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Sequence of Motor Nerve Terminal Involvement in Acrylamide Neuropathy

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Summary. Acrylamide neuropathy is characterized by distal multifocal axonal degeneration. In this condition long and large myelinated fibers are affected more than short and thin fibers. The purpose of the present study was to investigate the sequence of nerve terminal involvement. The study was limited to axons that belonged to one type of neuron, of approximately equal diameter but differing in length. Axon terminals from α motor neurons were investigated in five muscles from rats. The results show that the initial motor nerve terminal degeneration is widespread and not restricted to terminals of the longest axons with the largest volumes. It is suggested that the variation in degree of involvement of the motor nerve terminals is determined both by differences between endplates and the regenerative capacity of neurons.

Key words: Acrylamide neuropathy – Distal axonal degeneration – Axon terminals

A number of disorders of central and peripheral neurons are characterized by distal axonal degeneration. Pathological changes in these conditions develop initially in the peripheral parts of the axons. Long and large myelinated axons are often more involved than short and thin ones. It has been suggested that degeneration in these conditions commences at the distal tips of the longest and largest axons, to ascend from there in a centripetal direction. Endings of shorter and thinner axons become involved depending upon the rate of progress of the disorder (Cavanagh, 1964).

Investigations concerning this so-called dying-back type of degeneration have been performed mainly on experimental neuropathies induced by toxins such as triorthocresylphosphate, acrylamide, p-bromophenylacetylurea and aliphatic hydrocarbons (Spencer and Schaumburg, 1977). The dying-back character and the preferential degeneration of long and thick axons in acrylamide neuropathy have been established by histological and electrophysiological investigations (Fullerton and Barnes, 1966; Hopkins, 1970; Sumner and Asbury, 1975; Sumner, 1978). Schaumburg et al. (1974), however, when studying acrylamide neuropathy in cats observed two phenomena which were at variance with the commonly held views on "dying-back". Firstly, degeneration in distal parts of axons did not commence at one site but was multifocal from the start. Secondly, axonal degeneration in Pacinian corpuscles of fore- and hindpaws developed simultaneously though axons to the forepaws are approximately $^1/_3$ shorter than those to the hindpaws.

The aim of the present study was to obtain information on the sequential involvement of terminals from axons differing in length but of equal diameter in order to help clarify the significance of axonal volume in the pathophysiology of the dying-back neuropathies. Nerve terminals of one type of neurons were examined and a method was developed which enabled comparison of relatively large numbers of terminals of this type of neuron at different sites of the body.

Materials and Methods

Animals

Eighteen female albino Wistar rats of an inbred strain (140–160 g) were used. Doses of 50 mg/kg bodyweight of acrylamide (Serva, Heidelberg) dissolved in saline were injected intraperitoneally (i.p.) 3 times weekly. Before being killed each animal was examined for neurological dysfunction (Gipon et al., 1977). Prior to biopsy animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.).

Treatment Schedules and Histological Methods

Ten animals were used for treatment with cumulative doses of nil, 200, 400, 600, and 800 mg/kg, respectively (2 animals per treatment group). The biopsies of these animals were taken from the endplate regions of the tibialis anterior and plantar foot muscles of the right hind limb.

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From 6 animals (cumulative doses of nil, 400 and 600 mg/kg, $n = 2$) biopsies of endplate regions were taken from the tibialis anterior, psoas, flexor antebrachii and masseter muscles. The lengths of axons to these muscles are approximately in proportion of 1, $^1/_3$, $^2/_3$, and $< {}^1/_3$, respectively. All biopsies from endplate regions were stained with methylene blue according to the method described by Evans et al. (1970). These animals were killed by intracardiac perfusion. All of the following solutions were buffered with 100 mM phosphate at pH 7.4 and were warmed to body temperature. Following a prewash of the vascular system with saline, perfusion was accomplished with 4% p-formaldehyde and 100 ml of a 5% glutaraldehyde solution. After perfusion, muscle strips were excised from the endplate regions of not previously operated tibialis anterior muscles. Fixation of the muscle strips was then continued for 2 h in a 5% glutaraldehyde solution.

Two normal animals were killed by intracardiac perfusion with 4% p-formaldehyde solution for 30 s and 5% glutaraldehyde solution for 15 min. In one of these animals nerves entering the masseter, flexor antebrachii and tibialis anterior muscles were excised. In the other animal nerve twigs innervating the psoas muscle were sampled under the dissecting microscope. Tissues sampled for electron microscopy were cut into strips no longer than 3 mm and were postfixed in 1% osmiumtetroxide, dehydrated and embedded in epon. One µm thick micron sections were stained with toluidine blue. Thin sections were stained with uranyl acetate followed by lead citrate.

