Multivalent ligands such as immunoglobulins and lectins can cause their receptors to aggregate (*10*). Although α-bungarotoxin is a univalent ligand (*11*), the question arises whether it may have a similar effect on ACh receptors. To test this possibility, we conducted experiments which were based on the previous finding that prior fixation with paraformaldehyde at 37°C prevents the ligand-induced aggregation of concanavalin A receptors on fibroblasts (*12*). Denervated plantaris muscles were fixed for up to 90 minutes with 4 percent paraformaldehyde at 37°C before being exposed to fluorescent α-bungarotoxin. Although this fixation caused a nonspecific increase in background fluorescence, the typical discrete patches of fluorescent stain were still observed (Fig. 1B) and they had the same specificity as in the case when live muscles were stained with fluorescent toxin. These results indicate that the toxin did not cause ACh receptors to aggregate into high-density patches, but rather that the patches existed before the muscles were exposed to the toxin.

Patches of ACh receptors were also observed when denervated mouse plantaris muscles were exposed to ^{125}I-labeled α-bungarotoxin and examined by autoradiography (Fig. 2A). The ratio of the grain densities in the patches compared to adjacent extrajunctional regions on the same fibers was 21.5 ± 3.1 (mean ± standard error; N = 11) after 9 days of denervation and 17.9 ± 4.5 (N = 9) after 20 days of denervation. The corresponding ratios for endplates (Fig. 2B) relative to extrajunctional regions (not including patches) were 26.0 ± 2.7 (N = 19) and 29.5 ± 4.2 (N = 18). Grain density should be directly proportional to receptor density in extrajunctional regions where the sarcolemma is not folded, and it therefore follows from the present findings that the density of ACh receptors in patches is about 20 times greater than along the rest of the fiber. Values for grain density at endplates are less readily converted to relative receptor density. On the one hand the branching patterns of endplates are not revealed by autoradiography and, as a result, relative receptor density will be underestimated. On the other hand, the folding of the sarcolemma at the endplate will lead to an overestimate. If one assumes that these opposing errors approximately balance each other, our autoradiographic measurements suggest that the receptor density in patches may be close to that in the subsynaptic membrane.

Patches of high ACh receptor density thus develop not only on embryonic muscle cells in culture, but also on adult denervated muscle fibers in vivo. Since denervated muscle fibers can become reinnervated at sites other than the original endplate (*13*), the present findings are consistent with the notion that the patches may be preferred sites of innervation. However, even if they are not targets for growing nerve fibers the mechanisms involved in their formation may likewise participate in the development of a high receptor density at synapses. One possibility is that the patches reflect regions of sarcolemma where the rate of receptor degradation is particularly low, as is known to be the case for junctional receptors (*14*). In addition, many studies have revealed that surface membrane proteins can move laterally within the membrane (*15*), and that their mobility is subject to modulation (*10*). Recently, evidence has been obtained that the development of high densities of ACh receptors in patches and at sites of innervation on cultured amphibian muscle cells involves a process of receptor redistribution (*16*). It is therefore reasonable to assume that ACh receptors can move within the sarcolemma and that at sites of high packing density their mobility is significantly restricted.

P. K. Ko
M. J. Anderson*
M. W. Cohen
Department of Physiology, McGill University, Montreal, Canada H3C 1Y6

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* Present address: Department of Neurobiology, Salk Institute, San Diego, Calif. 95112.

1 November 1976

Partial Denervation Affects Both Denervated and Innervated Fibers in the Mammalian Skeletal Muscle

Abstract. *Partial denervation of the rat extensor digitorum longus muscle was performed by sectioning only one of the sciatic nerve roots. Measurements of spike resistance to tetrodotoxin in individual muscle fibers revealed denervation changes not only in the denervated fibers but also in the adjacent innervated ones. The results support the concept that products of nerve degeneration play a role in the origin of muscle changes induced by denervation.*

