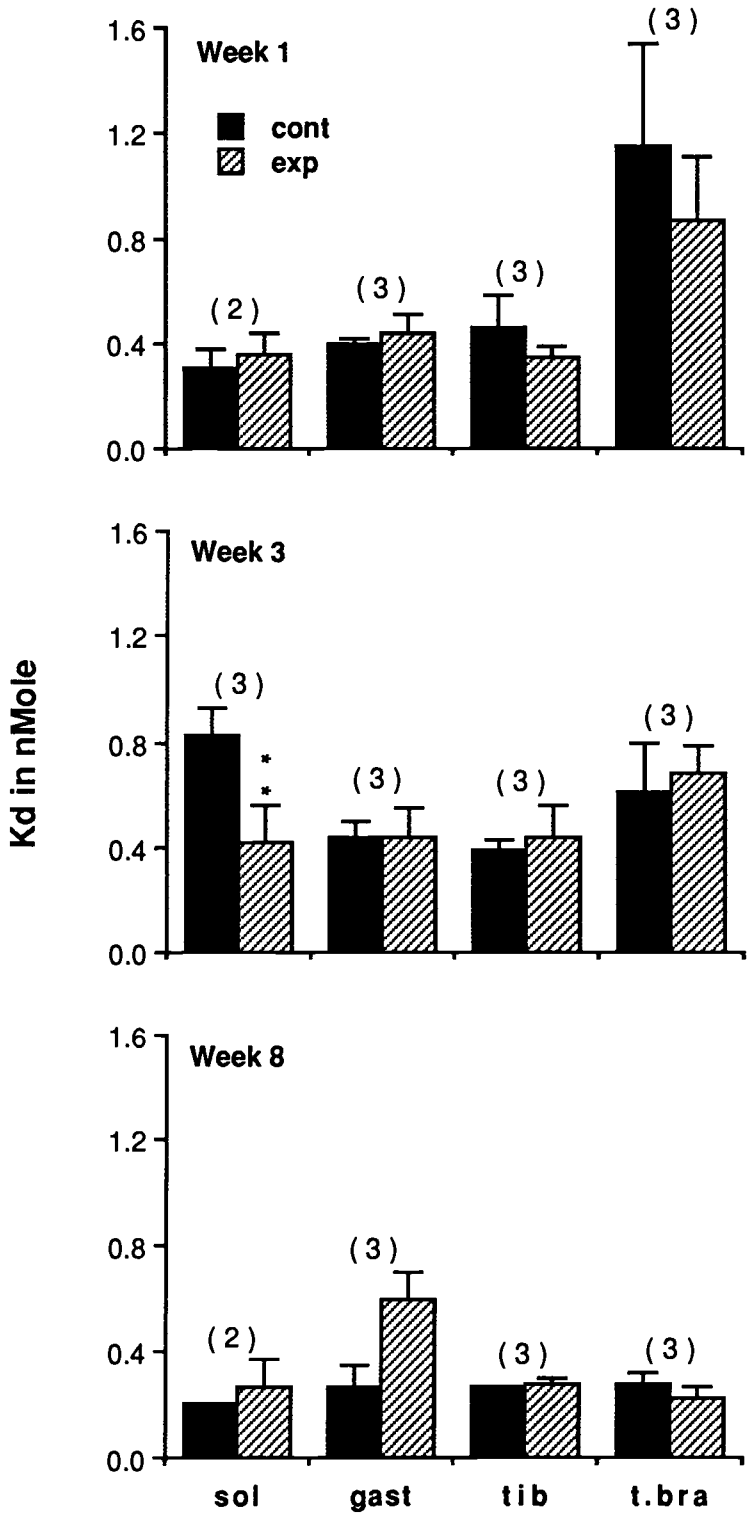
Three wk soleus and triceps brachii and 8 wk gastrocnemius B_{max} values expressed as fmol bound/mg protein, /gm tissue and /whole muscle. In the 3 wk soleus there appears to be a concentrating effect (i.e. an increase in fmol/gm tissue) but no real increase (fmol/muscle). In both the 3 wk triceps Brachii and the 8 wk gastrocnemius there is a true increase in receptor number (fmol/mus) in suspended animals. N values are as described in table II.4 note. Values are presented as the mean of percent of control. Error bars are SE. Comparisons done with a Student's paired t-test; * $P < .05$, ** $P < .01$, or *** $P < .001$.

3 wk and again between 3 and 8 wk. Fmol bound/gm tissue followed the same pattern as fmol/mg protein in control muscles but was increased only between 1 and 8 wk in experimental animals and in control and experimental whole muscle.

Receptor binding affinity (K_d) was different between control and experimental animals only in the soleus at the 3 wk time period (Fig. II.3). In analysis between time groups, 3 wk control soleus K_d was found to be greater than that of 1 or 8 wk control animals. Both control and experimental animals' triceps brachii K_d's were decreased between 1 and 8 wks. No other differences in K_d's between time groups were found.

Figure II.3. Receptor binding affinity (K_d) in nM over time for each of the four muscles studied. Error bars are ± SE. (N) values are as described in table II.4 note. Comparisons between control and experimental done with a Student's paired t-test, ** $P < .01$.

Fig II.3.


Week 1
cont
exp
Week 3
Week 8
Kd in nMole
1.6
1.2
0.8
0.4
0.0
(2)
(3)
(3)
(3)
(3)
(3)
(3)
(3)
(2)
(3)
(3)
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sol
gast
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Discussion

In our study of suspension-induced muscle atrophy, we found no change in the soleus in receptor number (B_{max}) when binding was expressed as fmol bound per mg protein in our purified membrane preparation. To take into consideration any changes in the ratio of muscle membrane to muscle fiber mass, we examined binding in whole tissue as a possibly better indication of tissue receptor levels. B_{max}, expressed as fmol bound per gm wet weight tissue, was increased two-fold in the soleus after three weeks of suspension. A similar increase in binding/gm of tissue was reported by Gupta, et al. (1985). However, in this study, when the decrease in muscle mass was considered and binding was expressed in terms of the whole muscle, the apparent increase disappeared. This suggests a concentrating effect on receptor population in the soleus, but no real increase in total number. Our data on protein content in the purified membrane preparation support the idea of increased receptor containing membrane proteins relative to whole muscle mass in the soleus. We found a two-fold increase in tissue protein at the three week time period which matched the apparent two-fold increase in receptor number. These data are supported by studies on suspended (Jaspers, et al., 1985) (Jaspers and Tischler, 1984) and flight animals (Musacchia, et al., 1987) (Steffen and Musacchia, 1985) which showed losses in total protein content in the soleus, with myofibrillar proteins affected preferentially over sarcoplasmic proteins.

The B_{max} in the gastrocnemius was increased after eight weeks of suspension whether expressed as fmol bound per mg protein, per gm wet weight tissue or per whole muscle. Thus, in this muscle, there is upregulation in receptor population. Denervation has long been known to result in upregulation. In addition, functional disuse caused by treatment with tetrodotoxin (Labovitz and Robbins, 1983) or blockade of ACh release by β-BuTX (Hoffman and Thesleff, 1972) leads to the formation of extrajunctional receptors. The chronic decrease in activity of these muscles could have been sufficient to initiate spread of extrajunctional receptors. The increased ratio of protein to wet weight that was found in the soleus was not evident, so if there is a loss in total protein, loss of membrane and myofibrillar or cytoplasmic proteins must be similar. Henricksen, et al. (1985) reported losses of total protein in the gastrocnemius from rats flown for 7 days on Spacelab-3 that paralleled weight loss (about 13%), but the decrease was not significant and no data was given on forms of the proteins in the gastrocnemius. Jaspers and Tischler (1984) reported that after six days suspension, of the four muscles in this study, only the gastrocnemius had a decreased wet:dry tissue weight ratio indicating a loss of fluids. They attributed the loss in mass to a loss of fluid, but saw no significant increase in protein concentration as might be expected if total protein content were maintained.

Analysis of tibialis receptor binding showed no difference between control and experimental animals in receptor population

at any time. In the triceps brachii, B_{max} in fmol per mg protein was increased at the three week time, but not significantly so. However, in experimental animals tissue levels (fmol/gm tissue) were significantly increased. The increase was maintained in the whole muscle (fmol/muscle), indicating a true increase in receptor number. It is unlikely that the increase in B_{max} seen in the forelimb weight bearing triceps brachii is due to the same factors as the increase seen in the unloaded gastrocnemius. Synaptic plasticity occurs under normal conditions. Terminal sprouting and endplate regeneration maintain neuromuscular junctions throughout life (Barker and Ip, 1965). Exercise leads to terminal sprouting in fish (Palacios-Pru and Colasanta, 1988) and rats (Stebbins, et al., 1985), and endplate size and complexity increase with increasing fiber size (Wigston, 1989) (Jans, et al., 1986) or activity (Lnenicka, et al., 1986) (Fields, et al., 1987). The potential for expanded endplates exists in the triceps brachii of suspended animals as they must assume the entire burden of locomotion in these animals. The increased protein concentration in the triceps brachii, where there was no decrease in muscle mass, is evidence of hypertrophy or growth in this muscle.

B_{max} patterns of change over time between muscles were individual and probably reflect differences in structure, function and/or maturational rates. There was very little difference in patterns over time between control and experimental animals' muscles whether binding was expressed as fmol per mg protein, per gm tissue or per whole muscle. The magnitude of change

tended to be less in experimental animals' muscles, but the direction of change was generally the same. This shows that growth and maturational changes in receptor population are maintained, if somewhat reduced. A study by Labovitz and Robbins (1983) on junctional ACh receptors also showed a conservation of maturational changes despite denervation or disuse. The only difference from controls found in between time analysis was in the experimental gastrocnemius, where B_{max} in the whole tissue or muscle did not decrease enough to be significantly different between three and eight weeks. This can be attributed to a combination of decreased muscle mass and increased receptor number.

We found the affinity (K_d) of the nicotinic acetylcholine receptor at the neuromuscular junction to be different from control animals only in the soleus. After three weeks of suspension, the K_d was decreased approximately 51%, indicating an increase in receptor affinity. An increase in acetylcholine receptor affinity at the neuromuscular junction has been reported after denervation. Almon, et al. (1974) found K_d to be decreased nearly ten-fold after denervation in both the slow twitch soleus and the fast twitch extensor digitorum longus. They attributed the decrease to the difference in membrane environment between junctional and extrajunctional receptors, as treatment of membrane preparations with the detergent Triton X-100 increased the affinity of receptors from innervated muscles to that of the denervated muscles. The difference in magnitude of change between muscles from our

suspended animals and the denervated muscles in Almon's study might be due to the difference in magnitude of disuse between suspension and denervation. That we do not see an increase in receptor number in our suspended animals' soleus muscles argues against the presence of extrajunctional receptors. Jones and Vrbova (1970) in their work with very young rat muscles (i.e., before endplate formation) gave evidence that muscle activity contributes to the inhibition, normally attributed to nerve impulse, of the spread of extrajunctional receptors in denervation. The residual muscle and nerve activity in the suspended animals' soleus muscles could have been sufficient to prevent an increase in receptor number. Denervation is known not to affect junctional receptor number, but replacement of fast-gating adult with slowly-gating fetal receptors and changes in endplate membrane folding, which might indicate changes in membrane structure, have been reported (Brenner and Rudin, 1989). The conversion from slowly-gating fetal to fast-gating adult ACh receptors is both nerve-evoked muscle activity and nerve-released trophic factor dependent (Brenner, et al., 1987). Alternatively suspension may be having a maturational effect. In the soleus of control animals, K_d increases between one and three weeks into the experimental protocol and decreases between three and eight weeks. In suspended animals there is no change in K_d during the course of the experiment. Remodeling of endplates with increasing fiber size (Wigston, 1989) and increasing speed of contraction between five and sixteen weeks of age in rat soleus muscles (Elder and

McComas, 1987) support age-related changes at the neuromuscular junction.

There is evidence for recovery or adaptation in some of the parameters measured in some of the muscles in our animals. Normalized soleus muscle weight percent of control increased between three and eight weeks of suspension (58% vs 69%) and there was no further decrease in the gastrocnemius during the same period. The increases seen in soleus and triceps brachii in mg protein/gm tissue at three weeks are lost at eight weeks. And the difference in soleus K_d at three weeks is nonexistent at eight weeks. However, in the gastrocnemius B_{max} is not increased until eight weeks of suspension, and likewise, the decrease in tibialis muscle mass is not apparent until then. The differences in effect or time course could be due to differences in muscle types, i.e. the unloaded, tonic soleus or weight-bearing, phasic triceps brachii vs the unloaded, phasic gastrocnemius or the posterior, extensor gastrocnemius vs the anterior, flexor tibialis.

Animal growth was decreased in our suspended animals and analysis of feeding data showed a decreased growth efficiency. That is, our suspended animals which ate as much or more than their pair-fed controls (which didn't always eat all they were given), gained less weight. As activity was, if anything, more restricted in the suspended animals, it is unlikely that exercise played a part in the decrease. This would seem to indicate an increased metabolic cost to suspension. Similar results have been reported by others in suspended and flight animals (Morey, 1979)

(Musacchia, et al., 1980). Trends in muscle mass loss also follow those reported by others for suspension and flight animals. That is, the soleus affected most, followed by the gastrocnemius with the tibialis and triceps brachii being affected least of all.

We found no evidence of thymic involution in our animals, although decreases in the thymus have been reported for suspended (Steffen and Musacchia, 1984) and flight (Gazenko, et al., 1980) animals. Both the thymus gland (Munck and Young, 1975) and skeletal muscle mass (Ramey, 1977) are decreased by elevated levels of circulating glucocorticoids from the adrenal glands. Adrenal hypertrophy has also been reported in both suspended (Musacchia, et al., 1983) and flight (Gazenko, et al., 1980) animals. Increased levels of plasma cortisol inflight and urine catecholamines and cortisol postflight have been reported in man (Leach and Rambaut, 1977). Also, increased levels of plasma and skeletal muscle glucocorticoid receptors have been reported (Steffen and Musacchia, 1987). However, Tischler, et al., (1985) combining adrenalectomy with suspension, report that glucocorticoids alone are insufficient to cause the decreases in muscle mass seen. We found slight adrenal hypertrophy in our experimental animals only after eight weeks of suspension. In another long term suspension study, Thomason, et al., (1987) found adrenal hypertrophy to be transient and dependent upon animal manipulation. Adrenal medullary homones have been used as markers for stress. Increased levels of epinephrine are associated with anxiety and increased levels of norepinephrine

with physical stress (Leach and Rambaut, 1977). The lack of thymic involution and adrenal hypertrophy (at least at the earlier times) in our study may be due to less stressed animals because of suspension protocol, cage dimensions (our cages were 61 cm^2) or handling.

