Nervous-system responses of rats to subchronic inhalation of n-hexane and n-hexane + methyl-ethyl-ketone mixtures

Altenkirch, H.[+)], Wagner, H. M.[++)], Stoltenburg, G.[+++)], Spencer, P. S.[++++)]

+) Department of Neurology, Klinikum Steglitz, FU Berlin, West Germany

++) Department of Biochemistry of Air Pollution, Bundesgesundheitsamt, Berlin, West Germany

+++) Department of Neuropathology, Klinikum Steglitz, FU Berlin, West Germany

++++) Institute of Neurotoxicology, Albert Einstein College of Medicine, Bronx, New York, USA

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7. Author(s) Altenkirch, H., H. M. Wagner, G. Stoltenburg, and P. S. Spencer

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16. Abstract (Limit: 200 words)

The nervous system effects of continuous inhalation exposures to concentrations of n-hexane (110543) (HEX) or HEX plus methyl-ethyl-ketone (78933) (MEK) mixtures were investigated in male Wistar-rats. Long term effects of intermittent exposures to the mixtures were also examined. In experiment-A, the animals were exposed for 24 hours/day, 7 days/week for 9 weeks to 500 parts per million (ppm) HEX, 700ppm HEX, 300ppm HEX plus 200ppm MEK, 400ppm HEX plus 100ppm MEK, 500ppm HEX plus 200ppm MEK, or hydrocarbon filtered air (HFA). In experiment-B the animals were exposed 8 hours/day, 7 days/week, for 40 weeks to 700ppm HEX, 500ppm HEX plus 200ppm MEK, or HFA. Solvent exposed animals from experiment-A showed marked reductions in weight. Those treated with the mixture of compounds demonstrated excessive hypersalivation, frequent orange/red discoloration of the skin and fur, and narcosis. All solvent exposed animals demonstrated limb weakness commencing by evasion of the hindlimbs and resulting in paralysis. Histological examination showed hexacarbon specific axonal lesions in peripheral nerves, particularly tibial branches to calf muscles, and in the gracile tract at cervical levels of the spinal cord. Those who survived experiment-B did not give evidence of narcosis and no depressed body weights were evident. Fur discoloration was restricted to an orange/red collar around the neck and appeared more often in animals exposed to the mixture than to hexane alone. The author recommends that exposure to the combination of HEX and MEK be avoided and that appropriate warning accompany these products.

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Introduction:

A high potential risk of a neurotoxic disorder may be involved in dealing with organic solvent mixtures containing n-hexane, methyl-n-butyl-ketone or other solvents that potentiate their effects. Such solvents have been widely used both in industry and in the household.

Both reversible and irreversible neurological syndromes have occurred in industrial workers exposed to solvent mixtures containing n-hexane (Yamamura, 1969; Herskowitz, 1971; Rizzuto, 1979; Spencer, 1980). Neurological damage involves the peripheral nervous system and, to a lesser extent, the central nervous system. Toxic neuropathies with a very severe clinical course and, in some cases, neuromyelopathies, have occurred in juveniles who repeatedly inhaled hexane-containing solvents as narcotics (Oh, 1976; Altenkirch, 1977; Goto, 1974; Korobkin, 1975). An outbreak of toxic neuropathy among Berlin sniffers correlated temporally with the addition of 11 % methyl-ethyl-ketone (MEK) to a hexane-containing solvent mixture with concomitant reduction of the n-hexane from 31 % to 16 %. No further neuropathies occurred after the removal of MEK from the solvent mixtures, although the sniffing abuse of the hexane-containing fluid has continued up to the present time. These data support the idea that the neurotoxic properties of n-hexane are considerably potentiated by methyl-ethyl-ketone (Altenkirch, 1977).