Quantitative Methods

The estimated frequency of pathological changes in motor nerve terminal arborizations was compared in tibialis anterior and in plantar foot muscles. Estimation was carried out as follows. In each of two specified muscles from two control animals 100 terminal arborizations were inspected by light microscopy using an objective of × 40. The variation in size of focal swellings in these arborizations was assessed by estimation. Subsequently, 100 terminal arborizations in each of two identical muscles from two intoxicated animals were inspected and the number of arborizations with abnormally large focal swellings per 200 was determined.

A semiquantitative method was used to compare pathological changes in terminal arborizations (TA) in the tibialis anterior muscle with those in the masseter, flexor antebrachii, and psoas muscles. Per muscle 100 arborizations were photographed, using a Leitz phase contrast objective × 40 and enlarged to a final magnification × 1,000. The size of the largest focal swelling (LFS) per arborization was determined by measuring its largest and smallest diameters and the mean value of the two was taken to be the size of the swelling.

Results

Neurological Assessment

Behavioral changes during acrylamide intoxication were described in a previous report (Gipon et al., 1977). The following aspects are relevant within the framework of the present study. Animals treated with a cumulative dose of 200 mg/kg had a normal gait. At 400 mg/kg the gait was slightly unsteady in some animals. At 600 mg/kg the animals were ataxic. Hindlimbs were obviously affected but forelimbs were not. Frequently, one or both hindlimbs were dragged for some time. At 800 mg/kg hindlimbs were dragged for most of the time but the animals could still move forward fairly rapidly with their forelimbs. No changes of mastication were observed.