In summary, we found growth and muscle mass losses similar to those reported for flight animals. Our animals did not exhibit the thymic involution or degree of adrenal hypertrophy reported by others. The difference is probably due to the protocols followed. Space flight would by necessity demand smaller cages and include the influences of takeoff and reentry. However, in a study to characterize only the effects of weightlessness at the neuromuscular junction, any decrease in external factors can only be an advantage. We found the nicotinic acetylcholine receptor number to be concentrated, but not increased, in the soleus after three weeks of suspension and an increase in receptor number in the gastrocnemius and triceps brachii at eight and three weeks respectively. We attribute the increase in the gastrocnemius to a probable upregulation due to decreased activity (similar to denervation-like changes) and the increase in the triceps brachii to growth effects as might be seen with fiber hypertrophy. We found receptor affinity to be different from controls only in the three week suspended soleus. The cause is unclear, but may be related to changes in endplate morphology or a possible change in growth or development patterns. In general we found suspension to have little effect on patterns of

change over time in receptor number or affinity. With the possible exception of the tibialis, evidence of recovery or readaptation is seen by eight weeks. Differences in individual muscle type responses in muscle mass, protein loss, receptor number or affinity or recovery indicate different sensitivities to the effects of this model for weightlessness. The differences could be due to differences in fiber type (slow vs fast), function, maturational state or normal neuronal input. That we see changes in these parameters support the hypothesis of a correlation between changes at the neuromuscular junction and space-induced muscle atrophy. That receptor number does not increase until after eight weeks of suspension shows that a chronic decrease in muscle activity can lead to changes similar to those seen in neuropathologic muscle and is a serious consideration for long-term space flights or habitation.

Chapter III. Rat Skeletal Muscle Acetylcholinesterase and Choline Acetyltransferase in a Model for Weightlessness

Introduction

Under the condition of weightlessness there occurs a loss of muscle mass and function, particularly of the antigravity muscles such as the soleus. These changes have been attributed to the fluid shifts from the periphery to the head and torso that occur and to the mechanical unloading of the muscles. However, a continued loss of mass is seen after the fluid shifts have occurred and despite vigorous exercise regimens (Moore and Thornton, 1987) (Thornton, et al., 1987). Analysis of Soviet and American astronaut urinary excretion show a loss of components indicative of muscle catabolism (Leach and Rambaut, 1979) (Rambaut, et al., 1979). Evidence of neurological impairment in electromyographs, reflex actions and contralateral movements have also been found (Kozlovskaya, et al., 1981) (Kenyon and Young, 1986). There are many reports of the interaction between nerve and muscle at the neuromuscular junction in maintaining muscle mass and function.

One such area of possible regulation is in the synthesis the neurotransmitter, ACh, by choline acetyltransferase (ChAT) and/or degradation by acetylcholinesterase (AChE). Both ChAT and AChE activity are decreased by denervation (Tucek, 1973) (Dettbarn, et al., 1987), or tetrodotoxin (TTX) nerve conduction blockade (Tucek, 1973) (Brockman, et al., 1984) and increased by nerve growth

factors (Takei, et al., 1988) (Lucas and Kreutzberg, 1985). Muscle activity has been shown to be necessary for accumulation of AChE at endplates (Lomo and Slater, 1980) and to increase AChE specific activity in dysgenic mice (i.e. mutant mice with action potential propagation but no muscle contraction) (Powell, et al., 1986). Reinnervating a slow muscle with a fast nerve results in accumulation of the forms of AChE (e.g. asymmetrical or globular) common to slow muscles showing muscles exert an influence here as well (Sketelj, et al., 1988). Excess AChE caused by AChE inhibitors has also been shown to lead to both presynaptic neuronal degradation (Hudson, et al., 1986) and to muscle necrosis (Kasprzak and Salpeter, 1985).

In our study we investigated ChAT and AChE activity in rat skeletal muscles using the Morey-Holton rat-tail suspension model to simulate weightlessness. This model reproduces the fluid shifts and muscle unloading (at least in the hindlimbs) common to weightlessness and to successfully mimic the muscle mass loss seen in flight animals (Morey, 1979) (Morey-Holton and Wronski, 1981).

Materials and Methods

Materials: Male Sprague-Dawley rats (mean weight 155.7 ± 2.1 gm) were from Simonsen Labs in Gilroy, CA. Acetylthiocholine iodide (ASCh), S-S'-dithiobis-2-nitrobenzene (DTNB), potassium phosphate monobasic (KH_2PO_4), cholinesterase, acetyl (AChE), choline oxidase, sodium chloride (NaCl), eserine, Triton X-100, bovine serum albumin, choline chloride (Ch), acetyl choline chloride (ACh), acetyl Co A, choline bromide, absolute ethanol, perchloric acid ($HClO_4$), maleic acid, gelatin, and Trizma base (tris(hydroxymethyl)aminomethane) were from Sigma Chemical, St. Louis, MO. Sodium phosphate dibasic (Na_2HPO_4), magnesium chloride ($MgCl_2$), glacial acetic acid, acetone, and ethyl acetate were from J.T. Baker Chemicals, Phillipsberg, NJ. Bromoacetylbromide was from Aldrich Chemical Co., Milwaukee, WI.

Suspension: Experimental and control animals were weight-matched at the beginning of each experimental period and pair-fed throughout. Experimental animals were suspended for 1, 3 or 8 weeks by the tail at a 30° head down tilt as previously described (Royland, et al., 1989) (Morey-Holton and Wronski, 1981). Controls were housed singly, unrestrained in identical cages.

Assay for acetylcholinesterase: Muscles analyzed were the hindlimb tonic, extensor soleus, the phasic extensor gastrocnemius and the phasic, flexor tibialis anterior and the forelimb phasic, extensor triceps brachii. Animals were killed by decapitation and muscles were dissected out into ice cold 10 mM phosphate

buffered saline within 15 minutes. Muscles were then quick frozen whole in dry ice and acetone and held at - 70° C until analyzed. Twenty mg tissue/ml phosphate buffer (0.1 M, pH 8.0) were homogenized with two 5 sec bursts on a Kinematic polytron at power level 6 and filtered through two layers of gauze to remove foam and large particulates. Samples were kept on ice until 15 minutes prior to reading when they were allowed to come to room temperature. Homogenate protein concentrations were determined by the method of Lowery, et al., (1951) using bovine serum albumin as the standard.

AChE was analyzed after the method of Ellman, et. al. (1961). In this photometric method the enzyme activity is measured by following the development of the yellow anion, 5-thio-2-nitro-benzoic acid, produced when the substrate, acetylthiocholine, reacts with dithiobisnitrobenzoate ion. Reaction rates were recorded at 412 mμ with a Gilford 2400 spectrophotometer and recorder. Two point six mls phosphate buffer (0.1 M, pH 8.0), 0.4 mls homogenate, 20.0 μls acetylthiocholine iodide substrate (0.075 M, made up in distilled water daily) and 100.0 μls dithiobisnitrobenzoic acid (0.01 M, made up in pH 7.0 buffer + 15 mg sodium bicarbonate/10 ml) were placed into a 1 cm quartz cuvette; mixed thoroughly by inverting several times; and placing in the spectrophotometer. During the first minute, recordings were erratic (bubbles rising, mixing effects, etc.) but were linear for the 6-7 minutes following. Values were calculated for the time interval from 2-5 minutes after placing the cuvette in the

spectrophotometer. Because low level absorbance was seen even without substrate, blank curves were run for each homogenate and subtracted from the sample absorbance.

Assay for choline acetyltransferase: ChAT activity in extracted homogenate was determined by measuring the production of ACh. ACh production was measured by high-performance liquid chromatography (HPLC) with a post-column enzyme reactor and electrochemical detection (Kaneda and Nagatsu, 1985). The contribution to ACh production by carnitine acetyltransferase was determined by calculating the difference between total production and the production in the presence of bromoacetylcholine (BrACh), a specific inhibitor of ChAT (Harris, 1987).

The HPLC system consisted of a Bioanalytical Systems (West Lafayette, IN) PM-30 dual-piston pump, 4B electrochemical detector and dual platinum electrode with a Rheodyne (Cotati, CA) 7125 injector with a 20 µl injection loop. Prior to the 10 cm small bore (2 mm ID) 5 micron C18 Adsorbosphere reverse phase analytical column were a silica presaturation column and a C18 pellicular-filled guard column (Alltech Assoc., Los Altos, CA). A Brownlee AX-300 anion-exchange column (Rainin Intsrument Co., Emeryville, CA) served as post-column enzyme reactor. Analytical and enzyme column temperature were maintained at 37° C with a water jacket and circulating water bath.

Data from the electrochemical detector fed into a SP4270 Spectra-Physics (San Jose, CA) integrator with computer link. Peak

height analysis was carried out with the Spectra-Physics' "WINner" software. Detection limits were 0.5 pmol ACh. Ch and ACh standards were run every 4-6 samples and corrections for changes in the detection system over time could be calculated.

The mobile phase was 25 mM tris-maleic buffer (pH 7.6) with 1 mM (tetramethyl)ammonium chloride, 200 µM sodium octyl sulfate and 100 mg/liter sodium azide added. The mobile phase solution was filtered and degassed daily. A flow rate of 0.3 ml/min gave retention times of approximately 2.3 and 6 min for Ch and ACh respectively.

Bromoacetylcholine perchlorate was synthesized after the method of Chiou and Sastry (1968). Choline bromide (10 mmole) was placed in a cooled flask, bromoacetylbromide (12 mmole) added and the mix stirred on ice for 30 minutes. Ice cold absolute ethanol (1.5 mls) was added and with continued stirring, a thick whitish precipitate formed. Seventy percent perchloric acid (1.125 mls) dissolved the precipitate temporarily but the solution reprecipitated immediately. This precipitate was filtered, cleaned and recrystalized as follows. The precipitate was dissolved in a minimum amount of hot acetone, just enough ethyl acetate was added to reinitiate precipitation, then hot acetone added dropwise to redissolve that precipitate. Cooling the solution to room temperature precipitated BrACh crystals. Analysis of the synthesized crystals gave a melting point of 103° C. This compares favorably with the published melting point of 103.5-104.5° C for BrACh, indicating a relatively pure compound.

As with the AChE assay, the soleus, gastrocnemius, tibialis anterior and triceps brachii muscles were assayed for ChAT activity. Tissues, 150 mg/ml, were homogenized with ground glass homogenizers in an extraction solution consisting of 200 mM NaCl, 10 mM $MgCl_2$, 0.2 mM eserine and 0.5% Triton X-100 in 40 mM sodium phosphate buffer, pH 7.4 (Harris, 1987). Protein analysis (Lowery, 1951) was done on the rough homogenate. This homogenate was centrifuged at 20,000 x g for 60 min at 4° C and the supernatent used for the enzyme solution. Eighty-five µl of enzyme solution were preincubated for 15 min at room temperature with either 15 µl 2 µM BrACh (inhibited) or 15 µl 40 mM sodium phosphate buffer (total). One hundred µl of substrate solution consisting of 560 mM NaCl, 12 mM $MgCl_2$, 0.4mM eserine, 0.6% Triton X-100, 1 mg/ml albumen, 1 mM acetyl CoA and 10 mM choline chloride in 24 mM sodium phosphate buffer, pH 7.6 was added and the mixture incubated for 45 min at 37° C. The reaction was stopped with 50 µl 1 M perchloric acid and 10 min in an ice bath. The mixtures were centrifuged at 15,000 RPM in a Beckman microcentrifuge for 10 min and the supernatent saved for HPLC determination of ACh. Total µmole ACh synthesized per hour per gm protein, per gm tissue or per muscle minus ACh synthesized in the presence of BrACh (i.e. ACh synthesized by carnitine acetyltransferase) gave the measure of ChAT activity.

Statistics: Differences between control and experimental values were analyzed using the Student's t-test. Analysis of

variance was used to determine differences between time groups and Fisher's LSD test used to test which groups differed.

Results

Experimental animals' soleus and gastrocnemius muscle weights (normalized as % of body wt) were decreased over those of control animals a maximum of 32% and 22% respectively. Tibialis muscle weights were generally unaffected showing only a slight decrease (about 5%) after 8 wks of suspension. The triceps brachii muscles were hypertrophied after 1 wk suspension, but had normalized by 3 wks. There was no change in protein concentration (mg/gm tissue) in any muscle at any time. Total protein content (mg/muscle) was decreased at all times examined in the soleus, after 3 or 8 wks suspension in the gastrocnemius, and was increased in the 1 wk triceps brachii. No changes in total protein were found in the tibialis (Table III.1).