Experimental studies with laboratory rats have shown that chronic exposure to n-hexane produces a peripheral neuropathy characterized by distal retrograde axonal degeneration in the spinal cord tracts and peripheral nerves (Schaumburg, 1976; Spencer, 1980). Methyl-ethyl-ketone, a compound unable to produce an experimental neuropathy, nevertheless potentiates the neurotoxic (neuropathy-inducing) properties of n-hexane when laboratory animals

are simultaneously exposed to mixtures of the two solvents at high concentrations (Altenkirch, 1978). Organotypic tissue cultures have also demonstrated that MEK can induce hexacarbon-specific pathological alterations in cultures treated with sub-neurotoxic doses of n-hexane and speed the onset of pathology in cultures treated with neurotoxic doses (Veronesi, Lington, Spencer, 1981).

The present study deals with two problems:

1. The effects on the nervous system of rats continuously exposed by inhalation to concentrations of n-hexane/MEK mixtures roughly corresponding to threshold limit values under occupational conditions.
2. The long-term effects of intermittent exposure to n-hexane/MEK mixtures.

Material and methods:

Male Wistar rats (Hannover strain), aged 4 months and initially weighing 300 - 400 g, were used for this study. Animals were kept in plastic chambers made of polymethacrylic acid esters, each chamber containing 5 animals. Solvent vapors were generated by perfusor pumps (Braun) which passed the solvent over a glass frit through which an air stream was conducted. Solvent concentrations within the exposure chambers were determined by

a) Draeger indicator tubes for n-hexane, and
b) flame-ionization-detection measurements (Beckman FID).

FID measurements were performed intermittently and the chamber concentration calibrated daily. Chamber solvent concentrations were also calculated from solvent consumtion and air flow per unit time. Ammonia concentration (from decomposition of urine and feces) was reduced by placing blotting paper impregnated with NaH_2PO_4 on the chamber floor.

Animals were fed between exposure periods with Altrumin A containing a standardized vitamin content. Water was provided ad libitum. Body weight was monitored weekly and compared with normal weight tables. Clinical status was evaluated two to three times per week by examination of:

a) general condition,
b) motor performance and
c) hindlimb toe-spreading reflex.

In set A of the experiments, the mode of exposure was 24 hours a day, 7 days a week up to a total period of 9 weeks. The actual exposure time amounted to about 22 hours per day, since the animals had to be taken out of the chambers for feeding, cleaning and examination. Groups of animals were exposed to the following nominal solvent concentrations:

500 ppm n-hexane
700 ppm n-hexane
500 ppm n-hexane + 200 ppm methyl-ethyl-ketone
400 ppm n-hexane + 100 ppm methyl-ethyl-ketone
500 ppm n-hexane + 200 ppm methyl-ethyl-ketone.

A further group of five animals was kept under the same conditions but without solvent exposure. They were housed in a chamber and received hydrocarbon-filtered air.

After conclusion of the experiments in set A, the experiments in set B were performed. In this case, the mode of exposure was 8 hours per day, 7 days per week up to a period of 40 weeks and 1 1/2 days. Two groups of animals were exposed to 700 ppm n-hexane or 500 ppm n-hexane plus 200 ppm MEK. Again, a third group received hydrocarbon-filtered air.

After the treatment period, the animals were anesthetized with sodium barbitone and perfused through the heart for 10 minutes with 5 % glutaraldehyde solution in phosphate

buffer (pH 7.4). Following perfusion, tissue was removed from certain vulnerable areas of the brain, spinal cord and peripheral nerves. Tissue segments were post-fixed in 1 % osmium tetroxide in phosphate buffer (pH 7.4), dehydrated stepwise and embedded in Araldite. One-micrometer cross and longitudinal epoxy sections were cut from hardened blocks and stained with methylene blue or toluidine blue for examination by bright-field microscopy. At least two sections were examined for each sampled area.

Since age-dependent morphological alterations had to be taken into account because of the long exposure time in set B, a special method was used in the experiment for histopathological assessment. Single-blind evaluation of the tissue sections was conducted by two examiners. The controls consisted of nerve tissue from age-matched and non-age-matched animals. Slides were randomly selected and qualitatively examined with X 40 and X 100 objective lenses. The degree of pathological change was scored by each observer on a scale from 0 (no change) to 5 (maximal change). The scores were averaged, grouped and analyzed after all data had been collected.

Results:

Exposure status:

Excursions in solvent concentration were not detected at any time during the exposure period. Solvent concentrations were maintained to or at the desired levels.

