An electron microscopic study of peripheral nerve damage in mice induced by repeated subacute exposure to endrin

by James John Walker

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
Endrin is a chlorinated hydrocarbon insecticide that has been used worldwide. Although acute endrin exposure produces neurological symptoms, there is generally a lack of morphological data documenting its cellular effects along the neuromuscular axis. This study was designed to characterize the morphological effects of endrin on peripheral nerve, optic nerve and muscle. Mice were given 20 daily intraperitoneal injections of endrin in sesame oil at subacute doses that increased from 1.5 mg/kg to 4.0 mg/kg. Controls were given the same intraperitoneal volume without endrin. Animals were sacrificed after 4, 7, 14, and 20 days of exposure and 14 and 92 days after the last injection. Sciatic nerve, optic nerve, skeletal muscle and cardiac muscle tissue were examined using both light and electron microscopy. Daily behavioral/neurological tests were used to assess general effects on the nervous system. Animals exposed to endrin were hyperactive, hypersensitive to stimuli, had difficulty maintaining their position on a rod and displayed signs of piloerection. Optic nerve, skeletal muscle and cardiac muscle appeared unaffected as were myelinated nerve fibers from sciatic nerve also appeared unaffected. However, unmyelinated axons in the sciatic nerve of exposed animals showed various changes which included axonal swelling, dissolution of microtubules and neurofilaments, axonal and Schwann cell vesiculation, and axonal vacuolation. Some vesicles were present in scattered rows along the axon or within the axon while others were present in pockets which often appeared continuous with the periaxonal space and often invaginated the axolemma. Some such pockets contained an amorphous material as well as vesicles and some regions within the Schwann cell that had contained axons appeared completely filled by vesicles or flocculent background debris. The results of this study indicate that repeated subacute doses of endrin produce morphological alterations in unmyelinated peripheral nerve fibers and their associated Schwann cells and thus could probably cause changes in neurological functions such as pain perception and autonomic activities related to temperature and blood pressure regulation.

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

September 1984

APPROVAL

of a thesis submitted by

JAMES JOHN WALKER

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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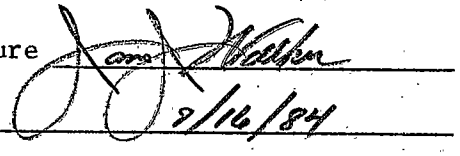
Signature 

Date 9/16/84

ACKNOWLEGMENT

I would like to express my sincere appreciation and deepest respect to Dr. Dwight Phillips for his guidance and encouragement throughout this study as both an advisor and friend. I would also like to thank Dr. James McMillan, Dr. William Dorgan and Dr. Robert Moore for serving as my graduate committee.

A special thanks goes to Caren and Jimmy for their patience, understanding, and support and especially for the sacrifices they have made for the betterment of my graduate studies.

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ABSTRACT

Endrin is a chlorinated hydrocarbon insecticide that has been used worldwide. Although acute endrin exposure produces neurological symptoms, there is generally a lack of morphological data documenting its cellular effects along the neuromuscular axis. This study was designed to characterize the morphological effects of endrin on peripheral nerve, optic nerve and muscle. Mice were given 20 daily intraperitoneal injections of endrin in sesame oil at subacute doses that increased from 1.5 mg/kg to 4.0 mg/kg. Controls were given the same intraperitoneal volume without endrin. Animals were sacrificed after 4, 7, 14, and 20 days of exposure and 14 and 92 days after the last injection. Sciatic nerve, optic nerve, skeletal muscle and cardiac muscle tissue were examined using both light and electron microscopy. Daily behavioral/neurological tests were used to assess general effects on the nervous system. Animals exposed to endrin were hyperactive, hypersensitive to stimuli, had difficulty maintaining their position on a rod and displayed signs of piloerection. Optic nerve, skeletal muscle and cardiac muscle appeared unaffected as were myelinated nerve fibers from sciatic nerve also appeared unaffected. However, unmyelinated axons in the sciatic nerve of exposed animals showed various changes which included axonal swelling, dissolution of microtubules and neurofilaments, axonal and Schwann cell vesiculation, and axonal vacuolation. Some vesicles were present in scattered rows along the axon or within the axon while others were present in pockets which often appeared continuous with the periaxonal space and often invaginated the axolemma. Some such pockets contained an amorphous material as well as vesicles and some regions within the Schwann cell that had contained axons appeared completely filled by vesicles or flocculent background debris. The results of this study indicate that repeated subacute doses of endrin produce morphological alterations in unmyelinated peripheral nerve fibers and their associated Schwann cells and thus could probably cause changes in neurological functions such as pain perception and autonomic activities related to temperature and blood pressure regulation.

INTRODUCTION

Endrin is a chlorinated hydrocarbon pesticide that has been used in the U.S. for agricultural and public health purposes since its introduction by the Velsicol Company in 1951. It is the most acutely toxic hydrocarbon insecticide known (Gaines,1969) and belongs to a group of structurally related cyclodiene compounds which includes aldrin, chlordane, chlordecone (kepone), dieldrin, heptachlor, isodrin, and telodrin. Although animals and humans acutely exposed to endrin display neurological symptoms which include hyperexcitability, hypersensitivity to stimuli, bradycardia, hypertension, increased rectal temperature, increased vascular resistance, tremor, and convulsions (Treon,1955; Emerson et al.,1964; Emerson and Hinshaw,1965; Reins et al.,1964; Coble et al.,1967; Weeks,1967), morphological documentation of such neurological symptomology is generally lacking. Since some of these symptoms are indicative of changes within the peripheral nervous system and since exposure to chlordecone has been shown to damage unmyelinated peripheral nerve fibers (Phillips and Eroschenko,1982), the present study was undertaken to examine peripheral nerve from animals exposed to endrin.

While endrin is toxic to all animals, the lethal toxicity to one half of a group of animals (LD50) varies depending on sex, method of

administration, and species variation. The acute oral toxicity of endrin and a number of related compounds is summarized in Table 1.

Table 1. Acute oral toxicity of selected chlorinated hydrocarbon pesticides in rats. (Reference: Gaines,1969)

Compound	LD50(mg/kg)		Lowest dose to kill a rat (mg/kg)	
	Males	Females	Males	Females
Aldrin	39	60	25	40
Chlordane	335	430	250	350
DDT(technical)	217	-	150	-
Dieldrin	46	46	30	30
Endrin	18	7.5	10	6.0
Heptachlor	100	162	50	100
Kepone	125	125	100	125

The excretion of endrin and its metabolites takes place primarily via the liver, bile and feces (Cole et al.,1968). Animals exposed to carbon fourteen labeled endrin excrete approximately 50-60% of the dose in feces within 24 hours (Cole et al.,1968; Ludwig,1966, as cited in Soto,1967; Hunter,1960, as cited in Soto,1967; Bedford et al.,1975a), while very little endrin or its metabolites are excreted in the urine (Hutson et al.,1975)

Endrin does not appear to accumulate in the tissues of exposed animals, rather, a plateau level of storage is reached after 6-10 days

(Brooks,1969; Korte,1967 and 1970, as cited in Donoso, 1979). Detectable levels of endrin are generally found only in animals receiving doses of 0.25 ppm or more, indicating that a threshold level of intake is necessary before the compound can be detected (Terriere al.,1958; Kiigemagi et al.,1958; Ely and Moore,1957; Moubry et al.,1968). In dogs acutely exposed to endrin there appears to be no relationship between the blood concentrations of the compound and those in the non-fat tissues (Richardson et al.,1967).

Humans do not tend to accumulate significant quantities of endrin. Endrin could not be detected in the blood of occupationally exposed workers except in those who had been over exposed (Jager,1970). The biological half life of endrin in human blood is about 24 hours (Jager,1970). Endrin was detected in the urine and blood of patients acutely poisoned by ingesting contaminated bread although blood and urine samples were normal within one week after poisoning (Curley et al.,1970; Coble et al.,1967).

Endrin is an environmentally persistent compound. Forty one percent an original application of technical endrin persisted in the soil for up to 14 years (Nash and Woolen,1967). Endrin is one of the least water soluble insecticides and is extremely persistent in an aqueous environment with as much as 80% of the initial endrin concentration (2 ppm) remaining in water samples after 16 weeks (Sharom et al.,1980).

Its acute toxicity and environmental persistence have led to limitation of endrin's use in the past several years. Massive fish kills in the Mississippi river system in the early 1960's led to the initial restricted use of endrin which culminated in the Environmental Protection Agency's ban of its use east of the Mississippi in 1979. It is still used in the Plains States for the control of cutworm in grain and in the Pacific Northwest for the control of voles in apple orchards. Heavy use of endrin in Montana in 1981 led to public and governmental concern over detected residue accumulation in wildlife, resulting in hunting season restrictions and further restrictions on its agricultural use.

Histopathological data from animals exposed to endrin is scarce. Diffuse degenerative changes have been reported in liver and kidneys (Treon,1955; Reins et al.,1964; Boyd and Stefec,1969; Reuber,1979), adrenal glands (Treon,1955; Reuber,1979), heart (Treon,1955; Reuber,1979), spleen (Reins et al.,1964; Boyd and Stefec,1969; Reuber,1979), brain (Treon,1955; Boyd and Stefec,1969; Reuber,1979), thymus (Boyd and Stefec,1969) and lungs (Treon,1955; Reins et al.,1964; Boyd and Stefec,1969) of laboratory animals exposed to endrin. Other histopathological changes included local irritation of the gastrointestinal tract, capillary venous congestion in the brain, heart and lungs, and depletion of secretions in the salivary glands (Boyd and Stefec,1969),

Neurological symptomology in endrin exposed animals, as

manifested by convulsions, tremor, increased salivation, and hypersensitivity, has led to the belief that endrin acts chiefly on the nervous system (Emerson et al.,1964; Hinshaw et al.,1966). Activation of the sympathetic and parasympathetic nervous systems by endrin has been proposed after studies of acutely exposed dogs (Emerson et al.,1964 and 1966; Reins et al.,1964 and 1966; Hinshaw et al.,1966). Bradycardia, copious mucoid salivation, hypertension, and convulsions following lethal exposure to endrin (10 mg/kg) suggest hyperactivity of both the sympathetic and parasympathetic nervous systems (Emerson et al.,1964). Endrin exposed animals develop increased renal resistance due to possible sympatho-adrenal stimulation and subsequent increase in circulating catecholamines (Reins et al.,1964) and increased venous return due to a massive sympathetic discharge leading to the release of blood stores from the liver and spleen (Hinshaw et al.,1966).

Other physiological changes induced by endrin exposure in dogs include, left heart failure, acidosis, hypoxia, increased rectal temperature, increased hemoconcentration, increased leukocyte concentration, increased peripheral resistance, decreased glomerular filtration, increased cerebral spinal fluid pressure and increased cerebral venous pressure (Emerson et al.,1964; Emerson,1965; Emerson and Hinshaw,1965; Reins et al.,1966; Hinshaw et al.,1966). The suggested physiological action of endrin in the dog is summarized in Figure 1. Most past studies dealing with endrin have examined

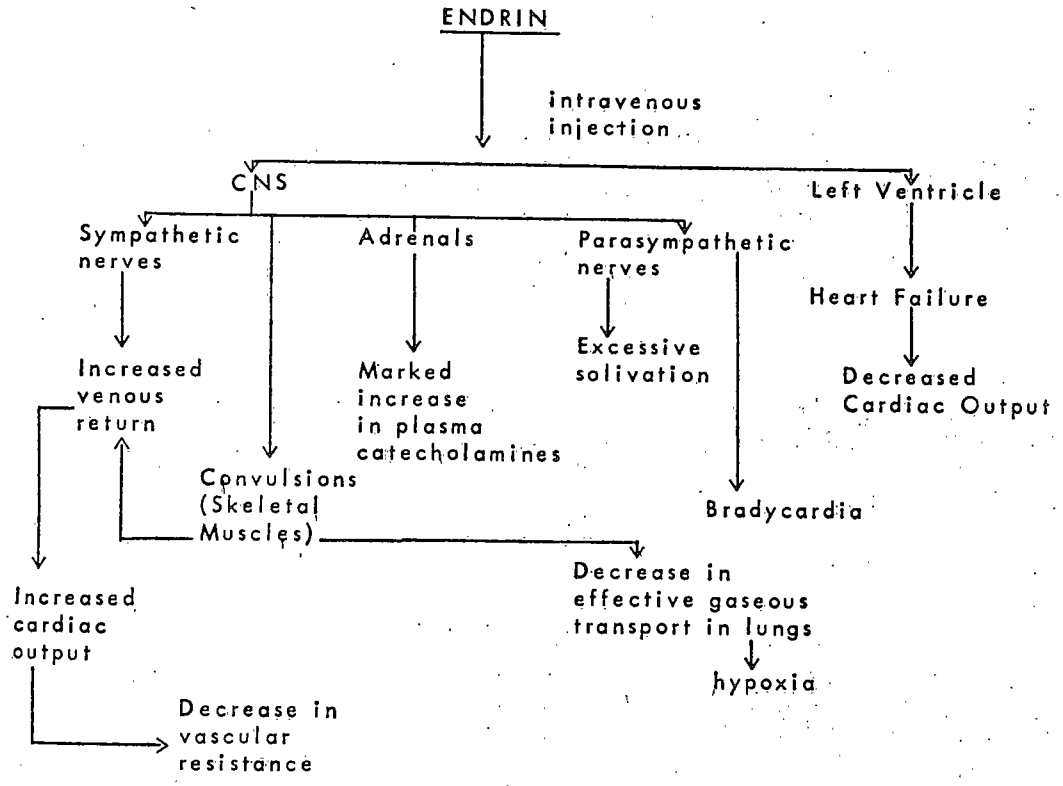


FIGURE 1. Summary of the effects of endrin in the dog.

metabolism, hepatic and renal damage, changes in the cardiovascular system, reproductive abnormalities and its acute and chronic toxicity. While much speculation exists in the literature concerning its effects on the mammalian nervous system, little morphological data exists to support such speculation even though the most profound symptoms are neurological. Most research up to this point involving the effects of endrin on the nervous system has been related to studying the physiological aspects of convulsions, hypertension, and bradycardia while the morphological basis for the development of such symptoms has been ignored. Thus, there is a need for morphological studies to characterize the effects of endrin along the neuromuscular axis. Because previous studies in this laboratory (Phillips and Eroschenko,1982) have shown that subacute exposure to the related compound chlordecone damages unmyelinated peripheral nerve fibers, and because endrin exposure has been speculated to cause autonomic dysfunction, it was of interest to determine the effects of endrin on peripheral nerve in animals exposed to repeated subacute doses of the compound. In addition, studies of some behavioral/neurological parameters were initiated to determine if changes similar to those observed in chlordecone exposed animals (Phillips and Eroschenko,1982; Jordan et al.,1981) and in animals acutely exposed to endrin (Treon,1955; Emerson et al.,1964; Reins et al.,1964) occurred in animals receiving subacute doses of endrin.

LITERATURE REVIEW

CHEMICAL AND PHYSICAL PROPERTIES OF ENDRIN

Endrin is the common name for 1,2,3,4,10,10,-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethano-naphthalene. It is a compound containing at least 92% of the endo-endo stereoisomer of dieldrin and is an epoxide of isodrin. Pure endrin is a white crystalline solid that is stable in the presence of wetting agents, emulsifiers, alkaline oxidizing agents and basic reagents but decomposes when treated with acids or heated above 200 C. Endrin, like other cyclodiene compounds is soluble in most organic solvents but is insoluble in water. It is unlike these compounds in that it has a much smaller partition coefficient (the tendency to distribute between polar and non-polar substrates), which limits its rate of decomposition and retention in exposed organisms (Montana Dept. of Agriculture,1983).

Pure endrin is stable in air and light but undergoes isomerization, rearrangement, or decomposition upon exposure to heat, acids or ultraviolet light (Soloway, et al.,1960; Burton and Pollard,1974; Rosen et al.,1966). These processes, plus microbial metabolism (Matsumura et al.,1971) are the primary means of degradation of the parent compound and play an important role in its environmental persistence. The principle products of such breakdown

are shown in Figure 2. Soto and Diechmann (1967) studied the acute toxicity of these metabolic products in rats and found the endrin aldehyde to be essentially non-toxic while the endrin ketone was approximately 1/4 as toxic as endrin itself. Susceptability of the endrin aldehyde appeared greater in females than males.

Photochemical degradation is an important factor in the persistence of endrin on the surface of plants. Harrison et al., (1967) showed that apple foliage sprayed with endrin retained only 13% of the initial treatment after one week and only 2% after 7 weeks. A monitoring program initiated by the Montana Department of Agriculture revealed that endrin residues on wheat foliage measured 2 days after application, had degraded by 99.9% after 10 weeks and that soil residues had degraded 67% during the same time. Detectable levels of the insecticide were still present 55 weeks after application (Montana Dept. of Agriculture,1983).

USES OF ENDRIN

Endrin has been used throughout the United States as an avicide, and rodenticide, but its major use has been as an insecticide. It was used throughout the 1950's on crops such as included alfalfa, eggplant, lettuce, potatoes, peppers, tomatoes, strawberries, corn, sorghum, sugar beets, cotton and small grains. It was used primarily in southeastern U.S. during the 1960's and 70's to control

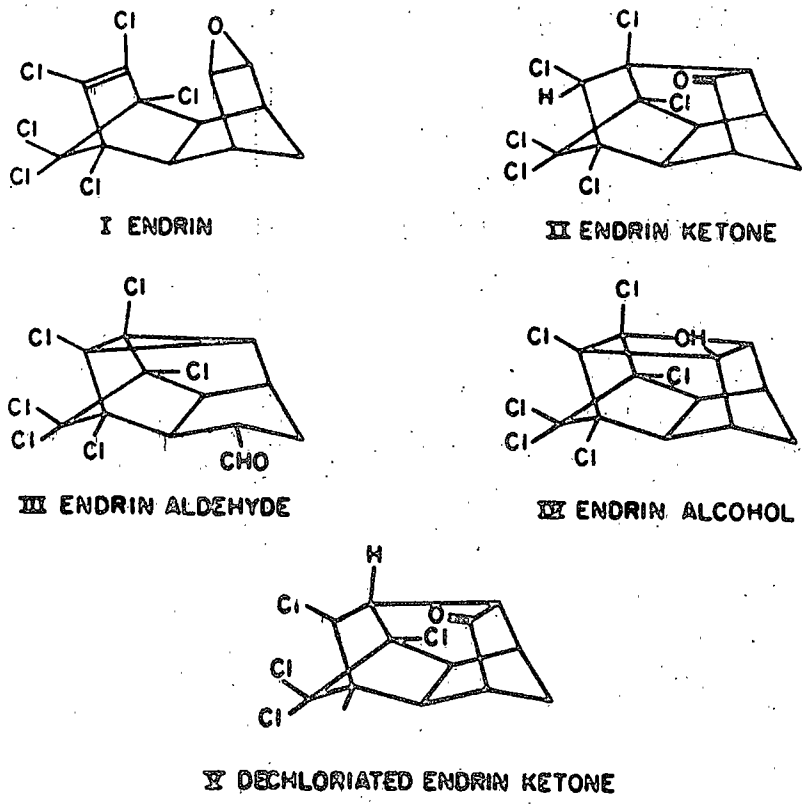


FIGURE 2. Products of endrin degradation.

lepidopterous larva in cotton crops. In 1971, over 75% of the endrin used was for treatment of cotton and 99% of this use was in the southeastern and delta states (Donoso et al.,1979).

