A MORPHOLOGICAL STUDY OF THE EFFECTS OF CYCLOLEUCINE ON THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS OF THE MOUSE

by

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ABSTRACT

Cycloleucine (CL) is a very potent inhibitor of S-adenosyl methionine transferase causing a rapid decline in CNS S-adenosyl methionine levels and increase in methionine in the nervous system. CL interrupts the transmethylation reaction in the CNS similar to that caused by deficiency of vitamin B12 or folic acid.

CL was administered as a single dose intraperitoneally (2mg/g body weight) to mice of varying ages (21 days - 5 months). The 21 day old mice given CL 2mg/g body weight showed evidence of toxicity within 24 hours and thereafter developed progressive muscle weakness and ataxia. Animals did not survive longer than 1 week. Light and electron microscopic examination of the central and peripheral nervous systems, from 12 hours to 7 days post-injection showed that intramyelinic vacuolation developed in the white matter of brain and cord within 12 hours but no myelin vacuolation occurred in peripheral nerves. Axonal lesions in the distal parts of motor nerves occurred within 12 - 24 hours resulting in degeneration of intramuscular nerve fibres and terminals. Motor end-plates became denervated. There was no degeneration of motor or sensory nerves in the muscle spindles. Later there was evidence of axonal degeneration in tibial and sciatic nerves. Many dorsal root ganglion cells became vacuolated or necrotic three days after administration of CL and numerous degenerated fibres were noted in the white matter of the spinal cord, especially in funiculus gracilis. The intramyelinic vacuolation in the white matter of brain and cord persisted and became more severe during the course of the experiment.

In adult mice (6 weeks - 5 months) the pathology consisted mainly of distal motor axonal degeneration which developed at 1 - 2 days. Only slight intramyelinic vacuolation in white matter was noted. At 3 days later, numerous degenerated fibres were seen in the

posterior columns of cervical cord, especially in the funiculus gracilis.

In a second series of experiments valine (3 mg/g body weight) was given every 12 hours for 6 doses beginning either 24 or 48 hours after CL. Valine (VL) is a very effective antidote against cycloleucine and reversed its toxicity. The intramyelinic vacuolation disappeared from the white matter in the CNS leaving little residual pathology. Regenerating axons and remyelinated fibres were found in previously degenerated distal peripheral nerves and denervated motor end-plates. The reinnervation took place more rapidly in animals given VL from 24 hours after CL than 48 hours after CL.

In conclusion it can be stated that CL causes a distal motor axonopathy and sensory ganglion cell necrosis in addition to the previously reported myelinic vacuolation in the CNS. The mechanism of toxicity of CL is likely to be related to the failure of transmethylation processes affecting particularly myelin basic protein and perhaps membrane turnover at active sites of transmitter release such as the neuromuscular junction.

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Chapter 1

INTRODUCTION

Since the first description of the association of pernicious anaemia with disease of spinal cord by Leichtenstern (1884), there has been more than one century of scientific effort to try to unravel the intriguing mystery of this disease. Russell, Batten and Collier (1900) gave it the name 'Subacute combined degeneration of the spinal cord' to make it a distinct morbid entity and distinguished it from other diseases. The discovery of the effectiveness of liver extract and later of Vitamin B12 were landmarks in the history of clinical neurology and therapeutics. Although it is now treated effectively with vitamin B12, the pathogenesis of subacute combined degeneration is still not clear. It is widely accepted that in the methyl transfer pathway vitamin B12 is a crucial coenzyme of methionine synthetase which is essential for regeneration of methionine ~~synthetase~~ from homocysteine. Vitamin B12 deficiency leads to the failure of transfer of methyl groups from methyl-cobalamin to methionine via methionine synthetase (Chanarin, Deacon, Lumb, Muir and Perry, 1985). Thus the decrease of methionine levels causes the decline of S-adenosyl methionine (SAM) levels in the CNS and results in failure of transmethylation in the nervous system.

Cycloleucine (1-aminocyclopentane-1-carboxylic acid - (CL)), a synthetic non-metabolisable amino acid, is known to be a potent inhibitor of SAM-transferase which is thought to be essential for production of SAM (Lombardini, Coulter and Talalay, 1970). SAM is known to be the sole donor in many important methylation reactions

in the nervous system, involving neurotransmitters, amines and polyamines, membrane phospholipid, protein and nucleoprotein (Baldessarini, 1987). SAM is also thought to be necessary for the development and maintenance of myelination in the central nervous system. CL has been used to produce an experimental model of subacute combined degeneration of the cord (Gandy, Jacobson and Sidman, 1973). The neuropathological lesions caused by CL have been reported as vacuolation of myelin sheaths in the spinal cord in the developing rat (Ramsay and Fischer, 1978) and chicks. This is associated with a reduction in myelin basic protein (Small, Carnegie and Anderson, 1981).

Recent work by Dr Robert Surtees, Institute of Child Health, formed the starting point of this study. In his studies of children with inborn errors of the methyl transfer pathways, involving disorders of folate and vitamin B12 metabolism and deficiency of S-adenosylmethionine transferase, Surtees found that the biochemical feature common to a number of conditions was the severe reduction in CSF levels of SAM. Successful treatment of these patients resulted in restoration to normal levels of SAM. Following these observations, and in the course of developing micro-methods for the assay of SAM in CSF, Surtees administered CL, because of its known inhibition of SAM-transferase, to young mice, following the dosage used by Gandy et al (1973). These mice developed ataxia and biochemical estimations indicated a fall in SAM content of the spinal cord. In order to correlate the biochemical changes with the morphology of myelin of the spinal cord a collaborative study was started in which the light and electron microscopy would be done in the Department of Neuropathology, Institute of Neurology. It was observed that the clinical illness caused in the mice by CL was characterized by progressive ataxia. It was noted that the ataxia was not spastic but seemed to be at least partly due to a progressive

weakness of distal muscles particularly affecting the hind limbs and feet. A preliminary study of the histology of CL mice indicated that there was some pathology of the peripheral nervous system as well as of the previously reported CNS abnormalities. These observations indicated that the neurotoxicity of CL was worthy of reinvestigation in detail.

Aims of the present study:

The published studies of the effects of CL did not include detailed electron microscopy of the white matter of the CNS and although some degeneration in the sciatic nerve was described by Nixon (1976) its pathogenesis was not determined. Since no studies of the neuromuscular junction or muscle in CL-treated animals had ever been reported it was decided to examine the pathology of both central and peripheral nervous systems with a wide range of neurohistological light microscopic techniques and also by electron microscopy.

The purpose of this study is to investigate the neuropathological effects of CL in mice and to compare these with methionine and SAM levels in the central nervous system. A parallel electrophysiological study of the nervous system is also under way by Mr Jonathan Edwards.

The effect of valine in CL-treated mice is of interest. Valine was noted to be a very effective antidote against CL (Ruelius et al. 1973, Nixon et al., 1973, Nixon 1974) and could reverse its toxicity. We found that mice became active and normal again after valine was injected after CL and one of the aims of the study was to examine the central and peripheral nervous systems during the reversal of the toxic effects of CL.

Animal Models of Neurological Disease:

Animal models of neurological disease have been known to provide valuable information for clinicians and research workers to understand human neurological disease. Naturally occurring mutant animals and laboratory-induced neurological diseases not only showed their similarity to human neurological illness but also offered new insights into pathogenesis and rational modes of treatment.

For the pathologist, specimens from biopsy or post-mortem for the diagnosis of human neurological disease only can provide limited material at a very restricted period in the time course of any disease process or just its end-stage. By using a suitable animal model, the research worker can examine the earliest pathological findings and learn how the disease is developing until its final stages.

In recent years there has been an upsurge in the use of neurotoxins as tools to produce models to study the degeneration and regeneration in the neurons. Enzyme inhibitors have also been used to produce animal models to try to mimic the metabolic problems of young babies with congenital enzyme deficiencies as well as adults with nutritional or metabolic problems.

Mice were chosen as the experimental animal for several reasons. As an experimental animal, the mouse is small, easily and cheaply bred. It will permit sufficient numbers of animals at successive stages of the experiments to allow for the range of variation which is to be expected in biological systems. Another reason for using the mouse is that in such a small animal it is possible for a more comprehensive histological examination of both central and peripheral nervous systems as well as skeletal musculature to be made than would be feasible in a larger animal. Especially in these experiments

it offers advantages for the study of motor end-plates and intramuscular nerve fibres at electron microscopic level.

The experiments described in this thesis provide a more thorough and extensive reappraisal of the ultrastructural and neurohistological studies of the morphological effects of CL in the central nervous system than were previously done. They also demonstrate a new observation of distal axonopathy, predominantly motor, and degeneration of dorsal root ganglion cells in the mice. They thus shed some light on the neuropathological effects of failure of transmethylation reactions in the central and peripheral nervous system.

PART 1: Review of the literature of 1-Aminocyclopentane,1-carboxylic acid (ACPC or Cycloleucine)

Since this thesis concerns a study of the effects of 1-aminocyclopentane, 1-carboxylic acid (ACPC or CL) on the central and peripheral nervous system in the mouse, it seems appropriate to begin this review with detailed consideration of the nature, historical development and usage of this synthetic compound.

1-Aminocyclopentane 1-Carboxylic Acid(ACPC), usually referred as CL, is a synthetic, non-metabolisable amino acid. It was first prepared by Zelinskii and Stadnikov (1912) with the now classical hydantoin synthesis. Bucherer and Lieb (1934) also described a modification of this method to synthesize it.

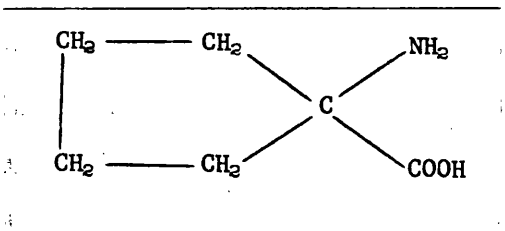

Fig. 1.1 Cycloleucine (1-aminocyclopentane-1-carboxylic acid)

CL is a white crystalline solid, hygroscopic sweet compound and has a melting point of 328-329°c. Its solubility is 5 g. in 100 ml. water. The mobility of CL in paper chromatography is strikingly different from that of the natural occurring amino acids. Leucine has the most similar R_f value but the two are readily distinguishable (Ross et al., 1961).

Antitumour activity of CL

Little attention was paid to this compound until in the late 1950's, Noll (see Ross et al, 1961) submitted a considerable number of compounds to the Cancer Chemotherapy National Service Centre in the United States for antitumour evaluation. Among these

compounds, solely by chance, was the amino acid, CL, which was assigned the accession number NSC-1026. Noll had become interested in this compound and in the alpha-substituted alpha-amino acids generally from the viewpoint of the physical properties of this unusual amino acid and the hydrolytic reaction rates of the corresponding precursor hydantoins.

Animal experiments: CL was evaluated in the Cancer Chemotherapy National Service Centre primary screening test systems which were composed of the sarcoma 180, carcinoma 755 and leukaemia 1210 mouse tumours. CL was most effective in the carcinoma 755 test. Significant inhibition of tumour growth was observed without undue weight loss. With respect to the animal test system used, it had been thoroughly established that substitution on the cyclopentane ring destroyed activity and that the N-derivatives, with the exception of the hydroxylamino and N-glycyl peptide, did not permit activities. Even as simple a derivative as the copper salt effectively prevented the appearance of biological activity in the rodent tumour systems studied.(Ross et al., 1961)

Simultaneously and independently, CL was synthesized and studied by Conners et al. (1960) in the Chester Beatty Institute, London (CB 1639) for trial as a potential anticancer agent as an outcome of interest in alpha-substituted amino acids as growth inhibitors. As a logical extension, the two alkyl chains were joined to provide first the cyclopropane amino acids and then other cycloaliphatic acids including the CL. Significant anti-tumour activity was exhibited only by compounds closely related to CL.

Inhibition of the growth of the Walker carcinoma 256 and some inhibition of the transplantable mouse sarcoma 180 and a transplantable mammary tumour in C-Bagg albino mice was

demonstrated by CL and its ester and peptide derivatives. The considerable effect of CL on haemopoiesis ~~of CL~~ was a fall in the number of circulating neutrophils which was described as a complete disappearance from circulation by the sixth day after administration of CL 750mg/kg in 4 male rats and was also accompanied by a less drastic fall in the blood platelets. The most marked physiological effect of CL was a prolonged inhibition of growth. This appeared to be largely due to loss of appetite and reduced food and water intake. Ulceration of the stomach in some of the rats was also reported (Connors et al., 1960).

With the aim of understanding the mechanism of the antitumour activity of CL, Berlinguet et al. (1962) injected C14 labelled CL subcutaneously into normal mice and cancerous mice with Ehrlich ascites tumour and sarcoma 180. With the technique of whole body autoradiography, they noted that C14 labelled CL was found to be selectively and intensely concentrated by the pancreas, tumour tissue and bone marrow.

The length of time which elapsed between injection and sacrifice of mice did not alter the distribution of CL whether the animals were sacrificed 1 hour, 24 hours and even 10 days after injection. Berlinguet et al. also put the supernatant fractions of tissue homogenates from various organs and tumours for paper chromatography study. Only one spot corresponding to the unchanged CL was found in the chromatograms. This proved that no metabolites from CL could be detected. Since CL has never been found to be incorporated into proteins, the autoradiography was due to entirely the unchanged free CL.

Clinical trials: In June, 1958, preliminary studies on CL were begun by a group of Veteran Administration hospitals in a series of 71

patients with a variety of far- advanced malignancies, including carcinoma of stomach and bowel, bronchogenic carcinoma, malignant melanoma, osteogenic sarcoma, hypernephroma, epidermoid carcinoma of the tongue, teratoma, carcinoma of bladder, rhabdomyosarcoma, carcinoma of parotid, and carcinoma of the prostate. There was little or no response noted in any patient. Only one case of malignant melanoma showed a decrease in one neck mass and an improvement in his performance status. Toxicity consisted of anorexia, nausea and vomiting in some patients. There was no evidence of marrow depression (Cancer Chemotherapy Reports,6,59-60,1959).

Bergsagel et al. (1962) reported that a group of eleven patients with multiple myeloma were treated with CL in a dose 80 mg/kg/day for 10 days and marked pain relief was reported by three of five patients who complained of this symptom. Otherwise,virtually no beneficial effects were observed of these patients.

Aust et al. (1970) administered CL at a dose level of 300mg/kg for 8 days to 147 patients with measurable tumours which were inoperable and had histologic proofs of diagnosis. Each patient had a defined lesion measured either directly by calliper or by a ruler on X-ray examination. Measurable response within 95% confidence limits ranged from 7 to 20% overall. The low response rate led them to believe that CL was not a useful chemotherapeutic agent for most form of malignant disease. Only sarcomas, especially leiomyosarcomas, showed the most promise with responses in six of fourteen patients having a measurable response of their disease and the others had signs of an initial response. Toxic effects of CL including anorexia and vomiting, progressive weakness, and of most concern, central nervous toxicity, i.e. dizziness, lethargy, slurred speech, numbness in extremities, hallucinations, ataxia, dysphonia,

staggering gait, convulsive seizure, tingling, shock, confusion, delirium, and vertigo were observed in their patients after administration of CL. All of toxic effects were reversed after the drug was stopped. Because of the intolerance to the compound, its use as an anti-neoplastic drug was abandoned.

Anti-malaria effect of CL

In 1960s much effort was done in the search for new antimalarial drugs by the Walter Reed Army Institute for Research. Several groups of compound were under consideration, including sulfones against leprosy; the sulfonamides and pyrimidine against gram-positive bacteria; hexachloroparaxylene against intestinal helminths; nitrophenyl urea against toxoplasmosis; pteridines as diuretics; and also CL as anti-neoplastic drug.

An anti-malaria effect was reported by Avido and Reutter (1969). CL could suppress parasitemia and prolong the survival time by the doses of 10, 50 and 100 mg/g in mice infected with not only the chloroquine-sensitive but also chloroquine-resistant strains of *Plasmodium berghei*. CL was one of the least toxic compound as compared with other new antimalarial drugs encountered in their research in that time. However, after these initial short reports I have not come across any further publication dealing the use of CL as an anti-malarial drug.

Immunosuppressive effects of CL

The immunosuppressive effects of CL were demonstrated by Frisch (1968) who pretreated Swiss mice with CL and thus prevented the synthesis of haemagglutinins and haemolysins for sheep erythrocytes by reducing the number of plaque-forming cells in the splenic pulp. As an immunosuppressant, CL acted on the preinduction phase, in contrast to thioquanine or cyclophosphamide which

interfered with the induction phase of humoral antibody synthesis. But the inability of CL to inhibit the secondary antibody response made it a less potent immunosuppressive drug.

Brambilla et al. (1972) also reported the significant inhibition the primary immune response to sheep blood cells and the allograft reaction in mice treated with half of the LD50 dose of CL . They found that the compound was effective in reducing the number of haemolysin-producing cells when it was administered from 21 days before to 1 day after the sheep erythrocyte antigen; it was most active in mice treated on 1 day before sheep erythrocyte antigen. The greatest effect against the reaction elicited by transplantation antigens of weak strength was found in mice receiving CL 24 hours after grafting.At this low dose level the animals did not show signs of neurotoxicity and there was some delay in the rejection of the skin graft.

Biochemical studies of CL

By trying to find the steric, electronic, and conformational of analogues of methionine essential for their function as substrates or inhibitors of the methionine adenosyltransferase reaction (with partially purified preparation of transferase from Escherichia coli, bakers' yeast, and rat liver), Lombardini, Coulter and Talalay (1970) found that CL was a very powerful competitive inhibitor of ATP;L-methionine S-adenosyltransferase(EC 2.4.2.13) (Fig. 1.2). The surprising finding that CL was a powerful inhibitor was of considerable interest. Since this cyclic amino acid lacked a region of electronegativity and had no alkyl group which corresponded in space to the S-methyl group of methionine, it must be assumed that the rigid cyclic structure provided the possibility for highly efficient van der Waals binding that could compensate for the lack of the two above mentioned features.

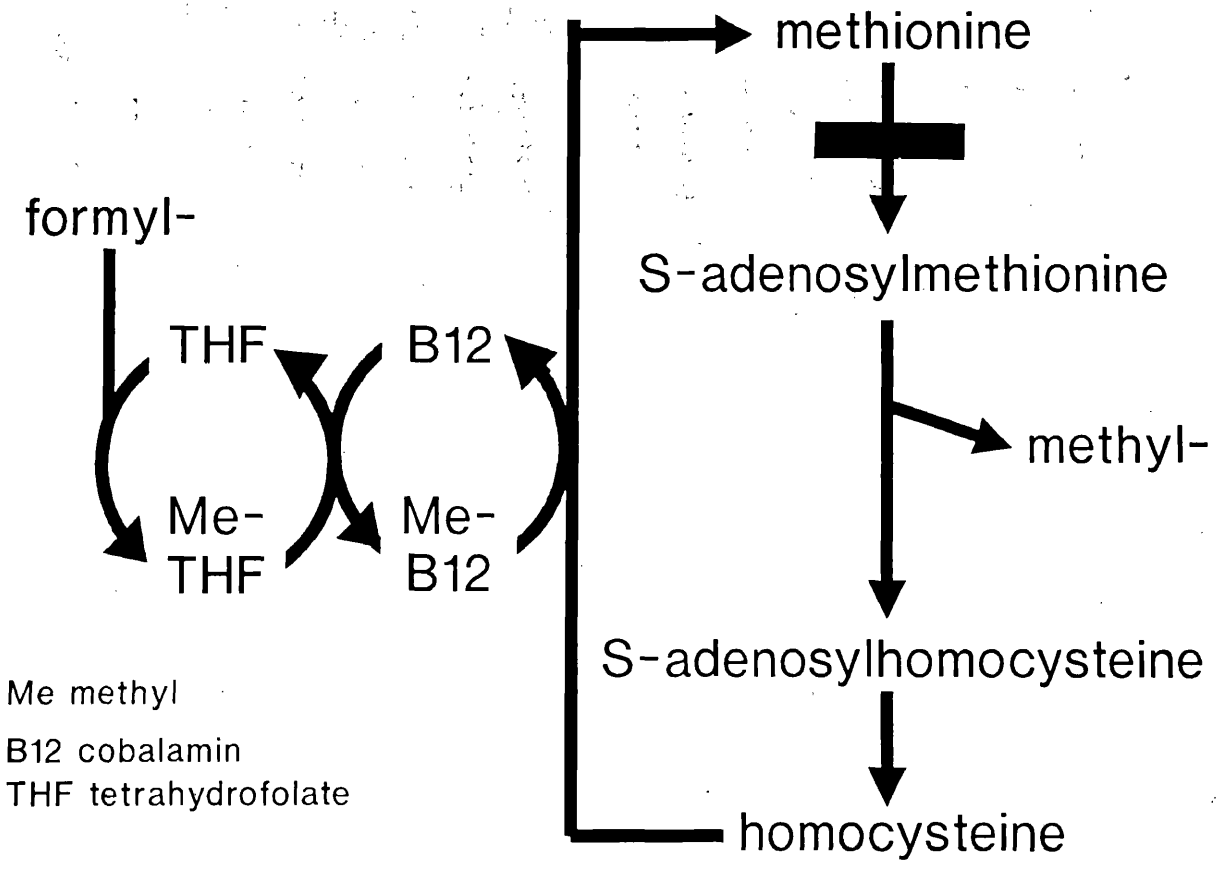

Fig.1.2 The methyl-transfer pathway, the ▬ block shows the enzyme inhibited by cycloleucine (methionine adenosyltransferase).

They thought the presence of amino and carboxylic groups on a 5-membered cyclopentane ring appeared to be a necessary condition for inhibition of the synthesis of S-adenosyl-L-methionine(SAM) in competition with L-methionine and the ring size of cyclic compounds was also absolutely critical.

Lombardini and Talalay (1973) injected CL intraperitoneally into rodents with transplantable neoplasms, including the Walker 256 tumour of the rat, and the Lewis lung tumour and B-16 melanoma of C57 black mice, and found that the enzymatic activities of S-adenosylmethionine transferase were significantly inhibited. A dramatic elevation of L-methionine levels was noted in all tissues examined, i.e. liver, spleen, kidney, brain, plasma, Walker 256 tumour, and Lewis lung tumour. There was also a 20-40% depression of SAM levels in most normal and malignant tissues, including spleen, kidney, pancreas, brain, adrenal, Walker 256 tumour of rats, and Lewis lung tumour and L1210 tumour of mice. In contrast to the finding in other tissues, the SAM levels of liver were invariably increased.

Relationship between CL effects and vitamin B12

For many years it has been known that subacute combined degeneration of the spinal cord in man, usually associated with megaloblastic anaemia, was due to a deficiency of vitamin B12 and/or folate. By an accidental finding when they were doing the study of the blocking of biosynthesis of lecithin in the lung, Jacobson, Gandy, and Sidman (1973) found the experimental mice showed a neurological disease. After a single injection of CL (8-40 mg per adult mouse) the mice became less active and then "impaired proprioception" became manifest. When moving on a inclined surface their toes failed to grip. Then the gait became ataxic even on a flat surface. Finally, motor strength decreased "without paralysis". The

disease progressed uniformly, irrespective of strain and sex. Death occurred in all animals between one to seven weeks and was dose dependent.

Jacobson et al. (1973) thought the histopathology of the central nervous system of CL-treated mice was indistinguishable from that found in human subacute combined degeneration. They observed that the myelin sheaths of thickly myelinated fibres in the spinal cord degenerated with swelling of the axons. There was no cellular infiltration and no glial infiltration. The cell bodies remained intact. Degeneration of axons might occur later. Those lesions were patchy in distribution, but often symmetrical. The same type of lesion was also found scattered in the white matter of medulla, cerebellum, brain stem and cerebrum. Thus a single dose of CL could cause a myelopathy with the characteristic features of subacute combined degeneration of the spinal cord, both in symptoms and microscopic pattern. But no electron microscopy was done in this study.

Jacobson, Gandy and Sidman noted the similarity between the effects of the absence of vitamin B12 and the enzyme blocking effect of CL. Both caused a reduction in synthesis of SAM, CL by its inhibition of SAM transferase, as shown by Lombardini, Coulter and Talalay (1970) and the human disease of subacute combined degeneration being the result of inhibition of methionine synthetase because vitamin B12 acted as a co-factor for this enzyme (fig. 1.2). The details and discussion of this study was published by Jacobson and Gandy (1979).

Gandy, Jacobson, and Sidman (1973) presented another paper of this model to a meeting of the Physiological Society. They suggested that CL by preventing the regeneration of methionine from homocysteine resulted in interference with the formation of SAM.

This might disrupt the cycle of transmethylation reaction, resulting in lack of methyl groups to synthesise choline. Choline is an essential component of lecithin, which is an important constituent of myelin.

Nixon, Jacobson, and Sidman (1973) demonstrated that L-valine administered intraperitoneally completely reversed the toxic effects of CL and reduced tissue levels by completely inhibiting CL reuptake by renal tubules. They speculated that with block of methyltransferase(s) either directly or via elevated S-adenosyl-homocysteine levels, transmethylation reactions crucial for myelin maintenance might be impaired. Among other effects phosphatidyl-choline catabolism might exceed synthesis.

Nixon (1976) reported primary degeneration of axons in the cerebellum, rostral spinal cord and peripheral nerves in CL-treated mice in which significant protein loss was noted, particularly that of cerebellum and cervical spinal cord following 2 mg/g of CL. The sulfatide content of spinal cord and peripheral nerves was reduced but that of brain was normal. The sphingomyelin level was increased. Nixon, Suva, and Wolf (1976) found that developing unmyelinated cerebellar cultures were more sensitive to CL than mature cultures and newly myelinated axons appeared to be particularly affected. By supplementing the medium with amino acids but not vitamins, the developing unmyelinated and newly myelinated axons were protected from toxicity at lower concentrations of CL and afforded considerable protection against the highest concentration. Nixon et al. speculated that the protective effect of amino acids could not be accounted for by inhibition of intracellular CL transport, but they thought the results were considered in terms of other evidence indicating defective protein metabolism in CL-treated mice.

Crang and Jacobson (1980) extended their previous studies of

acute effects of CL in the CNS in mice to the subacute effects of multiple, well-spaced sub-lethal doses (0.3mg/g) and described the action of CL on myelin basic protein (MBP) arginine methylation in the CNS. They thought that this would enhance more similarity with the human SCD than the myelin lesion in mice given lethal doses of CL. By extending the survival of mice given repeated sub-lethal doses of CL, the development of neurological signs was more protracted than observed in mice given lethal doses(>0.5mg/g). However, the extent of the histopathological lesions was similar to that found in their acute toxicity studies. Parenteral administration of vitamin B12 and folic acid had no effect on the development of the condition. By labelling the methyl groups with C14 in methionine and SAM, Crang and Jacobson demonstrated that the methylation *in vitro* of arginine residue in the MBP by mouse spinal cord cytoplasmic enzymes was inhibited by CL when the source of labelled methyl groups was methionine, but not when the source was SAM. They proposed that CL was capable of inhibiting MBP methylase from mouse spinal cord by depleting the levels of SAM synthesised from methionine and ATP.

Ramsay and Fischer (1978) administered CL to developing rats. With electron microscopy they found considerable myelin splitting in the spinal cord in one set of animals first receiving CL at 7 days of age, while the spinal cords from animals initially injected with CL at 4 or 22 days of age appeared comparable to control tissue. They suggested that the age at onset of treatment may play an essential role in the formation of a morphological lesion. They also noted the decrease in ethanolamine phospholipid levels, especially ethanolamine plasmalogen, and a relative increase in the saturated fatty acid content of ethanolamine phospholipid which had been also noted in a variety of demyelinating diseases.

Ramsay and Fischer (1978) also discussed the biochemical

model of vitamin B12 deficiency. There were at least two vitamin B12 dependent mammalian enzymes known. One was 5-methyltetrahydrofolate methyl transferase (SAM: L-homocysteine-S-methyltransferase. EC2.1.1.10), which required the methylcobalamin form of vitamin B12 and which CL blocked. The other was methylmalonyl-CoA mutase (Methylmalonyl-CoA: CoA-carbonylmutase. EC5.4.99.2), which required the 5-deoxy-adenosylcobalamin form of vitamin B12 and which, if inhibited, increased propionyl-CoA and methylmalonyl-CoA and the corresponding free acids. This enzyme did not appear to be affected by CL. Inhibition of methylmalonyl-CoA mutase resulted in an increase of propionic acid which could serve as a substrate for odd-chain fatty acid synthesis. Some of the vitamin B12 deficiency patients were reported to have elevated amounts of odd-chain fatty acids. By the evidence that administration of massive amounts of sodium propionate in addition to CL did not result in an odd-chain fatty acid increase in the CNS, Ramsay and Fischer suggested that the lack of methyl transferase, but not the blockage of the methylmalonyl-CoA to succinyl-CoA reaction, in the CNS that was responsible for lesion in the human subacute combined degeneration.

Small, Carnegie, and Anderson (1981) injected large doses of CL into chickens and with electron microscopy they observed the presence of clear vacuoles in many of the myelinated fibres throughout the brain. The myelin vacuoles were noted to be formed by splitting in the myelin sheaths at the intraperiod line. Myelin basic protein content of whole brain in CL treated chickens was 50% lower than that in the controls. The methylation of arginine was also 50% lower than in the controls. Myelin basic protein has been proposed as a "structural cement" in maintaining apposition of the cytoplasmic surface of the oligodendrocyte plasmalemma in myelin . Small and Carnegie (1981) suggested that a possible biochemical cause of the

myelopathy associated with vitamin B12 deficiency was the inhibition of methylation of myelin basic protein, not the inhibition of methylation of phosphatidylethanolamine proposed by Jacobson, Gandy and Sidman (1973).

Deacon et al. (1986) injected CL to rats and also found the expected fall in methylation of residue 107 arginine in myelin basic protein but not in the rats exposed to nitrous oxide for 7 days or fruit bats fed vitamin B12 free diet.

Pathology of cycloleucine-treated animals reported previously

Ross et al. (1961) administered CL to different animals and observed the following results. In the mice treated with 1000mg/kg single dose and sacrificed 6 days later, the histological examination showed cloudy swelling to marked fatty degeneration in the liver, tubular degeneration in the kidney, thymic atrophy in one animal, slight bone marrow atrophy and congestion. In the rats with 500 mg/kg i.v. single dose, histology showed atrophy of bone marrow, liver and pancreas, slight atrophy of testes, adrenal cortical hypertrophy and focal myocardial degeneration.

Given repeated doses of CL, rats showed atrophy of bone marrow, liver and pancreas, adrenal congestion and marked erythroid shifts with myeloid/erythroid ratios of approximately 1:8; dogs showed slight demyelination of cerebellum, splenic haemosiderosis, a myeloid/erythroid ratio of 5:1, atrophy of bone marrow, liver, pancreas and testes, moderate damage to the kidneys and adrenals, and large stomach ulcers.

Monkeys treated with repeated doses of CL showed petechial haemorrhages in the mucosa of the cardiac stomach, atrophy of the

spleen, intussusception of the ileum, haemorrhagic lesions in the ileum both above and below the obstruction and slight erythroid depression.

Pharmacological studies of cycloleucine

Ross et al. (1961) reported pharmacological studies of CL and certain of its derivatives in mice, rats, guinea pigs, dogs, and monkeys, and found the LD50 to be 309 mg/kg for mice, 290-340 mg/kg for rats, 140 mg/kg for guinea pigs and 300 mg/kg for dogs. It was comparatively well tolerated in the rhesus monkey.

CL has also been used as a model to study the transport of amino acids by Christensen and Jones (1962) and they observed that it was not metabolisable in vivo and had a very low urinary excretion rate. Ruelius et al. (1973) administered large and frequent doses of L-valine to CL pretreated mice resulting in a great increase in urinary excretion of CL significantly reducing its toxicity considerably. Blasberg and Lajtha (1965) suggested that many toxic effects of CL related to its competitive interactions with other large neutral amino acids, like phenylalanine, leucine, isoleucine, valine, tryptophan, and methionine. CL was noted to interfere with intracellular transport of these amino acids (Oxender and Christensen 1963) and impair food intake which further altered tissue amino acids pools . Sterling and Henderson (1962) studied the mechanism of action of CL in ascites tumour cells *in vitro* and found that it did not replace natural amino acids in protein synthesis nor did it interfere with the endogenous synthesis of natural amino acids from glucose. CL inhibited the uptake of DL-leucine and DL-valine in a competitive manner. High concentrations of CL partially inhibited the incorporation of amino acids into proteins in ascites tumour cells *in vitro* possibly due to inhibition of the transport of the amino acids into the cells.

Nixon (1975) has reviewed the pharmacological studies of CL in mice. In his experiments, he found the LD 50 range for CL lay between 0.35-0.40 mg/g and the latter was also the concentration required for behavioural signs of neurotoxicity. Most mice died in the fourth and fifth week following 0.4-0.6 mg/g or in the second week following 1 mg/g or more.

The half-life of CL in the plasma was extremely long. At the threshold lethal concentration (0.4-0.5 mg/g) the plasma CL level at 24th days was not significant different from that at the first day. The highest CL doses of 1-3 mg/g resulted in nearly similar CL levels by the fourth day.

The CL distribution ratio in brain and spinal cord was not significantly from unity. Accumulation against a concentration gradient occurred in kidney, in spleen to a greater degree, and to a much greater degree in pancreas than those reported by Berlinguet et al. (1962) in normal and cancerous mice. The distribution ratios for CNS tissue, kidney and spleen did not change as a function of plasma CL concentration or of time between 4 to 40 days after its administration.

The elimination of CL in the urine in the mice administered given less than 0.5 mg/g resulted in extremely low level in the urine. The urinary CL level was considerably higher at 1 mg/g but still represented a small fraction of the injected dose. The 2-3 mg/g doses of CL marked a turning point in the renal handling of CL in that large quantities appeared in the urine following these doses. Peaks of urinary CL concentration were observed at 0-6 hour and 9-20 hours, occurring concomitantly with elevations of plasma CL.

The brain and spinal cord tissue did not accumulate CL against a concentration gradient but did achieve levels equivalent to the plasma concentration, consistent with recent evidence that "large neutral" amino acids were transported in neutral tissue by facilitated diffusion (Richelson 1974).

Systemic toxicity was found to be highly correlated with hepatic CL levels. But it is unlikely that neurotoxicity was secondary to hepatotoxicity since it was toxic to organotypic cerebellar cultures at concentrations comparable to in vivo brain concentrations measured in this study (Nixon et al., 1976).

PART 2: The diseases caused by failure of transmethylation

1.Vitamin B12 deficiency

It has been more than one hundred years since the first time Leichtenstern (1884) described the association of pernicious anaemia with the disease of the spinal cord although he mistakenly interpreted it as the anaemia developing in the course of tabes dorsalis. Lichtheim (1887) later recognised this disease was a distinct morbid entity and considered it as an instance of cord changes associated with anaemia. The spinal lesions of pernicious anaemia were different from those of tabes. The lesions had a subacute course and involved the dorsal and anterolateral tracts with abundant lipophages in the degenerated tracts of the cord; there was no thickening of the leptomeninges and only slightly atrophy of the dorsal roots in the lumbar region. Minnich (1892) also reported slight degeneration in the posterior and lateral columns in five fatal cases of ~~pervious~~ pernicious anaemia with no neurological symptoms or signs.

Subacute combined degeneration of spinal cord: In the beginning of this century, the cause of this disease was still enigmatic. With the strong feeling of the need to give a proper name of this disease to distinguish from the other terms i.e. "Ataxic paraplegia", "Friedreich's Ataxy", "Cerebellar Heredo-Ataxy" and "General Paralysis of Insane", Russell, Batten and Collier (1900) named the disease "Subacute combined Degeneration" to establish the affection as a separate and distinct morbid entity. They thought the " Subacute" would be sufficient to exclude most other forms of combined degeneration of spinal cord and facilitated its study and freed it from the entanglements by which it was hampered, in consequence of the tracts that so many different diseases of the spinal cord were characterised by combined degeneration of the tracts in the cord of different function.

Clinical presentation: Russell et al. (1900) thoroughly investigated 9 clinically diagnosed patients in the wards of the National Hospital, Queen Square for three years with post-mortem examination of 7 of them and further three other cases in the appendix. They summarised three stages of the disease as:

(1) A stage of slight spastic paraplegia with slight ataxy and marked subjective sensation in the lower limbs.

(2) A stage of severe spastic paraplegia with marked anaesthesia of leg and trunk.

(3) A stage of complete flaccid paraplegia; absent knee jerks; absolute anaesthesia; rapid wasting and loss of faradic excitability in the muscles of the paraplegic region; increase of superficial reflex excitability; absolute incontinence of both sphincters and oedema of the lower extremities and trunk.

Pernicious anaemia in some of these cases was a striking feature in the symptomatology, while in others, it was slight and unobtrusive.

In that time they could not find the cause of the disease and all the treatments including mercury, iron, iodide of potassium, arsenic, strychnine, quinine, salicylate, and various animal extracts which had been given, were not beneficial to the patients.

Pathology: With the post-mortem examination of seven cases, Russell et al. (1900) noted that the disease mainly involved the mid-thoracic segments of the cord, where a zone of destruction of white matter might extend all around the cord, affecting not only the long tracts but endogenous and exogenous fibres alike. in severe cases only fibres adjacent to the grey horn were spared. Degeneration diminished towards cervical levels of the cord, particularly in the anterolateral columns. in the upper cervical segments only the posterior columns, especially the funiculus gracilis, and the

spinocerebellar tracts were demyelinated. At lower lumbar levels, lesions affected only the pyramidal tracts. This descending tract was usually unaffected in the region of the medullary pyramids, although some degeneration was occasionally seen at this level.

Russell et al. found that the earliest process that took place was swelling of the myelin sheaths, the axis-cylinders remained apparently unaltered. Then the swollen sheaths underwent fatty degeneration, and this in turn became absorbed, and at the same time the axis cylinders disappeared, a space being thus formed, simply surrounded by the glial tissue of the cord. This space was often of considerable size from the fact that the individual spaces became fused into one another, and in those portions of the cord where destruction had been most acute nothing was left but the glial tissue which formerly supported the now atrophied fibres forming the vacuolated areas. The axonal degeneration was seen at the primary site of the lesion (usually the mid-thoracic segments), leading to subsequent Wallerian-type degeneration of the long tracts in cervical and lumbar segments. Loss of fibres resulted in the formation of the spaces, giving the tissue a loose, vacuolated appearance.

The neuropathology of Vitamin B12 deficiency has been reviewed by Duchen and Jacobs (1984). The distribution of the lesions is remarkably constant. The earliest changes occur in the centre of the posterior columns, producing irregular oval-shaped areas of pallor. At the same time, or slightly later, lateral column lesions appear at the surface of the cord immediately anterior to the posterior horns. More often, small distinct foci of degeneration appear on the surface of the cord or in the lips of the anteromedian fissure. Early lesions in the posterior and anterolateral columns do not have the distribution of tract degeneration, but may eventually involve much of the posterior columns and the pyramidal and spinocerebellar tracts.

The peripheral nerves were thought to be normal or only slightly degenerated by Russell et al. (1900) in their patients, either by the Marchi or Weigert-Pal methods. Only in one case, there was very considerable degeneration of the nerve fibres - a change that not only occurred in the large nerves, but which also involved the finer branches of the nerves within the muscles, and which appeared to affect not only the motor nerves but also those of sensory origin within the muscle spindle. The degeneration of the nerves in this case Russell et al. considered as a late manifestation of the disease, as this patient lived a long time after the disease was diagnosed. There is still much controversy about the peripheral pathology in the vitamin B12 deficiency patients. Pallis and Lewis (1973) emphasised that the sensory neuropathy could not be explained by the spinal lesions only but also by peripheral neuropathy. After an extensive review of the literature and of his own cases, Victor (1984) concluded that the neurological manifestations of pernicious anaemia are due primarily to the spinal lesions. In the course of the myelopathy the peripheral nerves may also be involved, at least in humans, but the latter affection is much less frequent than the former and less significant clinically.

The muscle fibres in these patients showed a considerable variation in their degree of atrophy. In the most extreme case the muscle fibres were much reduced in size and had lost their striations. Increase of connective tissue was noted, but fatty degeneration was exceptional. In contrast to the atrophied condition of the extrafusal muscle fibres, the muscle spindles appeared remarkably well preserved. The capsule and general appearance of the spindle was normal; the intrafusal muscle fibres were of normal size, retained their striation, and only a few exhibited slight fatty degeneration (Russell et al. 1900).

The discovery of Vitamin B12: The progress of the understanding of this disease was very slow. A quarter of a century later, Minot and Murphy (1926) found that oral administration of liver had a beneficial effect in the patients with pernicious anaemia. Then by the empirical discovery of the value of liver in the treatment of pernicious anaemia, Lester-Smith (1948) purified two anti-pernicious anaemia factors from ox liver. From four tons of liver, barely one gram of red material which was an amorphous solid and had about the colour of cobalt salts was extracted. It was also effective in three cases of subacute combined degeneration of the spinal cord. Independently isolating an anti-pernicious factor, Rickes et al. (1948) found a crystalline red compound which was highly active for producing haematological responses in patients with pernicious and other anaemias. This compound was called Vitamin B12. They found that Vitamin B12 appeared to be a cobalt coordination complex which, having 6 groups about the cobalt atom, could involve one or more organic moieties. The red colour of Vitamin B12 appeared to be at least in part associated with its cobalt-complex character.

The finding of the Vitamin B12 marked the culminating point of a great scientific achievement and reduced the mortality due to the neurological effects of deficiency by prevention, by cure of early disease and by arrest and reversal of the fully developed disease.

Biochemistry:

Vitamin B12 is a corrin ring containing coenzyme which is required for the normal growth and development of the nervous system. There are only two Vitamin B12 requiring enzymes which have been identified in mammalian systems. They are methionine synthetase, which regenerates methionine from homocysteine, and methylmalonyl CoA mutase, which converts methylmalonyl CoA to succinyl-CoA.

Methyl Transfer Pathway: Vitamin B12 has been known to be a crucial coenzyme of the methyl transfer pathway which is regarded as very important in many areas of metabolism in the central nervous system. Single carbon groups enter the pathway as formal group (CHO) and bound to tetrahydrofolate (THF) and are reduced in a number of steps to methyl-THF, the methyl group then being transferred to homocysteine catalysed by the cobalamin (B12)-dependent enzyme methionine synthetase. Methionine is activated to form SAM in a reaction by SAM-transferase. SAM acts as the sole methyl donor in reactions catalysed by a variety of methyltransferases. The purine and pyrimidine, which are essential to the synthesis of DNA and RNA for highly proliferative tissue such as haemopoietic tissue and epithelial surfaces i.e. the tongue and gastrointestinal tract, are also known to be derived from tetrahydrofolate in this methyl transfer pathway.

SAM: SAM is known to be the sole methyl donor in many important methylation reactions in the nervous system, involving neurotransmitter, amine and polyamine, membrane phospholipids (Hirata and Axelrod 1980), protein and nucleoprotein (Baldessarini 1987). SAM is intimately linked to the folate cycle and vitamin B12 metabolism, which provides many of the methyl groups required for the SAM mediated methylation reactions, via the synthesis of methionine by the enzyme methionine synthetase and then the SAM by the enzyme SAM transferase. Blocking of either of the methionine synthetase by lack of folate or vitamin B12, or by inactivation of vitamin B12 by nitrous oxide, or inhibition of the SAM transferase by CL will lead to significant decline of SAM and thereby inhibit the transmethylation reaction in the nervous system. SAM was also proposed to be a methyl group donor which was essential in the conversion of phosphatidylethanolamine to phosphatidylcholine. Lacking of SAM caused failure of methylation and resulted in blocking

of biosynthesis of phosphatidylcholine which required three methyl groups donated from SAM (Gandy,Jacobson and Sidman 1973). Phosphatidylcholine was known to be an important component of myelin and plasma membrane. Methylation of phospholipids in plasma membrane by SAM was reported to decrease the membrane microviscosity and normalise aged membranes. The biochemical importance of SAM was discussed by Baldessarini (1987).

SAM was also considered to be the crucial methyl donor for transmethylation of arginine 107 in myelin basic protein and to maintain the normal configuration of the myelin sheath. Reduced SAM levels can be achieved by CL which was noted to cause inhibition of methylation of arginine of myelin basic protein in chicks and thereby caused demyelination (Small and Carnegie 1981).

Coenzyme of Methylmalonyl-CoA Mutase: It was thought that a deficiency of methylmalonyl-CoA mutase may be the cause of the neurological disease in vitamin B12 deficiency. An accumulation of methylmalonyl-CoA(Cox and White 1962) and its immediate precursor, propional-CoA was found and was considered to cause the incorporation of small amount of odd- and branched- chain fatty acids into myelin. The presence of these abnormal fatty acids in the myelin was thought to be responsible for the degeneration of myelin structure. However,there is no convincing evidence that this is the case.(Siddons, Spence and Dayan, 1975; Ramsay and Fischer, 1978; Small and Carnegie, 1981).

Source of Vitamin B12: Vitamin B12 can only be synthesised by microorganisms which live in water, soil, sewage and the intestine of man and animals. Almost all products of animal origin contain deoxyadenosyl-B12, the major dietary form of the vitamin. In clean food of vegetable origin the amount of vitamin B12 is negligible. Man

is entirely dependent on his food for vitamin B12. Because bacterial synthesis of vitamin B12 only happens in the caecum and colon, the specific receptors involving the absorption in the terminal ileum are situated too proximally to absorb vitamin B12 with intrinsic factor. The absorption of vitamin B12, which is called extrinsic factor, needs the intrinsic factor, a mucopolysaccharide from gastric parietal cells, to form a complex to be absorbed in the ileum.

Causes of Vitamin B12 Deficiency:

Vitamin B12 deficiency of dietary origin is rare. It may occur in certain very strict vegetarians termed "vegans" who do not eat eggs or milk products or some Indian vegetarians on both religious and economic grounds.

Conditions such as gastrectomy,gastritis, intestinal diseases, folate deficiency of nutritional origin, and iron deficiency can also affect the absorption of vitamin B12. Addisonian pernicious anaemia which is due to a failure of production of intrinsic factor by autoimmune atrophic gastritis has been known as the commonest cause of B12 deficiency with neurological disease. Other diseases, i.e. total and subtotal gastrectomy,and organic diseases of the intestine, including stricture, fistulae, diverticula,tuberculous ulceration, tumour, regional ileitis,and idiopathic and tropical sprue, may also be causative factors (Pallis and Lewis, 1973).

Habitually abusing nitrous oxide has been reported (Layzer et al. 1978) to cause a disabling peripheral neuropathy. Previously healthy dentists and hospital technicians developed mainly sensory peripheral neuropathy with numbness and a reverse Lhermitte sign with the signs of spinal cord involvement. Nerve conduction studies suggested an axonal rather than demyelinating neuropathy.

Congenital deficiency or abnormal low activity of some enzymes involving the methyl transfer pathway in some young patients was known as a cause of subacute combined degeneration. Dayan and Ramsey (1974) reported a 7-year-old girl with an error of vitamin B12 metabolism, which led to the intracellular deficiency of cobalamin, including the two coenzyme forms, methyl- and adenosyl- cobalamin. This girl presented with megaloblastic anaemia and severe retardation of intellectual and motor skills, but no obvious loss of sensation in the limbs or trunk. Post-mortem examination of the brain showed multifocal areas of perivascular demyelination and vascular damage in the white matter of the centrum ovale. The small and presumably the earliest lesions consisted of groups of fibres with ballooned disintegrating myelin sheaths around an arteriole or pre-capillary, which were associated with a few foamy, lipid-laden macrophages. Silver staining showed relatively less damage to axis-cylinder than to myelin sheaths.

2.Folate deficiency:

Methyl-tetrahydrofolate (methyl-THF) was known to play a regulatory role in the methyl transfer pathway. This occurs via the transfer of a methyl group to methionine via cobalamin and methionine synthetase then ultimately to SAM. Deficiency of folate causing a disturbance of the methylation reaction in the nervous system probably underlies subacute combined degeneration of the cord as well as vitamin B12 deficiency.

The commonest cause of folate deficiency is inadequate dietary intake. Malabsorption such as untreated coeliac disease, untreated tropical sprue, anticonvulsant drugs and alcoholism may also cause folate deficiency. Inborn errors of folate metabolism in children were also noted to cause severe and progressive disease of the CNS.

Congenital deficiency of 5,10-methylenetetrahydrofolate reductase in a 2-year-old girl was reported by Clayton et al. (1986). The patient presented with delay in development after 1 year-old and became ataxic, stopped talking, crawling, smiling and feeding herself and became incontinent. There were also fine, semi-purposeful movements in the limbs and a parkinsonian tremor in the arms with cogwheel rigidity and pill-rolling. Total folate concentration in serum, red cells and CSF were markedly reduced whereas concentrations of vitamin B12 were normal. Post-mortem examination of this patient showed the most prominent pathological changes in the white matter with numerous small perivascular foci of demyelination. Myelin staining stopped at the edge of this zone within which there were numerous macrophages, hypertrophied astrocytes and well-preserved axons. Demyelination was patchy in the brain stem but more severe in the cerebellar white matter. Cerebellar cortex was normal. The white matter of the spinal cord was particularly affected. Coalescence of demyelinated patches in anterior, lateral and posterior columns gave the typical appearance of subacute combined degeneration. The lesion was most extensive in the thoracic cord. At the margins of the demyelinated areas myelin sheaths showed spongy degeneration. There was accompanying gliosis but preservation of axons in all but the most severe degenerated regions. Spinal grey matter, in particular anterior horn cells appeared normal. Dorsal root ganglia and peripheral nerves were within normal limits and showed no evidence of demyelination. A fibre density count on a fascicle of femoral nerve was normal. Muscles and end-plates were not examined.

Hyland et al. (1988) measured the metabolites in the CSF from four patients with demyelinating disease and 5,10-methylenetetrahydrofolate reductase deficiency; one patient died and had a full postmortem examination mentioned previously (Clayton et

al, 1986), and three patients with neurological deterioration halted by the administration of betaine. Methionine and SAM concentrations were undetectable in the first patient. In those receiving betaine, methionine concentration was proportional to the dose administered and SAM concentration was near normal. Their results provided the first evidence for the association between defective SAM metabolism and demyelination in humans.

Lever et al. (1986) reported a clinical case of 53-years-old woman with 2 years history of progressive leg weakness, paraesthesia of hands and feet, dyspnoea on exertion, and 6 months of confusion. She also had horizontal nystagmus, an ataxic broad based gait, symmetrical grade 3 pyramidal weakness of her legs, absence of knee and ankle reflexes and bilateral extensor plantar response.Investigation showed she had a macrocytic anaemia and the CSF SAM level was one of the lowest of their patients. They also reviewed the previously reported cases of folate deficiency who presented with complications of vitamin B12 deficiency and only 5 met the following criteria: Pyramidal and dorsal column signs, low serum and red cell or CSF folate, megaloblastic marrow, normal vitamin B12 levels of absorption, and reversibility of neurological signs by folate, but not by Vitamin B12.

Deficiency of vitamin B12 or folate has been known to cause certain well-defined clinical manifestations, involving the haemopoietic system, epithelial surfaces and the nervous system. The first two have been extensively studied, but the effects on the nervous system still have much controversy to be clarified.

3.Other factors causing failure of transmethylation

HIV Human immunodeficiency virus (HIV) infection involving the

central nervous system includes subacute encephalitis and vacuolar myelopathy. The latter was reported to bear similar pathological changes to those of subacute combined degeneration of the cord; however, serum levels of B12 and folic acid were found to normal (see Gray, Gherardi and Scaravilli, 1988).

Recently, Surtees, Hyland and Smith (1990) measured the levels of ~~of~~ 5-methyltetrahydrofolate, methionine and SAM by high-performance-liquid-chromatography from 6 children with congenital HIV infection and neurological complications. In all six patients concentrations of one or more methyl-group carriers were lower than those in a reference population of children, and all of the five in whom CSF neopterines were measured had higher than normal levels. Surtees et al. suggested that defective methylation may play a part in the neurological damage caused by HIV infection.

Keating, Trimble, Mulcahy, Scott and Weir (1991) also measured SAM and S-adenosyl-homocysteine (SAH) in the CSF of 20 HIV seropositive patients and 30 HIV seronegative patients. They found that the HIV seropositive patients had significantly lower CSF concentrations of SAM and significantly higher concentrations of SAH than the controls. There was therefore a significantly lower methylation ratio (SAM/SAH) than the controls. Keating et al. suggested that HIV affected the brain from a very early stage of the infection. In HIV-infected patients a reduced brain methylation ratio would inhibit methyltransferase enzymes, which could lead to hypomethylation in the central nervous system and ultimately to neurological lesions. The HIV patients in this study were vitamin B12 and folate replete, which suggested a different cause for the low methylation ratio.

Methotrexate Methotrexate is a folic acid antagonist which has been used as a chemotherapeutic agent and is usually given intravenously or intrathecally combined with cranial or craniospinal irradiation to treat patients with acute leukaemia. The pathology caused by methotrexate includes leucoencephalopathy affecting particularly the periventricular regions with coagulative necrosis or extensive demyelination particularly in the centrum ovale (Rubinstein et al 1975; Jacobs and Le Quesne, 1984).

Experimentally induced failure of the transmethylation reaction in animals:

The animal models of subacute combined degeneration of the cord include:

(1) Prolonged feeding of a Vitamin B12 free diet:

Krohn, Oxnard and Chalmer (1963) reported that two of the monkeys in their colony developed paralysis which they thought was similar to subacute combined degeneration in man. They then measured the vitamin B12 levels in the 21 monkeys which had been kept in their colony for 1 to 15 years and they found the mean vitamin B12 level was much lower than in the other mammals and the normal range of man. Oxnard and Smith (1966) found that 5 of 15 rhesus monkeys maintained on a very strict vegetarian diet spontaneously developed a disease called cage paralysis. The spinal cords showed spongiform demyelination of posterior and lateral columns without significant gliosis. Severe demyelination of peripheral nerves, more markedly distal, was found in three of the animals. Four paralysed animals given cyanocobalamin showed clinical improvement. Torres, Smith and Oxnard (1971) showed that segmental demyelination was the main lesion with less axonal degeneration.

Siddons, Spence and Dayan (1975) reviewed the literature on

animal models of vitamin B12 deficiency and stressed the difficulty in finding a proper model. This was due to: (1) Both animals and man carry very large body stores of cobalamine which amount to several years' requirement. (2) Intestinal synthesis of vitamin B12 by the organisms in the normal gut flora which takes place in the large bowel. Absorption is very limited except in the instances of coprophagy. (3) Adventitious sources, such as (a) contamination of diets by traces of vitamin, or (b) the experimental animals are facultative carnivores and catch and eat insects, birds or rodents as the opportunity arises.

The interference of absorption can be achieved by removal of intrinsic factor-producing part of the stomach or excision of the ileum from which the absorption of vitamin B12 occurs, but all these are prone to cause more extensive abnormality and may involve essential trace elements and other fat- or water-soluble vitamins, such as folic acids. It is impossible to produce 'pure' vitamin B12 deficiency by the surgical method which cause disturbances in the metabolism due to lack of these unknown materials or other less-well-identified factors (Siddons, Spence and Dayan, 1975). The approach of using Vitamin B12 antagonists to produce an animal model also aroused many basic questions about specificity of action, lack of additional effects, and degree and duration of inhibition.

Siddons, Spence and Dayan (1975) maintained groups of 3 to 11 baboons on vitamin B12 deficient diet for periods of 1 to 4 years. These included five groups: vitamin B12-deficient basal diet alone; basal diet with added vitamin B12 supplement; basal diet and subtotal hepatectomy; basal diet plus ampicillin 50mg/kg/day; and a low fat diet with added sodium propionate to increase the metabolic demand for the vitamin. All the baboons given the vitamin B12 deficient diet remained healthy and active. No clinical signs of neurological damage

or haematological abnormalities were found; however, baboons given the vitamin B12 deficient diet did develop low serum and tissue vitamin B12 levels and excreted increased amount of methylmalonic acid. Pathological examination of nervous system and internal organs did not reveal any lesions attributable to vitamin B12 deficiency.

Siddons, Spence and Dayan (1975) administered 2-methyl-2-aminopropanol-B12 to 3 baboons which were vitamin B12 depleted. All 3 baboons developed paraparesis after 4 to 5 months and necropsy was done 9 to 12 months after dosing had commenced. Neuropathological examination showed extensive, scattered areas of demyelination in the cerebral hemispheres. The lesions appeared to be centered around small vessels. The brain stem and basal ganglia appeared normal. The cerebellar white matter showed less marked multifocal demyelination. In the spinal cords, there was demyelination and a lesser amount of axonal loss in the posterior and lateral columns at all levels. Anterior horn cells, spinal nerve roots and blood vessels appeared normal. Peripheral nerves of one baboon showed occasional scattered lesions of de- and remyelination and a few fibres showed axonal degeneration; the peripheral nerves appeared normal in the other baboon examined.

Later, the animal model by Oxnard and Smith (1966) was challenged by Agamanolis et al. (1976) who thought the cage paralysis might be due to deficiency of other vitamins and nutrients rather than vitamin B12 only. Agamanolis et al. maintained rhesus monkeys on a defined diet under controlled conditions. Five years after the institution the vitamin B12 deficient diet, the morphology and counts of peripheral blood and bone marrow were normal. Gross visual impairment appeared in five of the monkeys at 33 to 45 months. Subsequently, in three of the visually impaired monkeys, a gradually progressive spastic paralysis of their hindlimbs developed.

Agamanolis et al. (1978) published the ultrastructural studies of these monkeys using perfusion-fixation techniques and found the lesions were indistinguishable from those of human subacute combined degeneration. The mildest changes were of a focal spongy nature, caused by distension of myelin sheaths, wide separation of myelin lamellae forming extensive vacuoles or the formation of periaxonal spaces. The extracellular space was also increased. At later stages, myelin sheaths showed a honeycomb-like breakdown and other types of degenerative change. Axons disappeared from some fibres with abnormalities, but a great many remained intact, although denuded of myelin, embedded in a dense network of astrocyte processes. A substantial number of axons contained accumulations of dense bodies and mitochondria, often causing axonal swelling.

By keeping Egyptian fruit bats (*Rousettus aegypticus*) on a pest-free, all fruit diet and clean tap water for more than 200 days, Green et al. (1975) showed that the bats could be rapidly depleted of vitamin B12. Very low vitamin B12 concentrations in serum developed as well as neurological disturbances such as ataxia, loss of proprioceptive sensation in the lower limbs and inability to disengage their claws from the wire mesh during climbing. The flying cycle of these bats was noted to be severely affected. In the histological sections of 4 of 5 affected fruit bats which were killed at 200 days patchy spongy change in the white matter of the lower cervical and upper thoracic region was found, mainly affecting the lateral and ventrolateral columns and suggestive of demyelination. No anaemia was found in these fruit bats.