Clinical status:

Set A:

All animals survived the exposure period. The solvent-exposed animals showed a marked reduction in weight compared to control animals. The rats treated with n-hexane/

MEK showed an excessive hypersalivation. Orange-red discoloration of the skin and fur was also seen in all rats with the exception of the control animals. Narcosis was commonly seen.

All solvent-exposed animals developed limb weakness commencing by evasion of the hindlimbs leading to paralysis, and eventuating in quadriplegia. Hindlimb paralysis as shown in Fig. 1, was regarded as the unequivocal functional manifestation of a neuropathy. These clinical signs occurred at different times in the individual groups. A complete hindlimb weakness was exhibited in the ninth week by all animals exposed to 500 ppm n-hexane, and in the eighth week by groups treated with 400 ppm n-hexane and 100 ppm methyl-ethyl-ketone, as well as with 300 ppm hexane and 200 ppm methyl-ethyl-ketone. In contrast, animals exposed to 500 ppm n-hexane and 200 ppm methyl-ethyl-ketone or 700 ppm n-hexane displayed hindlimb paralysis in the fourth week of intoxication (Fig. 2).

Set B:

All animals survived the exposure period. Narcosis was not observed, and body-weight gain was the same in controls and treated groups. Solvent-treated animals showed fur discoloration, which was restricted to an orange-red collar around the neck. This discoloration appeared more marked in animals exposed to n-hexane/MEK-treated mixtures than in the rats exposed to n-hexane alone. Abnormal clinical signs were not detected in any animals.

Neuropathological findings:

Set A:

Light microscopic examination of peripheral nerves revealed the characteristic pattern of scattered multifocal giant axonal swellings. These alterations were mainly localized to the branches of the tibial nerve supplying the calf muscles but also seen in other portions of the ischiatic

nerve (see Fig. 3). Paranodal myelin retraction was evident in the area of the axonal swellings. Breakdown of axons and myelin degradation (myeling ovoids) was visible distal to the axonal swellings (see Fig. 4). Axonal swellings were also observed in the gracile tract of the spinal cord at cervical levels (Fig. 5). All these pathological changes were visible irrespective of the degree of clinical neuropathy. Ultrastructural examination of the axonal swellings revealed intra-axonal accumulations of 10-nm neurofilaments (Fig. 6). Glycogen granules free in the axoplasm or delimited by a single membrane were scattered in affected axons (Fig. 7).

Light-microscope and ultrastructural examination of the parotid and salivary glands showed, particularly in the animals treated with MEK/n-hexane, a fatty degeneration and widening of the glandular ducts, which could be associated with the excessive hypersalivation of the animals.

Set B:

CNS and PNS areas

Special attention was given to/which are specially vulnerable in n-hexane intoxication, i. e. the terminal portion of the gracile tract in the medulla oblongata and cervical cord, the ventro-medial tracts of the lumbar spinal cord, and the tibial nerve at the knee. Pathological changes characterized by scattered nerve-fiber degeneration, mainly in the form of fiber destruction and ovoid formation, were detected variably at these loci both in solvent-exposed and control animals . Axonal swellings were not found in the peripheral nervous system but were apparent in the gracile tract of the spinal cord, especially at the level of the gracile nucleus in medulla oblongata. The younger control animals were largely free of scattered peripheral nerve-fiber degeneration.

Discussion:

Our previous clinical observations and experimental animal studies led to the conclusion that n-hexane, although definitely a neurotoxin with a specific mechanism of action, has a low neurotoxic level which can be potentiated under certain conditions. Such a potentiator is methyl-ethyl-ketone (Altenkirch, 1977). Mixtures of MEK and hexacarbons have been frequently implicated in outbreaks of neuropathy (Table 1).