During the period from 1957 to 1966, the lower Mississippi river system had the highest levels of endrin contamination of any surface waters in the U.S., primarily as a result of runoff from crops and fields in this area. Such contamination resulted in continual restriction of its use nationwide although in 1981 over 27,000 lbs. of endrin was used for the control of rodents in the state of Washington (Eaton,1982) while in the same year over 200,000 acres of grain was treated in Montana (Montana Dept. of Fish, Wildlife and Parks,1983).

Monitoring programs involving State and Federal agencies during the heavy use in Montana revealed widespread contamination of fish and wildlife (primarily documented in birds). Several years of controversy and studies by the Montana Department of Fish, Wildlife and Parks have finally culminated in a recent (July,1983) Montana Department of Agriculture prepared EPA Environmental Impact Statement which has resulted in the Department of Agricultures' recommendation to " suspend the sales and use of endrin and to cancel endrin registrations when alternatives are registered by the Environmental Protection Agency " (K. Kelly,1984, personal communication).

METABOLISM AND ELIMINATION

The metabolism of endrin in mammals has been well studied (Terriere et al.,1958; Cole et al.,1968; Bedford et al.,1975a and 1975b; Soto and Deichmann,1967; Brooks,1969; Hutson et al.,1975). Hutson et al.(1975) dosed rats of both sexes for 2 weeks and found endrin and its major metabolites in feces in the following proportions : endrin, 11%; anti-12-hydroxyendrin 83%; syn-12-hydroxyendrin, <0.01%; 3-hydroxyendrin, 5%; 12-ketoendrin, 1%; and delta-ketoendrin, <0.01%. Bedford et al.(1975b) reported that the oral administration of endrin to rats resulted in the production of three metabolites, all of which were more acutely toxic than the parent compound, with 12-ketoendrin being the most acutely toxic. Other investigators have reported that the metabolites of endrin are less acutely toxic than is endrin (Klein et al., 1968, as cited in Donoso,1979). The greater susceptibility of females appears related to the fact that they metabolize endrin more slowly than do males (Korte, 1967, as cited in Brooks 1969) and tend not to excrete 12-ketoendrin (Hutson et al.,1975).

The accumulation of endrin in the rat varies with the sex (Hutson et al.,1975). These authors found that the tissue residue retention was higher in females and was composed primarily of unmetabolized endrin while 12-ketoendrin was the main tissue residue found in males. They found 12-ketoendrin was the major metabolite in the fat, liver and kidneys of males while unmetabolized endrin was the main

component in fat and kidneys of females. Endrin and 12-ketoendrin were detected in the liver of females although 70% of the original radioactively labeled compound could not be accounted for in their analysis.

The metabolic fate of endrin in the rabbit is markedly different from that in the rat. Bedford et al.(1975a) reported that almost half of the administered dose of endrin in the rabbit is excreted in the urine, compared to only 2% in the rat. This study also found excretion to be almost complete within 24 hours and that the major fecal component was unchanged endrin. The metabolites of the two species is similar although 12-ketoendrin is not a significant excretion product in the rabbit.

Endrin tends to reach a steady state condition in the tissues of repeatedly exposed animals. Rats fed carbon fourteen labeled endrin for 12 days tended to reach a plateau level of storage after 9 to 10 days (Brooks, 1969) although in another study, oral administration of endrin to rats resulted in a steady state of storage after about 6 days (Korte,1967 and 1970, as cited in Donoso et al., 1979). Steers, lambs, and hogs fed endrin for 12 weeks had detectable levels within the tissues only in those animals receiving a dose higher than 0.25 ppm (Terriere et al.,1958). In animals allowed to recover for 6 weeks, steers had a 60% reduction in the endrin content in their fat while no detectable levels were found in lambs and hogs. In a similar study Kiigemagi et al.(1958) fed dairy cows endrin and reported

detectable concentrations in milk only from animals receiving doses of 0.25 ppm and greater. They also noted that increased dietary levels had no effect on the milk secretion concentration of endrin. Other studies also indicate that a threshold level of intake is necessary before measurable amounts of the compound can be detected (Ely and Moore,1957; Moubry et al.,1968). Richardson et al.(1967) demonstrated in dogs that, excluding fat there is no relationship between blood concentrations of endrin and those in the tissues.

Most endrin and its metabolites are excreted in the feces (Cole et al.,1968), while very little endrin or its metabolites are excreted in the urine (Hutson et al.,1975). Cole et al.(1968) administered carbon fourteen labeled endrin intravenously to rats with and without bile fistulas. They found that over 50% of the daily dose was excreted within 24 hours and that 90% of the excreted compound was in the feces. In the same study, in an isolated perfused liver, 50% percent of the labeled endrin was excreted in the bile within one hour. In pigs fed endrin, 47% of the administered compound was recovered from urine, feces, stomach, gut, liver, fat, and blood within 24 hours while 94% had been recovered after 6 days (Hunter 1960, as cited in Soto and Diechmann,1967). Intubated rats fed endrin for 8 days excreted 60-70% of each daily dose in the feces (Ludwig, 1966, as cited in Soto and Deichmann, 1967). The first day, 30% of the amount excreted contained pure endrin and 70% was metabolites. Cessation of dosing resulted in 93% of the amount excreted as

metabolites. He also reported that of the amount excreted in urine, 82% consisted of metabolites and 18% was endrin. Rabbits given two oral doses of carbon fourteen labeled endrin 14 days apart excreted 37.3% of the first dose via the urine and 49.6% in feces during days 1-13. The second dose was eliminated in a similar fashion (Bedford et al.,1975a). Subsequent recovery of 96.7% of the radioactivity in urine and feces was achieved by day 49.

TOXICITY

Numerous studies have examined the toxicity of endrin in laboratory animals. Treon (1955) studied the toxicity of endrin in a number of laboratory animals utilizing various modes of administration. Continual application of endrin (dry powder) to the skin of rabbits for 24 hours resulted in development of convulsions in the most severly poisoned animals, while intermittent application for 2 hours on each of 5 days per week caused death in animals receiving as little as 19 applications. Symptoms displayed by these animals included convulsions, tremors, and facial twitching. Oral administration of 1 mg of endrin on each of 5 days per week to rabbits and rats of both sexes resulted in abdominal distention in rabbits, and hypersensitivity to stimuli in rats. In another experiment, the same author fed rats endrin at various doses over a period up to 2 years and reported convulsions and hypersensitivity to stimuli in

those animals fed diets with the highest concentration of endrin. Bedford et al.,(1975a) also reported signs of toxicity in rats such as ataxia followed by tonic convulsions. Treon (1955) also found that dogs given lethal injections of endrin regurgitated their food, became lethargic, salivated, developed respiratory distress and central nervous system symptoms that included hypersensitivity to stimuli, tremors, twitching, and severe convulsions.

Seizures developed in mice following intravenous injections of endrin (Walsh and Fink,1972). Graves (1965) gave intraperitoneal injections of endrin to mice and reported no mortality at doses of 1 or 2 mg/kg but all animals died at exposure levels of 10 mg/kg or more. Similar results were reported in bats exposed to various concentrations of endrin over a 28 day period (Luckens and Davis,1965). They found complete mortality at a dose of 12 mg/kg. Animals receiving 50 mg/kg developed tremors within one hour and died by 2 2/3 hours after exposure.

Endrin in also extremely toxic to birds and fish. Quail and pheasants develop symptoms of intoxication within 48 to 72 hours after being given endrin in their diets (DeWitt,1956). Initially these birds displayed a lack of muscular coordination, occasional tremors, and stiff-legged, hesitating movement in walking. Later they made spasmodic leaps and went into violent cartwheels. In another study (DeWitt,1955), it was reported that quail developed severe tremors, loss of muscular coordination and "extreme nervousness" within two

hours after being fed endrin. All birds exposed to endrin in this group died within 48 hours.

Numerous studies have documented the toxic effects of endrin on fish. Signs of intoxication include ineffective feeding (Grant and Merhle,1970), swimming in whirling patterns when disturbed (Hermanutz,1978), hypersensitivity to sudden noise (Grant,1976; Argyle et al.,1973), respiratory difficulties, sluggishness (Johnson,1968), and hyperexcitability (Grant and Mehrle,1973).

Symptoms of endrin poisoning in humans have been well documented. While death has been reported only in extreme cases, the initial symptoms after less severe exposures include headache, weakness, nausea, and abdominal discomfort while individuals more severely poisoned often exhibit convulsions, unconsciousness, and frothing at the mouth (Weeks,1967; Coble et al.,1967). Acutely poisoned infants displayed early symptoms such as tonic and clonic convulsions, slight trismus, unconsciousness, tachycardia, an elevated body temperature, respiratory distress and cyanosis (Jacobziner and Raybin,1959; Hayden et al.,1965). During the next several days these individuals developed symptoms such as vomiting, difficulty in swallowing, convulsions, limbs in a state of permanent contraction and pupilary dilation. Neurological examination of one child revealed signs of "diffuse encephalopathy with focal motor parietal discharge on the right side resulting in decerebrate rigidity and brain stem signs and vasomotor instability and hypertension due to involvement of

the cardiovascular centers in the medulla and pons" (Jacobziner and Raybin,1959). A hyperactive startle reaction and the alternating extension and flexion of the upper limb led these investigators to speculate extensive diencephalon involvement although movements due to basal ganglia involvement were not noted.

Four outbreaks of acute poisoning from endrin contaminated bread were reported in Saudi Arabia in 1967 (Weeks,1967). Within hours after ingesting contaminated bread, individuals developed abdominal pain, nausea, vomiting, lethargy, mental confusion, unconsciousness and convulsion. In many instances improvement was rapid, often within 2-5 hours. Seven people died in one incident within 12 hours after the onset of symptoms. Similar incidents in Egypt (Coble et al,1967) and Wales (Davies and Lewis,1956, as cited in Weeks,1967) have been described, all involving ingestion of endrin contaminated bread. Symptoms of intoxication included convulsions, facial contortions, frothing at the mouth, lethargy, headaches, mental confusion, beating the head on the floor, hyperactive reflexes and periods of semiconsciousness. Recovery in these poisonings was rapid, generally occurring within 1-7 days, depending on the severity of exposure.

CARCINOGENICITY

Endrin has been reported to be carcinogenic to rats (Reuber,1979). Rats exposed to concentrations as low as 0.1 ppm

developed significant incidences of carcinomas in liver, lung, thyroid, mammary gland, and adrenal cortex. Females tended to be more susceptible to the development of neoplasms in endocrine and reproductive organs. In other studies of male and female rats fed endrin in concentrations varying from 1 to 100 ppm over a period of 2 years no increased incidence of tumors was reported in experimental animals (Treon,1956, as cited in Jager,1970). Similar studies in mice and dogs were inconclusive (Reuber,1979). Reuber (1979) states that "sufficient documentation is available on qualitative extrapolation of animal data that one must conclude that finding of carcinogenicity in one mammalian species should be deemmed to have relevance in other mammalian species-including man".

Reuber's findings (1979) have not been accepted by the Environmental Protection Agency's Carcinogen Assessment Group who reviewed studies of the U.S. Food and Drug Administration, the National Cancer Institute, the University of Cincinnati and the University of Miami. This group concluded that ".... the weight of evidence is that endrin is unlikely to be a human carcinogen" (Albert,1978, as cited in Montana Dept. of Agriculture,1983). Endrin is also not on the list of carcinogens reported by the World Health Organization (Spencer,1981, as cited in Montana Dept. of Agriculture,1983).

TERATOGENICITY and MUTAGENICITY

Endrin has been shown to be teratogenic in hamsters and mice as well as causing weight reduction and a significant increase in fetal mortality (Ottolenghi et al.,1973). Anomalies in the offspring of endrin treated hamsters included open eye, webbed foot, and cleft palate. In another study, Chernoff et al.(1979a and 1979b) reported fused ribs, meningoencephaloceles, reduced skeletal ossification, increased mortality and fetal weight reduction. In the offspring of exposed mice, there was no effect on survival or weight and only a few incidences of cleft palate, open eye, microcephaly and exencephaly (Ottolenghi et al.,1973).

Endrin has been shown to be mutagenic in rats following testicular injections (Dikshith and Datta,1973). They found chromosomal changes that included fragmentation and the formation of single and double bridges with acentric fragments, unequal distribution of anaphase chromosomes, and transformation of chromatin into an amorphous lump.

PHYSIOLOGICAL EFFECTS

Physiological studies involving endrin exposed animals have been well documented and while little data is available concerning its mode of action within the nervous system, pharmacological studies indicate

that it is not a cholinesterase inhibitor (Colvin and Phillips,1968).

Ryan and Shankland (1971) explored the synergistic action of DDT and endrin on the giant axons of the cockroach central nervous system. They found that when given individually neither compound produced physiological abnormalities over a period of 3-5 hours. However pretreatment of the axons with DDT followed by exposure to endrin led to instability followed by complete blockade of axonal conduction. They concluded that endrin alone had no toxic action on the axonal membrane of the cockroach and the that apparent synergistic action of these compounds cannot be attributed to either compound alone.

Joy (1976) studied the convulsive properties of a number of chlorinated hydrocarbon insecticides in the cat central nervous system. Animals injected with endrin at doses of 1-2 mg/kg developed spontaneous seizures within 5-15 minutes and at higher doses developed seizures in 0.5-2.0 minutes. Hypotension, which was followed by hypertension and cardiac arrythmias were observed in some animals. Following administration of endrin, any type of sensory stimulation, especially tactile or auditory, would evoke a seizure. The author speculated that the chlorinated hydrocarbon compounds act directly on the central nervous system and that they do not have to be converted to an active metabolite to produce toxic effects (Joy,1976).

Seizures were produced in all parts of the pigeon telencephalon after intravenous administration of endrin (Revzin,1966). The ectostriatum appeared to be particularly sensitive and because it is a visual projection area in birds, it is probable that such exposures could result in visual deficits.

MATERIALS AND METHODS

EXPOSURE AND TISSUE PREPARATION

Ten week old, male, ICR mice (Charles River) with an average weight of 34 grams (range 28.5-39.4) were divided into control and experimental groups. Experimental animals were given daily intraperitoneal injections of endrin in sesame oil (0.2 ml) at doses which increased from 1.5 mg/kg on days 0-3, to 2.0 mg/kg on days 4-11, and finally to 4.0 mg/kg on days 12-19. This dosing protocol (Figure 3) was employed in an attempt to maintain a maximum sublethal exposure, in animals that may have adapted to the previous level of exposure. The initial daily dose of 1.5 mg/kg represents approximately 27% of the acute LD50 for mice while the final daily dose represents approximately 71% of the acute LD50 (Graves,1964). The cumulative dose in those animals receiving 20 consecutive injections was 54 mg/kg (approximately 10 times the acute LD50). The solutions were prepared with endrin (99% purity) supplied by the Velsicol Company (Chicago). Control animals received the same volume of sesame oil without endrin. Four experimental and two control animals were sacrificed after 4, 7, 14, and 20 days of exposure (d o e) and after 14 and 92 days of recovery (d o r).

Each animal was weighed on a beam balance immediately preceding sacrifice. The animals were anesthetized with ether and, following

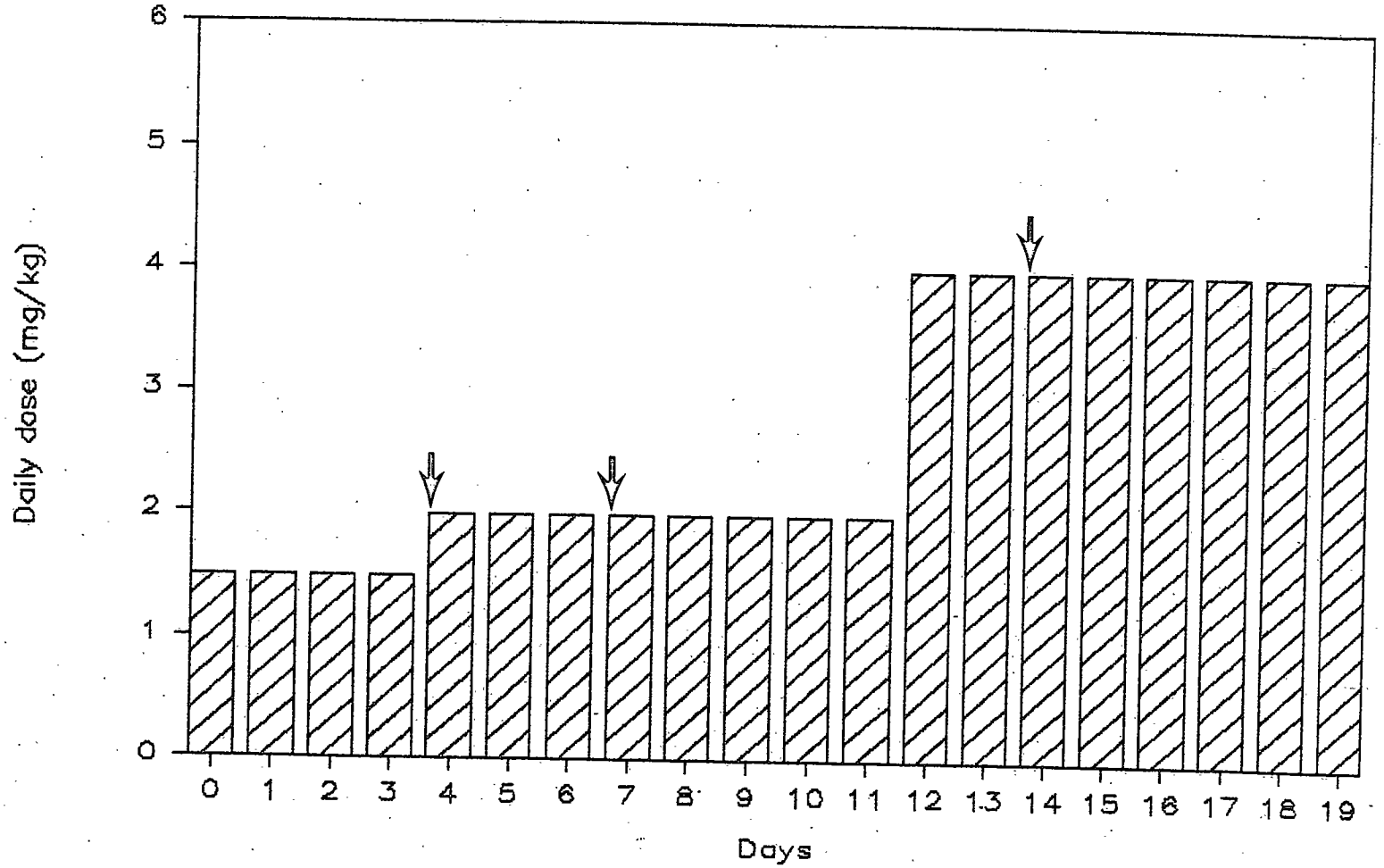


FIGURE 3. Dosing Protocol. Arrows indicate days of sacrifice.

lateral incisions, the rib cage was reflected superiorly. The pericardial sac was opened and a 21 gauge needle was inserted into the left ventricle. The right atrium was cut and the animal was perfused with 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) at a pressure of 80-100 mm Hg for 5 minutes. Sciatic nerve, optic nerve, gluteal and cardiac muscle were removed, placed in fixative and diced into 2 millimeter cubes. Following 2-3 hours in cold fixative, the tissue was washed in buffer and stored overnight. The next day the tissue was post-fixed in buffered 1% osmium tetroxide, then dehydrated in a graded ethanol series and propylene oxide and embedded in Spurr's resin. Tissue sections 1-2 um (micrometers) thick were cut from several blocks of each animal and stained with basic toluidine blue (Meek,1976) for light microscopic examination and subsequent orientation of thin sections. Light micrographs were taken with an Olympus PM-6 camera mounted on an Olympus BH-2 light microscope. Thin sections were cut with glass knives on an LKB Ultratome III, mounted on uncoated copper grids, stained with alcoholic uranyl acetate (Weakley,1981) and lead citrate (Venable and Coggeshall,1965) and examined and photographed with a Zeiss 9S-2 electron microscope.