(2) Exposing the animals to nitrous oxide: Nitrous oxide was known to be an inhibitor of methionine synthetase(EC 2.1.1.13) by inactivation of vitamin B12. Scott et al.(1981) maintained four pairs of monkeys in an atmosphere of nitrous oxide. One of each pair of

animals was supplemented with methionine, the other was not. In every case the monkey without the supplement became ataxic at around 10 weeks and the disorder progressed for another 2-3 weeks until the monkey became moribund. The monkey given methionine supplement did not show any clinical change. Histological examination of the monkeys without methionine supplement showed spongy degeneration in the posterior columns, anterior and lateral corticospinal tracts, which correlated closely to their clinical state. Severe demyelination of peripheral nerves were also noted. In contrast, the monkeys with methionine showed no or slight changes only. The histological appearance of the bone marrow in both groups were normoblastic and no anaemia was noted. Scott et al. suggested that inability to resynthesise methionine from homocysteine led to subacute combined degeneration, the root cause of which was by the inhibition of the vitamin B12 dependent enzyme methionine synthetase not by the inhibition of methylmalonyl CoA mutase.

Van der Westhuyzen et al. (1981) administered nitrous oxide to fruit bats and produced severe neurological impairment leading to ataxia, paralysis and death of these animals. This procedure took 6 weeks in bats depleted of B12 by dietary deprivation and 10 weeks in B12 repleted animals. They found supplement of the diet with folate caused acceleration of the neurological impairment, but supplement of the diet with methionine protected the bats from neurological impairment. Methionine supplementation also protected against the exacerbating effect of folate, preventing the development of neurological change. They thought that these findings would lend support to the hypothesis that the neurological lesion in cobalamin deficiency may be related to a deficiency in the methyl donor SAM which followed diminished synthesis of methionine.

Weir et al. (1988) treated the pigs with nitrous oxide and the

pigs failed to gain weight, developed progressive ataxia, and spinal neuropathy. The ataxia was totally, and the neuropathy partially, preventable by dietary methionine supplementation. There was a marked elevation of S-adenosylhomocystine (SAH) in the neural tissues and a concomitant failure of SAM to rise and thus maintain the methylation ratio (SAM/SAH), except when supplementary dietary methionine was added. Weir et al. suggested that the neuropathy is caused by raised SAH levels in neural tissues and the methylation ratio was inverted and SAM-dependent methylation reactions were inhibited.

Hakim et al. (1983) measured local cerebral glucose utilization by using the 2-deoxy-D-(1-14C)glucose technique to detect cerebral activity of methionine synthetase in rats treated with nitrous oxide and CL. They found that rats treated with nitrous oxide showed a depression, in whole brain activity, of the vitamin B12-dependent methionine synthetase but not the rats treated with CL.

(3) Administration of CL: This was used by Jacobson, Gandy and Sidman (1973) and has been reviewed above.

Chapter 2

MATERIALS AND METHODS

Animals:

More than 100 mice (3-25 weeks old) both male and female were used. Most of them were BALB/c mice but some were from non-inbred strains. There were no differences detected in the experimental results which might be attributable to the differences in sex or strain. They were caged in groups of 3-7 and were fed on solid pellets (CRM diet-Labsure) and tap water ad libitum. The food pellets were soaked in tap water and spread on the floor of cage to facilitate feeding and access by behaviorally affected mice.

*Mouse diet: Vitamin B12: 13.4 mcg/kg, Folic acid: 0.7 mg/kg.

Cycloleucine (CL): (Sigma) 1-aminocyclopentane-1-carboxylic acid

500 mg was dissolved in 10 ml. sterile distilled water.

Valine (VL): (Sigma)

VL 500 mg was dissolved in 10 ml. sterile distilled water.

Dose:

CL was injected intraperitoneally in a single dose of 0.5-2 mg/g. of body weight.

VL, 3 mg./g. of body weight, was administered intraperitoneally every 12 hours for up to six doses.

Experiments:

Experiment 1: Mice were given CL (0.5-2.0mg/g) intraperitoneally in a single dose. These animals were divided into 3 groups, **Group A**: 21 young mice of 21 days old treated with CL 2mg/g, **Group B**: 34

adults aged 6-10 weeks treated with CL 2mg/g and **Group C**: 7 adults aged 25 weeks treated with CL 0.5 mg/g. The young animals were allowed to survive from 12 hours to 7 days. Several animals, not included in these studies, died before 7 days and none survived longer than 7 days. The adult animals were allowed to survive from 1 - 14 days (see Table 2.1).

Experiment 2: 12 young (21 days old) and 6 adult (6 weeks old) mice were given CL 2mg/g. intraperitoneally. Then, beginning24 hours later (6 young mice, Group A) or 48 hours later (6 young, Group B and 6 adult mice, Group C), VL 3mg/g was started. This was injected every 12 hours for up to 3 days. Animals were killed from 24 hours to 20 days after the commencement of VL (i.e. 48 hours - 22 days after CL)(see table 2.2).

Controls:

An intraperitoneal injection of 0.4-1.0 ml distilled water was given to 16 mice. They were allowed to survive up to 13 days. In addition 4 normal mice were killed by perfusion for electron microscopy.

Fixative:

For light microscopy:

Formal-calcium (F-Ca) :

Formaldehyde 40 %	10 ml
Distilled water	90 ml
Calcium acetate	1 g

The fixative was used for all animals to be embedded in paraffin.

Formol-acetic-methanol (FAM) :

Formaldehyde 40%	10 ml
Glacial acetic acid	10 ml
Absolute methanol	80 ml

This fixative , which was introduced for demonstration of Golgi

Table 2.1

Age, dose and survival times of CL mice examined by light and electron microscopy (Experiment 1)

Group	(A)21 days old		(B)Adult(6-10 weeks)			(C)Adult(25w/♀)	
Dose	2mg/g			2mg/g		0.5mg/g	
	Paraffin	EM		Paraffin	EM	Paraffin	EM
12 hrs	-	2	1 day	1	2		
24 hrs	1	3	2 days	1	2		
2 days	-	4	3 days	5	3		
3 days	1	3	4 days	3	1	-	2
4 days	0	2	5 days	1	-	2	-
5 days	2	0	6 days	3	3		
7 days	1	2	7 days	1	-	2	1
			8 days	1	-		
			9 days	1	1		
			10 days	1	-		
			11 days	-	2		
			12 days	-	-		
			13 days	1	-		
			14 days	-	1		
Total 21 mice			Total 34 mice			Total 7 mice	

Table 2.2

Age, dose and survival times of CL then VL treated mice examined by electron microscopy (Experiment 2)

	21 days old		Adult(6w/o)
Group	A	B	C
1 day	*		
2 days	1	#	#
3 days	1	1	1
4 days	1		1
5 days			
6 days			1
7 days		2	
8 days	1		
9 days			1
12 days	1	1	
13 days			1
14 days	1		
15 days		2	
22 days			1
Total	6	6	6

(*) Group A: Starting point of administration of VL 3mg/g every 12 hours up to 6 doses started 24 hours after CL 2mg/g.
(#) Group B & C: Starting point of administration of VL 3mg/g every 12 hours up to 6 doses started 48 hours after CL 2mg/g.

apparatus in the central nervous system (David, Mallion and Brown 1960), penetrates tissue rapidly and prevents shrinkage during dehydration and embedding in paraffin wax. It was used as post-fixative for all material to be embedded in paraffin, after perfusion with F-Ca.

For electron microscopy:

The fixative was freshly prepared modification of Karnovsky's (1965) fluid.

Glutaraldehyde 25%	12 ml
Paraformaldehyde 10 %	20 ml
Sodium Cacodylate buffer (pH 7.3-7.5)	40 ml
Distilled water	28 ml
Anhydrous Calcium Chloride	50 mg

This fixative was freshly prepared before use.

Perfusion technique:

Under ether anaesthesia the mice were put in the supine position. The four extremities were fastened with pins to a cork board. The hind feet were secured at 90° to the lower legs to slightly stretch the calf muscles.

Under a Zeiss dissecting microscope, the chest wall was cut opened through both side of ribs laterally and diaphragm surface. After an incision was made in the right atrium, the heart was gently held in place with blunt curved forceps and a shortened and resharpened 21 Gauge Gillette needle was pushed through the wall of left ventricle. The fixative was injected from a plastic 20 ml syringe twice. The time between opening of the thorax and the end of the perfusion was usually 15 - 30 seconds.

Successful perfusion could be recognised by muscle contractions causing twisting and rapid stiffening of the body and tail and diffuse pallor or yellowish discoloration of the viscera.

Histology in paraffin sections:

After perfusion and post-fixation tissues were decalcified *en bloc* in a Formic Citrate solution (98% Formic acid 35 ml, 20% Trisodium Citrate 65 ml). The carcasses were then washed for about 1 hour in running tap water and stored in 10 % Formal-Saline (40% Formaldehyde 10 ml, 0.9 % Saline 90 ml). The limbs were separated from the head and trunk and dehydrated in ascending alcohols, cleared in chloroform and impregnated with paraffin wax.

The left limbs were then bisected longitudinally and the two halves embedded. The right limbs were cut into 2-3 mm slices following standardised anatomical levels and sequentially embedded in a single paraffin block. The head and trunk were cut into transverse slices 3-5 mm. thick following the serial block technique of Beesley and Daniel (1956). With the use of this method the entire brain and spinal cord of 14 animals were examined in serial blocks. The segments were dehydrated in alcohol, cleared in chloroform and paraffin-embedded in order of from anterior to posterior or from proximal to distal so that many levels of the head and trunk could be examined in a single paraffin block.

This serial block technique (Fig. 2.1), which has been routinely used in our laboratory, has been very useful in the studies of mutant mice and also in evaluating the general effects of neurotoxins in mice. The blocks from the head are cut in the coronal plane and shown from the anteriormost region (snout) to the posterior part (neck) in

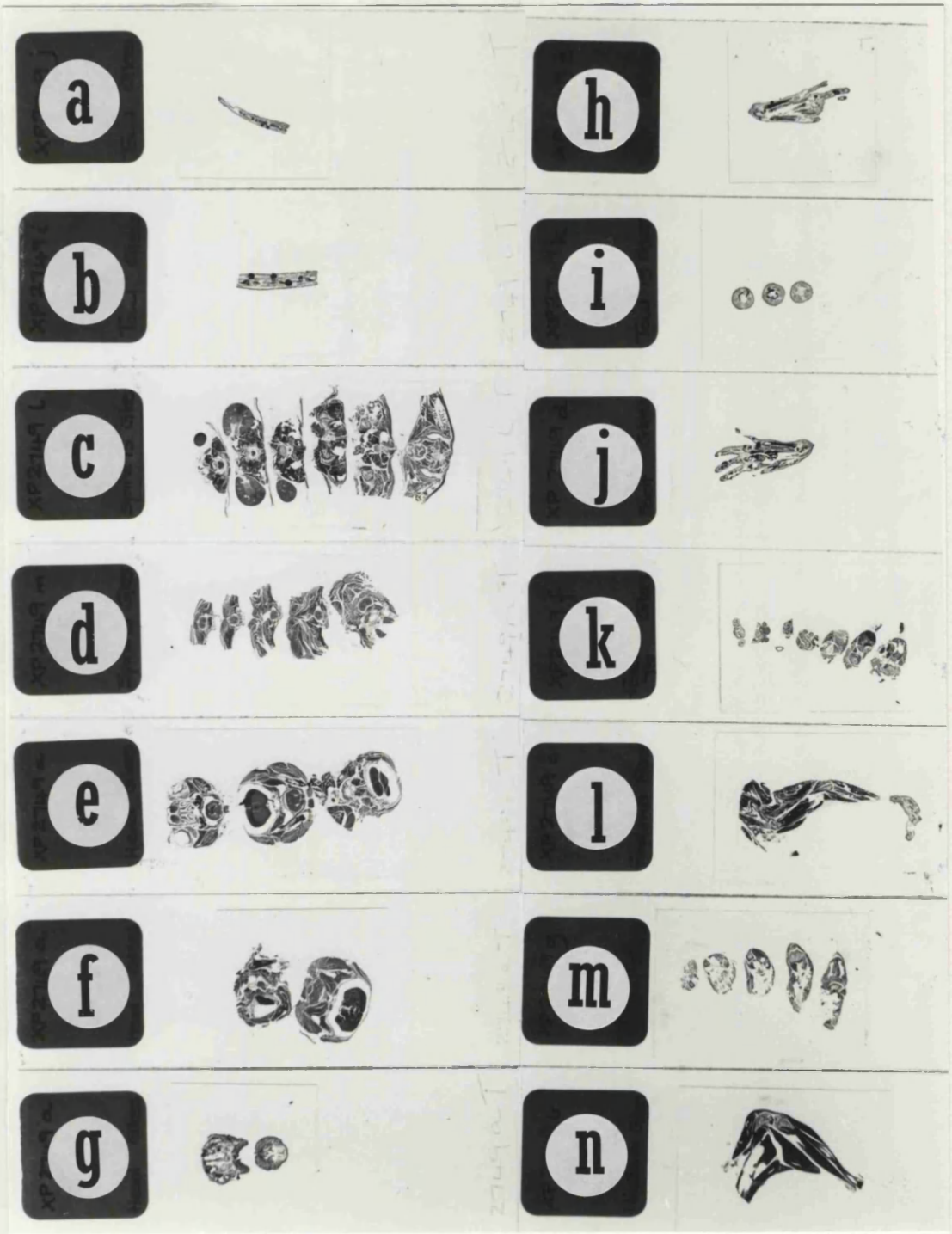

Fig. 2.1 Paraffin sections of mouse tissues stained by Glees silver method to show serial transverse section (TS) blocks and longitudinal sections (LS). a),b) and i): distal and proximal tail blocks. c) and d): serial blocks involving spinal cord vetebrae and axial muscles. e) - g): serial blocks of head. h) and j): hind feet embedded flat. k) and l): serial TS and LS blocks of fore limbs. m) and n): serial TS and LS blocks of hind limb.

seven levels . Thus nerve endings in the skin, muscle fibres and nerves in tongue, retina and optic nerves of eyes, extraocular muscles, inner ears, masseters, and entire brain can be examined systematically. The axial blocks contain neck, thoracic, lumbar and sacral regions in several levels which can be used to look for any pathology in the different levels of the cord, dorsal root ganglia and sensory and motor roots as well as the parascapular and paraspinal muscles; moreover, retroperitoneal organs such as kidneys, adrenals and colon can also be examined. The transverse and longitudinal blocks of the limbs and hindfeet provide a good chance to see the size and shape of muscle fibres, nerve bundles, motor end plates, muscle spindles and a good comparison of the morphology between proximal, i.e. biceps brachii, rectus femoris and distal muscles, i.e. soleus, extensor digitorum longus (EDL) and plantar muscles of hindfoot (including interosseous, flexor digitorum brevis, flexor hallucis brevis, flexor quinti digiti brevis and lumbricales). The anatomy of mouse is similar to that described in the rat by Greene (1955).

Paraffin sections cut at 5μm were stained with Haematoxylin-eosin (HE), Haematoxylin Van-Gieson (HVG). 14 μm. sections were stained for axons by the silver impregnation method of Glees (1946) modified by Marsland, Glees, and Erikson (1954) and others were stained for myelin with Luxol-fast-blue combined with Cresyl violet for nerve cells (Kluver and Barrera, 1953). Some visceral organs such as heart, lung, liver, spleen, stomach, intestine and kidney of 5 CL-treated mice were also included for histological examination to evaluate general toxicity of CL.

Electron microscopy:

All mice used for electron microscopy were perfused with 40 ml fresh cold Karnovsky's solution (4°C) . Immediately after perfusion

0.3-0.5 ml. Karnovsky's solution was injected into the intermuscular planes in the right thigh, calf muscles, anterior tibial muscle and plantar muscles of foot for rapid penetration and better fixation of nerves and muscles.

Under a dissecting microscope, the skin was removed and transverse cuts in cervical spine and lumbar spine were made and spinal cord was exposed to get better and quicker penetration of fixative. The mice were left overnight in a cold room at 4°C fully immersed in fixative.

Blocks were taken from soleus, extensor digitorum longus, plantar muscles of foot, tibial nerve, sciatic nerve, spinal cord at different levels, dorsal root ganglia, optic nerve, cerebellum, midbrain, and cerebrum. After being carefully labelled, the blocks were transferred to 1% aqueous osmium tetroxide for 2-4 hours at 4°c, dehydrated in grades of ascending ethanol, then transferred to propylene oxide for 15 minutes twice, and then transferred to a 1:1 mixture of propylene oxide and resin mixture(Araldite 20ml, DDSA 25ml and DMP30 0.8ml) placed on a rotator for half to one hour. Then they were transferred to a pure resin mixture and placed on a rotator overnight. The next morning the blocks were embedded in fresh resin mixture and polymerised for 15-40 hours at 60°c.

Sections were cut at 1 μm. thickness with a glass knife and stained with 1 % Toluidine blue in 1 % aqueous Borax for light microscopy. The sections were examined under the light microscope to select fields and blocks were then trimmed with a razor-blade for subsequent ultrathin sectioning.

All ultrathin sections were cut at the thickness of silver-greyish reflection with a diamond knife. They were collected on bare copper

grids and stained with saturated uranyl acetate in 50 % methanol and lead citrate (Reynolds, 1963). The grids were then examined and photographed in a JEOL 100 CX electron microscope at an accelerating voltage of 80 KV.

Biochemical study

Biochemical study was in collaboration with Dr.R.Surtees to measure the methionine and SAM levels in brain of thirty mice of age 21 days treated with CL 2mg/g by an electrochemical method of HPLC which was described in his paper (Surtees and Hyland, 1989).

To assay the brain concentration of methionine, brains from 9 mice were frozen in solid CO_2/acetone immediately after removal, thawed as soon as possible, and homogenised in ice-cold methanol (10 ml methanol to 1 g of tissue) and spun down the protein. Then 20 μl of supernatant was mixed with 100 μl O-phthalaldehyde-2-mercaptoethanol reagent (50 mg O-phthalaldehyde dissolved in 1 ml methanol, 40 μl 2-mercaptoethanol, 10 ml 0.2 M borate buffer pH 9.5) for 1 minute at room temperature and 2 μl of this mixture was injected onto the HPLC (Surtees and Hyland, 1990).

21 mice brains were used to assay the brain concentration of SAM. Mice brains were frozen in solid CO_2/acetone immediately after removal, thawed as soon as possible, and homogenised in 2 ml/g wet weight 0.4 M perchloric acid. The supernatant was neutralised with 0.5 vol. 0.4 M K_3PO_4. One hundred μl of neutralised mouse brain extract, 20 μl of 1 mM 3,4-dihydroxybenzylamine (DHBA), and 20 μl 1.6 M Tris, pH 8, containing 1.5 mM $MgCl_2$ and 1 mM dithioerythritol were added to 10 μl of the Catechol O-Methyltransferase (COMT) solution. The reaction mixture was incubated at 37°C for 1 hour. Excess DHBA was removed at the end of reaction by mixing for 15

minutes with 25 mg alumina. After centrifugation the supernatant was stored at -20°C until analysis.

HPLC was performed using a Spectra Physics SP8770 isocratic pump, a Rheodyne 7125 injector, and an Apex 5-μmODS (25 x 0.45 i.d. cm) reversed-phase column. Electrochemical detection was performed by an ESA 1011 coulometric dual-cell detector in redox mode with analytical electrodes set at +0.5 and -o.45 V. The mobile phase consisted of 100 mM sodium dihydrogen orthophosphate, 20 mM citric acid, 1.5 μM EDTA, 10% methanol, and 17.5% acetonitrile; the final apparent pH was adjusted to 3.2 with ortho phosphoric acid. The flow rate was 1.0 ml/min. The column was kept at a constant temperature of 30°C.

Assessment of Sensation

Assessment of sensation was done by using a small sharp-pointed forceps to pinch snout, trunk, fore limbs, hind limbs, hind feet and tail. This usually evoked a strong and prompt reaction in normal control mice.

Chapter 3

RESULTS: PART 1

Experiment 1: Effects of cycloleucine

Clinical Observations

The mice were assessed clinically before and immediately after the intraperitoneal injection of CL and then checked twice daily until they were sacrificed or died. The normal control mice were injected with 1 c.c. distilled water without CL.

Normal control mice

The control mice were active and showed no signs of muscle weakness at any time. When they were placed on a wire grid (the grid squares being about 2 x 2 cm), they were be able to grip the wire firmly with all four feet, and walked hardly ever losing their grip. When picked up by the tail, they would have the hind limbs fully extended and straight, and the toes of each forepaw and hind foot widely spread. Muscle power in all four limbs was strong and they clung to the bars of the wire grid very firmly when picked up.

Cycloleucine-treated mice

Group A (21 days old):

The mice looked normal and active immediately after the injection. At 12 hours the mice still did not show any signs of neurotoxicity. No weakness of any limbs or difficulty in walking on the wire grid were noted. At 24 hours mice were obvious ill and had

a staring ruffled coat. They could not groom themselves well. Some of the mice were not able to open their eyes. They already showed weakness of hindfeet. There was no weakness of forelegs and no apparent disturbance of sensation.

By 36 hours mice were ataxic and there was clearly weakness of the hindlegs and hindfeet. When lifted by tail, they did not extend the hindlimbs fully and were not able to spread the toes of the feet. When placed on a wire grid, they could not walk on the grid properly and only the forepaws could hold the bars firmly but not the hindfeet.

At 48 hours after the injection, the mice looked very ill and loss of body weight was apparent. The mice lost their liveliness and stayed quietly and responded sluggishly when touched. They tended to huddle together and remained immobile, with ungroomed fur and thick catarrhal secretion from the eyes. When stirred they would not walk more than a few inches with a very wobbling gait.

By 72 hours some mice died. The surviving mice showed severe weakness of hindlimbs and almost complete paralysis of the foot muscles. Misplacement and dragging of hindlimbs were noted in most mice and crawling was accomplished only with the forelegs. When placed on a wire grid, they could hold the bars only with the forepaws. The hindfeet were not able to grip the bars and fell into the spaces between them. Sensation was apparently intact. There was also paralysis of abdominal and respiratory muscles, and costal recession was apparent.

At 96 hours, the effects of CL still progressed. Several mice had died by this time. Mice looked very weak, showing paralysis of hindlimbs and marked costal recession. All the 21 day old CL-treated mice died within 7 days. (See Fig. 3.1)

Fig. 3.1 10 weeks old mouse 4 days after CL 2mg/g. When placed on a vertically held wire grid the hind feet do not grip the bars. Fore paws can still grip. The animal uses the hind limbs as hooks to cling to the grid bars, the proximal muscles being less affected than the distal.

Morphological Observations

Controls

The normal appearances, studied by a variety of techniques using light and electron microscopy are illustrated in Figs. 3:2 - 3:8.

Experimental Group A (21 days old)

12 hours

Two mice were studied at this time post-injection (p.i.). They were both perfused with Karnovsky's fixative and blocks processed for electron microscopy. Blocks studied in either or both of toluidine blue-stained sections or in the electron microscope included cerebellum, thoracic and lumbar spinal cord, optic nerve and muscles of the leg and foot.

Central Nervous System

Study of the toluidine blue-stained 1μm araldite sections (Figs. 3.9) showed pathological changes in the white matter of the cerebellum and spinal cord. In all the sections of white matter there were vacuolar changes (which by electron microscopy are clearly intramyelinic in origin). In the 1μm sections the vacuoles could be seen best in the fibres which are sectioned transversely, i.e. in the spinal white matter and in the cerebellar folia rather than in deeper areas. The vacuoles mostly had the shape of a crescent partly enclosing the axon, or were irregular spaces one or two times the diameter of the axon which could be seen at one edge, partly enclosed in a myelin sheath.

The distribution of vacuoles was not evenly diffuse. In the cerebellum the fibres in the folia were more affected than deep white

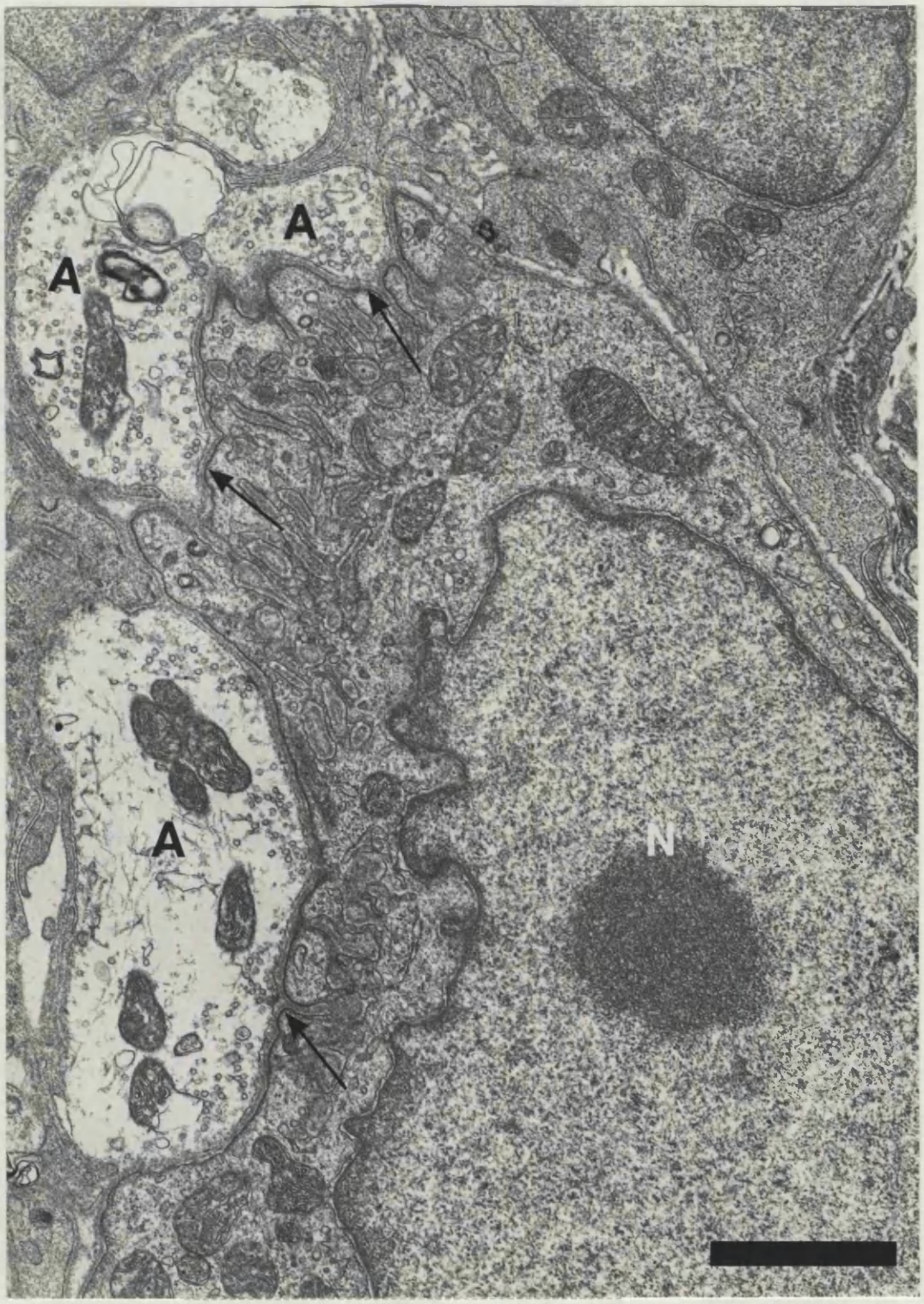

Fig. 3.2 Motor end-plate of soleus of 21 days-old normal mouse. Axonal terminals (A) contain clear synaptic vesicles, cisternae, filaments and mitochondria. The post-synaptic membrane with its specialized folds (arrows) is closely covered by the axonal terminals, separated from them by basal lamina. Schwann cell processes cover the external surfaces of terminals. N = sole plate nucleus of muscle fibre. Bar = 1μm (Neg. 91564).

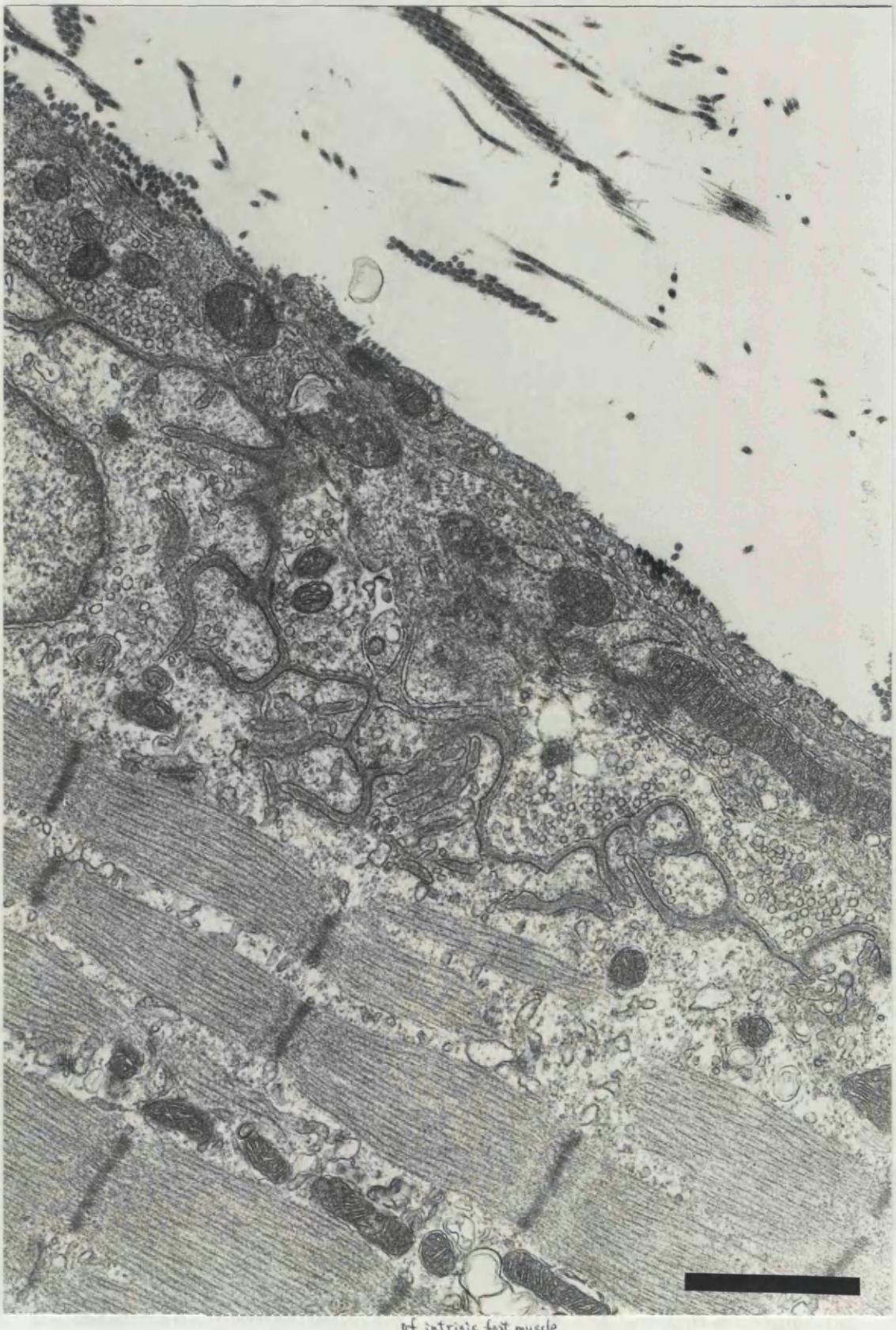

Fig. 3.3 Axonal terminals of intrinsic foot muscle contain vesicles and mitochondria and cover the postsynaptic membrane. The appearances are similar to the end-plates in soleus though the terminals tend to be smaller and the synaptic cleft shallower. Bar = 1μm (Neg. 91562).

Fig. 3.4 Myelinated intramuscular nerve fibres in foot muscle of normal 21 days old mouse. Axons contain well-organized neurofilaments, microtubules mitochondria and smooth endoplasmic reticulum. Myelin lamellae are compact. These fibres each lie singly within Schwann cell cytoplasm and there is a delicate perineurial sheath. They may be preterminal, close to the end-plates. Bar = 1μm (Neg. 91559).

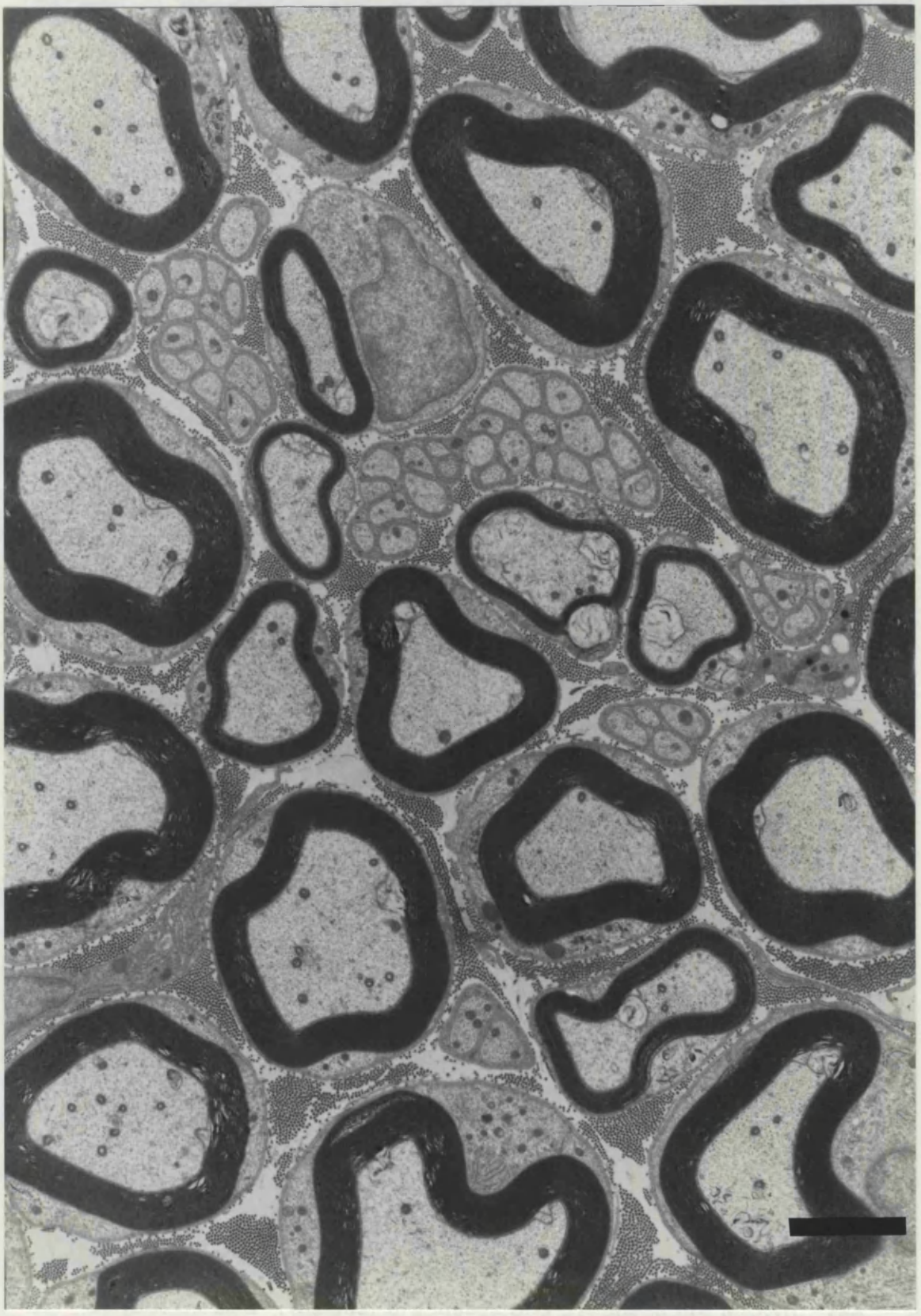

Fig. 3.5 Transverse section of sciatic nerve of normal control mouse, to show normal range of axonal diameters and myelin sheath thickness. No degeneration seen. Groups of unmyelinated fibres present. Bar = 2μm (Neg. 97714).

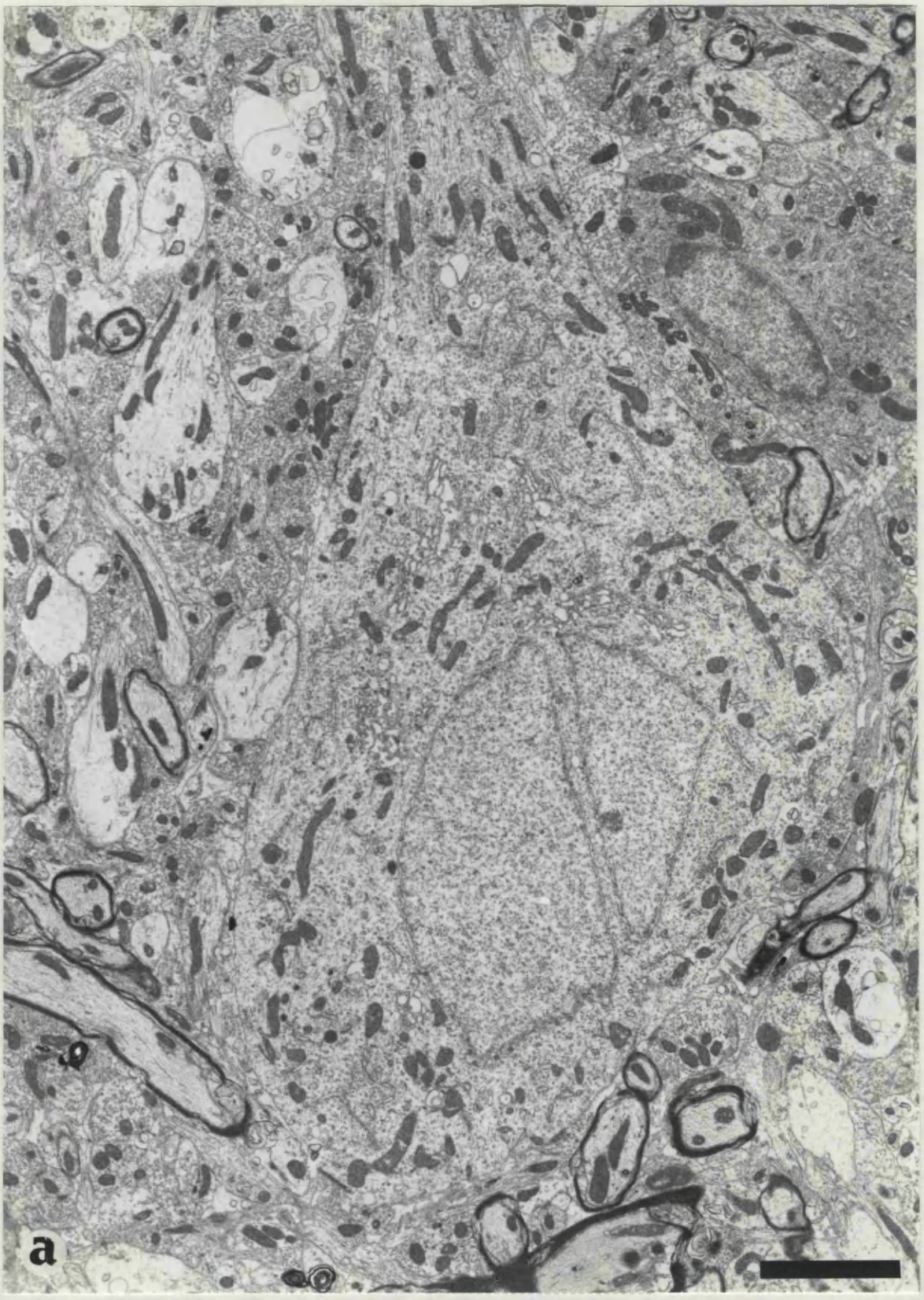

Fig. 3.6a Anterior horn cell of lumbo-sacral cord of 21 days old control mouse. Nucleus, organelles and synapses are shown. The cytoplasm contains abundant rough endoplasmic reticulum and ribosomes but these are not organized into stacks forming Nissl bodies (see Fig. 3.6b). Abundant mitochondria and smooth ER of Golgi apparatus present. Bar = 3μm (Neg. 91567).

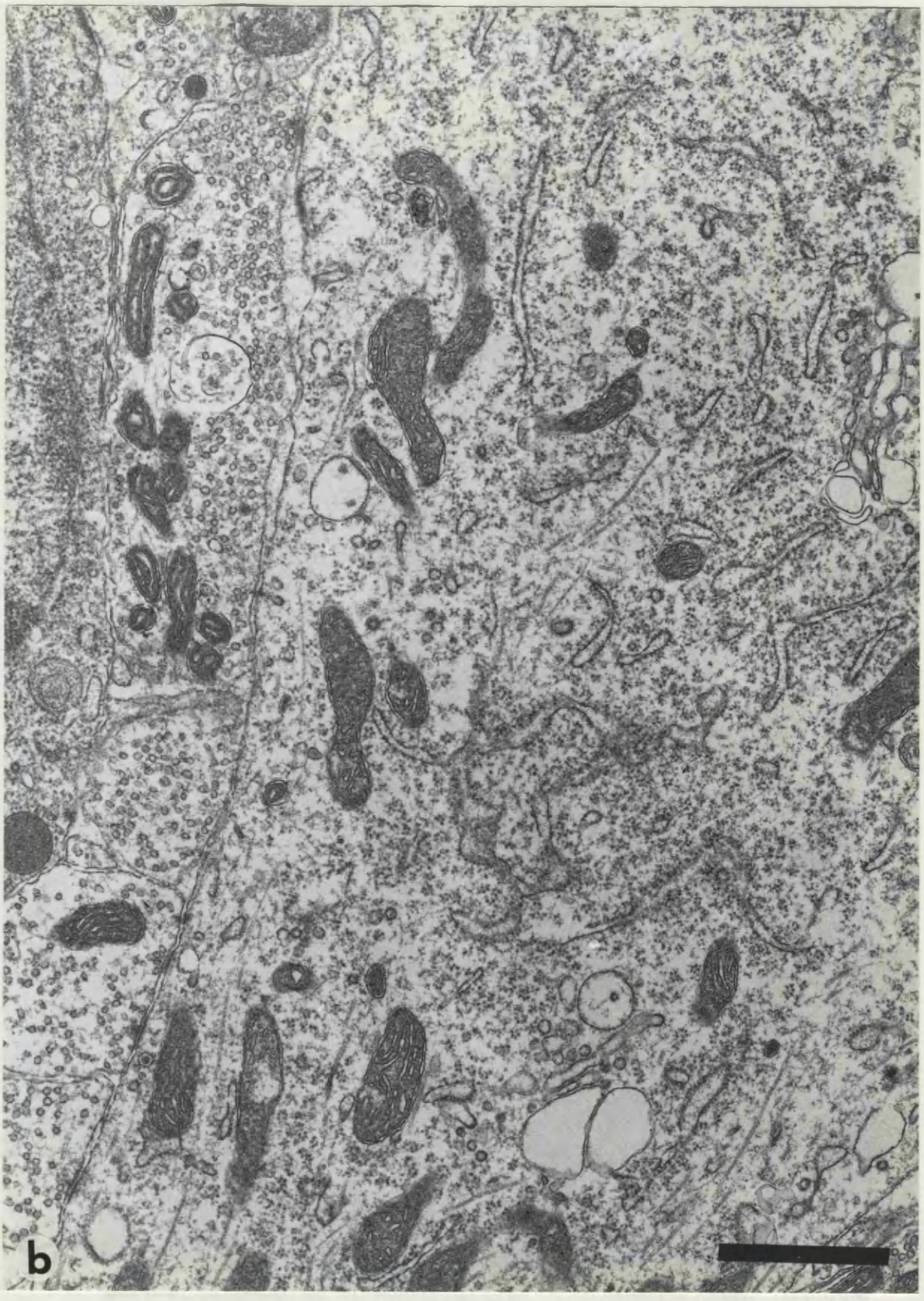

Fig. 3.6b Detail of neuron shown in Fig. 3.6a. Perikaryon contains rough ER, polyribosomes, mitochondria and Golgi membranes. No organized Nissl bodies seen. Numerous axonal terminals form axo-somatic synapses. Bar = 1μm (Neg. 91568).

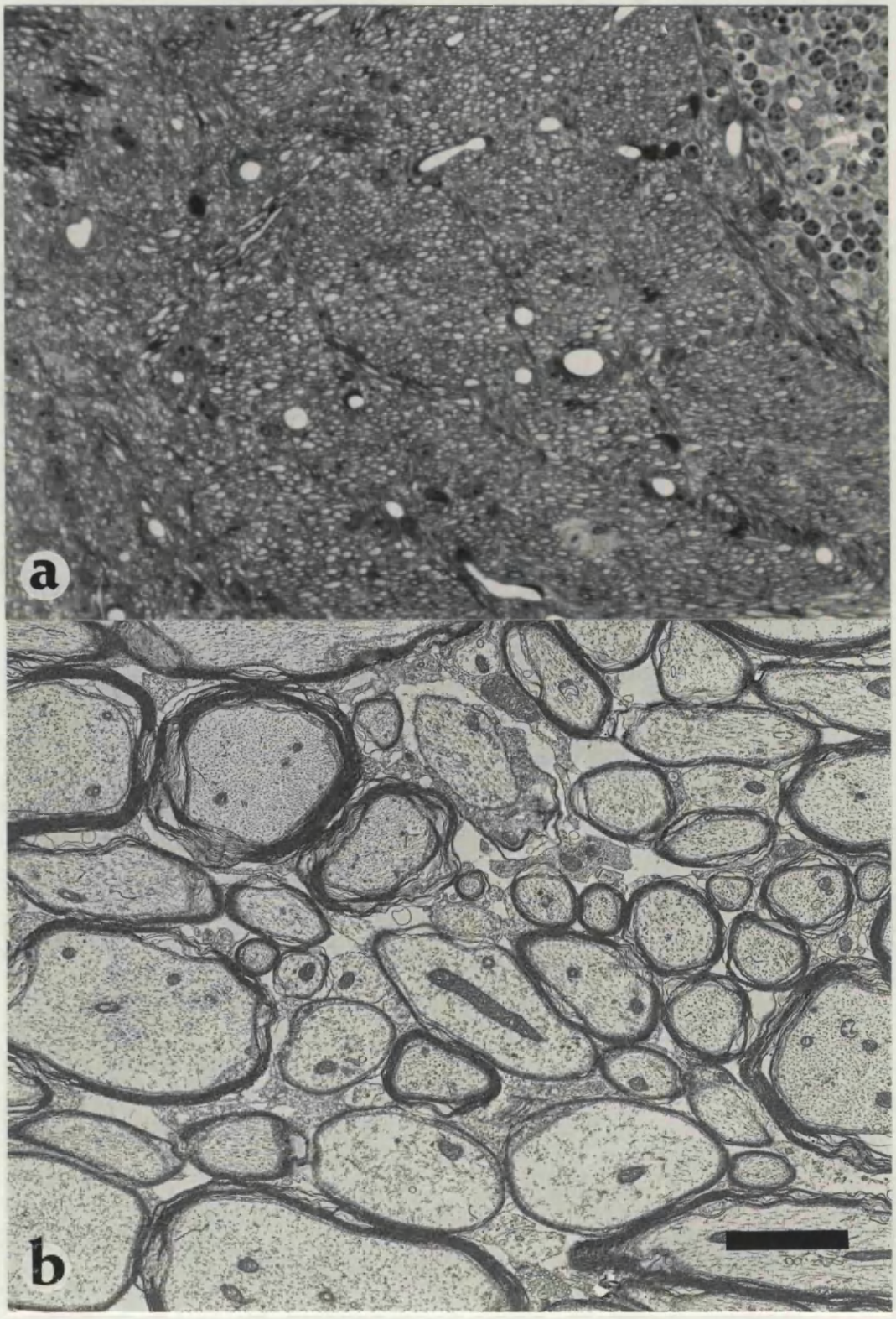

Fig. 3.7 (a) 1μm plastic section of cerebellum of control 21 days old mouse, showing white matter. No vacuolation is seen. Toluidine blue x 400. (b) EM of cerebellar white matter of same mouse as Fig. 3.7a, showing myelinated fibres and little extracellular space. Splitting and bubbles in myelin sheaths are fixation artefacts. Bar = 2μm (Neg. 91285).

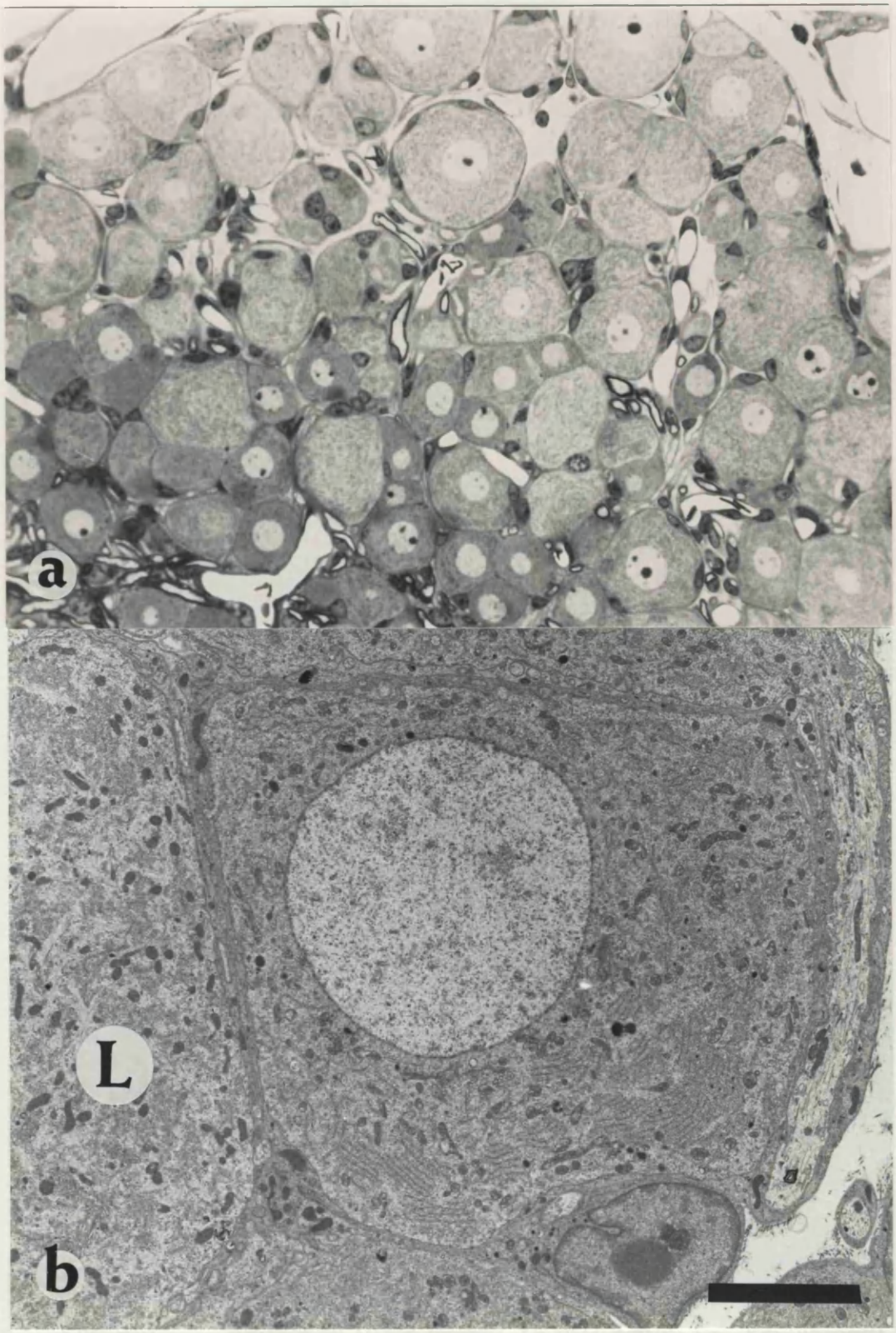

Fig. 3.8 (a) Dorsal root ganglion of normal control mouse. 1μm plastic section. Toluidine blue x 400. (b) EM of normal dorsal root ganglion cells. Rounded central nuclei mitochondria and Nissl bodies seen. Each cell is enclosed by a thin rim of satellite cell cytoplasm. These are probably small dark neurons. Part of a light cell (L) shows. Bar = 5μm (Neg. 97715).

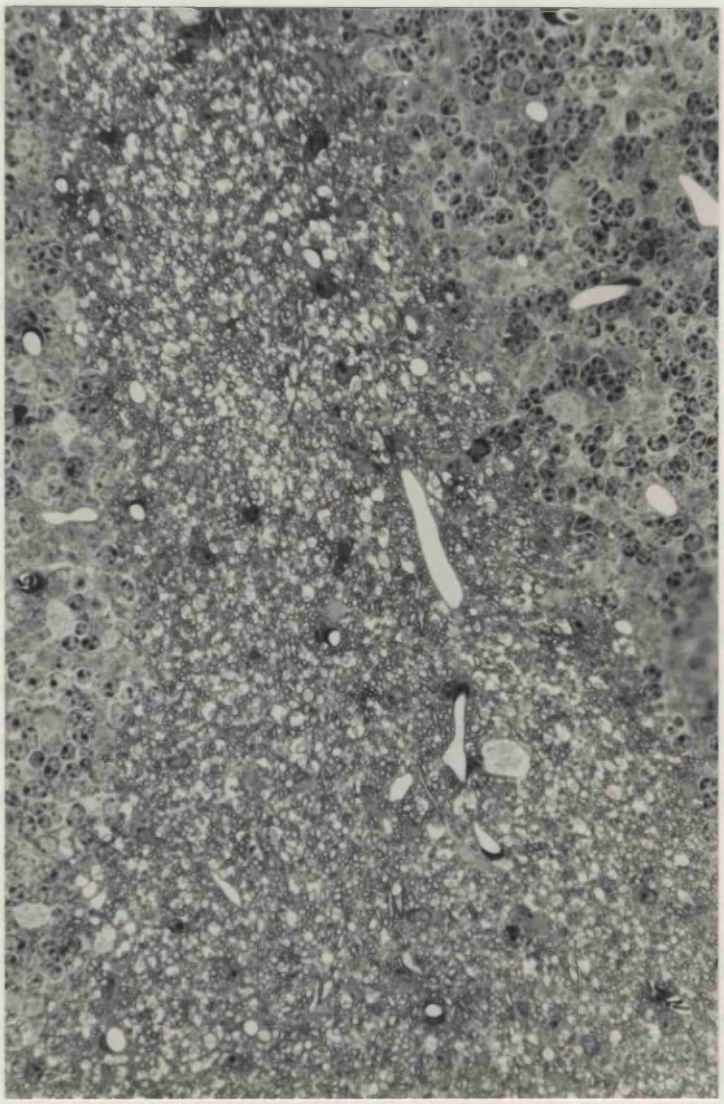

Fig. 3.9 Cerebellar white matter 12 hours after CL Vacuolation is already apparent. 1μm plastic section. Toluidine blue x 400.

matter and in the cervical spinal cord the dorsal columns were more affected than lateral or anterior white matter. In the thoracic cord only dorsal columns showed vacuolation. It also seemed that most vacuoles affected small myelinated fibres. Very few large myelinated fibres with vacuoles were seen and these were mostly in the dorsal columns. No vacuolation was visible in the molecular or granular layers of cerebellum and granule cells, Purkinje cells and neurons of the dentate nucleus were of normal appearance. In the spinal cord neurons of the grey matter were normal in appearances. Both motor and sensory roots were visualized and these did not show vacuolation or other abnormality. No dorsal root ganglion cells were seen in these animals. Optic nerve appeared within normal limits and no vacuolated fibres were seen.

Electron microscopy: Ultrathin sections were prepared from two cerebellar and thoracic and lumbar cord blocks. The vacuolation is illustrated in Figs. 3:10 - 11. With EM it can be clearly seen that the vacuolation is intramyelinic and arises by splitting at the intraperiod line. In some fibres there seemed to be several splits in the myelin sheath. Affected fibres tended to be of the order of 1 - 2 μm diameter though occasionally larger calibre fibres with vacuoles were seen. There was no significant difference in the morphology of the vacuoles at different sites. They appeared to be empty so presumably any content had been removed during processing, perhaps water. Fixation in the white matter of the ~~cars~~ mice was never satisfactory and some artefactual splitting of myelin was always seen. However the vacuolation was clearly significant, particularly in view of the findings with longer survival. The axons of fibres showing myelin vacuolation did not appear abnormal. Axonal microtubules and filaments, mitochondria and cisternae of smooth endoplasmic reticulum all appeared to be within normal limits. Oligodendrocytes did not show degenerative changes or vacuolation. Neurons in

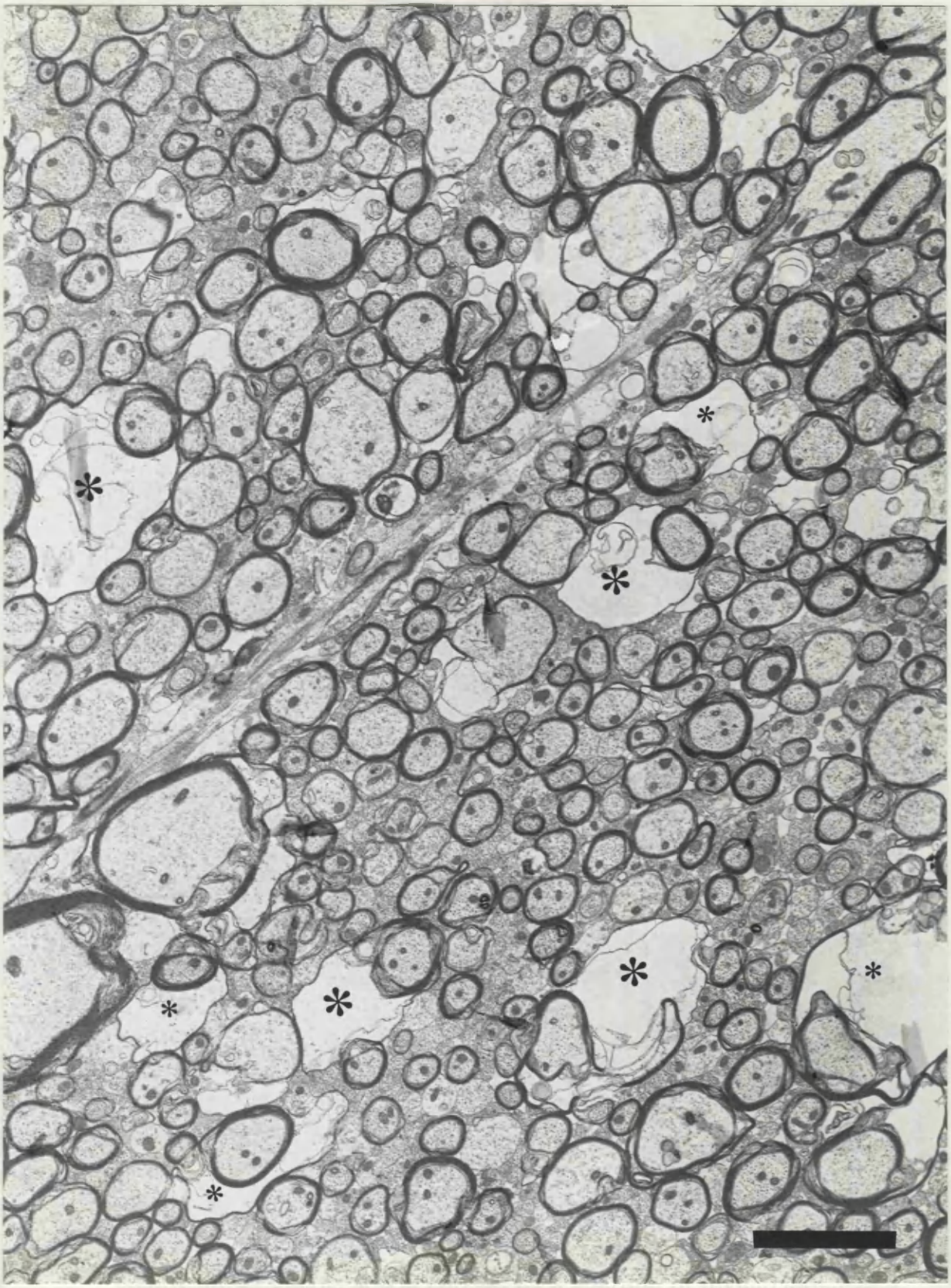

Fig. 3.10 Thoracic cord of the same mouse as in Fig. 3.9. Intramyelinic vacuoles (*) are already present in dorsal column fibres. No axonal degeneration. Bar = 3μm (Neg. 99426).