While the purpose of earlier experiments (Altenkirch, 1978) was to reproduce in the experimental animal model the conditions of very high concentrations occurring in solvent abuse, the present study examines the effects of solvent concentrations which roughly correspond to reported occupational exposures. The results in set A show that n-hexane-induced hindlimb weakness manifests after 9 weeks of continuous exposure to 500 ppm n-hexane, while a complete hindlimb paralysis is already visible after 4 weeks with a mixture of 500 ppm n-hexane and 200 ppm MEK. Paralysis also occurred after 4 weeks of continuous exposure to 700 ppm n-hexane. Thus a clear and unequivocal MEK potentiation of n-hexane neurotoxicity has been demonstrated under these conditions. This statement only applies for the conditions of continuous exposure provided in set A. Though the threshold limit values have been set for an 8-hour working day, this is exceeded in a number of occupational situations in Europe, e. g. in the leather-processing industry in Italy, Spain, Portugal and Austria where working place and household are frequently combined and a more or less continuous exposure has occurred. A large number of the more than 400 cases of occupational neuropathies among Italian shoemakers apparently developed under such conditions. Moreover, affected individuals often sustain additional skin exposure to the solvent mixture which is totally ignored by the concept of the threshold limit value.

Inoue and co-workers assume that a hexane-neuropathy is induced in workers after 3 to 7 months of daily 8-hour exposure to 500 to 1000 ppm. In this connection, the risk in the hobby and household area, where continuous long-term exposure is not uncommon, has so far been totally disregarded.

Pathological changes which typify the onset of subclinical n-hexane neuropathy consist of giant axonal swellings filled with 10-nm neurofilaments appearing concurrently in the gracile tract of the medulla/cervical spinal cord and tibial nerve at the knee (Spencer and Schaumburg, 1977). Evidence that n-hexane has induced the neuropathy is dependent on the identification within individual animals of giant axonal swellings at all of these sites. At later stages, axonal swellings appear more proximally in the affected nerve tracts, so that they are also found in the proximal section of the sciatic nerve as well as in the gracile tract in the thoracic or lumbar region of the spinal cord. Furthermore, animals with clinical signs of a neuropathy also display specific pathological changes in the ventro-medial tract of the spinal cord as well as in the cerebellar vermis and distal optic tracts. The examination described in set B has not evaluated the temporal development of morphological changes in exposed animals and is limited to the assessment of pathological findings at a single time-point. A potential difficulty with this approach is that the nervous system of rats displays an increasing incidence of pathological change with increasing age. These changes are not only similar (upon light-microscopic evaluation) to those which occur in hexacarbon neuropathy, but are also localized in those nerve tracts vulnerable to hexacarbon intoxication. Age-matched animals in the present study displayed scattered nerve-fiber degeneration in the same loci and to a similar degree as in solvent-exposed animals. Age-associated and hexacarbon-

specific changes can be distinguished from each other by four factors:

a) Axonal swellings filled with neurofilaments occur contemporaneously in vulnerable loci of the PNS and CNS in n-hexane neuropathy but not in normal aged animals, where they only appear in the CNS.

b) Late stages of nerve-fiber degeneration in n-hexane neuropathy are always accompanied by earlier phases (e. g. axonal swellings) in both the CNS and PNS.

c) Aged animals show specific pathological changes (not seen in younger animals) in the axons (e. g. metachromatic bodies and other dark-staining material) as well as in the neurons (pigmentation); these occur in aged (but not young) hexacarbon-treated animals (Spencer et al., 1980).

d) Ultrastructurally, hexacarbon-specific axonal swellings are filled with neurofilaments; whereas age-associated swellings contain large numbers of tubulo-vesicular profiles.

Based on these pathological criteria, it can be concluded from the present observations that age changes were prominent in all solvent-exposed animals and that no hexacarbon-specific abnormalities were detected in any of the sampled tissue. This interpretation of the morphological data is substantiated by the absence of abnormal clinical signs and an abnormal rate of body-weight gain. The fur discoloration was the only positive sign which distinguished exposed and control groups. The significance of this observation is unknown, but the results in set B as well as previous studies using high solvent concentrations have demonstrated that fur discoloration, failure to gain weight normally and hypersalivation precede and accompany the development of the neuropathy. The present study also shows that the degree of fur discoloration is more extensive in animals treated with MEK and n-hexane than in animals exposed to n-hexane alone.