BEHAVIORAL/NEUROLOGICAL TESTS

Behavioral/neurological tests were performed within a 30 minute time span immediately preceding the dosing of the control and

experimental animals, excluding the days in which those animals were to be sacrificed. The tests were designed to assess changes in the animal's behavior approximately 24 hours after the previous injection. The tests were performed daily during the 20 days of dosing (days 0-19) and thereafter at 1, 8, 12, 14, 22, 29, and 92 d o r. Each animal was removed from their cage and tested with minimal space restrictions. Each animal was tested and observed as follows:

GAIT: The gait of individual animals was visually evaluated as they moved about a wooden surface. Animals were observed for any changes in gait such as splaying of either the front or hind limbs and for signs of unsteadiness and/or immobility.

TREMOR: Animals were observed for any sign of tremor.

TAIL FLICK: This test was used to evaluate each animal's response to noxious thermal stimuli (Janssen et al.,1963). Approximately one half of length of the animals tail was dipped in a water bath maintained at a temperature of 56-56.5 C and the time required for the animal to remove its tail completely from the water was recorded to the nearest tenth of a second.

STARTLE RESPONSE: The reactivity of each animal to a blast of air into their face was categorized as being hypoactive, normal or hyperactive. Each animal was tested using an aerosol can of compressed air, adjusted to the high setting and directed with an extension tube.

ACTIVITY: A subjective evaluation of activity was made based on visual observation of each isolated animal as they moved about a wooden surface. A general description was made of each animal (from extremely hypoactive to extremely hyperactive).

ROD TEST: This test checked for the animal's ability to maintain its position when placed on a fiberglass rod and was an indirect measure of balance and grip strength. A uniform fiberglass rod 43.0 cm long and 8.0 mm in diameter was suspended diagonally across a box 29.0 cm from the bottom. The animals were held by the tail and lowered onto the rod such that all 4 paws gripped the rod securely and the animal appeared stable. The animal was quickly released and the time interval between release and complete separation from the rod was recorded to the nearest tenth of a second.

RESULTS

SCIATIC NERVE

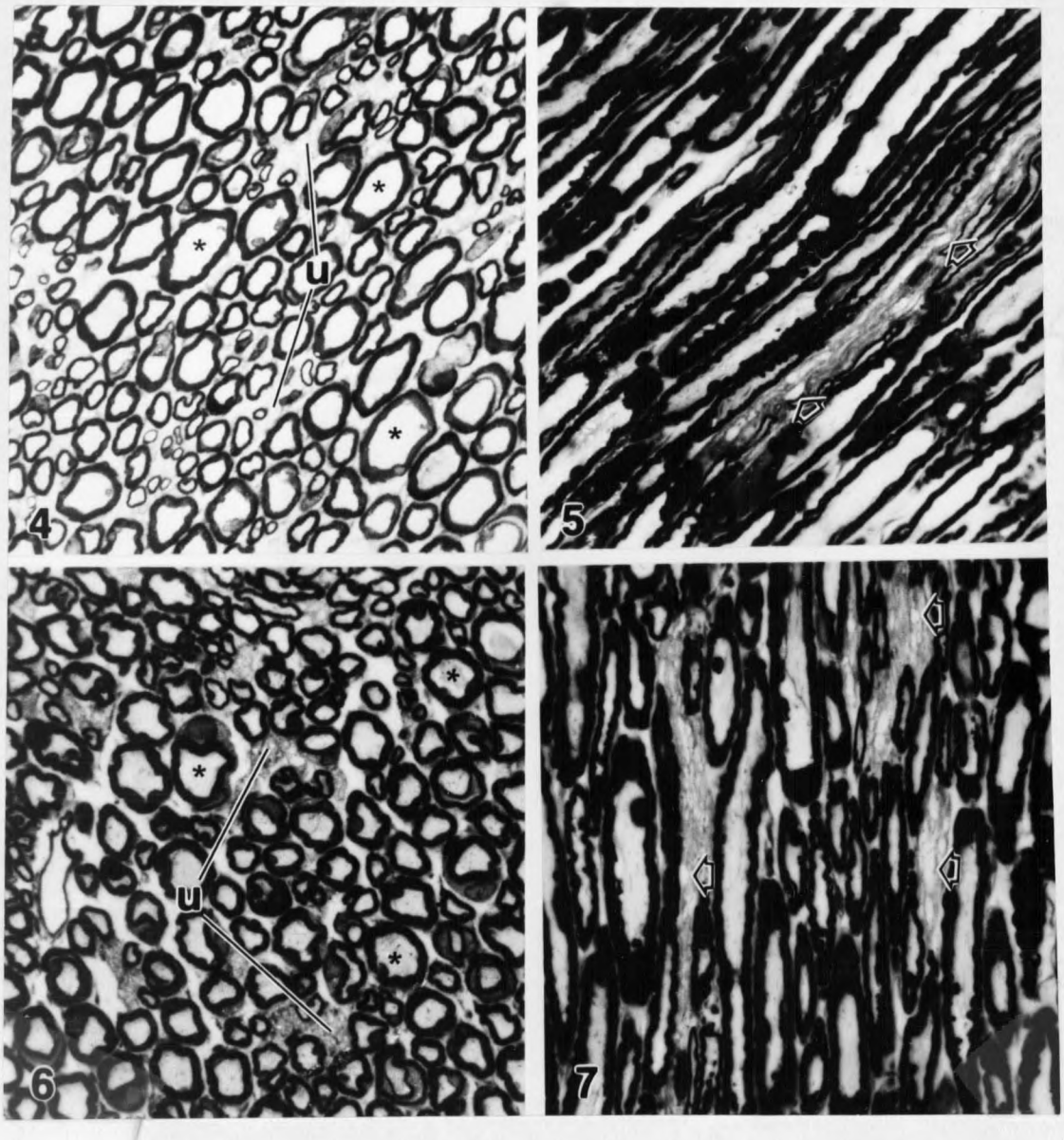
Myelinated peripheral nerve fibers, myelin, and myelin producing Schwann cells in endrin exposed animals appeared similar to those in control animals at the light (Figures 4-7) and electron microscopic levels (Figure 8) and resembled those described as normal in the literature (Peters et al.,1976; Landon and Hall,1976). In both control and experimental tissue, preparative artifact was commonly observed as focal interperiod swellings within the myelin (Figures 8 and 9).

Unmyelinated peripheral nerve fibers from control animals appeared similar to normal fibers described in the literature (Ochoa, 1976; Peters et al.,1976). These fibers were found throughout the entire cross-sectional area of the sciatic nerve and the actual number of unmyelinated axons always outnumbered the myelinated fibers. The unmyelinated axons were generally 0.5-0.6 um in diameter with an axonal membrane (axolemma) 7-8 nm thick. The axonal cytoplasm (axoplasm) contained an accumulation of microtubules (20-25 um in diameter), neurofilaments (10 um in diameter), mitochondria, and sparce smooth endoplasmic reticulum (Figures 8, 10, 11). The mitochondria were often thin and elongate in longitudinal section and almost round in cross section (Figures 8 & 10). They were often several micrometers long and contained cristae that typically

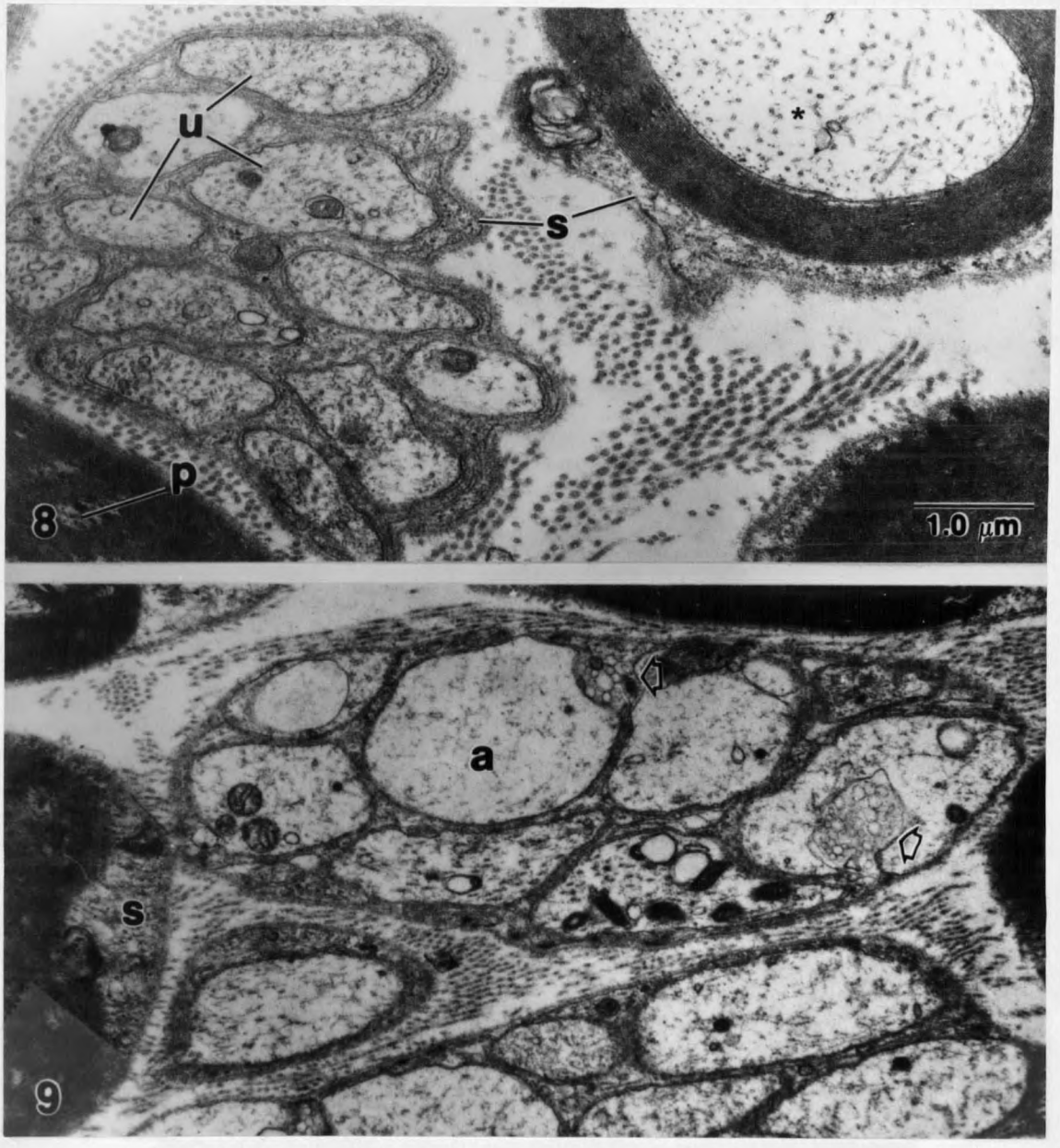
paralleled the length of the axon. The smooth endoplasmic reticulum was located between the microtubules and neurofilaments and was usually represented by a few irregular vesicles or tubules that also paralleled the length of the axon. While vesicles in unmyelinated axons are not typically described in the literature other than near the axon terminal, occasional vesicles were observed in unmyelinated fibers from control animals in this study. Such vesicles were round to hexagonal in shape, 0.1-0.2 um in diameter, and they generally occurred in groups of 2 or 3.

Unmyelinated peripheral axons were invested by Schwann cell cytoplasm, and it was not uncommon to find 10-15 axons per Schwann cell (Figures 8 & 10). The Schwann cell cytoplasm contained a variety of cellular organelles, most of which were near the perinuclear area. Typically microtubules, neurofilaments, and mitochondria were seen elsewhere in transverse sections. A basal lamina enclosed the Schwann cell (Figures 10 and 11). Collagenous fibers were frequently encountered as part of the endoneurium between the Schwann cell bundles of unmyelinated axons (Figures 8, 10, 11).

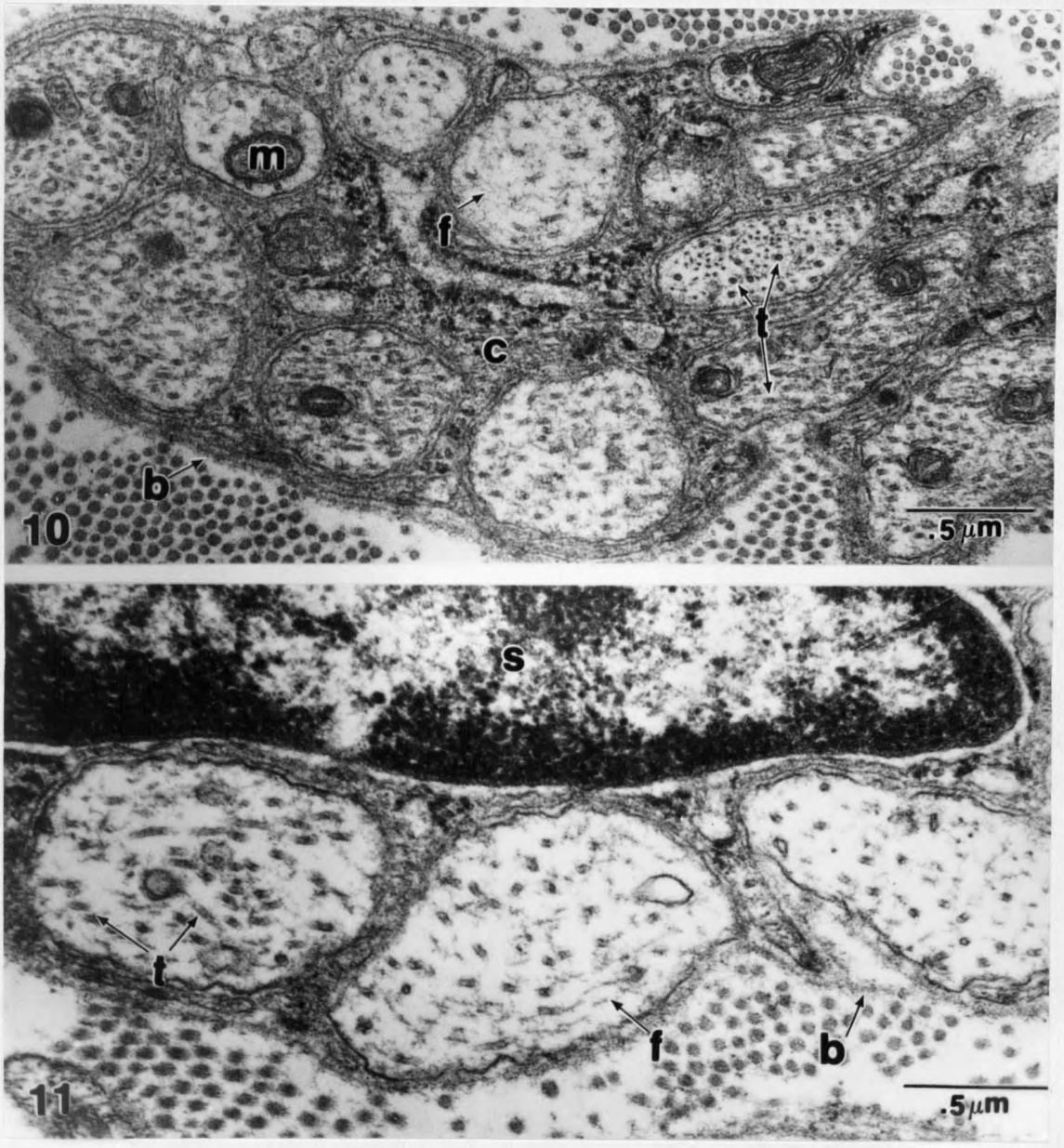
Changes were apparent in unmyelinated nerve fibers from endrin exposed animals and were most obvious after 4 days of exposure. At that time approximately 40% of the unmyelinated axonal profiles examined showed morphological changes that commonly included dissolution of microtubules and neurofilaments, axonal swelling and vesicle accumulation. It was not unusual to find all of these changes



FIGURES 4-7. Light micrographs of sciatic nerve tissue from 4 and 20 day control and experimental animals showing fields of myelinated (*) and unmyelinated (u) axons. x1500. FIGURE 4. 4 day control animal. FIGURE 5. 4 day experimental animal. Some swelling can be seen in unmyelinated axons (arrows). FIGURE 6. 20 day control animal. FIGURE 7. 20 day experimental animal. Note the swelling in some of the unmyelinated axons (arrows).



FIGURES 8 & 9. Electron micrographs of sciatic nerve from 4 day control and experimental animals showing fields of myelinated (*) and unmyelinated (u) axons and their associated Schwann cells (s). Areas of interperiod swelling (p) can be seen in some myelin sheaths. FIGURE 8. Control animal. x16,400. FIGURE 9. Experimental animal. Note the swollen axon (a) and the pockets of vesicles (open arrows). x18,200.



FIGURES 10 & 11. Electron micrographs of sciatic nerve from 4 day control animals. A normal accumulation of microtubles (t), neurofilaments (f), and mitochondria (m) can be seen within the axoplasm. Note the Schwann cell cytoplasm (c) between the axons and a basal lamina encircling the Schwann cell (b). FIGURE 10. X35,000. FIGURE 11. s = Schwann cell nucleus. x40,000.

occurring within the same axon or associated group of axons . Similar changes were observed, although less frequently, after 7, 14, and 20 d o e and after 14 d o r. At 92 d o r, most axons appeared similar to controls. These variations in ultrastructure could not be detected at the light microscopic level except for occasional swelling of unmyelinated axons (Figures 4-7).

The dissolution of microtubules and neurofilaments was frequently observed in all experimental animals sacrificed up to 14 d o r. The normal arrangement of the microtubules and neurofilaments within the axoplasm was replaced by an irregular dispersion of aggregated filamentous material throughout the axoplasm or along the periphery of the axon (Figures 9, 12-17). These filamentous "aggregates" could not be specifically identified as to their origin nor was it possible to determine if there was a specific sequence to the dissolution (tubules versus filaments). It was not uncommon for markedly swollen axons to contain very little, if any, of this material (Figures 12, 13, 17).