In the soleus, after 1 wk of suspension, AChE specific activity (Fig. III. 1), measured as μmoles ACh hydrolyzed/min/gm protein, was increased approximately 33% over controls. This increase disappeared when activity was determined for the whole muscle (μmole/min/muscle). No differences were found in the soleus after 3 wks of suspension, but AChE activity was decreased 47% in the whole muscle (μmol/min/mus) after 8 wks. Gastrocnemius AChE activity in suspended animals was different from controls only after 3 wks where a 33% decrease in total muscle levels was found. In the tibialis, AChE specific activity was decreased 26% after 3 wks suspension, and total muscle activity was decreased 33% and 26% after both 3 and 8 wks respectively. The triceps brachii had a 24% decrease in total activity after 3 wks and a

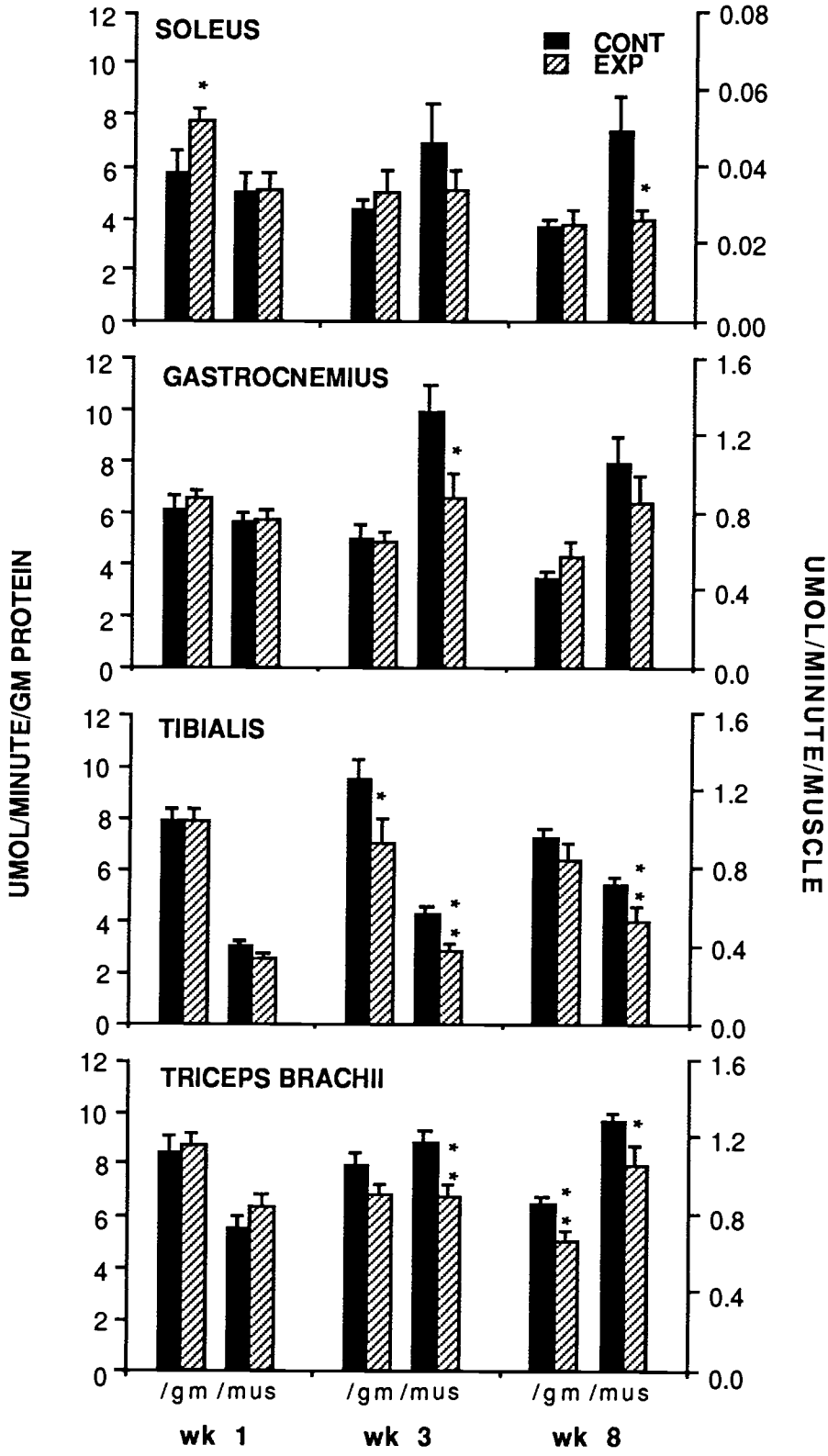
Table III.1 Muscle weights and protein content per gram of wet weight of tissue and per whole muscle in suspended rats expressed as percent of control.

week	SOL	GAST	TIB	T. BRA
		MUSCLE WEIGHTS		
1	78.1***	91.0**	100.8	111.8**
3	72.0***	80.9***	101.3	97.2
8	68.1***	78.3***	94.8**	102.4
		MG PROTEIN/GM TISSUE		
1	98.4	102.3	91.1	107.0
3	100.8	103.8	99.8	101.4
8	91.4	97.3	95.8	111.0
		MG PROTEIN/MUSCLE		
1	77.8**	93.8	91.0	118.7*
3	72.2**	85.3*	100.0	100.8
8	63.8***	76.6**	87.3	113.6

Note: Percents calculated from means of normalized data (i.e. % body wt) for muscle weights. N = the # of pairs. For the sol, n = 12, 20 & 9 for 1, 3 & 8 wks respectively. For the others, n = 16, 23 & 12 for 1, 3 & 8 wks respectively, except for 1 wk gast where n = 15. Comparisons done with the Student's t-test; *P < .05, **P < .01, ***P < .001.

Figure III.1. Acetylcholinesterase activity over time in control and suspended animals expressed as specific (μmol/min/gm protein) or total activity(μmol/min/muscle). N as described in table III.1 Within time analysis was by Student's t-test (* $P < .05$, ** $P < .01$). Differences between time groups were determined by analysis of variance (ANOVA). If ANOVA analysis showed a difference, the Fisher's LSD test was used to determine which groups differed (see results).

Figure III.1.


SOLEUS
CONT
EXP
GASTROCNEMIUS
TIBIALIS
TRICEPS BRACHII
UMOL/MINUTE/GM PROTEIN
UMOL/MINUTE/MUSCLE
/gm /mus
/gm /mus
/gm /mus
wk 1
wk 3
wk 8

decrease in both specific (22%) and total (18%) AChE activity after 8 wks of suspension.

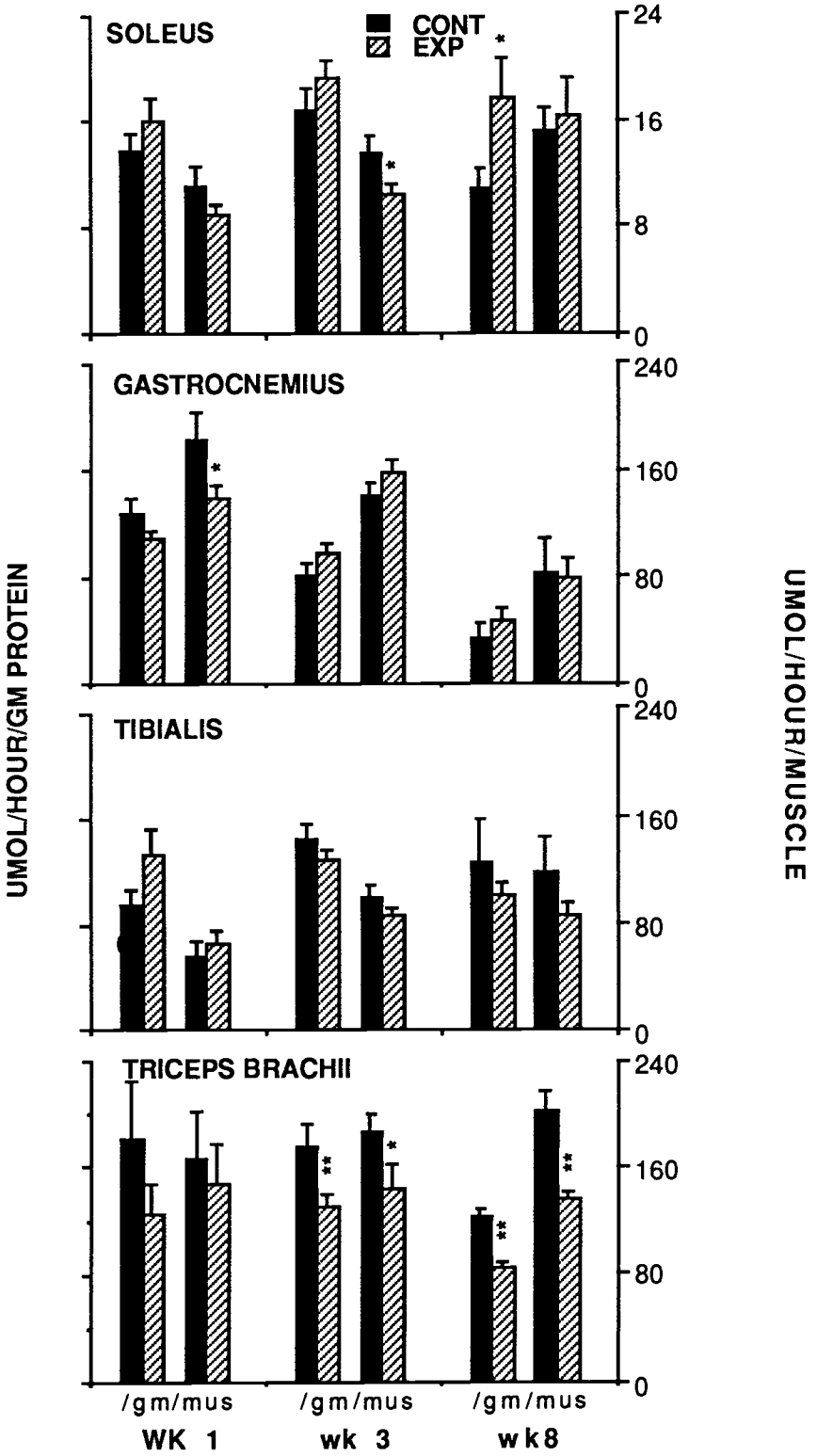
In control animals, AChE activity per gm protein was unchanged in all four muscles between 1 and 3 wk into the experimental protocol. Specific activity was decreased in the gastrocnemius ($P < .05$) and tibialis ($P < .01$) between 3 and 8 wk. There was no significant decrease between 3 and 8 wk in the soleus and triceps brachii, although there was a decrease from 1 to 8 wk ($P < .05$). AChE total activity was unchanged over time in the soleus. Total activity increased from 1 to 3 wk in the gastrocnemius ($P < .01$), tibialis ($P < .01$) and triceps brachii ($P < .001$) and continued to increase from 3 to 8 wk in the tibialis ($P < .01$) while remaining the same in the gastrocnemius and triceps brachii.

The patterns in AChE activity in experimental animals' muscles were generally different. The specific activity was decreased in the soleus ($P < .05$), gastrocnemius ($P < .05$) and triceps brachii ($P < .01$) between 1 and 3 wk and in the triceps brachii ($P < .01$) between 3 and 8 wk. There was no difference in experimental tibialis specific activity over time. Total activity was not different in any muscle, except an increase between 1 and 8 wk in the tibialis ($P < .05$).

ChAT activity in the soleus was unchanged from controls after one wk of suspension (Fig. III.2). After 3 wks, a 22% decrease only became apparent when total muscle activity was considered. In the 8 wk suspended animals, soleus ChAT specific

Figure III.2. Choline acetyltransferase activity over time in control and suspended animals expressed as specific (μmol/min/gm protein) or total activity (μmol/min/muscle). N as described in table III.1. Within time analysis was by Students t-test (* $P < .05$, ** $P < .01$). Differences between time groups were determined by analysis of variance (ANOVA). If ANOVA analysis showed a difference, the Fisher's LSD test was used to determine which groups differed (see results).

Figure III.2.


SOLEUS
CONT
EXP
GASTROCNEMIUS
TIBIALIS
TRICEPS BRACHII
UMOL/HOUR/GM PROTEIN
UMOL/HOUR/MUSCLE
0
8
16
24
80
160
240
/gm/mus
/gm/mus
/gm/mus
WK 1
wk 3
wk8

activity was increased 62%, but the increase disappeared when expressed as total muscle activity. The only difference from controls in gastrocnemius ChAT specific activity in suspended animals was a 24% decrease in total activity after 1 wk. Tibialis ChAT activity was unchanged by suspension whether expressed as specific or total activity. We found no difference in ChAT activity in the triceps brachii after 1 wk of suspension, but both specific (24 and 23%) and total (32 and 33%) activity levels were lower than control levels 3 and 8 wks into the experimental protocol. We found no difference in control animals' soleus or triceps brachii ChAT specific or total activity between 1, 3 and 8 wk into the experimental period.

Discussion

Enzyme activity is dependent on both age and muscle type and these factors affect AChE and ChAT differently. Also the direction and magnitude of changes are dependent whether the activity is expressed as specific or total activity. For example, in the gastrocnemius, comparison between control animal time groups shows that AChE specific activity, decreases significantly only between 3 and 8 weeks into the experimental period, while total activity, increases significantly between 1 and 3 weeks. In contrast ChAT specific activity in the gastrocnemius decrease continuously from 1 to 8 weeks, and was decreased rather than increased when expressed as total activity. This is opposite to the increasing total ChAT levels in developing rat soleus muscle reported by Harris (1987). The difference may be due to the size of the animals used (i.e., < 80 gm in Harris' study vs > 150 gm in our study). The decreases in our study probably reflect dilution due to growth of muscle fibers, while the increases reflect increases in total muscle size. Relative differences over time between muscles could result from maturational differences in muscle growth and/or enzyme synthesis rates. AChE specific and total activity, as well as the forms of the enzyme (e.g. globular or asymmetric), have been shown to vary between fast and slow muscles (Lomo, et al., 1985) (Gisiger and Stephens, 1988).

The patterns in suspended animals' muscles also are often different than those seen in control animals. AChE specific activity in the gastrocnemius was decreased between 1 and 3 weeks in

experimental animals as opposed to between 3 and 8 weeks in controls. This switch could result from a slight increase over controls in AChE specific activity after 1 week of suspension and a decrease in muscle mass in the suspended animals at the later times decreasing any dilution effects. As opposed to control gastrocnemius, triceps brachii and tibialis, an increase in total AChE activity was found only in the tibialis in experimental animals, even though all muscles increased in size over time. ChAT specific and total activity in the soleus of control animals showed no significant change over time, while in suspended animals the tibialis showed no change.

Presumably the same effects of dilution and increased muscle size due to growth, though reduced, would occur in suspended animals as occurs in controls. Possibly changes in muscle activity affecting muscle development had an effect on growth related patterns. Suspension causes a stunting of growth in fast muscles such as the gastrocnemius (Jaspers, et al., 1985). Lomo and Slater (1980) have shown that muscle activity is necessary for the accumulation of AChE at endplate regions during synapse formation. Also, in reinnervation studies the time course of AChE recovery differs between the fast extensor digitalis longus and the slow soleus, suggesting different developmental sensitivities to neural signals, whether trophic or electrical (Dettbarn, et al., 1987). ChAT activity is generally considered to be independent of muscle activity (Haubrich, 1976). ACh levels, however, would affect the mass action equation, can act as an

inhibitor to ChAT, and its synthesis and release can be affected by muscle activity patterns (Browning, 1976) (Whittaker, 1986). Also Brenneman (1986) found ChAT activity to be decreased 32% in spinal cord-dorsal root ganglia in culture after 7 days nerve conduction blockade with tetrodotoxin.