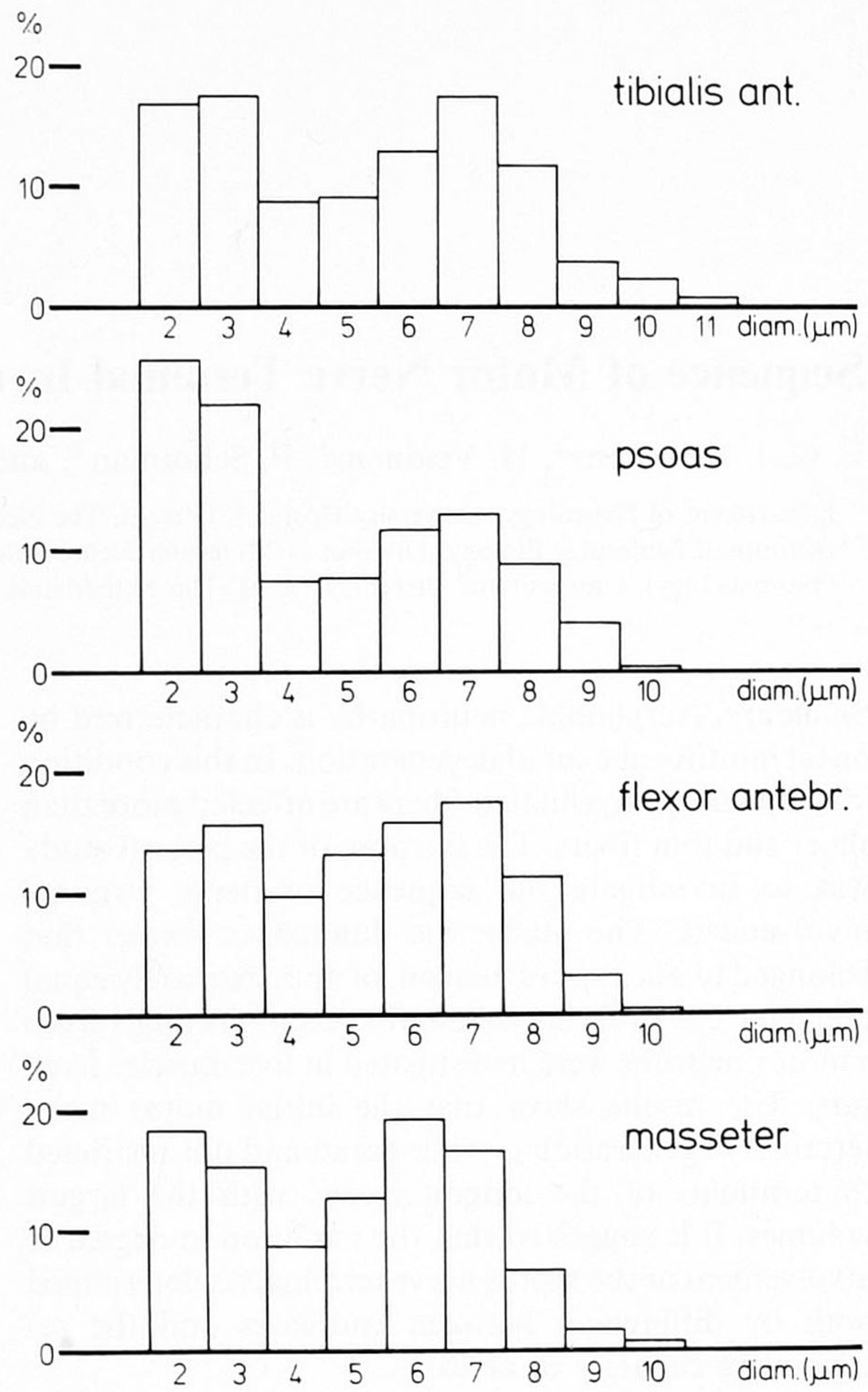

Fig. 1. Frequency histograms of nerve fiber diameters in muscle nerves. Between 480 and 500 myelinated fibers were measured in each nerve

Comparison of Nerve Fiber Diameters in Normal Rats

Frequency histograms of fiber diameters of nerves entering the tibialis anterior, psoas, flexor antebrachii, and masseter muscles were bimodal (Fig. 1). Similar peaks of small and large fiber groups were found irrespective of the axon lengths in the various nerves. The percentage of small fibers in the nerve entering the psoas was relatively high. This may be caused by a slight variation in the dissection and biopsy procedure used. As the psoas is pierced by a number of nerve trunks belonging to the lumbosacral plexus, the innervating nerves had