Axonal swelling was a consistent finding in experimental animals from 4 d o e to 14 d o r, and was most obvious after 14 d o e (Figures 24 and 25). The swelling was often accompanied by dissolution of microtubules and neurofilaments (Figures 12-14, 17, 19) and frequently involved numerous axons ensheathed within the same Schwann cell (Figures 9, 12, 13). In some cases, longitudinal sections revealed alternating swollen and constricted regions within the same axon with the constrictions often containing dense accumulations of filamentous

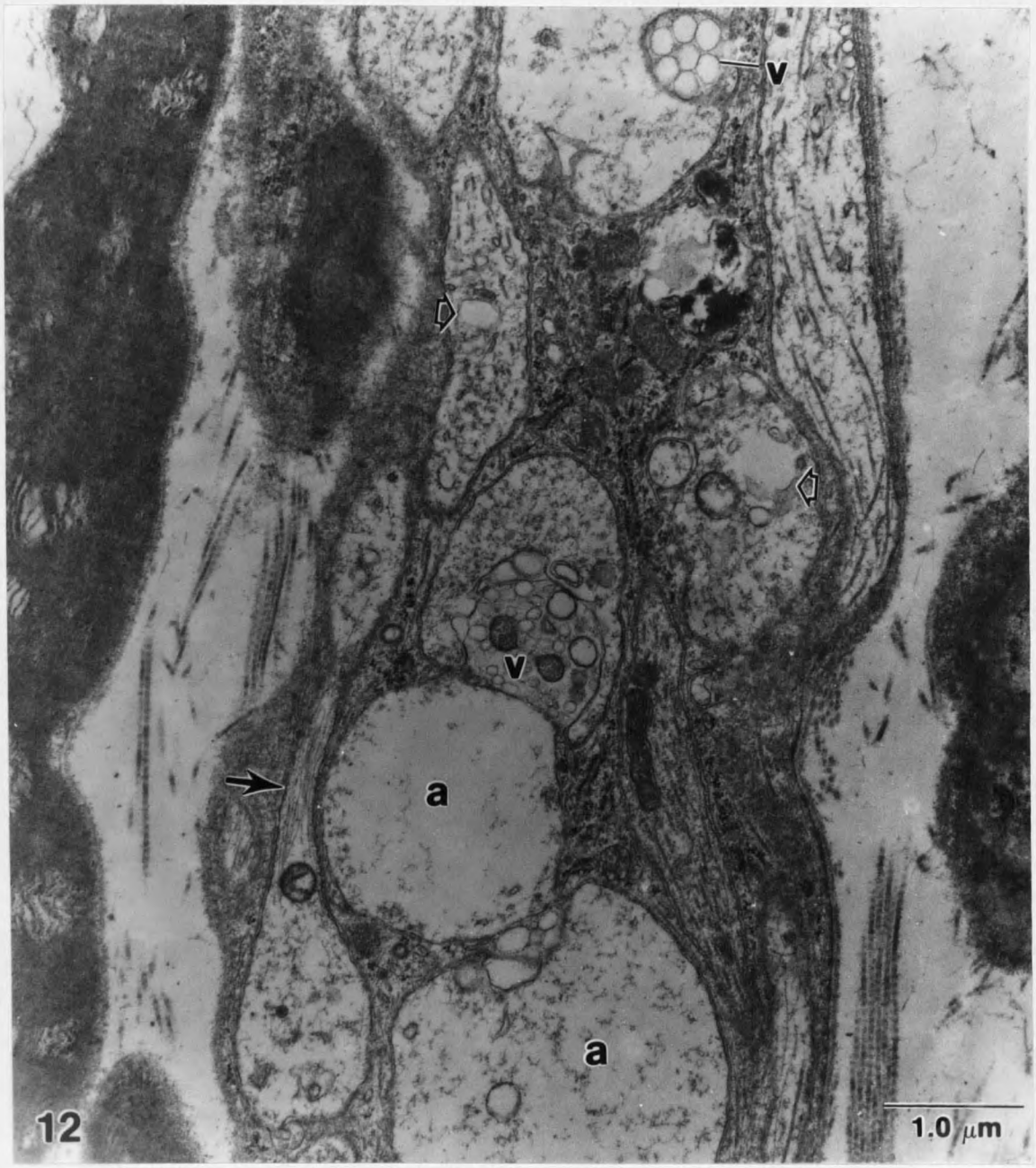
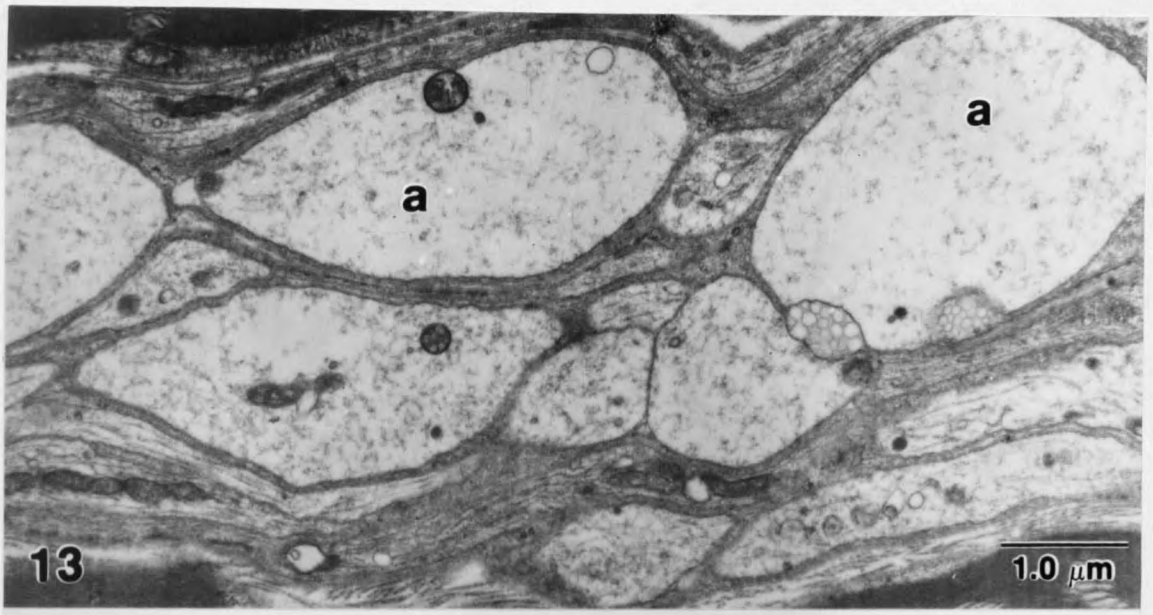
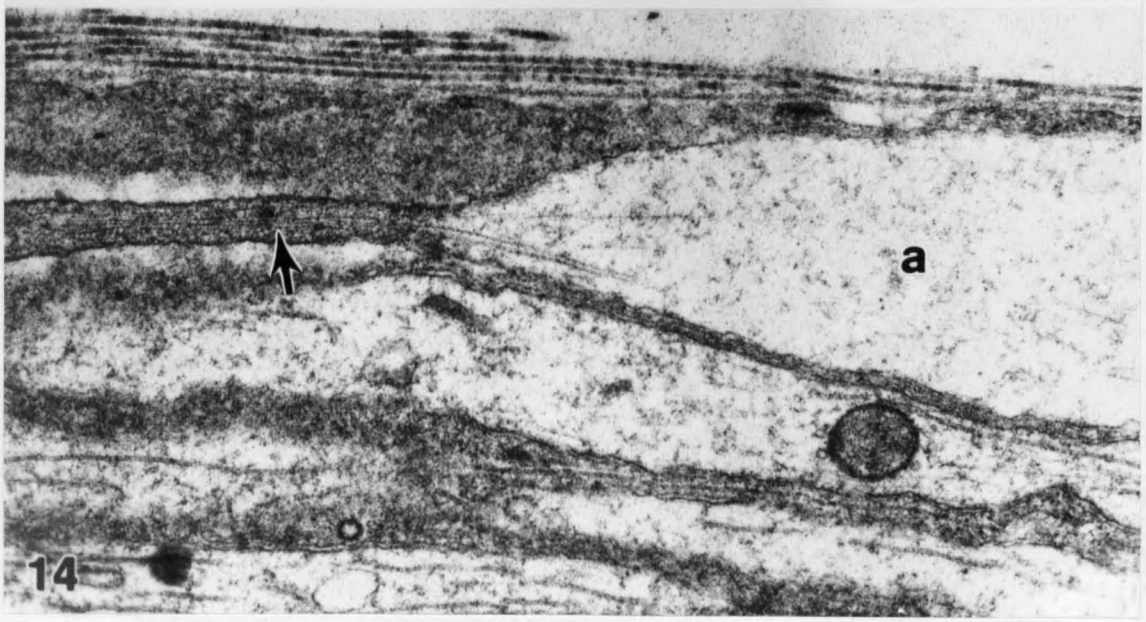
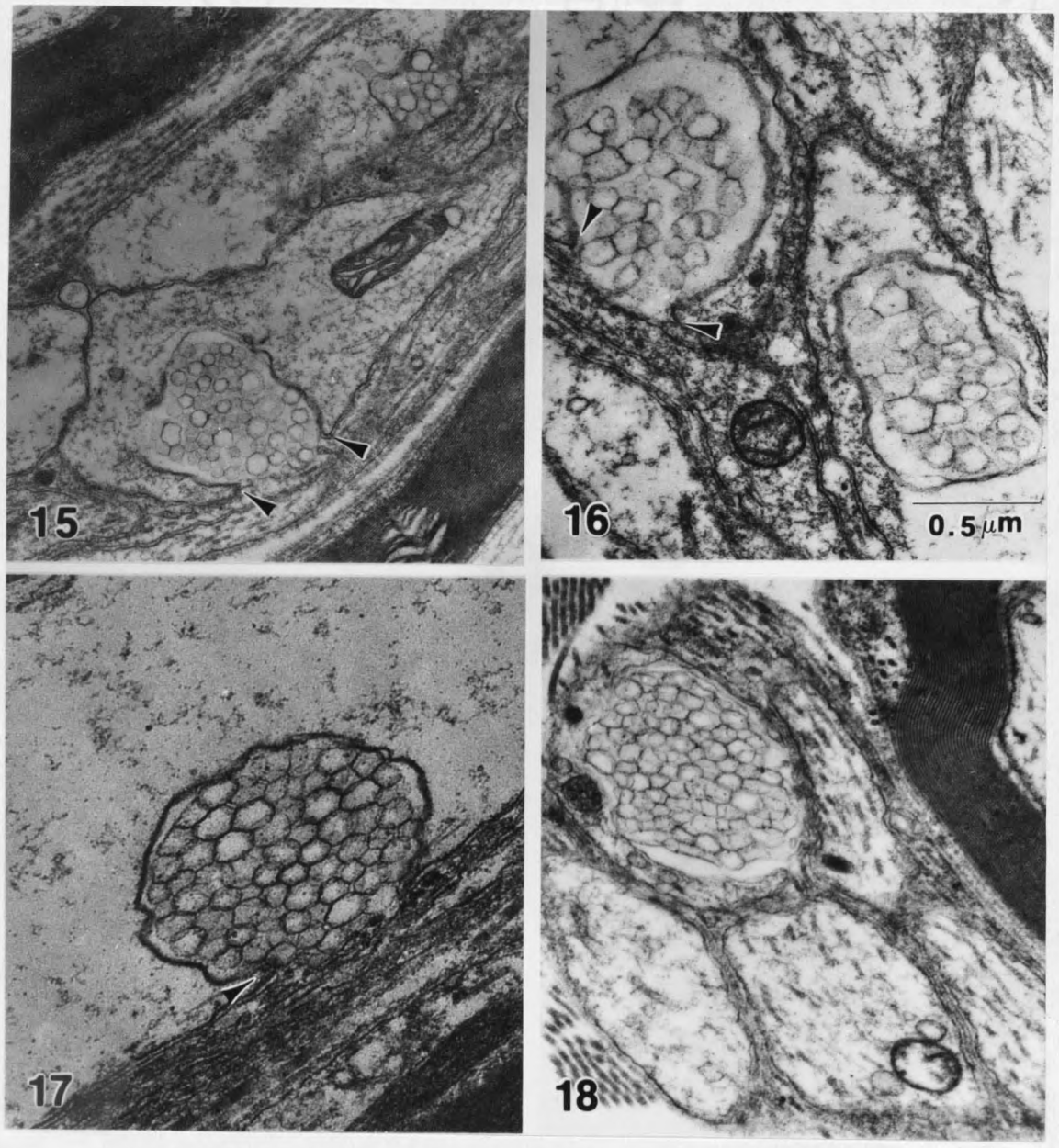


FIGURE 12. Sciatic nerve from a 4 day experimental animal showing various examples of pathology in unmyelinated axons. Note the dissolution of microtubules and neurofilaments within the swollen axons (a), the accumulations of vesicles (v) and the swollen SER (open arrows). Part of one axon shows a constricted region with a high density of microtubules and neurofilaments (arrow). x20,000.





FIGURES 13 & 14. Sciatic nerve from 4 day experimental animals. FIGURE 13. Note the swollen axons (a) and accumulations of vesicles. x16,000. FIGURE 14. A swollen axon (a) and adjacent constriction (arrow) with a dense accumulation of microtubules and neurofilaments. x28,000.



FIGURES 15-18. Sciatic nerve from experimental animals showing examples of vesiculation associated with unmyelinated axons. Note the accumulations of vesicles and the invaginated axolemma (arrows). FIGURE 15. 4 day animal. X24,000. FIGURE 16. 4 day animal. x32,000. FIGURE 17. 4 day animal. x40,000. FIGURE 18. 7 day animal. x28,000.

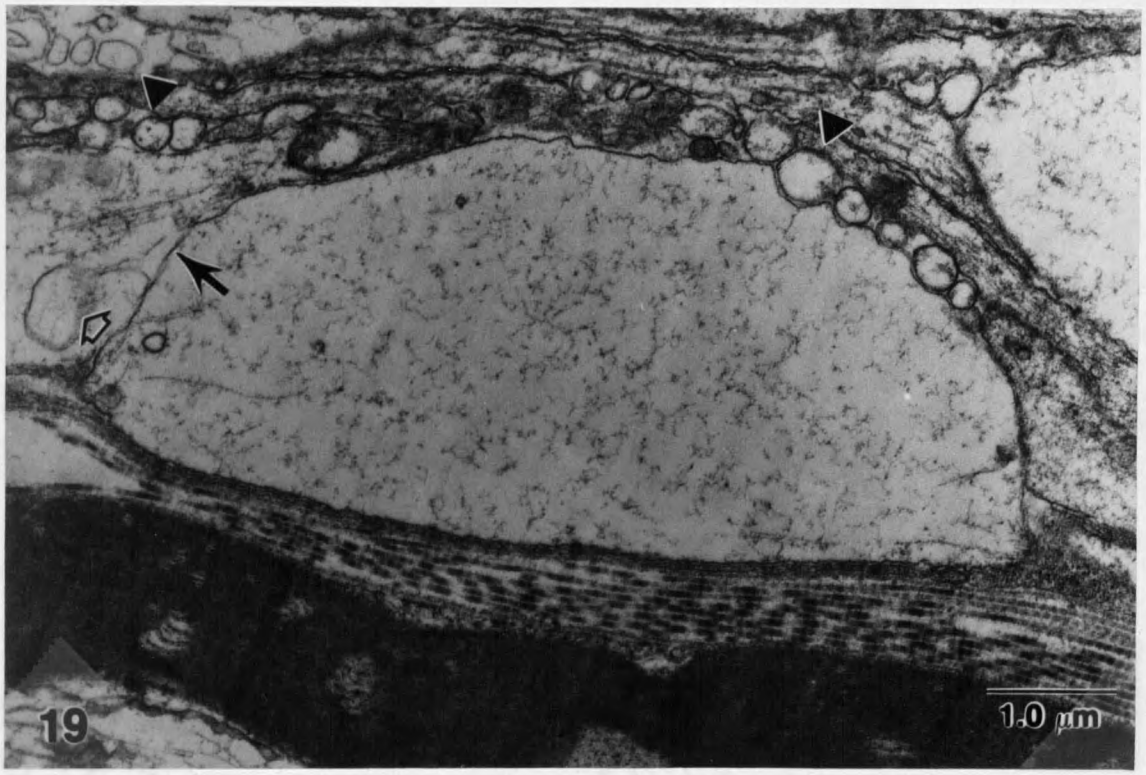
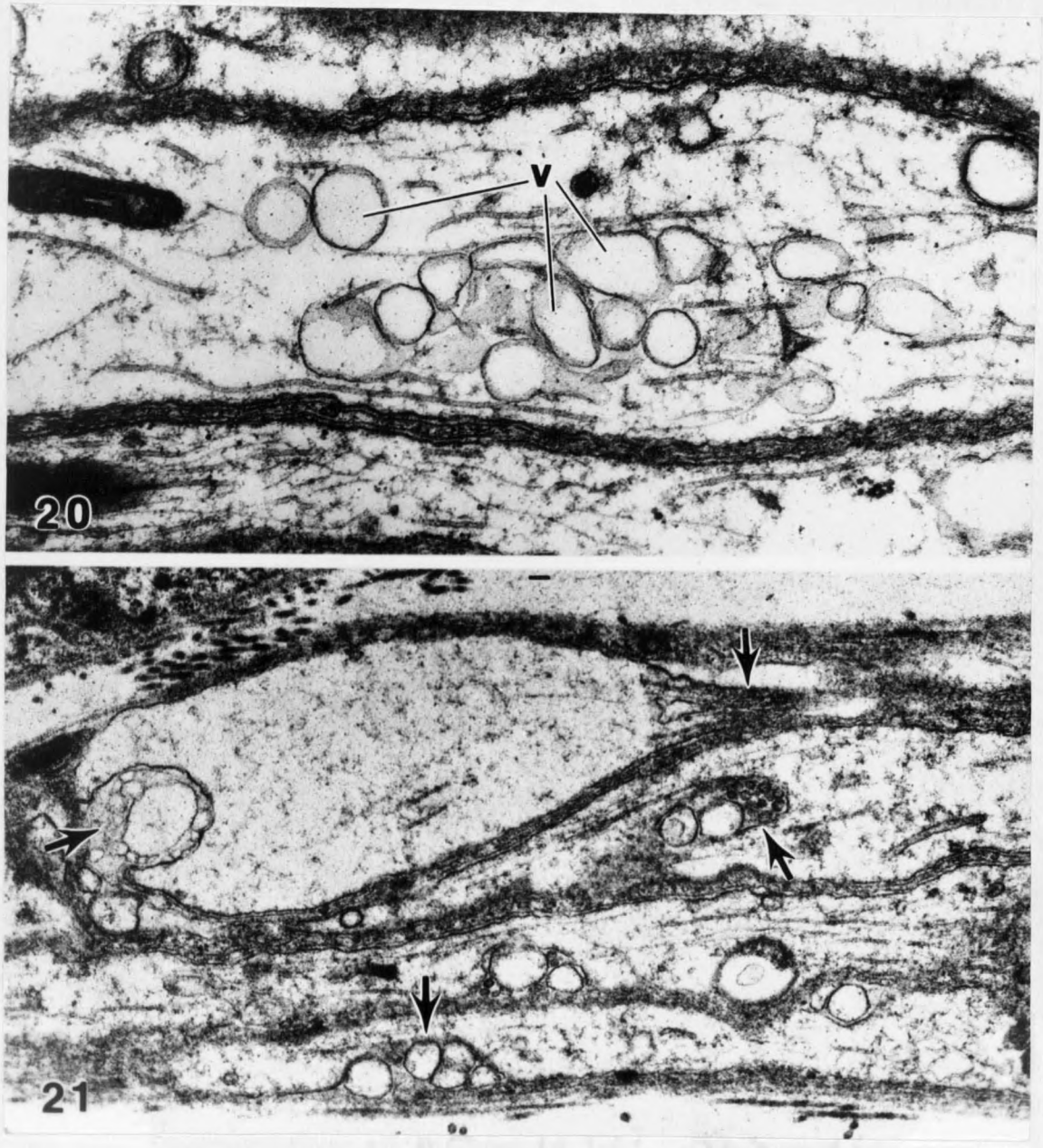