Sectioning of the motor nerve to skeletal muscles is followed by a number of striking changes in the electrical properties of muscle fibers, such as the appearance of sensitivity to acetylcholine (ACh) in the extrajunctional membrane (*1, 2*), the development of resistance of the action potential to tetrodotoxin (TTX) (*3*), a decrease in resting membrane potential (*4*), and spontaneous spike activity (fibrillation) (*5*). Most of these changes occur shortly (about 1 day) after the onset of nerve degeneration which follows the separation of peripheral axons from their cell bodies. Denervation changes have been attributed either to the lack of a hypothetical "neurotrophic factor" for muscle (*2*) or to the absence of nerve impulses (*6–8*). That two factors must be involved in the origin of denervation changes is now well established. On the one hand, there is no doubt that nerve impulses play a prominent role, because continuous direct stimulation of denervated muscles prevents or represses denervation changes (*7–9*). On the other hand, the participation of a factor different from nerve impulses is strongly suggested by experiments of partial denervation of the

doubly innervated frog sartorius muscle fibers (*2*), and by the importance of nerve stump length in determining the time of onset of denervation phenomena (*10*). The hypothesis that such an impulse-independent factor is a substance carried by axoplasmic transport has been tested by applying colchicine and vinblastine to the nerve. Although changes similar to those occurring after muscle denervation have been shown to occur after this procedure (*11–13*), the interpretation of these findings is not straightforward because colchicine can affect muscle fibers by a systemic direct action (*12–14*) and without producing a measurable block of axonal transport (*13, 15*).

Furthermore, although muscle inactivity is sufficient to produce denervation-like changes (*7, 16*), it has recently been demonstrated that it is not quantitatively as effective as denervation (*17, 18*). This again may suggest the participation of a "neurotrophic factor" which would still function when the muscle was not in use. However, another interpretation is possible, namely, that in the denervated muscle there is an additional factor that causes changes similar to those of inactivity. This factor might be released by the degenerating nerve (*19, 20*).

To test this hypothesis we have studied the effect of partial denervation on the extensor digitorum longus (EDL) muscle of the rat. If products of axonal degeneration could induce changes similar to those of denervation, they could affect not only the denervated muscle fibers but also the adjacent innervated ones. Partial denervation was accomplished by sectioning L5, that is, the most rostral of the two major roots that give rise to the sciatic nerve (*21*). This root was proved in preliminary experiments to give the greatest contribution to the innervation of the EDL muscle (*22, 23*).

At various times (up to 84 hours) after root section, adjacent EDL muscle fibers (30 to 50 per muscle) were examined in vitro for resistance of their action potential to TTX($10^{-6}M$) by impaling the fibers at the end-plate region with stimulating and recording micropipettes (*24*). Simultaneously, the fibers were labeled as innervated or denervated on the basis of the presence or absence, respectively, of miniature end-plate potentials (MEPP's). Our main finding was that between 56 and 72 hours after root section almost every tested fiber, whether denervated or innervated, had become to some extent resistant to TTX (see records in Fig. 1). Resistance to TTX appeared concomitantly in denervated and innervated fibers at about 48 hours and increased thereafter, with the denervated fibers soon attaining higher values (see Fig. 1G). The results obtained from all fibers tested (698 fibers, 19 muscles) between 48 and 72 hours are also summarized in Fig. 2, A and B. The class distribution of resistances to TTX are shown for the innervated (Fig. 2A, open columns), and for the denervated fibers (Fig. 2B) and compared to a population of 148 fibers tested in 13 normal control muscles (Fig. 2A, dashed columns). While the normal fibers have essentially no resistance to TTX, after partial denervation the innervated fibers behave in a way similar to denervated ones, in that they acquire various degrees of resistance to the poison.

These results are consistent with the interpretation that some signal released either by the degenerating axons or by the denervated muscle fibers somehow affects the adjacent non-denervated fibers. Alternatively, the presence of signs of denervation, such as resistance to TTX, in fibers which are innervated, could be interpreted as the result of fresh reinnervation of initially denervated fibers, by collateral sprouting. Extensive reinnervation would be required to account quantitatively for our observations, and this seems unlikely considering the short delay after denervation. Nevertheless, we sought additional criteria to rule out the above possibility. A