Fig. 3.11 EM of cerebellar white matter. The vacuolation is clearly intramyelinic and is caused by a splitting of myelin lamellae at an intraperiod line (arrow). Bar = 0.2μm (Neg. 99610).

cerebellum and in spinal cord as well as axonal terminals, dendritic profiles and synapses, e.g. cerebellar glomeruli, showed no abnormalities.

Peripheral Nerve and Muscle

Toluidine blue-stained 1μm sections were examined from 3 blocks of soleus and 2 blocks of foot-muscle (either interossei, lumbricals or flexors of the digits) from two animals. Many myelinated nerve fibres in peripheral nerve bundles or lying singly innervating end-plates or muscle spindles were seen and were apparently in–tact. No fibre or myelin degeneration could be identified.

By electron microscopy of ultrathin sections many myelinated fibres were seen, both arranged in intramuscular nerve bundles as well as singly. No clear abnormalities were seen (Figs. 3:12 and 13). Occasional collections of membrane or distended cisternae may well be artefactual and are similar to those seen occasionally in normal nerves.

Motor end-plates were seen in foot muscles. At least 9 end-plates were photographed. Axonal terminals were intact and lay in contact with the post-synaptic surfaces of the muscle fibres. They contained abundant clear as well as coated vesicles. Mitochondria looked normal (Fig. 3.14). In two end-plates (Figs. 3.15 and 3.16) there were electron-lucent areas which may have been distended cisternae or some rarefaction of the axoplasm but these may have been artefactual. However in view of later events these may represent very early significant pathological changes.

24 hours

At this stage after injection of CL four mice were examined

Fig. 3.12 12 hours after CL. Intramuscular nerve bundle in foot muscle appears normal. Bar = 2μm (Neg. 98078).

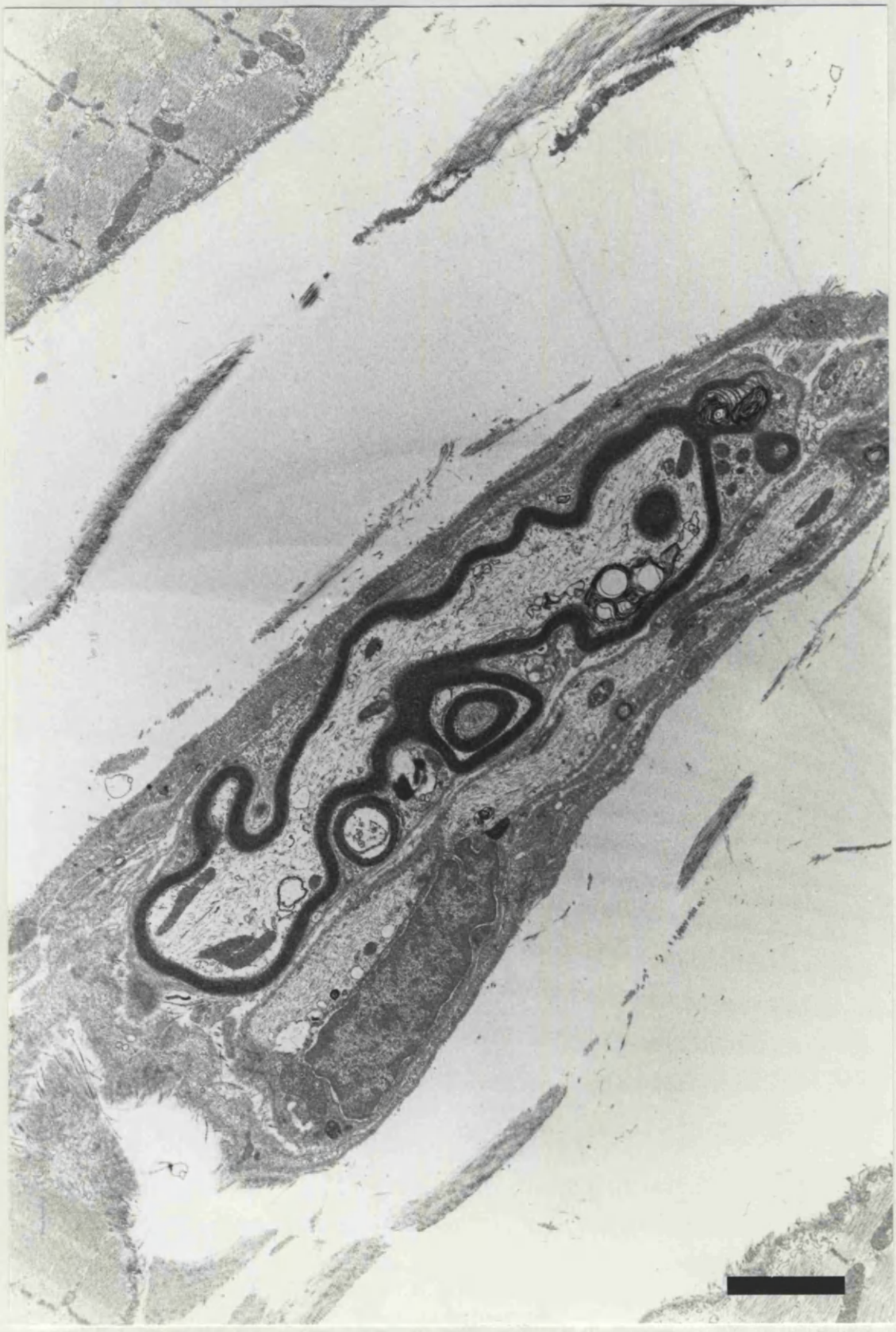

Fig. 3.13 Another intramuscular nerve in same mouse as Fig. 3.12. Nerve fibres are normal except for occasional collection of membrane and distended cisternae which are sometimes seen in normal control nerve. Bar = 2μm (Neg. 98083).

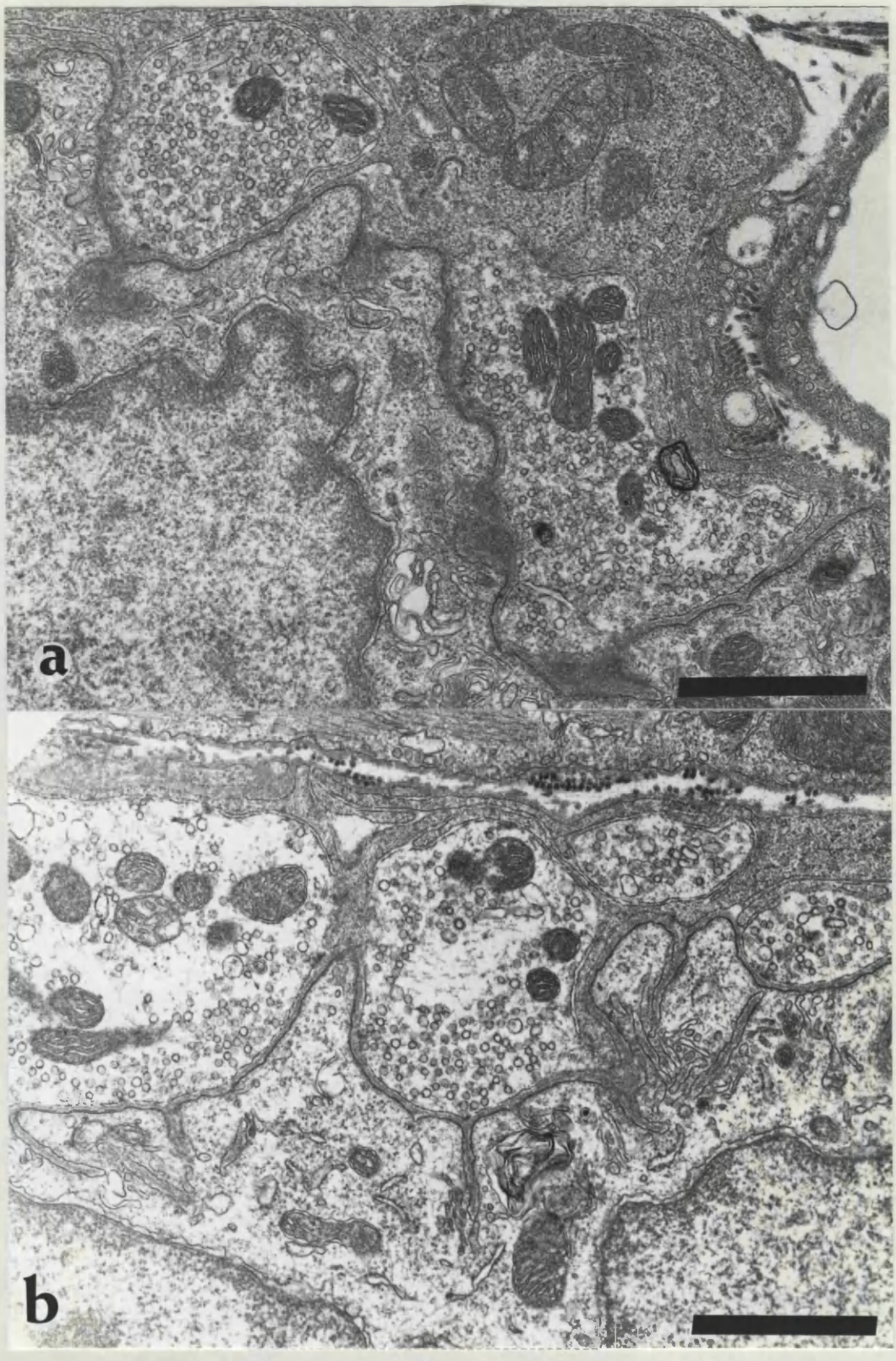

Fig. 3.14 a and b Motor end-plates in foot muscles at 12 hours (different mice). Appearances of these end-plates seem to be within normal limits although without quantitative analysis of the organelles this remains uncertain. Appearances of axolemmal membrane, vesicles and mitochondria are normal. Bar = 1μm (Negs. 91460 and 98080).

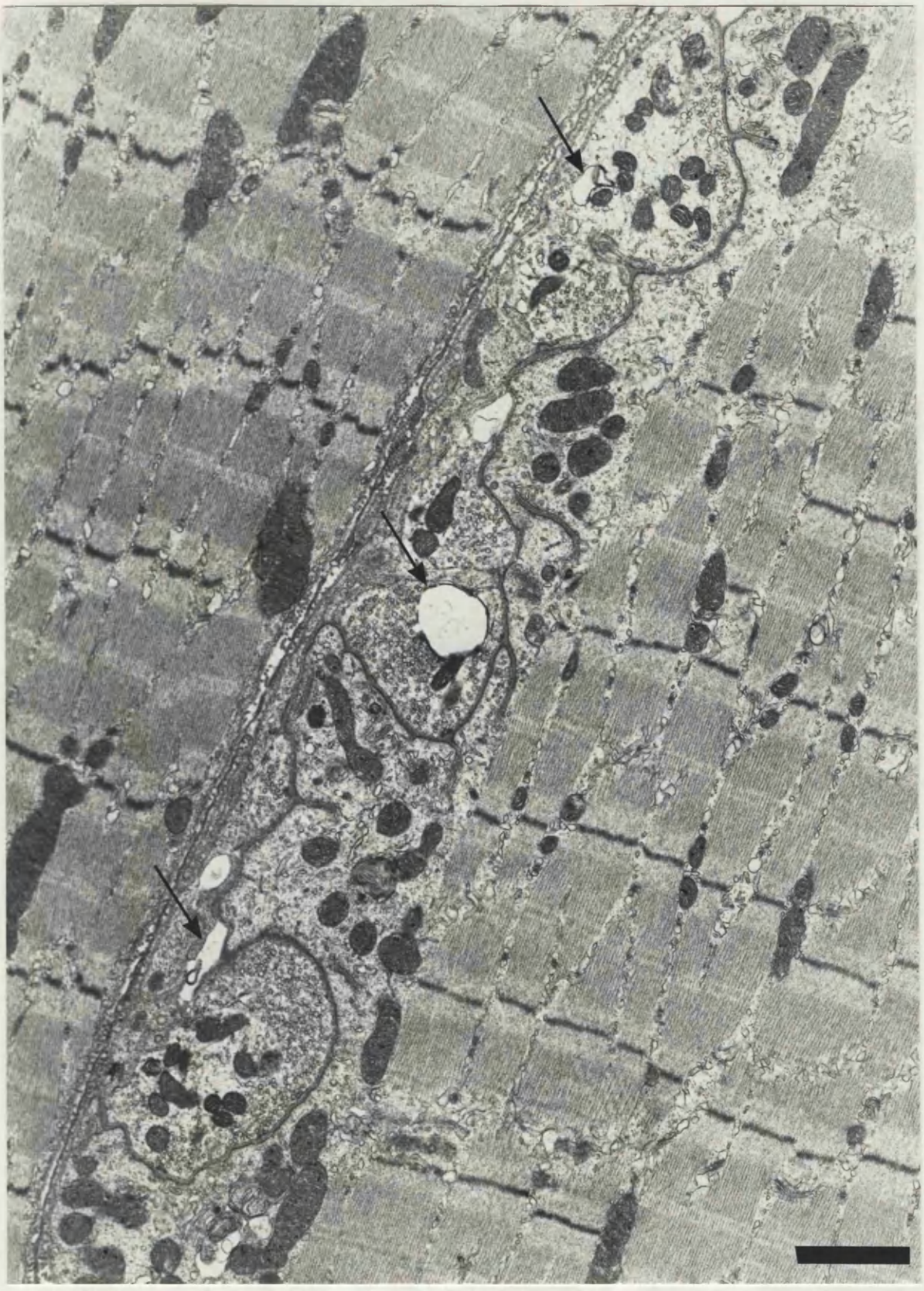

Fig. 3.15 Motor end-plate at 12 hours. Membrane bound vacuoles (arrows) in or close to axonal terminals may be artefactual but are probably real abnormalities in view of later degeneration. Bar = 1μm (Neg. 98093).

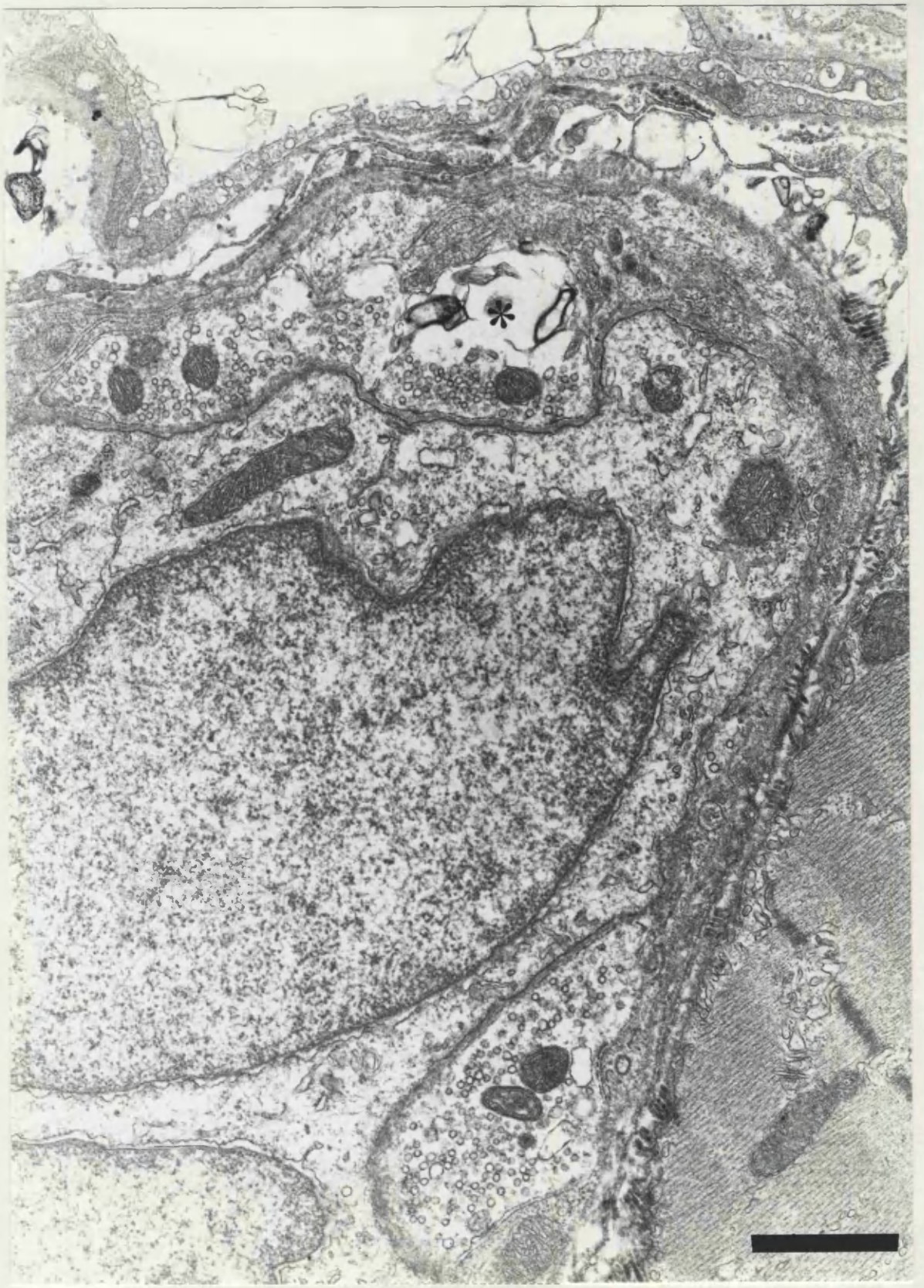

Fig. 3.16 Motor end-plate of foot muscle of same mouse as in Fig. 3.15. Electron-lucent areas (*) in axonal terminal may be an early sign of abnormality. Bar = 1μm (Neg. 98081).

histologically, three in plastic embedded blocks from both central and peripheral nervous system and one in paraffin embedded blocks from the hind limb and hind feet only.

Central Nervous System

Blocks from inferior frontal cerebral hemisphere, optic nerve, cerebellum, medulla and cervical, lumbar and sacral spinal cord were examined in 1μm toluidine blue-stained sections. Vacuolation of the white matter was fairly generalized affecting cerebral as well as cerebellar tissue. Deep as well as foliar white matter showed intra-myelinic vacuolation and in the spinal cord the vacuolation which at 12 hours was largely confined to the dorsal columns was now more widely distributed in anterior and lateral as well as dorsal columns. Vacuoles also tended to be larger than before and were mostly rounded when seen in transverse sections, with the axon and the inner part of the myelin sheath lying at one side. Fibres of all sizes seemed to be affected unlike the previous impression at 12 hours of small fibres being mainly involved. Large calibre fibres in all parts of cerebellar and spinal white matter were vacuolated. The optic nerve, however, was not affected. Neurons of anterior and posterior horns were of normal appearance and spinal roots were not vacuolated.

Electron microscopy. The vacuolation was now widespread and more obvious than at 12 hours. Vacuoles varied in size but it seemed that most were rounded rather than crescentic and many were multiloculated (Fig. 3.17) and several times the diameter of the axon with which they are associated. Some dendritic profiles contained electron lucent rarefied patches or dilated cisternae but these may be artefactual. Neurons of anterior horn and synapses were normal in appearance (Fig. 3.18) and there was no sign of axonal or nerve fibre degeneration. Some oligodendrocytes were seen (see Fig. 3.19) and were normal in appearance and there were few astrocyte nuclei and

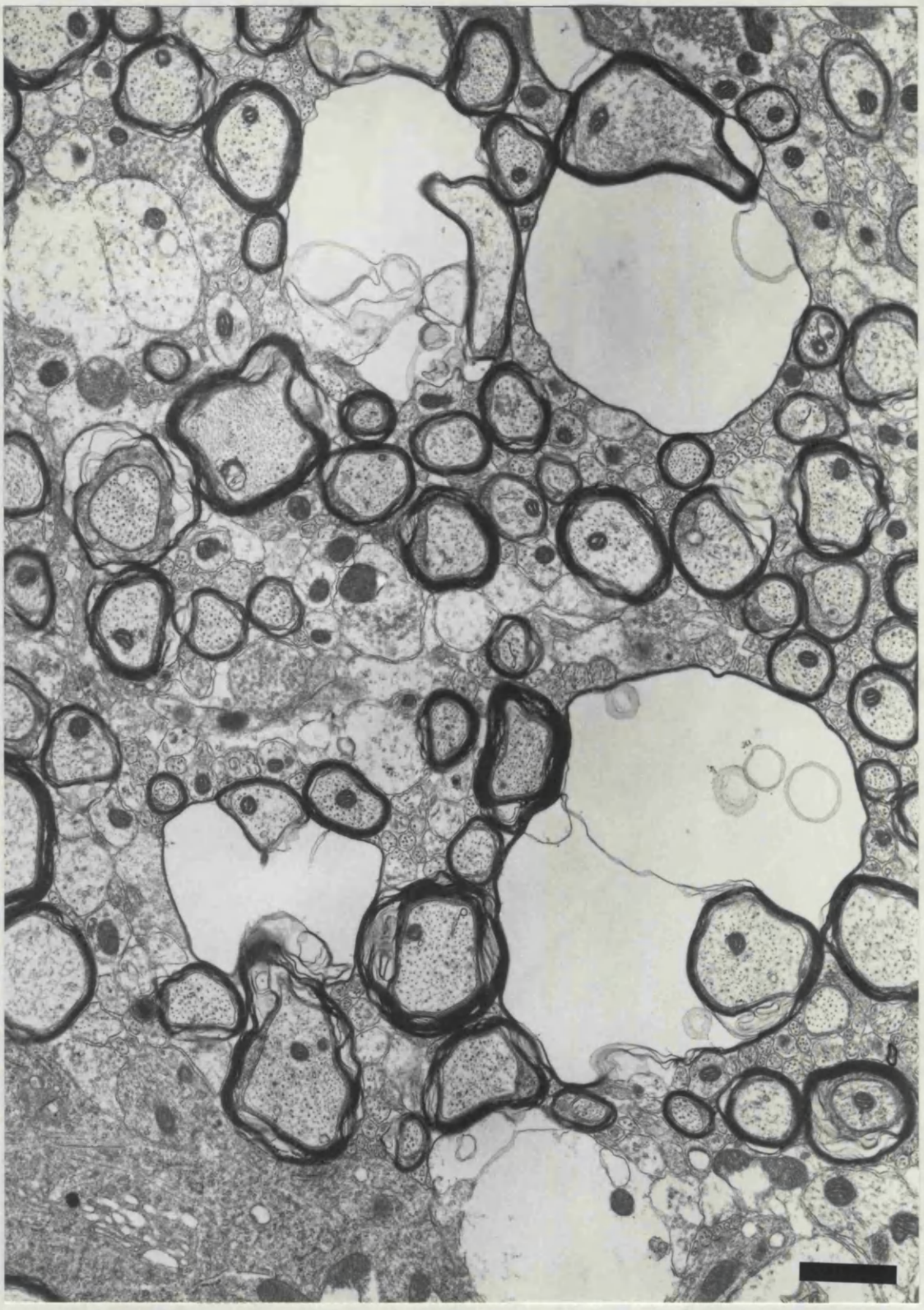

Fig. 3.17 Lumbar spinal cord at 24 hours. Intramyelinic vacuoles are larger and more numerous than at 12 hours. They are multiloculated and bounded by a few lamellae of myelin. Bar = 2μm (Neg. 89356).

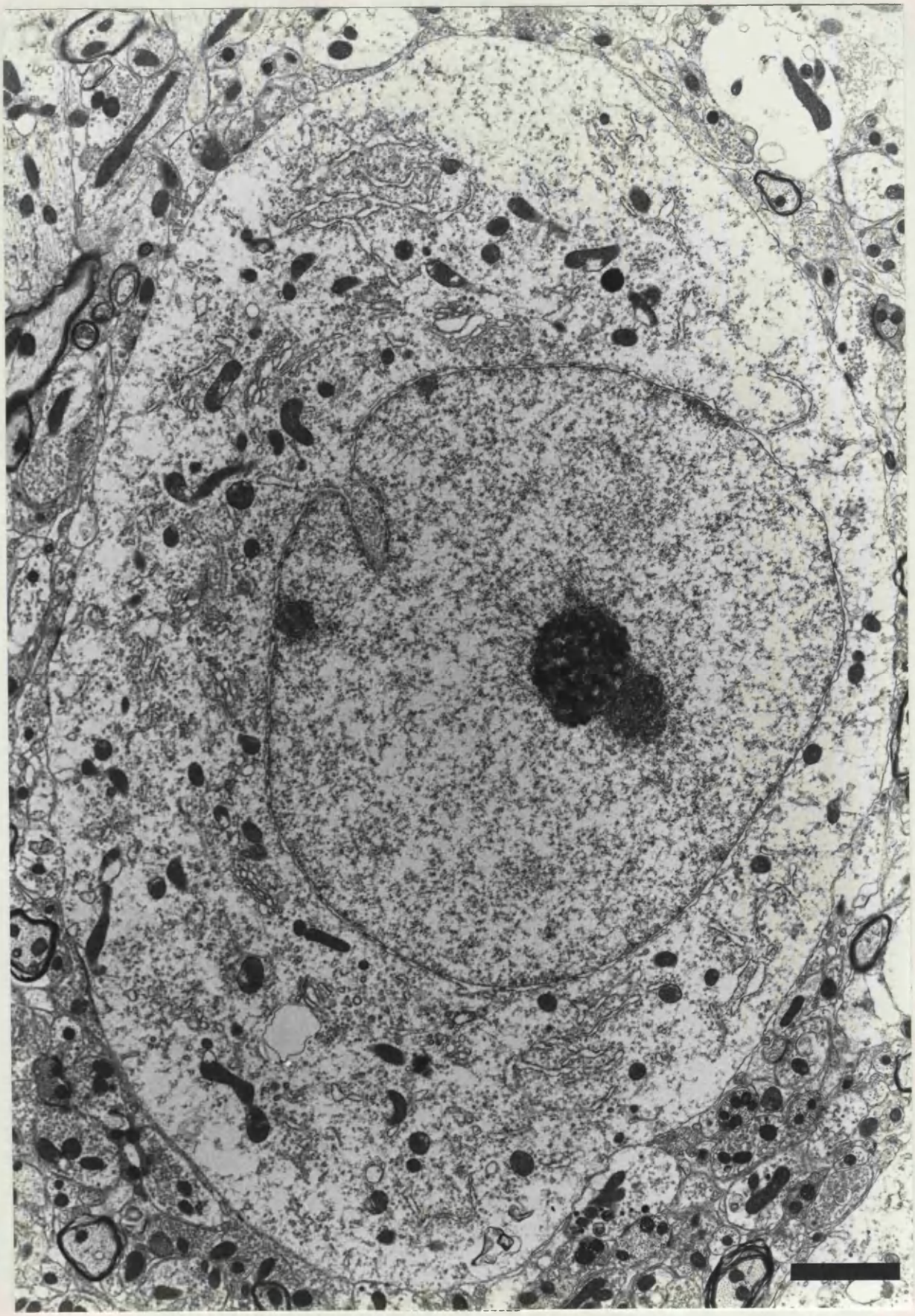

Fig. 3.18 24 hours. Lumbar anterior horn cell does not show significant abnormality. Bar = 2μm (Neg. 98071).

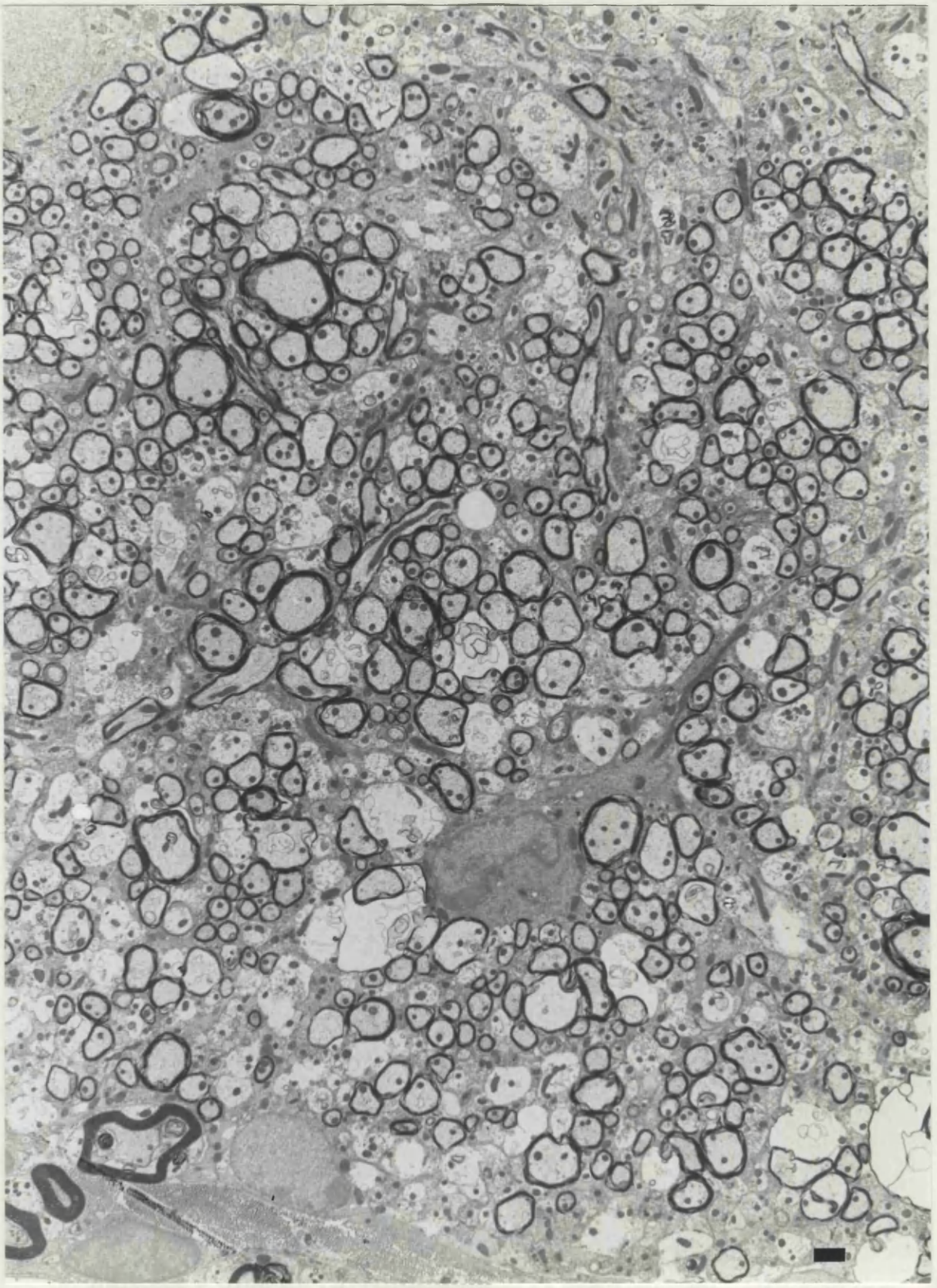

Fig. 3.19 White matter of lumbar cord at 24 hours. An oligodendrocyte appears normal. Several intramyelinic vacuoles are shown. Bar = 1μm (Neg. 98388).

few glial processes seen.

Peripheral Nervous System and Muscle

Paraffin sections of the hindlegs and hind feet were examined with various histological staining methods. The most useful of these was the silver impregnation by the modified Glees method. In these sections the intramuscular nerves were well visualized and a varied picture was seen. The larger nerves such as posterior tibial and some of the intramuscular nerve bundles were of normal appearance and many single preterminal axons and end-plate arborizations were seen and looked normal. However there was also evidence of axonal degeneration in some small intramuscular nerves and end-plates. In some small nerves only an occasional axon was present or none at all, and the presence of argyrophilic granules either within the nerve sheath or at the end-plate was evidence of axonal fragmentation (Fig. 3.20).

The innervation of the skin of the foot and toes, presumably sensory innervation, was seen in abundance and no axonal degeneration was present.

Examination of the toluidine blue-stained 1μm sections of soleus, EDL and foot muscles confirmed that some intramuscular nerves were normal and an occasional one contained degenerating nerve fibres which were fragmented and represented by myelin ovoids.

Electron microscopy Ultrathin sections were examined of foot muscles, soleus, EDL and posterior tibial and sciatic nerves. In the muscles a total of 16 motor end-plates were studied. Of these one could be described as normal (Fig. 3.21) and two were questionably within normal limits (Figs. 3.22 and 3.23), containing electron lucent

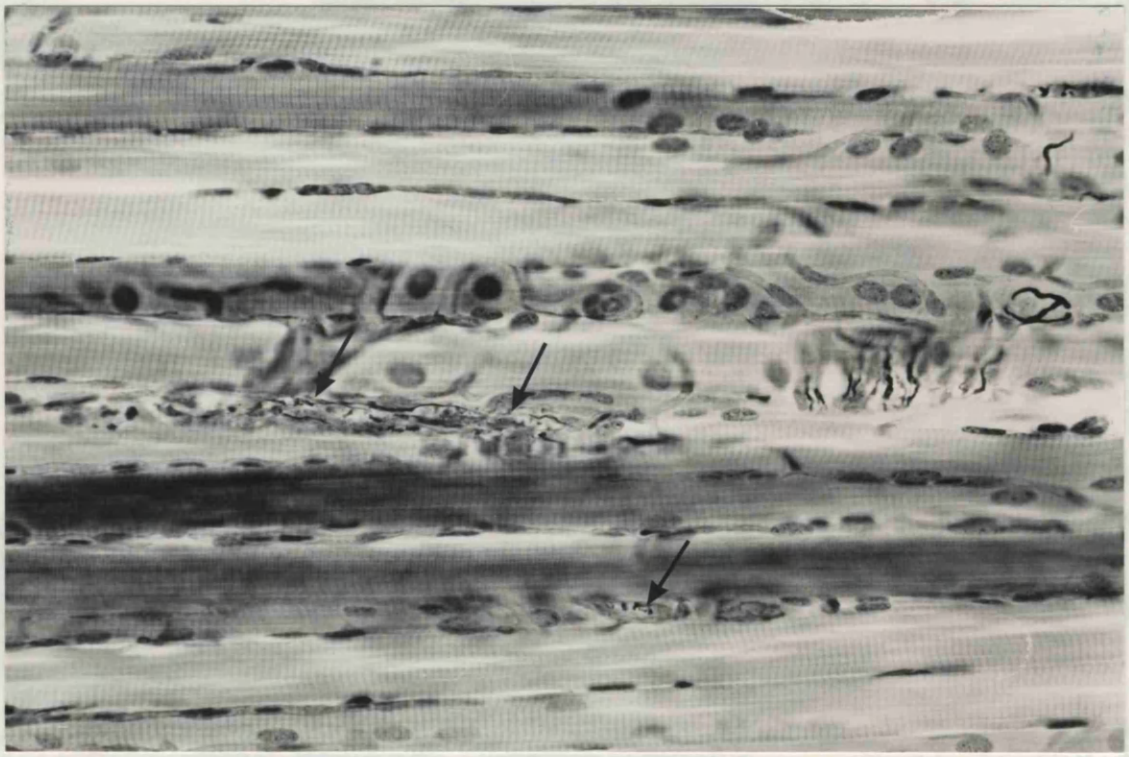

Fig. 3.20 24 hours. Paraffin section of lower leg muscle showing intramuscular nerves and end-plates. The presence of argyrophilic granules (arrows) is evidence of axonal degeneration. Glees silver method x 400.

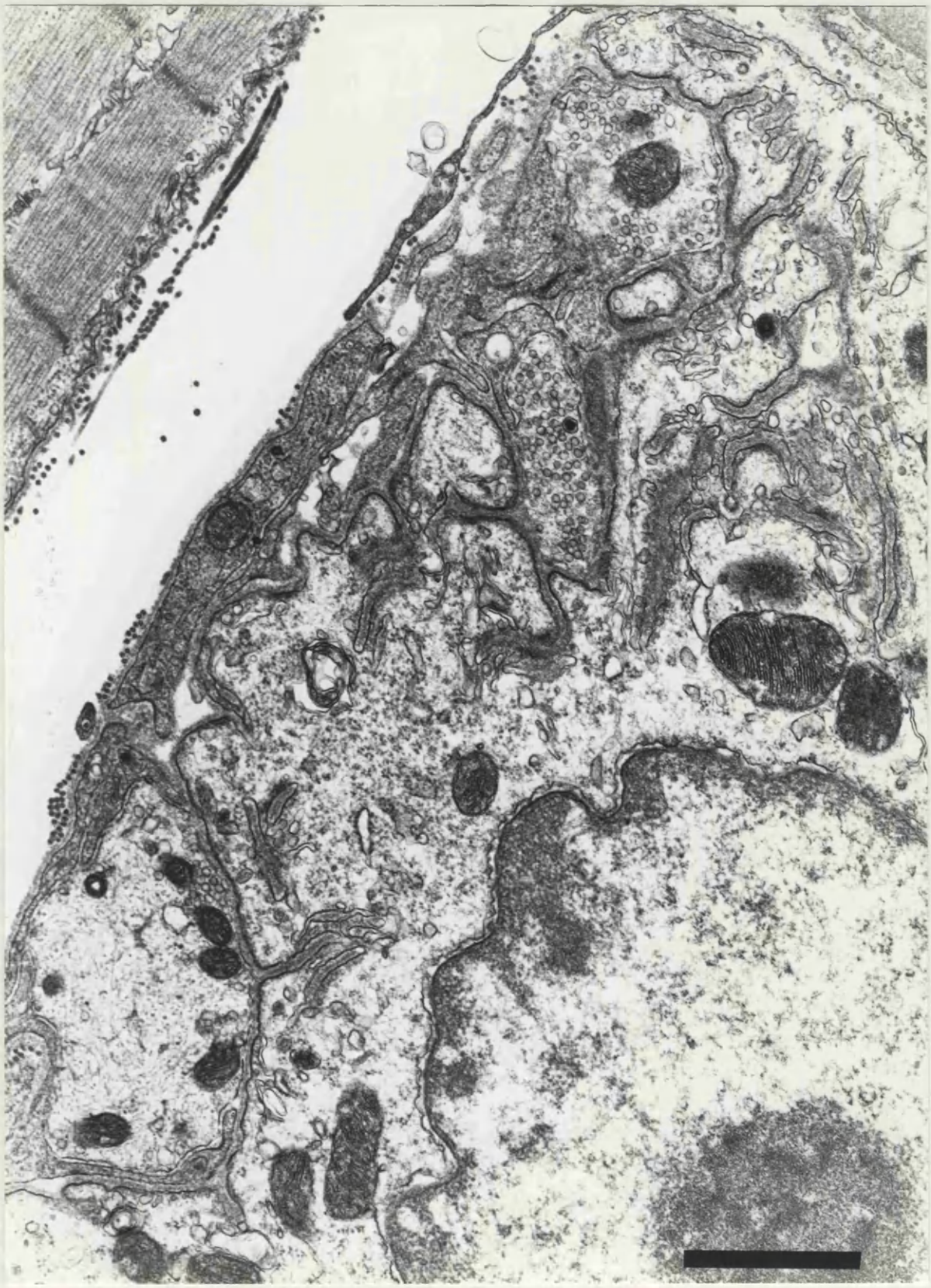

Fig. 3.21 Foot muscle at 24 hours. In this motor end-plate the appearances of nerve terminals is within normal limits. Bar = 1μm (Neg. 98052).

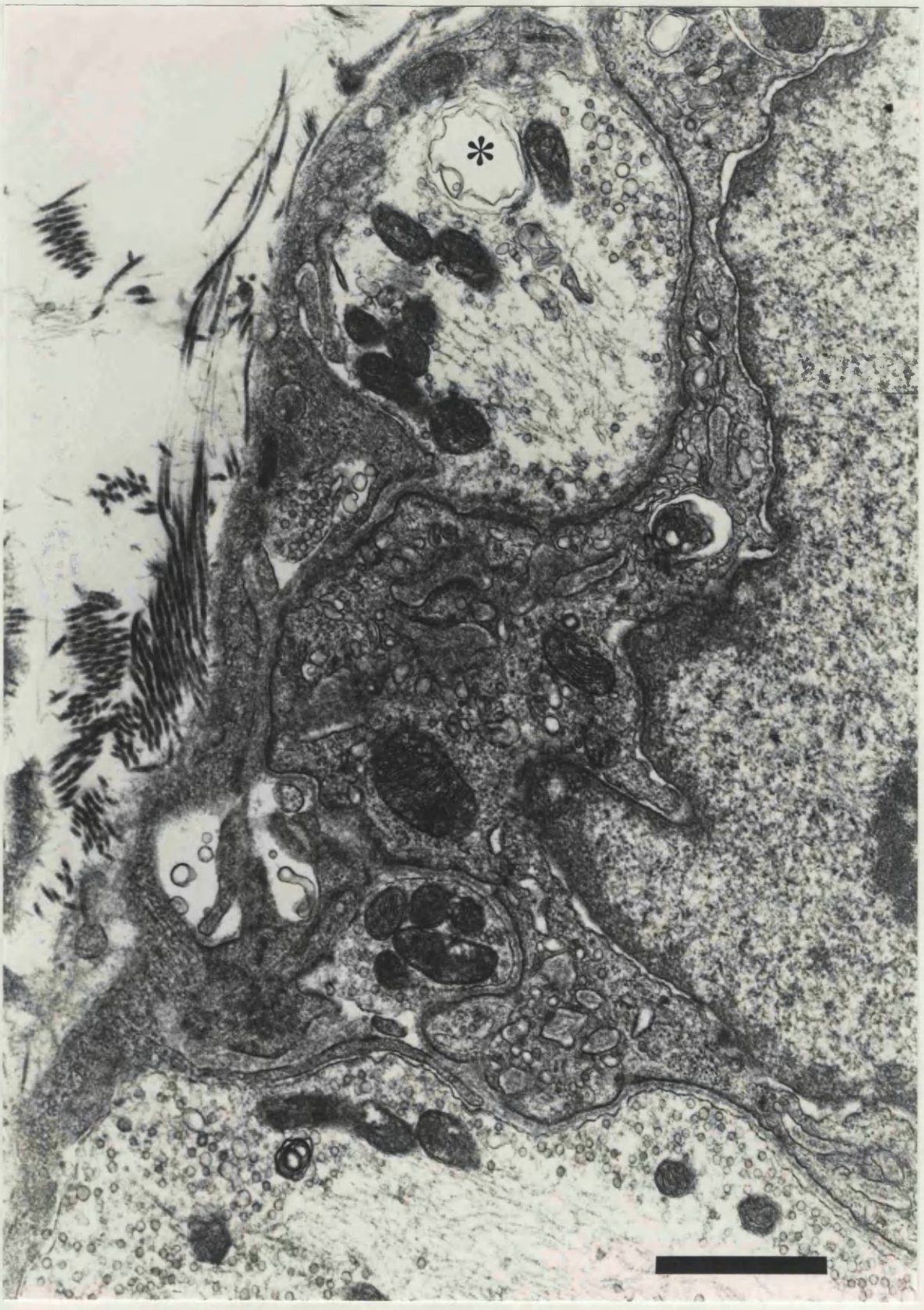

Fig. 3.22 Foot muscle at 24 hours. A membrane bound vacuole (*) may be abnormal. Other organelles do not appear to be abnormal. Bar = 1μm (Neg. 98050).

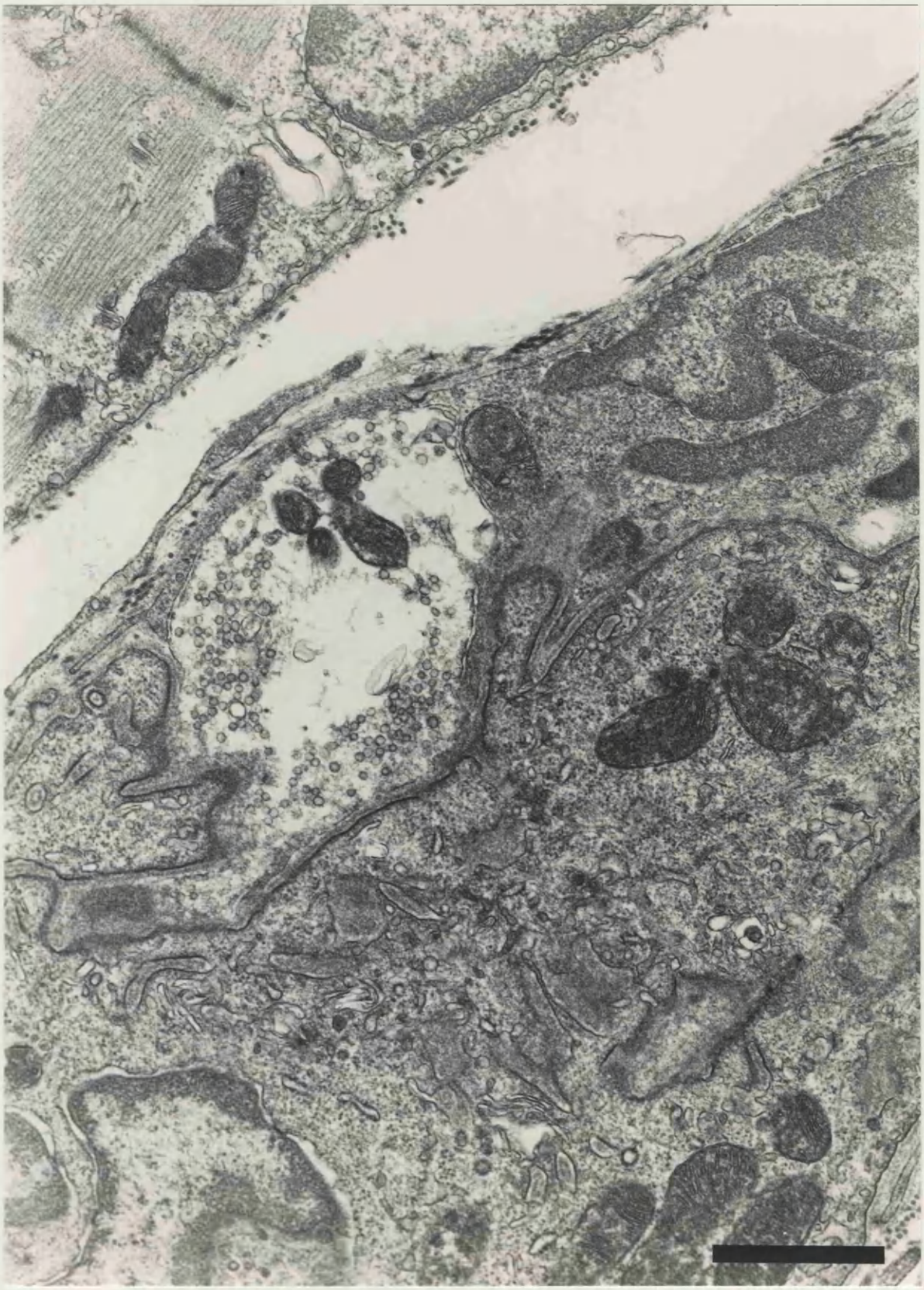

Fig. 3.23 Foot muscle. 24 hours. An area of rarefaction in the axoplasm of a terminal may be an early abnormality. Bar = 1μm (Neg. 98054).

rarefied part of axonal terminals or dilated membrane-bound cisternae which can sometimes be seen in control material. All the other end-plates showed evidence of abnormality. In some (Fig. 3.24, 25, 26, 27) the axonal terminals were rarefied and contained few vesicles in an electron-lucent axoplasm in which mitochondria were swollen and rounded. Processes of Schwann cell cytoplasm interposed between axon terminal and post-synaptic surface of the muscle fibre and the nucleus of the Schwann cell lay close to the synaptic area of the muscle fibre, an unusual position for the nucleus. In other end-plates some axonal terminals looked normal whilst some were degenerating. The end-plate illustrated in Fig. 3.28 is an example in which one (or part of one) axonal terminal is relatively normal; one contains a rounded clear space and a degenerating mitochondrion; another contains degenerating mitochondria and a fourth contains clusters of dense degenerative vesicles, mitochondria and dense bodies. Whether these are all separate terminals or are part of the same terminal is not known.

At this stage there were no completely denervated end-plates. Muscle fibres showed no significant changes.

Electron microscopy of peripheral nerves included lumbar motor and sensory roots and sciatic and posterior tibial nerves. The appearances of all these were within normal limits. There was no degeneration or vacuolation of axons or myelin sheaths, Schwann cells were normal and there was no endoneurial oedema or excess collagen. Unmyelinated axons were also normal in appearance.

In the smaller distal nerves, however, there were abnormalities in the axons. These were seen in intramuscular nerve bundles and in single preterminal myelinated axons in soleus and foot muscles (Figs. 3.29, 30, 31) and took the form of rarefaction of the axoplasm or

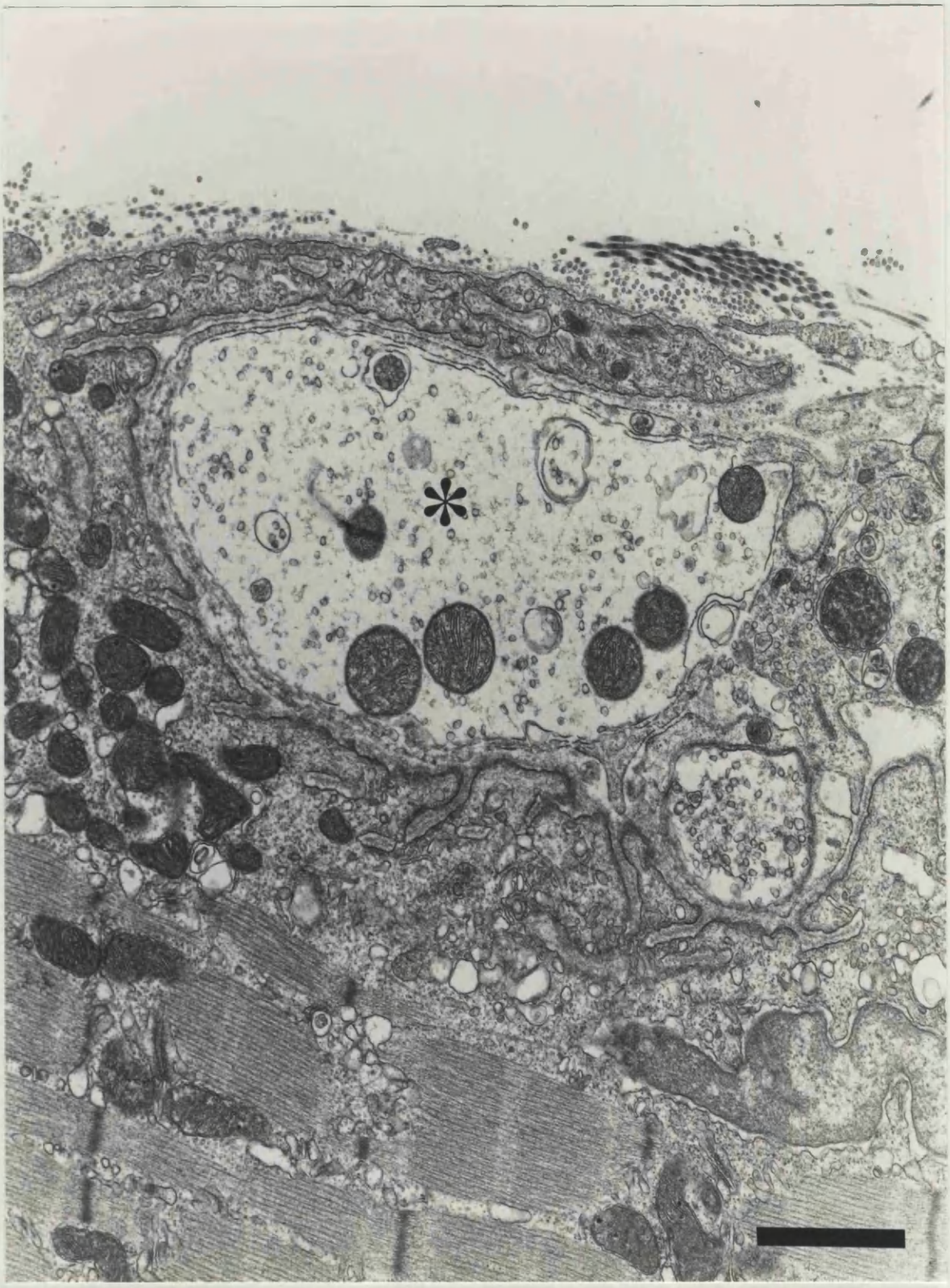

Fig. 3.24 Foot muscle of same mouse as Figs. 3.22 and 3.23, 24 hours after CL This end-plate clearly is abnormal. One axonal terminal (*) contains rarefied electron-lucent axoplasm, rounded degenerated mitochondria and few widely-dispersed vesicles. An adjacent terminal contains degenerated dense mitochondria. The terminals are being separated from muscle membrane by processes of Schwann cell cytoplasm. Bar = 1μm (Neg. 98063).

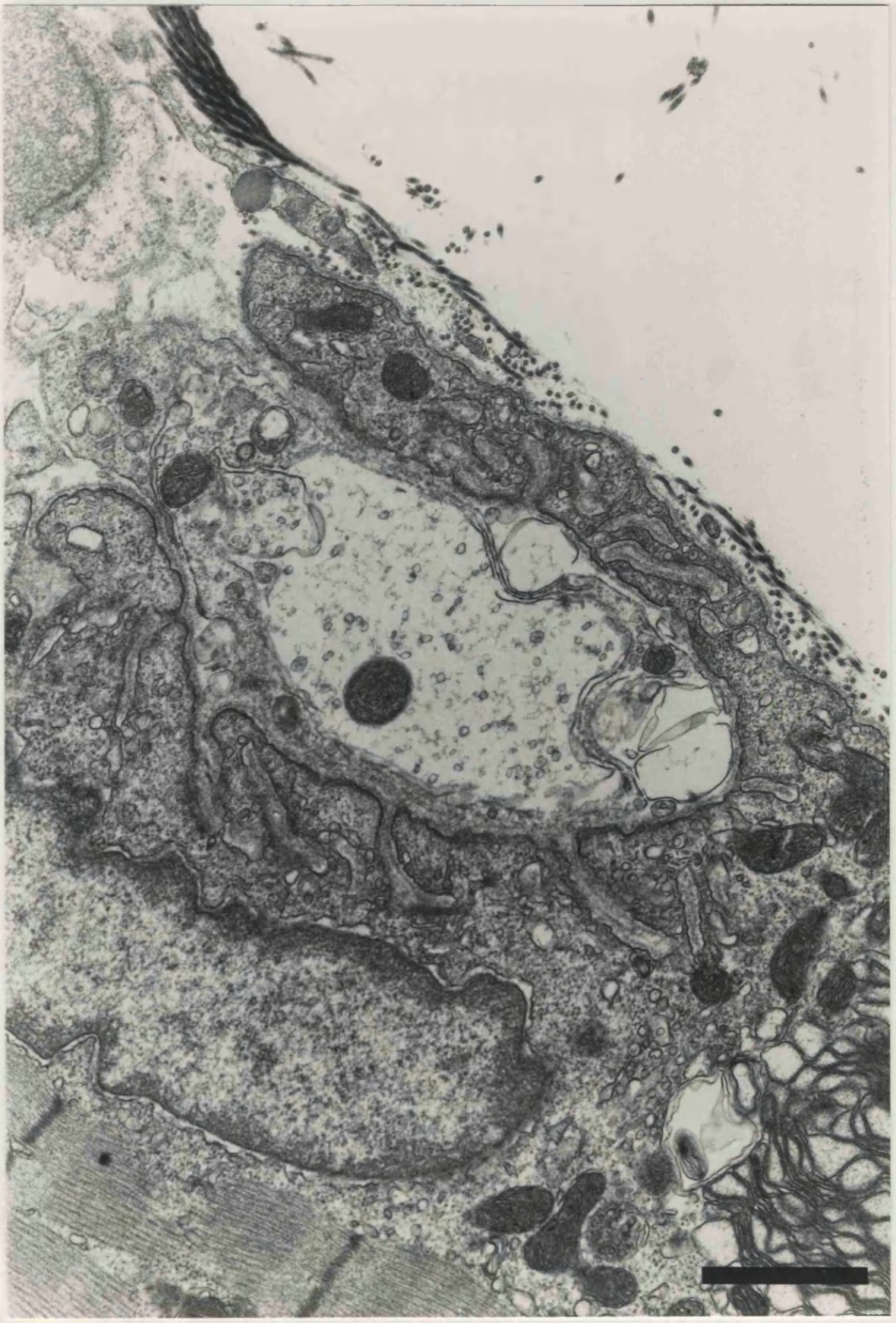

Fig. 3.25 Foot muscle at 24 hours. Early degeneration of an axonal terminal similar to that in Fig. 3.24 which is from the same animal. Bar = 1μm (Neg. 98060).