to be dissected and were excised slightly further into the interior of the muscle than the nerves of the three other muscles (Boyd and Davey, 1968). The results showed that nerve fiber diameter in proportion to axonal length did not markedly vary and was certainly not larger in nerves with short axons than in nerves with long axons.

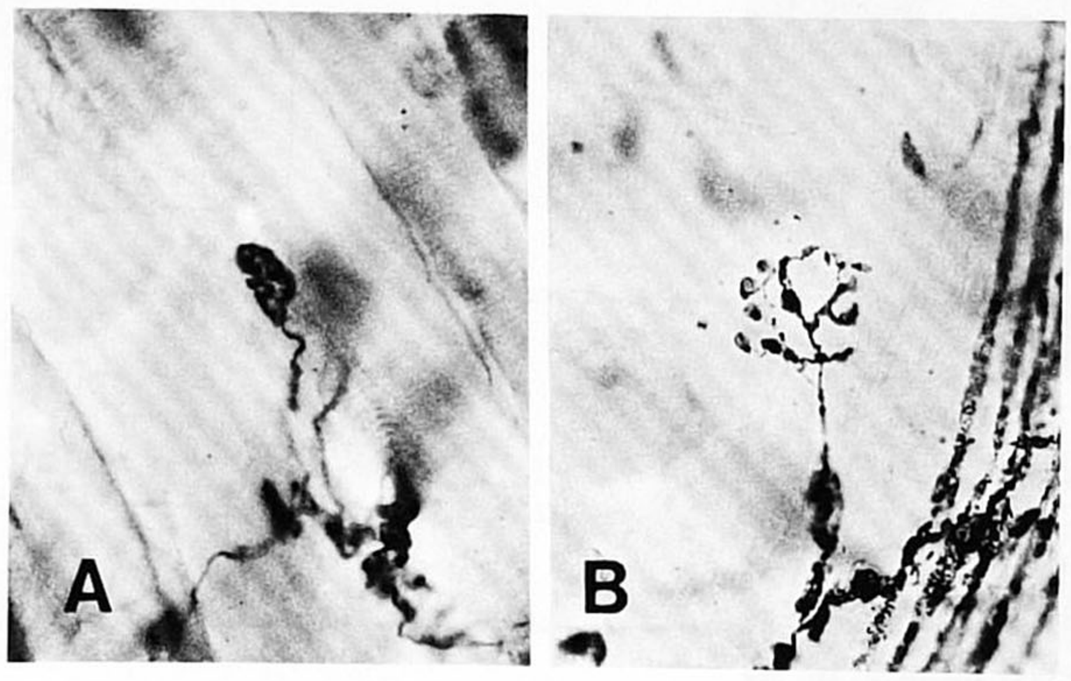

Fig. 2. Examples of terminal arborizations in flexor digitorum brevis (**A**) and tibialis anterior (**B**) muscles of normal rat. Methylene blue, × 320

Table 1. Mean diameters of LFS (μm) in 200 TA's in four different muscles from two normal rats. One hundred TA's per muscle, per rat

Muscle	Mean		S.D.
Tibialis anterior	3.42	±	0.92
Psoas	3.44	±	1.03
Flexor antebrachii	3.32	±	1.04
Masseter	3.13	±	0.99