Changes in muscle protein content would also affect apparent enzyme activities. Six days suspension lead to decreased protein synthesis and increased protein degradation in the soleus. Sarcoplasmic protein concentration was increased with no effect noted in myofibrillar proteins (Jaspers, et al., 1985). As protein synthesis was decreased, this suggests myofibrillar proteins to be more sensitive to suspension. However, they found no increase in total protein concentration (sarcoplasmic plus myofibrillar) in the soleus, gastrocnemius, tibialis or triceps brachii. The decreased muscle mass of the soleus combined with no increase in total protein concentration (mg protein/gm tissue) indicates a loss of total muscle protein. Our data support this hypothesis. However, we found hypertrophy of both protein concentration and total protein in the weight-bearing triceps brachii. Of the four muscles, only the gastrocnemius has been shown to have a decreased wet:dry tissue weight ratio indicating a loss of fluids (Jaspers and Tischler, 1984), which could have contributed to the decreased muscle mass when compared to controls. The lack of an increase in protein concentration, combined with a decrease in muscle size and a decrease in total protein, as seen in our 3 and 8 week

gastrocnemius data indicates a loss of total protein in this muscle as well.

Data on 7 day flight animals from Spacelab-3 showed similar trends. Muscle fiber diameter was decreased with no change in total number of fibers, indicating a loss of fluids and cytoplasmic proteins (Musacchia, et al., 1988). Both soleus and gastrocnemius showed losses in total protein (Musacchia, et al., 1987). In the soleus, myofibrillar proteins were found to be most affected (Steffen and Musacchia, 1985). DNA concentration was increased with no increase in total levels, again supporting the idea of loss of cell volume with no loss in cell number. Both RNA concentration and total levels were decreased, indicating decreased synthesis (Steffen and Musacchia, 1985).

Analysis of control vs experimental enzyme levels requires taking into consideration differences in muscle mass to obtain a true picture of functional enzyme activity. The importance of this is seen in evaluating data for the soleus, the muscle most affected by weightlessness and the suspension model. One week into the experimental protocol it appears that AChE activity per gm protein is increased in suspended animals. Similar increases have been reported by others (Dettbarn, et al., 1987) (Gupta, et. al., 1985). However, in this study, when the decrease in muscle mass is considered, the increase disappears. A loss of total muscle protein, most likely correlating with a loss of myofibrillar proteins, made an actual unchanged enzyme level appear elevated. After 3 weeks suspension, AChE specific activity starts to drop and by 8 weeks no

apparent difference from controls in specific activity is actually a significant decrease in total enzyme activity. ChAT activity patterns are different. At 3 weeks we found only total activity to be significantly decreased. At 8 weeks an apparent significant increase in specific activity (as in the 1 week AChE activity) is counterbalanced by the decrease in muscle mass to show no real difference from controls in total activity.

The gastrocnemius follows a pattern similar to that of the soleus, but the changes are more subtle and the time course is earlier. At 3 weeks into the experiment, no difference in AChE specific activity again reflects a significant decrease in whole muscle enzyme activity. By 8 weeks total AChE activity in suspended animals has increased until there is no difference from controls. ChAT total activity is decreased after 1 week of suspension, and is apparently recovered by the third week. Gupta, et al., (1985) showed ChAT total activity in the soleus increased after 2 or 3 weeks of suspension (93 and 108% respectively compared to controls) and the extensor digitorum longus (45% and 67% respectively), but did not correct for carnatine acetyltransferase activity (CarAT). CarAT can contribute more than 50% to ACh synthesis, and apparent ChAt activity, in skeletal muscle (Tucek, 1982) (Harris, 1987). We found CarAT activity to range from 30-60% of total muscle ACh synthesizing activity depending on muscle and time into the experimental protocol (data not shown).

Enzyme levels in tibialis anterior and triceps brachii muscles, the mass of which are either unaffected or increased by weightlessness and/or suspension, follow a similar, though generally more pronounced pattern. Tibialis AChE specific and total activity is decreased after 3 weeks suspension and remains significantly decreased in the whole muscle after 8 weeks. Triceps brachii AChE total activity is significantly decreased at 3 weeks and both specific and total activity are decreased at 8 weeks. The tibialis showed no significant changes from controls in ChAT activity at any time studied. The triceps brachii had decreased ChAT activity after both 3 and 8 weeks of suspension whether expressed total or specific activity. That the weight bearing triceps brachii shows changes similar to the unloaded hindlimb muscles is of interest. The hypertrophy of the suspended animals' triceps brachii, due to their increased function in locomotion, probably results from hypertrophy of existing fibers rather than growth of new fibers. This being true, the innervation would remain unchanged and the decreases seen between control and experimental animals would reflect the same sort of dilution effects as seen in specific activities between control animals of succeeding experimental times. That the total activity remains less might be attributed to a decrease in experimental animals in the ratio between dilution and growth, so that compared to controls, they show a loss of total activity.

Despite variation between animals and suspension groups, overall the pattern is clear. Suspension causes decreases in both

AChE and ChAT total activity in the soleus and gastrocnemius. The effect is much less pronounced than for denervation and the time course is longer, but the trend is the same. It is recognized that differences between suspension and denervation have been reported. For example, denervation in some muscles results in changes in the forms and distribution of AChE found (Fadic and Inestrosa, 1989) (Lomo, et al., 1985), while suspension does not (Dettbarn, et al., 1987). However human skeletal muscle does not have the uneven distribution of the forms of AChE found in adult rats, so the importance of this difference is unclear (Sketelj and Brzin, 1985). Changes in muscle mass and protein content similar to those found in flight animals support, though do not prove, the hypothesis of similar changes occurring inflight.

Chapter IV. Rat Skeletal Muscle Acetylcholine and Choline in a Model for Weightlessness

Introduction

Weightlessness causes the unloading of weight-bearing and anti-gravity muscles and results in loss of muscle mass (Moore and Thornton, 1987) and function (Oganov, et al., 1980). A portion of the mass loss can be attributed to the fluid shifts from the periphery to the head and torso that occur, but urinary excretion of nitrogen, phosphorous and certain amino acids give evidence of muscle catabolism (Rambaut, et al., 1979) (Leach and Rambaut, 1979). It is well accepted that changes in neural input affect muscle function and integrity. Studies on denervation (Cangiano, 1984), cross-reinnervation (Foehring, et al., 1987a), nerve conduction blockade (Bray, et al., 1979) or axonal transport blockade (Hofmann and Thesleff, 1972) show changes in muscle mass, receptors, membrane potential, fiber types and post-synaptic components (e.g., acetylcholinesterase levels). Likewise muscle properties affect the function of motoneurons (Hill and Bennett, 1986) (Smith, et al., 1986) (Foehring, et al., 1987b).

A range of reports on astronauts and cosmonauts suggest a neurological component to this space induced muscle atrophy (Baker, et al., 1977) (Kozlovskaya, et al., 1981a and b) (Kenyon and Young, 1986). Changes in electromyographs, vibrosensitivity thresholds, reflex actions, and contralateral movements show that

changes in motor units, proprioreception, and efferent and afferent signals have occurred.

In our study of the effects of weightlessness at the neuromuscular junction we examined endogenous levels of the neurotransmitter, acetylcholine (ACh) and its precursor, choline (Ch). ACh and Ch have been shown to be sensitive to variations in neuronal activity. ACh synthesis and release increases with increased activity (Browning, 1976) and Ch uptake by synaptic terminals depends on electrochemical gradients driven by depolarization (Tucek, 1985). Nerve activity also affects the availability of ACh for release by affecting its exchange between intraterminal vesicular and cytoplasmic pools (Whittaker, 1986) (Molenaar, et al., 1987). In our study we used the Morey-Holton rat-tail suspension model. This model mimics the changes in bone metabolism, electrolyte balance, renal function and the losses in muscle mass and protein seen in flight animals (Morey, 1979) (Morey-Holton and Wronski, 1982) (Musacchia, et al., 1987).

Materials and Methods

Supplies: Reagent grade ACh chloride, Ch chloride, acetylcholinesterase (AChE) (activity approx. 1200 units/mg solid), choline oxidase (activity approx. 12.8 units/mg solid), 30% hydrogen peroxide (H_2O_2), sodium azide, Trizma base (tris(hydroxymethyl)aminomethane) and maleic acid were purchased from Sigma Chemical, St. Louis, MO. (Tetramethyl)ammonium chloride was purchased from Aldrich Chemical Co., Milwaukee, WI and sodium octyl sulfate was from Bioanalytical Systems, West Lafayette, IN. All other chemicals were reagent grade. Millipore filters, 0.22 μm, were from Millipore, Bedford, MA. Male Sprague-Dawley rats, mean starting weight 140.9 ± 1.1 g, were from Simonsen Labs, Gilroy, CA.

Suspension: Animals were weight-matched at the beginning of the experiment and pair-fed throughout. Suspension was carried out as previously described (Royland, et al., 1989) (Morey-Holton and Wronski, 1981) for 1, 3 or 8 weeks. Experimental animals were suspended by the tail at a 30° head down tilt from an apparatus constructed in this lab that allowed for 360° freedom of movement within a 60 cm diameter circle. Control animals were housed singly, unrestrained in an identical cage. Daily records of food consumption and weekly records of weight were kept.

Solutions: The mobile phase was 25 mM Tris-maleic buffer (pH 7.6) with 1 mM (tetramethyl)ammonium, 200 μM sodium octyl sulfate and 1.54 mM sodium azide added. The mobile phase was filtered through 0.22 μm filters and degassed daily.

The enzymes were made up in distilled water to give concentrations of approximately 680 and 630 U/ml for choline oxidase and AChE respectively. The AChE was diluted 1:1 with distilled water. One hundred µl of the choline oxidase solution was injected directly onto the anion-exchange column (i.e. no analytical column online) followed by 200 µl of the diluted AChE.

Ch and ACh standards were made up in 0.1 mM acetic acid to give 500 and 100 pmol/20 µl respectively. Hydrogen peroxide (500 pmol/20 µl) for enzyme efficiency determinations was made up in distilled water. Standard curves were linear up to 1000 pmols.

The extraction solution consisted of 1:6, vol:vol, 0.1 M acetic acid:acetonitrile.

Tissue preparation: Animals from the 1 and 3 week suspension groups were killed by a 2.5 sec burst at 45% power output to the head from a S6F focal microwave (Cober Electronics, Stamford, Conn.). Immediately after, animals were reversed in the holder and a second burst given to the back half of the animal to microwave the hindlimbs. The larger 8 week animals were handled similarly but except they received two bursts to both the head and hindquarters. Lag time between front and back bursts was 59 ± 4 secs. Analysis of AChE activity by the method of Ellman, et al., (1961) was carried out on various muscles to check for enzyme inactivation. After microwaving, the animal was cooled with ice and the gastrocnemius, soleus, tibialis anterior and

triceps brachii muscles dissected out. Muscles were quick frozen with dry ice and acetone and stored at -70° C. until analyzed.

Tissues were homogenized 1:10, wt:vol, with two, 15 sec bursts on a Polytron tissue homogenizer at power level 6. A 2.5 ml aliquant of homogenate (corresponding to 250 mg tissue) was placed in a centrifuge tube, incubated on ice for 30 min, and spun for 30 min at 20k x g. The supernatant was double extracted with an equal volume of hexane. The organic phase was discarded. Extracted supernatent was evaporated to dryness with air and a heating block set at 37° C. If necessary, dried samples could be stored at -4° C. Evaporated samples were dissolved in 200 µl 0.1 mM acetic acid prior to injection on the HPLC. Homogenate protein concentration was determined by the method of Lowery (1951). Standard protein curves were linear from 10 - 100 mg/20 µl injected.

HPLC system: The system consisted of a PM-30A dual-piston pump, 4B electrochemical detector, and dual platinum electrode from Bioanalytical Systems (West Lafayette, IN) with a Rheodyne 7125 injector with a 20 µl loop (Cotati, CA). A presaturation column filled with 50 micron silica was placed between the pump and the injection port and a C-18 pellicular silica filled guard column preceded the analytical column. The analytical column, a 10 cm smallbore (2 mm ID) 5 micron C18 Adsorbosphere reverse phase analytical column (Alltech Assoc., Los Altos, CA), was followed with a Brownlee AX-300 anion-exchange column (Rainin Instrument Co., Emeryville, CA) acting as the postcolumn

enzymatic reactor. System flow rate was 0.3 ml/min. Column temperatures were maintained at 37° C with a water jacket and circulating water bath.

Data from the electrochemical detector fed into a SP4270 Spectra-Physics (San Jose, CA) integrator with direct computer link. Peak analysis was carried out via Spectra-Physics' "WINner" software. Detection sensitivity was 0.5 pmol of ACh. A standard mix of 500 pmol Ch and 100 pmol ACH was run every 4-6 samples and corrections made for changes in the detection system.

Enzyme efficiency: With the analytical column removed, equimolar amounts of Ch, ACh and H_2O_2 were injected directly onto the enzyme column. The stoichiometry of the breakdown of ACh or Ch by AChE and/or choline oxdidase results in 2 moles of H_2O_2 formed for every mole of reactant. Enzyme conversion efficiencies were calculated from peak areas. Efficiencies were greater than 90% on freshly loaded enzyme columns and were greater than 70% after 4 weeks of use.

Statistics: Data was analyzed with the Student's t-test for comparison of means.

Results

Suspended animals grew more slowly than their weight matched and pair-fed controls. Muscle weight normalized to body weight show the unloaded (i.e. suspended) tonic soleus muscle to be the most adversely affected at all times examined, followed by the unloaded phasic gastrocnemius muscle. In contrast, the unloaded phasic tibialis and the weight-bearing triceps brachii of suspended animals show an increase in muscle mass and were affected only at the 1 week time period. (Table IV.1).