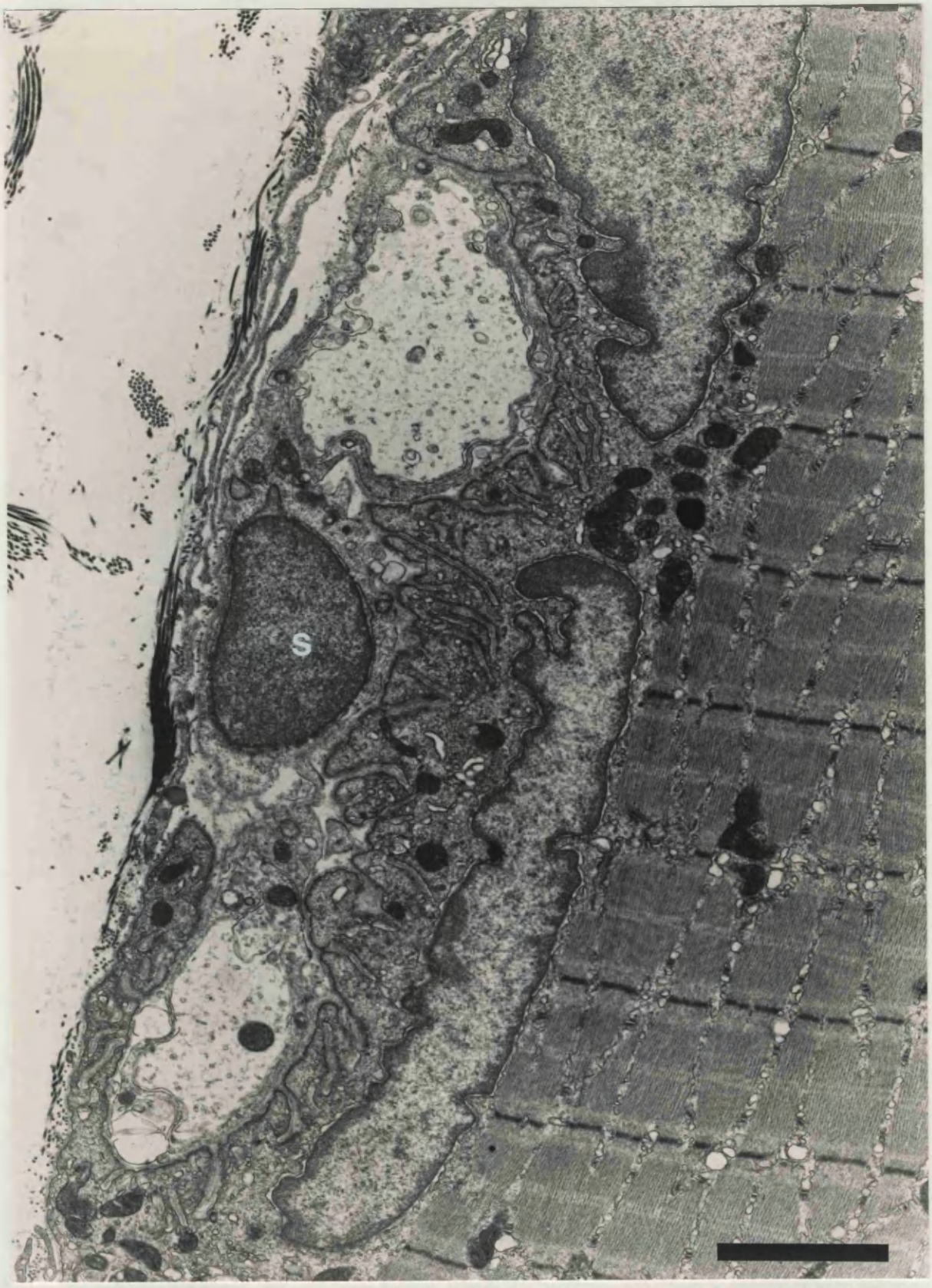

Fig. 3.26 Foot muscle at 24 hours (same muscle as in 3.23 and 3.24). Axonal terminals are rarefied, contain few vesicles and are wrapped in Schwann cell processes. The nucleus of the Schwann cell (S) lies close to the post-synaptic membrane. This is an unusual position for the nucleus. Bar = 2μm (Neg. 98061).

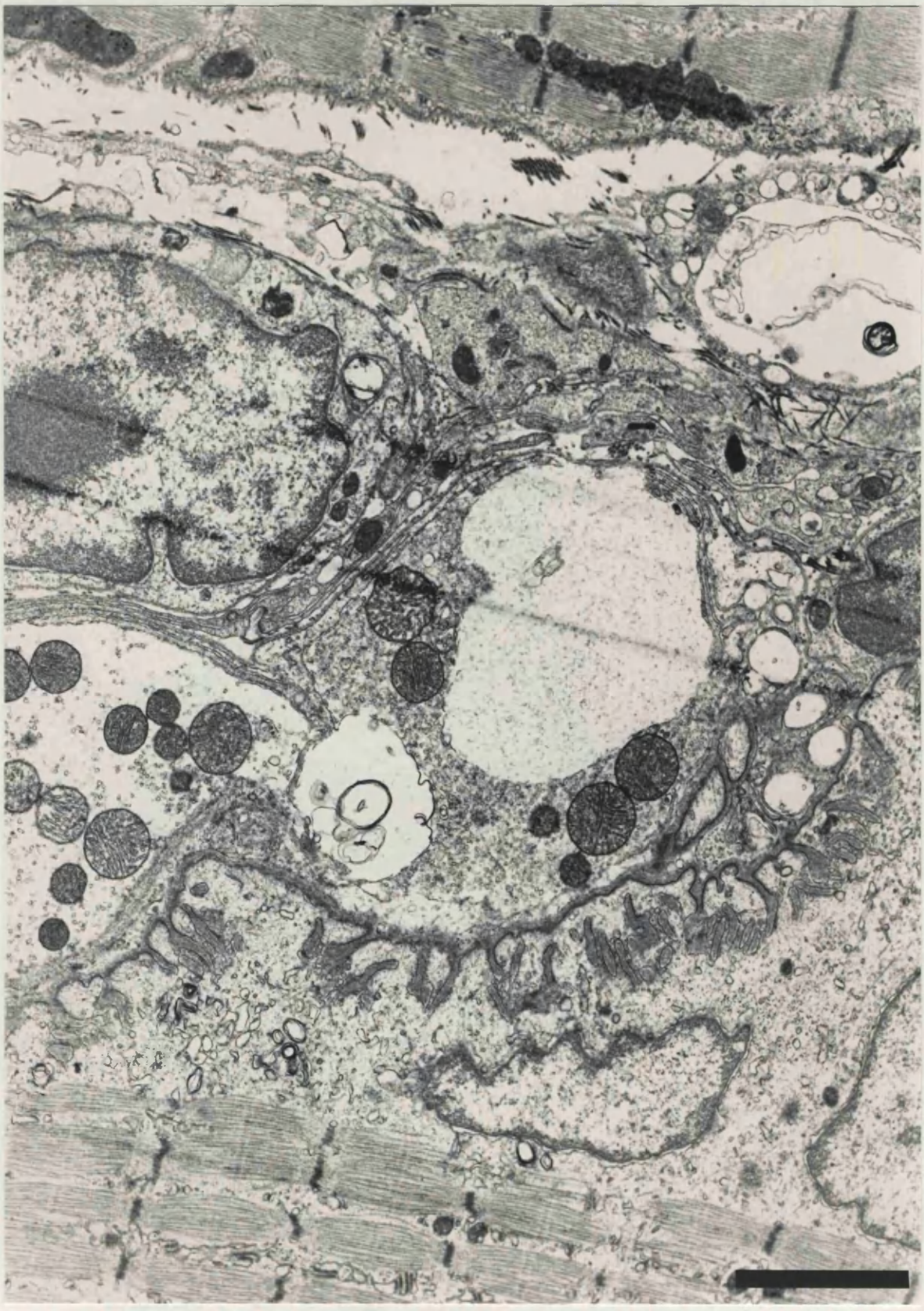

Fig. 3.27 End-plate in soleus at 24 hours (same animal as in Figs. 3.24-26). Several large vacuoles may be intra-axonal or peri-axonal. Rounded mitochondria have disrupted cristae. There is clearly early degeneration at this end-plate. Bar = 1μm (Neg. 98414).

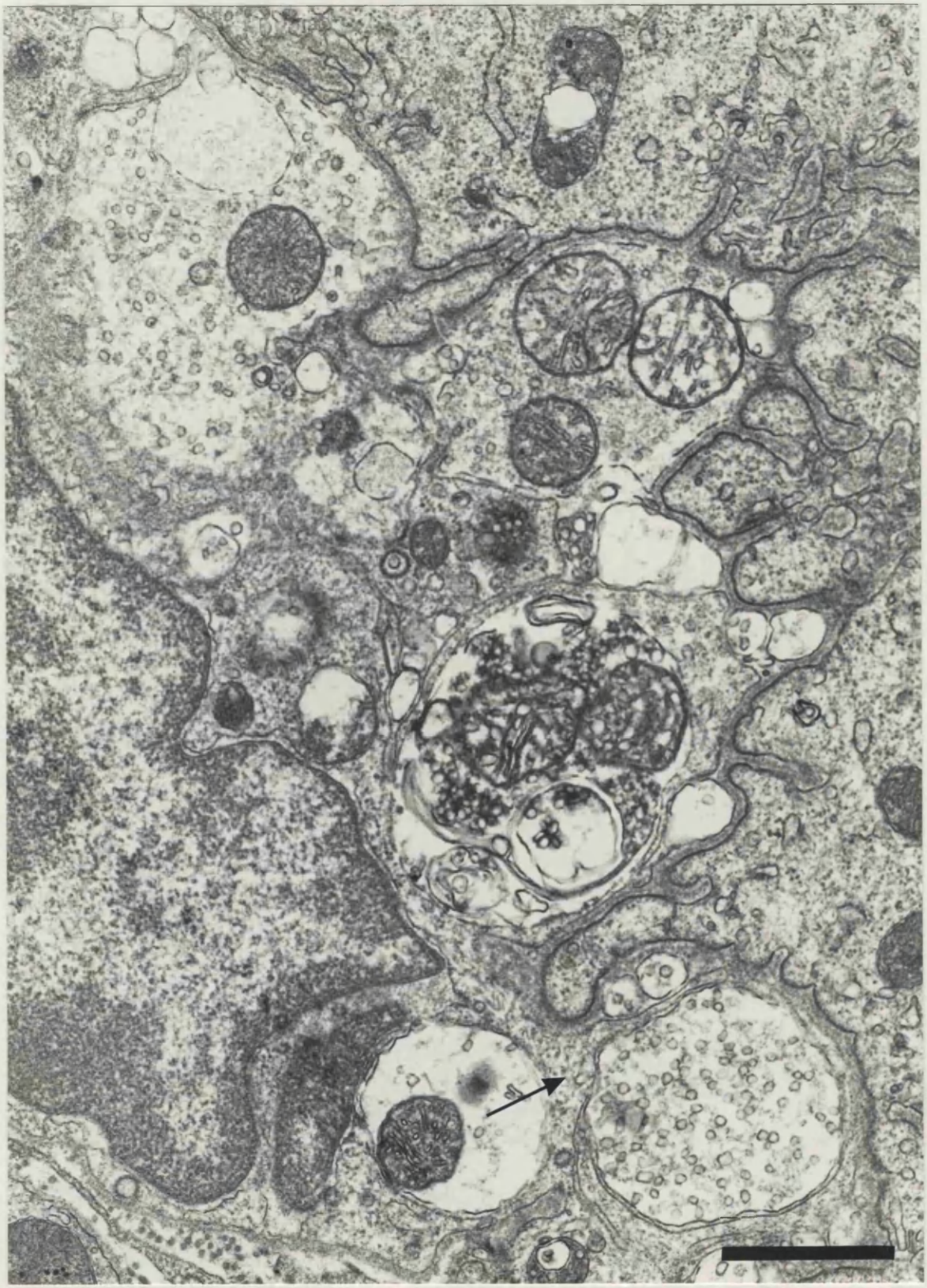

Fig. 3.28 Motor end-plate in soleus at 24 hours. There is clear evidence of axonal degeneration, in the form of accumulations of dense bodies, clumps of dense vesicles, and mitochondria which are rounded and have disrupted cristae. Processes of Schwann cell cytoplasm (arrow) are surrounding one terminal. Bar = 1μm (Neg. 98411).

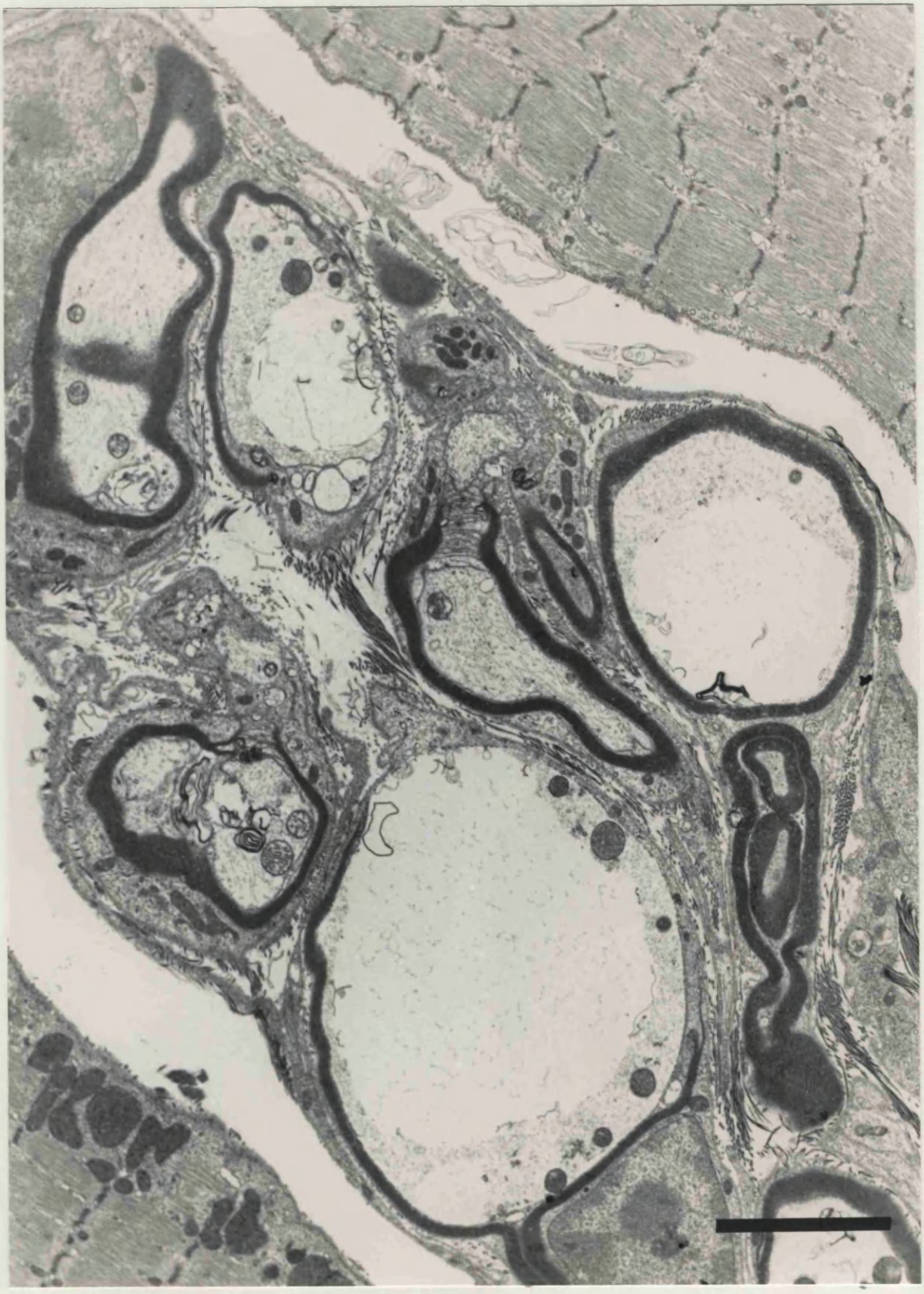

Fig. 3.29 Intramuscular nerve in soleus at 24 hours. Vacuoles or areas of axoplasmic rarefaction cause distension of myelinated nerve fibres and are early signs of axonal degeneration. There is no intramyelinic vacuolation. Bar = 3μm (Neg. 98412).

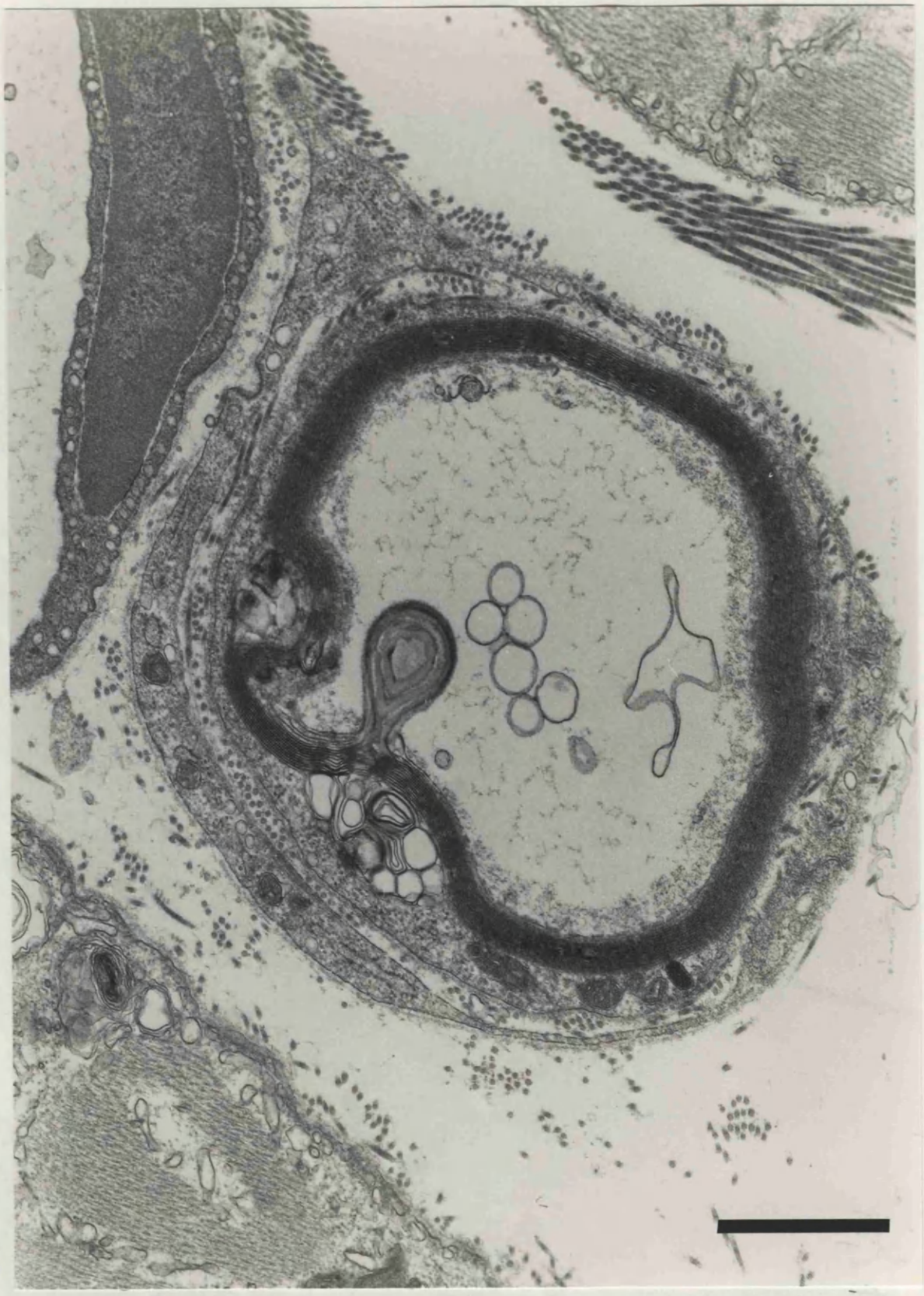

Fig. 3.30 Myelinated nerve fibre in foot muscle at 24 hours, probably close to an end-plate. The axoplasm is rarefied and contains little vacuoles and granular amorphous material. The axolemma may be breaking down. Bar = 1μm (Neg. 98066).

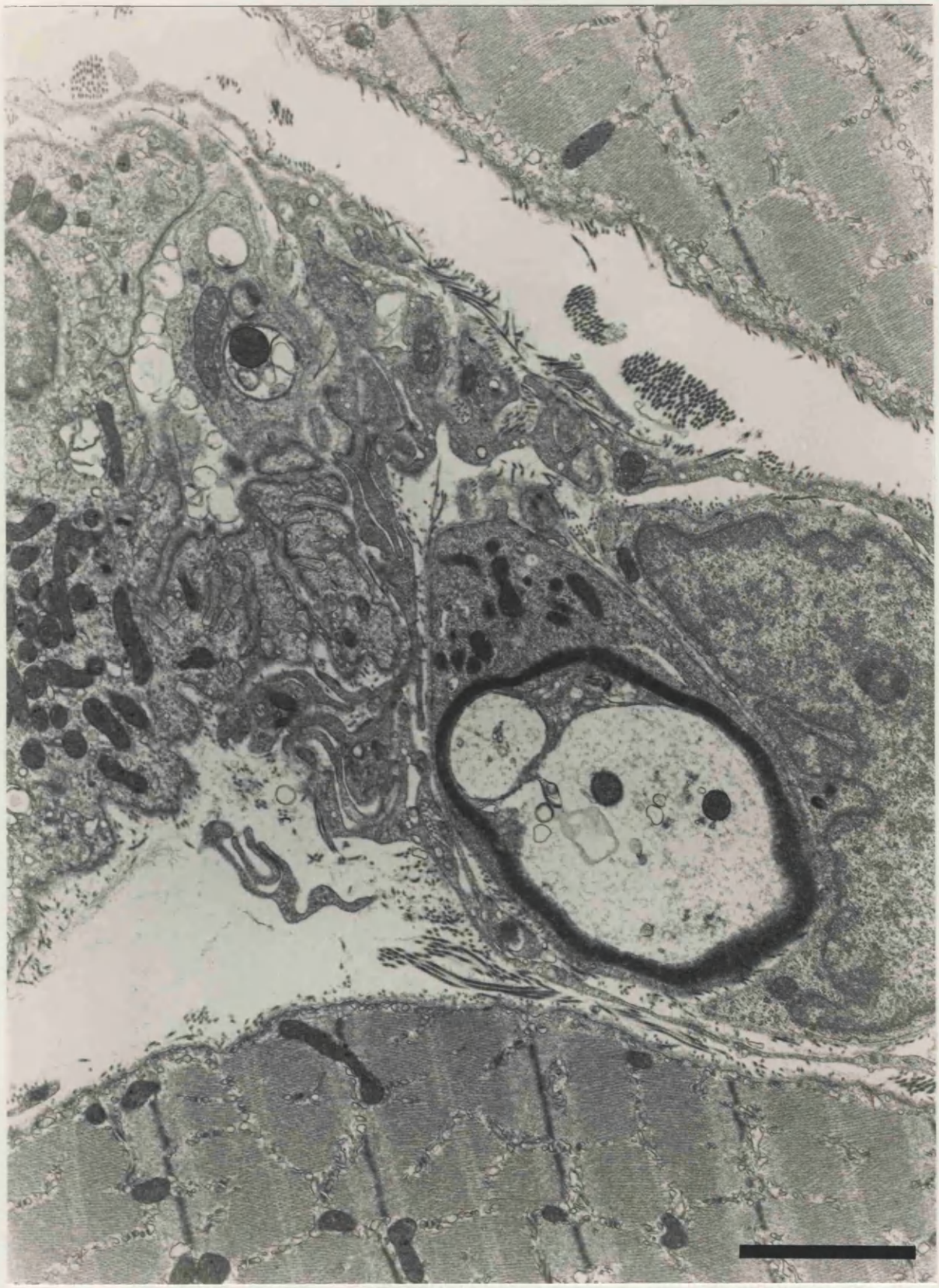

Fig. 3.31 A preterminal axon in foot muscle at 24 hours. There is axonal degeneration similar to that in Fig. 3.30. Bar = 2μm (Neg. 98064).

perhaps of development of a membrane-bound vacuole. In some of the axons the membrane appears to be becoming fragmented. Although the paraffin silver preparations showed axonal fragmentation in some small intramuscular nerves these were not visualized in the EM preparations.

Sensory root ganglia were examined with EM and the appearance of the ganglion cells was within normal limits (Fig. 3.32).

48 hours

Material available for examination at 48 hours, from four mice, included blocks of cerebral cortex and white matter, basal ganglia, cerebellum, pons, spinal cord, peripheral nerves and muscles.

Central nervous system

The vacuolation of white matter in the cerebral hemisphere and in cerebellum was very severe (Fig. 3.33). The tissue had a diffusely spongy appearance and only in some small areas were myelinated fibres spared. Vacuoles were mostly rounded and large - three or four times the diameter of the axon. Some myelin could be seen immediately surrounding the axon. The vacuolation of white matter in the spinal cord was not very striking, being mostly confined to the dorsal columns. Some regions of anterior columns were slightly affected.

Electron microscopy (Figs. 3.34 and 35) showed the marked intramyelinic vacuolation without axonal degeneration in the cerebellum. The vacuoles were multilocular, traversed by lamellae of myelin. Axonal terminals were intact and synaptic thickening present as in the normal. Oligodendrocytes were of normal appearance. In the dorsal columns of the spine there was an occasional degenerating nerve fibre.

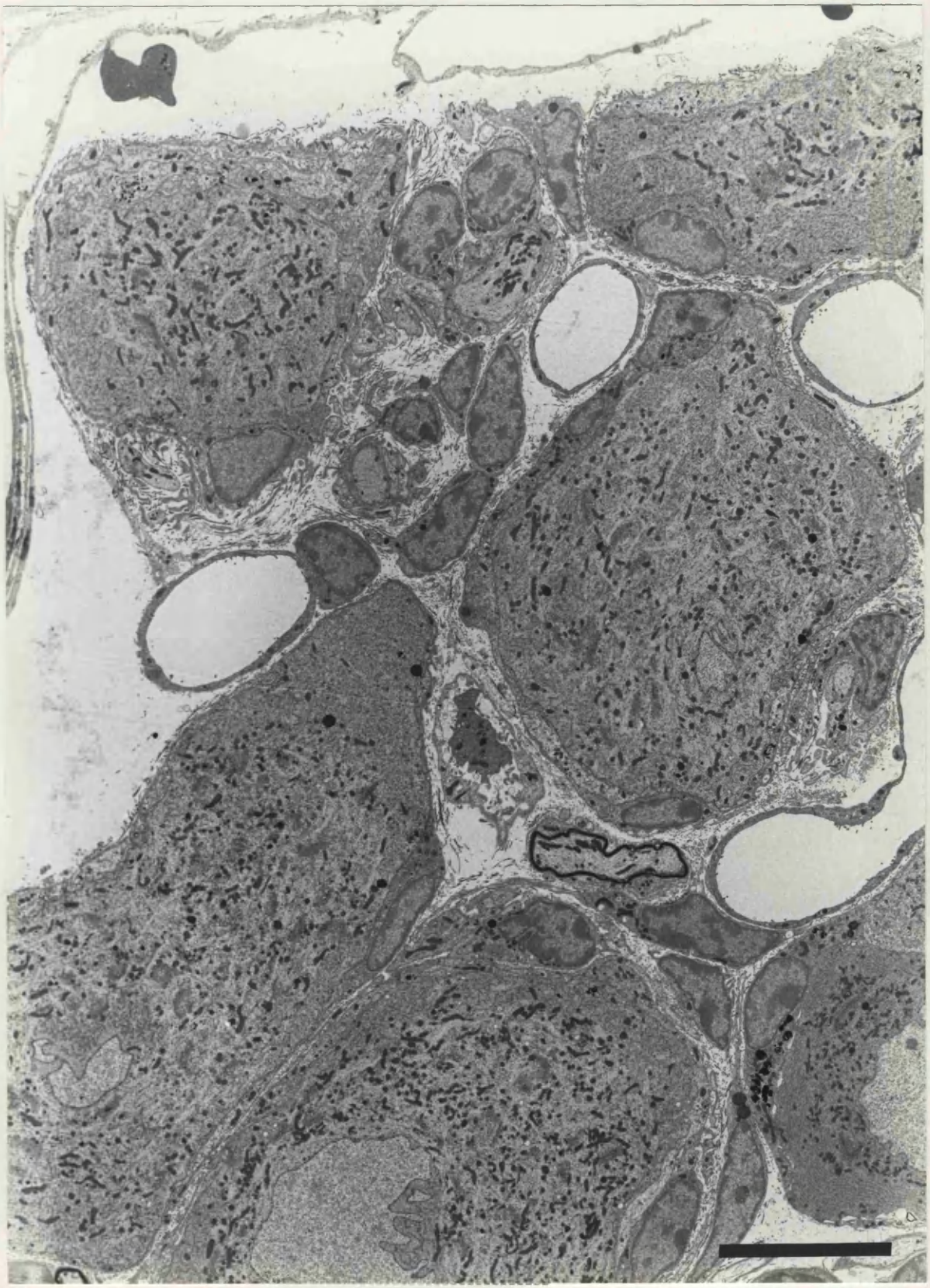

Fig. 3.32 Lumbar dorsal root ganglion at 24 hours. The ganglion cells appear normal. Bar = 10μm (Neg. 98073).

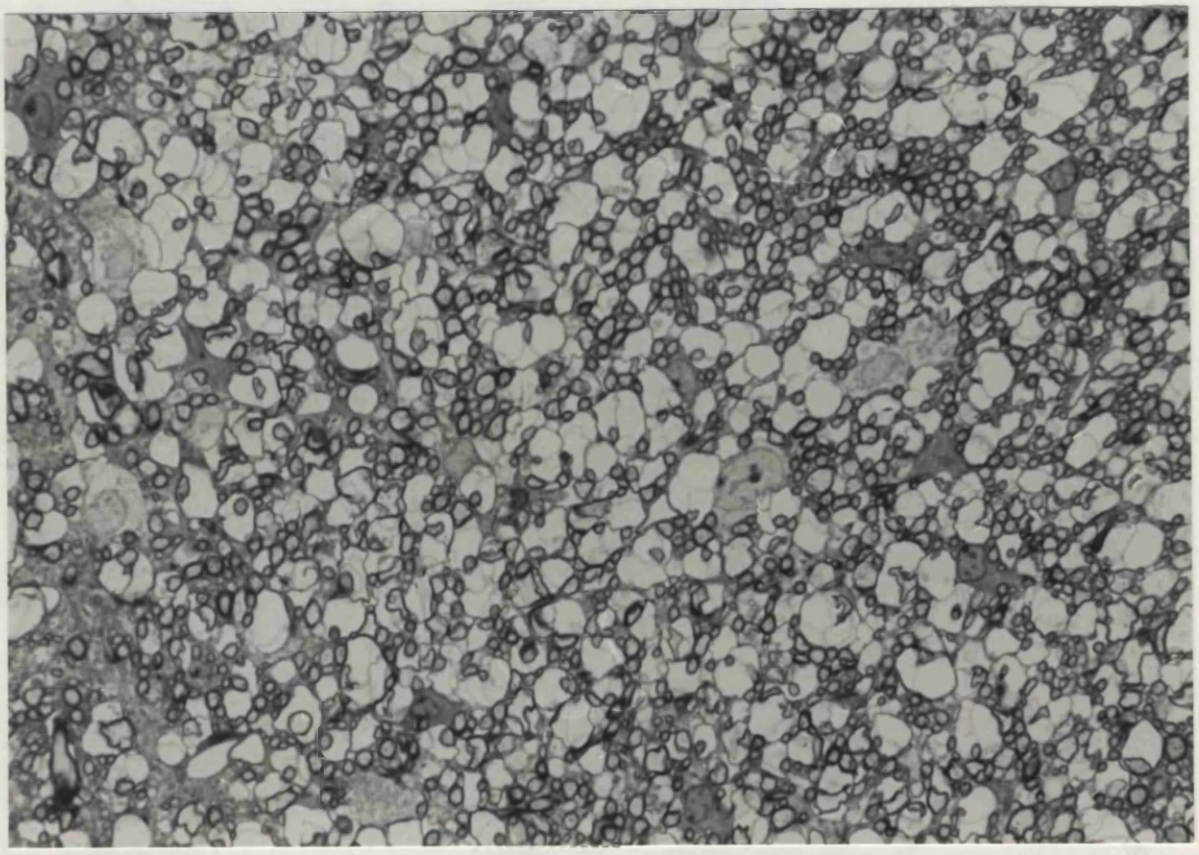

Fig. 3.33 Cerebellum at 48 hours. White matter shows severe vacuolation giving the tissue a spongiform appearance. 1μm plastic section. Toluidine blue x 400.

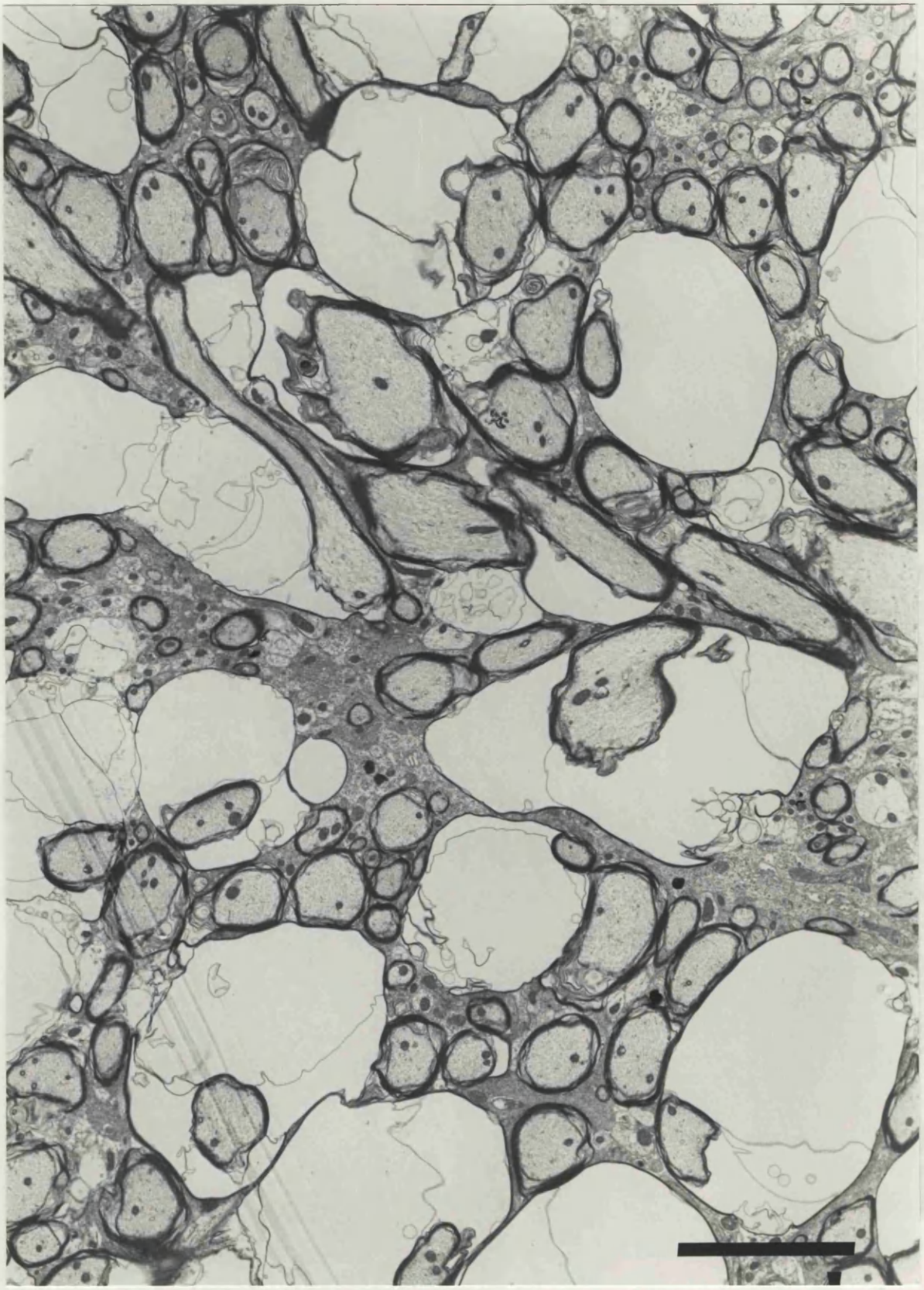

Fig. 3.34 Cerebellar white matter at 48 hours showing the marked intramyelinic vacuolation. No axonal degeneration. Vacuoles are multilocular and traversed by lamellae of myelin. Bar = 5μm (Neg. 99600).

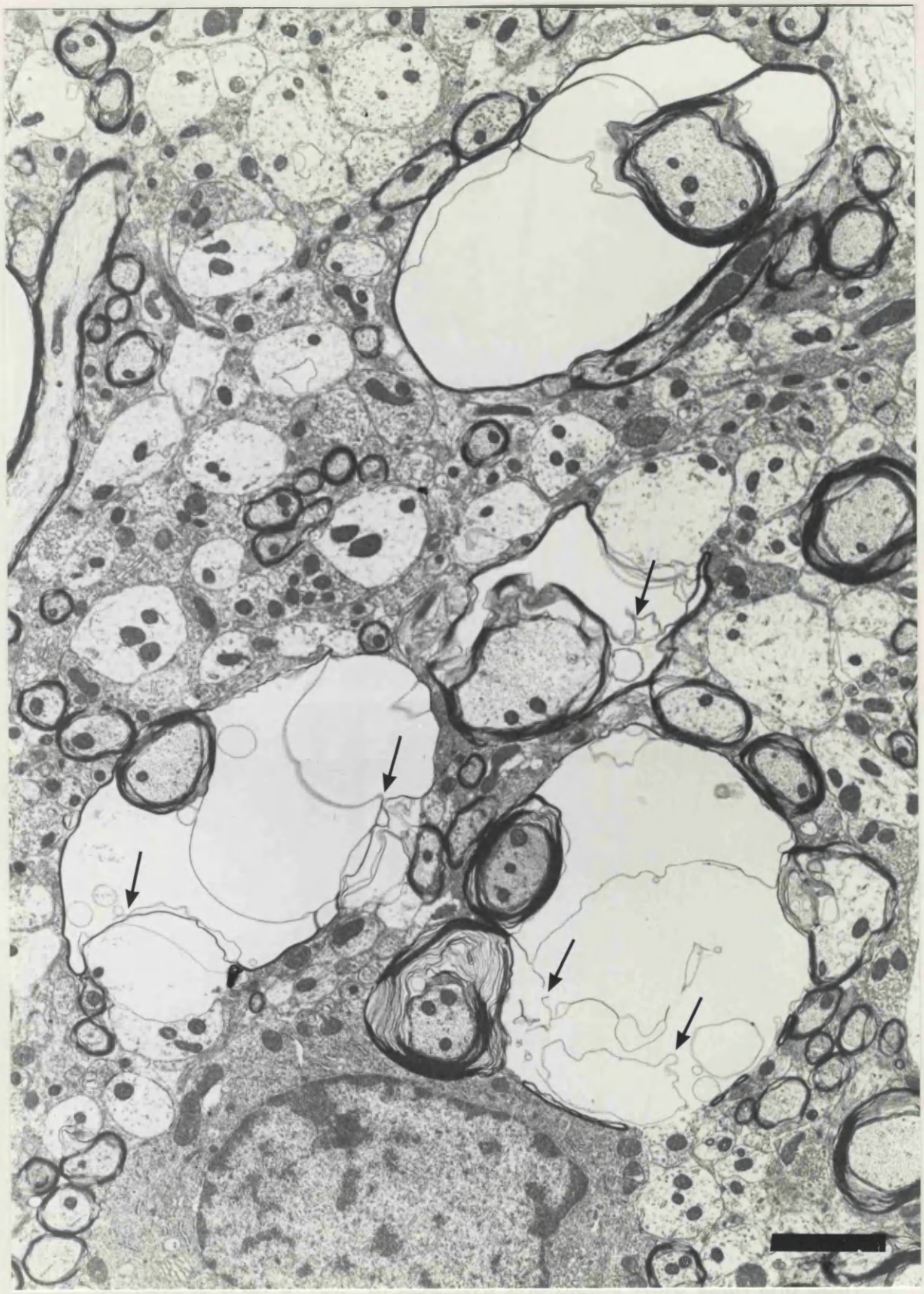

Fig. 3.35 Cerebellum at 48 hours. Several large multilocular intramyelinic vacuoles shown. The part of the myelin sheath forming the outer wall of the vacuole seems to be breaking down (arrows) with fragments of myelin lamellae forming irregular membranes across the vacuoles. Dendrites, axons, axonal terminals, synapses and an oligodendrocyte appear normal. Bar = 2μm (Neg. 99602).

Peripheral nerve and muscle

Electron microscopy of tibial (Fig. 3.36) and sciatic nerves showed no significant abnormality. The axons and myelin sheaths were of normal appearance and there was no vacuolation. An occasional nerve fibre in the tibial nerve showed degeneration of the axon (Fig. 3.37), the myelin sheath being still intact.

In the intramuscular nerves there was now abundant evidence of degeneration of myelinated fibres (Figs. 3.38 - 39). Many nerve fibres lacked axons and myelin ovoids or irregular dense fragments remained. In other fibres the axoplasm was electron lucent and contained vacuoles or membrane-bound cisternae (Fig. 3.40) and the axonal organelles seemed to have disintegrated and mitochondria were rounded and abnormally dense.

The contrast between nerves innervating extrafusal muscle fibres and those innervating muscle spindles is shown in Fig. 3.41. The extrafusal nerve shows severe degeneration while the intrafusal myelinated fibres are normal in appearance. In keeping with this observation is the absence of pathological changes in the sensory innervation of intrafusal fibres (Fig. 3.42), the terminals of the annulospiral ending looking normal.

The innervation of extrafusal muscle fibres was now very abnormal. Fifteen motor end-plates were visualized in foot muscle, soleus and EDL. In the foot muscles, all 10 end-plates seen were denervated and Schwann cell cytoplasm covered the post-synaptic membrane (Fig. 3.43). In EDL two end-plates were seen and both were denervated while in soleus two end-plates were innervated by normal looking axonal terminals and one was denervated. It seems that the more distal, the more severe and complete the motor neuropathy.

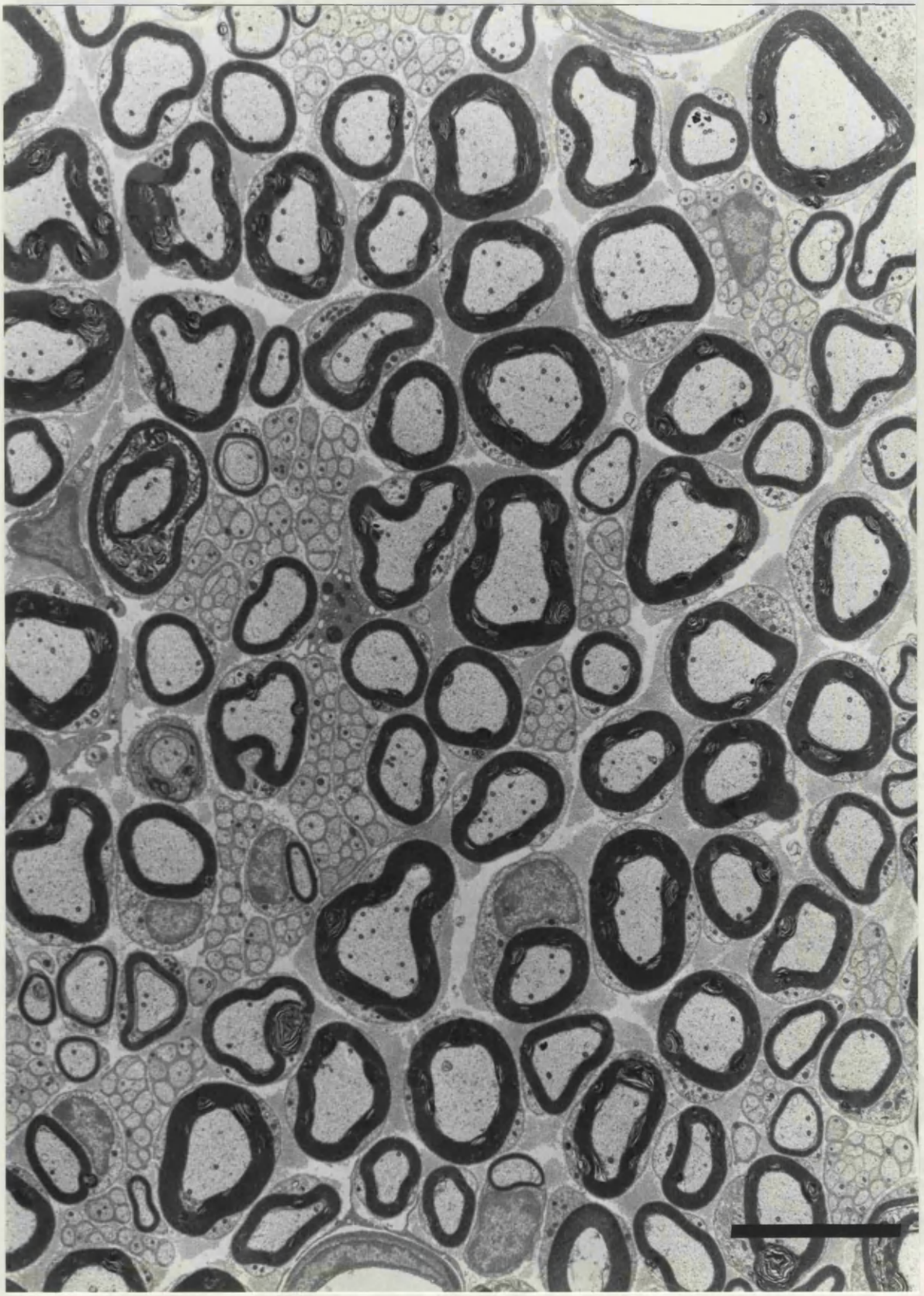

Fig. 3.36 Posterior tibial nerve at 48 hours. Appearances are within normal limits. There is no vacuolation of myelin. Bar = 6μm (Neg. 98563).

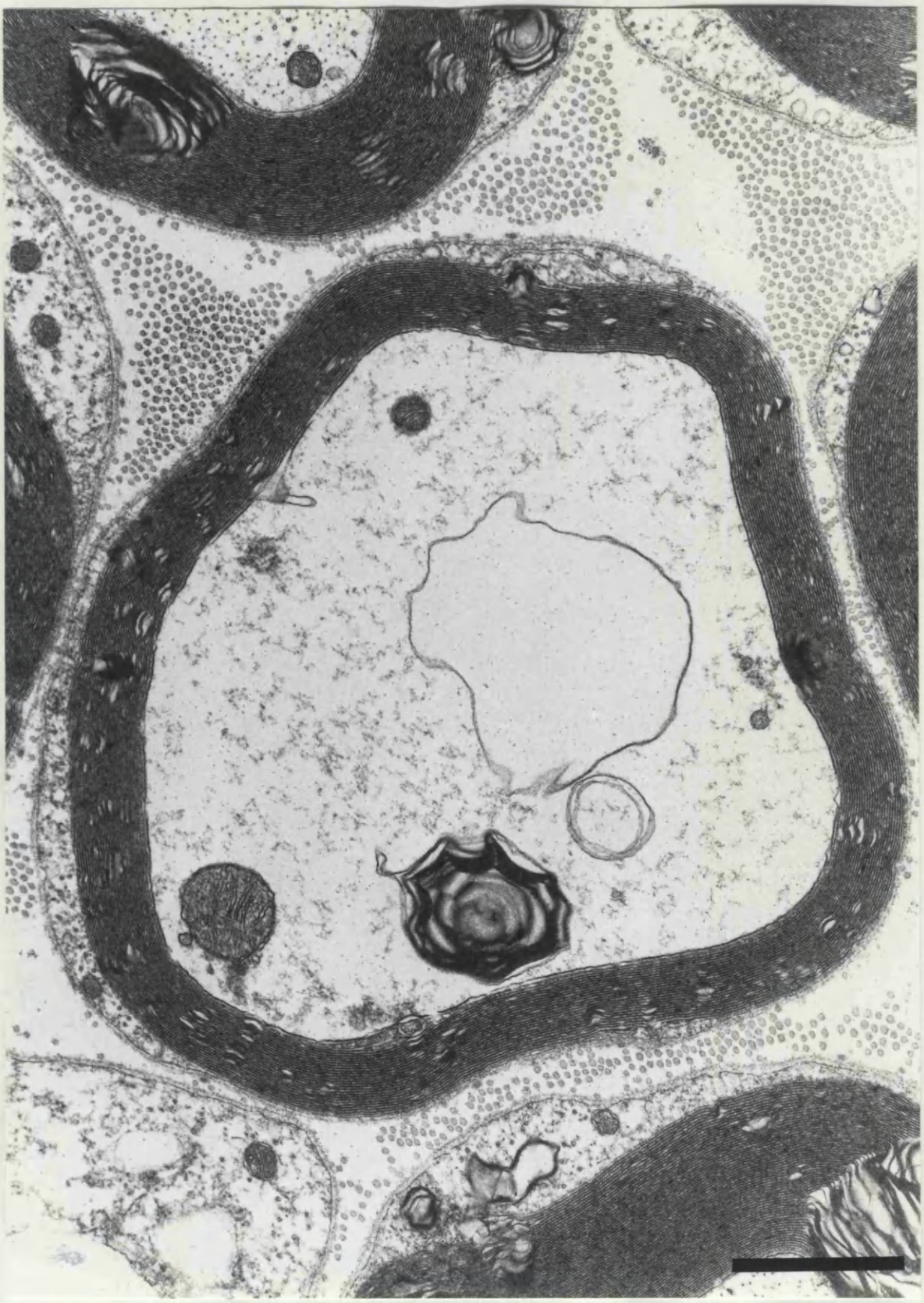

Fig. 3.37 Posterior tibial nerve at 48 hours. Occasional nerve fibres show axonal degeneration. The axonal organelles are disintegrated and a vacuole is present in the axoplasm. The myelin sheath is still intact. Bar = 1μm (Neg. 98561).

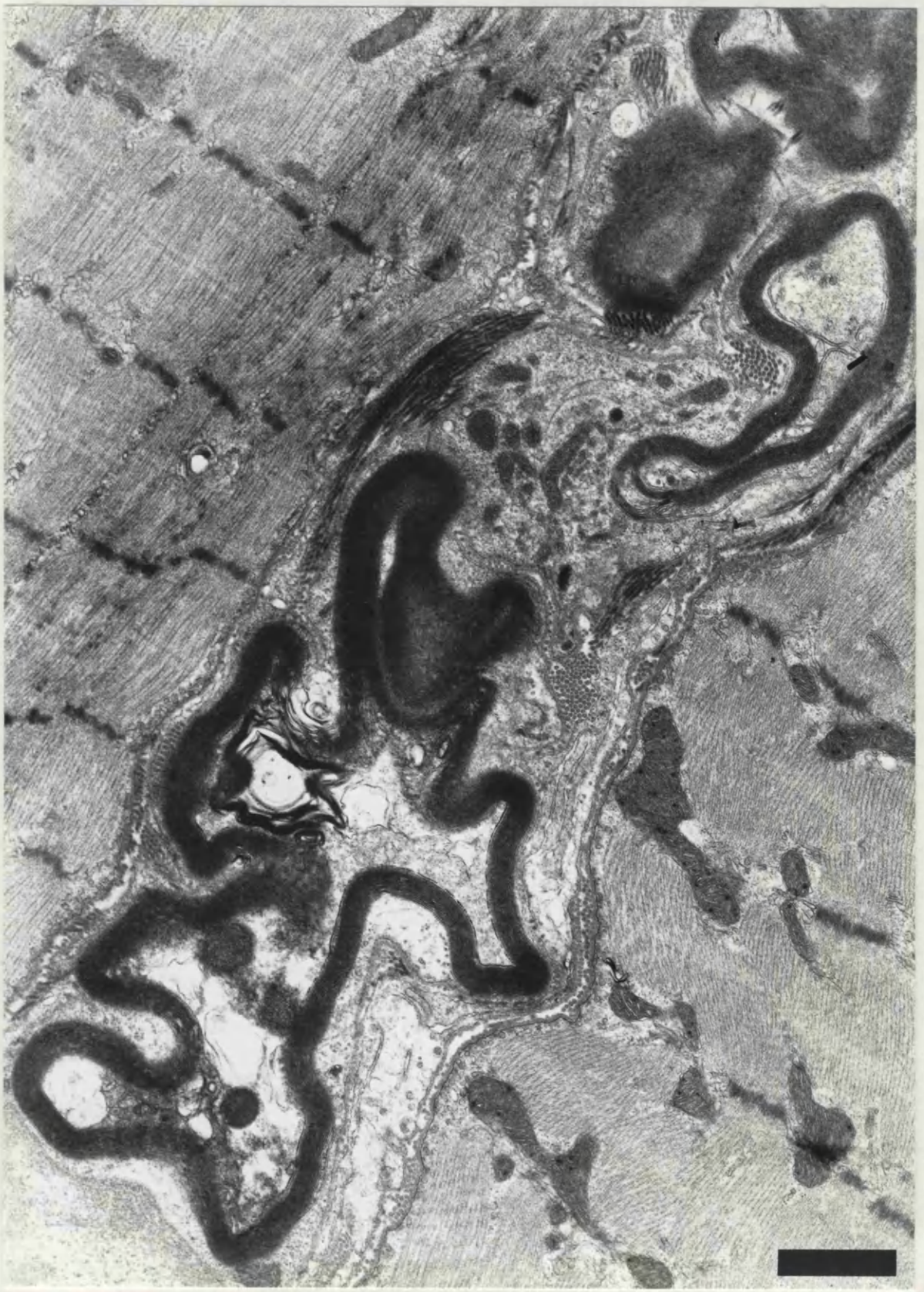

Fig. 3.38 Soleus at 48 hours. Intramuscular nerve bundle consists of fibres showing axonal degeneration and breakdown of myelin. Bar = 1μm (Neg. 98490).

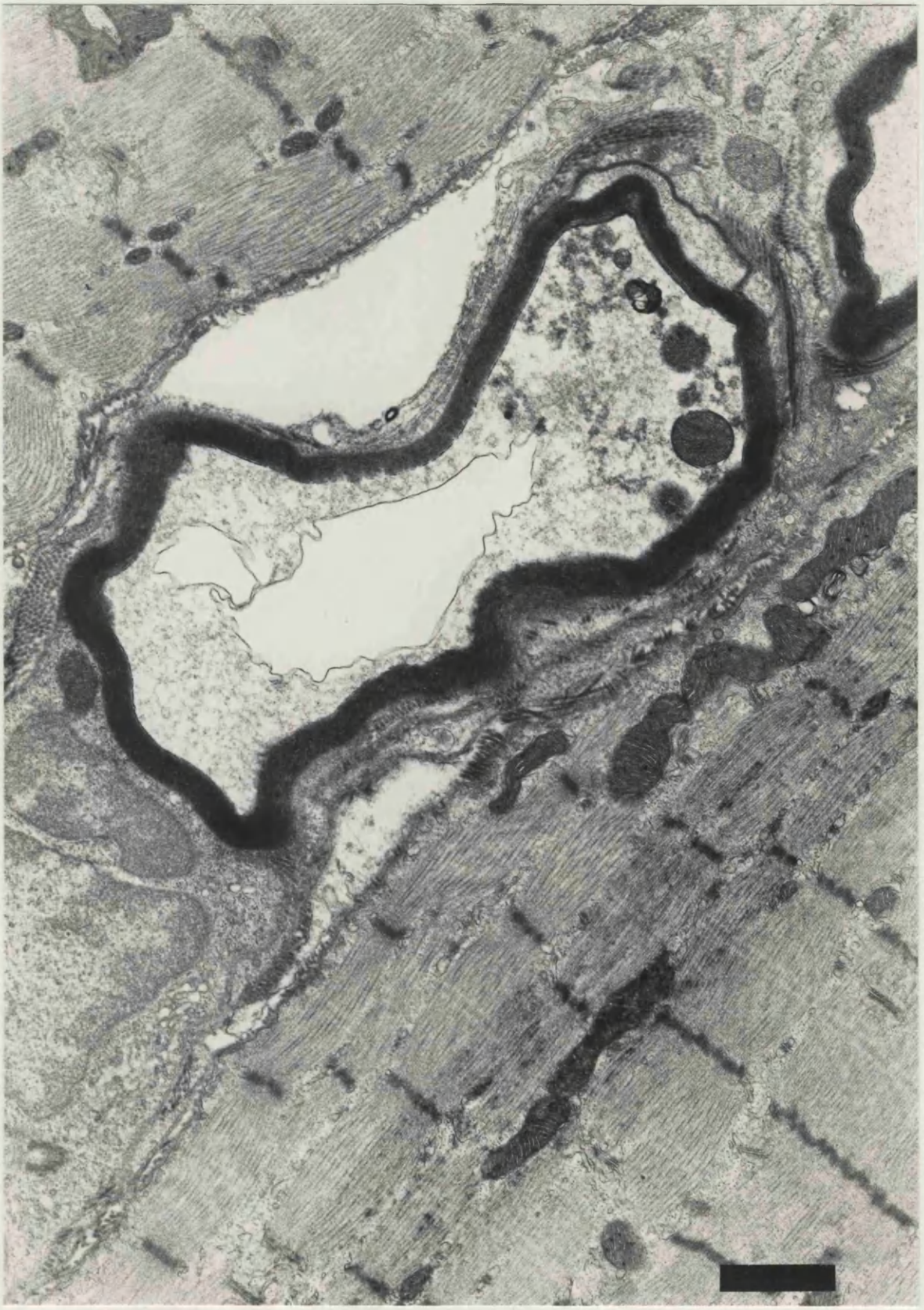

Fig. 3.39 Axonal degeneration in intramuscular nerve fibre at 48 hours. Axoplasm composed of granular amorphous debris and degenerated mitochondria and a vacuole are present. The myelin is still intact. Bar = 1μm (Neg. 99619).