Terminal Arborizations in Normal Rats

The sizes of the TA's were estimated to be somewhat larger in the tibialis anterior and psoas than in the flexor antebrachii and masseter. TA's in the plantar foot muscles were smaller than in all other examined muscles (Fig. 2). The myelin sheaths terminated at variable distances from the TA's. The branches of the TA's were often gracile but this again varied. In general, the presynaptic axonal swellings on the branches could be discerned without difficulty. LFS were measured in 200 TA's in each of the above mentioned muscles – the plantar foot muscles excepted – from two normal rats, 100 TA's per muscle and per rat. The smallest size was taken to be 2 μm as below this size, measurement was not accurate. Mean values differed slightly, from 3.13 in the masseter to 3.44 in the psoas (Table 1).

Terminal Arborizations in Intoxicated Rats

Changes in the TA's of the five examined muscles were qualitatively similar but differed in degree. The initial change on the light microscopical level consisted of enlargement of focal swellings. Abnormal swellings were localized at the branching sites, in the branches themselves or at the tips of the branches (Fig. 3).

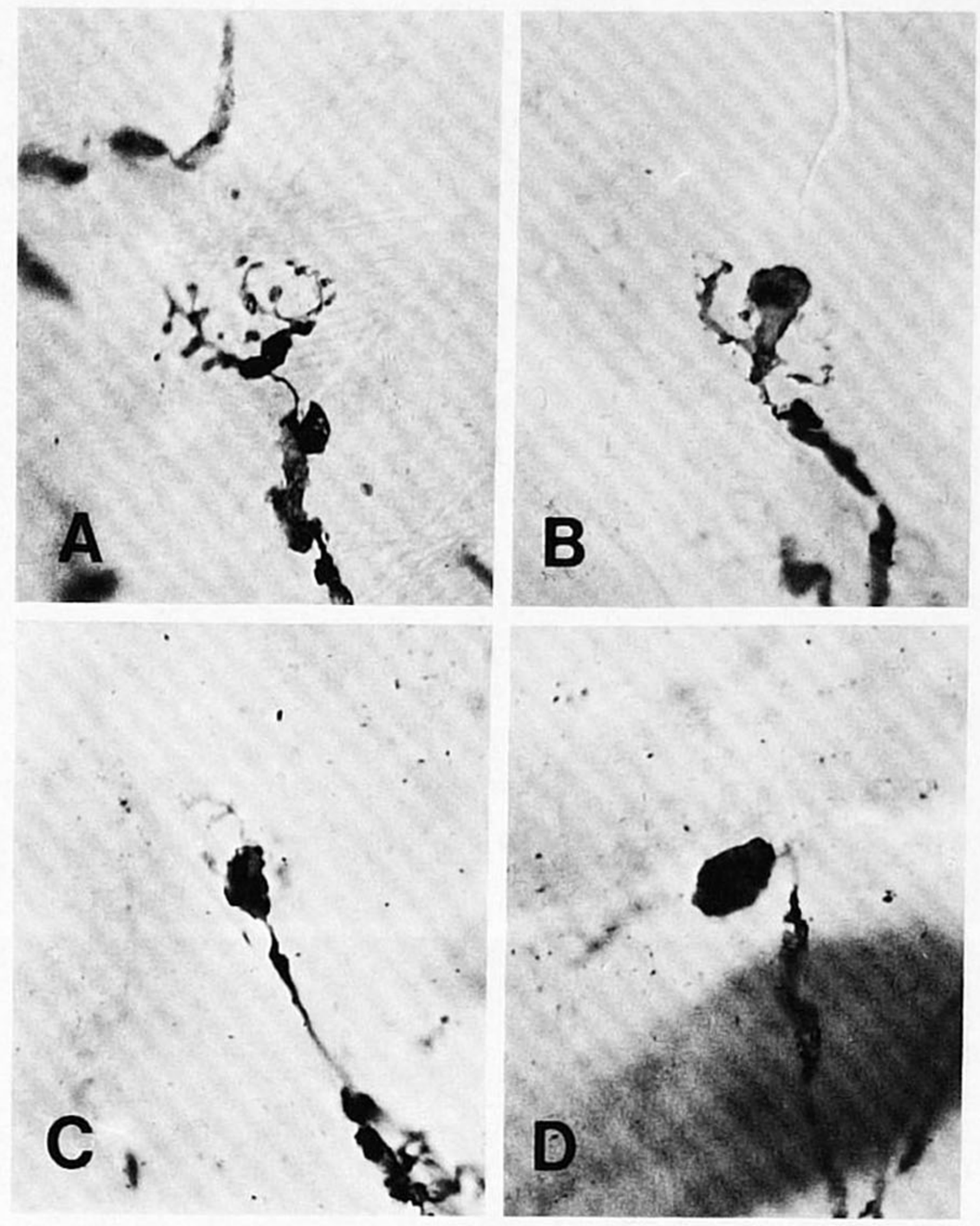

Fig. 3A—D. Terminal arborizations in tibialis anterior muscles of intoxicated rats. Acrylamide cumulative dose 600 mg/kg bodyweight. Methylene blue, × 320. **A** Slightly enlarged focal swelling at branching site. **B** One branch is fully swollen. **C** Atrophy of branches distal to enlarged focal swelling. **D** Branches distal to enlarged focal swelling have disappeared