Microwaving the animals as described under "Materials and Methods" resulted in all muscles looking as if cooked medium rare. There was no detectable ACHE activity in the gastrocnemius, soleus, triceps brachii, tibialis or jaw muscles of a microwaved animal (body wt. = 159.6 g). There was no difference in muscles' percent of body weight between microwaved animals and comparable nonmicrowaved animals, indicating no loss of muscle fluids due to microwaving.

ACh levels, expressed as pmol/mg protein, were significantly increased only in the soleus and gastrocnemius and only after 3 weeks of suspension. Decreased ACh levels were found only in the tibialis after 3 weeks of suspension and in the triceps brachii after 1 week (Fig IV.1). Except for the 3 week soleus value, expressing ACh in terms of the wet weight of tissue or of whole muscle (pmol/gm tissue or pmol/muscle) did not change the above results. When muscle mass was considered and levels were expressed as

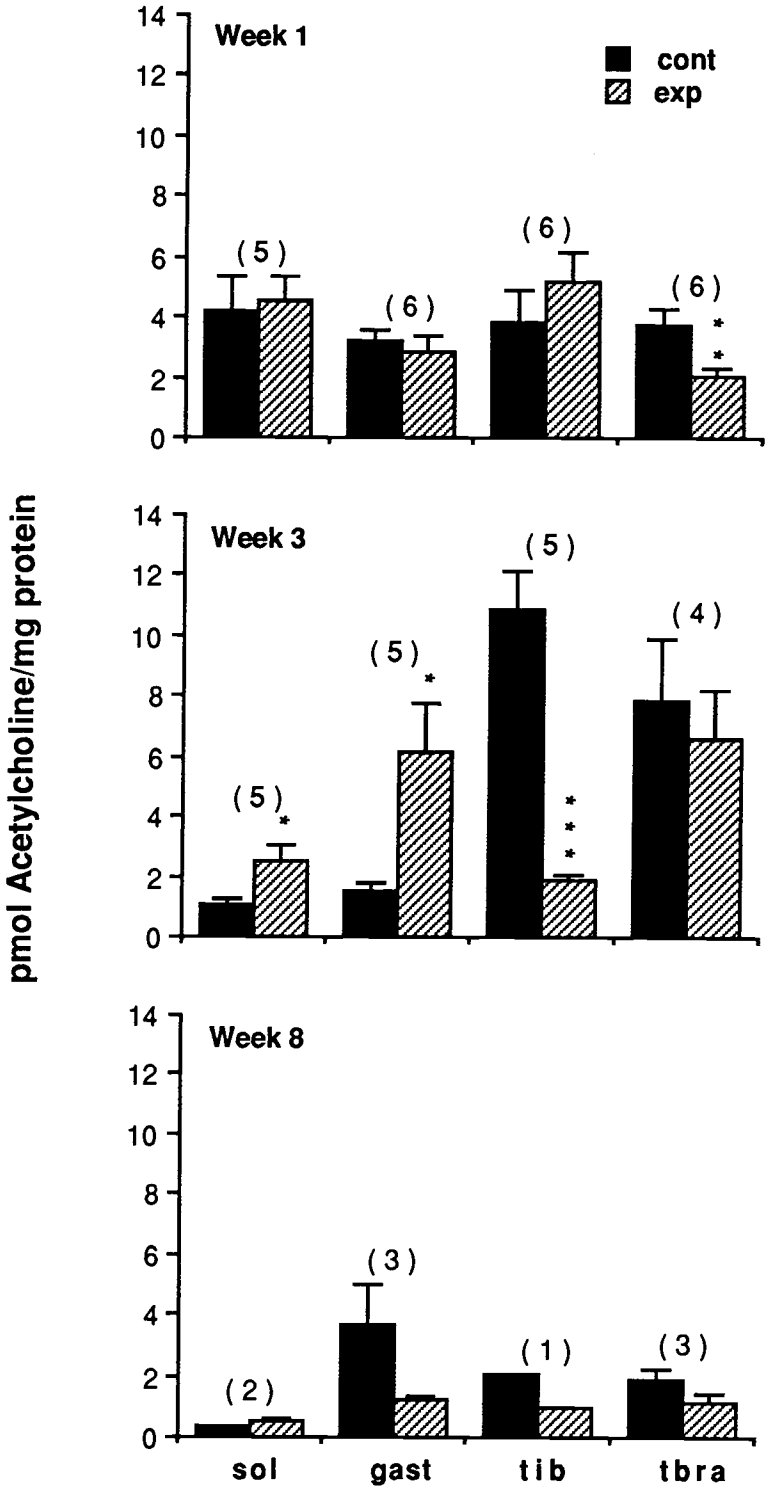
Table IV.1 Body and muscle weights of suspended rats over time expressed as percent of control.

wk	n	body wt	SOL	GAST	TIB	T. BRAC
1	6	97.5[a]	54.2**	85.5**	110.6**	110.2**
3	6	96.2[a]	48.6***	76.8***	100.5	98.9
8	3	93.1	69.6*	80.7*	91.1	81.0

Note: Except where noted (see below), n = # of pairs of control and experimental animals. Comparison done with Student's t-test; *P < .05, **P < .01, ***P < .001. Percents calculated from weights normalized to body wt (i.e. % of body wt). [a]n = 15 and 9 for wk 1 and wk 3 body wt values respectively.

Figure IV.1. ACH levels (pmols/mg protein) in experimental vs control rats in each of the muscles studied after 1, 3, or 8 weeks of suspension. (N) equals the number of pairs of control and experimental animals. Statistical analysis was by the Student's t-test: * $P < .05$, ** $P < .01$, or *** $P < .001$.

Figure IV.1.


Week 1
cont
exp
Week 3
Week 8
pmol Acetylcholine/mg protein
sol
gast
tib
tbra

pmol/muscle, the increase seen in the 3 week suspended soleus was lost (Table IV.2). Ch levels were significantly decreased in the soleus after 1 week of suspension, in both the soleus and gastrocnemius after 3 weeks, and unaffected in the triceps brachii and tibialis whether expressed /mg protein, /gm tissue or /muscle (Fig IV.2). No other significant differences were noted.

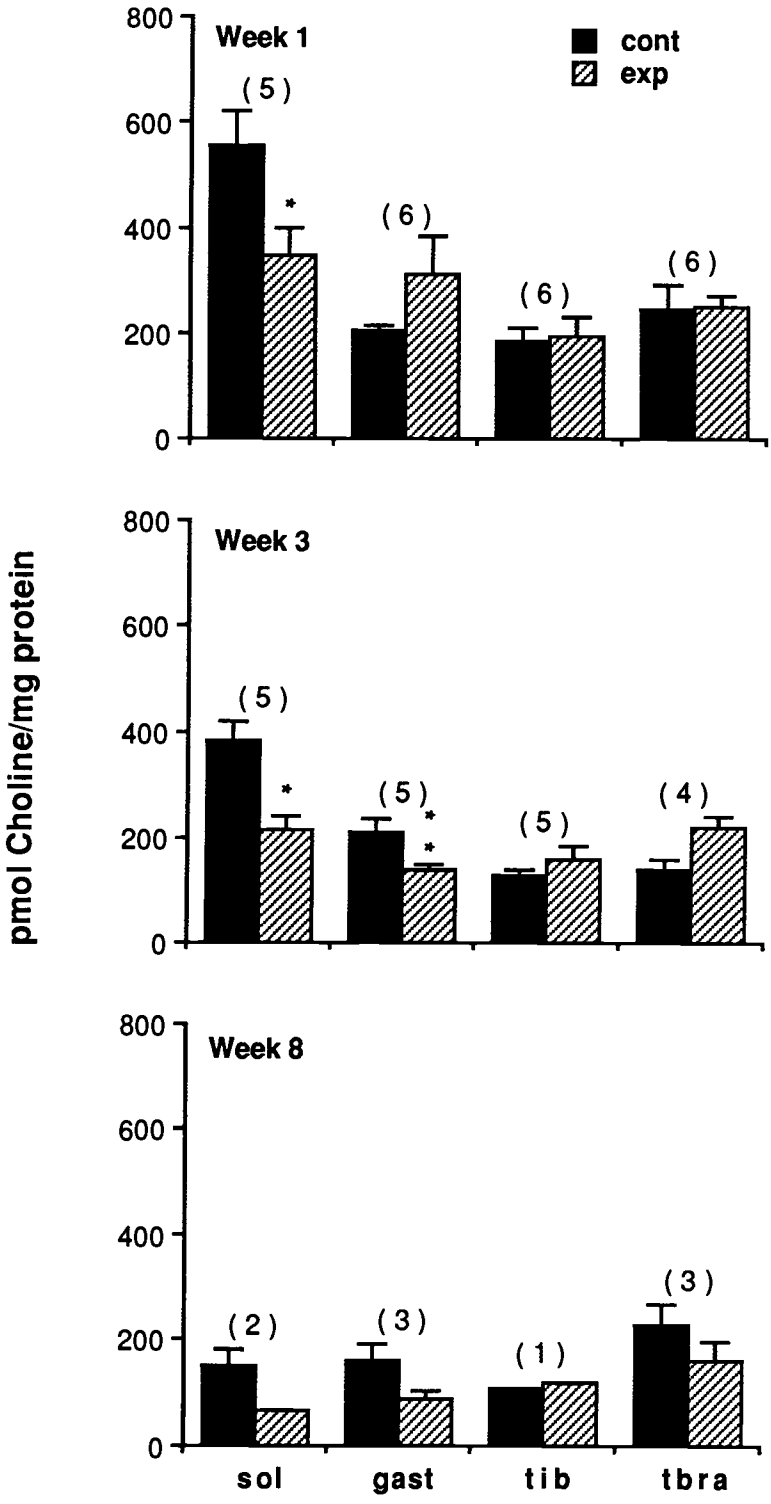
Table IV.2. Comparison between rats suspended for 3 weeks and their age-matched controls in ACh tissue levels in the soleus expressed per mg protein, per gm wet weight of tissue or per whole muscle.

	control	suspended
pmol/mg prot	1.11 ± .16	2.51 ± .48*
pmol/gm tiss	160.11 ± 17.88	422.52 ± 61.97**
pmol/muscle	13.70 ± 1.48	16.27 ± 2.56

Note: N = 5 pairs of control and experimental animals. Comparisons done with a Student's t-test; *P < .05, **P < .01.

Figure IV.2. Ch levels (pmol/mg protein) in experimental vs control rats in each of the muscles studied after 1, 3, or 8 weeks of suspension. (N) equals the number of pairs of control and experimental animals. Statistical analysis was by the Student's t-test: *$P < .05$ or **$P < .01$.

Figure IV.2.


Week 1
cont
exp
(5)
(6)
(6)
(6)
Week 3
(5)
(5)
(5)
(4)
Week 8
(2)
(3)
(1)
(3)
800
600
400
200
0
pmol Choline/mg protein
sol
gast
tib
tbra

Discussion

The gastrocnemius is one of the most affected muscles in both suspension and space. Its ACh level at the three week time period is greater than that of control rats. An apparent similar increase is seen in the soleus muscle after three weeks of suspension when ACh level is expressed as pmol per mg protein or per gm tissue, but the increase disappears when the loss in muscle mass is considered and level is expressed as pmol ACh per muscle. This shows, that at least in the soleus, ACh total muscle levels are maintained, but that there is a concentrating effect due to tissue loss. In the unloaded, flexor, phasic tibialis and the loaded triceps brachii, ACh levels are decreased from controls.

ACh levels have been linked to Ch uptake (Lindmar, et al., 1980). Ch uptake is activity dependent (Murrin and Kuhar, 1976) and thought by some to be rate-limiting in ACH synthesis (Guyenet, et al., 1973) (Yamamura and Snyder, 1973). The suspension model induces hypokinesia in the unloaded hindlimbs by removing their weight-bearing function. Dettbarn, et al., (1987) report that with suspension, EMG activity of the soleus and the extensor digitorum longus, a phasic muscle, becomes "silent". The decreases we found in the unloaded soleus after one week and in both the unloaded soleus and gastrocnemius after three weeks of suspension support activity dependent Ch uptake. We found no evidence, however, of a Ch related effect on ACh, as ACh levels in the hypokinetic gastrocnemius is increased despite the decrease in Ch level.

ACh synthesis is accelerated with increased neuronal activity (Browning, 1976). That ACH is synthesized in the cytoplasm and released from vesicles suggests that synthesis could be tied to vesicular uptake and release. If this is true, then the decreased activity in the suspended animals' muscles should result in decreased or no change in ACH levels. However, work done by Collier, et al., (1987) with the drug AH5183, which blocks uptake of ACH by synaptic vesicles, showed that synthesis and uptake can be independent of release.

Under normal conditions, ACH levels are unaffected by neuronal firing. The ACH packaged in the rapidly recycling subpopulation of vesicles is replaced as fast as it released. These vesicles are only partially filled with transmitter and are about 20% smaller than non-recycling reserve vesicles. With high levels of stimulation, the recycling vesicles may be depleted but the reserve vesicles maintain their ACH stores (about 80% of the total). In the resting terminal, recycling vesicles are converted over about a 24 hr period to reserve vesicles (Tucek, 1985) (Whittaker, 1986). In the long-term passive unloaded muscles decreased release would allow for more recycling vesicles to age to reserve vesicles resulting in a higher steady state ACH level, such as seen in the three week gastrocnemius of suspended animals. In opposition to this idea is the work done by Baranski, et al., (1979) that showed soleus synaptic vesicles from rats flown on Biosputnik 936 to be decreased in both size and number. This implies a decrease in ACH levels not seen in our experimental protocol. However, Thomason,

et al., (1987) report that firing in the soleus becomes more phasic with long term suspension. Compared to tonic muscles, the quantal content of endplate potentials is greater and replacement slower in phasic muscles (Storella and Baker, 1988). Thus the switch from slow to fast under suspension or weightlessness could cause an increase in number of the smaller recycling vesicles and a decrease in total vesicle number due to increased release and slower replacement.

The decrease in tibialis ACH levels may be due to a switch in function to something resembling the tonic action of the soleus. Activity-dependent pharmacologic differences that indicate differences in transmitter release between the tibialis and the soleus have been found (Storella and Baker, 1988). Also, several electromyographical studies have shown activity in the soleus to be greatly reduced while activity in the tibialis becomes tonic under conditions of hindlimb suspension (Alford, et al., 1987) (Dettbarn, et al., (1987), atmospheric parabolic flight (Clement and Andre-Deshays, 1987), and space flight (Kozlovskaya, et al., 1981) (Lestienne and Gurfinkel, 1988).