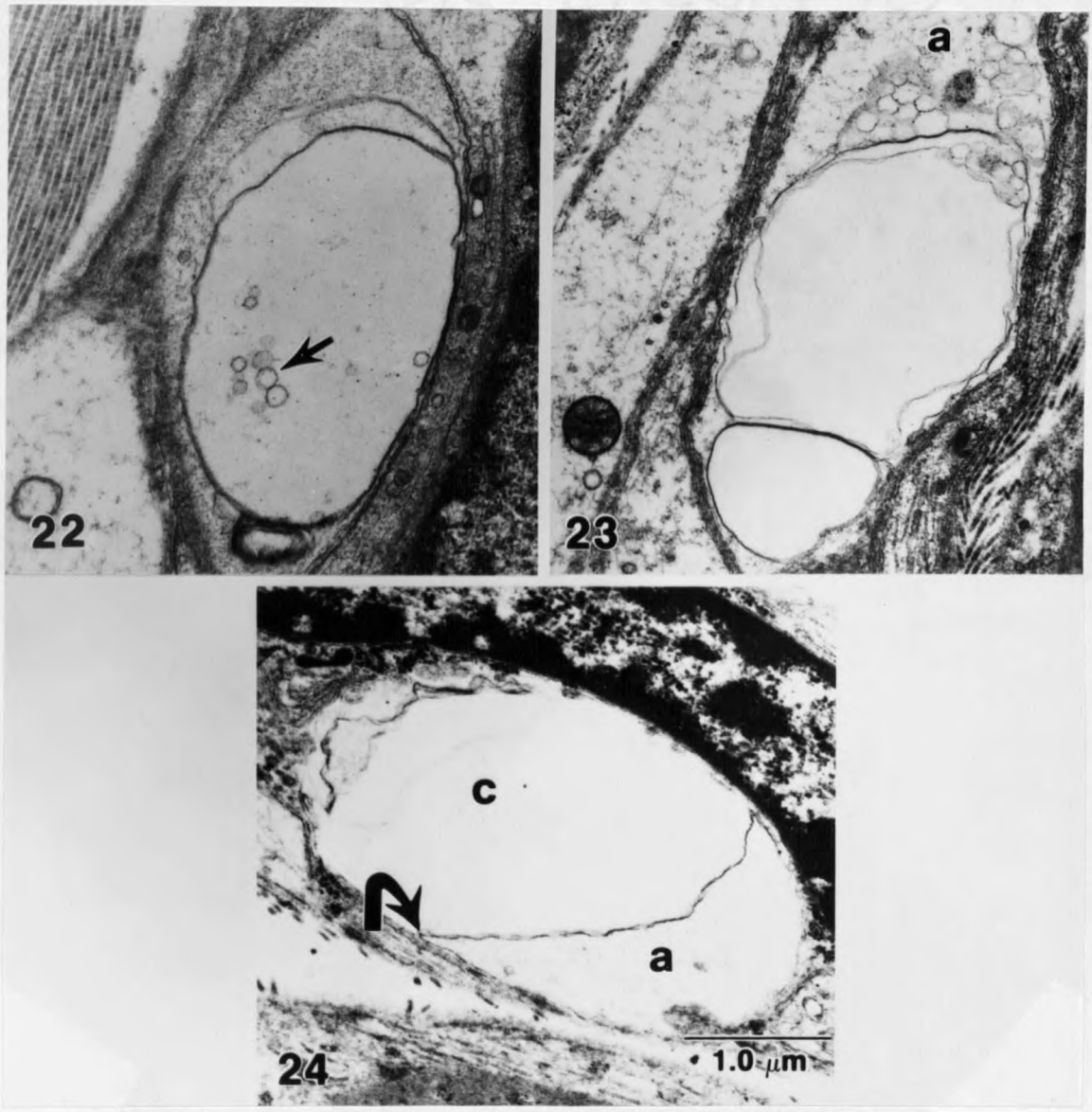
FIGURE 19. Sciatic nerve from a 4 day experimental animal. Note the location of the vesicles (triangles), the membranous transection of the axon (arrow) and the axonal vesicle (open arrow). x16,400.

material (Figures 12, 14, 21). Many of the swollen regions were as large as 2.5-3.0 um in diameter (Figures 12, 13, 16, 24, 25).

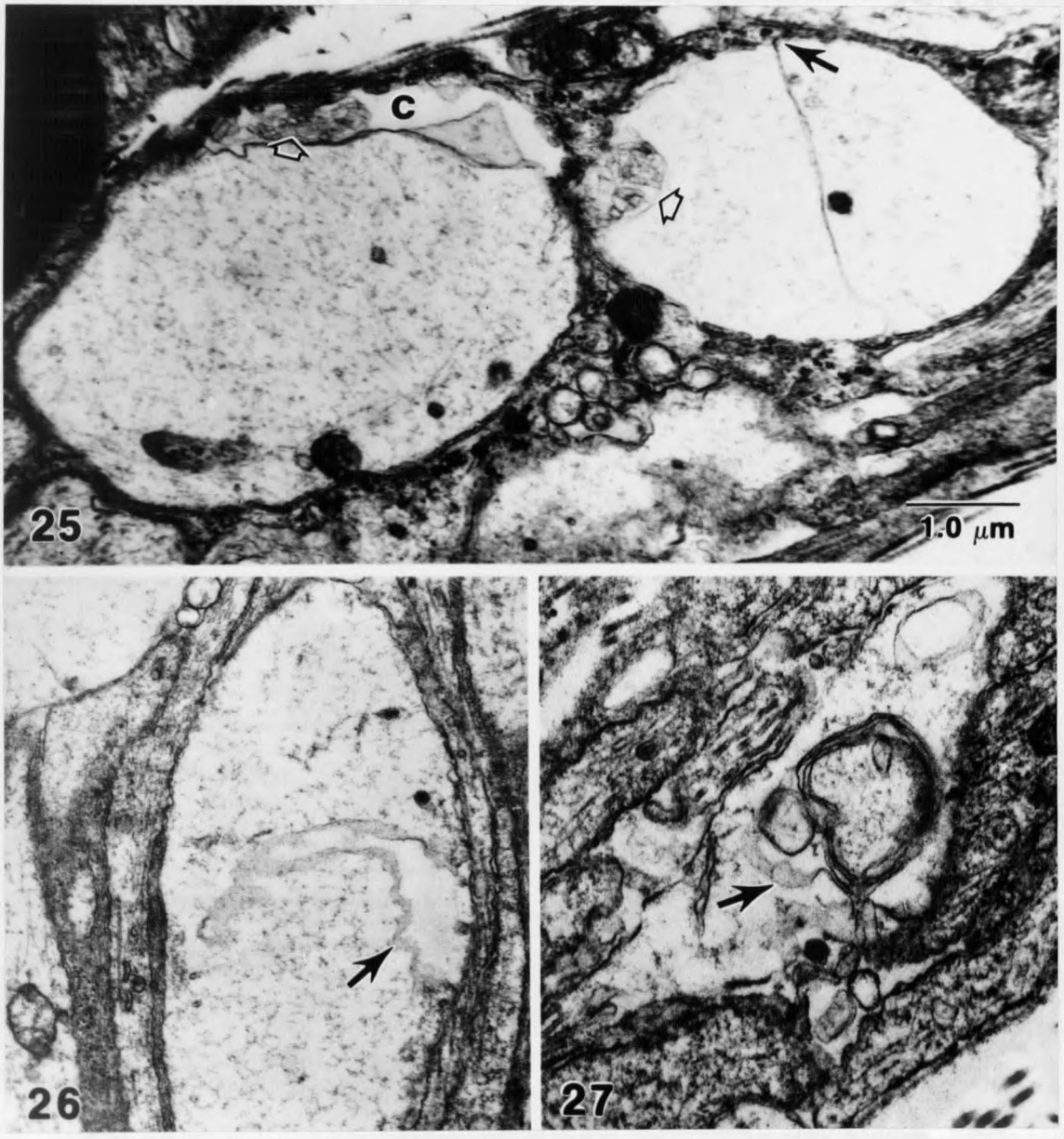
Membrane bound vesicles were often observed in association with affected axons. Pockets or aggregates of densely accumulated vesicles were often seen in the Schwann cell cytoplasm or in the adjacent periaxonal space (Figures 9, 12, 13, 15-19, 21, 25, 26, 28). Such pockets of vesicles frequently invaginated into the axolemma and/or Schwann cell membrane (Figures 9, 12, 13, 15-17, 21, 28, 30). The number of such vesicles in these pockets ranged from as few as 3-4 to as many as 40 or more. The vesicles were 0.1-0.2 um in diameter, round, oval, or hexagonal in shape, and contained an electron lucid matrix (Figures 12-17). An occasional vesicle contained a core material of intermediate electron density (Figures 9, 15, 18, 28). Others had a clear core and were invested by a thick electron dense "halo" of material (Figures 15 and 16). The pockets of vesicles occured singly (Figures 9, 15-17), as several pockets within the same axon (Figure 13), or as multiples within the same associated group of axons (Figures 12, 15, 16). Some accumulations of these vesicles almost completely filled the axonal profile or area that appeared to have previously contained an axon (Figure 18). In other examples the vesicles were present in rows beside the axon but appeared to be located either within the periaxonal space or within the Schwann cell cytoplasm (Figures 19, 21, 25). These vesicles were also 0.1-0.2 um in diameter but had an electron lucid core occasionally containing



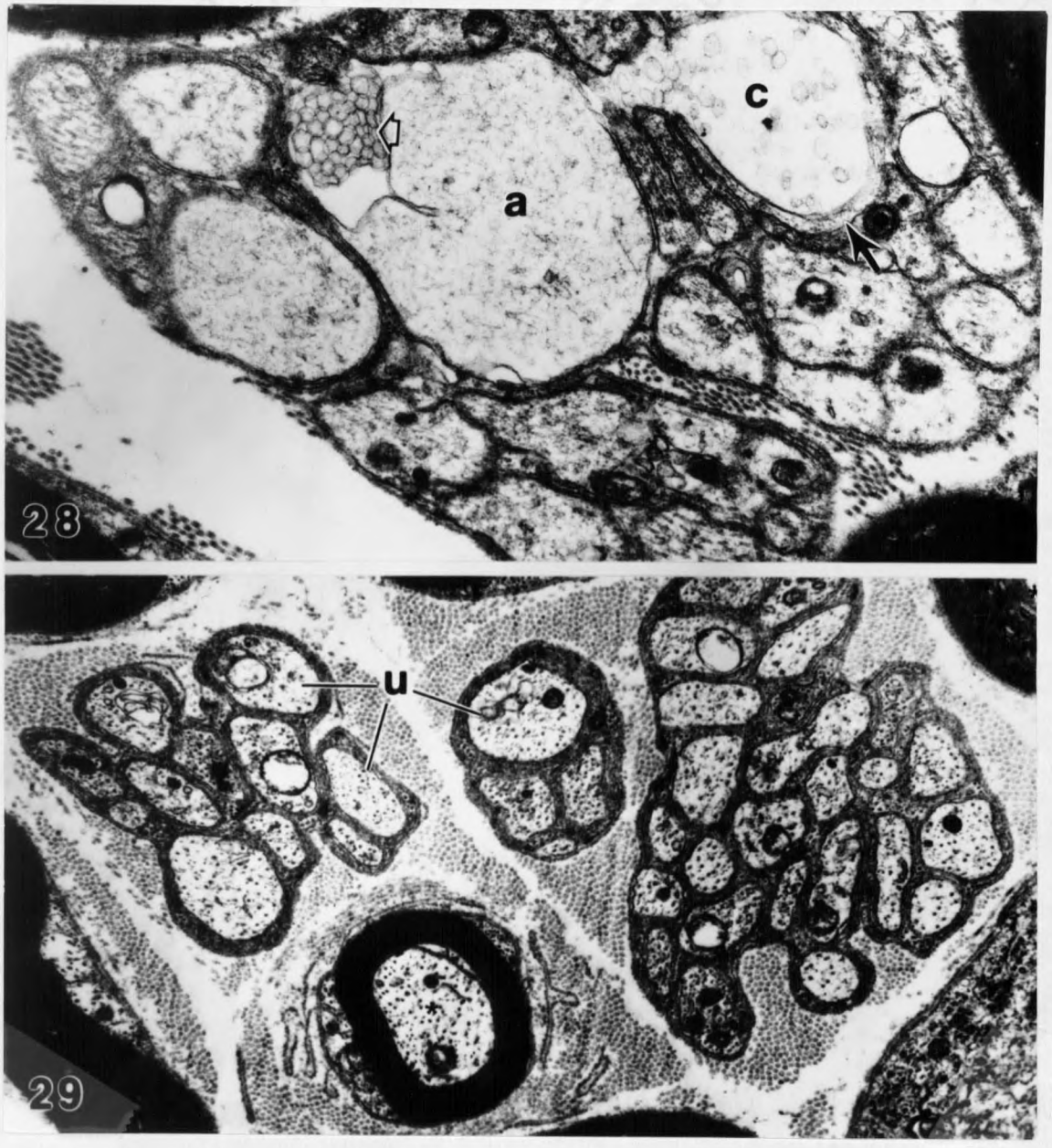
FIGURES 20 & 21. Sciatic nerve from 4 day experimental animals. FIGURE 20. An example of an accumulation of axonal vesicles. x36,000. FIGURE 21. Numerous examples of endrin induced changes (arrows) within a group of associated axons. x28,000.



FIGURES 22-24. Sciatic nerve from experimental animals showing various examples of vacuolation of unmyelinated axons. FIGURE 22. 4 day animal. Note the small vesicles within the vacuole (arrow) x28,000. FIGURE 23. 4 day animal. Note the accumulation of vesicles within the vacuole and adjacent axon (a). x24,000. FIGURE 24. 14 day animal. Note the clear vacuolated area (c) which appears to displace the axon (a). The axolemma appears invaginated (arrow). x20,000.



FIGURES 25-27. Sciatic nerve from experimental animals. FIGURE 25. 14 day animal. Note the transection of the axonal profile and the invagination of the axolemma (arrow). A small vacuole (c) which contains vesicles (open arrows) and other debris can be seen crowding the axon profile. x15,500. FIGURE 26. 4 day animal. Note the swollen membranous profile (arrow) within the axon. x24,000. FIGURE 27. 20 day animal. Note the swollen membranous profile (arrow) within an apparent axonal area. x36,000.



FIGURES 28 & 29. Sciatic nerve from experimental animals. FIGURE 28. 34 day animal. Note the clear vacuolated area containing small vesicle (c) within the area previously occupied by the axon. The axon appears to be displaced (arrow). An adjacent axon (a) is swollen and the axolemma is invaginated by a pocket of vesicles (open arrow). x16,400. FIGURE 29. 112 day animal. The unmyelinated (u) and myelinated (*) axons generally appear normal except for an accasional clear vesicle. x24,000.

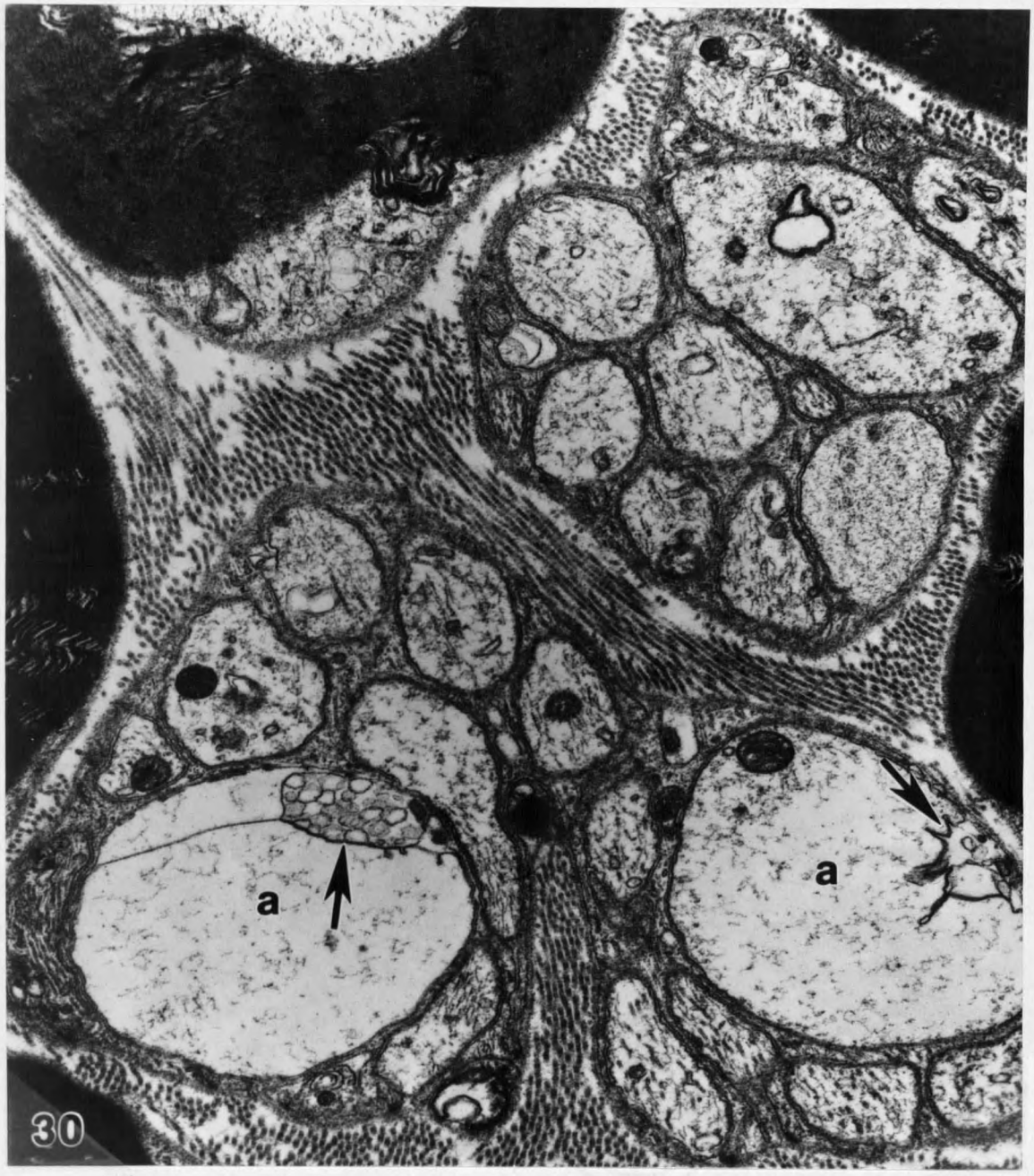


FIGURE 30. Sciatic nerve from a 112 day experimental animal. Note that most unmyelinated axons appear normal although two axons (a) are swollen and are invaginated by pockets of vesicles (arrows). x20,000.

isolated accumulations of electron dense material (Figures 19 and 25). The vesicles within the axon were encountered less frequently than those in other locations. The vesicles were often of irregular shape with a thick ring of intermediate electron density surrounding a clear core (similar to those previously described). They were approximately 0.2-0.4 um in diameter and occurred singly or in groups (Figures 9, 20, 23, 24). It was not uncommon for a particular axon or group of axons to exhibit various combinations of these types of vesicle accumulations (Figures 9 and 21).