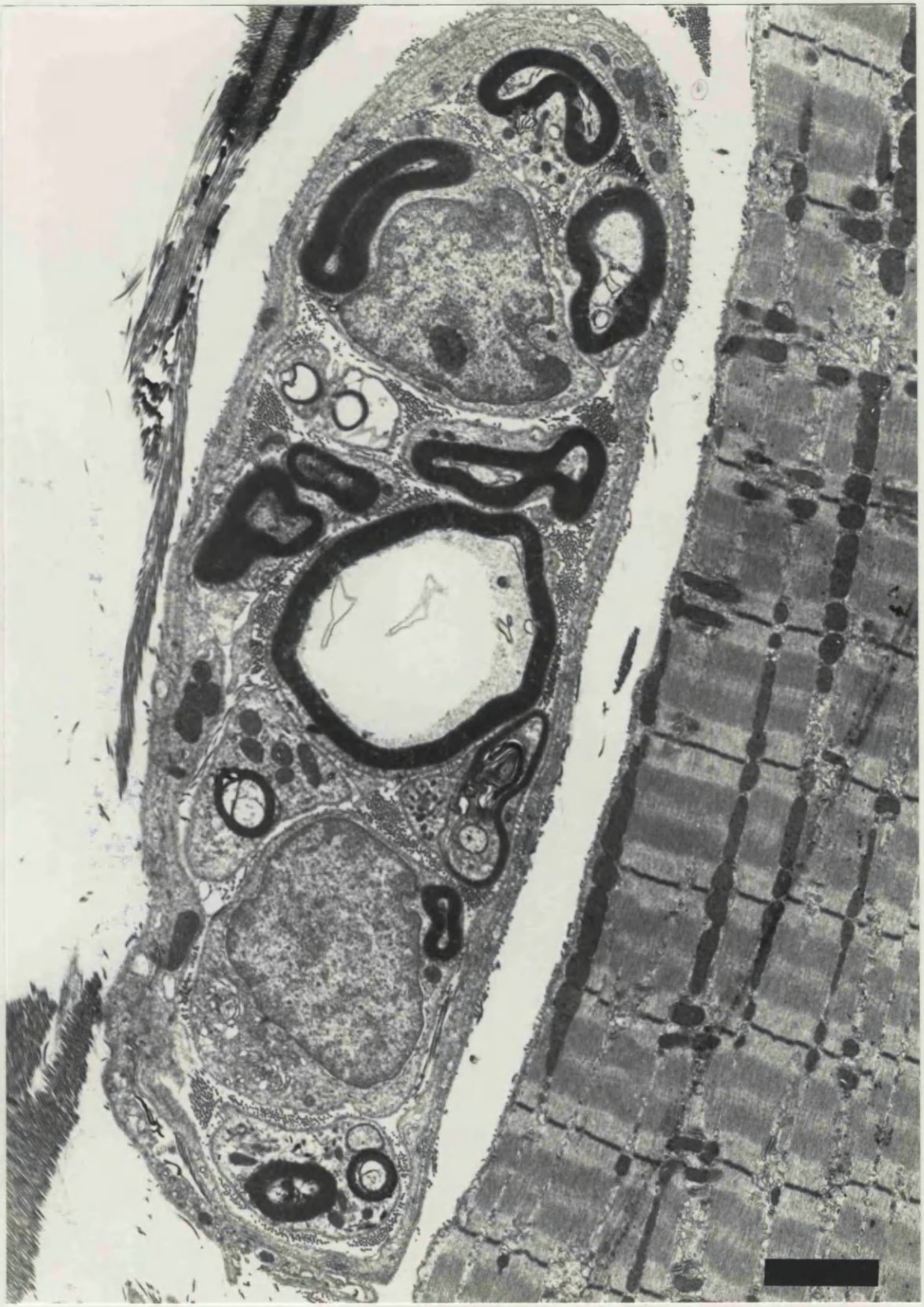

Fig. 3.40 Degeneration of intramuscular nerves in foot muscle at 48 hours. The distended axoplasm of one fibre is largely replaced by clear space, perhaps a breaking down vacuole. All the fibres in this nerve show axonal degeneration with early breaking up of myelin sheaths. Bar = 2μm (Neg. 99619).

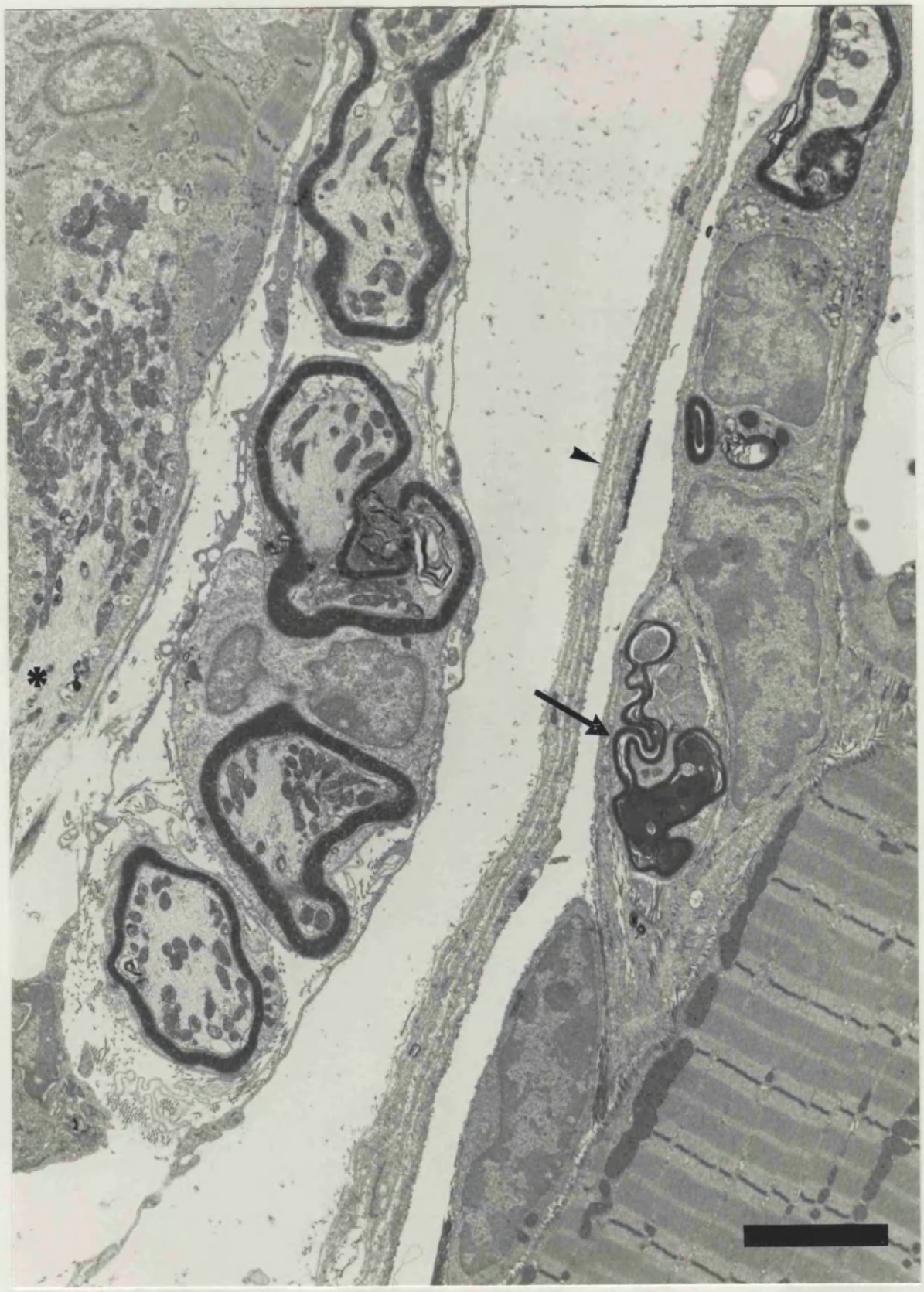

Fig. 3.41 A foot muscle at 48 hours, showing a degenerating extrafusal nerve fibre (arrow) contrasted with the intact myelinated nerve fibre and sensory terminal (part of the annulospiral ending - *) inside the spindle capsule (arrowhead). Bar = 3μm (Neg. 99615).

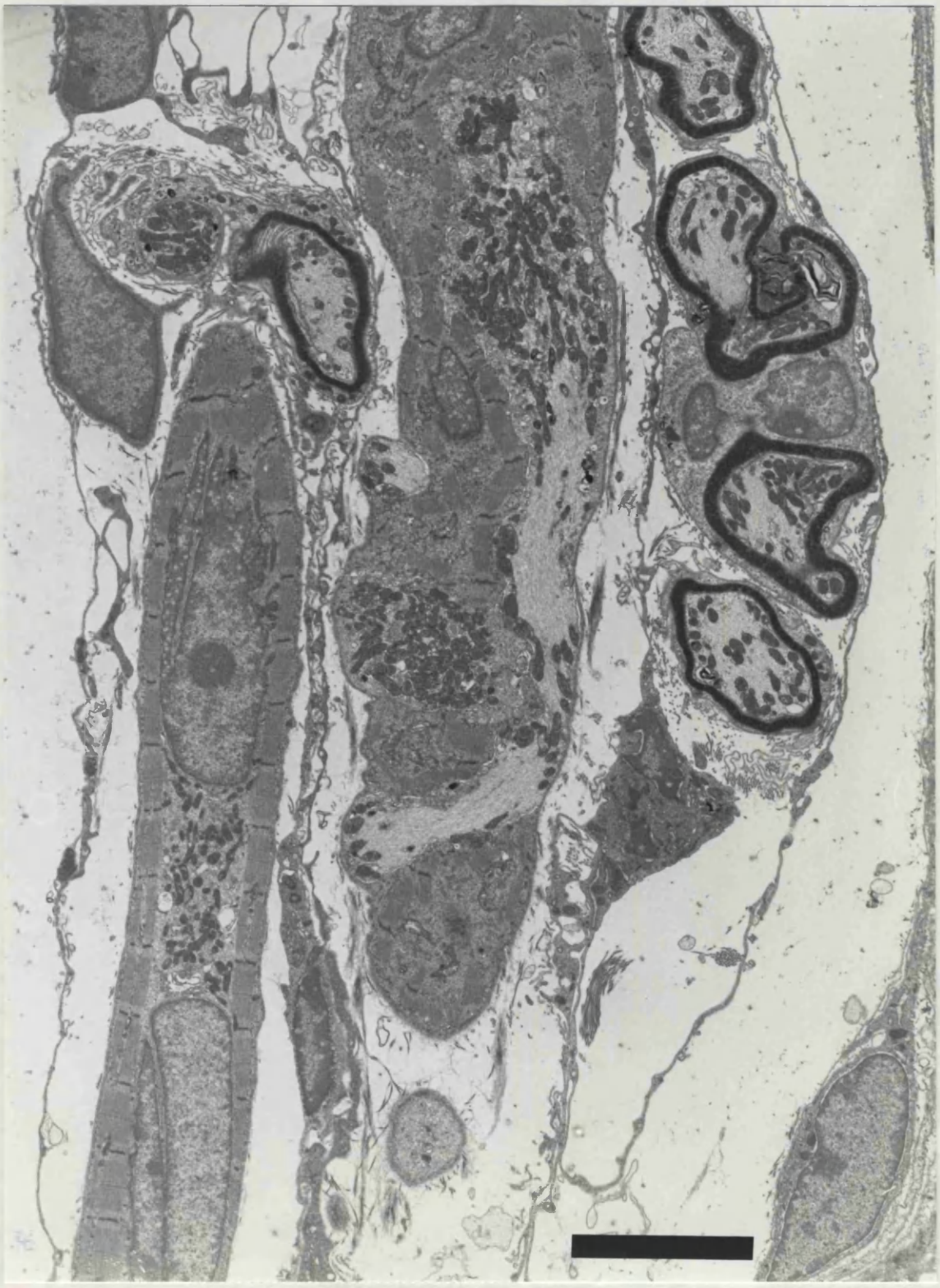

Fig. 3.42 A muscle spindle in foot muscle at 48 hours. Nerve fibres and terminals in the annulospiral ending are normal. Bar = 5μm (Neg. 99614).

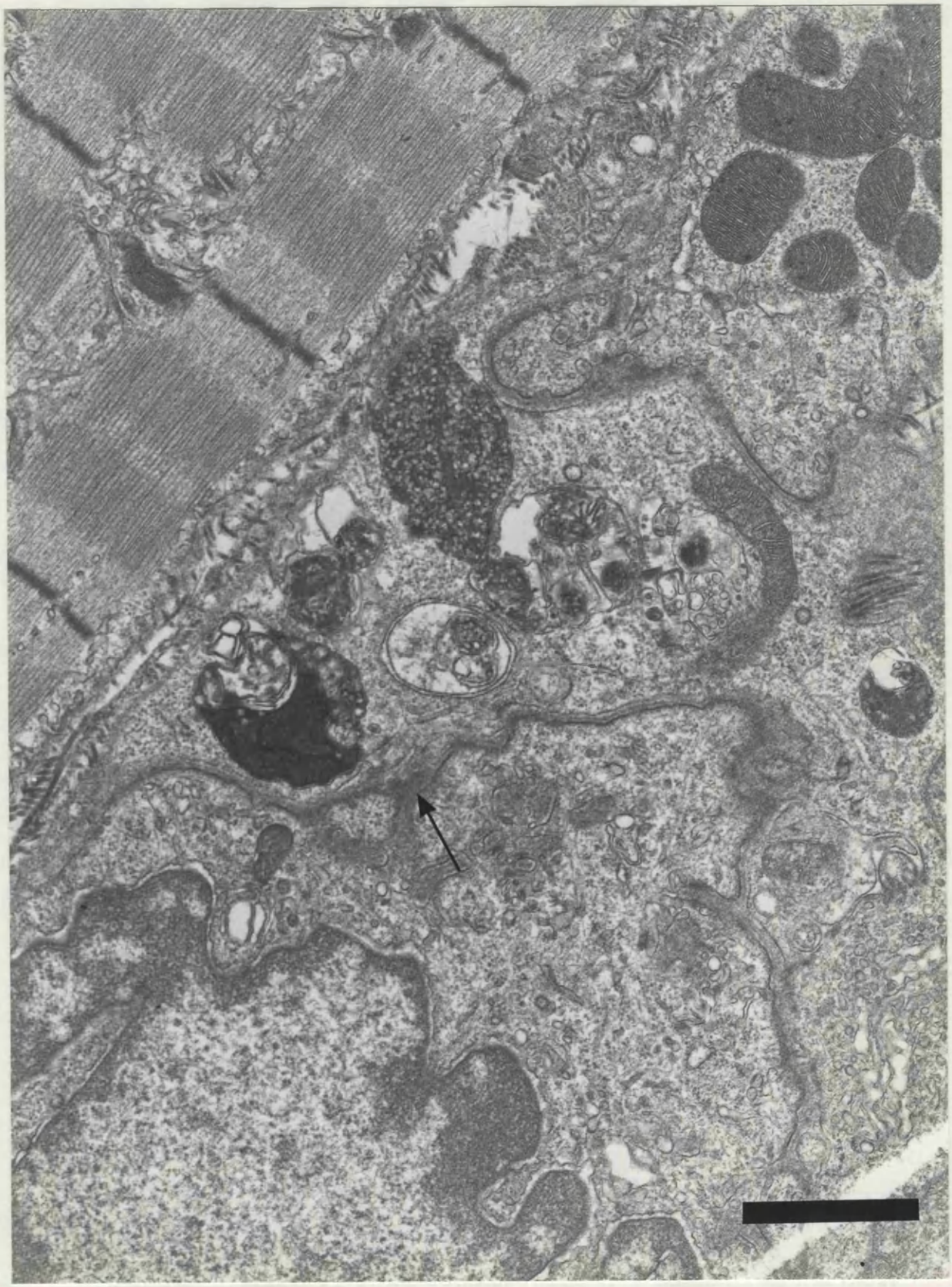

Fig. 3.43 Motor end-plate of foot muscle at 48 hours. No intact axon remains. Debris lies in cytoplasm of Schwann cell which covers the postsynaptic membrane (arrow). Bar = 1μm (Neg. 99618).

72 hours

By this stage all mice were severely ill as described previously.

Central nervous system

Examination of 1μm toluidine blue-stained sections was done on blocks of cerebellum, pons, medulla and spinal cord at several levels. It was apparent by light microscopy that there was considerable variation among the three animals studied in the degree of severity of the myelin vacuolation. One mouse (EM82/89) was very severely affected. White matter in all regions examined was almost spongy in appearance. The other animals (EM83/89 and 7/90) were less affected, though even in these there was variation from one site to another. The cerebellum showed a modest degree of white matter vacuolation. The medulla showed marked vacuolar changes in myelin particularly in the decussation of pyramidal fibres. In cervical spinal cord, very few vacuoles were seen in anterior and lateral columns but the corticospinal fibres in the dorsal columns were moderately severely affected. In thoracic cord only deeper parts of myelinated regions contained vacuolated fibres. Some degenerating fibres were present in fasciculus gracilis at cervical level.

In these 1μm sections of spinal cord some vacuolated fibres were seen cut longitudinally (Fig.3.44). Here it could be seen that the vacuoles could be intermittent. Some large myelinated fibres were seen in the grey matter and vacuoles were clearly separated from each other in the long axis of the fibre. It could not be seen whether the vacuoles extended the full length of the internode.

Electron microscopy of ultrathin sections was confined to the most severely affected animal (EM82/89). Vacuolation was severe in that the individual vacuoles were large, irregular in shape, coalescent in

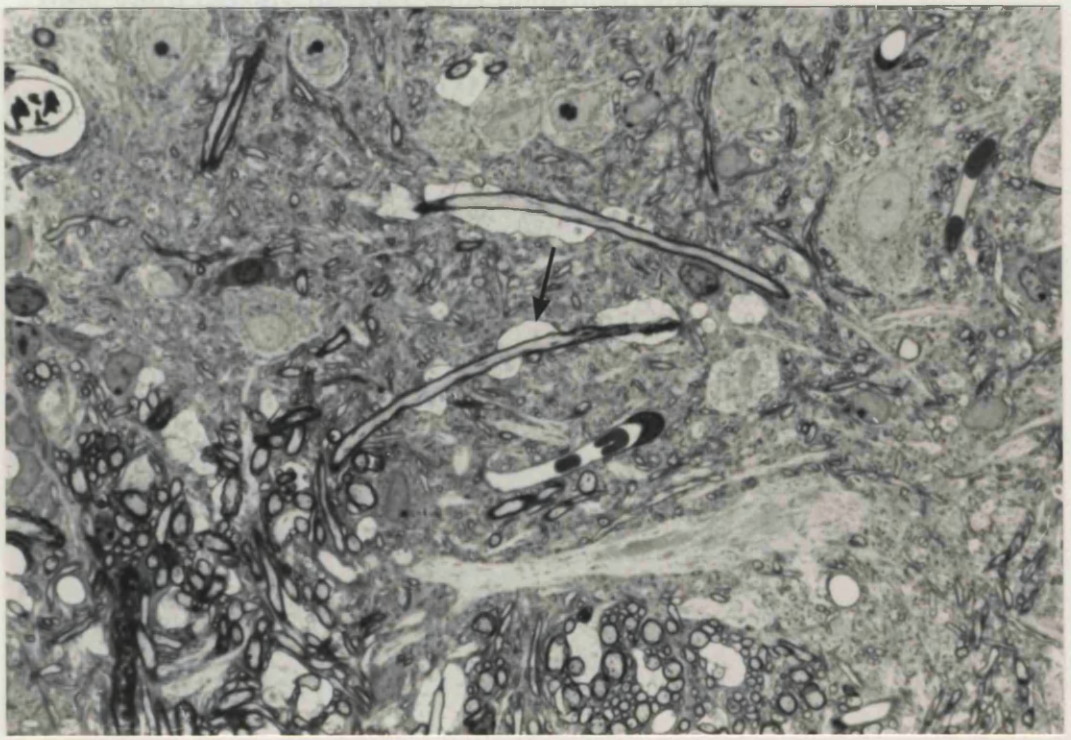

Fig. 3.44 Sacral spinal cord at 72 hours. A myelinated nerve fibre, cut longitudinally, shows intermittent intramyelinic vascuolation (arrow). Anterior horn cells look normal. Toluidine blue x 400.

places with membranes apparently beginning to breakdown (Fig. 3.45). Vacuoles which were not bounded by a clear membrane were quite numerous. Whether this breaking down of limiting membranes is artefactual or not is not certain, but the numbers of these non-bounded spaces lying between identifiable profiles in fairly well-fixed areas seemed to indicate a real pathology. Vacuoles were also seen within axons and in unidentifiable structures which may have been dendritic in nature. Some axons appeared somewhat abnormal in that they were closely packed with neurofilaments.

Peripheral nerve and muscle

Thoracic and lumbar dorsal root ganglia were examined in toluidine blue-stained 1μm sections (Fig. 3.46) and by electron microscopy. They were very abnormal. Many of the dorsal root ganglion neurons were shrunken and very darkly stained while many of the large neurons contained rounded large vacuoles in their cytoplasm. In some neurons there were several vacuoles but mostly only a single large one was seen. In a few neurons the cytoplasm was diffusely filled with many small vacuoles and the borders of these cells were poorly defined as if they were undergoing dissolution.

By electron microscopy it could be seen that the small densely blue-stained cells were also markedly electron dense (Fig. 3.47 -49), the cytoplasm being packed with laminated or concentrically arranged layers of degenerated rough endoplasmic reticulum together with dense aggregates of ribosomes, dense bodies, membranous bodies, vesicles and degenerating mitochondria. About 10% of neurons showed this type of degeneration.

The dorsal root ganglion neurons which contained large vacuoles could not be photographed in their entirety under the EM,

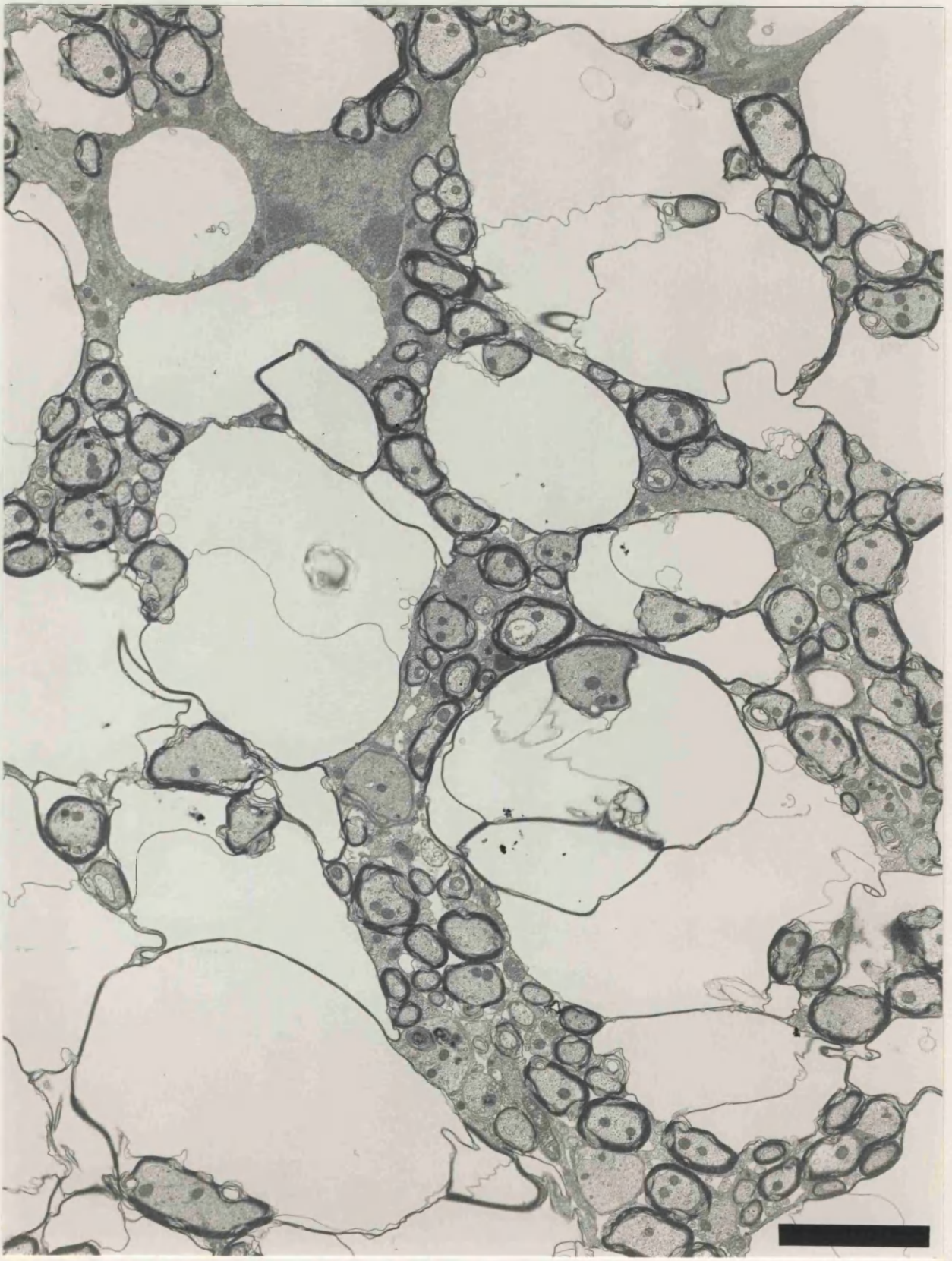

Fig. 3.45 Cerebellar white matter at 72 hours. There is severe vacuolation, vacuoles being large with membranes beginning to break down. Bar = 3μm (Neg. 91472).

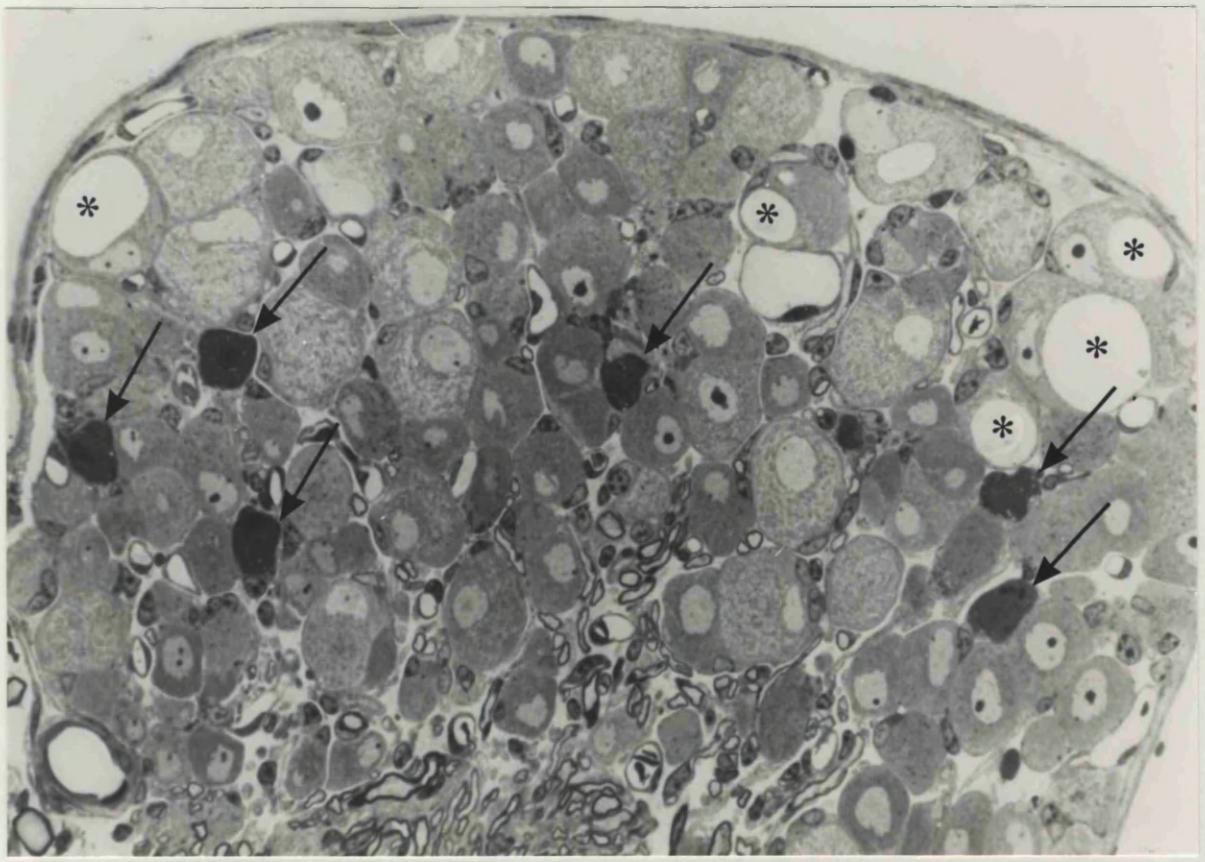

Fig. 3.46 Thoracic dorsal root ganglion at 72 hours. Numerous shrunken darkly stained neurons (arrows) are present whilst some large light ganglion cells contain rounded vacuoles (*) in their perikarya. 1μm Toluidine blue x 400.

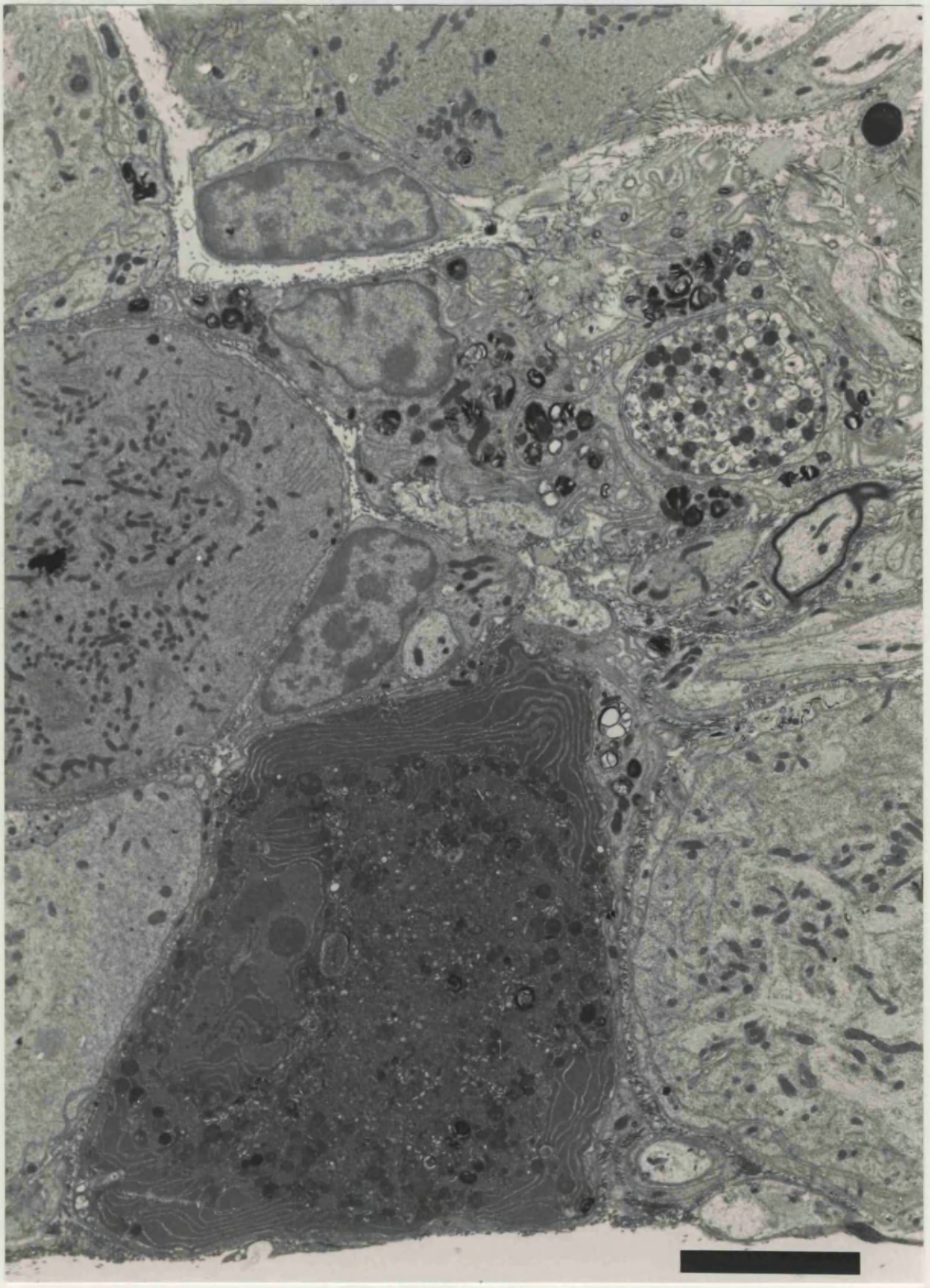

Fig. 3.47 EM of same ganglion shown in Fig. 3.46. One electron dense neuron contains layers of endoplasmic reticulin, dense bodies and degenerated organelles. A small profile (? axon) contains membranous bodies and mitochondria. Bar = 5μm (Neg. 97275).

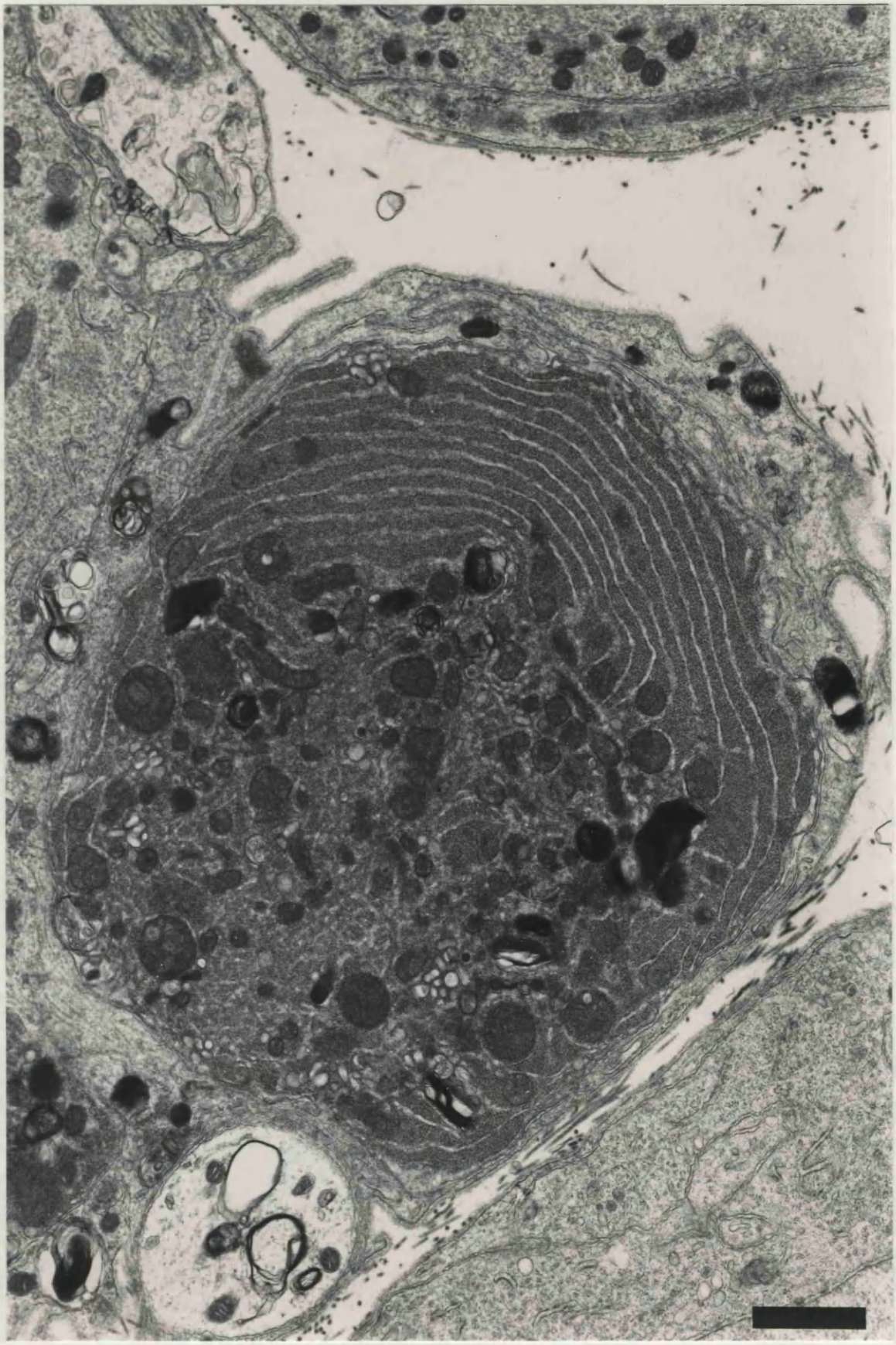

Fig. 3.48 A necrotic dorsal root ganglion cell at 72 hours. The concentric layers of degenerated rough ER are shown, together with dense bodies which pack the rest of the cell. Bar = 1μm (Neg. 97384).

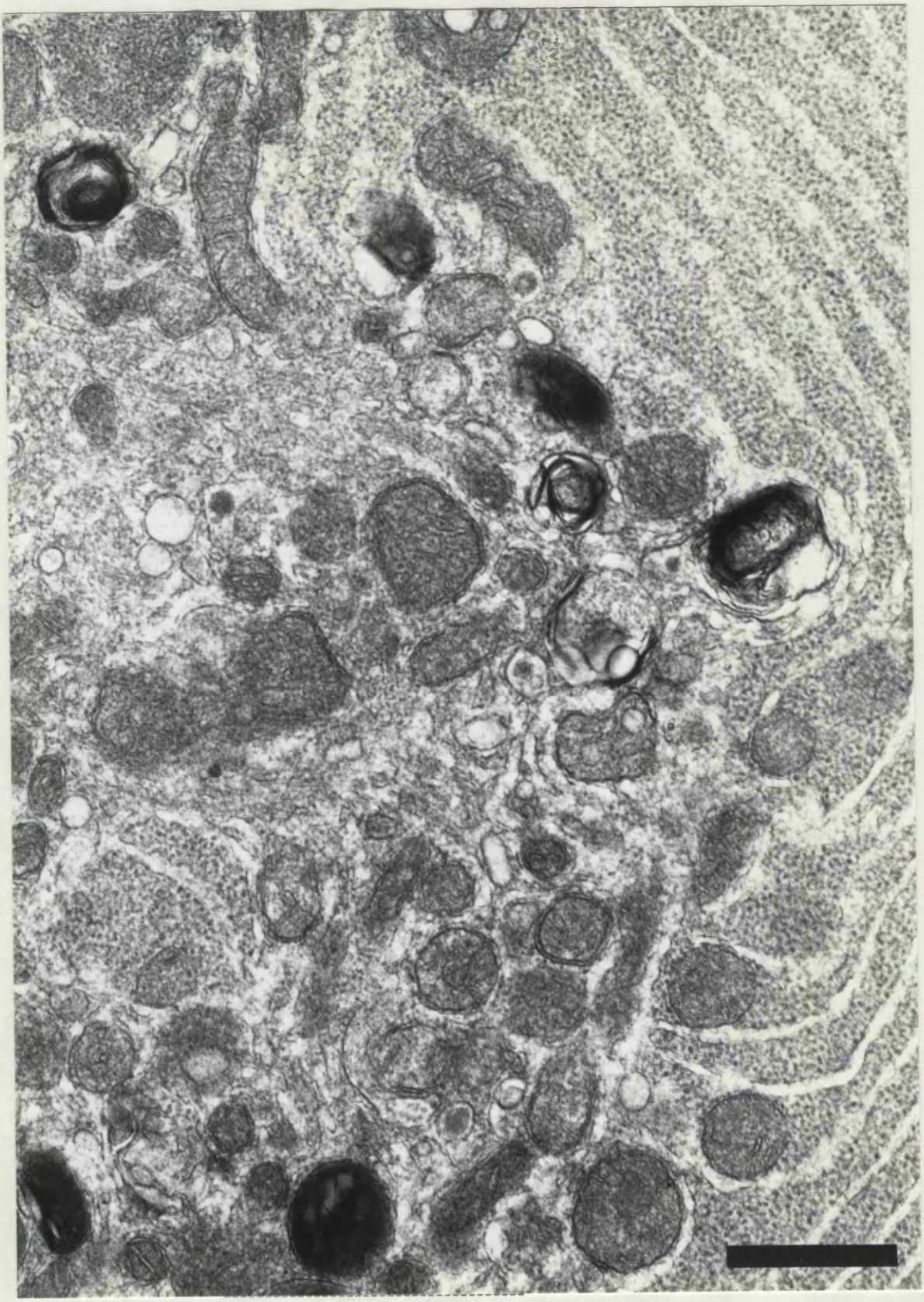

Fig. 3.49 Detail of ganglion cell shown in Fig. 3.48. The layer of rough ER and ribosomes together with other degenerated organelles are shown. Bar = 0.5μm (Neg. 97385).

because of their size. They did not contain identifiable material and they were bound by a single membrane. The neurons in which these vacuoles were seen did not otherwise show evidence of degeneration. The significance of these large vacuoles is uncertain. Similar vacuoles can be seen in occasional dorsal root ganglion neurons in normal control mice. However in these CL - 72 hours survival animals there were more cells affected that in the normal. Counts were made in two ganglia. About 4% of neurons contained large empty vacuoles.

The other type of degenerating neuron consisted of replacement of the perikaryon by masses of vesicles, multivesicular bodies, dense bodies and amorphous granular material. It seemed possible that some of this material extended into 'axonal' processes.

Silver impregnation of paraffin sections of the limbs showed a contrast between fore and hind limbs in respect of the state of innervation of their muscles. In the hind limb muscles, only larger nerve bundles contained intact axons. There was extensive axonal fragmentation in smaller intramuscular nerves and preterminal fibres and almost no intact terminal arborizations were seen. Innervation of spindles seemed to be within normal limits, annulospinal endings being preserved. In the fore limbs there was little or no evidence of axonal degeneration. Occasional end-plate aborizations consisted of rather thick argyrophilic terminals and a few may have been lost, since a few preterminal axons ended blindly without innervating an end-plate. this, however, could have been an effect of the plane of section and may not be significant.

Electron microscopy of the muscles and nerves showed that all end-plates visualized in soleus and in foot muscles were denervated (10 end-plates found by EM). Processes of Schwann cell cytoplasm,

some containing fragments of debris, lay in contact with postsynaptic membrane (Fig. 3.50). Muscle fibres generally were of relatively normal appearance, but since it seemed clear that the primary pathology affected the motor nerves and that any muscle changes would be secondary, not much attention was given to the state of the muscle fibres.

One muscle spindle was found in soleus by electron microscopy. The sensory terminals were intact and, in contrast with extrafusal motor innervation, an intact motor end-plate was observed (Fig. 3.51).

Of peripheral nerves studied by electron microscopy the smaller intramuscular nerves showed the most severe abnormalities. Small nerves in soleus, EDL and foot muscles were almost totally degenerated (Fig. 3.52). Nerve fibres were devoid of axons, they were fragmented and myelin ovoids or debris were all that remained. In larger nerve bundles such as plantar (Fig. 3.53) or posterior tibial or sciatic nerves only a few degenerated fibres were present (Fig. 3.54). No vacuolar or degenerative changes were noted in the motor roots. In the sensory roots occasional vacuoles were present very close to, or in the region of, the root entry zone (Fig. 3.55). No vacuoles were present in the sections of the rootlets which were clearly lying further away from the cord.

4 days (96 hours)

At this stage 2 mice aged 21 days were perfused for electron microscopy and blocks taken from many regions of central and peripheral nervous systems.

Central Nervous System

Toluidine blue-stained 1μm plastic sections showed marked

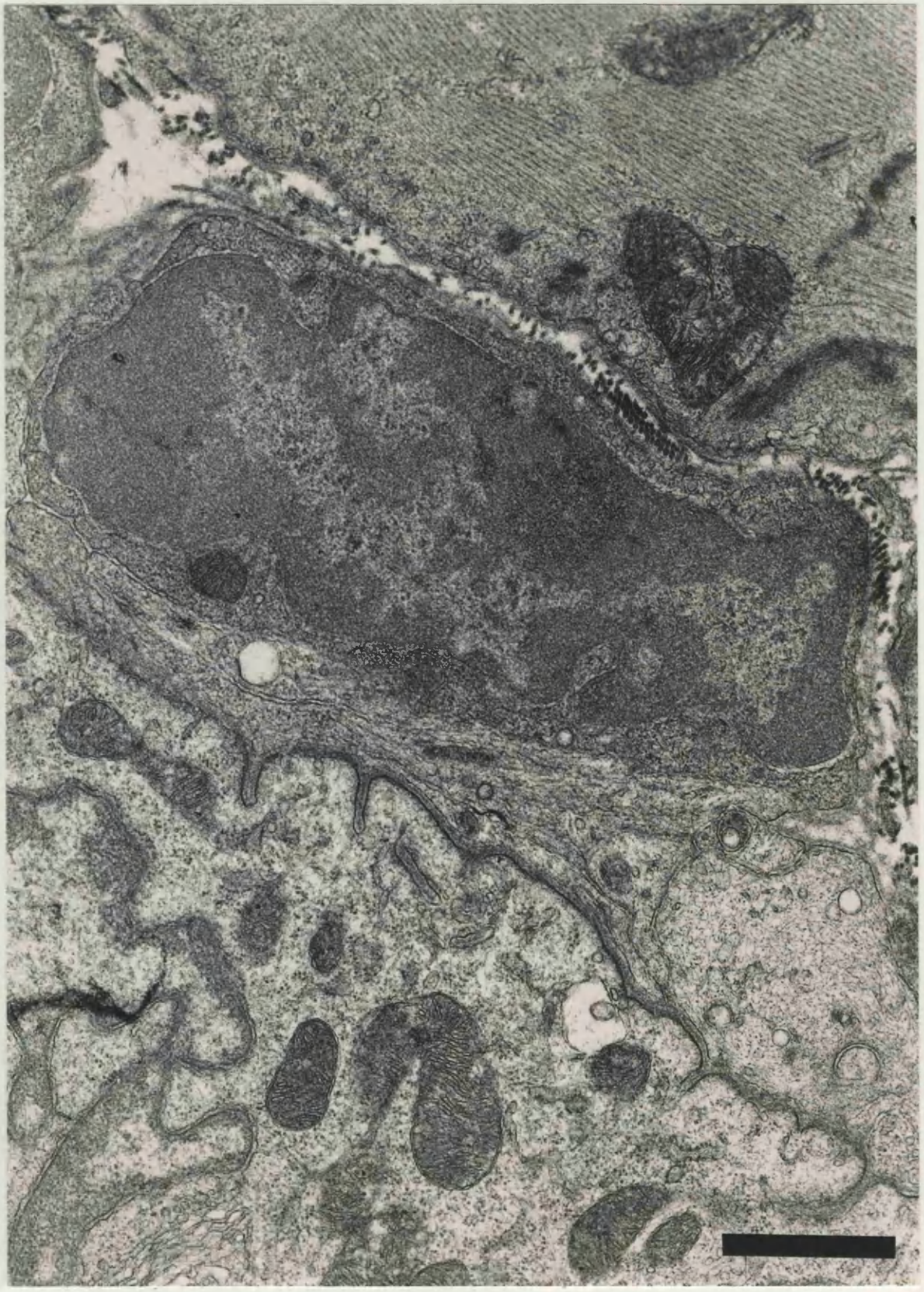

Fig. 3.50 Soleus at 72 hours. The motor end-plate is denervated and Schwann cell cytoplasm covers the postsynaptic membrane. Bar = 1μm (Neg. 97833).

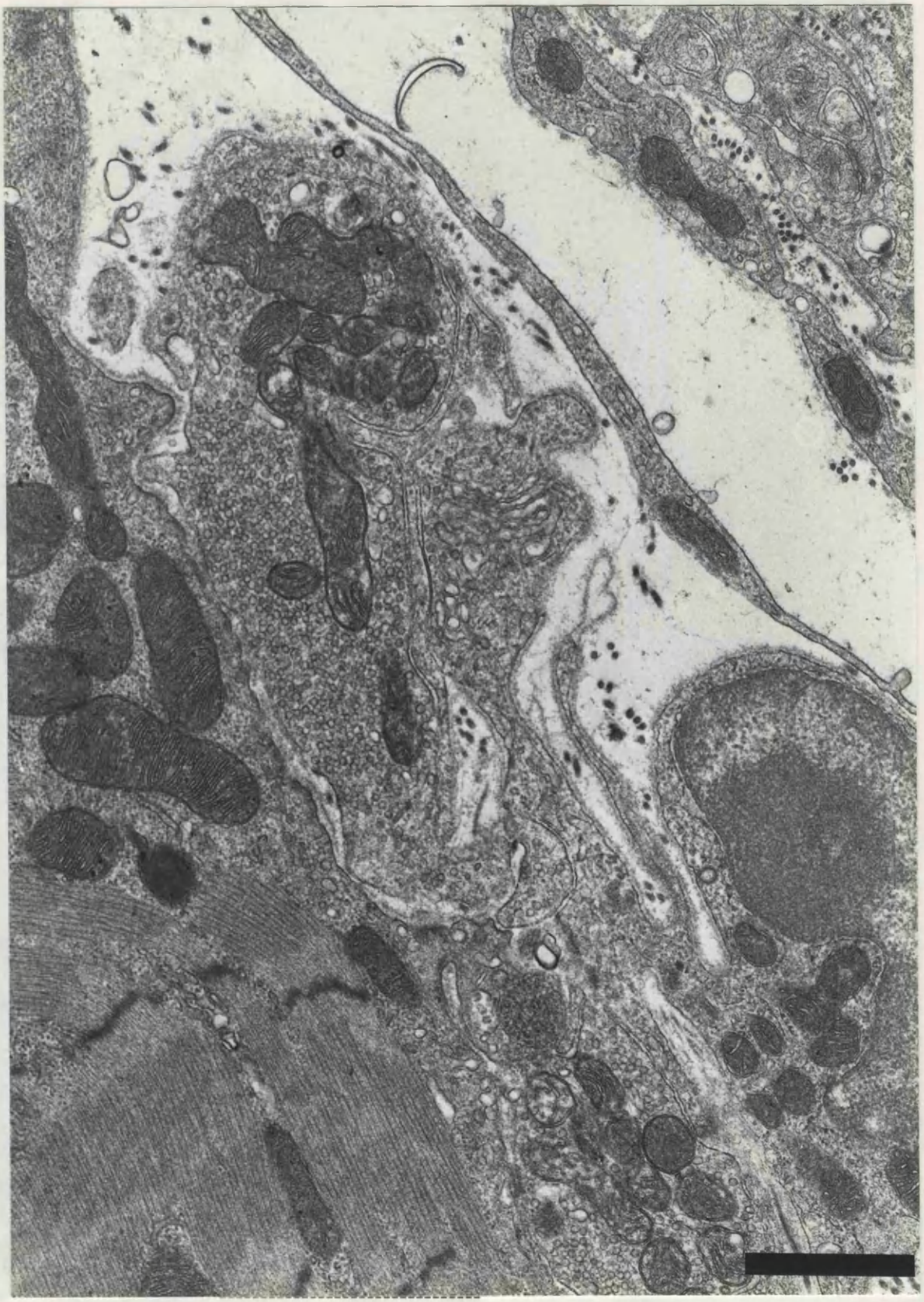

Fig. 3.51 Intrafusal motor end-plate at 72 hours is normal in appearance in contrast to the extrafusal end-plates. Bar = 1μm (Neg. 97836).

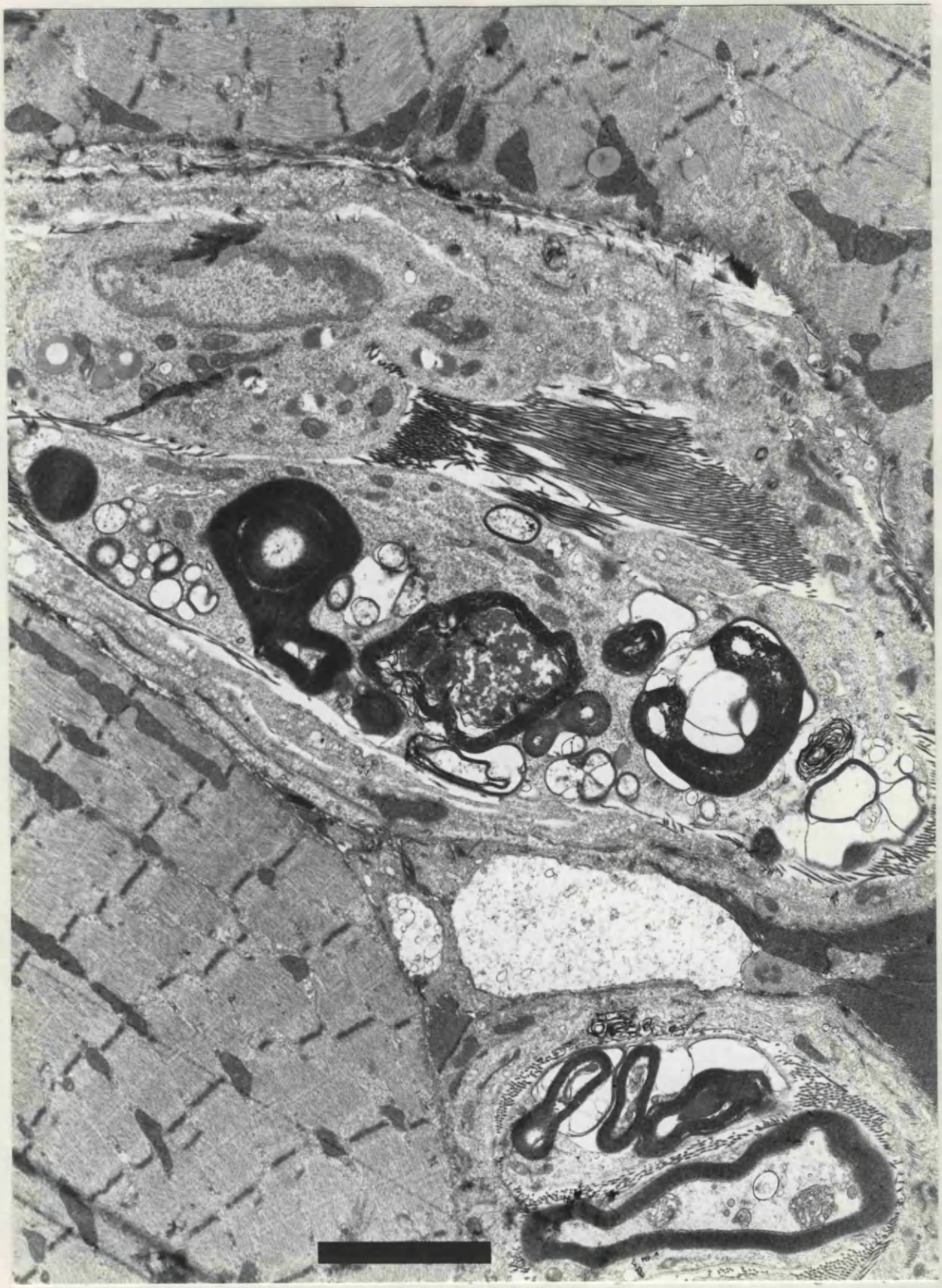

Fig. 3.52 Soleus at 72 hours. All nerve fibres in an intramuscular nerve are degenerated and myelin sheaths are breaking down. Bar = 3μm (Neg. 97839).

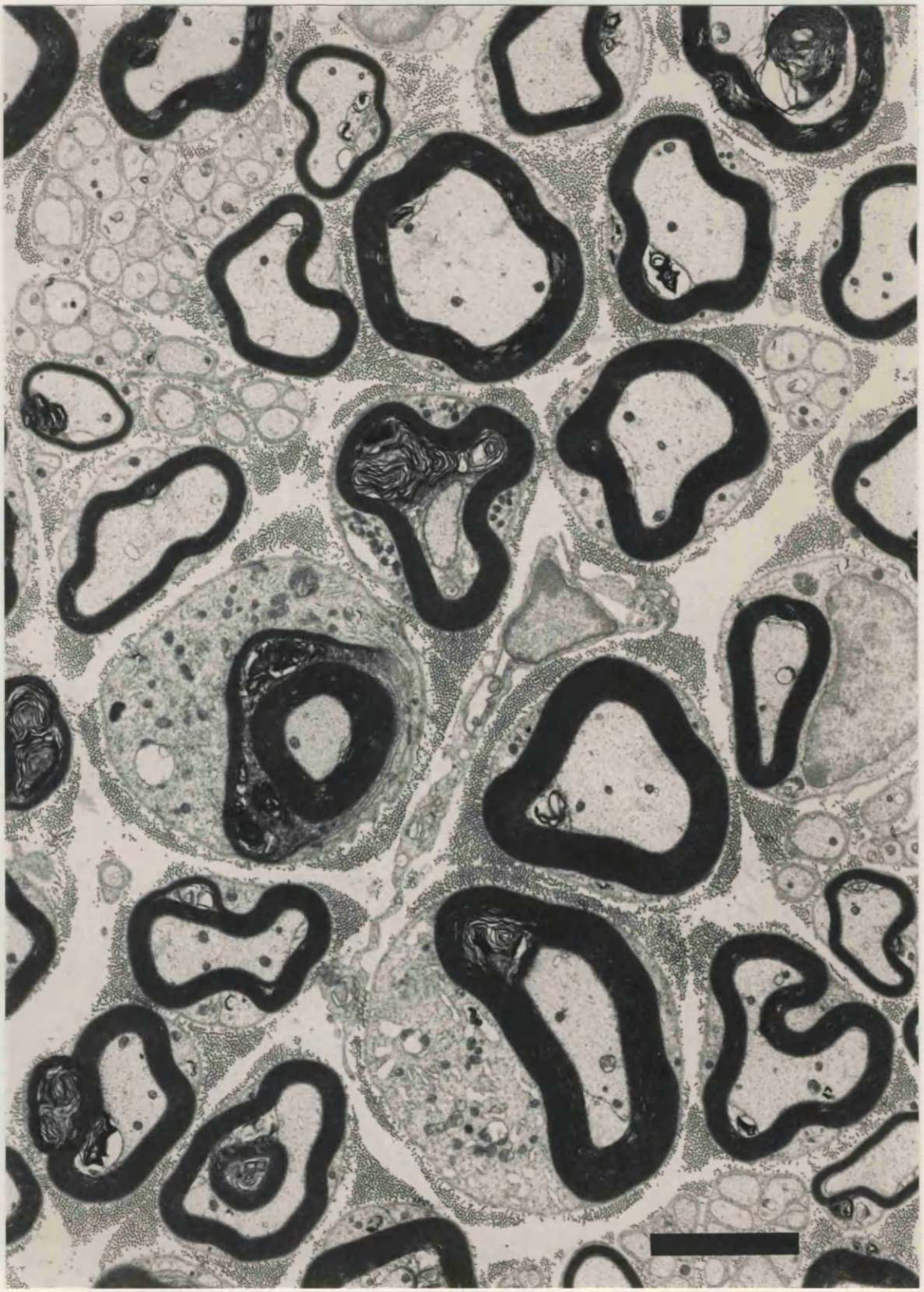

Fig. 3.53 Plantar nerve at 72 hours. Appearances are within normal limits in this field. Bar = 3μm (Neg. 97574).