It can be said in summary that animals exposed 8 hours a day, 7 days a week for 40 weeks and 1 1/2 days to either 700 ppm n-hexane or 500 ppm n-hexane + 200 ppm MEK showed no neuropathological or clinical signs of neuropathy, whereas animals continuously exposed to the same concentrations 24 hours a day developed a clinically manifest neuropathy with hexacarbon-specific morphological alterations after 4 weeks. The results in set B of the experiments do not rule out the possibility that neuropathy would have developed if exposure times (both daily and total exposure) had been longer. This is of great importance with regard to use of these solvent mixtures in industrial working situations or in the household. A clear potentiation by methyl-ethyl-ketone of hexacarbon neurotoxicity can be observed upon continuous exposure to MEK/n-hexane mixtures, even at low concentrations. In view of all available clinical and experimental data, the combination of MEK with hexacarbon should be avoided in commercial solvents, and appropriate warnings should accompany these products.

Summary:

The effects of long-term continuous and intermittent inhalation exposure to selected concentrations of n-hexane and mixtures of n-hexane and methyl-ethyl-ketone (MEK) on the nervous system of rats were investigated. Animals exposed continuously (24 h/d, 7 d/week) to 500 ppm n-hexane displayed complete hindlimb paralysis after 9 weeks. Histological examination showed hexacarbon-specific axonal lesions in peripheral nerves, particularly tibial branches to calf muscles, and in the gracile tract at cervical levels of the spinal cord. Similar clinical pathological signs of neuropathy appeared one week earlier in animals treated with a mixture of 500 ppm n-hexane/MEK (4:1 or 3:2) and 5 weeks earlier with 700 ppm n-hexane/MEK mixture (5:2) or 700 ppm of n-hexane alone. Rats exposed to the latter concentrations intermittently (8 h/d, 7 d/week) for 40 weeks did not develop clinical or morphological signs of a hexacarbon neuropathy.

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Table 1

Presence of MEK in solvent mixtures in cases of "hexane neuropathy" reported in the literature

Goto, 1974	sniffer's neuropathy	MEK, n-hexane, toluene
Oh, 1976	Huffer's neuropathy	MEK, MIBK, toluene, acetone
Gaultier, 1973	industrial solvent neuropathy	MEK, cyclohexane, toluene, industrial benzine, type C
Vaucher, 1972	industrial solvent neuropathy	MEK, cyclohexane, toluene, industrial benzine type C
Carapella, 1975	industrial solvent neuropathy	MEK, n-hexane, toluene, in several analyses
DiBosco, 1974	industrial solvent neuropathy	MEK, n-hexane, toluene, in several analyses
Lima, 1977	industrial solvent neuropathy	MEK, MIBK, toluene, acetone

Fig. 1:

Severe hindlimb paresis in an animal into
n-hexane + MEK. Only animals at this stag
nal impairment were considered clearly ne