Occasionally large membrane bound clear vacuoles were observed within the axon. Such vacuoles often occupied a large part of the axonal profile, commonly displacing much of the axon (Figures 22-24). In some instances these vacuoles contained small circular vesicles (Figure 23), accumulations of irregularly shaped vesicles, or other debris of unknown origin (Figures 24 and 25) within a clear, apparently watery, matrix. The source of the membrane enclosing such vacuoles could not be determined, but in some cases it appeared to include the axolemma (Figures 24 and 25).

Sometimes a thin "membrane" appeared to traverse the entire swollen axonal profile (Figures 19 and 25) and appeared to be an infolding of the axolemma. Swelling of the smooth endoplasmic reticulum (Figure 12) and membranous profiles (Figures 26 and 27) were infrequently encountered in affected axons. The appearance of the mitochondria in unmyelinated axons from experimental animals appeared

similar to controls in all tissues examined (Figures 9, 14-16)

By 14 d o r much of the tissue appeared to resemble that from control animals although changes similar to those encountered at earlier times were still present. These included axonal swelling, dissolution of microtubules and neurofilaments and the appearance of accumulations of membrane bound vesicles. In one instance an area thought to have previously contained an axon was completely devoid of axoplasmic material and contained only small vesicles that appeared to be extruding from the area (Figure 28). It is possible that the axolemma was infolded in this area due to the presence of a large vacuole that has displaced the axon.

By 92 d o r, most tissue from the experimental animals was similar to that in controls (Figure 29) although isolated instances of axonal swelling and vesicle accumulation were still present (Figure 30). It could not be determined if permanent axonal degeneration had taken place although there appeared to be no indication of such.

OTHER TISSUES

Light and electron microscopic examination of skeletal (gluteal) muscle, cardiac muscle and optic nerve from animals after 4 and 20 d o e revealed no obvious morphological changes when compared to that of control tissue (Figures 31-39).

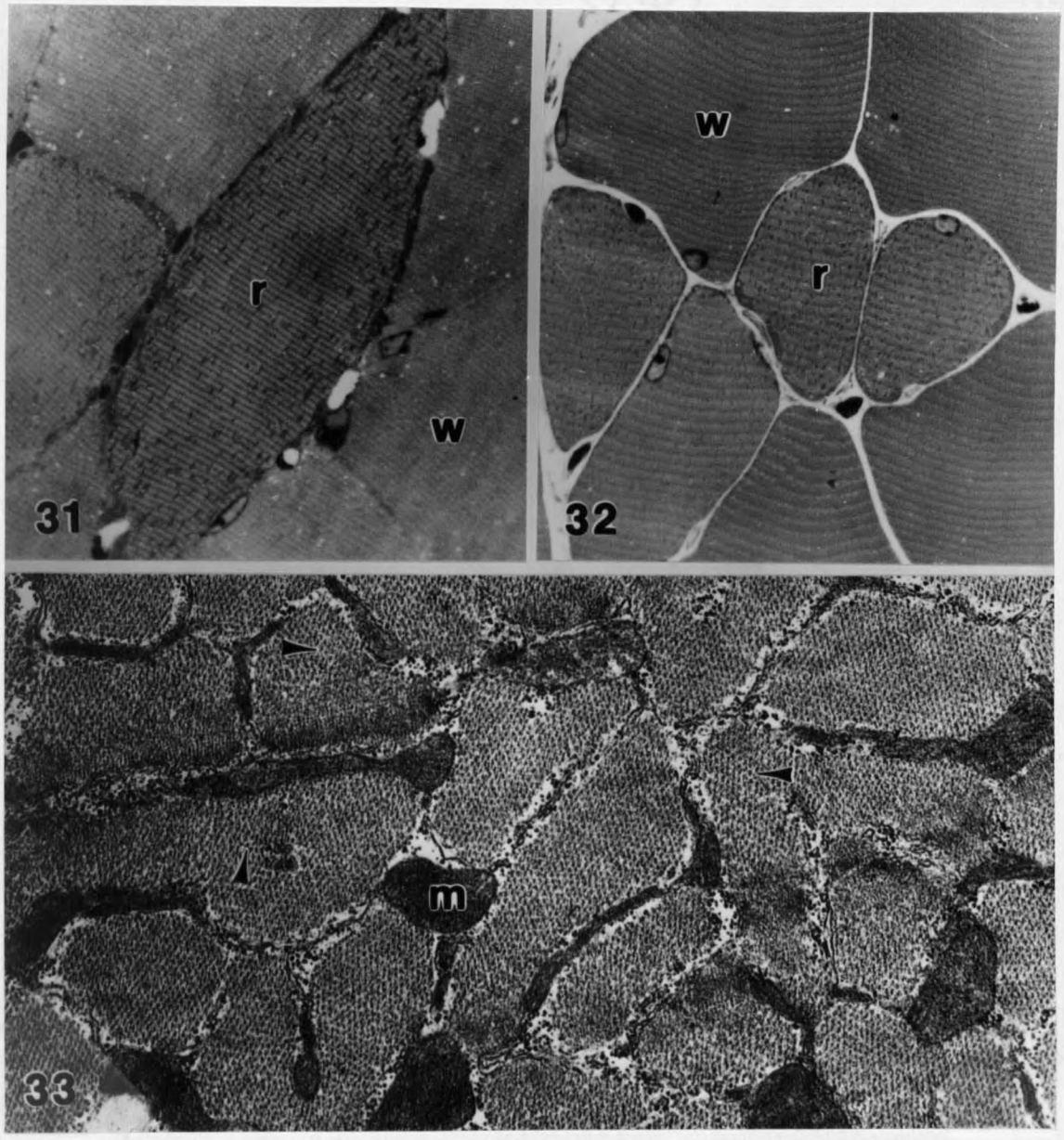
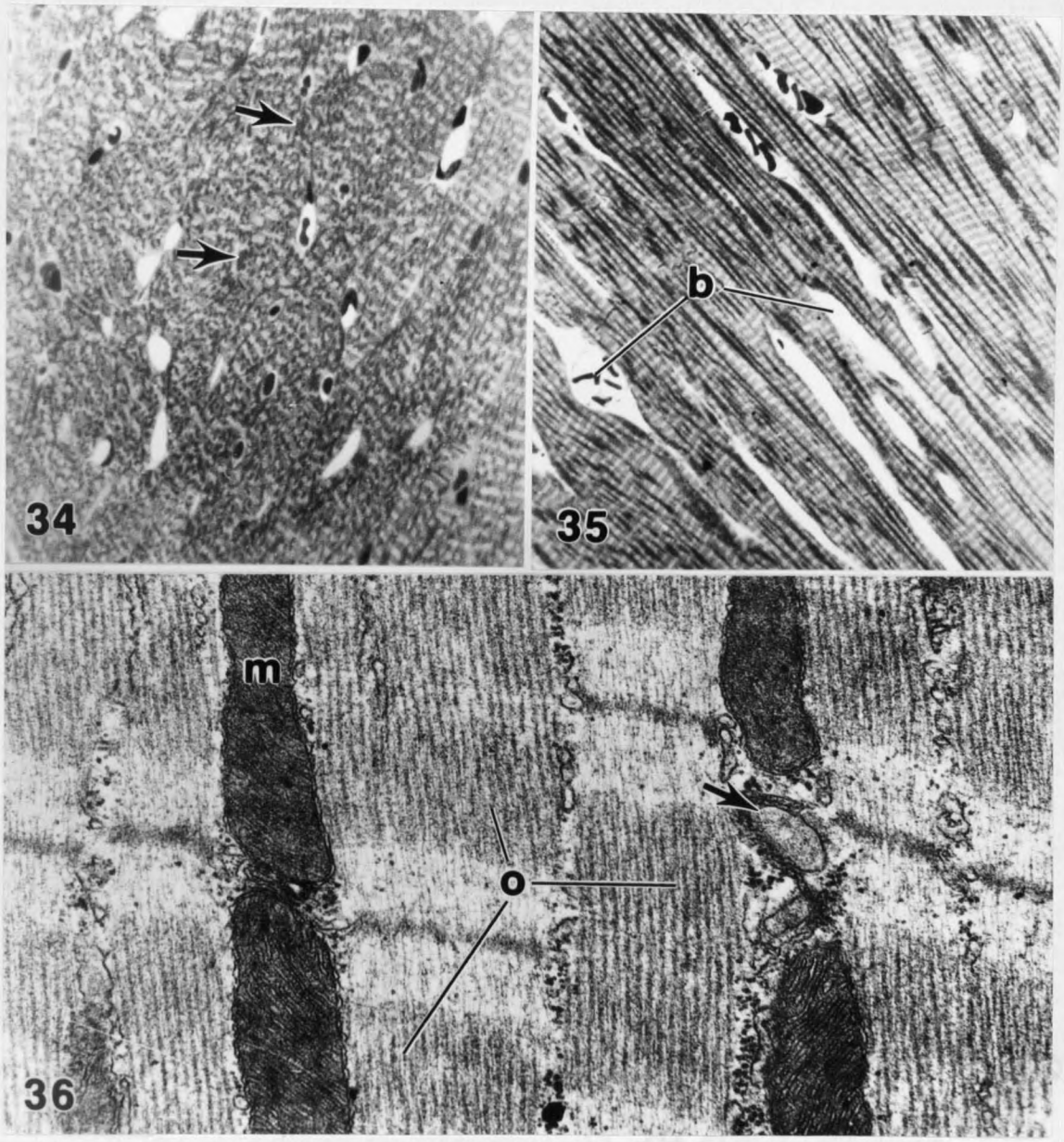
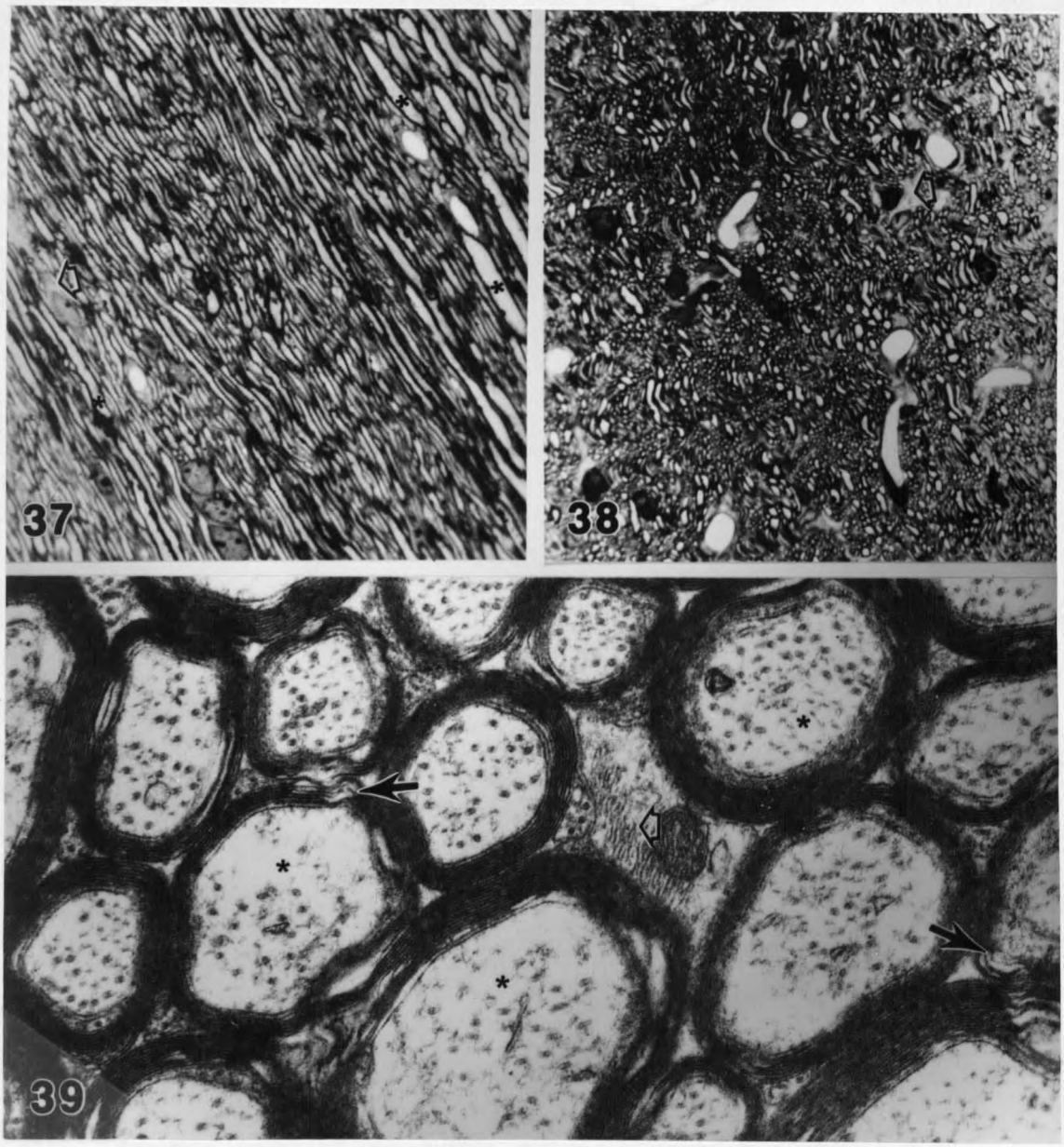


FIGURE 31-33. Light and electron micrographs of skeletal muscle from 20 day animals. Note the resemblence of the red (r) and white (w) muscle fibers in experimental tissue to those in the controls. FIGURE 31. Control animal. x1500. FIGURE 32. Experimental animal. x1500. FIGURE 33. Normal appearing tissue from an experimental animal. Note the dense accumulation of mitochondria (m) and myofilaments (arrows) in this red muscle fiber. x26,600.



FIGURES 34-36. Light and electron micrographs of cardiac muscle from 20 day animals. Note the resemblence of the experimental tissue to that of controls. Numerous blood vessels (b) can be seen throughout the field. FIGURE 34. Control animal. Note the dense accumulation of mitochondria within the muscle bundles (arrows). x1500. FIGURE 35. Experimental animal. x1500. FIGURE 36. Experimental animal. The mitochondria (m), myofilaments (o) and t-tubules (arrows) appear normal and glycogen is prominent. x33,300.



FIGURES 37-39. Light and electron micrographs of optic nerve from 20 day animals. The field contains numerous myelinated axons (*) and glial cell processes (open arrows). FIGURE 37. Control animal. x1500. FIGURE 38. Experimental animal. x1500. FIGURE 39. Experimental animal. Some preparative artifact can be seen in the form of interperiod swelling in the myelin sheaths (arrows). x36,000.

BEHAVIORAL/NEUROLOGICAL EFFECTS

Experimental animals appeared hyperactive within 24 hours after the first injection and this symptom continued throughout the exposure phase of the experiment and for over 2 weeks into the recovery phase. Hyperactivity was most obvious 4-5 hours after each injection and tended to diminish slightly over time until the next injection. Such behavior was characterized by animals running constantly about the cage and regularly climbing the wire cage lid and moving back and forth or circling on it while hanging upside-down. In addition, during the time the animals were being injected or weighed, animals climbed out of the cage at every opportunity. However, when the animals were placed in an isolated setting, usually following handling or after their initial response to a startle stimulus, they exhibited a hypoactive and timid behavior. Such animals often tended to remain stationary for 10-15 seconds. It was not uncommon for animals to remain this way until forced to move, at which time they exhibited only a timid exploratory behavior. Frequently the animals displayed a change in posture characterized by a hunching of their thoracic spine, both while stationary, and while walking. This stationary activity and postural change was obvious after 6 d o e and was still evident after 14 d o r.

Piloerection was apparent in 35% of the experimental animals by 16 d.o.e.and a similar percentage of animals remained so throughout the remainder of the exposure period.

The gait of experimental animals appeared similar to controls until 13 d o e when some animals exhibited a general splaying of the hindlimbs. This was most obvious at 17 d o e when approximately 65% of the animals were involved, but decreased to 28% by 18 d o e, 23% at 20 d o e and continued to decline until the gait appeared normal in all animals by 14 d o r. Control animals never displayed any signs of gait abnormality. An erect tail posture was observed in exposed animals and was consistently noted between 1 and 15 d o e. The tail in these animals was stiff and seldom contacted the ground whereas the tail of control animals was flaccid and in contact with the walking surface.

Tremors or convulsions were never observed in either control or experimental animals. Two animals developed what appeared to be hiccups. In one animal these "hiccups" were obvious at 19 d o e, 12 d o r and 29 d o r, at which time it was noted that the animal appeared very withdrawn and hypoactive. The other animal exhibited similar behavior after 14 d o r and developed shakes of short duration immediately following the "hiccup".

Control animals showed very little response to startle stimuli, commonly only reacting with a slight blinking of their eyes. The number of animals exposed to endrin that displayed a hyperactive startle reaction increased during the exposures. After 1 d o e 31% of the animals were hyper-responsive and by 17 d o e, 88% were

hyper-responsive. That percentage declined to 25% by 29 d o r, and finally all animals resembled controls by 92 d o r. In extreme instances a puff of air directed at animals in their open cages caused the animals to jump completely out of the 6 inch high cage.

The results of the tail flick test are summarized in Figure 40. There appears to be no significant or consistent trend to the data. Only after 8, 12 and 20 d o e was there a statistically significant (Student t-test) difference between controls and experimentals and in all three cases the experimental animals responded more quickly than controls.