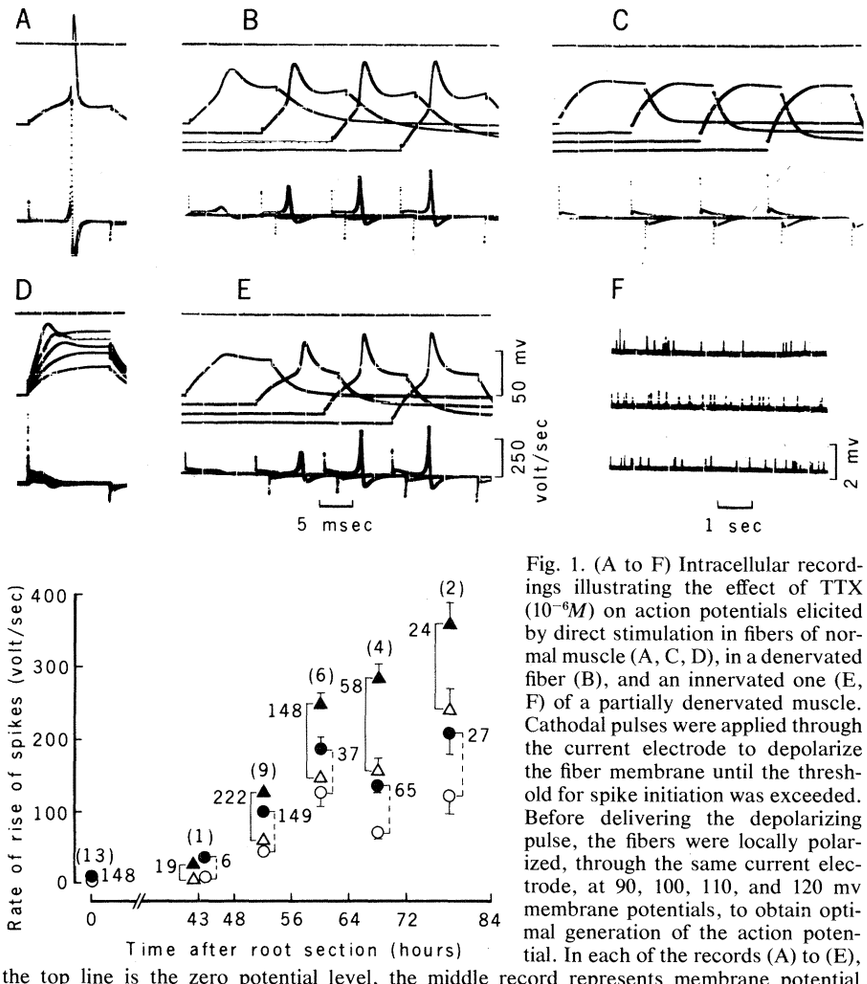

Fig. 1. (A to F) Intracellular recordings illustrating the effect of TTX ($10^{-6}M$) on action potentials elicited by direct stimulation in fibers of normal muscle (A, C, D), in a denervated fiber (B), and an innervated one (E, F) of a partially denervated muscle. Cathodal pulses were applied through the current electrode to depolarize the fiber membrane until the threshold for spike initiation was exceeded. Before delivering the depolarizing pulse, the fibers were locally polarized, through the same current electrode, at 90, 100, 110, and 120 mv membrane potentials, to obtain optimal generation of the action potential. In each of the records (A) to (E), the top line is the zero potential level, the middle record represents membrane potential changes, and the bottom record is the first derivative of the action potential obtained with an RC circuit (100 kohms, 100 pFarad). (A) Spike in a normal fiber, before TTX. (D) Normal fiber under TTX, showing complete blockade of any regenerative response at various levels of cathodal depolarization. Note the delayed rectification observed with the highest levels of depolarization. (B) Superimposed responses at 90, 100, 110, and 120 mv polarization in a denervated fiber of a partially denervated EDL muscle at 70 hours, under TTX. Greater polarization did not further improve the regenerative response. (C) Normal fiber of control muscle under TTX, showing that no regenerative response can be obtained at any level of membrane potential up to 120 mv. (E) Innervated fiber of a partially denervated EDL muscle at 54 hours, showing a regenerative response under TTX. (F) MEPP's recorded from the fiber in (E). (G) Time course of development of resistance to TTX in denervated (triangles) and innervated fibers (circles) of the EDL muscle after L5 root section. Values of rate of rise of spikes elicited at 100 and 120 mv membrane potentials are indicated by open and closed symbols, respectively. Bars indicate standard error of the mean. The number of fibers tested is shown on the side of each set of values, whereas the number of muscles is indicated in brackets.