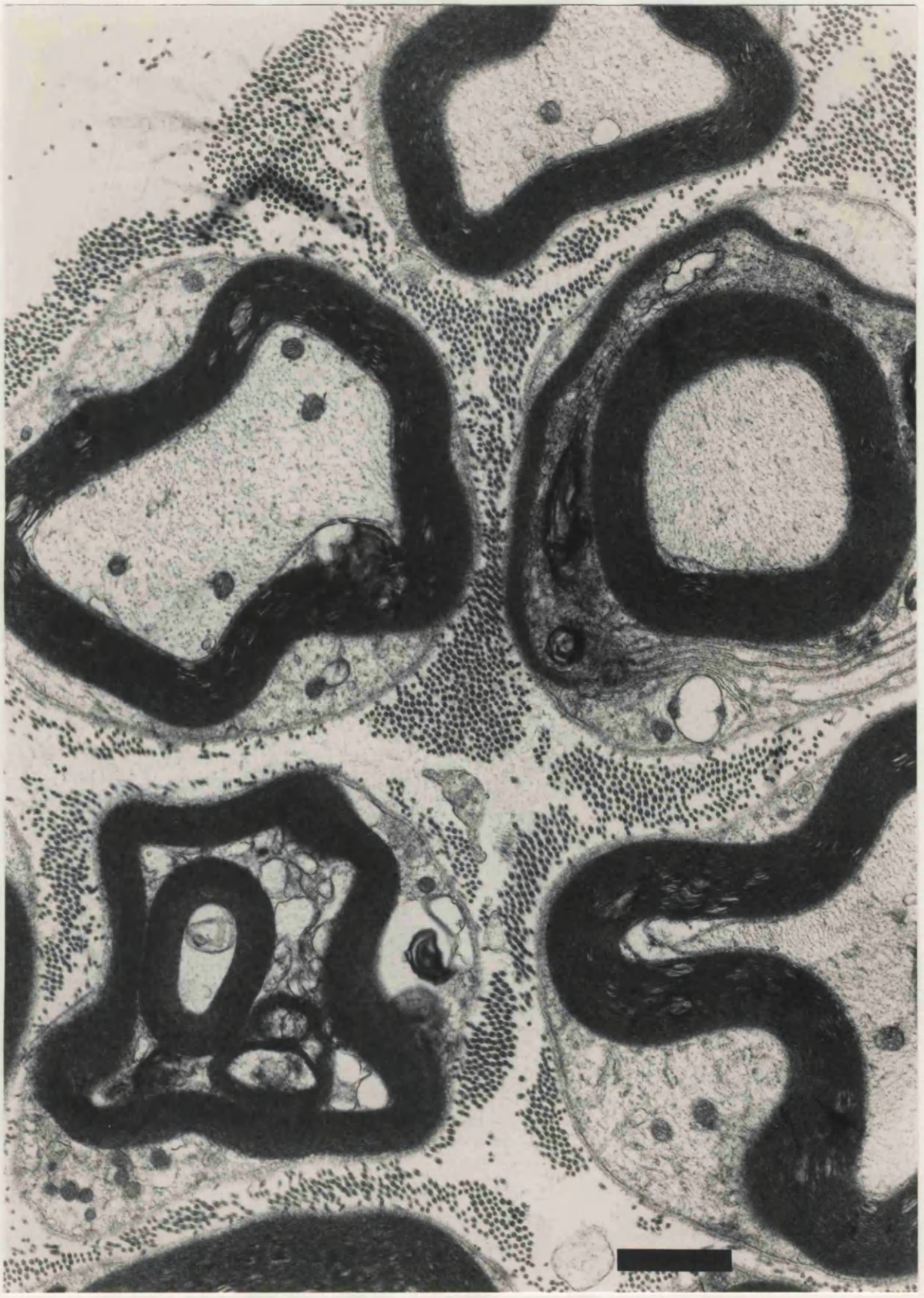

Fig. 3.54 Another area of the plantar nerve shown in Fig. 3.53. Degeneration of the axon of one fibre with early breakdown of the myelin sheath is shown. Bar = 1μm (Neg. 97576).

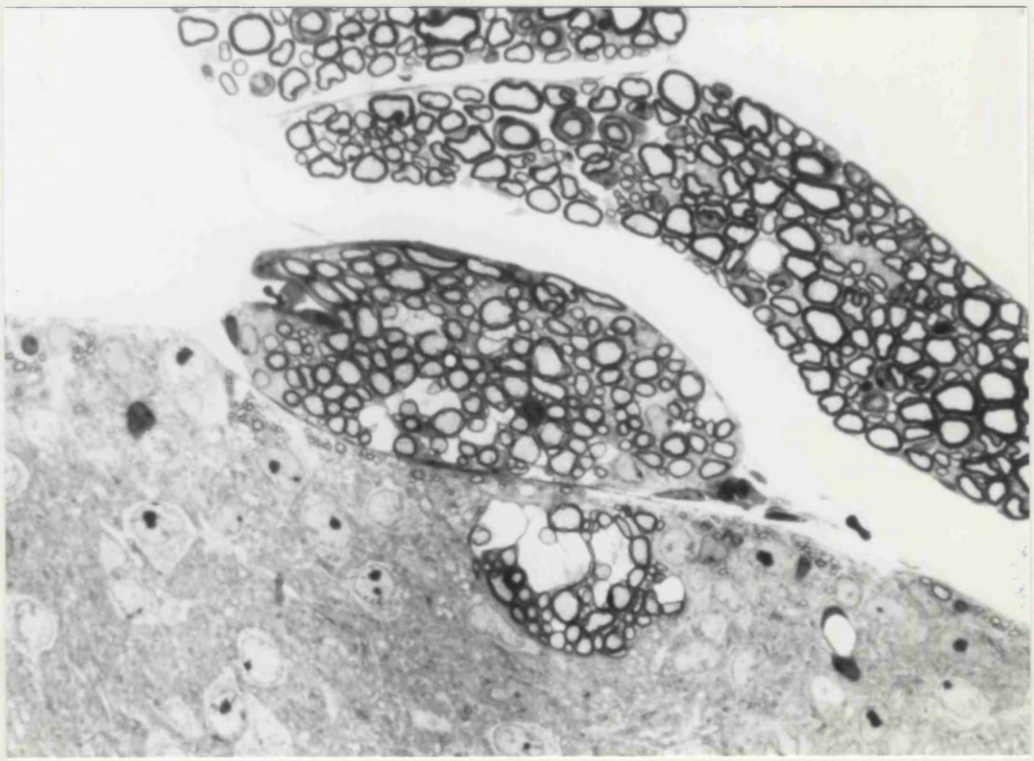

Fig. 3.55 Sacral cord at 72 hours. Occasional myelinic vacuoles are present in or close to the root entry zone. 1μm Toluidine blue x 400.

variation of white matter in different areas. There was severe vacuolation in the cerebral hemisphere particularly of the caudate-putamen and thalamus. The cerebellum, pons and medulla were also severely affected but cervical cord showed less marked vacuolation which was mainly present in the corticospinal part of the dorsal column and in deeper parts around the grey matter. The lumbo-sacral cord, however, was very severely vacuolated. Roots looked normal. At cervical level there were scattered degenerating fibres in the white matter, more numerous in the dorsal columns.

Electron microscopy of the CNS blocks showed that the vacuoles were irregular in shape and seemed to be confluent and multilocular (Fig. 3.56). Loose membranes were common and some were breaking down (Fig. 3.57). There was evidence of degeneration now taking place. Some myelinated axons contained dense bodies and aggregates of tubules whilst some profiles, which could have been axonal or dendritic, appeared to be electron lucent and disintegrating, forming irregularly shaped extracellular spaces. Neurons appeared normal.

Peripheral nerve and muscle

Toluidine blue-stained 1μm plastic sections of sciatic, tibial and plantar nerves, dorsal root ganglia, biceps brachii, soleus, EDL and foot muscles were examined.

Dorsal root ganglia contained degenerative neurons. In one lumbar dorsal root ganglion about 300 neurons were counted in one section. Of these there were 12 shrunken darkly stained cells and 3 containing either large or small vacuoles. By EM the necrotic neurons were seen to be packed with densely stained material, some appearing to be lamellae of rough endoplasmic reticulum together with dense bodies and membranous bodies lying in vacuoles. Some

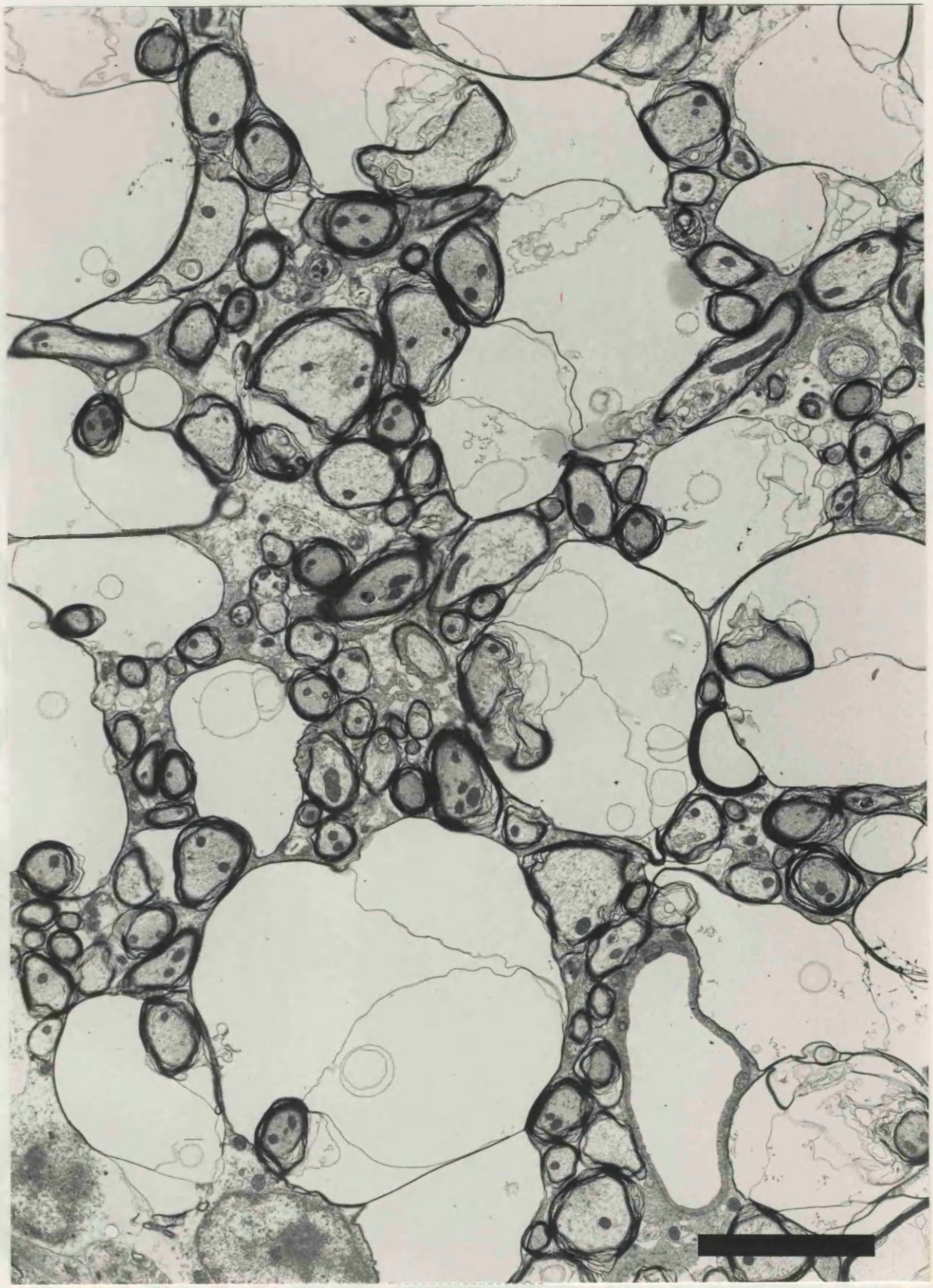

Fig. 3.56 White matter of cerebellum at 4 days, showing very marked intramyelinic vacuolation. Bar = 5μm (Neg. 97607).

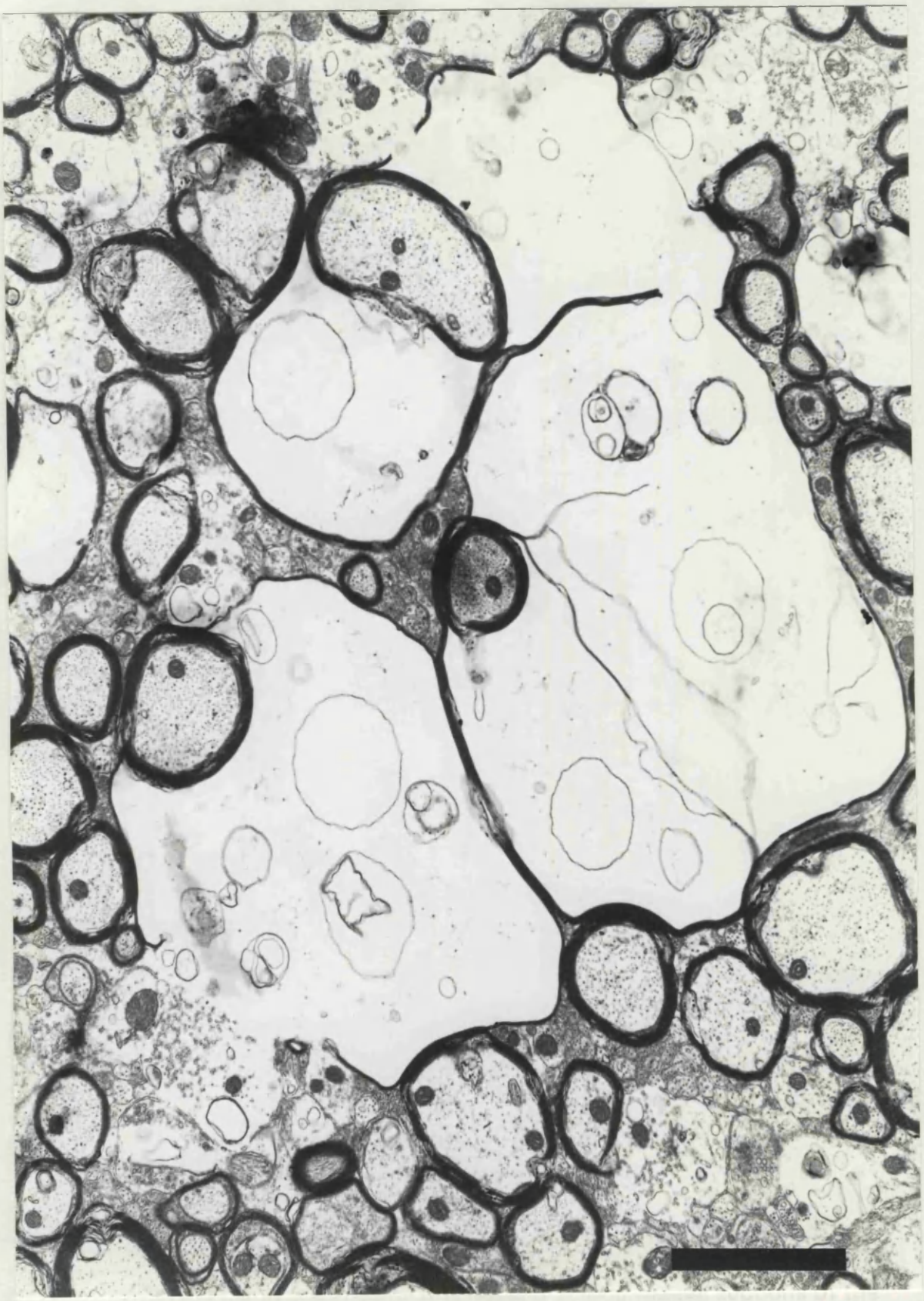

Fig. 3.57 White matter of lumbar cord at 4 days. In several large intramyelinic vacuoles the membranes seem to be breaking down in places. Bar = 2μm (Neg. 84728).

of the debris was lying in the cytoplasm of satellite (Schwann) cells (Fig. 3.58).

In the sciatic and tibial nerves there were many degenerating fibres (Fig. 3.59) which showed collapse and varying degrees of honey-comb appearance or fragmentation of myelin around a central space devoid of axon (Fig. 3.60). Debris lay within the cytoplasm of Schwann cells. Although some unmyelinated axons contained occasional dense bodies or whorls of membrane there was no convincing evidence of degeneration of these unmyelinated fibres. Examination of muscles in 1μm plastic sections showed a striking contrast between the upper fore limb muscle biceps brachii and the muscles of the leg and foot. In biceps brachii intramuscular bundles of myelinated fibres were of normal appearance, no degeneration was seen and there were normal looking motor end-plates easily distinguishable. In soleus, EDL and foot muscles intramuscular nerve branches showed severe, almost total degeneration.

Electron microscopy confirm these findings (Fig. 3.61). Motor end-plates in biceps brachii were also of normal appearance by EM in contrast with those of soleus and foot muscles where no normal end-plates were seen (Fig. 3.62a and b). Seven end-plates were identified in foot muscles and all were denervated. The post-synaptic membranes were covered by layers of Schwann cell cytoplasm which contained occasional dense bodies or other debris.

5 days

Two mice were examined in paraffin sections. Sections of serial blocks of head, trunk and limbs and longitudinal sections of the fore and hind feet, embedded flat, were stained by a variety of neurohistological methods including silver impregnation.

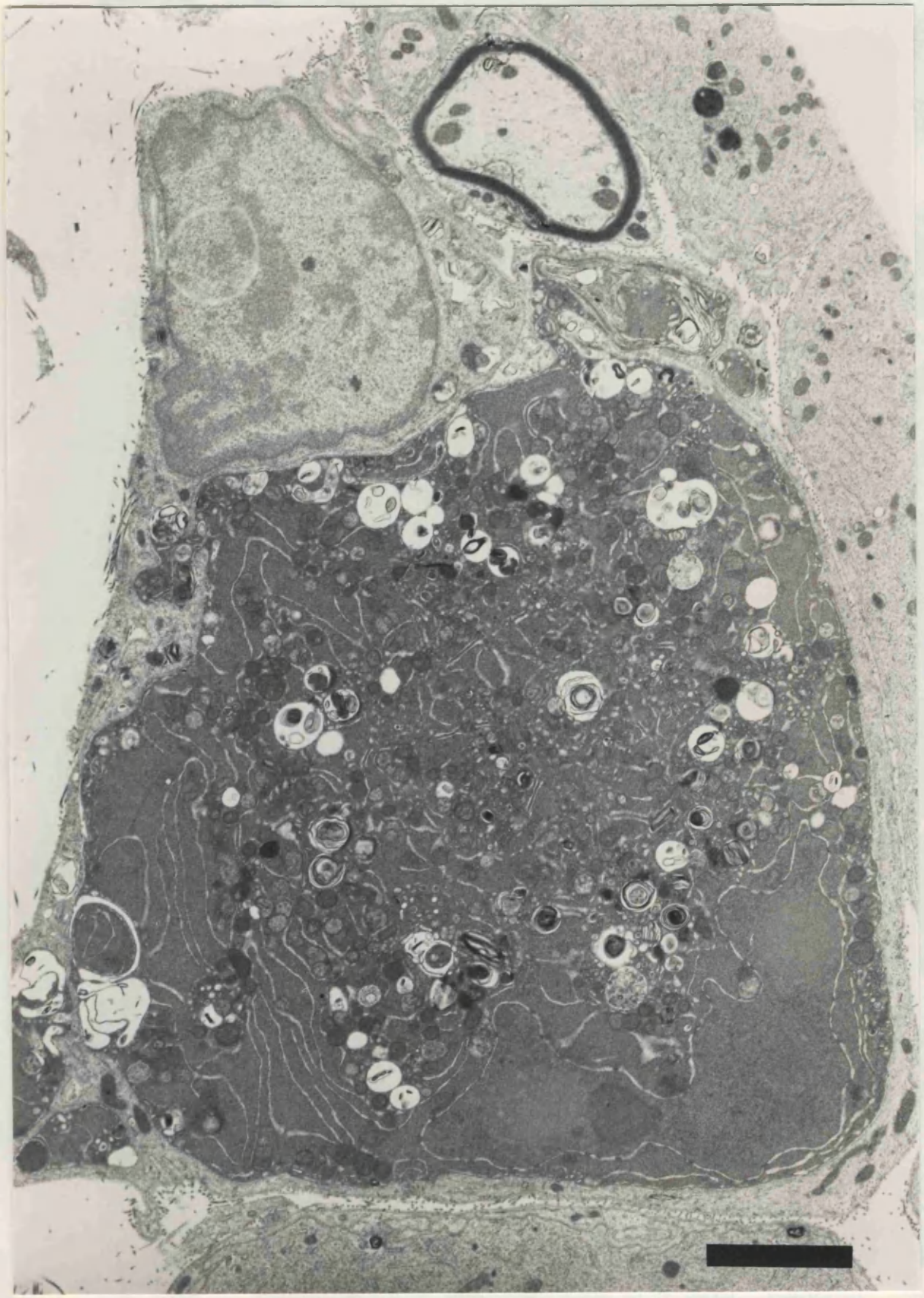

Fig. 3.58 Densely stained necrotic ganglion cell in dorsal root ganglion at 4 days. Appearances are similar to those seen at 3 days (Figs. 3.46-8). Bar = 2μm (Neg. 97659).

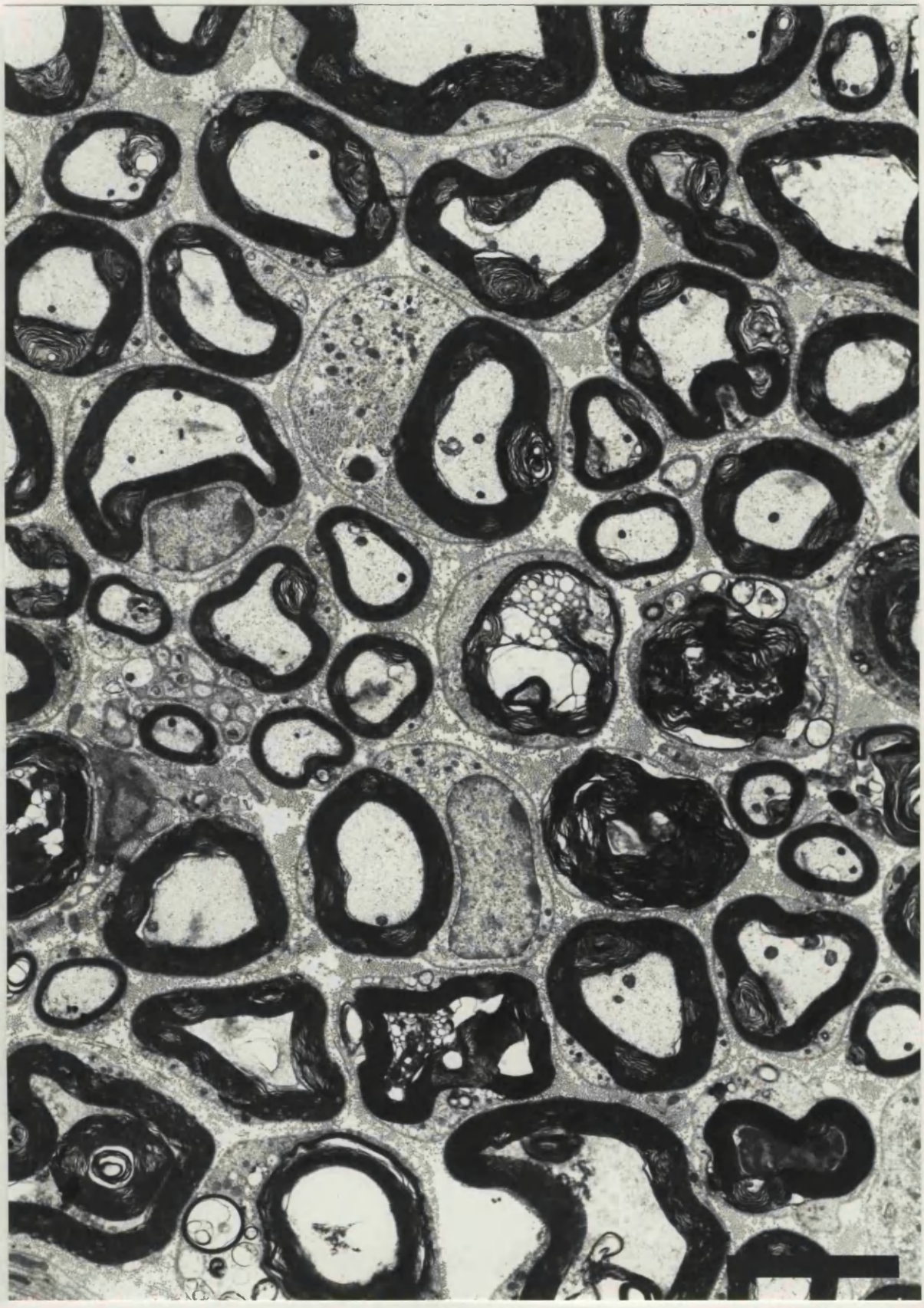

Fig. 3.59 Sciatic nerve at mid-thigh level at 4 days. Fibres in varying stages of degeneration, primarily due to axonopathy, are shown. Myelin sheaths are breaking down. Bar = 5μm (Neg. 97537).

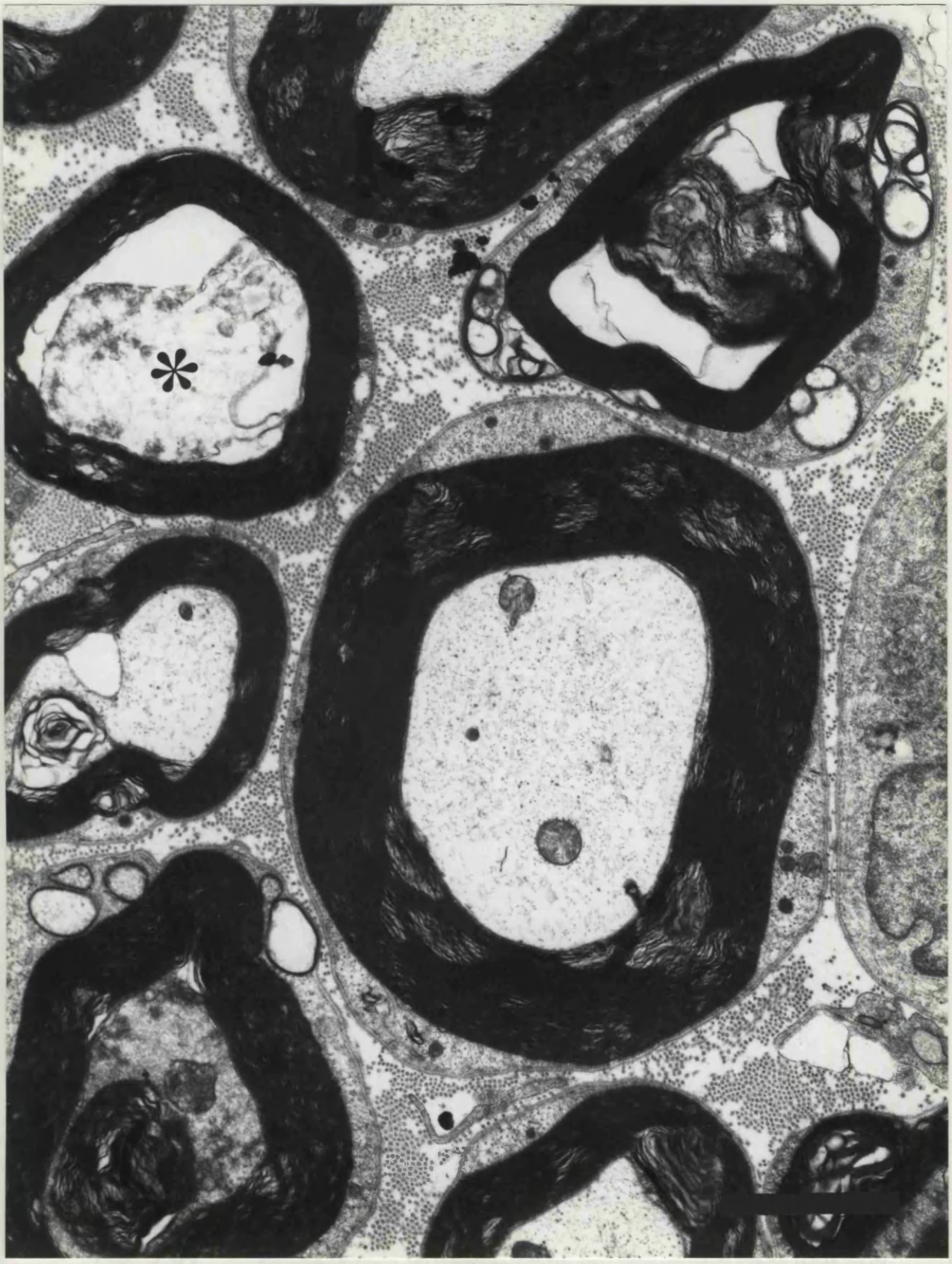

Fig. 3.60 Degenerating fibres in sciatic nerve at 4 days. Disintegration of axoplasmic organelles (*) before the myelin sheath breaks down indicates a primary axonopathy. Bar = 2μm (Neg. 97555).

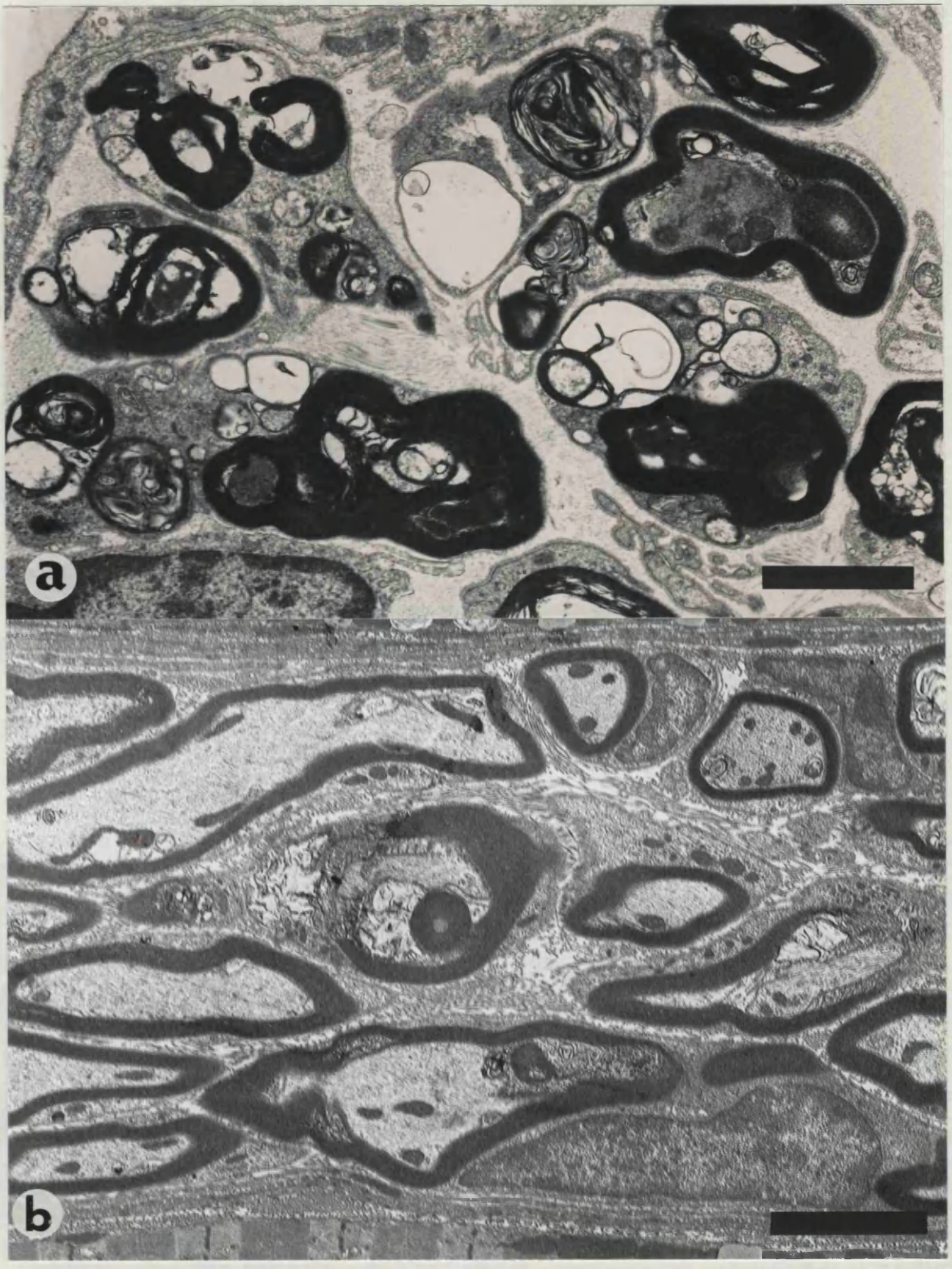

Fig. 3.61 The contrast between distal nerves of the hind foot and proximal nerve in biceps brachii at 4 days is shown. The distal intramuscular nerve (a) shows severe degeneration affecting almost every fibre. The proximal nerve in biceps brachii (b) has a relatively normal appearance. Bar = 3μm (Negs. a) 97320, b) 99541).

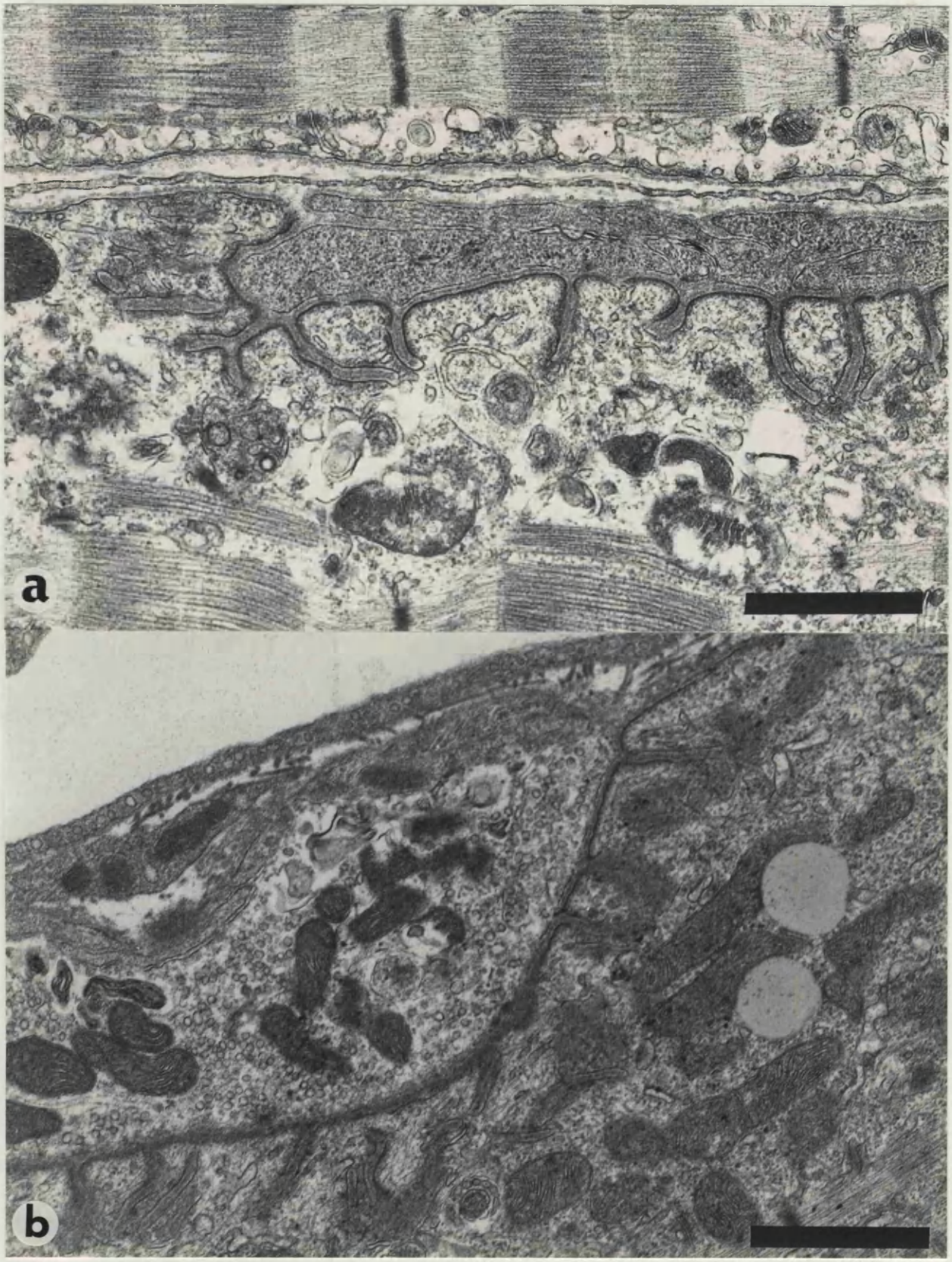

Fig. 3.62 End-plates in foot muscle (a) are denervated at 4 days but in biceps brachii (b) are intact and normal in appearance. Bar = 1μm (Negs. a) 97314, b) 99565).

Vacuolation of white matter was widespread throughout the CNS. Of interest was vacuolation in the optic tracts but not in the optic nerves. Necrosis of ganglion cells could not be identified with certainty in dorsal root ganglion or sympathetic ganglia in paraffin sections. Some cells appeared small and darkly stained but no cellular reaction could be identified and it can only be thought likely that they correspond to the pyknotic cells seen by EM at day 3.

The silver impregnated sections were most useful for seeing the state of innervation of widely dispersed areas. In the sections of the snout region it could be seen that the nerves to the whiskers were intact and throughout the head, neck and trunk sections the innervation of muscles was normal. No degeneration of large nerve trunks, intramuscular nerve bundles or of preterminal fibres and terminal arborizations was seen. Spinal roots and roots of cranial nerves were also normal.

The innervation of the forelimb was also studied in the silver preparations and was found to be normal. Sensory innervation of the skin of the fore foot and of muscle spindles was abundant and nerve fibres were intact. Main nerve trunks and intramuscular innervation, including the end-plates, were entirely within normal limits (Fig. 3.63a).

In the hindlimbs the innervations of the pelvic and thigh muscles was within normal limits but in the leg muscles (gastroenemius, soleus, EDL) degeneration of axons in the intramuscular bundles became visible. Only few end-plates with normal arborizations could be demonstrated and in most of these muscles the preterminal fibres ended blindly (Fig. 3.63b). In the foot the innervation of the muscles was severely devastated, few axons remained in intramuscular nerves and no intact end-plates could be

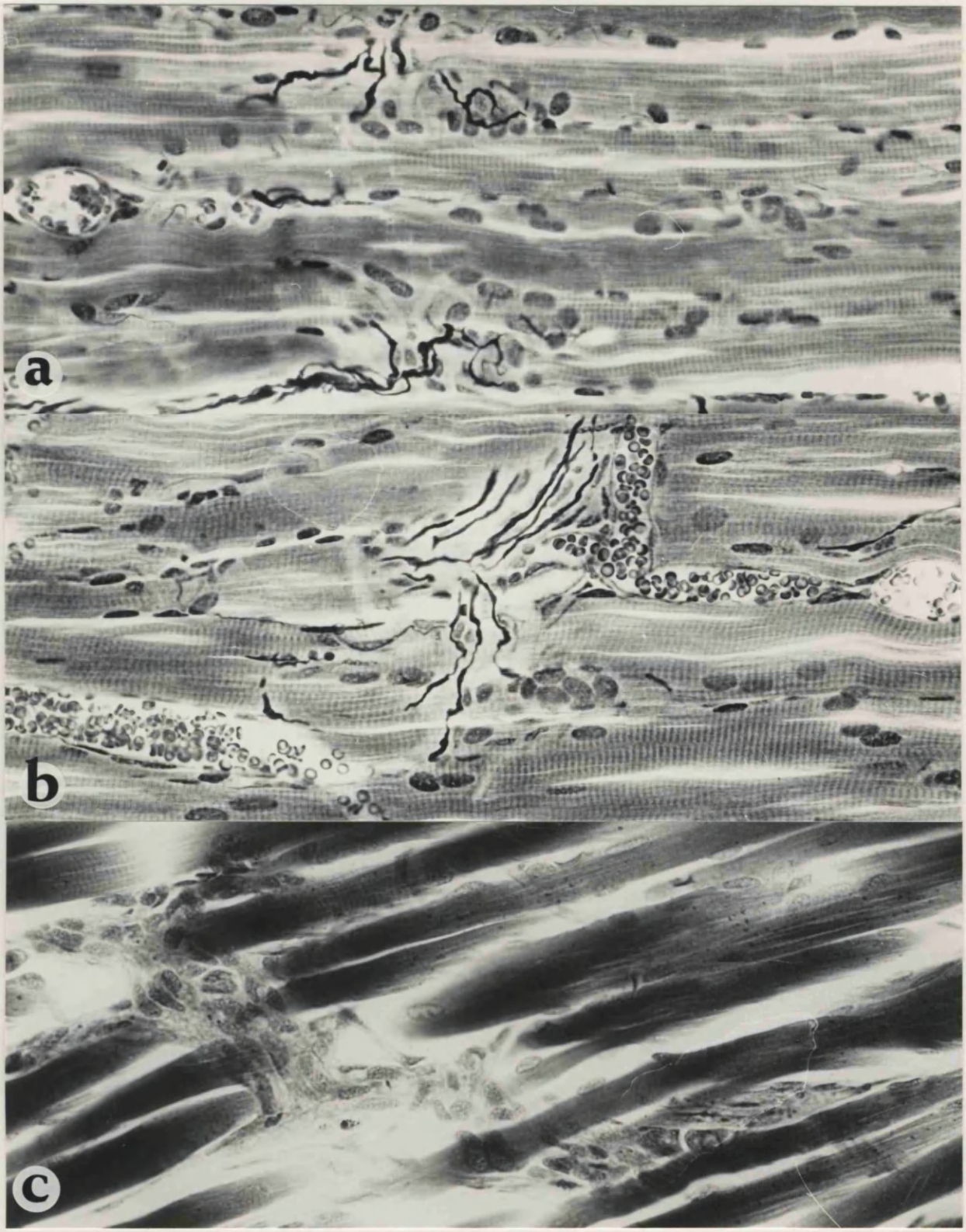

Fig. 3.63 Innervation of muscles in paraffin sections at 5 days. (a) section of proximal hindlimb muscle shows normal preterminal axons and terminal arborizations. (b) in gastrocnemius the preterminal axons are present but not the arborizations. (c) in the foot muscles almost all axons have disappeared from the intramuscular nerve. Glees silver method x 400.

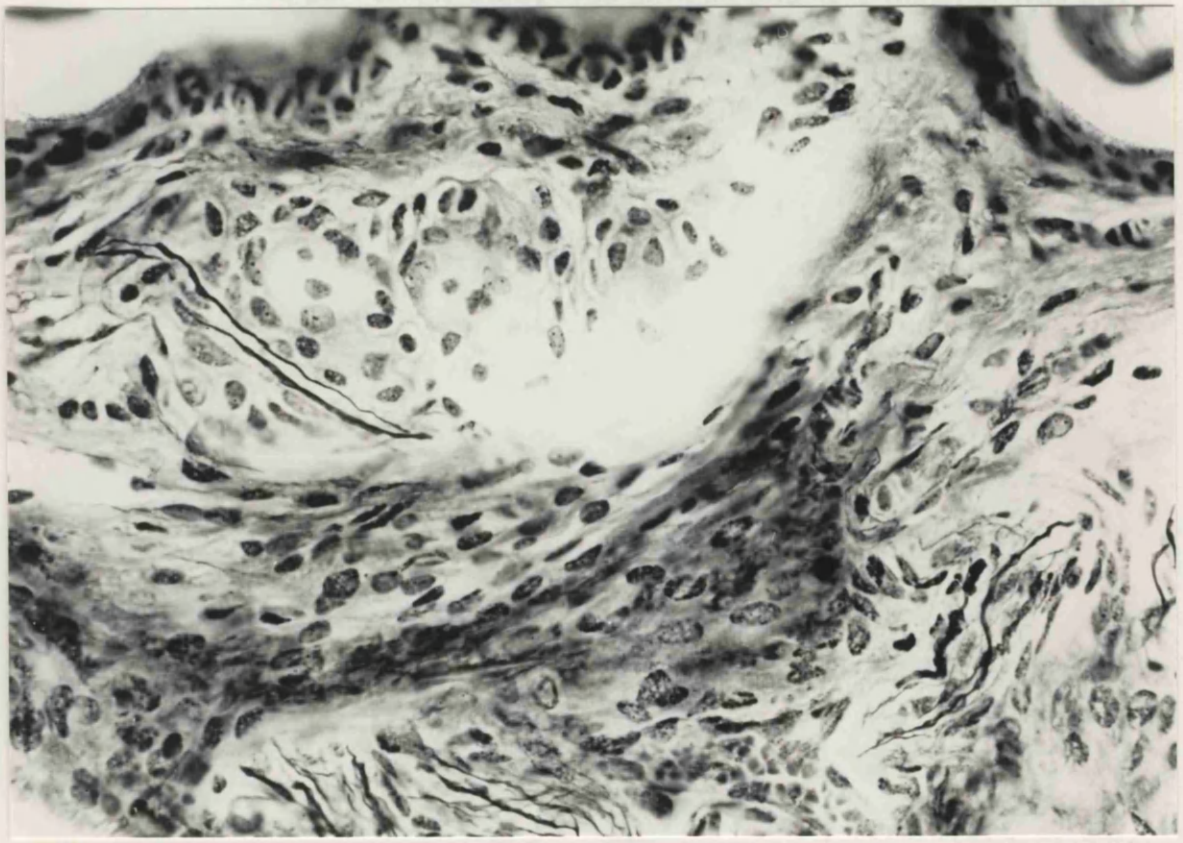

Fig. 3.64 Section of skin of hind foot at 5 days. There are abundant nerve fibres, probably mostly sensory and no sign of axonal degeneration. Glees silver method x 400.

identified (Fig. 3.63c). The innervation of the skin of the foot and of the sensory receptors such as Pacinian corpuscles was apparently normal (Fig. 3.64)No electron microscopy was done on the 5 day mice.

7 days

This part of the study was difficult and little material was put through for either paraffin or plastic embedding. This was because so few animals of the 21 days of age group survived for this length of time. The very few that did so were not as severely ill as the others which were killed at earlier stages if they looked as if they would not survive overnight.

In the peripheral nerve and muscle the material then examined in paraffin sections included only one mouse. Silver impregnation showed some preservation of the innervation of the fore limb and paw although even here there was some indication of abnormality in that some of the terminal arborizations may have degenerated. The larger intramuscular nerve bundles and nerve trunks were normal in appearance.

In the hind limb, in both paraffin and plastic sections, the innervation was much as described for earlier stages with almost total disintegration of intramuscular nerve bundles, preterminal axons and almost no end-plate arborizations identified. This was particularly apparent in the foot muscles. Sensory innervation of the skin seemed intact.

A limited amount of electron microscopy was done. Sections of the foot muscles were examined and most end-plates were denervated. In one end-plate however some tiny axonal profiles were

observed in contact with the post-synaptic membrane (Fig. 3.65). In one intramuscular nerve bundle there were, in addition to degenerated nerve fibres and myelin debris, some tiny unmyelinated axonal profiles. These suggested that regeneration may already have been starting at this time.

Ultrathin sections of sciatic nerve were also examined in the EM and occasional degenerative fibres were present. There were also a very few thinly myelinated axons, again suggesting some recovery process taking place.

In the central nervous system there was much less extensive vacuolation than at earlier times, particularly in the spinal cord, although some, as in the cerebellar white matter, were very large. An appearance not seen at earlier stages in CNS white matter was the presence of large irregular spaces lying extracellularly. These contained amorphous granular material or seemed otherwise empty. The most likely interpretation of these spaces is that they are derived from broken down vacuoles that were originally intramyelinic. In addition there were degenerating axons and other degenerative profiles. Nerve cells in brain stem, cerebellum and spinal cord, best seen int he plastic sections, did not look abnormal. There did not seem to be excessive numbers of astrocytes or glial fibres. It is hoped that further work on these longer surviving animals may be possible in the future.

Group B (6-10 week old):

Clinical observations

Although there was some variation in the severity of effects of CL from mouse to mouse, in general, they were similar to, but

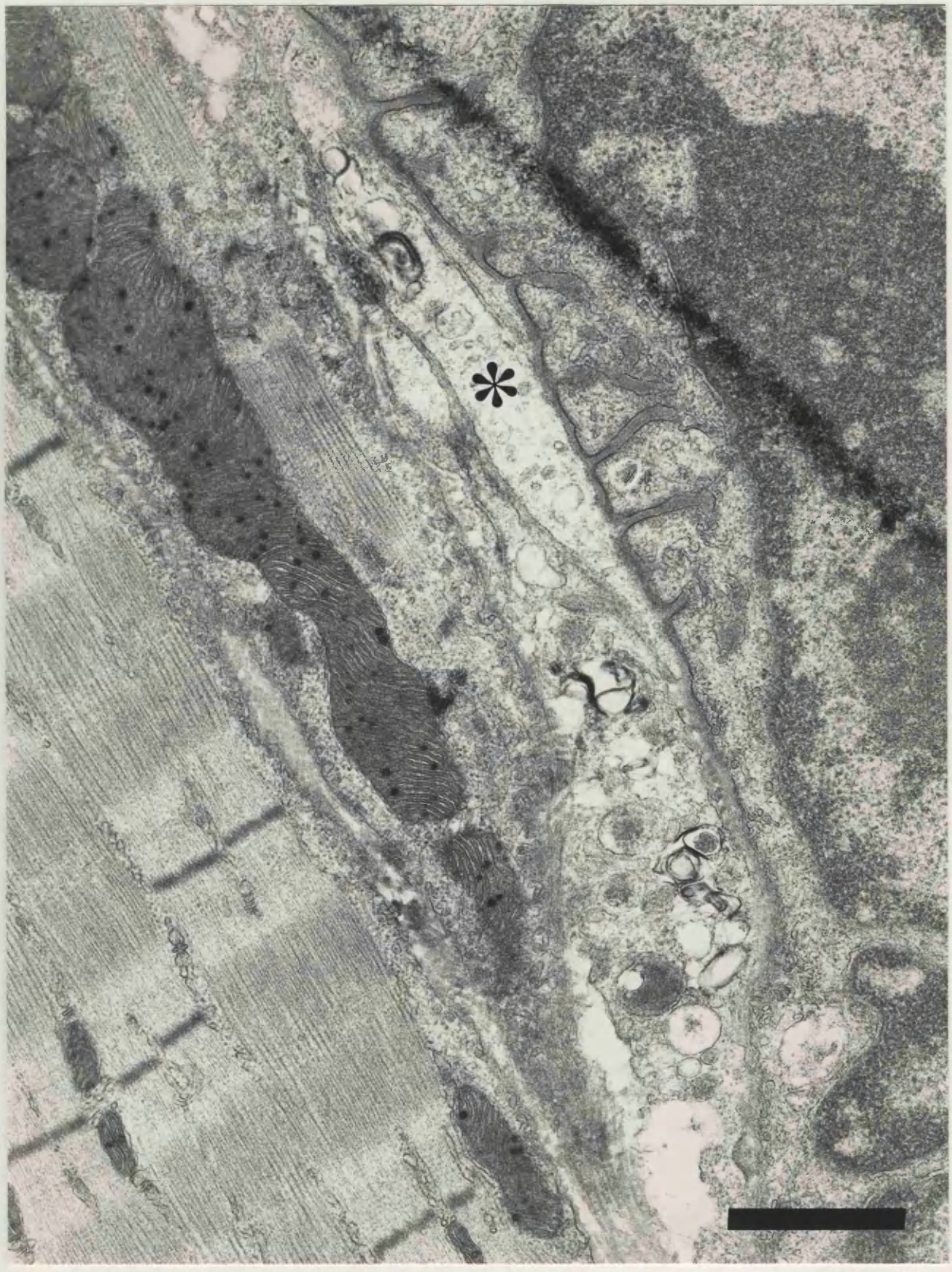

Fig. 3.65 A motor end-plate in foot muscle at 7 days. There are some tiny axonal profiles (*) in contact with the postsynaptic membrane as well as debris nearby in Schwann cell cytoplasm. Bar = 1μm (Neg. 90356).

developed slower than, those of 21 day old mice. The adult mice showed the same behavioral changes as those of the younger age group but the toxicity developed later. Animals looked normal and active immediately after the injection. At 24 hours they showed few signs of neurotoxicity. No weakness of any limbs or difficulty in walking on the wire grid were noted. At 48 hours they were obviously ill and some of them had a staring ruffled coat. Some were not able to open their eyes. They already showed weakness of the hind feet. There was no weakness of forelegs and no apparent disturbance of sensation could be detected by the reaction to pin-prick on the snout, fore and hind limbs, trunk and tail. By 3 days mice were ataxic and there was clearly weakness of the hind legs and hind feet. When lifted by tail, they did not extend the hindlimbs fully and were not able to spread the toes. Clasping of hind limbs was noted. They could not walk on the wire grid properly and only the forepaws could hold the bars firmly.

By 4 days after the injection, the mice looked very ill and loss of body weight was apparent. The mice stayed quietly and responded sluggishly when touched. They tended to huddle together and remained immobile, with ungroomed fur and thick catarrhal secretion from the eyes. When stirred they would not walk more than a few inches with a very wobbling gait. There was also paralysis of abdominal and respiratory muscles, and costal recession was apparent. By 5 days they showed more severe weakness of hind limbs and almost complete paralysis of the foot muscles. Misplacement and dragging of hindlimbs were noted in most mice and crawling was accomplished only with the forelegs. By 6 days, the effects of CL were still progressive. Several mice had died by this time. Survivors looked very weak, showing paralysis of hind limbs and marked costal recession. After 7 days the general condition of the surviving adult mice did not seem to deteriorate, but the severe

weakness of hind limbs persisted with some fine tremor of the body. Marked decrease of body weight was more apparent. By 8 days there were evidences of the progress of neurotoxicity with muscular weakness and paralysis of the hind limbs. Several mice died and the surviving mice showed almost total paralysis and dragging of hindlimbs by 10 days some mice developed the muscular weakness and ataxia more slowly and animals survived up to 14 days.

Morphological Observations

Central Nervous System:

In the 6 weeks old mice there were some patches of intramyelinic vacuolation in the cerebellar and pontine white matter and very occasional vacuoles could be seen in the spinal white matter by 24 hours. The severity of vacuolation was much less than those of 21 days old mice. In the 10 weeks old mice only very occasional vacuoles could be found in the CNS. There was no evidence of degeneration of nerves cells such as Purkinje cells, neurons of the dentate nuclei and anterior horn cells. By 48 hours there were more vacuoles in the cerebellum but very few in the spinal white matter in the 6 weeks old mice. Myelin lamellae formed the outer walls of the vacuole and also separated them into multilocular cysts. In the 10 weeks old mice, intramyelinic vacuoles were very rare.

By 3 days, patches of vacuolation was seen in cerebellar white matter in the 6 weeks old mice but not in the spinal white matter. Occasional degenerated nerve fibres appeared in the spinal white matter, especially in the fasciculus gracilis. There was no obvious evidence of intramyelinic vacuolation in the white matter of the spinal cord of 10 weeks old mice.

By 6 days there were more numerous vacuoles in the cerebellar

white matter but very few in the spinal cord in the 6 weeks old mice. In the 10 weeks old mice no intramyelinic vacuolation could be found. Many degenerated nerve fibres appeared in the dorsal columns in both age groups, especially in the fasciculus gracilis. Anterior horn cells, motor and sensory roots were normal.

At 9 days, only the 10 week old animals surviving more than 6 days, very occasional vacuoles were seen in the spinal cord. There were many degenerated myelinated fibres in the dorsal columns and also occasional degenerated fibres scattered in the rest of the spinal white matter. Nerve cells appeared normal. The appearances were similar at 11 and 14 days.

Peripheral Nervous System and Muscle:

At 24 hours most axonal terminals and nerve fibres in the muscles appeared normal or very slightly affected. An occasional intramuscular fibre showed axonal rarefaction. By 48 hours many intramuscular nerve fibres contained severely degenerated axons. In some of them the axons appeared electron dense and some showed rarefaction of the axoplasm. The myelin sheaths showed only slight changes. The muscle fibres were normal.

By 3 days the peripheral nerves showed severe distal axonopathy without intramyelinic vacuolation. 4 mice were studied by the serial block technique to evaluate the distribution of neurotoxicity. The nerve fibres and axonal terminals in the muscles of head, trunk, forelimbs and proximal part of hindlimbs appeared normal and the arborization of terminal axons was clearly demonstrated by silver impregnation. In the foot muscles and those of the leg, marked degeneration of axonal terminals and intramuscular nerve fibres were noted. The arborization of motor end-plates could not be identified.

Muscle spindles were normal. The innervation of the skin appeared normal. Electron microscopy showed axonal degeneration of nerve fibres in the soleus. In the foot muscles, there was also severe degeneration of nerve fibres and denervation of motor end-plates.

By 6 days there were more degenerated nerve fibres with numerous myelin ovoids in the legs and feet. Muscle fibres and spindles looked normal. By 9 days the axonal degeneration had progressed with much myelin debris. Some argyrophilic debris in the posterior tibial nerve could be demonstrated by silver impregnation. By 11 days the axonal degeneration did not seem to be more severe. Some myelin debris was noted in the soleus and foot muscles. At 14 days in the foot muscles there were some normal looking motor end-plates and some in which small axonal terminals were packed with clusters of vesicles suggesting progressive degeneration of axonal terminals. Sensory ganglion cells were not studied in this group.

Group C (25 week old with CL 0.5 mg/g)

Clinical Observations

7 mice were included in this group (Table 2.1). These mice were normal and active after CL administration. At 24 hours they still looked normal and no signs of weakness could be found. At 48 hours mice started to show some weakness of the hind limbs but this was not severe. By 72 hours there was marked weakness and dragging of the hind limbs. Mice were ill with ungroomed fur and thick catarrhal secretion of the eyes. No sensory disturbance or fore limb weakness could be detected. By 4 days they were very ill and loss of body weight was apparent. By 5 days there was almost total paralysis of hind limbs. Sensation was apparently intact. Paralysis of abdominal and respiratory muscles was also noted. By 6 days the toxicity had

progressed. Mice appeared very weak with paralysis of hindlimbs and costal recession. By 7 days all mice were very ill and dying. None survived longer than this.

Morphological Observations

Central Nervous System

By 4 days there was slight vacuolation in the pons but these were very rare in the spinal white matter. Some degenerated fibres were seen scattered in the spinal white matter, especially in the fasciculus gracilis. Nerve cells and motor and sensory roots were normal. By 7 days some intramyelinic vacuoles were found in the pontine white matter but very few in the spinal cord. In the dorsal columns of the cervical cord there were some degenerated myelinated fibres. No evidence of degeneration of nerve cells was seen.

Peripheral Nerves and Muscle

By 4 days there was severe axonal degeneration in intramuscular nerve fibres in the foot muscles. Some axons were electron dense, others electron lucent. Motor end-plates in the foot muscles were mostly denervated. Muscle fibres and spindles appeared normal. By 5 days paraffin sections with silver impregnation showed that the arborization of motor end-plates in legs and feet were not intact. The motor end-plates in the muscles of trunk. fore limbs and thighs were normal. By 7 days silver impregnation of paraffin sections showed degeneration of axonal terminals and intramuscular nerve fibres in muscles of the legs and feet. Electron microscopy showed many axonal degenerated nerve fibres in the foot muscles. Muscle fibres and spindles were normal.