Results of the rod test are summarized in Figure 41. The inability of experimental animals to maintain their position on the fiberglass rod was a consistent finding. The number of animals immediately falling off the rod was much greater in the experimental group than in controls from 1 to 4 d o e and then again on days 8-20. After day 13, none of the controls fell off immediately. By day 15 none of the experimental animals could maintain their position on the rod although some could still grasp the rod and hang there until finally falling off. By day 16, 56% of the experimental animals fell off the rod immediately. Most experimental animals trembled when placed on the rod. By 14 d o r, most of the experimental animals could maintain their balance on the rod for 2-3 seconds before falling off and by 29 d o r all animals tested had no trouble maintaining their position on the rod for up to 10 seconds which was the duration

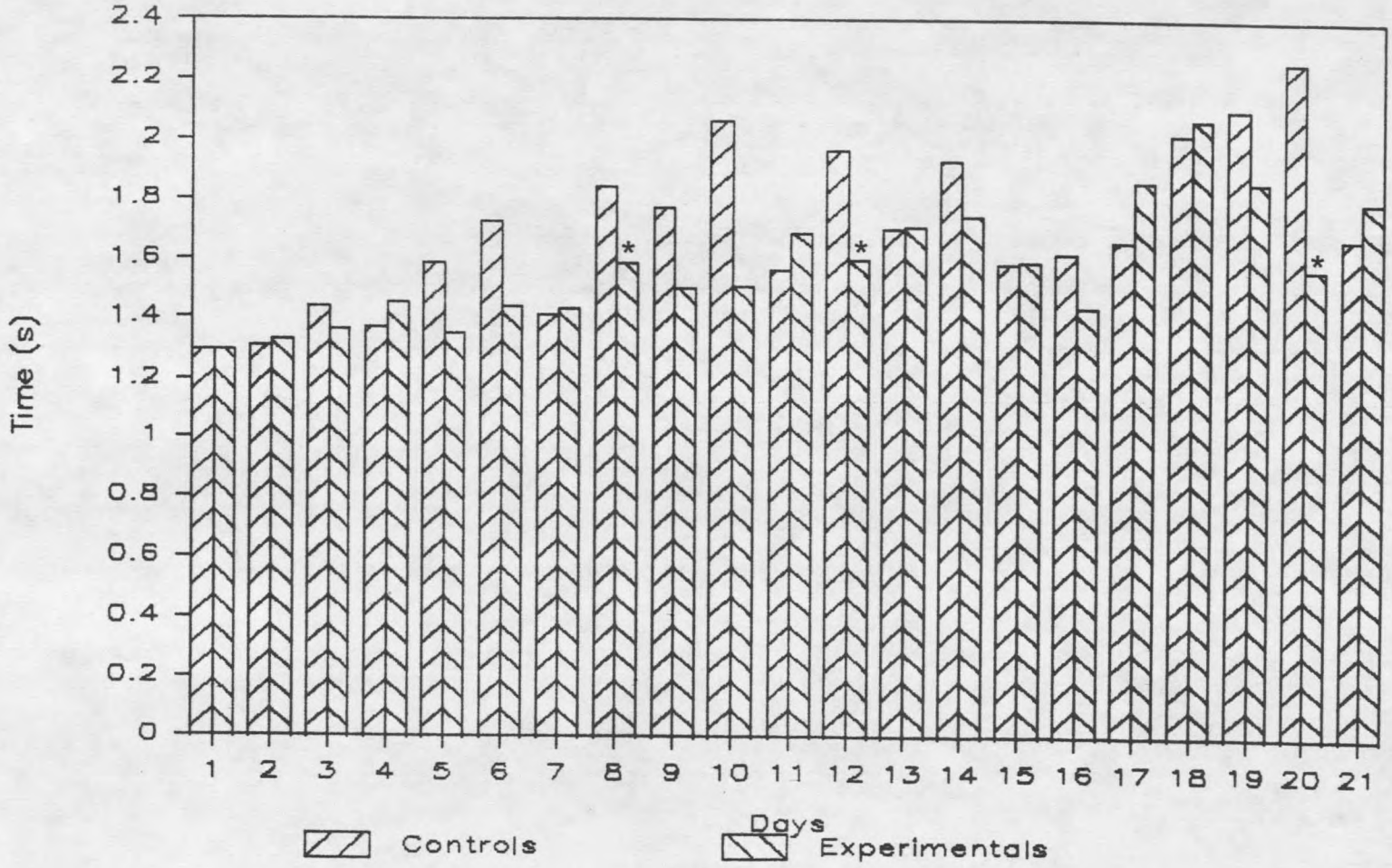


FIGURE 40. Summary of the tail flick test. * indicates a statistically significant difference.

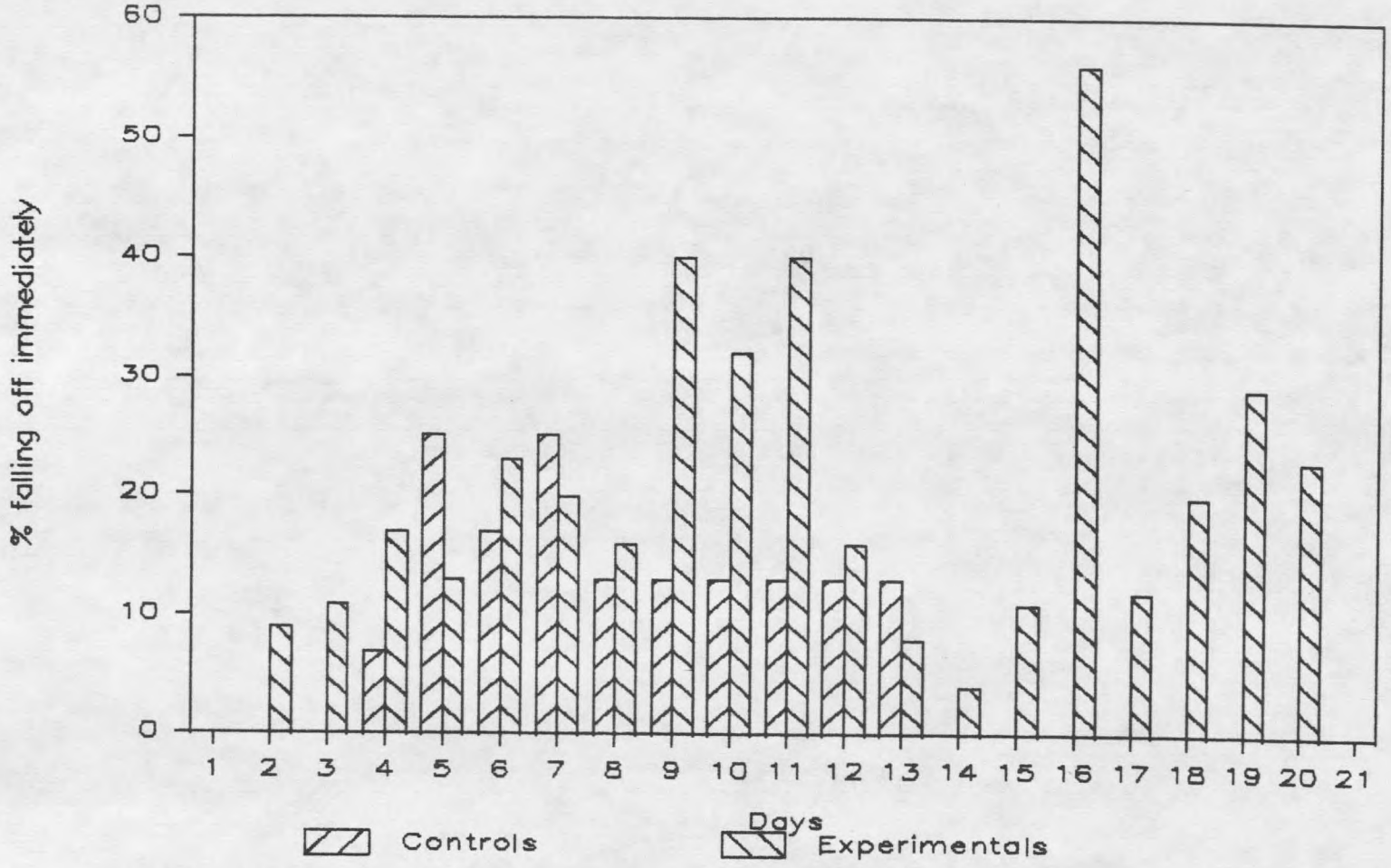


FIGURE 41. Summary of the rod test. No control data indicates that no animals fell off immediately.

of the test.

The percent of change in the animals weight over the 112 day study period is depicted in Figure 42. The experimental animals do not appear to gain weight as rapidly as the controls although the only statistically significant (Student t-test) difference was on day 20.

Thirty-five experimental and 15 control animals were used at the start of the study. One control animal, representing 6.7% of the control animals died on day 8 of the study. Eight endrin exposed animals (23%) died during the 20 days of exposure, 5 between days 17 and 20. There was no mortality in any animals after the exposure regime was finished. On day 12, approximately 4 hours after increasing the dosage from 2.0 mg/kg to 4.0 mg/kg, two animals died. Figure 43 shows the number of animals that died as a percentage of those remaining. The greatest mortality occurred on day 18, when 19% of the animals died. Postmortem examination of dead animals revealed no sign of death due to hemorrhage caused by injection.

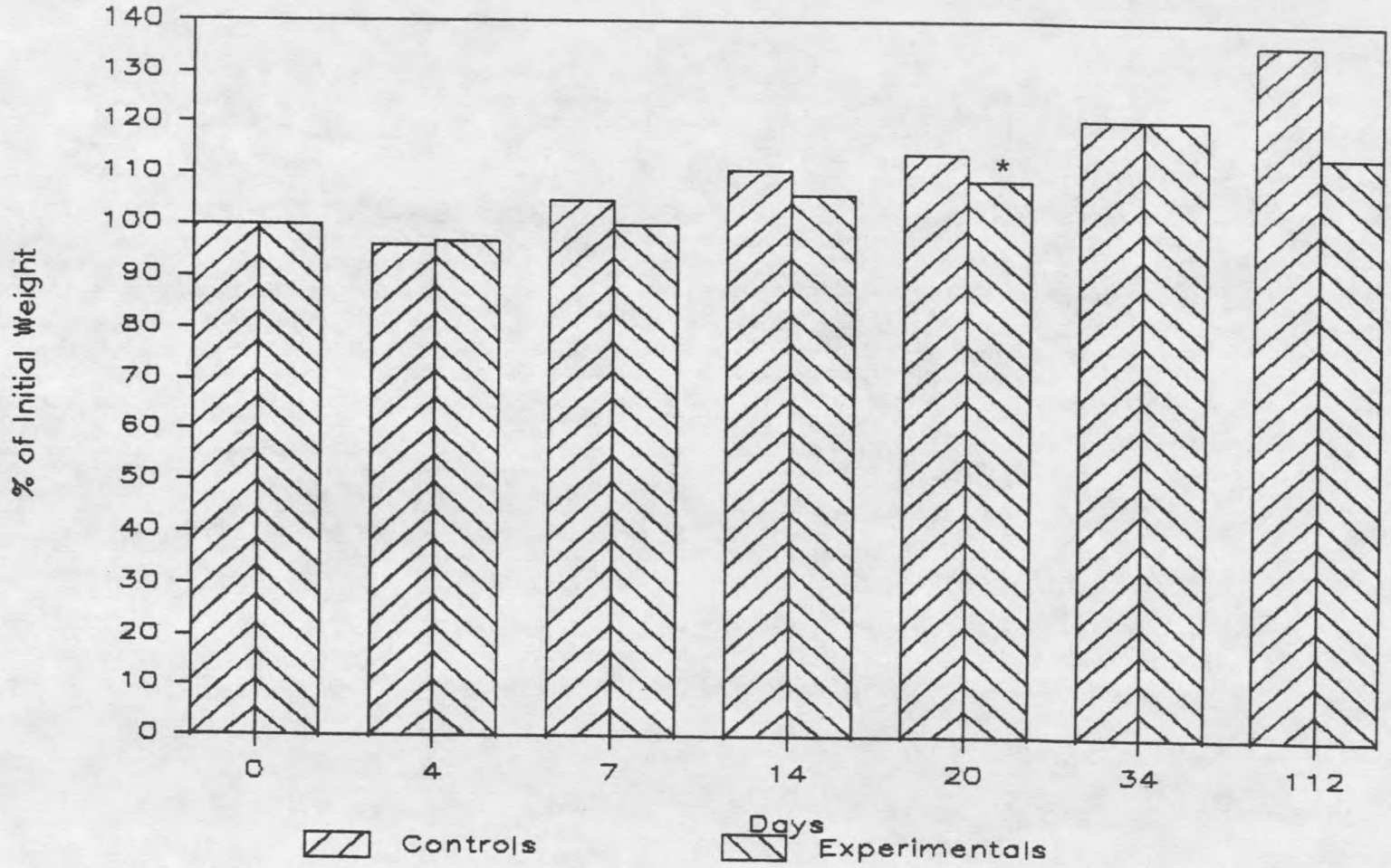


FIGURE 42. Animal weight changes. * indicates a statistically significant difference.

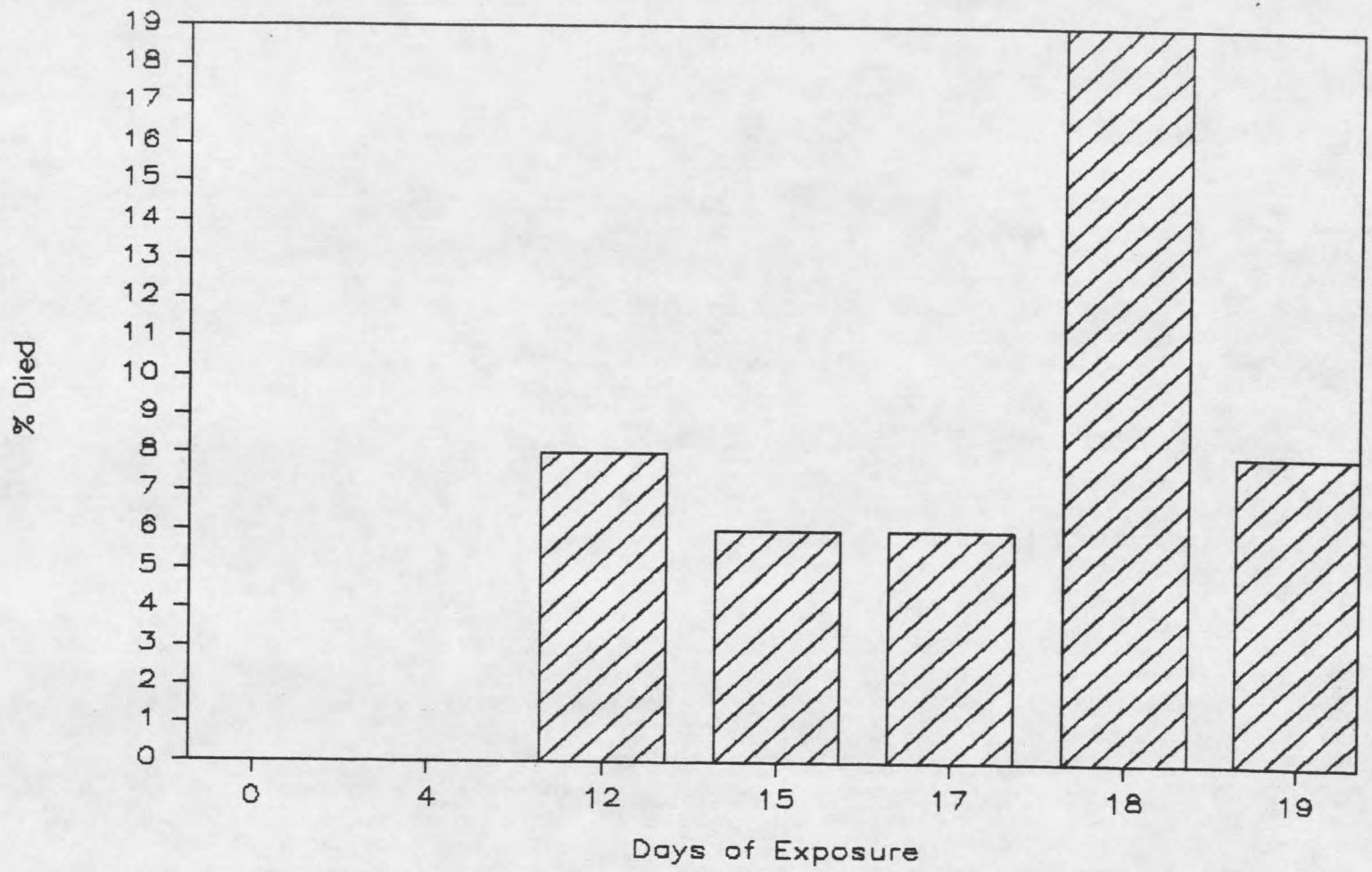


FIGURE 43. Animal mortality.

DISCUSSSION

SCIATIC NERVE

Electron microscopic studies of peripheral nerve in animals exposed to repeated subacute doses of endrin reveal a pattern of degenerative changes in unmyelinated axons characterized by axonal swelling, dissolution of microtubules and neurofilaments, and the appearance of vesicles within the axon and adjacent Schwann cell cytoplasm. Endrin does not appear to continuously accumulate significantly within the tissues of chronically exposed animals (Cole et al.,1968) but rather tends to reach plateau storage levels (Brooks,1969). This results in a limiting upper level of accumulation that declines with time when exposure ceases. According to Donoso et al.(1979) "it appears that, initially, endrin is rapidly absorbed, but a "coping" mechanism is developed that may allow metabolism or excretion of endrin, resulting in a concentration drop. A gradual increase in endrin follows, until the contending processes of uptake and excretion reach their respective levels and an equilibrium is attained." The greater amount of damage observed after 4 days of exposure, may indicate that the animals have have not yet reached such an equilibrium and that the levels of endrin within the tissues exceeds the plateau level that will be reached later. At such a time, the concentration of the toxin within the tissues reaches a level that

will not be exceeded unless the daily doses is markedly increased. A further indication of such adaptation to a new level of exposure was observed as a higher frequency of axonal swelling observed on day 14, shortly after the increase in dose from 2.0 mg/kg to 4.0 mg/kg on day 12.

Peripheral nerve damage, induced by chemical or physical means is often accompanied by axonal swelling (Dyck and Hopkins,1972; Hendelman and Mire,1968; Sima and Robertson,1979; Bray et al.,1972; Behse et al.,1975; Schlaepfer and Hasler,1979b). The focal dilations and constrictions reported here appear nearly identical to those reported by Mire et al.(1970) following nerve transection and nutritional deprivation. Previous morphological studies by those authors suggest that an ionic imbalance involving the ionic sodium-potassium ATPase may lead to abnormal accumulation of axonal sodium ions and water leading to axonal swelling (Hendelman and Mire,1968; Mire el al.,1970). This ATPase system is energy dependent and requires ATP as a substrate to achieve the extrusion of sodium from the axon. Therefore, any process that inhibits the production of ATP could lead to swelling. The swelling reported in the present study could very well be the result of such an ionic imbalance. Mire et al,(1970) found, in axons grown in culture, that 90-100% of the swelling disappeared within 30-90 minutes following either refeeding with normal medium, supplementing the medium with glucose, or opening the culture to room air. They suggest that these manipulations may lead

to production of ATP within the axon resulting in increased activity of the ionic sodium-potassium ATPase. In another study (Webster and Ames,1965) deprivation of oxygen and glucose to isolated rabbit retina resulted in organelle swelling that was reversible only if such deprivation was not prolonged. Those authors suggest that such changes may be related to the availability of high energy phosphate bonds that may be responsible for the active maintenance of osmolarity across the membrane. The periodic swelling of the smooth endoplasmic reticulum, the swollen membranous profiles and the presence of "watery" vacuoles observed in this study may be the result of such changes in osmolarity reflecting the possible decrease in ATP availability in animals exposed to endrin.