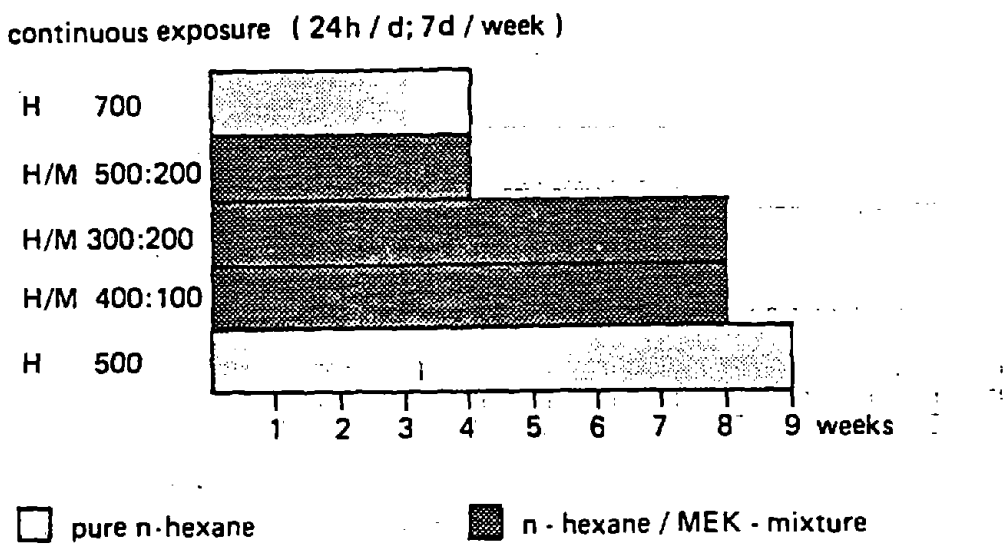

Fig. 2:

Time of onset of severe functional impairment as shown in Fig. 1, following exposure to different solvent mixtures.

Fig. 3: [illegible]

Paranodal axonal swelling with prominent myelin retraction in the paranodal area and slightly increased density of axoplasm. Methylene blue, x 1.500. n-hexane/MEK, 500 : 200 ppm, 4th week.

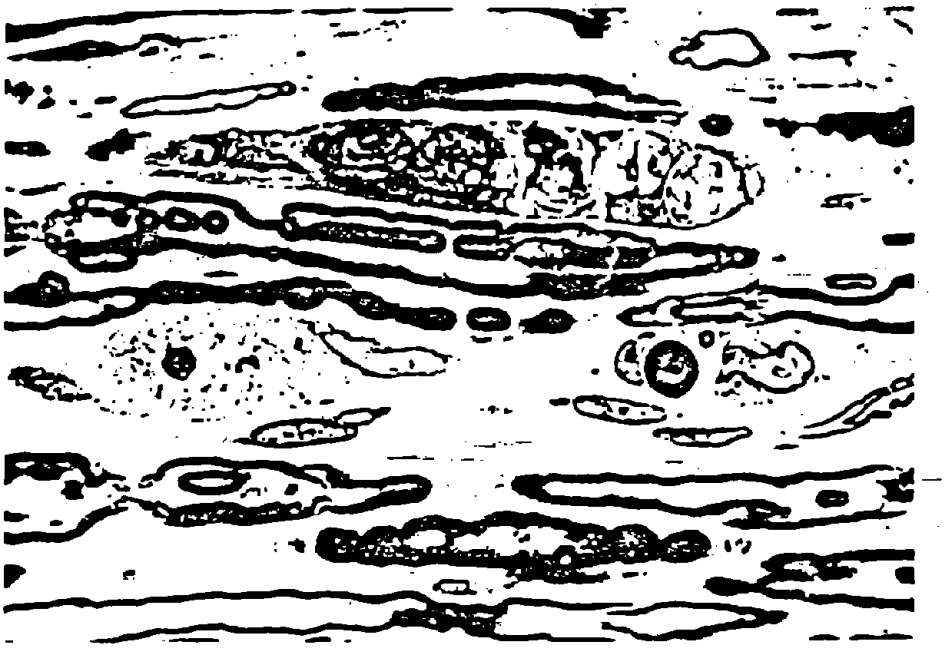

Fig. 4:

Ovoid formation distal to axonal swellings. Methylene blue, x 1.500. n-hexane/MEK, 500 : 200 ppm, 4th week.