first criterion is represented by the frequency of MEPP's, which is known to be very low in freshly formed neuromuscular junctions and which requires several days to "mature" to normal values (*25*). However, innervated fibers, 48 to 72 hours after partial denervation, had a distribution of MEPP's frequencies similar to normal fibers (see Fig. 2C) (*26*). A second and independent criterion is given by the consideration that if all the fibers in question were reinnervated fibers, one should be able to find, in addition, a conspicuous population of innervated fibers showing no resistance to TTX, that is, those fibers which receive their nerve supply from the roots left intact. The results summarized in Fig. 2A indicate that this is not the case. We first determined the percentage of normal fibers, that is, innervated by the intact roots, that one should expect to find after partial denervation. This was done by measuring the percentage of fibers with MEPP's 26 to 30 hours after section of the radicular nerve L5 in a population of 779 fibers of the EDL muscles of 15 animals (*27*). The value observed amounted to 31 percent. We then attributed to these fibers the values of resistance to TTX (that is, no or minimal resistance) found in a large population of normal fibers (148) from 13 EDL muscles of intact control rats. These combined data were utilized to construct the class distribution of resistances of normal fibers with their expected percentage of occurrence after partial denervation (dashed columns in Fig. 2A). Because the distribution actually observed was that indicated in Fig. 2A by the open columns, we conclude that 48 to 72 hours after root section the population of fibers innervated by the intact roots is almost completely transformed into a population of fibers resistant to TTX.

We also considered the possibility that our findings could be explained by inactivity of the innervated fibers, but discarded it on the basis of a series of control experiments. For example, neuromuscular transmission of the action potential was tested in several partially denervated muscles before adding TTX to the chamber, and it was found normal in almost all fibers which exhibited MEPP's. Furthermore, a sham operation, that is, isolation of the radicular nerve L5 without sectioning it, did not produce fibers resistant to TTX in the EDL muscles of two rats. Likewise, the EDL muscle contralateral to the side where the radicular nerve L5 had been sectioned remained entirely normal in two additional rats and the same was true for the ipsilateral EDL muscle of two rats in which only the dorsal root L5 was sectioned intradurally central to the dorsal root ganglion.

The signal that induces resistance to TTX in the innervated fibers may arise either from those axons and axon terminals in the muscle which undergo degeneration or from the denervated fibers. The first explanation appears more likely because resistance to TTX begins simultaneously in the denervated and in the innervated fibers (Fig. 1G), suggesting a common causal factor. A more direct argument stems from a separate series of experiments in which we produced a partial compressive conduction block of the sciatic nerve by means of silastic cuffs of critical size, without producing axonal degeneration. After several days we found, in the EDL muscle, fibers showing no resistance to TTX, that is, normal fibers, adjacent to fibers with intense resistance (attributable to inactivity), all of them exhibiting MEPP's. Accordingly, while the latter fibers showed extrajunctional sensitivity to ACh, the former did not. This strongly suggests that the presence of denervation-like changes in the muscle fiber membrane does not itself constitute the signal for inducing similar changes in adjacent fibers, but that axonal degeneration is a necessary step in the production of such a signal.

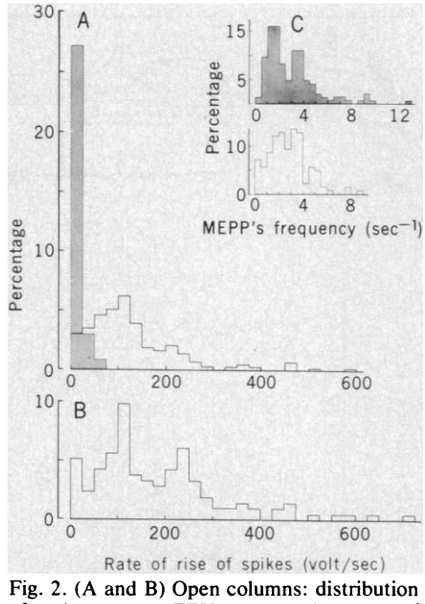

Fig. 2. (A and B) Open columns: distribution of resistances to TTX, measured as rate of rise of the action potential, in a population of 698 fibers from 19 partially denervated muscles at 48 to 72 hours. (A) Open columns: innervated fibers (19 fibers with MEPP's frequencies lower than 0.5 sec^{-1}, presumably reinnervated, are not included). (B) Denervated fibers. The number of fibers in each class is expressed as the percentage of the total number of fibers tested (that is, both innervated and denervated). (A) Dashed columns: distribution of resistances in normal fibers (see text for explanation). Action potentials elicited in all fibers at 120 mv membrane potential. (C) Distribution of MEPP's frequencies in 143 fibers from 15 normal muscles (gray columns) and in 264 innervated fibers from 19 partially denervated muscles at 48 to 72 hours (open columns).