Transection or crush of peripheral nerve can result in the decreased activity of ionic sodium-potassium ATPase (Bachlard and Silva,1966). Mitochondrial (oligomycin-sensitive) ionic magnesium-ATPase in the rat brain is inhibited by endrin (Mehrota et al.,1982) and the related compound chlordecone (Jordan et al.,1981). Mehrota et al.(1982) suggest that such inhibition could impair availability of ATP to the ionic sodium-potassium ATPase, thereby limiting its normal function and resulting in an abnormal sodium gradient across the membrane. One can speculate that the axonal swelling observed in this study could very well be the result of such impairment. The high degree of swelling on day 14 could indicate a peak in the animal's inability to produce adequate amounts of ATP. The

animals may subsequently adapt to a new level of toxicity, and as the endrin level in the tissues concomitantly drops, the degree of inhibition of the sodium pump declines. This could decrease in the amount of swelling . Colvin and Phillips (1968) speculated that endrin and its analogues bind to the lipid-rich structural components of mitochondria and certain other organelles and that this binding may affect the catalytic activity of enzymes associated with these lipid-rich fractions.

Dissolution of microtubules and neurofilaments is one of the earliest signs of axonal damage, either following mechanical injury (Schlaepfer and Hasler,1979a; Mire et al.,1970; Dyck and Hopkins,1972; Bray et al.,1972), systemic neuropathy (Behse,1975; Sima et al.,1979) or chemical exposure (Phillips and Eroschenko,1982). These organelles are replaced by an amorphous, flocculent and granular material (Schlaepfer and Hasler,1979b; Schlaepfer and Micko,1978). Clumping of the axoplasmic contents (Dyck and Hopkins,1972), and granular disintegration of axoplasm were accompanied by swelling (Schlaepfer,1977). The results presented here are consistent with these findings.

The dissolution of microtubules and neurofilaments is thought to involve increased membrane permeability to calcium and subsequent axoplasmic alteration (Schlaepfer,1979; Schlaepfer and Micko,1978; Schlaepfer and Hasler,1979a) via a calcium activated protesase (Schlaepfer,1979). Any process that could lead to the loss of

selective permeability of the axolemma could result in such breakdown. The structural and chemical disruption of neurofilaments is accelerated by energy deprivation or increased calcium concentrations in culture (Schlaepfer,1979).

Degenerative axoplasmic changes are more widespread in small myelinated and unmyelinated fibers than in large myelinated fibers after incubation in media containing calcium (Schlaepfer and Hasler,1979a). These authors also found the smaller fibers to be more prone to breakdown induced by calcium indicating the possibility of a higher concentration of axonal proteases. They speculate that these proteases exist in an inactive form, and are activated by release of calcium from within the axon or by influx across the axolemma, possibly as a result of deficiencies in the metabolic control of calcium permeability.

Studies involving similar pathologies have shown that the disappearance of microtubules precedes the loss of neurofilaments (Schlaepfer,1974 and 1977). The progression of such dissolution could not be determined in the present study, perhaps because of the time lapse between the days of sacrifice.

Empty areas, devoid of axoplasmic material, and thought to have previously contained an axon, were occasionally observed within the Schwann cell cytoplasm of experimental animals. Sometimes these areas contained only a few scattered vesicles while others were almost

completely filled with vesicles. Behse et al.(1975) reported the appearance of empty axonal areas in the Schwann cell cytoplasm in sural nerve biopsies from patients with systemic neuropathy. They referred to these areas as "empty Schwann cell sub-units". These authors suggested that such empty areas may be due to either the survival of the Schwann cell after the loss of the unmyelinated axons or to the proliferation of the remaining Schwann cells. Similar findings were reported by Dyck and Hopkins (1972) following crush injury of unmyelinated fibers. They found one third of the unmyelinated fibers were represented by empty spaces which showed a watery appearance. The axolemma was discontinuous or had disappeared. The findings reported in the present study could in fact be a result of axonal loss although the low frequency of such changes makes interpretation difficult. A comprehensive morphimetric analysis involving counts of unmyelinated fibers from experimental animals could confirm such speculation.

One of the most consistent observations in sciatic nerve from animals exposed to endrin was the appearance of vesicles in unmyelinated axons, adjacent periaxonal spaces, or within the Schwann cell cytoplasm. The origin and fate of these vesicles is not apparent from this study, since it could not be determined if the vesicles were moving into the axon or being extruded from it. Phillips and Eroschenko (1982) have reported similar pockets of vesicles in unmyelinated axons following chlordecone exposure and observed that

some of these axons contained pockets of vesicles within the Schwann cell cytoplasm which had invaginated the axolemma. Spencer and Thomas (1974) described Schwann cell cytoplasmic invaginations in physically damaged myelinated fibers. These invaginations were invested by an infolding of the axolemma and enclosed electron lucid areas of axoplasm in a "honeycomb" configuration which they termed "Schwann cell/axon networks". They reported large clusters of clear and dense core vesicles surrounded by a microfilament meshwork and speculated that the cytoplasmic invaginations are a means by which the axon sequesters and phagocytoses degenerate or undesirable axoplasmic debris such as unusual or abnormal organelles (vesicles or dense bodies). A similar hypothesis has been presented by Schlaepfer and Hasler (1979b), who observed that the granular axoplasmic disintegration coincided with the appearance of floccular, and granular material of moderate electron density within the endoneurium. Such changes were attributed to a process of externalization of axonal breakdown products in degenerating nerve fibers. Further evidence relating the morphological changes to such externalization has been reported by Sima and Robertson (1982), who found irregular conglomerates of membranous profiles in swollen unmyelinated axons from mutant diabetic mice. They also reported the extrusion of axoplasmic debris into the Schwann cell cytoplasm or adjacent periaxonal space, and in longitudinal sections observed the invagination of the Schwann cell cytoplasm into the axoplasm forming "honeycombed Schwann cell axon complexes". Vesicles (smooth membrane

profiles) have been observed by Wood and Engel (1976) in the inner Schwann cell cytoplasm of 4-6 day old neonatal rats during active sciatic nerve myelination. They found that these profiles were usually enclosed within a larger smooth membrane structure and occasionally the entire complex could be seen fusing with the axolemma. They speculated that these vesicles may be involved in an exchange of membrane material between the Schwann cell and the axolemma. These authors point out that such vesicles were seen at other stages of early development but were never observed in sciatic nerve from adult animals.

The vesicles observed in the present study could represent accumulations of abnormal axoplasmic material leaving the axon, fluid, or even as an accelerated means of normal membrane exchange. At no point was there any evidence of endoneurial deposition of axoplasmic debris. Vesicles were observed within the axon, along the axon, in the Schwann cell cytoplasm, and in the periaxonal space indicating there may have been some type of externalization (or internalization) of these vesicles from the axon but the directionality of such movement could not be verified. These vesicles could in fact play a role in membrane exchange in swollen axons where more membrane would be needed to accomodate the enlarged axon or they could provide a method of fluid removal from such axons. The clear core vesicles containing a "halo" of material of intermediate electron density were most often observed within the axon and may be accumulations of fluid

destined for removal from the axon. Vesicles similar to the axonal vesicles observed in this study have been found in dendrites and nuclei of substantia nigra neurons in mice exposed to chlordecone (Van Natta and Phillips, in preparation) and in the developing cuneate nuclei of the rat (David and Nathaniel,1978).

Thin cytoplasmic invaginations of the axolemma similar to those observed in unmyelinated nerve fiber from experimental animals have been previously reported in degenerating myelinated nerve fibers (Spencer and Thomas, 1974) possibly as a means of sequestration of axonal debris by the adjacent Schwann cell. Such invaginations have also been reported in myelinated fibers from animals exposed to ethyl n-butyl ketone and methyl ethyl ketone (O'Donoghue et al.,1984). In the present study, axolemmal invaginations differed from those reported by Spencer and Thomas and O'Donoghue et al. primarily by the degree of invagination since they were occasionally seen traversing the entire axonal profile, although they were never observed to have actively enclosed axoplasmic contents as has been proposed by these authors. Rather, they are most likely invaginations of the Schwann cell cytoplasm and axolemma, that as a result of the plane of sectioning, appear only as a thin cytoplasmic stalk.

The large membrane bound vacuoles occasionally observed within the area normally occupied by the unmyelinated axons often filled a significant area of the axonal profile and appeared to displace the axon to one side. Other reports of vacuoles similar to those observed

in this study have not been found in the literature. Large vacuoles have been observed in the neuropil of the central nervous system in patients with Creutzfeldt-Jakob disease (Foncin,1967; Hirano et al.,1972; Bignami and Forno,1970; Lambert et al.,1971; Gonatas et al.,1965). They are described as containing an electron lucid material which often contained smaller vesicles or membrane fragments and is bounded by a membrane that is frequently interrupted. Vacuolization has also been reported by Martinez et al.(1978) in unmyelinated and small myelinated axons from biopsied sural nerve of employees involved in the manufacturing of kepone. The vacuoles observed in the present study are possibly accumulations of a watery fluid in the periaxonal space that results in the displacement of the axon or more likely, fills the space previously occupied by a degenerating axon, only a portion of which is visible in some profiles. This may indicate a transient phase of axonal degeneration which finally results in an empty area within the Schwann cell cytoplasm.

OTHER TISSUES

The fact that skeletal and cardiac muscle from experimental animals appears similar to tissues from control animals does not rule out the fact that subtle biochemical or transient morphological changes could be occurring within these tissue. Alterations in

skeletal muscle which included changes in mitochondrial ultrastructure and lipid and glycogen stores have been reported in chlordecone exposed mice (Phillips and Eroschenko,1984). However, animals exposed to kepone develop severe tremors and gait difficulites resulting in extreme energy depletion which may account for the morphological changes observed in these animals. Since animals exposed to endrin were never observed to display such symptomology, the energy burden in these animals may not be as great and thus one might not expect to see such changes.

The optic nerve in all experimental animals appeared similar to that in control animals. If endrin does not damage myelinated peripheral nerve fibers then one might not expect to see changes in the optic nerve which is comprised almost entirely of myelinated fibers, although a more comprehensive examination of such tissue is needed for verification of these findings.

Mitochondria from all experimental tissues sampled resembled those of controls despite reports of biochemical changes in mitochondria in endrin exposed animals (Pardini et al.,1971; Mehrota et al.,1982). This in contrast to reports of mitochondrial changes in chlordecone exposed mice, characterized by paracrystalline inclusions in mitochondria of unmyelinated fibers, and ultrastructure alterations in skeletal muscle (Phillips and Eroschenko,1982; Phillips and Eroschenko,1984).

BEHAVIORAL/NEUROLOGICAL EFFECTS

It is generally believed that endrin stimulates central nervous system activity. Behavioral changes reported here appear to confirm such a hypothesis. Neurological symptomology displayed in the experimental animals in this study, including hyperactivity and hypersensitivity to stimuli have been well documented in the literature and such changes have been consistent findings in a variety of experimental animals including mammals, birds, and fish.

Hypersensitivity to startle stimuli was one of the earliest signs of exposure to endrin. Similar findings have been reported in chlordecone exposed rats (Reiter and Kidd,1978; Jordan et al.,1981) and mice (Phillips and Eroschenko,1982). Jacobziner and Raybin (1959) reported an exaggerated startle response displayed by a child acutely exposed to endrin and they felt that it was indicative of extensive involvement of the diencephalon. Joy (1976) found in rats exposed to endrin that sensory stimuli of any modality would elicit seizure activity. His study showed that peripheral nerve stimulation resulted in the enhancement of a central nervous system response to sensory stimuli at both the somatosensory and sensorimotor cortex resulting in a three to five fold increase in cortical motor outflow that could possibly contribute to the response to startle stimuli seen in exposed animals. A study involving animals exposed to dieldrin showed that such changes are related to an increased postsynaptic response of the

cortical cells and the author speculates that endrin acts in a similar fashion (Joy,1974, as cited in Joy,1976). Jordan et al.,(1981) suggested that a similar startle response in rats exposed to chlordecone was indicative of a abnormal activity in the auditory, reticular and/or motor system (dysfunction or abnormal excitation) and possibly generalized central nervous system involvement.

Piloerection was not a noticable effect in exposed animals until somewhat later during exposure. Similar findings have not been reported in the literature, although mice exposed to kepone become piloerected shortly after injection (Van Natta and Phillips, in preparation). It has been reported that kepone exposure leads to hyper-responsiveness of unmyelinated peripheral nerve fibers to stimulation (R. Weller, J. McMillan and D. Phillips, personal communication). It has also been suggested that endrin exposure results in sympathetic nervous system stimulation (Emerson et al.,1964; Hinshaw et al.,1966). If stimulation of unmyelinated nerve fibers and thus postganglionic sympathetic fibers does occur in endrin exposed animals, then it is likely that such stimulation would lead to piloerection.

Endrin causes damage to unmyelinated axons which include sensory or autonomic postganglionic fibers. Consequently such damage could result in impairment of autonomic functions related to temperature regulation, blood pressure maintenance, and a number of other visceral functions. Physiological manifestations of such impairment have been

documented in dogs and include, hyperthermia, hypertension, bradycardia and increased salivation (Emerson et al.,1963; Reins et al.,1964 and 1966; Emerson and Hinshaw,1965; Emerson,1965). Damage to these particular fibers could also result in changes in reflex responses and sensations related to noxious stimuli. It is probable that many of these changes could be be subtle and may affect humans or animals in doses well below those that would produce gross behavioral or morphological symptoms. Even if such subtle changes were detected, the cause of such changes would not likely be suspected. Regarding such subtle changes, a wildlife study by the Montana Department of Fish Wildlife and Parks (1983) suggests that "pesticide related causes for such changes could include reproductive impairment, increased neonatal mortality and physiological or behavioral changes that can affect survival".

Gait disturbances due to exposure to endrin, in this study, were minimal and all animals appeared to have recovered by 14 d o r. This is in contrast to the severe gait difficulties reported in mice exposed to chlordecone which included wide-based splaying, instability and inconsistent placement of the extremities (Phillips and Eroschenko,1982; Jordan et al.,1981; Egle et al.,1979). Ataxia has been reported in birds (DeWitt,1955) and rats (Bedford et al.,1975) exposed to endrin.

Results of the tail flick test are inconclusive since a statistically significant difference between controls and

experimentals occurred only on 3 days. It is likely that any change in pain perception due to damage to unmyelinated sensory nerve fibers would not affect tail withdrawl since such damage would most likely result primarily in changes involving second pain sensation (Price,1972).

Results of the rod test were interesting but difficult to interpret since a number of factors could influence the results noted. A significant change occurred in the ability of the experimental animal to maintain itself on the rod. Few animals could stand on the rod at all by 8 d o e and those that did often trembled markedly before finally falling off. It is necessary to point out that the animals total recorded time on the rod did not necessarily reflect the animals ability to maintain its position once placed there, since many times the animal would fall off the rod immediately only to grasp the rod and hang from it, thus resulting in assignment of a much longer recorded time. The basis for this change in behavior cannot be determined from this study although one could speculate as to the reasons for such changes. Disturbances in vestibular function, muscle coordination and/or muscle strength, sensory-motor dysfunction or timidity could account for the animals inability to stay on the rod. Loss of equilibrium has been reported in fish exposed to endrin (Mount,1962, as cited in Grant and Merhle,1973) but not in other animals. Ataxia has been reported in some animals exposed to endrin (DeWitt,1955; Bedford et al.,1975) while muscle weakness was a

consistent finding in acutely exposed humans (Jenkins and Toole,1964; Coble et al.,1967).

Convulsions and to a lesser extent, tremors, are consistent symptoms of acute endrin exposure reported in the literature. The absence of these symptoms in this study could be the result of species variation, dose and route of exposure, or duration of exposure.

Animal weight in control and experimental animals increased up to 14 d o r although the weight of the experimentals had decreased slightly by 92 d o r. The difference in weight gain between the controls and experimentals was statistically different only on day 20. However, the experimental animals consistently gained less weight than controls but at a rate not statistically different, possibly due to the high amount of activity displayed by these animals. Some of the weight gain in both groups of animals was likely due to the fact that these animals are still growing although a residual accumulation of sesame oil, evident upon sacrifice of all animals up to 14 d o r could account for some of this increase. Blus (1978) found that shrews exposed to endrin lost an average of 19-27% of their weight. On the other hand Treon (1955) reported that prolonged feeding of endrin to rats resulted in a weight gain equal to or greater than that of the controls except in males fed the highest dose (25 ppm). A single dose of endrin as high as 10 mg/kg had no effect on the weight of pregnant hamsters although animals receiving doses above 0.75 mg/kg/day for 10 consecutive days all had a reduction in weight (Chernoff et

al.,1979).

The rapid metabolic fate of endrin in exposed animals could explain why the severity of some symptoms diminish with time. It may also account for the changes in symptomology following a change in the dose and also the apparent recovery of exposed animals following cessation of dosing. It appears that cyclodienes can exist in the brain for periods of time without producing toxic symptoms, but at a later time the same concentration may produce severe symptoms. This suggests a time dependent mechanism within the central nervous system that determines when such symptoms will become apparent (Walsh and Fink,1972). The slight dimunition of hyperactivity and a pronounced startle response over a 24 hour period following injection could result from decreasing levels of endrin in exposed animals. The gait changes on day 13, one day following an increase in the dose (from 2.0 to 4.0 mg/kg) and their subsequent gradual decrease after day 17 could indicate that the animals had "adapted" to the previous dose, thus establishing a plateau level within the tissues but then had to readapt to the new dose. The inability of animals to maintain their balance during the rod test continued throughout the dosing portion of the experiment. Significant recovery displayed by animals in this test and the gradual disappearance of the other observed symptoms occurred over the 2-4 weeks following cessation of exposure indicating a rapid turnover and elimination of endrin in these animals.

Total animal mortality in this experiment was 23%. There were no

obvious indications for the cause of death, nor were there overt premortal symptoms indicating any of the animals were near death. The incidence of mortality was closely associated with the change in the dose concentration. There was no mortality at a dose of 1.5 or 2.0 mg/kg. Raising the dose to 4.0 mg/kg on day 12 resulted in the death of two animals within 4 hours after injection and the additional death of 6 more animals over the next 7 days. It was apparent that those animals that could tolerate a change in dose did so by a process of adaptation, probably through rapid metabolism to less toxic intermediates and subsequent elimination which ultimately resulted in their survival until the termination of the experiment.

SUMMARY

This study demonstrates that endrin does produce specific morphological changes in unmyelinated fibers of peripheral nerve although the mechanisms of such change remain to be elucidated. There is a need for further studies to substantiate the apparent specificity of endrin's toxicity to unmyelinated fibers in other areas of the peripheral nervous system and possibly within the central nervous system and to further characterize the damage occurring within these fibers. Such studies could include examination of specific populations of unmyelinated fibers (sensory and autonomic) from a number of locations to determine if these effects are localized or

widespread and to inspect the dorsal root and autonomic ganglia of fibers to verify possible involvement of the perikarya. In addition, it would be of interest to investigate the effects of endrin in neonatal animals since another neurotoxic compound, capsaicin, has been shown to selectively induce degeneration in unmyelinated nerves of neonatal rats (Jancso et al.,1977; Jancso and Kiraly,1981; Nagy et al.,1980; Nagy,1982). Studies are also needed to ascertain the threshold dose which will induce initial morphological and/or behavioral changes in exposed animals. Knowledge of such specificity could lead to the use of endrin as a valuable tool in neurological research.

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