In the triceps brachii, the early decrease in ACh level might be attributed to the initial increase in activity in that muscle as the animal adapts itself to the suspended condition. With the hindlimbs elevated the forelimbs take on the total burden of locomotion. At later time periods decreased overall activity and/or a stabilization of synthetic and storage mechanisms could

counteract the increased muscle function and return ACh endogenous levels to normal.

ACh levels, with the exception of the gastrocnemius, follow the same trends for the long-term suspension (i.e. eight weeks) as they do for the shorter suspension times. The generally lower values are probably due to growth effects as the muscles increase in size. That is a dilution of the neuronal ACh relative to whole muscle protein content or mass. In the eight week suspended gastrocnemius we see a reversal of the increased levels seen in the three week suspended muscles. The reason for this is not obvious, but could reflect a greater sensitivity of this primary weight-bearing muscle to chronic unloading. Ch levels follow the same patterns throughout the eight weeks of suspension. They also exhibit the generally decreasing levels as the muscles increase in size with time. Time related variation between muscles could be attributed to differences in growth and maturation rates.

In summary, we found the hindlimb suspension model to result in an increase in whole muscle ACh levels only in the gastrocnemius after three weeks. The increase could be due to changes in intra-terminal compartmental distribution. This increase is in contrast to data from flight animals that showed vesicular changes in the soleus that would most probably result in decreased ACh endogenous levels. However, our finding in the soleus do not necessarily contradict the flight data, as we found no difference from controls in total ACh level. Also, after eight weeks of suspension, the increase over controls seen in the three week

suspended gastrocnemius ACh level is lost. In the tibialis we found a decrease in ACH level suggestive of a functional or metabolic change to a tonically firing muscle. The loaded triceps brachii had a transitory decrease in ACH level probably attributable to increased use. Ch levels were affected only in the soleus and gastrocnemius of the suspended animals, where they were decreased. A decrease in the impulse dependent Ch active uptake of motorneurons could have contributed to the decreases found in these hypokinetic muscles.

These data show that suspension induced changes do occur in neuromuscular junction ACh and Ch levels. The changes vary depending on the muscle examined and the length of suspension, and are probably related to differences in muscle type and function both before and after suspension. These data support reversal of function in the soleus and tibialis reported in flight animals and astronauts and indicate a disruption of gastrocnemius function. We found no significant change from control values in our long-term suspended animals, but the reversal in gastrocnemius ACh levels compared to controls (i.e. from an increase to a decrease) that occurred between three and eight weeks suspension indicates ongoing changes.

Chapter V. Discussion

Growth in our suspended animals was less than that of pair-fed controls housed under identical conditions. Analysis of feeding records showed a decreased efficiency of growth, that is, the suspended animals gained less weight per gram of food consumed than the control animals did. A similar decrease was reported in flight animals (Morey, 1979). This decrease in growth efficiency could have had repercussions on development of muscles and testes (see appendix A) in these young growing animals. Possible evidence of maturational effect is seen in our data in the lack of change in the K_d of the experimental animals' soleus muscles over time, in the differences in the patterns of enzyme activities over time between control and experimental animals and in the size related effects in testes testosterone levels (see appendix A).

Of the unloaded hindlimb muscles, the mass of the tonic extensor soleus is decreased the most, followed by the phasic extensor gastrocnemius with the phasic flexor tibialis being affected the least. The loaded forelimb triceps brachii mass varies from slight hypertrophy (10%) to slight mass loss (10%) depending on the time and the data set, but in general it was affected very little. Our data on protein content of the purified membrane preparations used in the radioligand binding receptor studies support literature reports on suspended and flight animals of a preferential loss of myofibrillar over sarcoplasmic proteins in the soleus (Jaspers, et al., 1985) (Steffen and Musacchia, 1985).

In our hands, we saw almost no evidence of the adrenal hypertrophy or thymic involution used as indicators of stress and reported by others in suspended (Musacchia, et al., 1983) (Steffen and Musacchia, 1984) and flight rats (Gazenko, et al., 1980). This finding has both positive and negative connotations. On one hand, this decreases the concern about changes due to stress, but on the other hand does not agree with changes in these parameters seen in flight animals. How this relates to effects in man has not been investigated. Changes in astronaut adrenal hormone plasma and urinary levels have been reported, but are variable depending on exercise level and have been attributed primarily to increased work load and blood pressure and body fluid homeostatic mechanisms (Leach and Rambaut, 1977).

The effect of the rat tail-suspension on the parameters of the neuromuscular junction measured in this study varies depending on the muscle examined (Table V.1). In the soleus and the gastrocnemius the magnitude of the change is less and the time course is extended, but the overall pattern of the neuromuscular parameters measured is similar to what would be expected in a denervation study. We found an increased receptor population, decreased AChE and ChAT activity, and increased ACh and decreased Ch endogenous levels. Despite the greater affect of suspension on the soleus mass, the effect on the neuromuscular junction components was greater in the gastrocnemius. The increases in receptor population and ACh endogenous levels were seen only in the gastrocnemius. It is not clear whether the

Table V.1. A summary of the effects of the rat-tail suspension model at the neuromuscular junction expressed as percent difference from controls at the indicated time period.

Assay	Sol	Gast	Tib	T. Brac
B_{max}[a]	ns	+61% 8 wk	ns	+52% 3 wk
K_d[a]	-49% 3 wk	ns	ns	ns
AChE[c]	-47% 8 wk	-33% 3 wk	-34% 3 wk	-25% 3 wk
			-26% 8 wk	-18% 8 wk
ChAT[d]	-22% 3 wk	-24% 1 wk	ns	-23% 3 wk
				-33% 8 wk
ACh[e]	+126% 3 wk[f]	+312% 3 wk	-83% 3 wk	-44% 1 wk
Ch[e]	-38% 1 wk	-34% 3 wk	ns	ns
	-43% 3 wk			

[a] fmol ligand bound per muscle

[b] n M

[c] µmol ACh hydrolysed per min per muscle

[d] µmol ACh synthesized per hr per muscle

[e] pmol per mg protein

[f] note: This increase disappears when the decrease in muscle mass is considered (ie pmol/muscle--see fig III. 2).

course is extended, but the overall pattern of the neuromuscular seen only in the gastrocnemius. It is not clear whether the increase over controls in soleus receptor affinity is due to a disuse effect directly on the receptors or indirectly on junction maturation patterns. The differences between the soleus and the gastrocnemius or why the gastrocnemius appears to be affected more than the soleus may be due to the differences in muscle types. In denervation studies fast muscles like the gastrocnemius are affected more than slow muscles such as the soleus. Data from suspended and flight animals show a switch in the soleus from slow to fast muscle fiber types. Perhaps the greater resistance of the soleus at the neuromuscular junction is because the first response to weightlessness is to switch from a tonic to a phasic firing muscle.

The reaction of the tibialis to mechanical unloading or weightlessness is different. Electromyographical studies have shown that the tibialis, as the antagonist flexor muscle to the ankle extensor soleus, takes over the tonic firing pattern of the soleus in suspension or weightlessness (Dettbarn, et al., 1987) (Lestienne and Gurfinkle, 1988). The decreases seen in AChE and ACh levels could reflect a switch to levels more representative of a tonically firing muscle.

As a supposed control, the changes found in the loaded forelimb triceps brachii present a different problem in interpretation. The changes seen can be related to the increased use of the forelimbs in locomotion in the suspension model. The

increase in receptor number and the decrease in endogenous ACh levels can be attributed to increased work load. The cause of the decreases found in both specific (μmole/min or hour/gm protein) and total (μmol/min or hour/muscle) AChE and ChAT activity is less clear. Muscle fiber hypertrophy in suspended animal muscles could have resulted in a dilution effect, i.e. the same amount of enzyme in more tissue or protein.

The consequences of the decrease in testes size found in suspended animals to the effects seen at the neuromuscular junction needs to be considered. Testosterone action at sites distant from the testes would be caused by circulating levels of the hormone. As we saw no change in plasma testosterone level until eight weeks of suspension, any earlier changes at the neuromuscular junction would have been unaffected. Of the changes found after eight weeks of suspension, the decreases in the tibialis AChE activity and in the triceps brachii AChE and ChAT were already evident at the three week time period. AChE activity is also decreased in the soleus from animals suspended for eight weeks. Kostirova, et al., (1975) found castration to have no effect on soleus AChE activity, though activity in the hormone sensitive levator ani was decreased 79%. Tsika, et al., (1987) found androgen treatment of suspended female rats to decrease muscle mass loss in the fast-twitch plantaris but not the slow-twitch soleus. The soleus seems to be resistant to both decreases and increases in testosterone levels. The increased B_{max} in the gastrocnemius of eight week suspended animals we have

attributed to chronic disuse. Bleisch, et al., (1982) show testosterone to partially restore, in the levator ani, ACh receptors lost due to castration, suggesting testosterone levels can affect skeletal muscle receptor population. However, Bernard and Max (1986) show denervation of hindlimb muscles to lead to a redistribution of muscle testosterone receptors that could result in reduced muscle sensitivity to the hormone. High levels of testosterone have been reported to inhibit protein synthesis and total body growth (Kochakian, 1975), but the doses given were much larger than the increases we found in plasma levels (μg vs ng/ml quantities). It is impossible to exclude all effects of the hormone on muscle protein, motoneurons (Frolkis, et al., 1985) or body growth, but trends found before circulating levels increased and in opposition to reported effects (e.g. decreased enzyme levels when hormone levels were high) make it unlikely that increased testosterone and not decreased neural input is responsible for the long-term changes.

It is well documented that associative and feedback mechanisms exist between nerve and muscle. That intact neuromuscular interaction is required to maintain both nerve and muscle function and structural integrity. Changes typical of muscle atrophy can be produced by blocking all or part of this interaction. Denervation has been shown to result in extrajunctional nicotinic acetylcholine receptors, muscle fibrillation, increased electrical excitation and decreased resting membrane potential and acetylcholinesterase (AChE) activity (Cangiano, et al., 1984).

Blocking nerve conduction with the specific sodium channel blocker, tetrodotoxin (TTX), results in decreased fiber diameter, twitch response and twitch:tetanus ratios (LaPoint and Gardiner, 1984). Blocking nerve transmission with botulinum toxin results in the spread of extrajunctional receptors (Thesleff, 1960). Cross-reinnervation studies have shown muscle fiber type to be sensitive to changes in neuronal impulse pattern and motoneuron electrical properties to be sensitive to muscle activity patterns (Foehring, et al., 1987a and b). Trophic substances from the nerve influence muscle properties (Hasegawa, et al., 1982) and trophic substances from the muscle have been implicated in nerve cell body function (Czeh, et al., 1978).

Many factors could contribute to the changes in neuromuscular components found in this study. Fluid shifts and changes in electrolyte balance, stress or behavior alterations, or endocrine and growth functions could all have had an effect on the parameters measured. We examined the possibility of an anabolic hormone effect in the testosterone study and concluded that it is unlikely that this hormone played a major role. The changes we found at the neuromuscular junction of suspended rats are similar to changes reported in the literature in denervation and nerve blockade studies. Such changes could decrease function of the neuromuscular junction and contribute to muscle mass loss and dysfunction as reported in astronauts and flight animals. Thus, these data support our hypothesis of a neural component to space-induced muscle atrophy.

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APPENDIX

Appendix A. Testes Size And Testosterone Levels in a Model For Weightlessness

Joyce E. Royland, L. J. Weber, and M. Fitzpatrick

Contribution of authors: M. Fitzpatrick contributed to the radioimmunoassy for determination of testosterone levels.

Introduction

The physiological adaptation to the microgravity of space has been most successfully mimicked by the Morey-Holten rat tail-suspension model (Morey, 1979) (Morey-Holton and Wronski, 1981). This model mimics the fluid shifts and muscle unloading of weightlessness and results in metabolic changes in bone formation, renal function, electrolyte balance and muscle mass comparable to those recorded from biosatellite animals. Using the Morey-Holten model in our investi-gation of the effects of weightlessness at the neuromuscular junction, we observed that the testes of suspended animals were smaller than those of controls. We were concerned that this was an artifact of the model that would affect testosterone levels and influence muscle atrophy and events at the neuromuscular junction.

The testes are the major source of testosterone, which serves growth and maintenance functions for skele-tal muscle (Breuer and Florini, 1966) (Buresova and Gutmann, 1971) (Bergamini, 1975) (Capaccio, et al., 1987). Castration in rats leads to skeletal muscle atrophy in the hormone sensitive levator ani (Leonard, 1957) (Bass, et al., 1969) and in other non-sensitive striated muscles (Koenig, et al., 1980). Alternatively, high doses of testosterone inhibit muscle protein synthesis and cause growth inhibition in both normal and castrated rats (Kochakian, 1975). Testosterone also influences acetylcholine esterase (Gutmann, 1976) and choline acetyltransferase synthesis (Kostirova, et al., 1975) (Gutmann, 1976) and shows an effect on motoneuron axonal

transport (Frolkis, et al., 1985) and size (Kurz, et al., 1986). Tsika, et. al., (1987) in their hindlimb suspension study on the effects of the steroid, nandrolone decandate, on female rat skeletal muscle mass, found steroid treatment reduces the mass loss in the fast-twitch plantaris and slow-twitch soleus muscles; to partially spare plantaris but not soleus myofibril content; but to have no effect on the isomyosin pattern induced by suspension. The possibility that the decrease in testicular size is a mechanical artifact of the suspension model that might result in hormonal alterations of importance to our study needed to be examined. We therefore compared the levels of testosterone in testes and plasma in suspended and control animals.