Our findings and their interpretation based on the postulated ability of products of nerve degeneration to induce denervation-like changes, are also in agreement with recent experiments of Lømo and Westgaard showing that a transient ACh hypersensitivity develops after denervation in directly stimulated soleus muscles (*20*). As pointed out by these authors, previous findings, such as the effect of the nerve stump length on denervation changes (*10*) and the results of partial denervation in the frog sartorius muscle (*2*), formerly considered as strong evidence in support of "neurotrophic factors," can now be interpreted as being due to products of nerve degeneration. The same applies to the recently shown higher efficacy of denervation, as compared to inactivity alone, in producing resistance to TTX and fibrillation (*17*) or extrajunctional ACh receptors (*18*). A final consideration is that products of axonal degeneration might be of primary importance in the production of denervation-induced membrane changes (and subsequent reactive phenomena such as collateral sprouting) in neurons of the central nervous system: inactivity might be there a less important factor than in muscle because of the multiple synaptic drive.

A. CANGIANO, L. LUTZEMBERGER
Istituto di Fisiologia Umana,
Università di Pisa, 56100 Pisa, Italy

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21. Section of the radicular nerve was performed just outside the vertebral canal where it is easily accessible between the lateral vertebral processes, immediately cranial to the iliac spine. This is a simple procedure which avoids possible complications due to the opening of the dural space necessary to reach the ventral root.
22. The amount of denervation obtained in EDL muscles of different rats varied roughly between 40 and 90 percent, whereas there was much less variability between the two sides of the same rat. This was assessed by measuring tetanic tension following sciatic nerve stimulation at 100 pulses per second, 24 to 30 hours after root section. At this time practically all fibers innervated by the sectioned root must have been no longer responsive to indirect stimulation, because of the rapid degeneration of axon terminals consequent to axotomy (*23*). That this was the case also in our experimental condition, which was characterized by a long nerve stump, was demonstrated by sectioning all the radicular nerves giving rise to the sciatic nerve in several rats and measuring indirect tetanic tension 24 hours later. No tension was measurable in some cases whereas in others it was negligible. Accordingly, in a similar series of completely denervated EDL muscles we found no fibers, 20 hours after denervation, exhibiting MEPP's in experiments in vitro recording at the end-plate region (160 fibers tested in four muscles).
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24. The physiological solution bathing the muscles was equilibrated with 95 percent O_2 and 5 percent CO_2 (*p*H 7.2) and maintained at 28° to 29°C. The amount of resistance to TTX of each fiber was characterized by the rate of rise of its action potential given by the amplitude of the electronically recorded first derivative of the action potential.
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26. Average values were 2.86 ± 0.18 (standard error) for the junctions of normal control muscles and 2.71 ± 0.10 for the innervated junctions of the partially denervated muscles. In the latter a few fibers (19 out of 270) had indeed a MEPP's frequency that was lower (range, 0.08 to 0.43 second^{-1}) than in any of the normal fibers (see Fig. 2C) and they must represent freshly reinnervated fibers. This is also indicated by the fact that they had a resistance to TTX that was higher than that of the remaining population of innervated fibers (means, 276.8 ± 42.0 volt/second and 126.1 ± 6.1, respectively; $P < 0.001$) and even higher than that of the denervated fibers (mean, 183.5 ± 6.6; $P < .02$).
27. At this time new junctions cannot have formed, whereas MEPP's have disappeared in all denervated fibers (*22*). To minimize variability of innervation, most of the muscles (12) of this group were from one side of animals in which TTX resistance was measured later (that is, at 48 to 72 hours) on the opposite side.
28. This investigation was supported by research grants from the Muscular Dystrophy Association of America and from the Consiglio Nazionale delle Ricerche of Italy.