Branches distal to large swellings were often atrophic or not present. Some TA's consisted of only a few large swellings. This was observed most frequently in the most intoxicated animals (800 mg/kg bodyweight). Preterminal nerve fibers of TA's with enlarged focal swellings appeared sometimes fully normal. Changes in preterminal nerve fibers comprised focal swellings and irregularities of the myelin sheaths. In general, such changes were not present in nerve fibers with normal TA's. Nerve fibers in the most intoxicated animals occasionally terminated in one large swelling. It was impossible in some of these cases to decide whether the swelling was the last remnant of a TA or the most distal structure of a preterminal nerve fiber whose terminal part had disappeared. Ultrastructural studies of enlarged focal swellings in TA's, showed them to contain large masses of neurofilaments (intermediate filaments) and occasional proliferated vesicular endoplasmic reticulum profiles (Fig. 4). The number of synaptic vesicles was less than normal. Remaining vesicles were gathered at one or a few sites, usually near the presynaptic membrane.

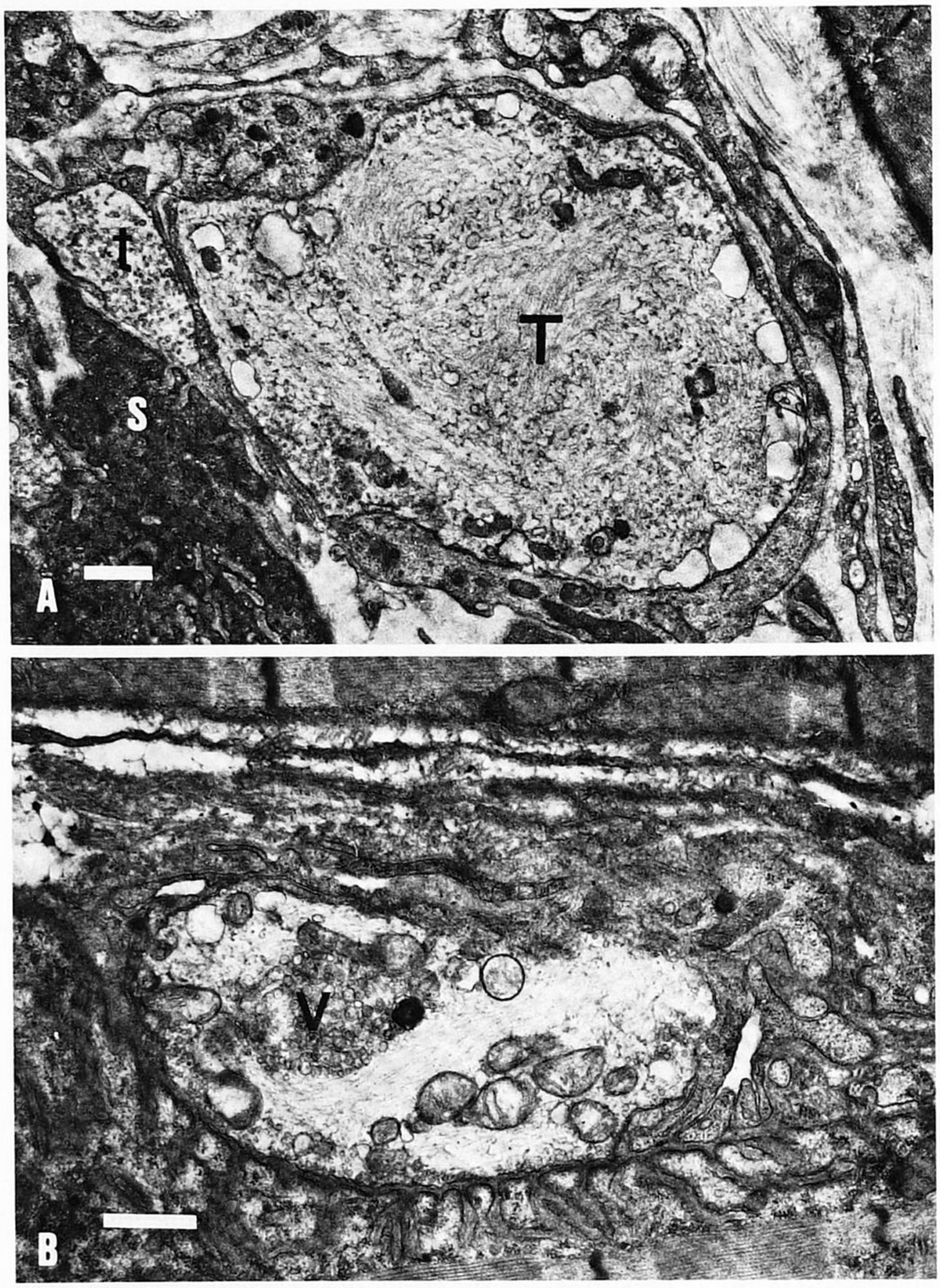

Fig. 4 A and B. Ultrastructure of enlarged focal swellings in terminal arborizations of tibialis anterior muscle of intoxicated rats. Acrylamide cumulative dose, 600 mg/kg bodyweight. The white bar represents 1 µm. **A** Swollen terminal (*T*), filled mainly with neurofilaments. Part of another terminal (*t*) containing synaptic vesicles, and a few secondary clefts (*s*) are also visible. **B** Terminal containing vesicular endoplasmic profiles (*V*) and neurofilaments

Estimated Frequency of LFS Enlargement in Terminal Arborizations of Tibialis Anterior and Plantar Foot Muscles

Less than 10 per 200 TA's in tibialis anterior muscles of normal animals showed LFS which were considered as abnormally large. Differences from normal animals, in rats treated with cumulative doses of 200 mg/kg were not seen in this muscle. At the 400 mg/kg dose level, the number of TA's with enlarged LFS had increased to approximately 20 per 200. This number was approximately 100 in rats treated with 600 mg/kg and 150 in rats treated with 800 mg/kg bodyweight. Reliable estimates of the frequency of TA's with enlarged LFS in plantar foot muscles could not be made in view of the small size of these TA's.

Measured Frequency of Enlarged LFS in Terminal Arborizations of Psoas, Flexor Antebrachii, and Masseter as Compared to Tibialis Anterior in Intoxicated Rats

In each muscle LFS were taken to be abnormally large if their size was larger than the mean diameter plus twice the standard deviation of the largest swellings in 200 TA's in similar muscles from the two normal animals (Table 1). According to this criterion, LFS of 6 µm or more were considered enlarged in all four muscles. Frequency histograms of LFS sizes in tibialis anterior were unimodal irrespective of the degree of intoxication (Fig. 5). Peak frequencies in normal rats and in rats treated with 200 mg/kg bodyweight were at 3 µm. The numbers of enlarged LFS per 200 TA's were