Materials and Methods

Materials: Male Sprague-Dawley rats (Simonsen labs: Gilroy, CA), starting age 5 - 6 weeks (X body weight, 151.0 = 1.9 gm) were used throughout. Supplies for suspension were as follows: Compound benzoin tincture, Whiteworth, Inc (Garden, CA); Fas-Trac adhesive traction strip, DePuy, Inc (Warsaw, IN); and 1/2 inch waterproof medical tape, Johnson & Johnson Products, Inc (New Brunswick, NJ). The testosterone antisera No. T3-125 was from Endocrine Sciences (Tarzana, CA). The radioimmunoassay employed is sensitive down to 2 pgm testosterone. Dihydrotestosterone, delta-1-dihydrotestosterone, and delta-1-testosterone cross-react significantly with the antibody (44.%, 18.%, and 41.% respectively). Budget Sol scintillation fluor was from Research Products International. All other chemicals were reagent grade. Suspension: To take into account possible growth effects, a control animal was weight-matched to each experimental animal at the beginning of the experiment and pair-fed throughout the experimental time period. Experimental animals were suspended for one, three or eight weeks at a 30 degree head-down tilt as described by Morey-Holten and Wronski (1981). Unanesthesized animals' tails were cleaned with soap and then 70% ethyl alcohol to remove dirt and oils. The tail was swabbed with tincture of benzoin to toughen the skin. Starting at the base of the tail, a strip of Fas-Trac orthopedic tape 5 mm wide was run 2/3d's of the way up one side of the tail, a small loop formed at the top, and then continued back down the opposite side. A strip of adhesive

medical tape was loosely spiraled up the tail. Care was taken not to wrap the tape too tightly and constant watch was kept on the tail tips to insure that good circulation was maintained. A hook in the previously mentioned loop was used to suspend the animal from a chain attached to the suspension apparatus. The chain allowed for adjusments in hindlimb suspension height to maintain a 30 degree headdown tilt as the animal grew.

The suspension apparatus is designed to allow the animal unrestricted movement to all but the corners of the cage. The apparatus consisted of an L-shaped metal strip bolted to the side of a 61 cm square, solid walled, meshed bottomed cage so that the long arm protuded 25 cm towards the cage center. Attached to the arm was a 9 cm rotating piece with a swivel suspended from the free end. The rotating piece allowed the animal to move in a 360 degree circle and the swivel allowed the animal to spin 360 degreees without becoming entangled. The above mentioned chain was attached to the swivel. Control animals were housed singly, unre-strained in identical cages.

All animals were weighed weekly and record was kept of daily food consumption. Experimental animals were fed ad libatum. Food lost through the cage floor was accounted for in the records. Control animals were fed the amount their paired experimental animal had consumed the previous day. Food lost through the cage floor was weighed and, if no food remained in the cage, an equal amount regiven to the animal. Excess food remaining at the end of 24 hrs was removed. As a measure of

stress in our animals, adrenals were weighed at the time of tissue sampling.

Sample Preparation: On the 8th, 22nd or 57th day of suspension, animals were killed by decapitation. Mixed venous and arterial blood from the severed neck was taken with a heparinized syringe for plasma analysis. The testes were removed into ice-cold 10 mM phosphate buffered saline. Samples were always taken between 9:30 and 10:30 a.m. (Kalra and Kalra, 1979). The testes were weighed, frozen whole with dry ice and acetone, and stored at -70°C until assayed. Plasma was also quick frozen and stored.

For testosterone extraction the testes were thawed, minced in 3:1, vol:wt, 10 mM phosphate buffered saline, and homogenized for two, 5 second bursts at power level 6 with a Kinematica polytron. Ten microliter samples of homogenate were double extracted by adding 2 ml anhydrous diethyl ether, vortexing for 30 seconds and freezing in acetone and dry ice for 5-6 seconds to solidify the aqueous portion. The ether with the extracted testosterone was decanted off and evaporated dry with N_2 gas at 45° C. The thawed aqueous portion was reextracted by again adding 2 ml anhydrous ether and repeating the above procedure. Twenty-five microliters of plasma were likewise extracted. The extracted dried testosterone was resuspended in 1 ml of phosphate-gelatin buffer (2.9 mM NaH_2PO_4, 5.9 mM Na_2HPO_4, 144 mM NaCl plus .12 gm gelatin/liter) by 10 seconds slow vortexing.

Radioimmunoassay: Two hundred microliter aliquots of the

extracted solution were assayed by the proceedure outlined by Sower and Schreck (1982) as modified by Fitzpatrick, et. al. (1986). All samples were run in duplicate. Experimental values were compared to simultaneously run standard curves to determine testosterone levels. Conjugated metabolites are not extracted by the nonpolar extraction solvent, therefore did not contribute to sample levels. Sample curves of testosterone levels for 5-50 microliters of extracted homogenate or plasma were parallel to standard curves and linear over the range tested. Extraction efficiency based on a known amount of ^{3}H-testosterone averaged 81% for the testes tissue homogenate and 85% for the plasma samples. Intra- and interassay coefficients of variation were 7.8% (n = 8) and 13.7% (n = 5) respectively.

Statistics: Growth, weight and testosterone levels within a suspension group were compared using the Wilcoxon signed rank test for paired samples. Testes size data within a suspension group but between treatments, were compared using the Mann-Whitney U test. Between group data was evaluated first by a Kruskal-Wallis one-way analysis to determine whether differences existed, and then by the Newman-Keuls multiple range test to determine which groups varied significant-ly from each other (Zar, 1974).

Results

Records of growth and food consumption show similar patterns in control and suspended animals. Beginning body weight was 152.7 ± 3.0 gm and 151.8 ± 2.8 gm for control and experimental animals (n = 38) respectively and increased steadily to 331.7 ± 7.1 and 292.8 ± 4.5 gm respectively at eight weeks (n = 6). Food consumption ranged from 151.8 ± 4.2 gm/wk for the first week (n = 38), to a peak of 215.6 ± 10.0 gm/wk in the fifth week (n = 6), to 184.4 ± 5.5 gm/wk during the eighth week (n = 6) in control animals. Suspended animals' consumption followed a similar pattern with values of 157.7 ± 4.6, 227.2 ± 7.2, and 203.2 ± 5.4 gm/wk respectively. Suspended animals showed less weight gained at 1, 2 and 4 thru 8 wks ($P < .01$ at wk 1 and 2; $P < .001$ at wks 4 - 8; ns at wk 3) and more food consumed ($P < .001$ at wks 1, 2, and 4 - 8; $P < .01$ at wk 3) per week than their controls, demonstrating a decreased growth efficiency as defined by weight gained in gms per gm of food consumed (Table A.1).

No evidence of adrenal hypertrophy was found in our suspended animals. Adrenal weights in experimental vs. control animals did not differ significantly ($P > .05$) at any time examined, whether compared as wet weight or normalized to body weight (Table A.2).

All testicular weights of experimental animals were less than those of control animals whether expressed as absolute weight or normalized to body weight ($\bar{X}$ % body wt) (Fig A.1). Suspension for

Table A.1. Effect of suspension on growth efficiency.

		Weight gain in grams per gram of food		
week	N	control	suspended	% of cont
1	38	.22 ± .06	.18 ± .05**	81.8
2	32	.25 ± .05	.19 ± .05**	76.0
3	32	.17 ± .05	.14 ± .04*	82.4
4-8	30[a]	.10 ± .04	.06 ± .03**	60.0

Note: Values represent the means ± SEM. P values determined by Wilcoxin signed rank test for paired samples; *P < .05, **P < .001. [a]N = 30 values determined for 6 animals over 5 weeks. At all other times N = number of animals = the number of values.

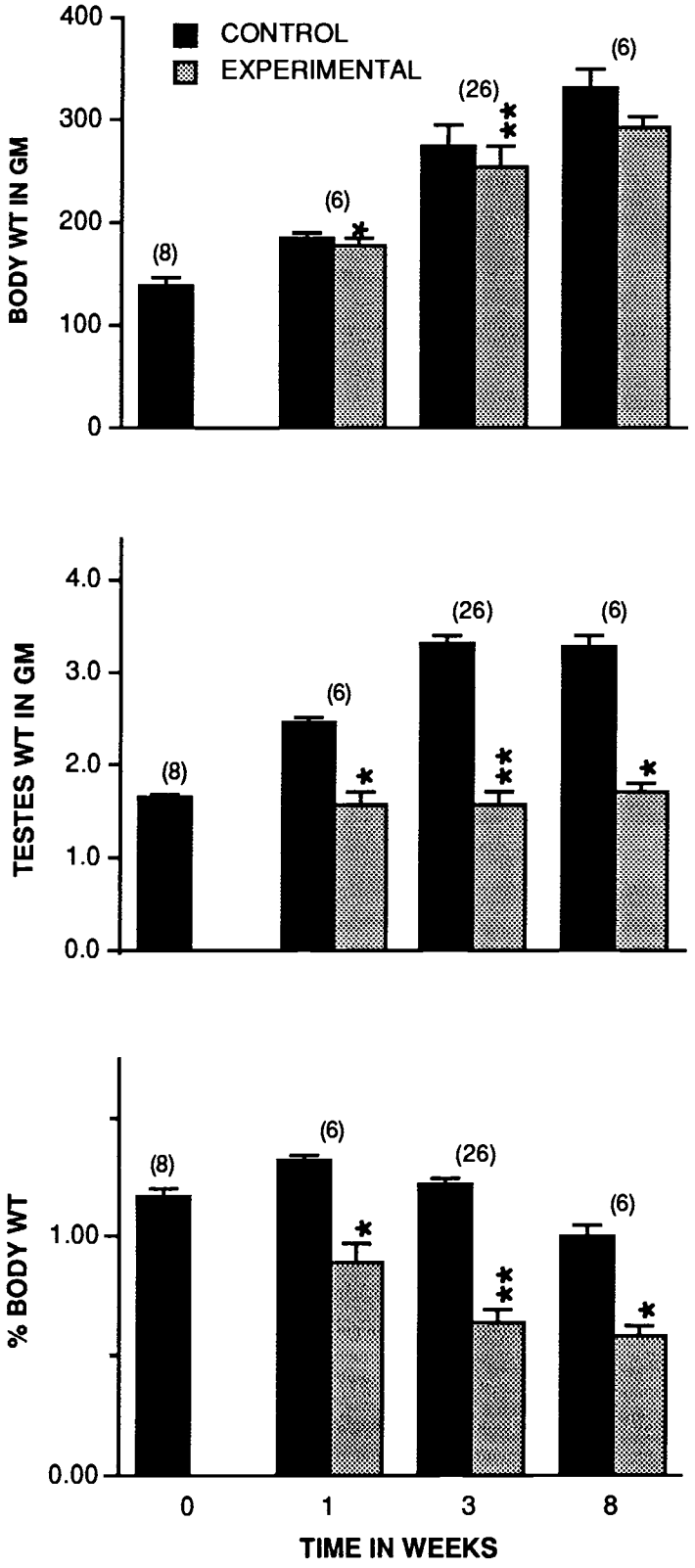
Table A.2 Adrenal weights in control and suspended rats over time.

Condition		N	Wet Wt in mgs	% body wt x 10^{-2}
1 wk	control	6	34 ± 4	1.8 ± 0.19
	suspended	6	34 ± 1	2.0 ± 0.07
3 wk	control	14	44 ± 2	1.6 ± 0.05
	suspended	14	42 ± 1	1.6 ± 1.02
8 wk	control	5	43 ± 2	1.3 ± 0.05
	suspended	5	41 ± 1	1.4 ± 0.04

Note: Values are means ± SEM.

Figure A.1. Comparison of control and suspended animals' body and testicular size over time. Values are $\bar{X}$'s ± S.E.. N's are in parenthesis. * $P < .05$, ** $P < .01$ vs control; Wilcoxon signed rank test for paired samples.

Figure A.1.


CONTROL
EXPERIMENTAL
BODY WT IN GM
400
300
200
100
0
(8)
(6)
(26)
(6)
*
**
TESTES WT IN GM
4.0
3.0
2.0
1.0
0.0
% BODY WT
1.00
0.00
0
1
3
8
TIME IN WEEKS

one, three or eight weeks resulted in testes of approximately 67, 52, or 58% of control testes' size respectively.

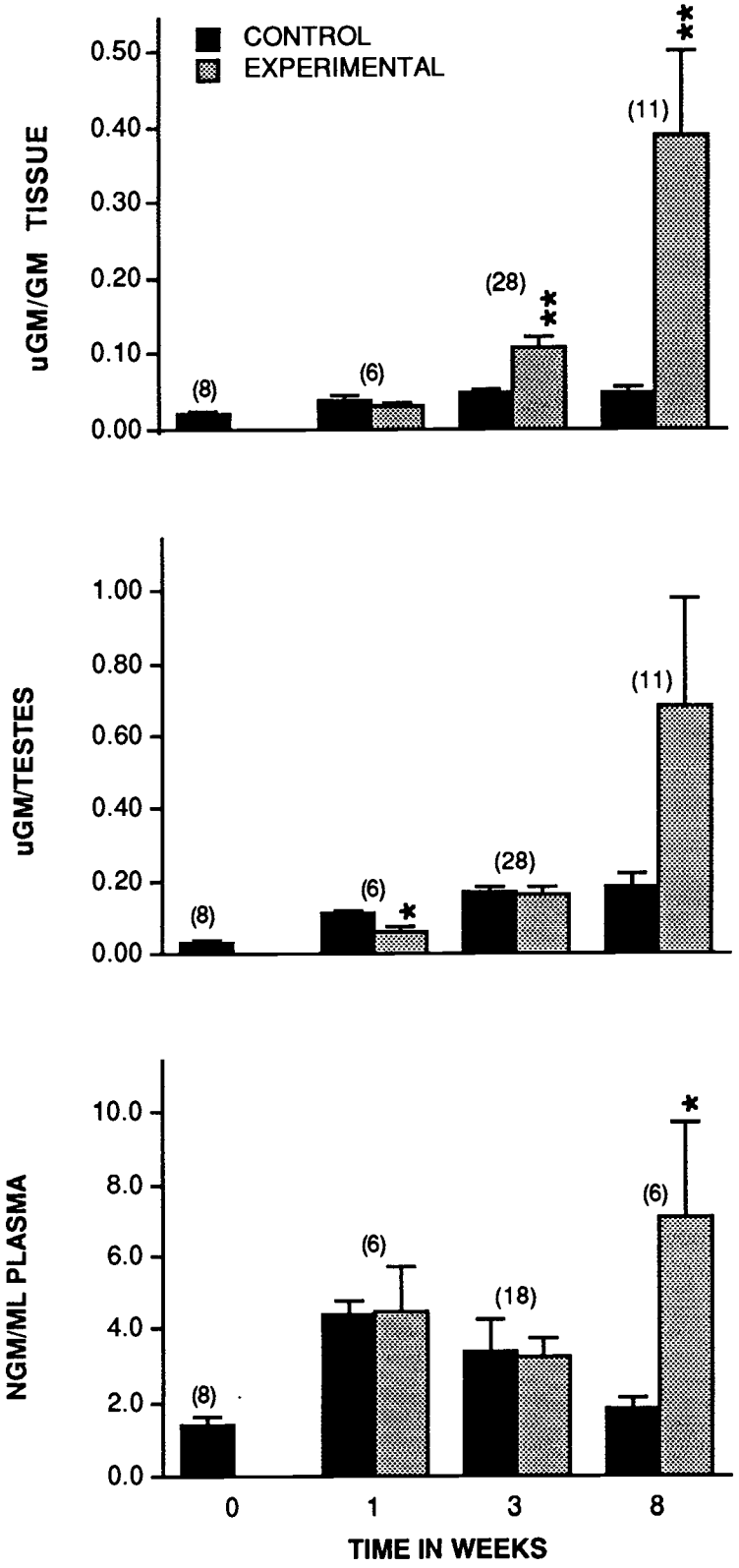
Testes wet weight of control animals sampled after one week increased significantly from time zero, and again between one and three weeks, but not between three and eight weeks. Normalizing testes weight to body weight, % body wt., showed no difference between zero or three weeks, but all other groups differed significantly. There was no difference among any experimental animal group in wet weight of testes. Percent body weight values showed week $8 = 3 < 1 < 0$.