30 July 1976; revised 20 October 1976

Pheromonally Induced Sexual Maturation in Females: Regulation by the Social Environment of the Male

Abstract. *Social subordination, which suppresses gonadal function in juvenile and adult male house mice, also suppresses the activity of an androgen-dependent urinary pheromone that accelerates the rate of sexual maturation in juvenile females. Pheromone production may also be suppressed by the presence of pregnant or lactating females. This suggests that the social environment may influence the fertility of population females by altering urinary pheromone activity in the male.*

Social stresses resulting from high population densities may be important factors regulating mammalian populations under natural conditions. Christian (*1*) proposed that ecological and social stresses associated with high or increasing populations would, by increasing adrenal function and reducing gonadal function, interfere with an organism's growth, reproduction, and general physiological homeostasis. Although the initial emphasis in this regulatory system was on the role of intensified male-male social interactions and the associated pathologies resulting from such stress (*2*), recent studies have emphasized the inhibition of reproductive function in both juvenile and adult females as primary correlates of increasing density in a population (*3*). The social environment of the female, however, may consist of two antagonistic influences affecting fertility. On the one hand, stimuli from other females act to reduce reproductive potential by inhibiting sexual maturation in juvenile females (*4*) or by inhibiting estrus in grouped adults (*5*). In contrast, adult males produce androgen-dependent urinary priming pheromones that accelerate sexual development in juvenile females and induce estrus in grouped, anestrous adults (*6*). From a theoretical perspective the interactions between male stimulation and female inhibition of reproductive function could be important determinants of female fertility in a population.

The capacity of the urine from male mice to stimulate puberty in females is quantitatively regulated by the androgen state of the male (*7*). The chemical stimulus (pheromone) responsible for this effect is associated with the protein fraction of urine and has a molecular weight of approximately 860 (*8*). We demonstrate here how social factors that alter gonadal function in the male also alter the activity of the male's urinary pheromone and thereby may supply a mechanism for the density-dependent regulation of female fertility by the males of a population. To do this we used two experimental conditions known to cause changes in testicular function: cohabitation of dominant and subordinate males (*9*) and cohabitation of juvenile with adult males (*10*). We hypothesized that urine from adult males subordinate to their cagemates or from juvenile males exposed to adult males would be less effective in stimulating female maturation than urine from either dominant males or urine from juvenile males housed with females of the same age.

The ability of urine from male mice to accelerate the onset of puberty in females was assayed by measuring the uterine weight of prepubertal females after application of 0.03 to 0.05 ml of male urine to the oronasal groove daily for 8 days. Mean uterine weight was used as an index of pheromonal activity (*11*).

To determine if social subordination could influence pheromone activity, 34 male mice were caged individually at 21 days of age. At 70 days, 17 of these males were randomly chosen and trained as fighters to create the dominant subjects (*12*). The remaining 17 males were paired continuously with one of the trained animals. Five pairs were eliminated from the study because dominance was reversed during the experimental period. After 1 week under these conditions, urine was collected daily for 8 days from each male, pooled by group, and delivered to corresponding groups of females for assay. A control group of females received water.

Urinary pheromonal activity of subordinate animals was significantly less than that of dominant animals (Table 1). There were essentially no differences in uterine weight between those females receiving urine from subordinate males and those receiving water. We killed the remaining pairs of males and weighed their adrenal glands, testes, and seminal vesicles. Subordinate animals had significantly heavier adrenals (5.94 ± 0.46 mg compared to 4.92 ± 0.29 mg; $P < .01$, *t*-test) and significantly lighter testes (211.9 ± 15.5 mg

Table 1. Mean body weight and uterine weight (± standard error) of juvenile female mice ($N = 15$ per treatment) exposed for 8 days to urine from dominant or subordinate males that had been paired for 1 week; N.S., not significant.

Treatment	Body weight (g)	Uterine weight (mg)
Water control	17.5 ± 0.3	45.8 ± 6.8
Urine, subordinate male	18.0 ± 0.4	47.6 ± 6.7
Urine, dominant male	17.9 ± 0.3	73.4 ± 7.8*
Analysis of variance (d.f. = 2, 42)		
	$F = 0.79$ N.S.	$F = 4.72$ $P < .025$

*Mean significantly different from those for controls and urine-subordinate males [$P < .05$, least significant difference method (*17*)]