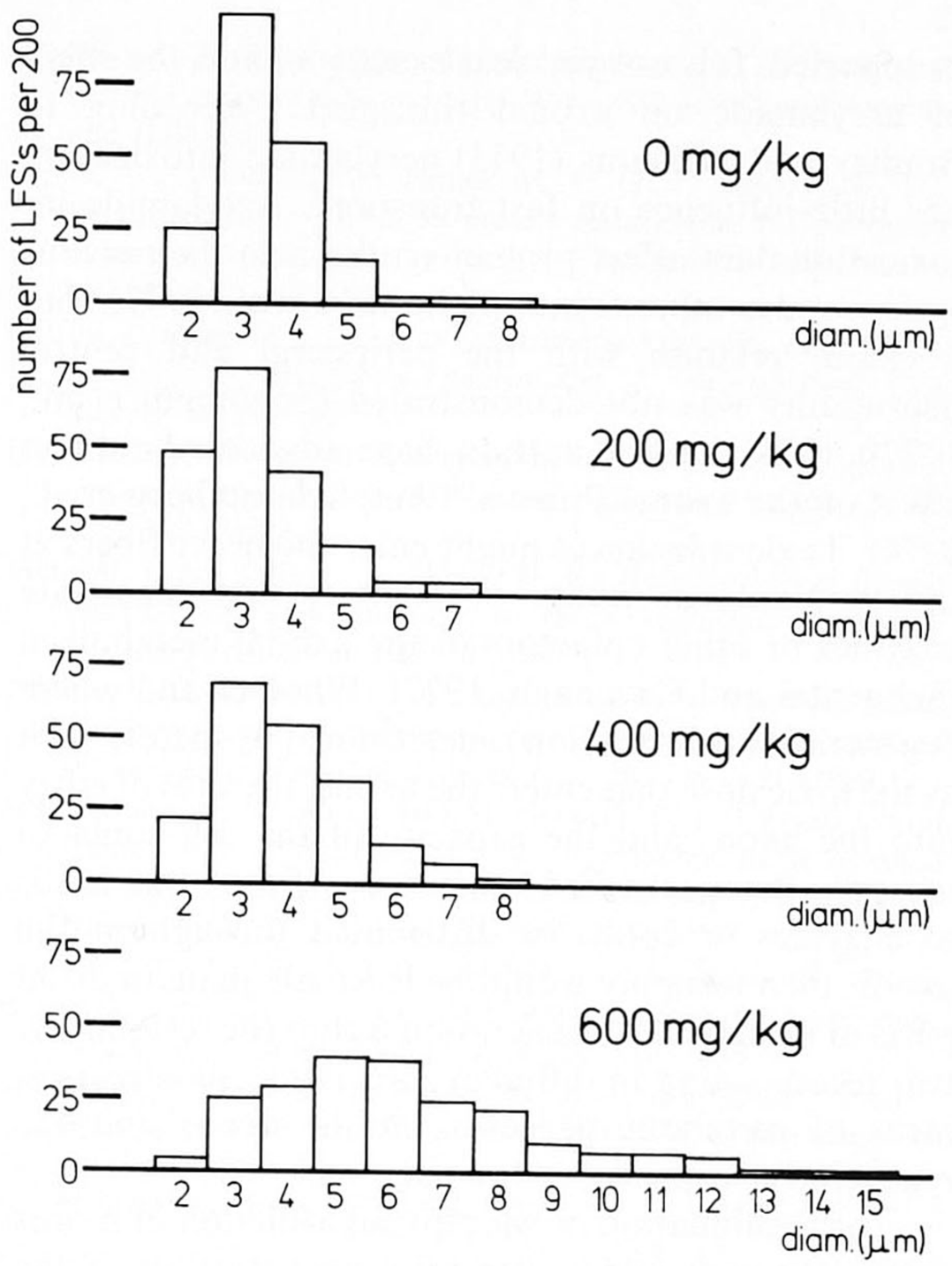

Fig. 5. Frequency histograms of LFS sizes in 200 TA's from tibialis anterior muscles. Two animals, 100 TA's per muscle and animal. Acrylamide cumulative doses in mg/kg bodyweight

6 and 8, respectively. At 400 mg/kg the peak at 3 µm was lowered and the histogram was slightly more skewed, the number of LFS in each of the size categories from 4–7 µm now being higher than in normal rats and in rats treated with 200 mg/kg. The number of enlarged LFS per 200 TA's had increased to 22 which is significantly higher than in normal rats (Table 2). At 600 mg/kg the histogram was flattened. The frequency of TA's with LFS of 2 or 3 µm had decreased markedly and peak frequencies were now at 5 and 6 µm. The frequency of TA's with enlarged LFS was nearly 6 times higher than at the 400 mg/kg dose level. Since there was no definite change from normal in tibialis anterior muscles of the two rats treated with cumulative doses of 200 mg/kg, the other muscles were not examined at this dose level. Number of TA's with enlarged LFS in rats treated with 400 mg/kg bodyweight had increased significantly in all three muscles, in comparison to normal rats. The frequency of TA's with enlarged LFS was now significantly higher in the psoas than in the tibialis anterior and masseter. Numbers in the flexor antebrachii and masseter were not significantly different from those in the tibialis anterior. Flexor antebrachii contained slightly more TA's with enlarged LFS than the masseter. In animals

Table 2. Numbers of TA's with enlarged LFS out of 200. Two animals, 100 TA's per muscle and per animal. Acrylamide doses in mg/kg bodyweight. Numbers at succeeding dose levels in similar muscles (horizontally) and numbers at similar dose levels in different muscles (vertically) are significantly different unless denoted otherwise. χ^2-test. Probabilities other than those <0.001 are given in the table

Muscle	0	200	400	600
Tibialis anterior	6 ns	8 0.01[a]	22	117
	ns			ns
Psoas	8		47	117
	ns			
Flexor antebrachii	6		34	81
	ns		0.05[a]	
Masseter	4	0.02[a]	18	54

ns = not significant
[a] probability

treated with cumulative doses of 600 mg/kg bodyweight, the frequency of TA's with enlarged LFS had again increased significantly in each of the three muscles when compared to similar muscles from animals treated with 400 mg/kg. The increase was largest in the tibialis anterior. The difference between the tibialis anterior and the psoas had evened out. The frequency of TA's with enlarged LFS in the flexor antebrachii was lower than in the tibialis anterior but it was definitely higher than in the masseter.