Biochemical Study

This study was done in collaboration with Dr Robert Surtees, Institute of Child Health and Hospital for Sick Children, Great Ormond Street and I am most grateful for his permission to include the results in this thesis.

Thirty young mice (21 days old) were used in this experiment. They were given an intraperitoneal injection of CL (2 mg/g of body weight) and were allowed to survive from 24 hours to 6 days. Animals were killed by cervical dislocation. The brains and spinal cords were rapidly dissected out and frozen in liquid nitrogen (see Method and Material) and then were prepared for HPLC study.

Methionine: In normal young mice the concentration of methionine in the brain is very low, being of the order of 0.37mmol/g. Within 1 day of CL administration the levels of methionine were increased to a mean of about 2.2mmol, and by 3 days post-injection the levels were increased to a mean of 4.3mmol/g, a rise of more than tenfold.

SAM: The estimation of SAM levels in normal mice gave a mean of almost 34 nmol/g. Within 12 hours the level of SAM had already fallen to approximately 25nmol/g, and within 24 hours to about 21nmol/g, a fall to about 60% of normal. Thereafter the fall was less steep, being about 20nmol/g at 2 days and 17nmol/g at 6 days, a fall to 50% of normal values. This accumulation of methionine and fall in the SAM content is due to the inhibition of SAM-transferase which normally converts methionine to SAM (see Fig. 3.66 and Table 3.1).

In these experiments the estimations were confined to brain and spinal cord. Other tissues are known to be affected by CL. Lombardini and Talalay (1973) found a similar severe reduction of

SAM in most viscera except the liver, and a marked increase in methionine levels in all tissues.

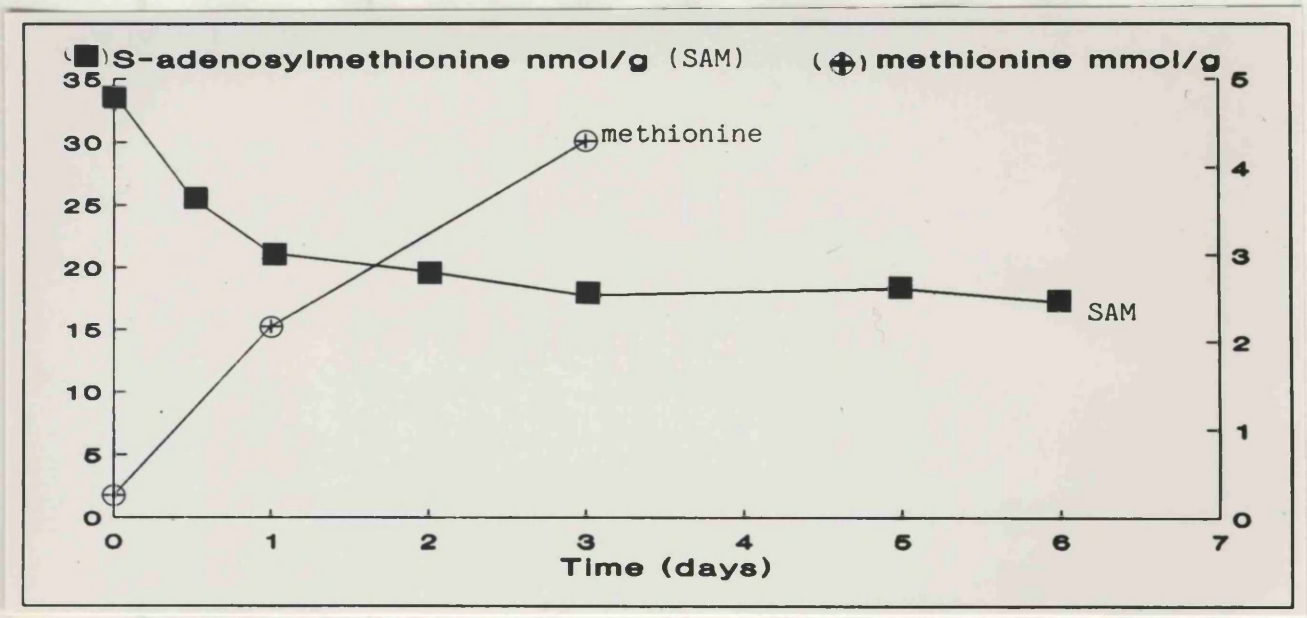

Fig 3.66

Brain concentration of S-adenosylmethionine and methionine in 21 days old mice given 20mg cycloleucine.

Each point on the graph represents the mean estimation in 3 mice.

Table 3.1

Days after normal controls	Brain methionine mmol/g		
	0.29	0.21	0.63
1	2.34	2.71	1.48
3	3.91	4.43	4.56
Time	Brain S-adenosylmethionine nmol/g		
Normal controls	33.96	34.81	32.70
0.5	27.21	24.16	25.14
1	20.01	21.12	22.25
2	19.86	20.41	18.81
3	16.71	18.67	17.94
5	18.92	18.30	17.64
6	17.40	16.81	17.40

Brain concentration of methionine and S-adenosylmethionine in 21 days old mice given 20mg cycloleucine.

Summary

CL was administered intraperitoneally (2mg/g body weight) to mice of varying ages (21 days-5 months). The 21 day old mice given CL 2mg/g body weight showed evidence of toxicity within 24 hours and thereafter developed progressive muscle weakness and ataxia. Animals did not survive longer than one week. Light and electron microscopic examination of the central and peripheral nervous systems, from 12 hours to 7 days post-injection showed that myelin vacuolation developed in the white matter of brain and cord within 12 hours. Axonal lesions in the distal parts of motor nerves occurred within 12-24 hours resulting in degeneration of intramuscular nerve fibres and terminals. Motor end-plates became denervated. There was no evidence of degeneration of motor or sensory nerves in the muscle spindles. Later there was evidence of axonal degeneration in tibial and sciatic nerves. Many dorsal root ganglion cells became vacuolated and necrotic three days after administration of CL and numerous degenerated fibres were noted in the white matter of the spinal cord, especially in funiculus gracilis.

In adult mice (6 weeks-5 months) evidence was found of only distal motor axonal degeneration which developed at 1-2 days. Very little intramyelinic vacuolation in white matter was noted. By 3 days numerous degenerated fibres were seen in the posterior columns of cervical cord, especially in the funiculus gracilis.

Chapter 4

RESULTS: Part 2

Experiment 2: Effects of Valine on CL-treated mice

The aim of this experiment was to study the effects of VL on the CL-treated mice in which the pathology has been described in the previous chapter. VL has been known to be the most effective antidote reversing the toxicity of CL in mice (Ruelius, 1973 and Nixon, 1974) by increasing the urinary excretion of CL and thus reducing its concentration to subtoxic levels.

VL 3 mg/g was administered intraperitoneally every 12 hours for up to 6 doses starting 24 hours after the injection of CL in 6 mice aged 21 days in **Group A**, 48 hours after the injection of CL in another 6 mice aged 21 days in **Group B**, and 6 mice aged 6 weeks in **Group C**. The animals were sacrificed from 3 to 22 days after the administration of CL (Table 2.2).

Clinical observations

In **Group A**, all the 6 mice aged 21 days were obviously ill and showed decrease of motility, loss of appetite and mild weakness of hindlimbs by 24 hours. VL was started 24 hours after the injection of CL. The toxicity was apparently reversed after 2-3 doses of VL. There was a dramatic improvement in the clinical appearance of these mice compared to those given CL alone which were very ill and showed severe weakness of the hind limbs. Three days after the

institution of valine, they already showed signs of gaining weight and there were almost no detectable signs of abnormality.

In **Group B,** by 48 hours after CL mice were severely ill and ataxic with a very severe weakness of the hind limbs. The general condition of mice was much improved and they became active within 24 hours after 2-3 doses of valine. There was still some weakness in the hind limbs. The motility of mice was restored, they could eat and groom themselves properly and showed obvious gain in weight after 6 doses of valine. The hindlimbs started to recover from the muscle weakness.

In **Group C,** 6 mice aged 6 weeks old were given CL 2mg/g and showed severe weakness of hindlimbs within 48 hours. They became active and started to eat and gain weight after 3-4 doses of VL. Some weakness of the hind limbs, was still noted. The general condition of each mouse was much improved after 6 doses of VL. They could groom themselves well and showed an apparent increase of body weight. Compared to the mice with CL only, the mice were much more active, moved around in the cage and had a shining smooth fur and bright eyes. They reacted very swiftly to any stimulation but there was still a slight abnormality of gait. These mice recovered progressively day by day and by 7 days after the start of administration of VL, the only abnormality which could be found was mild to moderate weakness of hindlimbs, in particular of the hind feet.

Morphological observations

Group A:

2 days (CL 1 day + VL 1 day). In the mouse which had received 2 doses of VL from 24 hours after CL, there were numerous intramyelinic vacuoles of the white matter in the cerebral hemisphere,

cerebellum (Fig. 4.1), medulla oblongata and spinal cord. The severity and distribution of the intramyelinic vacuolation of this mouse was comparable to that of mice 2 days after CL only. Blocks of soleus, EDL and foot muscles were examined in plastic 1μm sections stained with toluidine blue. Nerve fibre degeneration was found in many of the small intramuscular nerve bundles. Electron microscopy was done only on the foot muscle. No normal end-plates were seen. Four end-plates were found totally denervated (Fig. 4.2) without any residual axon terminals but with some debris lying within Schwann cell cytoplasm (see Table 4:1). No significant pathology was seen in sciatic and tibial nerves or in dorsal root ganglion cells.

At 3 days (CL 1 day + VL 2 days) in the mouse which had 3 doses of VL, there was intramyelinic vacuolation of the CNS white matter in cerebellum, medulla oblongata and spinal cord. The severity of the vacuolation of this mouse was much less than that of those mice 3 days after the administration of CL only. There were several fibres with degenerated axons in the dorsal columns of the cervical cord, especially in the fasciculus gracilis. Axonal degeneration with electron dense or rarefied axoplasm and breakdown of myelin sheaths were noted in intramuscular nerves in the soleus, EDL and foot muscles. Only 3 end-plates, all denervated were found on EM of foot muscles. No abnormalities were found in tibial or sciatic nerves examined by light or electron microscopy.

At 4 days (CL 1 day + VL 3 days) the mouse which had received 6 doses of VL still showed some vacuoles in the white matter in the cerebrum, cerebellum and cervical cord. In the cervical cord there were numerous degenerated fibres with fragmenting myelin sheaths in the dorsal columns; the large fibres in the fasciculus gracilis seemed to be most severely affected. In the soleus, EDL and foot muscles, there were many fibres in intramuscular nerves showing

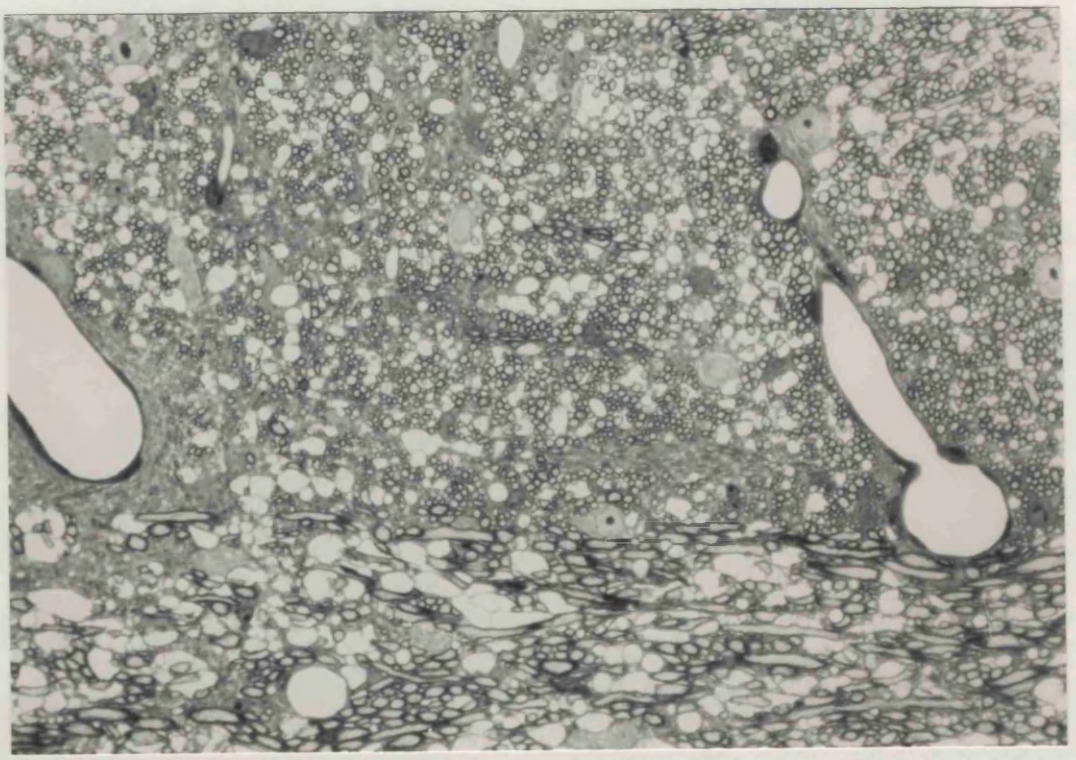

Fig. 4.1 Cerebellum in Group A 2 days (CL 1d + VL 1d). Some vacuoles can be seen in the white matter. 1μm Toluidine blue x 400.

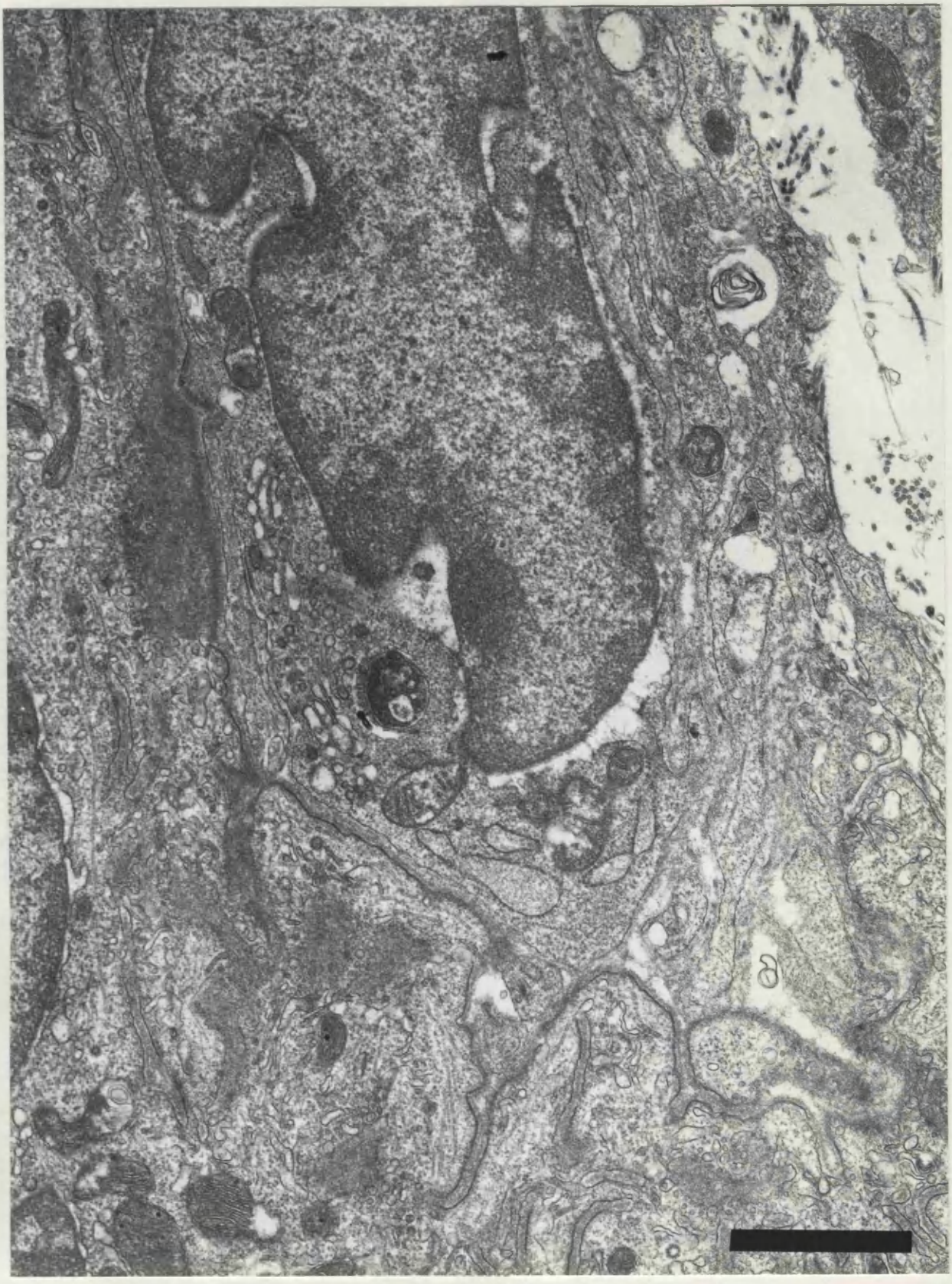

Fig. 4.2 Same mouse as Fig. 4.1. Foot muscle. A denervated motor end-plate is shown. The axonal terminal has degenerated and some dense debris lies in the cytoplasm of the Schwann cell. Bar = 1μm (Neg. 91501).

axonal degeneration with numerous myelin ovoids. All 6 neuromuscular junctions found were denervated and the postsynaptic folds were covered by Schwann cell processes. No significant pathology could be found in the posterior tibial nerve or sciatic nerve.

At 8 days (CL 1 day + VL 7 days) there was little intramyelinic vacuolation in the white matter of the cerebellum and spinal cord. In the intramuscular nerves in soleus and foot muscles, there were many un- or thinly- myelinated axons with electron-lucent axoplasm containing poorly organised neurofilaments, some vesicles and a few mitochondria (Fig. 4.3). In all 8 neuromuscular junctions found, small axons were present while the postsynaptic membrane was still partly occupied by processes of Schwann cell (Fig. 4.4). There was some myelin debris, Schwann cells and macrophages in the intramuscular nerve bundles, giving the impression of the remains of degenerated fibres as well as evidence of regeneration and new myelin sheath formation.

At 12 days (CL 1d + VL 11d) there was no intramyelinic vacuolation anywhere in the white matter of cerebellum, cervical, thoracic and lumbar cords. There were, however, some extracellular spaces and some swollen processes in the white matter which may have been the residual changes after recovery of previously vacuolated areas (Fig. 4.5). These 'extracellular spaces' tended to be round and were only partially enclosed by a single membrane. Some parts seemed to lack a membrane and some vesicular structures lay in these spaces. The spaces abutted myelin sheaths but no myelin lamellae enclosed them. It seemed likely that these were the remains of the large intramyelinic vacuoles. There were no macrophages, no signs of myelin debris and no astrocytic gliosis. There were numerous degenerated fibres in the dorsal columns in the cervical cord. There were many thinly myelinated fibres and regenerating

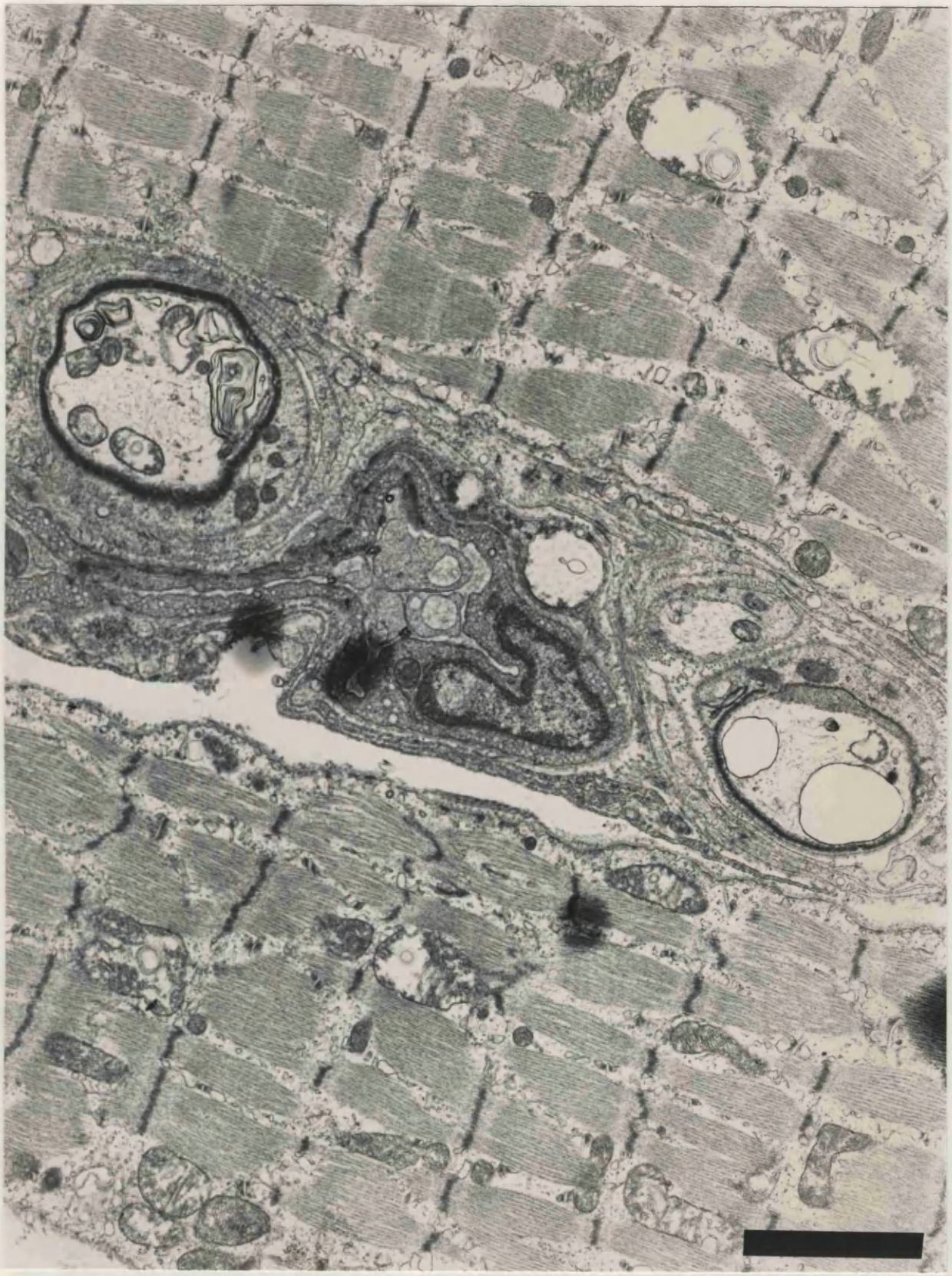

Fig. 4.3 Intramuscular nerve in foot muscle at 8 days (CL 1d + VL 7d). Several thinly myelinated nerve fibres are present. They contain poorly organized neurofilaments, some vesicles and a few mitochondria. Bar = 2μm (Neg. 99075).

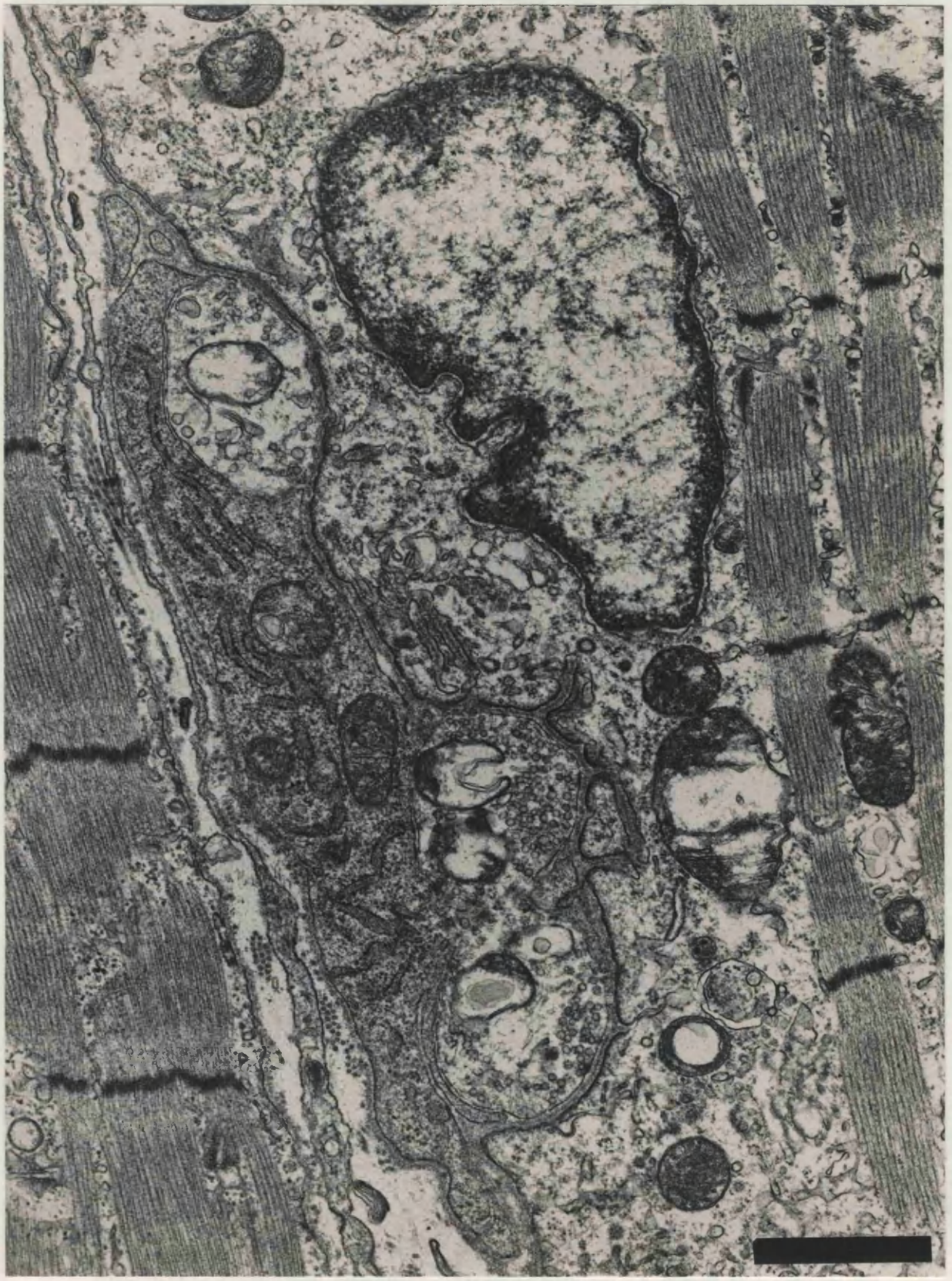

Fig. 4.4 End-plate in foot muscle, Group A at 8 days. Several small axonal terminals are present in this end-plate which is still partly covered by Schwann cell cytoplasm. Bar = 1μm (Neg. 99066).

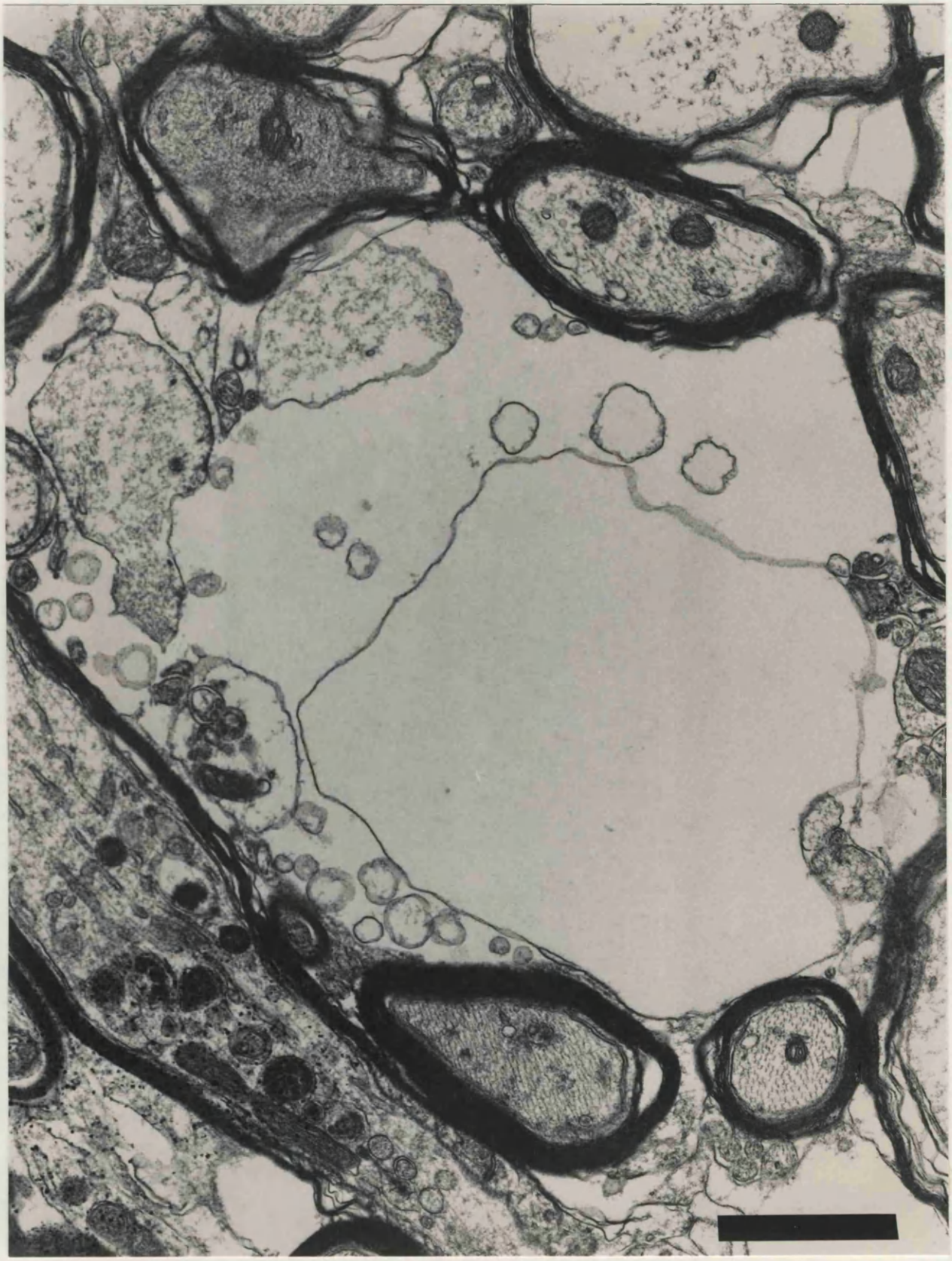

Fig. 4.5 White matter of cerebellum at 12 days (CL 1d + VL 11d). Some extracellular spaces incompletely enclosed by membrane may be the result of breaking down intramyelinic vacuoles. Bar = 1μm (Neg. 99009).

unmyelinated fibres in the intramuscular nerve bundles of the soleus, EDL and foot muscles (Fig. 4.6). All of the 20 motor end-plates found (see Table 4.1) were reinnervated by axonal terminals, many of which were small and contained vesicles and usually did not cover the entire post synaptic membrane (Fig. 4.7). Many terminals were quite large and looked very like the normal.

At 14 Days (CL 1d + VL 13d), there was no intramyelinic vacuolation in the CNS white matter. There were very occasional extracellular spaces the white matter similar to those seen in some control preparations. There was no significant pathology in the sciatic nerve but a few degenerated fibres in post tibial nerve. Some thinly myelinated fibres were seen in the soleus and foot muscles indicating regeneration of nerve fibres. The 2 neuromuscular junctions found were both innervated.

Group B

The mouse that received 2 doses of VL 2 days after CL was sacrificed on the **third day** (CL 2d + VL 1d) after the administration of CL. There was very severe intramyelinic vacuolation in the cerebellar and pontine white matter and a few vacuoles in the spinal white matter. Numerous degenerated myelinated fibres were noted in the dorsal columns of the cervical cord, especially in the fasciculus gracilis. A very few dorsal root ganglion cells were vacuolated; otherwise, no significant pathology was found. In the foot muscles, there was a very severe axonal degeneration of fibres in intramuscular nerve bundles. Denervation of motor end plates was noted in the foot muscles. All 5 end-plates found were affected.

At 7 days (CL 2d + VL 5d) there were still a few intramyelinic vacuoles in white matter of cerebellum and pons, and very few could be found in the cervical cord. There were many fibres showing

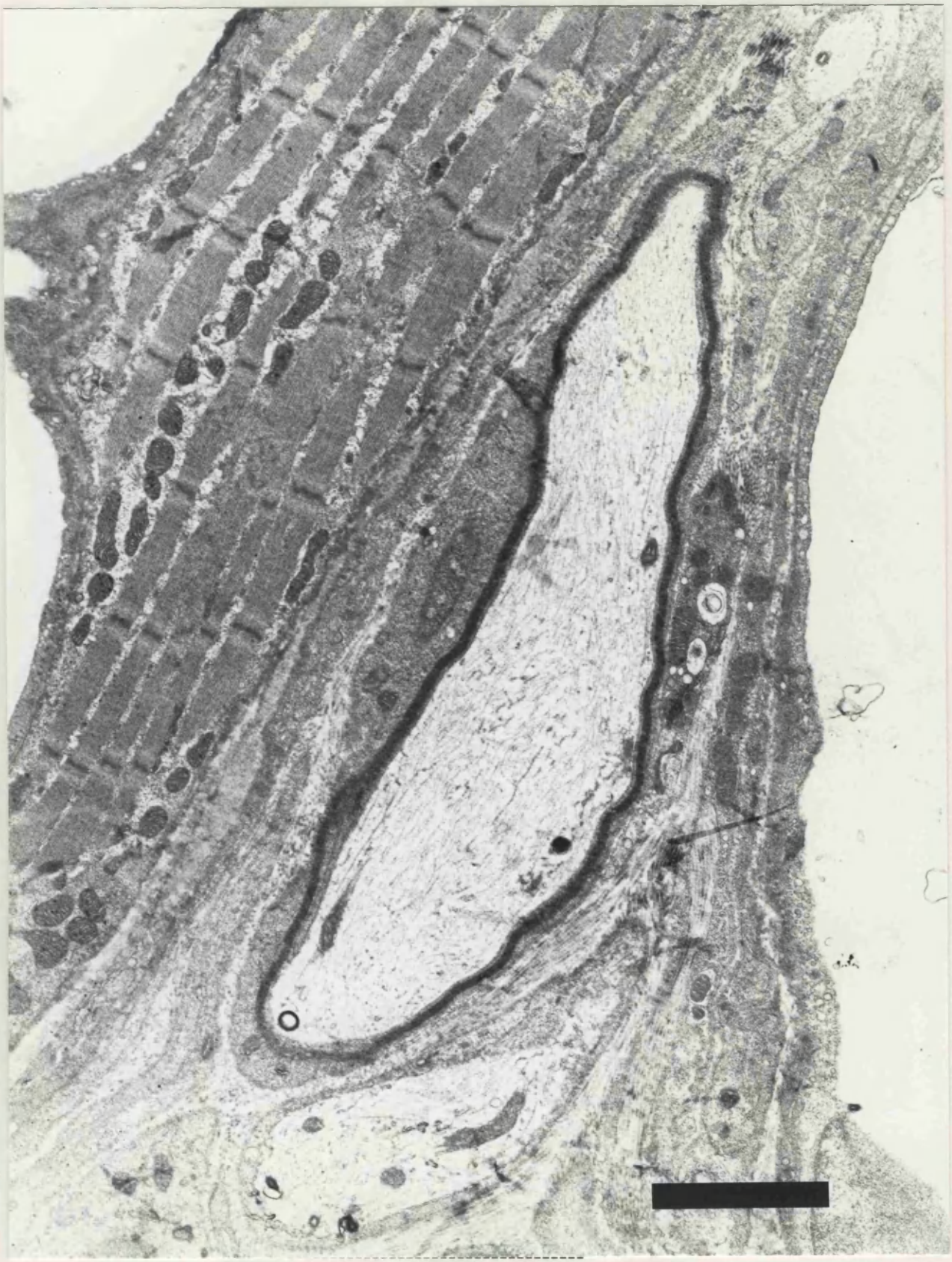

Fig. 4.6 Foot muscle at 12 days (CL 1d + VL 11d). A thinly myelinated and an unmyelinated fibre are shown. Bar = 2μm (Neg. 99204).

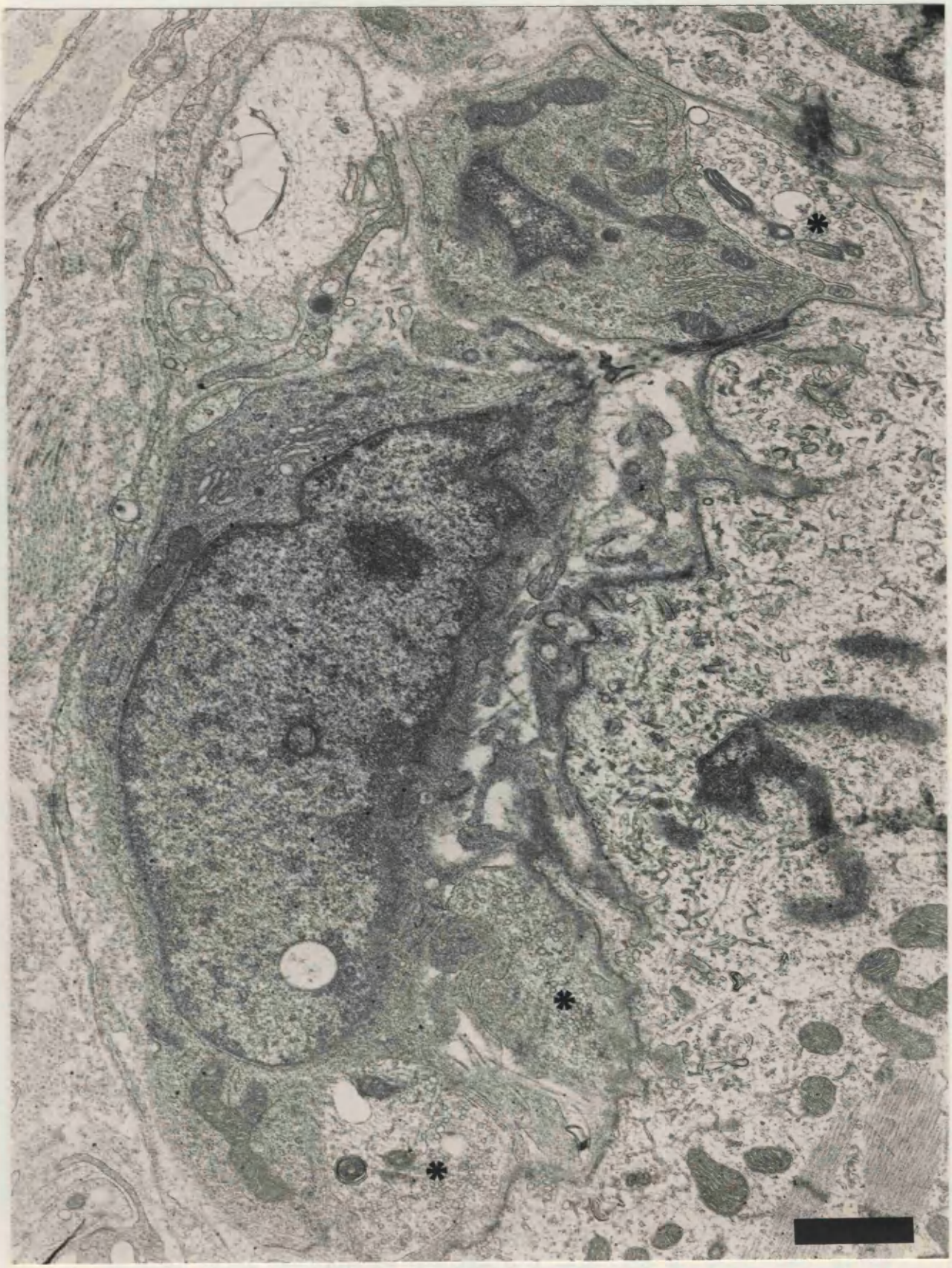

Fig. 4.7 A motor end-plate in foot muscle at 12 days. Several small axonal terminals (*) are present in contact with the post synaptic membrane. Bar = 1μm (Neg. 99211).

axonal degeneration in the dorsal columns. Occasional degenerated fibres were found scattered in the anterior and lateral columns. Some cells with dense cytoplasm, probably oligodendrocytes, with electron dense cytoplasm were noted to engulf myelin debris (Fig. 4.8). Nerve cells and motor and sensory roots were normal. In the lumbar dorsal root ganglia no vacuolation or necrosis of ganglion cells were present. There ~~was~~ were still many degenerated intramuscular nerve fibres with much myelin debris and also some un- or thinly myelinated fibres in the foot muscles. Muscle spindles appeared normal. 14 end-plates were found. They were all denervated (see Table 4.1).

By 12 days after CL (CL 2 d + VL 10 d) only very occasional intramyelinic vacuoles could be seen in the white matter of cerebellum and pons. This may indicate that most intramyelinic vacuoles which were seen in those animals with CL only or which were killed in the early stage of administration of VL after CL have disappeared and only an occasional extracellular space and membrane-bound vacuole were found in the white matter (Fig. 4.9). In the spinal cord, there were no intramyelinic vacuoles and many degenerated fibres were seen in the dorsal columns.

There was clear evidence of nerve fibre regeneration with numerous clusters of unmyelinated axons (Fig. 4.10) and many thinly myelinated fibres in the foot muscles. Reinnervation of motor end-plates was taking place with the presence of small axons in the previously denervated postsynaptic fold in 5 of the 11 end-plates found (Fig. 4.11).

At 15 days (CL 2 d + VL 13d) there was very little vacuolation in the white matter of cerebellum and spinal cord but some degenerated nerve fibres persisted in the fasciculus gracilis. In the posterior tibial nerves there were some fibres showing axonal

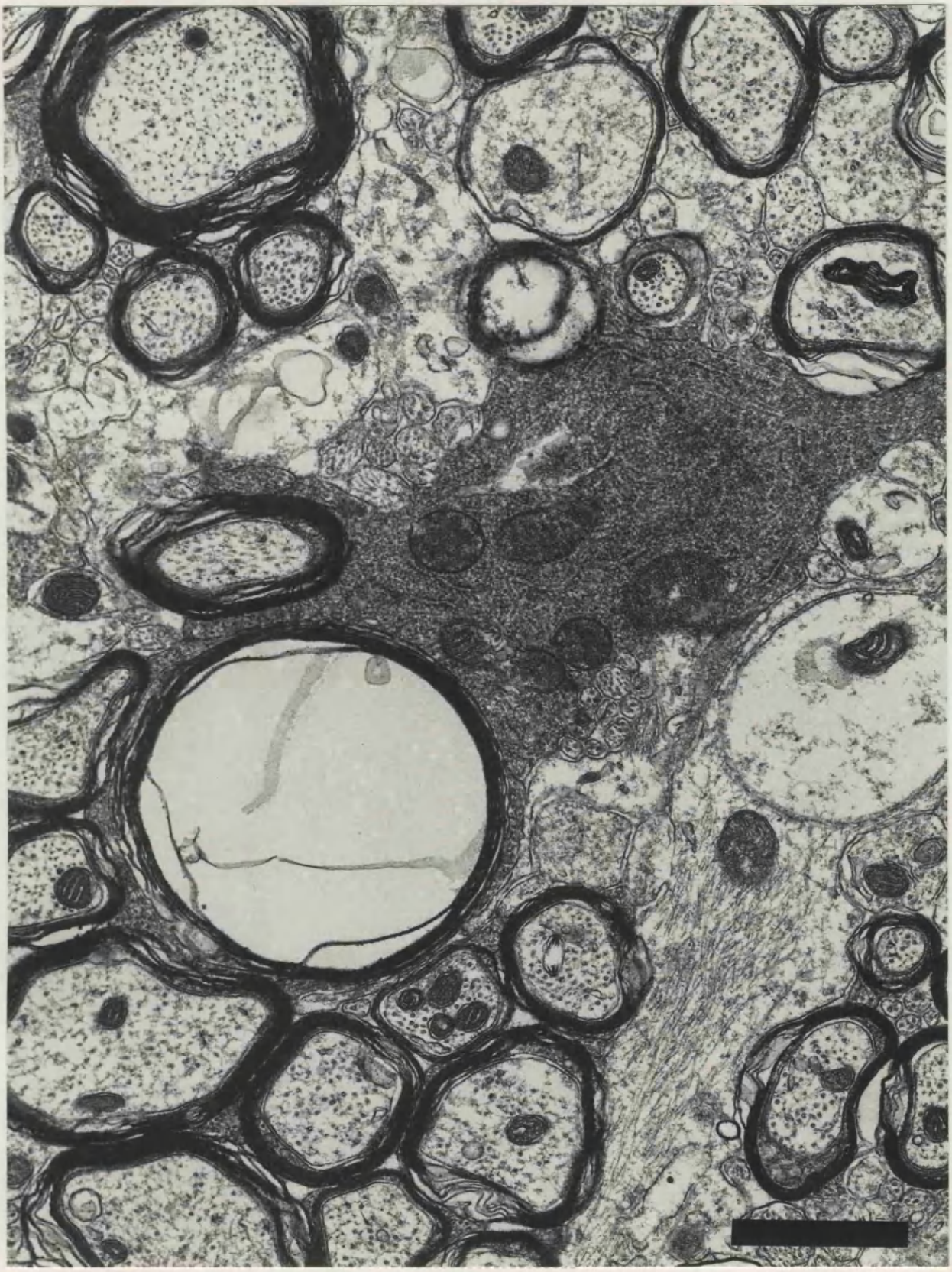

Fig. 4.8 Group B at 7d (CL 2d + VL 5d). Degenerated nerve fibres in dorsal column in cervical spinal cord. Myelin debris lies in the cytoplasm of a cell which seems to be an oligodendrocyte. Bar = 1μm (Neg. 91049).

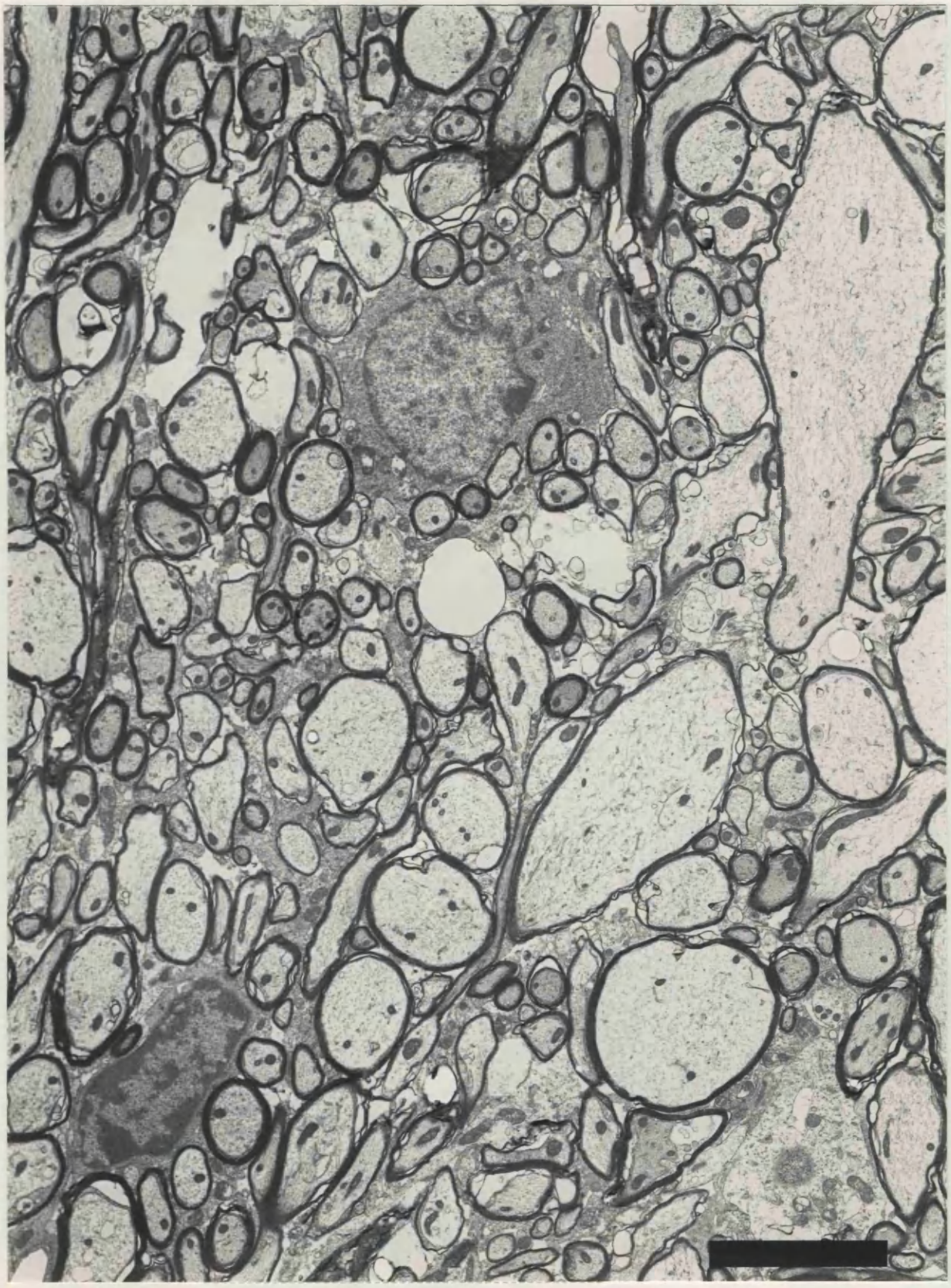

Fig. 4.9 Cerebellar white matter at 12d (CL 2d + VL 10d). There is little or no intramyelinic vacuolation and occasional extracellular spaces may not be significant. Bar = 5μm (Neg. 91095).

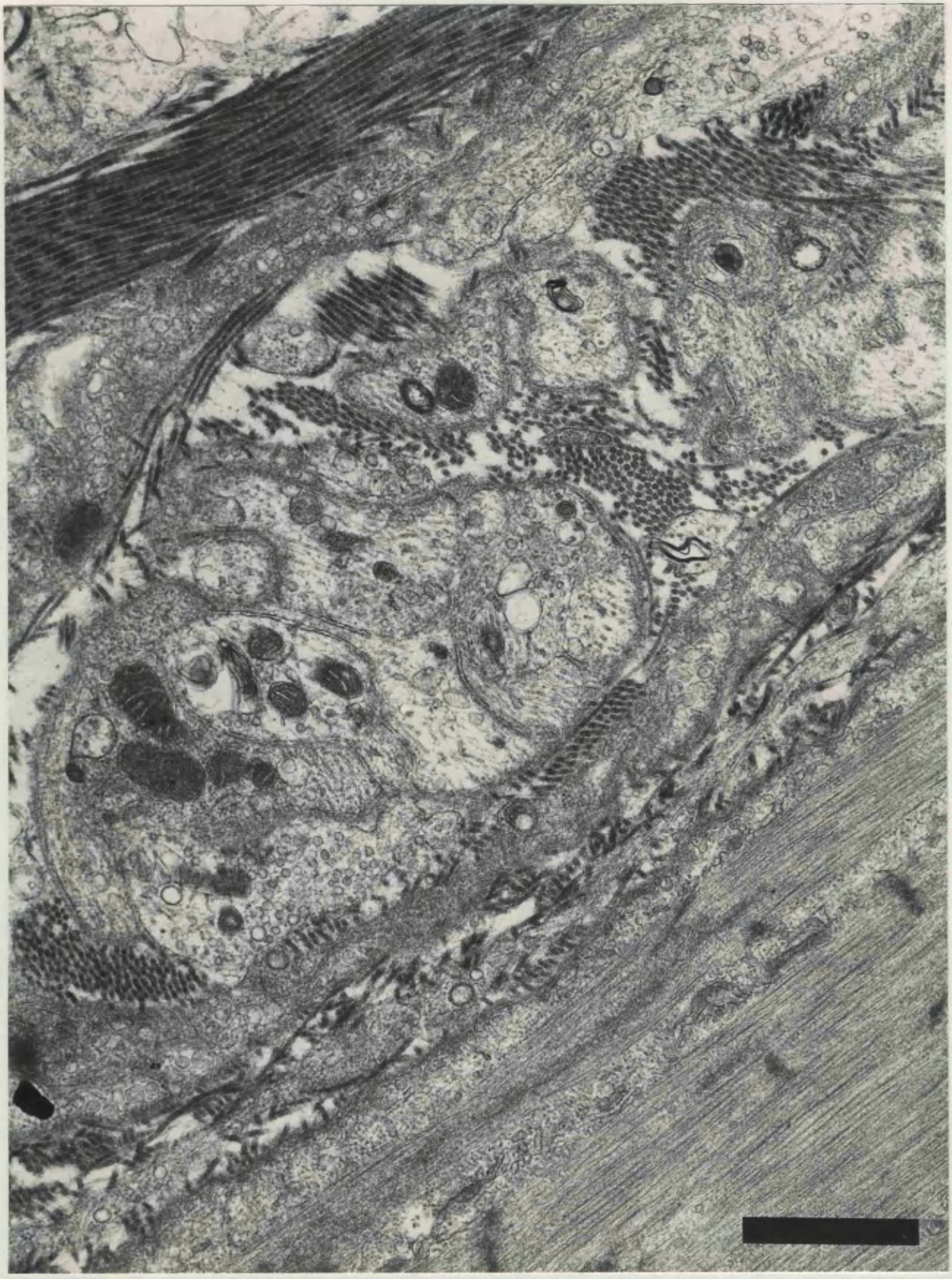

Fig. 4.10 Section of foot muscle of same animal as in Fig. 4.9. An intramuscular nerve is composed of a cluster of unmyelinated axons suggesting that regeneration is taking place. Bar = 1μm (Neg. 91101).

Fig. 4.11 Foot muscle of same animal as in Figs. 4.9 and 4.10. Axonal terminals are present (*). Bar = 1μm (Neg. 91109).

degeneration as well as thinly myelinated fibres. Many clusters of axonal sprouts and thinly myelinated fibres were noted in the foot muscle. Seven end-plates were found of which 5 were denervated and 2 innervated by small axonal terminals.

Group C

The mouse that received 2 doses of VL were sacrificed on the **third day** (CL 2d + VL 1d) after the administration of CL. There were some patches of vacuoles in the cerebellar white matter but these were very rare in the spinal white matter. No nerve cell degeneration was noted. In the foot muscles most intramuscular nerve fibres showed severe axonal degeneration with abundant myelin debris (Fig. 4.12). Muscle fibres appeared normal. Six motor end-plates were found and all were denervated (Fig. 4.13).

By **4 days** (CL 2d + VL 2d) the degree of vacuolation in the cerebellar white matter had markedly decreased compared to the previous mouse (sacrificed at 3 days) and those mice with CL only. There were some extracellular spaces, swollen axons and glial processes in the cerebellum. In the cervical cord, there were many degenerated myelinated fibres in the posterior columns, especially in the f. gracilis. There were also some occasional degenerated fibres scattered in the cerebellar white matter. The intramuscular nerve fibres in the soleus and foot muscles showed severe axonal degeneration, some contained electron dense axoplasm or showed rarefaction of axoplasm. Abundant debris was also noted. Three denervated motor end-plates with post-synaptic folds covered by Schwann cell processes were seen in foot muscles.

At **6 days** (CL 2d + VL 4d)only a few vacuoles could be seen in the cerebellar white matter and spinal cord. There were numerous degenerated fibres in the dorsal columns, especially in the fasciculis

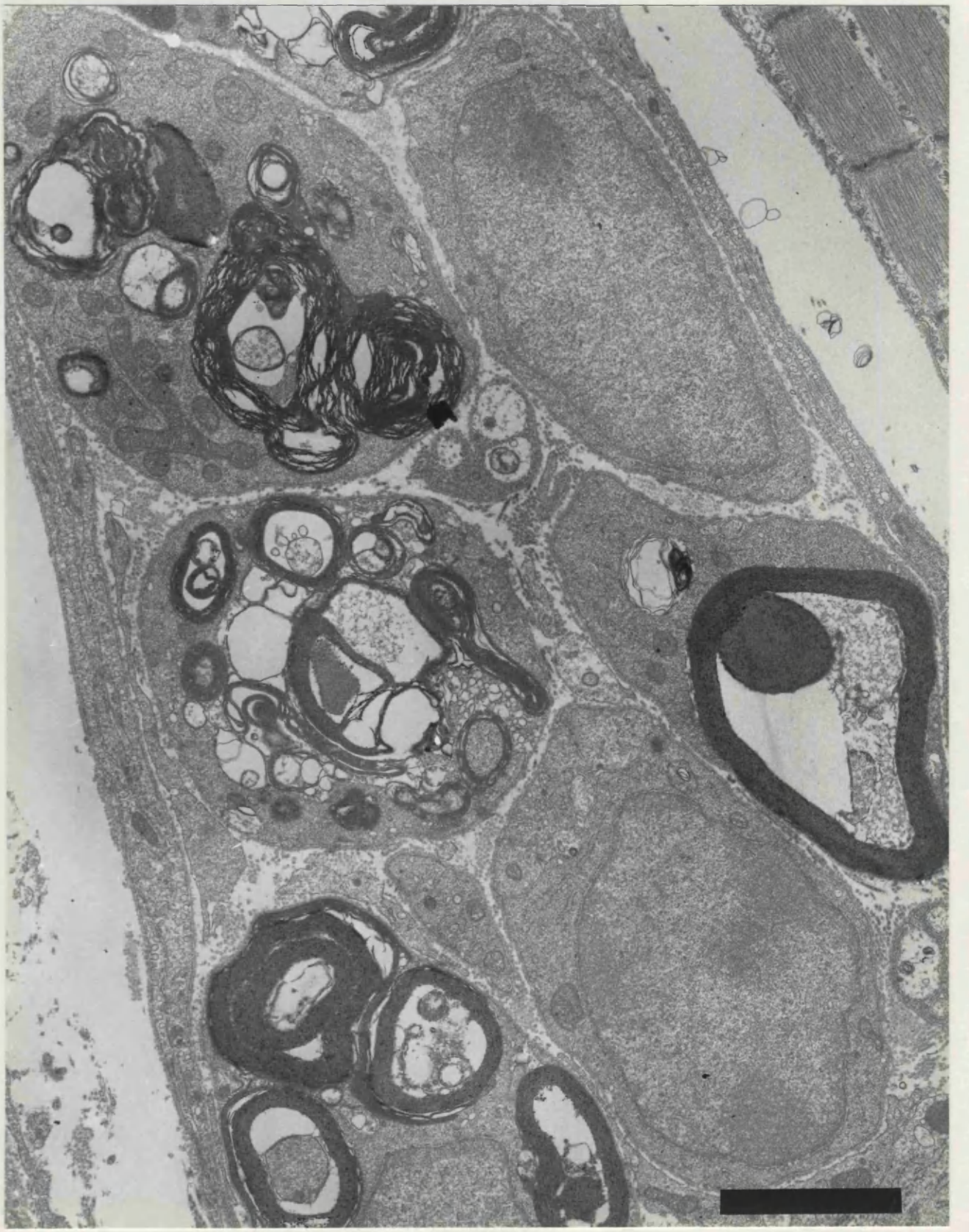

Fig. 4.12 Foot muscle of mouse in Group C at 3 days (CL 2d + VL 1d). An intramuscular nerve shows severe axonal degeneration of almost all nerve fibres. Bar = 2μm (Neg. 89315).