Testes testosterone levels were elevated over controls in animals suspended for three or eight weeks when measured as micrograms testosterone per gram tissue (Fig A.2). When testosterone levels were calculated as μgm testosterone/testes pair, experimental animals after one week of suspension had a decrease from controls in total tissue testosterone. Also, the apparent increase in tissue testosterone (μgm/gm) seen after three weeks of suspension, disappeared. Testosterone levels of control animals increased only between zero and one week whether analyzed per gm of tissue or per testes pair. In contrast, experimental animals showed significant increase only between three and eight weeks of suspension when analyzed either way.

Plasma testosterone levels were significantly different between control and experimental animals only after eight weeks of suspension. Analysis of eight week testosterone shows a positive

Figure A.2. Comparison of control and suspended animals' testes and plasma testosterone levels over time. Values are $\bar{X}$'s ± S.E.. N's are in parenthesis. * $P < .05$, ** $P < .01$ vs control; Wilcoxon signed rank test for paired samples.

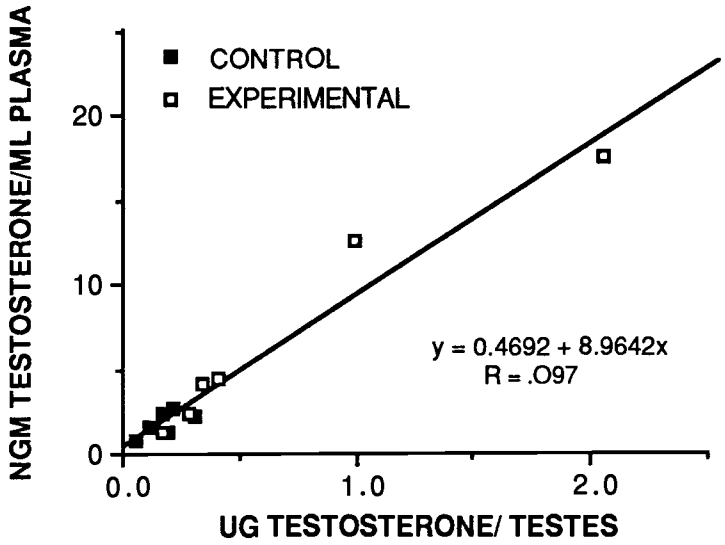
Figure A.2.


CONTROL
EXPERIMENTAL
uGM/GM TISSUE
0.50
0.40
0.30
0.20
0.10
0.00
(8)
(6)
(28)
(11)
**
**
uGM/TESTES
1.00
0.80
0.60
0.40
0.20
0.00
(8)
(6)
*
(28)
(11)
NGM/ML PLASMA
10.0
8.0
6.0
4.0
2.0
0.0
(8)
(6)
(18)
(6)
*
0
1
3
8
TIME IN WEEKS

correlation (r = 0.97) between concentrations in testes and circulating plasma (Fig A.3).

Comparing testes testosterone concentration and size led to data clumping into two main size groups regardless of treatment; group A with testis weighing less than 1.30 gms with higher concentrations and group B with testis weighing more than 1.30 gms with lower concentrations. Figure A.4 shows tissue testosterone levels (μgm testosterone/gm of tissue) relative to treatment, testis size and suspension time. After one week of suspension there was no difference between control and experimental testes testosterone concentration or size grouping. After three weeks of suspension, group A testes of experimental animals had significantly more testosterone per gram of tissue than group B, however, there was no difference in testosterone levels between testes of a similar size whether the testes were from control or experimental animals (group B). At eight weeks statistical comparison could not be done between group A control and experimental data or between control group A and group B because of only one nondescended control testis falling within the small size group.

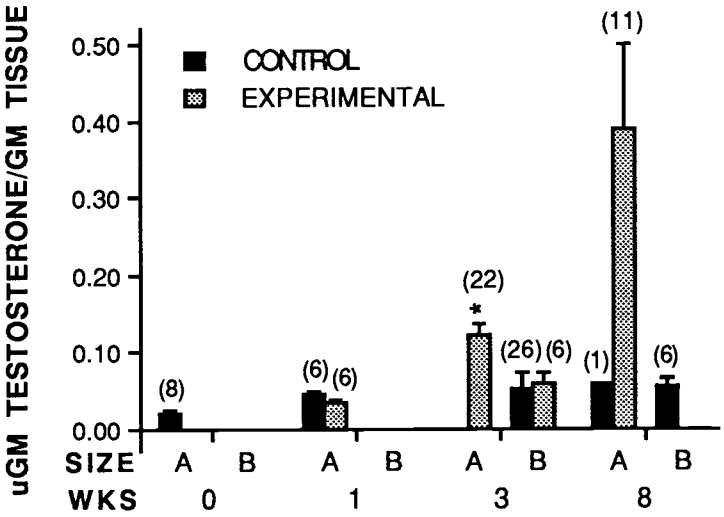
Figure A.3.



Plasma testosterone levels relative to total testes tissue testosterone levels at the 8 wk experimental time period. Line represents linear regression analysis of combined control and experimental data.

Figure A.4. Comparison of testis testosterone levels (µg m testosterone/gm tissue) to testis size, duration of suspension in weeks, and treatment (ie. control and experimental). Values are $\bar{X}$)'s ± S.E.. N's are in parenthesis. A = testis wt < 1.30 gms; B = tesies wt > 1.30 gms. Blanks indicate no testis of that treatment fell within that size grouping at that time. * $P < .05$, A vs B ; Mann Whitney test. Newman-Keuls multiple range test for between time groups: Control µg testosterone/gm tissue, wk 0 ≠ wk 1 = wk 3 = wk 8, $P < .05$; experimental µg/gm, wk 0 = wk 1 = wk 3 ≠ wk 8, $P < .01$.

Figure A.4.


uGM TESTOSTERONE/GM TISSUE
0.50
0.40
0.30
0.20
0.10
0.00
CONTROL
EXPERIMENTAL
(8)
(6)
(6)
(22)
*
(26)
(6)
(1)
(11)
(6)
SIZE A B A B A B A B
WKS 0 1 3 8

Discussion

Tissue testosterone concentration up to three weeks of suspension appears to be unrelated to treatment. Increased tissue levels in suspended animals are apparently accounted for by decreased testis size. Three different kinds of data suggest that increases in tissue testosterone levels are related to testis size and/or maturation state: 1) Testes of control animals continue to increase in size while testes of experimental animals do not; 2) the time course for increases in tissue testosterone level lags behind in experimental animals; and 3) testosterone levels in testes of comparable size from control or experimental animals are the same. However, after eight weeks of suspension, it becomes clear that something additional is occurring. Tissue testosterone levels increased in some animals several times more than can be accounted for in lost mass of the testes.

The plasma testosterone levels reported here are somewhat higher than the maximum level of 2 ngm/ml reported by Dohler and Wuttke (1975) and Resko, et al., (1968) but are in close agreement with values reported by other authors (Kalra and Kalra, 1979) (van Tienhoven, 1983). Differences may be due to time of sampling as levels may vary due to diurnal cycles. Or methodologically, radioimmumoassay cross-reactivity may also contribute to higher apparent levels (see methods). Following the sequence reported by Ramaley (1979) both control and suspended animals plasma levels peak 1 week into the experimental period (42 days postpartum) and then begin to drop off. At the 8 week

time period, control plasma levels continue to drop off, as expected, while experimental plasma levels increase dramatically. It is important to note that at eight weeks plasma testosterone levels are significantly greater than those of control animals, correlating with increased tissue levels. Since physiologic function is dependent upon circulating levels, this suggests that secondary growth effects could be affected.

Growth and weight data for the rats in this study are comparable to those found in previous suspension experiments and from the Cosmos 782 and 936 biosatellites (Morey, 1979). In each time interval analyzed, control animals offered the same amount of food that their paired experi-mental counterparts had consumed the previous day, ate less but gained more weight. The effect was most pronounced at the later time periods when growth rates decreased. These data indicate a higher metabolic cost to rats under the suspension or weightless condition (Morey, 1979), particularly as the animal approaches zero growth rate.

Several factors need to be considered when comparing testes size between suspended and control animals. The decrease in size may be due to the angle at which the animals were suspended. At a 30° head-down tilt, the tendency would be for gravity to pull the testicles into rather than out of the abdominal cavity. It is known that placing the testical into the abdominal cavity can result in atrophy (Moore, 1951). Also, non-descended or late descending testicles do not develop normally, therefore the smaller size may be due in part to lack of growth. The time of suspension in this

study ranges from about five to thirteen weeks of age. Resko, et al. (1968) report that testes weight more than doubles during this period with the greatest increase between five and seven weeks post partum. In our data control animal testicular growth followed this pattern, increasing in size from zero to three weeks, and leveling off between three and eight weeks of the experimental period or at approximately eight to thirteen weeks of age. In the experimental animals, there was no change in testes size throughout the eight week experimental period, suggesting delayed growth and/or development. The increased metabolic demand as suggested by the decrease in growth efficiency in suspended animals may have had greater consequences in a rapidly growing tissue such as the developing testes.

Normalizing testes size to body weight eliminated the increase observed in control animals sampled after three weeks, probably due to deferential maturing rates between the testes and the whole animal. Testes size relative to body weight decreased in experimental animals from zero to three weeks of suspension -- a not surprising result with the testes remaining the same size while the animal continued to grow. As growth rate began to level off 3 weeks into the experiment, there was no further significant decrease in testes percent of body weight.

Control animal testosterone content per gram of tissue or per testes pair showed a significant increase only between zero and one week or approximately 35-42 days postpartum. In experimental animals, a significant increase was found only after

more than three weeks of suspension. The different timing in tissue testosterone production between control and experimental animals again suggests a delay in testes development of suspended animals.

In normal development, the rat reaches puberty at 35 days post partum, the approximate age of our rats at the beginning of the experimental period. Testicular growth and development of the seminiferous tubules occurs with the increasing testosterone production that occurs at this time (Eik-Nes, 1975) (Rao et al., 1984). If the smaller testes in experimental animals is due to a stunting of growth, the decreased mass might be attributable to the failure of the seminiferous tubules to develop. Alternatively, it has been long known that placing the testes into the body cavity results in degeneration of the tubule epithelium (Moore, 1951). In either case, the apparent increase in tissue testosterone/gm tissue in rats suspended for three or eight weeks may result from a relatively greater proportion of testes weight being due to testosterone producing Leydig cells, rather than an actual increase in production. When one looks at total testes testoserone levels (μgm testosterone/testes pair), the apparent increase is counteracted by the decrease in testes size and becomes nonsignificant.

A recent report by Serova, et al., (1989) makes an histological comparison between flight and suspended animals' testes. Rats flown for 7 days onboard the biosatellite, Cosmos-1667, had no decrease in testes size. Animals flown for 13 days on

Cosmos-1887 had a less than 10% decrease in testes and epidymis mass which was attributed to water loss. However, data (interpolated from a bar graph) did show an approximate 25% decrease in spermatogonia. A similar loss in mass was found in animals flown for 7 days on Space Lab-3 (Philpott, et al., 1985). A 7.5% decrease in testes size correlated with a loss of spermatagonial cells, an effect similar to that found in experimentally produced cryptorchism (Moore, 1951). In contrast, in 14 day tail-suspended animals, Serova's group (1989) found testes mass to be less than 50% that of controls. There was a profound loss of spermatagenic cells (approx. 84%) and spermatazoa (approx. 98%) and evidence of cellular degeneration. Seratoli and Leydig cells were affected less (approx. 30% decrease).

The difference in magnitude of the testes mass loss between flight and suspended animals raises the question of model artifact. Serova's data supports testes degeneration due to cryptorchism mechanically produced by the suspension model. Delays in development due to nondescension may also have contributed to the large differences seen between control and suspended animals. Note that our animals do not show a loss in testes mass, only no increase. Flight animals are generally adults (Serova's are described as being from the breeding colony) while suspended animals, due to technical considerations, are generally immature. The loss in testes weight (> 50%) reported above compares with the 48% loss seen in our 21-day suspended animals. More mature flight rats would be at a less susceptible phase of testes

development during microgravity exposure. Though to a much lesser degree, changes do occur in the mass and germinal epithelium of the testes of flight animals similar to those found in suspended animals. Also, in contrast to the lack of difference in plasma testosterone levels in this study after 1 or 3 weeks of suspension or the increase found after 8 weeks, data from the 7 day Space Lab-3 mission showed significantly decreased (> 75%) plasma levels (Vasques, et al., 1988). Samples were taken 11 - 17 hours after reentry.

Our results suggest that, while testicular weight is reduced, short term use of the Morey-Holten rat tail-suspension model does not induce testosterone changes of consequence to a skeletal muscle study. For longer studies, however, consideration should be given to possible hormonal effects since by eight weeks of suspension one begins to see increases in tissue and plasma testosterone levels.

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