Discussion

Nerve fibers belonging to different populations of neurons have been reported to vary in susceptibility to acrylamide (Schaumburg et al., 1974; Sumner, 1978). This study was concentrated at the significance of axonal length and volume in the dying-back process and therefore only one type of neuron was examined. The nerve fiber diameters of the examined neurons were approximately the same (Fig. 1). We assume therefore that the differences in axonal volume of these neurons are proportional to the differences in axonal length.

Distal axonal degeneration in acrylamide neuropathy is multifocal in nature and involves the site chosen for study, i.e., motor nerve TA's. The methylene blue technique visualizes large numbers of preterminal and terminal axons and their arborizations. Inspection of single nerve fibers is possible from the site where the fibers leave the nerve fascicles. No attempt was made to quantify the changes in those stretches as the distances from fascicles to TA's are extremely variable.

The main ultrastructural features of the enlarged focal swellings within TA's of intoxicated animals are masses of neurofilaments, proliferated vesicular endoplasmic reticulum profiles and decreased numbers

of synaptic vesicles (Fig. 4). Branches distal to enlarged focal swellings tend to atrophy (Fig. 3) which may explain the observation of Tsujihata et al. (1974) of disappearence of axon terminals from some presynaptic regions and preservation of other terminals within the same endplate regions. Atrophy of nerve fibers distal to large focal swellings has been reported in hexacarbon neuropathies and is thought to be due to a local blockade of axonal transport (Griffin et al., 1977). Since there is no close correlation between the frequency of enlarged focal swellings in the TA's (Table 2) and the behavioral changes as described in this paper, other factors such as the involvement of preterminal motor axons, peripheral sensory neurons, and central neurons may be of equal importance for the development of the clinical signs of acrylamide neuropathy.

The present data on sizes of focal swellings in TA's were collected using the methylene blue technique. This technique does not allow precise measurement of actual size, as it involves the quenching of strips of muscle which by itself may cause deformation. Furthermore, the margins of the swellings as depicted on the photographs are not always sharply delineated. The method is useful, however, for size comparisons, as in the present study.

At the time intoxication causes the first obvious pathological changes in TA's of long axons in the tibialis anterior, similar changes are present in TA's of much shorter axons and these changes may even be more frequent (Table 2). Apparently, the sequence of appearence of pathological changes in rat motor nerve terminals is not determined – or not determined alone – by the length and volume of the axons. This observation confirms, for motor nerve terminals, the report by Schaumburg et al. (1974) on sensory terminals in Pacinian corpuscles. Widespread initial affliction of nerve terminals need not contradict the reported preference of the degeneration process for long and large nerve fibers (Fullerton and Barnes, 1966). It is suggested that the difference between the axons involved, may not so much depend upon the time of initial pathological changes in the terminals but rather on the rate of progress of these changes. The observation in this study that, following upon initial involvement of the motor nerve terminals, increase of TA changes occurred most rapidly in the tibialis anterior muscle, may be in support of this contention.

Distal axonal degeneration in acrylamide and other neuropathies has been suggested to result from (i) failure of the synthetic machinery in the neuronal cell bodies (ii) impaired axonal transport or (iii) direct compromise of metabolic processes in the axons (Spencer and Schaumburg, 1977). Investigations until now have been mainly concerned with the first two of these possibilities but confirmatory evidence has yet to be reported. It is not yet clear exactly what is the effect of acrylamide on axonal transport. According to Bradley and Williams (1973) acrylamide intoxication has little influence on fast transport. Acrylamide intoxication does affect protein synthesis in the nervous system and in other organs (Schotman et al., 1977a) but a causal relation with the peripheral and central neuropathy was not demonstrated (Schotman et al., 1977b, 1978). Several authors have advocated a direct effect on the axons (Prineas, 1969; Schaumburg et al., 1974). Toxic substances might enter the nerve fibers at the terminals or nodes of Ranvier and inactivate enzymes or other cofactors of the axonal metabolism (Schoental and Cavanagh, 1977). Whether and where degeneration will develop is determined by factors such as the toxic dose that enters the axons, the sites of entry into the axons and the capacity of the cell soma to resupply the inactivated substances. If the toxin binds to enzymes or cofactors distributed throughout the axons, then resupply would be least adequate in distal parts of the axons. Degeneration is thus the resultant of two forces acting in different directions: the derangement of metabolic processes in the axons and the regenerative capacity of the neurons.

The combination of widespread affliction of motor nerve terminals and preferential degeneration of the longest and largest axons is consistent with a multifactorially determined process and is as such readily explicable by the last hypothesis. According to this theory local action of acrylamide or its metabolites induces the changes in the motor nerve terminal. Endplates differ in size and in several other respects (Gauthier, 1976). It is suggested that the variation in degree of involvement of the motor nerve terminals is determined both by differences between endplates and in regenerative capacity of neurons.

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