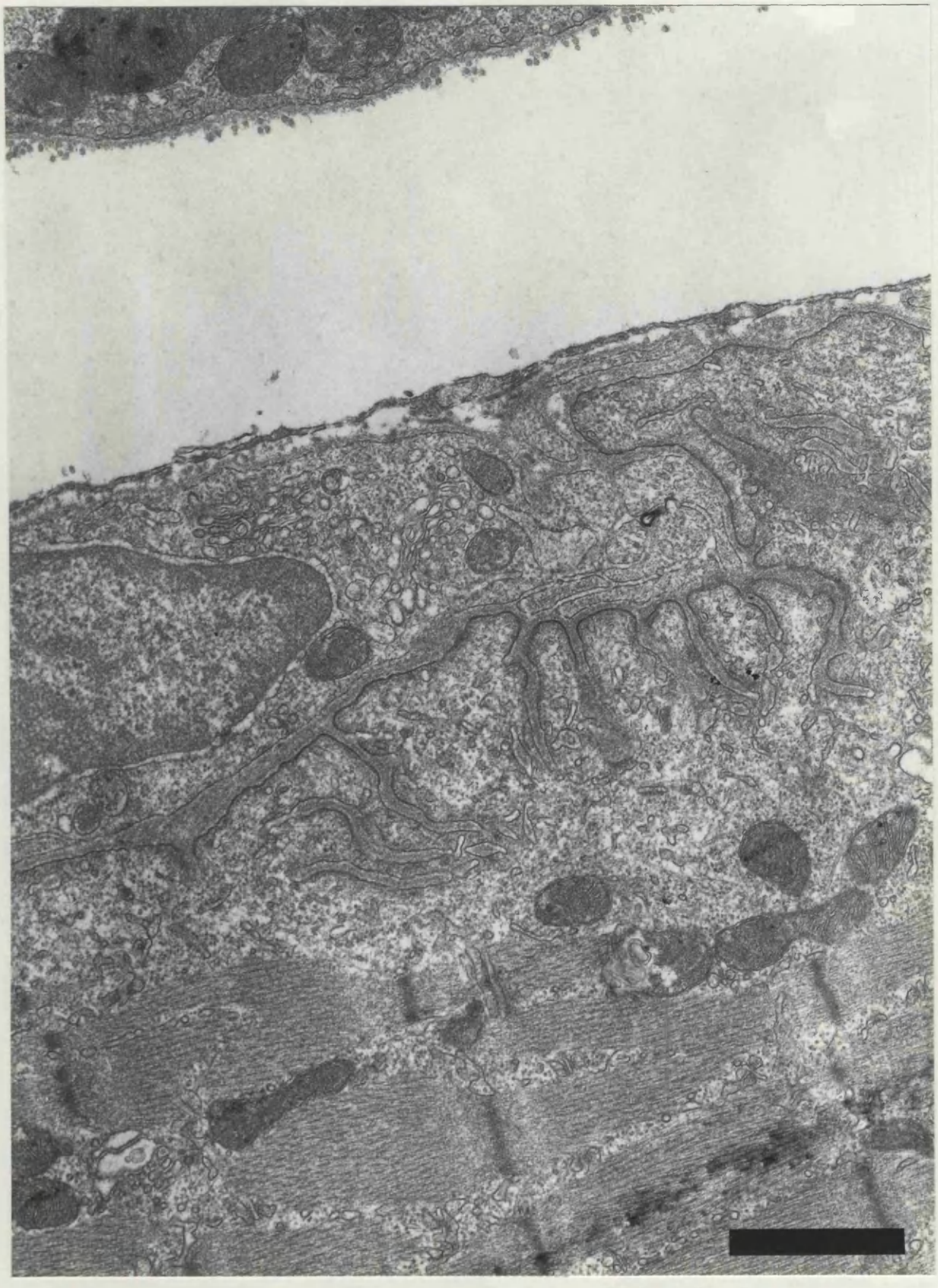

Fig. 4.13 End-plate of same animal as in Fig. 4.12 showing lack of axonal terminal (note that post synaptic folds are considerably deeper and more numerous at 6 weeks compared with 21 days of age). Bar = 1μm (Neg. 89320).

gracilis with a few scattered in the remaining spinal white matter. Many degenerated fibres with myelin debris were seen in foot muscles and soleus. There were also groups of unmyelinated fibres seen in nerves in soleus and foot muscle which may indicate the early regeneration (Fig. 4.14). All three end-plates found were denervated (Fig. 4.15).

By **9 days** (CL 2d + VL 7d) there were very few vacuoles in the cerebellar white matter and no vacuoles in the spinal white matter. Some degenerated myelinated fibres were seen scattered in the spinal white matter, especially in the fasciculus gracilis. Lumbar dorsal root ganglion cells appeared normal. There were many degenerated fibres as well as some clusters of unmyelinated and thinly-myelinated fibres in the foot muscles. The 2 end-plates found in foot muscle were innervated by small axons.

At **13 days** there were no intramyelinic vacuoles in the cerebellar white matter and spinal cord. Some degenerated myelinated fibres scattered in the spinal white matter were seen. In intramuscular nerve bundles, in addition to the debris of degenerated fibres, there were large unmyelinated fibres, some clusters of axonal sprouts and some thinly myelinated fibres. All 3 end-plates found were reinnervated by regenerating axons.

At **22 days** (CL 3d + VL 20d) there were no intramyelinic vacuoles in the CNS. Some degenerated myelinated fibres were noted scattered in spinal white matter. No evidence of degeneration of nerve cells was found. In the foot muscles, EDL and soleus there were many big unmyelinated axons and many thinly myelinated fibres (Fig. 4.16). 11 end-plates were found in the foot muscles, all of them innervated by axonal terminals some of which were small but others more normal in appearance (Fig. 4.17).

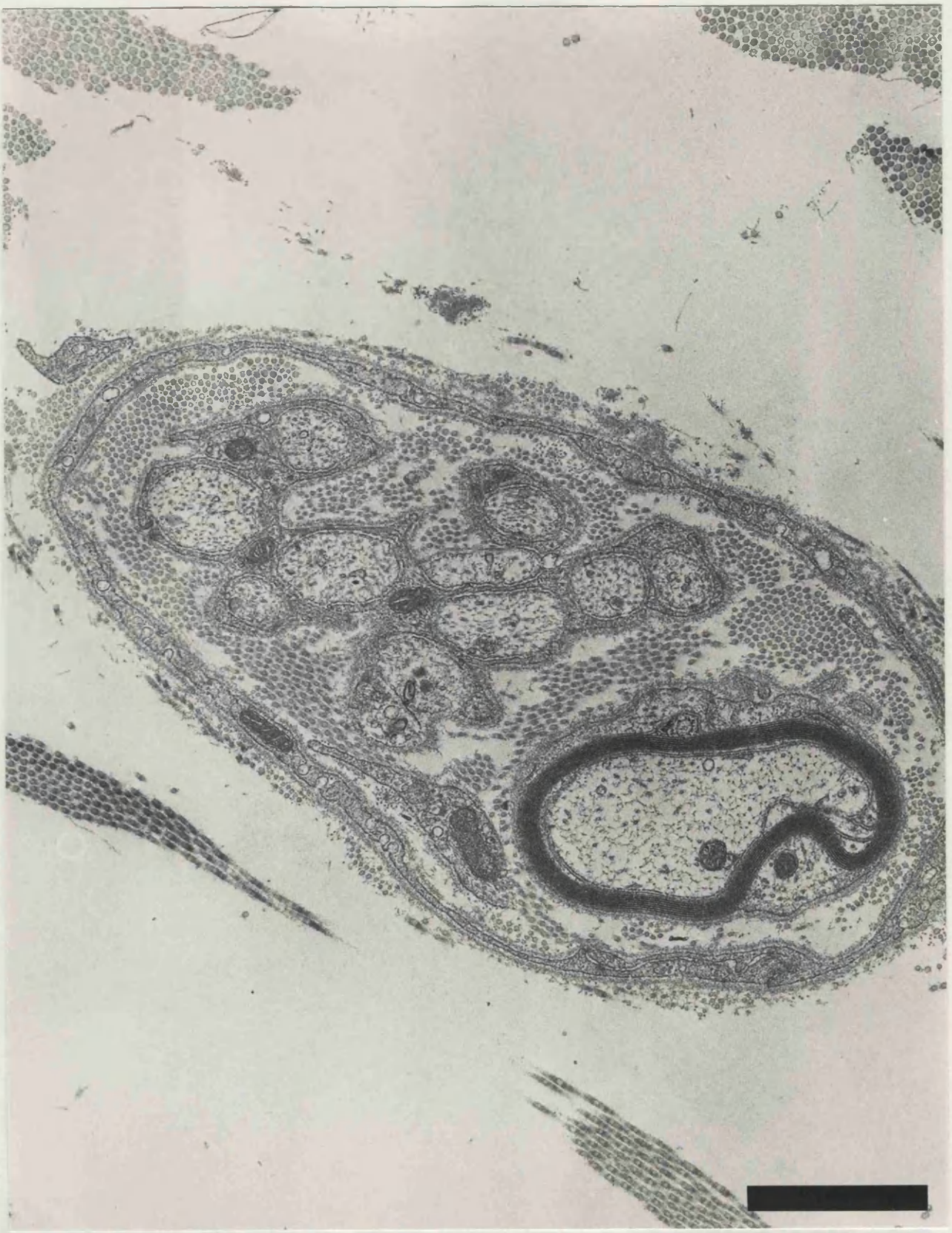

Fig. 4.14 Group C at 6 days (CL 2d + VL 4d). An intramuscular nerve in soleus contains a group of unmyelinated fibres, an appearance ~~suggesting regeneration~~ similar to the normal. Bar = 1μm (Neg. 99804).

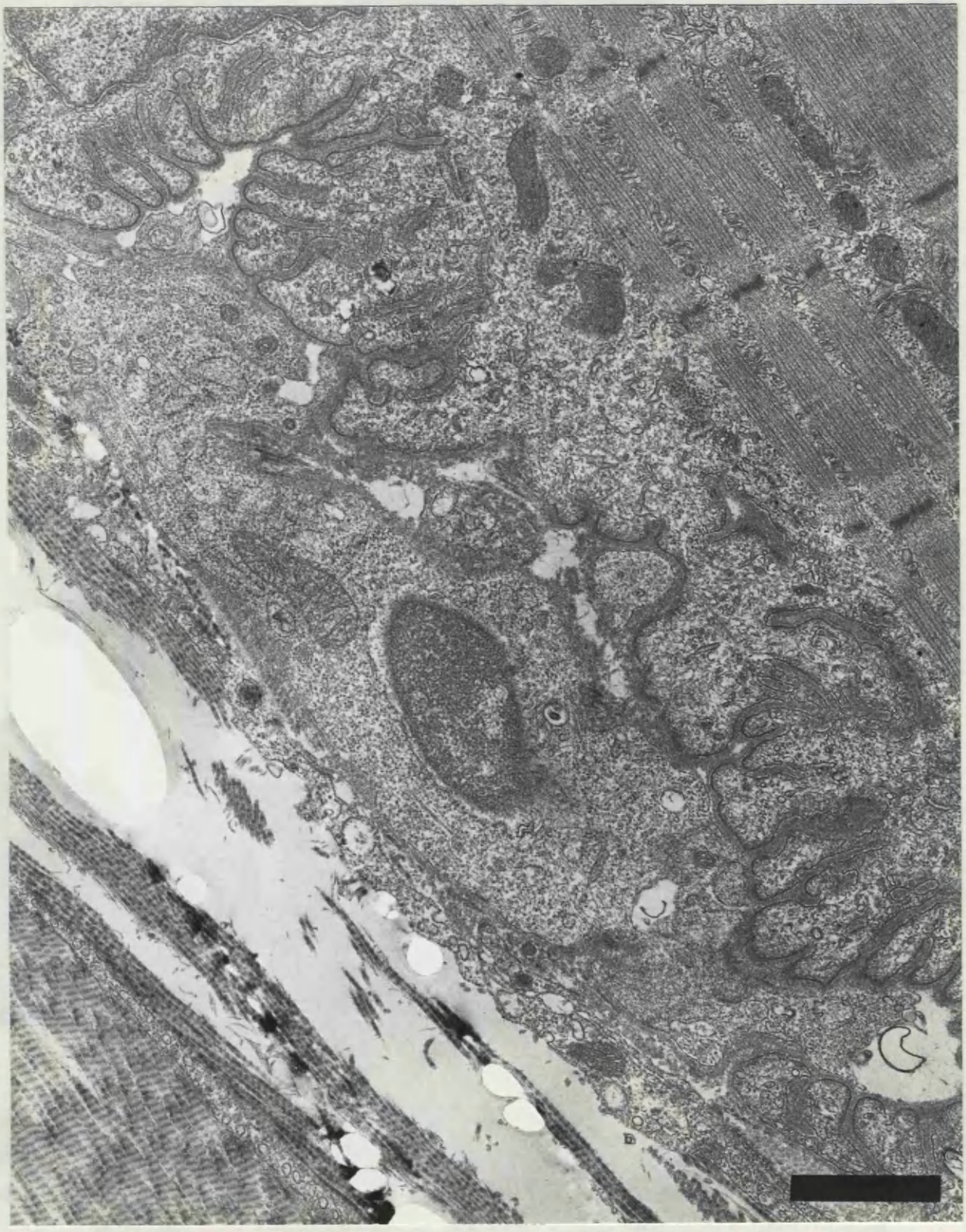

Fig. 4.15 Foot muscle from same animal as in Fig. 4.14 showing a denervated motor end-plate. Bar = 1μm.

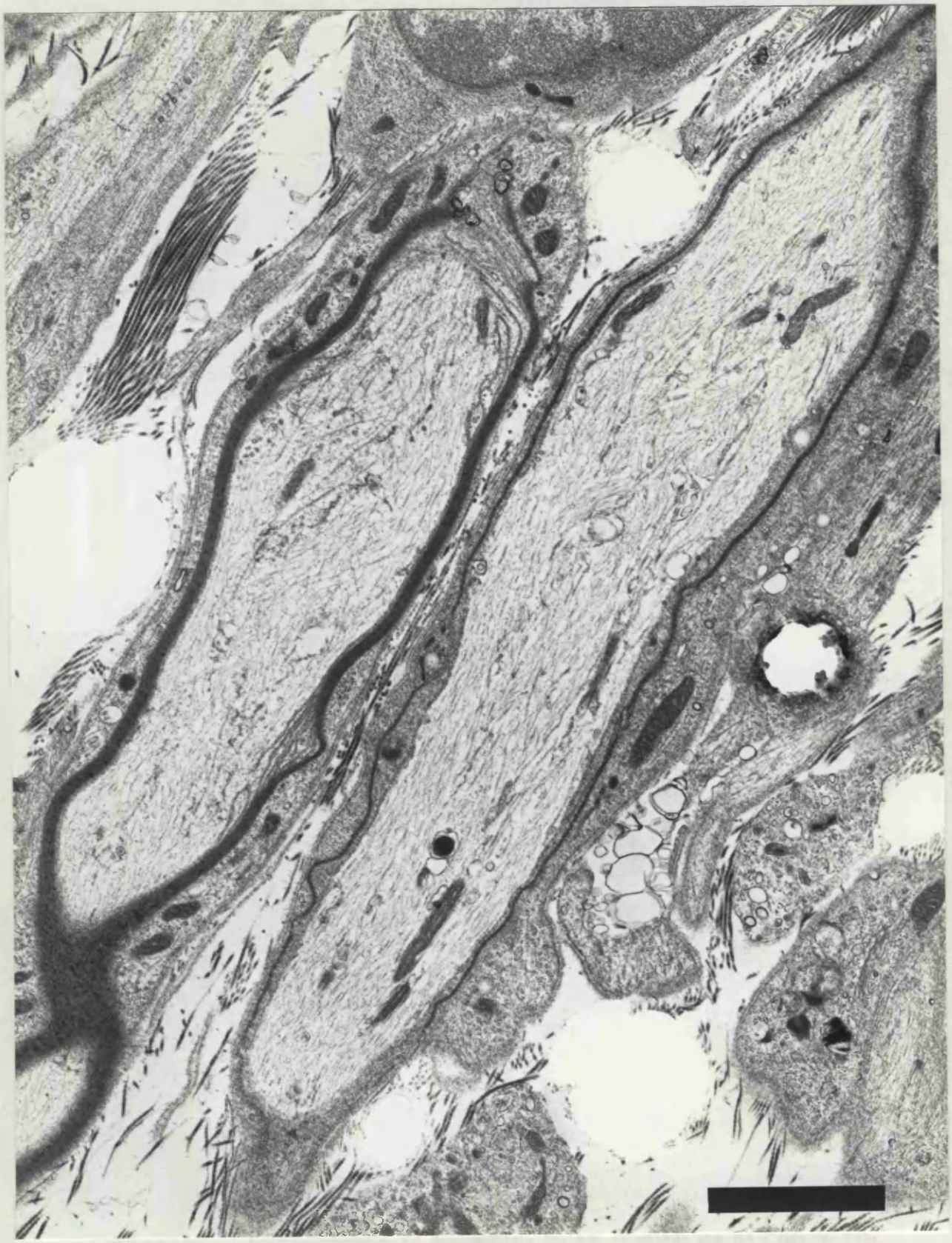

Fig 4.16 At 22d (CL 2d + VL 20d) these axons in a foot muscle are thinly myelinated indicating regeneration and remyelination. Bar = 2μm (Neg. 00008).

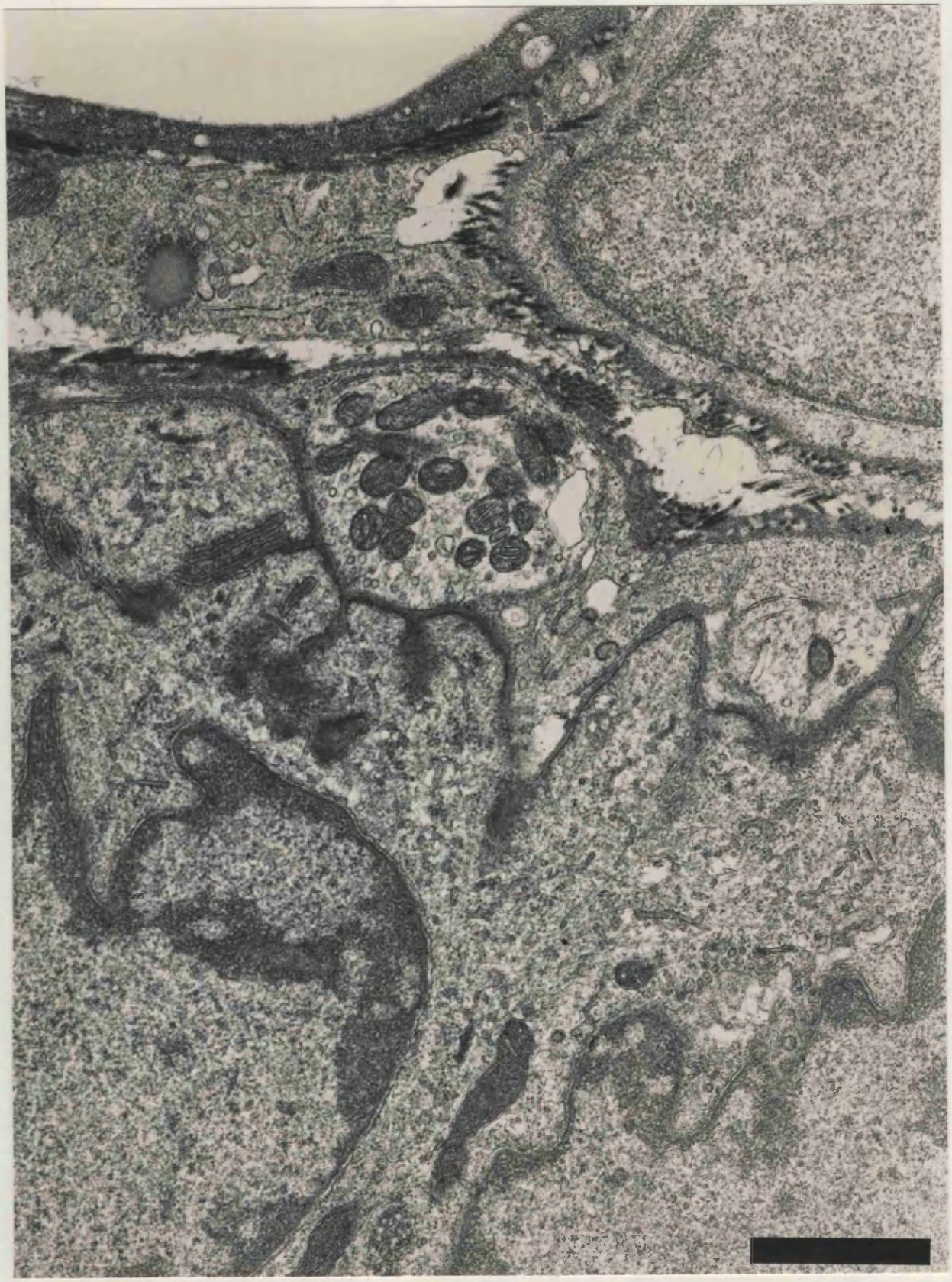

Fig. 4.17 Foot muscle at 22 days, same animal as in Fig. 4.16. A normal looking axonal terminal containing vesicles and mitochondria innervates this end-plate. Some Schwann cell cytoplasm still covers part of the postsynaptic membrane. Bar = 1μm (Neg. 00004).

Summary

In addition to confirming the observations of previous experiments by Ruelius et al (1973) and Nixon (1974) that VL is a very effective antidote of CL, this experiment also followed the morphological sequence of events taking place after the administration of VL in CL-affected mice. Vacuolation of myelin in the CNS subsided with little residual pathology. Some intramyelinic vacuoles seemed to become separated from their myelin sheaths and lay in the neuropil. Breakdown of their membranes led to the formation of irregularly shaped spaces which were extracellular and which gradually became less numerous with longer survival. There did not appear to be a significant astrocytic glial reaction during the time the animals were allowed to survive. There was no myelin debris.

Regeneration of peripheral nerve fibres led to reinnervation of end-plates which occurred within 7 days after the administration of VL. beginning 24 hrs after CL. In the animals given VL only 48 hrs after CL the regeneration and reinnervation of end-plates was slower and even 13 days after VL denervated end-plates were still to be found. This indicates that the CL caused more extensive damage over 48 than after 24 hrs and that the damage began in the most distal parts of the motor axons, i.e. in the terminals, later spreading proximally.

Table 4.1

Motor end-plates in soleus and foot muscles of mice given Valine 24 or 48 hours after Cycloleucine

Days after VL	21 days old mice				6 weeks old mice	
	Group A. CL 1d + VL		Group B. CL 2d + VL		Group C. CL 2d + VL	
	Denerv.	Reinnerv.	Denerv.	Reinnerv.	Denerv.	Reinnerv.
1	4	0	5	0	6	0
2	3	0	-	-	3	0
3	6	0	-	-	-	-
4	-	-	-	-	3	0
5	-	-	14	0	-	-
7	0	8	-	-	0	2
10	-	-	6	5	-	-
11	0	20	-	-	0	3
13	0	2	5	2	-	-
20	-	-	-	-	0	11

- = Not examined.

End-plates were counted in ultrathin sections. Each end-plate was counted only once even if seen in several sections. All end-plates identified in all groups of mice were devoid of axonal terminals between 1 - 5 days after valine (2 - 7 days after CL). All end-plates after day 7 at which axonal terminals were found were presumed to be reinnervated.

Chapter 5

DISCUSSION

The Effects of CL

These results show that in addition to the intramyelinic vacuolation and demyelination previously reported, CL also caused distal motor axonopathy and necrosis of dorsal root ganglion cells. These are entirely new observations. The intramyelinic vacuolation affected only the central but not the peripheral nervous system; and it occurred in young developing mice and not in the older mature mice. This indicates that the different components of myelin in the central and peripheral nervous systems and also the differences in the composition of myelin at different ages may play an important role in the formation of intramyelinic vacuoles.

Previous Animal Experiments in which Cycloleucine was used to produce Subacute Combined Degeneration of the Cord

CL was first used to produce an experimental model for subacute combined degeneration of the cord in mice by Jacobson, Gandy and Sidman (1973) and Jacobson and Gandy (1979). After a single injection of CL (8-40mg per adult mouse), the mice became less active and then "impaired proprioception" was noted, later the gait became ataxic. It was thought that motor strength decreased but "without paralysis" and that CL mainly affected the sensory nerves and dorsal columns of the spinal cord rather than the motor nerves.

This interpretation by Jacobson et al. is very different from

what was found in this experiment which showed a distal, predominantly motor, axonopathy with severe degeneration of axonal terminals in the neuromuscular junctions with preservation of nerve endings in the skin of feet and nerve fibres in the muscle spindles. Jacobson et al. considered that the histopathology of CNS of CL-treated mice was very similar to that found in human subacute combined degeneration and interpreted the degeneration of the myelin sheaths of thickly myelinated fibres in the spinal cord as being due to swelling of axons. No studies of peripheral nerves and muscles were done by Jacobson et al. No ultrastructural studies were reported. It seems very likely that the intramyelinic vacuolation which was seen in the present experiments also affected the animals in Jacobson et al's experiments and that their conclusions were incorrect.

Nixon (1974) found that in CL-treated mice significant protein loss was noted, particularly that of cerebellum and cervical spinal cord following 2 mg/g of CL. The sulfatide content of spinal cord and peripheral nerves was reduced but that of brain was normal. The sphingomyelin level was increased. Primary degeneration of Purkinje cell axons in the cerebellum, rostral spinal cord tracts and sciatic nerve was demonstrated by electron microscopy but no vacuolation of myelin was seen. Nixon's observations may have been the result of his using mature adult animals. On the present experiments no degeneration of Purkinje cells or their axons were found.

Ramsey and Fischer (1978) demonstrated the splitting of myelin sheaths in only one of three sets of developing rats administered CL with electron microscopy. They suggested that the age at which CL was given may be an important factor in the formation of the myelin vacuoles. They also found a decrease in ethanolamine phospholipid levels, especially ethanolamine plasmalogen, and a relative increase in the saturated content of

ethanolamine phospholipid. The pathogenesis of the splitting of myelin sheaths was still unknown.

Small, Carnegie and Anderson (1981) injected a large dose of CL into chicks and found clear vacuoles in the myelin sheaths in the brain; they also reported that CL caused a 50% decrease of myelin basic protein in the brain and a 50% inhibition of methylation of arginine in the myelin protein. They suggested that the myelopathy associated with vitamin B12 deficiency was caused by the decrease of SAM and inhibition of methylation of MBP.

Until now, detailed systematic descriptions of the lesions in the central and peripheral nervous system by electron microscopy have been lacking.

Methods used in this study

The serial block method and multiple sections of fore limbs and hind limbs of the mouse provides a very useful way to obtain a general overview of the neuropathology caused by a neurotoxin. The silver impregnation by Glees method is a good and reproducible method for the examination of the axons in the central and peripheral nervous system. In particular the terminal axons and their arborizations in the neuromuscular junctions can be well visualized. In this experiment, this method clearly demonstrated the distribution of the effects of CL in the nervous system. The motor fibres innervating extrafusal fibres were clearly affected in distal muscles with sparing of the nerve fibres in proximal muscles such as facial, truncal, forelimbs and thigh. It also showed the normal appearance of nerve endings in the skin, including the skin of hind feet.

The paraffin sections only show the very apparent and severe

changes in the nervous system but they are unable to show the early or detailed pathology, especially the changes in the neuromuscular junctions and the myelin sheaths. Electron microscopy has enabled us to study the degeneration of neuromuscular junctions and the axonal lesions as well as myelin changes which are not able to be seen clearly in paraffin sections.

The difficulty of the fixation of CNS myelin has been shown in the electron micrographs. Even in the mice with very nicely perfused and fixed tissues the separation of myelin lamellae was still present, although the degree is very different from the myelinic vacuolation caused by CL. The difficulty of fixation does pose some problems in the interpretation of subtle lesions in the CNS particularly during the recovery phase in the CL-VL experiment.

There was also considerable variation in the degree of toxicity in mice affected by CL. Some of young mice died very soon (within 2-3 days after CL injection) but some of them survived up to 7 days in better condition than those died earlier. The effects of CL in the adult mice were not so severe and rapid as in the young mice. The intramyelinic vacuolation developed in the CNS has been shown to vary from one mouse to another even in the same age group at the same length of time after injection of CL and also varied from area to area in the CNS. Usually the white matter in the cerebellar folia, basal ganglia and pons were more severely affected than in the spinal cord. And in each level of the spinal cord, the white matter in the dorsal columns seemed to be affected more severely than it in the anterior and lateral columns. It was thus necessary to examine many blocks from each mouse but this increased the amount of work needed and in some cases it was just not possible to be as thorough as I would have liked.

Perhaps this is the place in which to say that all the electron microscopic work - perfusion, processing of blocks, embedding, trimming, cutting and staining of 1μm and ultrathin sections as well as the photographic work - was done by myself.

Pathogenesis of Myelinic Vacuolation:

Two hypotheses have been proposed to explain how the effects of CL on the myelin sheaths. One was by Jacobson et al (1973). They thought that CL was a competitive inhibitor of S-adenosylmethionine transferase, which converted methionine to S-adenosylmethionine (SAM), a donor of methyl groups for numerous transmethylation reactions. Jacobson et al. proposed that CL caused degeneration of myelin by inhibiting the methylation of phosphatidylethanolamine leading to a decline of phosphatidylcholine levels which is an essential component of myelin. The other hypothesis, proposed by Small and Carnegie (1981), was that transmethylation of the arginine 107 residue in myelin basic protein is essential in maintaining the compaction of the myelin sheath. The inhibition of methylation of myelin basic protein (MBP) might be responsible for the splitting of myelin sheaths caused by CL.

Myelin Basic Protein:

MBP is a very hydrophobic protein and is known to be situated in the cytoplasmic interface of bilayers of myelin lamellae. MBP has been proposed as the "structural cement" in myelin formation and stabilisation. As MBP has been shown to cross-link lipid vesicles, it may stabilise the compaction of myelin by cross-linking the lamellae in a similar fashion. MBP is methylated at a single arginine residue by the enzyme, protein methylase 1, using SAM as substrate producing three forms of MBP, unmethylated, with monomethylarginine and

(*) The investigations of immunocytochemical localization of MBP were reviewed by Sternberger (1984) who concluded that the localization of MBP was more likely to be in major dense lines rather than in intraperiod lines. By using pre-embedding immunostaining techniques, Omlin et al. (1982) have shown that MBP was localized in the major dense line regions of well preserved central and peripheral myelin as well as in the cytoplasmic faces of oligodendroglial and Schwann cell membrane. This localization supported previous studies by Poduslo and Braun (1975) who used biochemical method and Kirschner and Ganser (1980) who used X-ray diffraction techniques.

It has been shown that CL inhibited methylation of the arginine residue in MBP in mice (Crang and Jacobson, 1980), rats (Deacon et al. 1986) and chickens with a 50 % decrease of content of MBP (Small et al. 1981). The splitting of CNS myelin was at intraperiod lines in CL-treated animals in the study by Small et al. (1981) and in the present experiments, but in the mutant mouse 'shiverer', which is known to lack MBP, the splitting is at major dense lines (Kirschner and Ganser, 1980). How the inhibition of methylation of the arginine residue in MBP leads to myelin vacuolation will need further investigation.

with dimethylarginine (Baldwin and Carnegie, 1971). Methylated arginine might play a role in stabilising the conformation of MBP by cross-chain reactions, since methylated arginine was more hydrophobic than arginine itself (Brostoff and Eylar, 1971). CL strongly depressed methyl incorporation into methylarginine in vivo (Small et al. 1981), and caused the inhibition of methylation of MBP thus leading to the splitting of myelin sheaths.

MBP in the CNS and PNS:

MBP is known to be a major protein (30-40% of total myelin protein) of CNS and only a minor component (about 18% of total myelin protein) of peripheral nerves. In the myelin of PNS, MBP is thought to be not so important in maintaining the compaction of myelin as in the CNS. The studies of the MBP in the CNS and PNS of mutant shiverer mouse showed that MBP may have different role in the PNS and CNS. In shiverer mouse MBP, which comprises 18% of the total protein in normal mouse PNS myelin, is missing but normal multilamellar myelin still exists in the PNS (Kirschner and Ganser, 1980; Ganser and Kirschner, 1980; Waehneldt and Linington 1980). Apparently, in PNS myelin, the MBP is not required for maintaining the compactness of myelin lamellae. In the shiverer CNS, on the other hand, which also lacks MBP, it may in fact be required for the formation of normal, compact myelin. The small amount of myelin that does form in the shiverer CNS is not compact at the cytoplasmic apposition. Neither the whole white matter nor the isolated myelin-like material has MBP in the shiverer.
(*)

MBP in Developing and Adult Mice:

The levels of MBP in whole brain increases as the rate of myelination increases (Benjamins, 1984). Analysis of the rates of

synthesis of the 14K and 18.5K MBP in mouse brain between 14 and 39 days (Campagnoni et al. 1978) showed a peak of synthesis of both proteins at 18 days of age, coincident with the period of maximal myelin deposition.

Des Jardins and Morell (1983) studied the comparison of phosphorylation and methylation of basic protein in vivo during development. They found the rate of methylation of MBP slowed with development and appeared to be proportional to the rate of synthesis of MBP. This suggested that methylation occurred only as MBP was being synthesised and that MBP molecules previously assembled into myelin were no longer available for methylation. This may explain why CL caused massive intramyelinic vacuolation in the CNS white matter in young developing mice but very little in older mature animals.

Mechanism of Subacute Combined Degeneration of the Cord:

Vitamin B12 is a corrin ring containing coenzyme which is required for normal growth and development of the nervous system. There are only two vitamin B12-requiring enzymes which have been identified in mammalian systems. They are methionine synthetase, which regenerates methionine from homocysteine, and methylmalonyl CoA mutase, which converts methylmalonyl CoA to succinyl-CoA. It is widely accepted that the pathogenesis of subacute combined degeneration of the cord is caused by the failure of transfer of methyl groups from methyl-cobalamin to homocysteine for the regeneration of methionine via methionine synthetase (fig. 1.2). To clarify the pathogenesis of the effects of vitamin B12 deficiency, Scott et al. (1981) treated monkeys with nitrous oxide, which is known to inactivate vitamin B12, and produced subacute combined degeneration of the cord. Simultaneous supplementation of

methionine in the diet significantly ameliorated the nitrous oxide-induced lesion in the monkeys. Scott et al. suggested that inability to resynthesise methionine from homocysteine led to subacute combined degeneration of cord and the root cause of subacute combined degeneration was likely to be by the inhibition of vitamin B12 dependent enzyme methionine synthetase not by inhibition of methylmalonyl CoA mutase. Van der Westhuyzen et al. (1981) administered nitrous oxide to fruit bats and produced subacute combined degeneration of the cord. They found supplements to the diet of folate caused acceleration of the neurological impairment, but supplementing the diet with methionine protected the bats from neurological impairment. They suggested that these findings would lend support to the hypothesis that the neurological lesion in vitamin B12 deficiency may be related to the deficiency in the methyl donor S-adenosylmethionine (SAM).

The fact that CL caused pathology similar to that of subacute combined degeneration in monkeys deprived of vitamin B12 (Agamanolis et al., 1978), lends support to the hypothesis that the neurological lesion of vitamin B12 deficiency is caused by deficiency of the methyl donor SAM which results in failure of transmethylation in the nervous system which leads to failure in maintaining the compactness of myelin sheaths.

Biochemical Studies of Cycloleucine in Mice:

In a parallel biochemical study by Dr Robert Surtees (Institute of Child Health) thirty 21 day old mice were used to assess the effect of CL on the concentrations of methionine and S-adenosylmethionine in brain from the day of injection to 6 days post-injection.

The brain levels of S-adenosylmethionine fell to about 60% within

one day and to about 50% of normal at 6 days. The brain levels of methionine underwent a five-fold increase within one day and 10-fold increase by 3 days. These results show that CL is a very potent inhibitor of methionine adenosyltransferase which leads to a significant fall of SAM levels in the CNS.

Other Causes of Myelinic Vacuolation:

The exact mechanism of separation of myelin lamellae is still unknown. The conditions which are known to cause splitting of myelin sheath in the central and peripheral nervous system were reviewed by Agamanolis et al.(1978). Compounds which are well-known to produce vacuolation in the CNS, include hexachlorophene (HCP), triethyl tin (TET), isoniazid (INH) and cuprizone.

Hexachlorophene: HCP is an antiseptic drug which had been widely used for sterilising the skin and has been reported to cause spongiform changes in the brain stem in premature infants who died after receiving multiple washings with a solution containing 3% HCP (Powell et al, 1973). HCP was noted to cause intramyelinic vacuolation in the peripheral nerves as well as in the CNS (Twofighi et al., 1973; Powell and Lampert, 1975). Powell et al. (1978) used a micropressure transducer developed for recording pressure in the microcirculation to measure the endoneurial pressure and found increased endoneurial pressure in the peripheral nerves in rats fed HCP and thus suggested that axonal degeneration in HCP neuropathy was caused by increased endoneurial pressure. The mechanism of intramyelinic vacuolation caused by HCP is still unclear.

Triethyl tin: TET is known to cause symptoms and signs of increased intracranial pressure such as severe and persistent

headache, photophobia, vomiting and drowsiness in patients treated with Stalinon in which TET diiodide was found in concentrations of up to 10%. The process by which myelin vacuoles form remain obscure. Torack, Gordon and Prokop (1970) found astrocyte swelling and suggested that this is possibly an intermediate step in the movement of fluid into the myelin sheath. In animal experiments using the rat, Jacobs et al. (1977) found that within 1-2 hours of intravenous injection of TET intramyelinic vacuoles appeared in the CNS white matter with no accompanying or proceeding changes in astrocytes or oligodendrocytes or extracellular space and they suggested that TET acted directedly on the membrane of the oligodendroglia myelin. No effects were found in peripheral myelin. TET is known to have a high affinity binding constant for myelin and this may explain the selective localisation of vacuoles (Lock and Aldridge, 1975).

Isoniazid (INH): INH is known to interfere with essential phosphorylation of vitamin B6 by inhibiting pyridoxal phosphate kinase and causes peripheral neuropathy in patients taking the drug (Cavanagh, 1973). Blakemore, Palmer and Noel (1972) administered INH to dogs and caused vacuolated lesions in the white matter. The vacuoles were shown by electron microscopy to arise at the separation of the intraperiod line of the myelin sheath in addition to some distension of the periaxonal space and swellings of the inner tongue of oligodendroglial cytoplasm and focal swellings of axons. Blakemore (1980) summarized the findings in the CNS in experimental INH intoxication and noted that intramyelinic vacuolation developed in chicks, ducklings and dogs.

In a number of post mortem studies of INH neuropathy axonal degeneration in peripheral nerves with associated atrophy has been described (see Jacobs and Le Quesne, 1984). Changes in the motor

end-plates and axonal degeneration of intramuscular nerve fibres and collateral sprouting as well as axonal degeneration of in the dorsal columns in the spinal cord have also been noted. Distal axonal degeneration, affecting motor fibres more severely than sensory, was found by Cavanagh (1967) and confirmed by Jacobs et al. (1979) in rats fed with INH. These appearances bear some similarities to the pathology caused by CL in the present experiment which showed intramyelinic vacuolation as well as distal, predominantly motor, axonal degeneration.

Cuprizone: Cuprizone caused intramyelinic vacuolation with separation at intraperiod lines (Suzuki and Kikkawa 1969). Blakemore (1972) considered that cuprizone acted directly upon myelin-forming oligodendrocytes since degenerate oligodendrocytes were found in cuprizone-treated mice at all stages of intoxication. Love (1988) also observed that cuprizone induced intramyelinic vacuolation but not demyelination in rats. He also found that cuprizone caused a distal peripheral axonopathy with degeneration of myelinated axons in the sciatic nerve but preservation of the spinal nerve roots, dorsal root ganglia, posterior spinal funiculi and anterior horn cells.

CL caused intramyelinic vacuolation only in the CNS myelin with no evidence of degeneration of oligodendroglia, and also a severe distal axonal, predominantly motor, neuropathy.

Mechanism of Degeneration at Neuromuscular Junctions and Peripheral Nerves:

The cause for the distal axonal, predominantly motor, degeneration caused by CL is enigmatic. The following hypotheses may be considered in relation to this observation:

(1) Dying Back Process: Distal axonopathy ('dying back') was thought to result from impaired metabolism of the perikaryon of the nerve cell (Cavanagh, 1964), Cavanagh (1979) classified distal axonal neuropathy into 3 groups; Group 1 neuropathies: Energy-dependent axonal neuropathy: This includes neuropathies caused by thiamine deficiency, alcohol, nitrofuran drugs, arsenic, thallium, tropical neuropathy, riboflavin deficiency, dinitro-o-cresol, tetraethyl thiuram disulfide and Friedreich's ataxia. The mechanisms of Group 1 neuropathies are thought to be, from a metabolic standpoint, caused by a defect of energy-producing pyruvate metabolism. The mechanism of chronic thiamine deficiency neuropathy is generally accepted as due to a defect in pyruvate decarboxylation that requires thiamine-pyrophosphate as its first step and thus leads to a diminution of the source of energy derived from the activities of the tricarboxylic acid cycle. Nitrofuran drugs are thought to competitively inhibit pyruvate breakdown and reduce available cellular energy. Arsenic is known to inhibit the disulfide cofactor, lipoic acid, required in the second step of pyruvate transformation. Thallium is known to be able to combine with riboflavin and causes reduction in the availability of flavoproteins that are required in the third step of pyruvate metabolism. Group 2 neuropathies: Pyridoxine dependent axonal neuropathy: This includes isoniazid, hydralazine, ethionamide and porphyria. The mechanisms of Group 2 neuropathies are thought to be due to a defect of pyridoxal phosphate metabolism. The pattern of degeneration of this group was distal (sensory greater than motor) in man with INH intoxication with the milder lesions; in more severely INH-poisoned animals, the degeneration reached more proximally in motor fibres than was ever encountered in the group 1 intoxications. Group 3 neuropathies: In this group the long spinal pathways also showed degeneration as well as peripheral nerve. There are two subgroups: (a) filamentous accumulations: This includes n-hexane, methyl butyl ketone, 2,5-hexane dione, carbon disulfide, acrylamide,

(b) vacuolo-membranous changes: This includes organophosphates, such as triorthocresyl phosphate(TOCP), p-bromophenylacetylurea and clioquinol.

The axonal neuropathy caused by CL has some similarity to Group 3 (b). Organophophates, which cause an inhibition of cholinesterase, lead to selective damage to large diameter, long fibres in the CNS and PNS. In the early stages, before wallerian-type degeneration, membranous accumulations were found in axons, an abnormality not found in CL-induced axonopathy. SAM is known to be the sole methyl donor in many transmethylation reactions in the nervous system, (Baldessarini 1987) and we know that CL inhibits S-adenosylmethionine transferase and leads to the decline of SAM levels; since SAM is essential for the methylation of membrane lipid (phophatidylcholine) and decreasing microviscosity and normalising cell membrane, failure of methylation of cell membrane will lead to their breakdown, especially in the presynaptic membrane of motor end-plates where there is a high membrane turnover during transmitter release (Heuser and Reese, 1973). The failure of methylation of RNA, neurotransmitter and choline may also play a role in the distal axonopathy and dorsal root ganglion cell degeneration.

Although the dying back hypothesis may be true for some human conditions, there are some in which the evidence that axons do not 'die back' from their ends, but develop focal and multifocal damage tending to occur at distal ends of axons which could also produce this change. Spencer and Schaumburg (1976, 1977) found that in hexacarbon (n-hexane, methyl n-butyl ketone and 2,5-hexanedione) neuropathy axonal swelling occurred distally, but multifocally, first appearing in large fibres which supply the calf muscles and later involving the longer plantar nerves. The filament-containing swellings tend to develop in the paranodal regions on the

proximal side of nodes of Ranvier. Bouldin and Cavanagh (1979a,b) studied the neuropathy caused by di-isopropylflurophosphonate(DFP) in cats and found that in teased-fibre preparation from the left recurrent laryngeal nerve was initially focal and nonterminal then the axonal degeneration subsequently spread in a somatofugal direction to involve the entire distal axon. Nerve fibre varicosities and paranodal demyelination preceded the axonal degeneration. The varicosities were associated ultrastructurally with intra-axonal and/or intramyelinic vacuoles and sometimes with collection of smooth endoplasmic reticulum. These studies reveal that the hypothesis that dying back neuropathies evolve from a retrograde axonal degeneration is probably not valid for hexacarbon and organophosphorous neuropathy.

In the present experiments the earliest pathology in peripheral nerves occurred within 24 hours and affected only the axonal terminals and preterminal axons. No abnormalities were seen in motor or sensory roots or major peripheral nerves. Later there was more severe and extensive degeneration of distal motor nerves and only at 2 - 3 days were degenerated fibres found in the tibial and sciatic nerves. By that time degenerative changes were present in sensory ganglion cells and some of the peripheral nerve fibre degeneration may have been related to this even though no actual degeneration of sensory innervation in skin and muscle spindles was found. Whether anterior horn cells in the spinal cord were abnormal within 24 hours is not certain and later in the experiment changes in these cells may have been part of a secondary phenomenon (the axonal reaction) due to the peripheral lesions. Therefore there is some doubt about whether this peripheral lesion is secondary to an abnormality of motor neuronal metabolism. Evidence points against this 'dying back' hypothesis being applicable to CL. Methylation phenomena and the effects of SAM deficiency may be more relevant to motor nerve terminals than to the perikaryon. However, why the

longest motor nerve fibres are preferentially affected is not at all clear. Physiological properties of these motor neurons with very long axons including the degree of their functional activity such as the rate of transmitter release are important. This is an area for further research.

(2) Lack of Blood-Nerve-Barrier: Blood-Nerve-Barrier is known to exist in the endoneurium of peripheral nerves but not at the neuromuscular junctions and dorsal root ganglia (Jacobs, 1980). CL causes distal axonal, predominantly motor, degeneration as well as necrosis of dorsal root ganglia cells, but not in the motor or sensory nerve endings in the muscle spindles. The spindle may be protected by its capsule which is continuous with, and embryologically derived from, the perineurium of the nerve supplying the spindle (Landon 1972). This may imply a lack of protection of Blood-Nerve-Barrier in neuromuscular junctions of extrafusal muscle fibres and dorsal root ganglia, which are known to be vulnerable to the effects of neurotoxins. However, it would be very difficult to explain why the CL affected the motor nerve terminals of distal muscles more than the proximal. It seems very unlikely that the axonal pathology is related to the presence or absence of barriers.

(3) Interruption of Axonal Transport: It is firmly established that there are well-organized transport mechanisms within axons capable of moving material both toward and away from the cell body, concurrently and at a variety of rates (see Thomas, Landon and King, 1984). In order to investigate the pathogenesis of selective axonal lesions caused by acrylamide and triorthocresyl phosphate, Pleasure, Mishler and Engel (1969) injected tritiated L-leucine intraperitoneally into cats and observed that axoplasmic flow of proteins was interrupted in cats with neuropathy induced by acrylamide, but not interrupted in normal cats and in those with neuropathy induced by

triorthocresyl phosphate.

Whether or not CL caused distal axonal degeneration as a result of the interruption of axonal transport will need further investigation. The use of radioisotopic tracers may help to clarify the pathogenesis.

Mechanism of the Degeneration of Dorsal Root Ganglion Cells:

The degeneration of DRG cells is an entirely new finding. It may partly account for the axonal degeneration in fibres in the spinal white matter which was especially numerous in the f. gracilis and to a lesser extent in other areas. The mechanism is still unclear. The putative explanations are as follows:

Dorsal root ganglia, as well as neuromuscular junctions, are known to lack a blood-nerve barrier and may be more vulnerable to the effects of toxins such as CL than other components of nervous system (Jacobs, 1980). The present experiments showed that DRG cells became vacuolated and necrotic within 3 days after the administration of CL. This happened much later than the degeneration of axonal terminals in the neuromuscular junctions.

Another possible explanation is that CL has also been shown to be a selective inhibitor of nucleic acid methylation in Chinese hamster ovary cells, an effect that is directly mediated through its ability to block SAM synthesis. This leads to a severe impairment in the global efficiency of ribosomal RNA maturation and influences the rate of cell growth. This may contribute to the degeneration of DRG cells which are not able to maintain the metabolism of cell bodies and leads to degeneration of distal sensory fibres in the posterior columns in the spinal white matter. However,if this is true, the reason why CL selectively affects DRG cells but not other nerve cells such as motor

neurons in the anterior columns of spinal cord, neurons in brain cortex and Purkinje cells is unknown. Furthermore the distal sensory innervation of receptors and skin seems to be unaffected. At present therefore there are no completely satisfactory explanations for the ganglion cell necrosis.

The Effects of Valine on the Cycloleucine-Treated Mice:

CL is known to be non-metabolisable and excreted very slowly by the kidney of the mouse due to the reabsorption of CL in the proximal renal tubules (Christensen and Jones, 1962). This accounts for the long biological half-life of CL. Ruelius et al.(1973) gave several large doses of VL 2 mg/g intraperitoneally each hour for 7 hours immediately after administration of CL 2 mg/g or VL 2 mg/g at 0, 24, 48 hours after administration of CL 0.4 mg/g and showed an increase of CL excretion with a concomitant decrease in the concentration of CL in the blood and tissues. The precise mechanism by which valine increases renal excretion of CL has not been determined, but Ruelius et al. suggested that the most plausible reason for their observation was based on a CL-VL interaction in the kidney tubules. Their interpretation took into account of the work of Christensen and Jones (1962) as well as Holtzapple et al. (1970). Christensen and Jones found that CL is excreted in the glomerular filtrate and reabsorbed in the proximal tubules, presumably by transport sites operative for certain neutral amino acids. Holtzapple et al. demonstrated that CL inhibited the uptake of valine and other amino acids. High concentration of VL would be expected to inhibit the uptake of CL if these two amino acids interacted at a common site in the kidney. In the studies of Ruelius et al. they found that in reversing the toxicity of CL, L-valine appeared to be the most effective antagonist of CL, followed by L-isoleucine and L-leucine. Basic amino acids were found to have no effect on the excretion of CL under their experimental

conditions.

Nixon (1974) also observed that some large neutral amino acids i.e. valine, leucine, isoleucine, methionine and phenylalanine caused a reversal of the toxicity of CL. The mechanism by which they acted was thought to be by inhibiting renal reabsorption of CL thus increasing its excretion. Nixon found that VL was the most effective amino acid in increasing the excretion of CL by more than a thousand fold. Most of CL affected mice recovered after the administration of massive doses of VL, but no morphological studies were done by Nixon.

Since the half-life of CL is known to be more than 21 days and its neurotoxic action is therefore very prolonged, VL can be used to cut short this persistent action, reverse the neurotoxicity of CL and thus allow tissues to recover rapidly. The present experiments have shown that CL-affected mice recovered rapidly after VL administration and only some slight weakness of the hindlimbs remained. The intramyelinic vacuolation was reabsorbed leaving little evidence of residual pathology and the peripheral motor nerve fibres regenerated and reinnervated muscles.

Disappearance of Myelinic Vacuolation: It is reasonable to think that in experiment 2, after massive doses of VL to the CL treated mice, the S-adenosylmethionine transferase was not inhibited any more and resulted in normalisation of methylation reaction in the nervous system, especially the methylation of arginine in MBP. The exact mechanisms by which the vacuolation develops and then disappears are not known with certainty, nor is the content of the vacuoles known. The intraperiod line is derived from the external membrane of the myelin sheath and the space formed by the vacuoles should be in continuity with the extracellular space in the CNS. Probably when

the vacuoles disappear their watery content is pushed out again into the extracellular space. Thus the compactness of myelin sheaths became restored with the removal of water from the intramyelinic spaces.

Regeneration of Peripheral Nerve: It is well-known that the peripheral nerves are capable of regeneration after crush injury or transection. The regeneration can be influenced by irradiation (Love 1983) or distal environment such as distal stump (Scaravilli 1984a and b). The mechanism of the regeneration of peripheral nervous system has recently been reviewed by Thomas (1989) and Hall (1989). Thomas concluded that satisfactory nerve regeneration depends on a highly complex cooperation between different cell types. This includes the endoneurial/perineurial fibroblasts which are probably necessary to produce an extracellular matrix conducive to colonization of Schwann cells and invasion by regenerating axons. In nerve regeneration chambers the initial fibrin/fibronectin matrix is haematogenous. This is thus invaded by fibroblasts and blood vessels before it is crossed by regenerating axons and Schwann cells. Hall (1989) thought that mammalian peripheral nerve fibres can regenerate after injury and are most likely to succeed if axons are simply crushed or have only very short (<0.5cm) interstump gap to cross. Reactive axonal sprouting appears to be an intrinsic neuronal response to injury. The subsequent organization of the axonal sprouts, in particular their orderly outgrowth in minifascicles toward a distal stump does not occur unless Schwann cells are present. During the injury response, Schwann cells proliferate and co-migrate with regrowing axons and respond to axonal cues by transient upregulation or re-expression of molecules which provide a favourable substrate for axonal extension; and attract bundles of regrowing axons and their associated Schwann cells across interstump gap up to 1 cm in length. Recruited macrophages remove myelin debris from Schwann cell tubes and

probably interact with Schwann cells in other ways during the injury response.e.g. by presenting mitogens and cytokines.

The evidence of the regeneration of peripheral nerves in CL mice after the administration of VL was found within 7 days after the start of VL. There were numerous axonal sprouts and some un- or thinly myelinated fires noted in the intramuscular nerve fibres. Within 14 days after the start of VL, there were already many more well-developed and myelinated fibres in the intramuscular nerve fibres. The rapidity of the regeneration of peripheral nerves could be partly due to the short length of the degenerated fibres affected by CL in two days and also by the preservation of the Schwann cell tubes and extracellular connective tissue matrix formed by endoneurial/perineurial fibroblasts.

Reinnervation of Denervated Neuromuscular Junctions: Bennett, McLachlan and Taylor (1973) found that regenerating axons in the adult mammalian muscle preferentially reinnervated the old end-plate region. The growth of regenerating axons was not oriented towards the end-plate zone but followed muscle fibres and blood vessels in random directions. Saito and Zacks (1969) found that after a sciatic nerve lesion in mid-thigh in the mouse axons were absent by the end of the first week with persistent postsynaptic folds. The primary synaptic clefts became shallow and the secondary synaptic clefts shorter and wider. The earliest returning axonal sprouts were first observed in foot muscles at three weeks and became numerous by four weeks in Saito and Zacks experiment and by 15 to 30 weeks they found that all neuromuscular junctions studied had a normal fine structure. They also suggested that the reinnervation of denervated neuromuscular junctions took place by return of axons to old junctional areas rather than by new junction formation. Sanes, Marshall and McMahan (1978) demonstrated that the basal lamina of

the end-plate play a decisive role in attracting regenerating nerve fibres to old synaptic site.

In CL treated mice, within 7 days after the start of VL administration, there were already many axonal sprouts and some thinly myelinated nerve fibres seen in the foot muscles. There were also some tiny axonal sprouts with electron lucent axoplasm containing a few mitochondria, neurofilaments and vesicles. Axons were noted to lie in close apposition to the residual, flattened postsynaptic fold of previously denervated motor-end-plates. This regeneration happened earlier than the study by Saito and Zacks (1969) and is comparable to the studies by Duchen, Gomez and Queiroz (1979, 1980) who found rapid regeneration of axons to reinnervate denervated neuromuscular junctions, the restoration of normal function within a few days after the local injection of Latrodectus spider venoms. This venom caused degeneration only of the axonal terminals. The rapid regeneration of peripheral nerves and reinnervation of neuromuscular junctions may be attributed to the integrity of the Schwann cell tubes and their lamina which were apparently not damaged by CL and the short distances involved.

Electrophysiological Studies of the Effects of Cycloleucine on the Neuromuscular Junction of Mice:

A parallel electrophysiological study has been undertaken by J. Edwards in our laboratory. The preliminary results showed that in 21 days old mice at 24 hours approximately 85% of muscle fibres in Soleus and EDL failed to show end-plate potential (EPP) compared with 5% in control muscle.

In the later stages of intoxication (more than 5 days) the characteristics of denervation, such as fibrillation potentials and a

decrease muscle resting membrane potential appeared.

In the adult mice similar results have been found; however, the adult mice are more resistent, at 24 hours 50% of fibres were found to show EPP.

At 24 hours, in both young and adult mice high frequencies of miniature end-plate potentials were recorded at many end-plates. These were 5 times more than control values.

Conclusions

The mechanism of toxicity of CL is most likely due to its inhibition of S-adenosyl methionine transferase, and as a result, of inhibition of transmethylation processes. The vacuolation in CNS white matter, its occurrence in the immature, but not in the adult, animal is a strong indication that the methylation of myelin basic protein is impaired and the vacuoles result from a loss of hydrophobicity of the MBP. The exact source of the contents of the vacuoles is not known with certainty.

The experiments described in this thesis also showed that CL causes a distal motor axonopathy and degeneration of sensory ganglion cells, in addition to the myelinic vacuolation. These changes have not been previously observed. The exact mechanisms for production of these lesions are not clear. The axonal degeneration clearly begins in the motor nerve terminals and most distal, preterminal, axons. The terminals are sites of great metabolic activity and membrane turnover and may be affected primarily because of these functions occurring as part of neuromuscular transmission. The motor neuron in the spinal cord does not degenerate and, as shown in the valine experiments, retains its capacity to regenerate when the toxicity of CL is removed. Exactly why the degeneration of motor nerve terminals occurs first at the ends of the longest nerve fibres is not clear and is a subject for further research. One of the questions that were considered was whether CL might also affect other cholinergic nerve terminals. The cholinergic terminals of preganglion sympathetic nerve fibres should be investigated with this in mind. In a very recent and limited experiment, the cervical sympathetic (middle and lower) ganglia were examined in toluidine blue and EM preparations in a 21 days old mouse 5 days after CL. No ganglion cell necrosis or degeneration of nerve terminals were seen, but

because of the limited nature of the experiment no firm conclusions could be drawn. This is an area in which research could be continued.

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