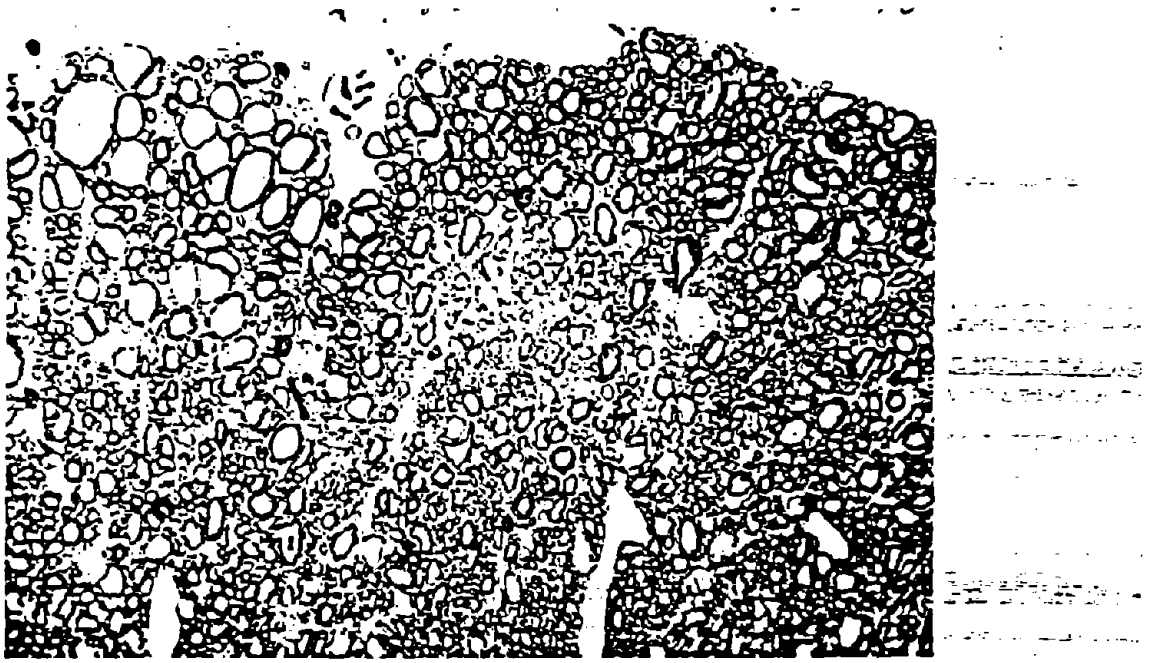

Fig. 5: Fig. 5:

Axonal swelling in gracile tract axons of the upper cervical level. Methylene blue, x 255. n-hexane/MEK, 400 : 100 ppm, 8th week.

Fig. 6:

Cross section of densely accumulated neurofilaments in an axonal swelling. The myelin sheath is abnormally thin (x 26.000). (n-hexane/MEK, 500 : 200 ppm; 4th week.

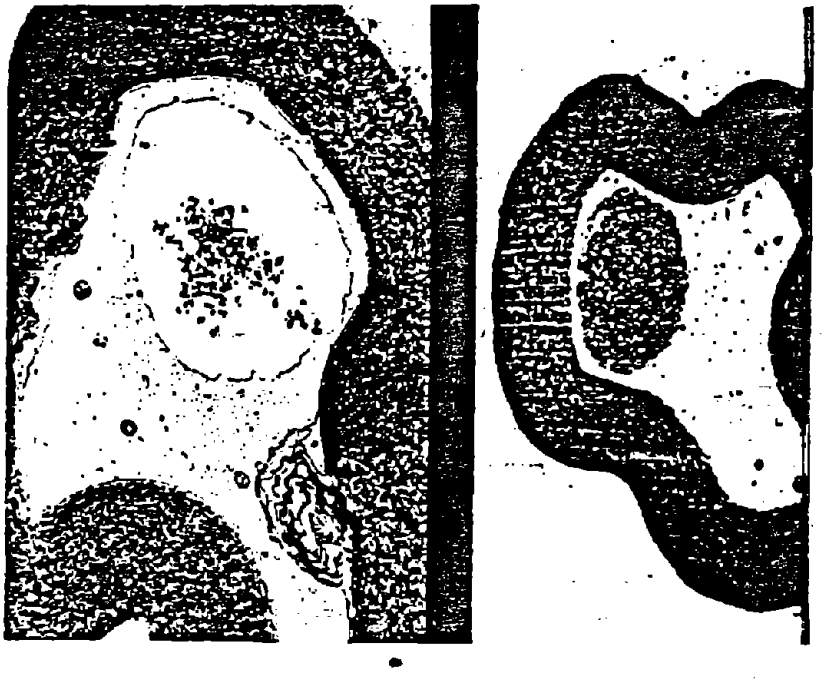

Fig. 7:

Circumscribed accumulation of glycogen granules in myelinated axons surrounded by a membranous structure (x 10.000).