MORPHOLOGIC ALTERATIONS PRODUCED BY COPPER IN NEURAL TISSUES WITH CONSIDERATION OF THE ROLE OF THE METAL IN THE PATHOGENESIS OF WILSON'S DISEASE*

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Plates 103 and 104

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Hepatolenticular degeneration or Wilson's disease, is regularly associated with altered copper metabolism (1). As is well known, an excess of the metal accumulates within the neural tissues and notable histologic alterations regularly appear in them (1–3). Yet, the precise role of copper itself in the production of these lesions remains obscure (1, 3).

Recent studies have made it clear that fish kept in water to which copper sulfate has been added take up and retain the metal within neurons, which undergo conspicuous cytologic changes (4). In the present study, minute quantities of copper were injected into the cerebral spinal fluid of cats. The metal accumulated promptly in the neural tissue, catastrophic functional disturbances appeared, and shortly thereafter, conspicuous morphologic alterations became evident. Studies of the pathogenesis of these lesions by histologic and histochemical methods and by chemical analyses have provided additional information about the neurotoxic properties of copper and its role in the pathogenesis of hepatolenticular degeneration.

Materials and Methods

A copper-albumin complex was made by equilibrium dialysis (5), and injected intraventricularly into cats. At regular intervals after the injection of graded dosages, the content of copper in different regions of the central nervous system was determined by the method of Eden and Green (6), and the cytologic changes were observed in tissues prepared by specific histological techniques. For control purposes, comparable studies were made upon the neural tissues of other cats injected intraventricularly with the same quantities of a 5 per cent albumin solution. In ancillary studies, copper sulfate, iron sulfate, and saccharated iron were injected intraventricularly into still other cats.

Animals.—Young, adult alley cats of both sexes were used. These were always held under observation for a period of 2 weeks or longer to ascertain that they were free from manifest disease.

Copper-Albumin Complex.—A 5 per cent solution of bovine albumin (Armour) was made in

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demineralized water, and 300 cc. was encased in cellulose tubing and placed for 6 hours in 3,000 cc. of a 0.0025 M copper sulfate solution (Mallinckrodt, analytical reagent grade). Dialysis in free flowing tap water was then carried out for 3 days. Analysis showed the solution to contain 208 gamma of copper per cc. To 100 cc. of this solution, and to the same quantity of one of 5 per cent albumin, to be used for control purposes, was added 0.9 gm. of NaCl. Each solution then had a pH of 5.8.

Chemical Analysis for Copper.—Duplicate samples of the copper-albumin solution were analyzed by the method of Eden and Green using the DU Beckman spectrophotometer (6). Duplicate samples of neural tissue, fixed in 10 per cent formalin, made with demineralized water, were analyzed by the same procedure. The values were expressed in gamma of copper per 100 mg. of wet tissue.

Injection Procedure.—Intraventricular injections were made slowly over periods of several hours through small burr holes in the calvaria of animals lightly anesthetized with nembutal. A 25 gauge needle was introduced to a depth of $\frac{5}{8}$ inch at the junction of a line drawn between the anterior edges of the ears and the upper insertion of the right temporalis muscle. This regularly provided free flow of fluid into and from the right lateral ventricle.

Histologic Procedures.—Most of the brain and spinal cord tissue was fixed in 10 per cent formalin. As routine, blocks were embedded in paraffin, sectioned at 5 microns, and stained by hematoxylin and eosin, Masson's trichrome method, Nissl's cresyl violet method for neurons, Weil-Loyez method for myelin, and Bodian's method for axis cylinders. Selected portions were fixed in rubeanic acid preparatory to histochemical stains for copper (7).

In Vitro Histologic Technique.—Discs of spinal cord tissue, 1 cm. in thickness, from normal cats were incubated at 37°C. in copper-albumin solution, and for control purposes, similar sections of spinal cord were simultaneously incubated in a 5 per cent albumin solution. Duplicate specimens were removed from each solution at regular intervals up to 24 hours thereafter, and when fixed in 10 per cent formalin and embedded in paraffin were sectioned at 5 microns and stained by hematoxylin and eosin, Weil-Loyez's method for myelin, and Bodian's method for axis cylinders.

RESULTS

Functional Disturbances in Cats after Injections of Copper.—All 18 cats that survived intraventricular injections of 52 to 208 gamma of copper promptly and regularly manifested total loss of voluntary motor activity in the extremities (Table I). Quadriplegia was manifest in 14 of the animals by the time the effects of the anesthesia had regressed, usually after 3 to 6 hours; the rest showed quadriparesis which regularly progressed to quadriplegia within several hours. Thereafter, the paralysis persisted without change until the animals were killed at intervals up to 9 days (Fig. 1). Consciousness was manifested by voluntary extraocular motion; nevertheless, the animals did not eat or drink, and they were sustained by intraperitoneal injections of 10 per cent dextrose solution in saline and by intramuscular injections of penicillin. Marked wasting occurred. The extremities were regularly in extension, held so by rigid musculature that firmly resisted passive motion. Moderately strong pain stimuli to the paws caused ineffectual withdrawal. Clonic and tonic generalized seizures appeared on the 2nd day in one animal and on the 4th in another, and in each instance persisted until death within an hour. Clonic movements were present in one or both hind limbs of five additional animals.

TABLE I

Effects of Copper-Albumin Complex Injected Intraventricularly in Cats

Animal No.	Copper injected as albumin complex	Changes in living animal	Survival time	Copper content of tissues	Essential microscopic changes in the central nervous system
	γ			γ/100 mg. wet tissue	
1	208	Quadriplegia with beginning recovering from anesthesia.	3 hrs.	Spinal cord 0.78	Hydropic swelling of all neural tissues in outer margins of cervical cord. No inflammation.
2	208	Weakness of limbs with beginning recovery from anesthesia.	3 hrs.	Spinal cord 0.98	Hydropic swelling of the parenchyma, particularly the myelin in cervical cord. No inflammation.
3	208	Quadriplegia persistent until death.	20 hrs.	Lent. nuc. 1.19 Caud. nuc. 0.75 Motor cort. 0.44 Thalamus 0.88	Early marginal necrosis about cord and medulla. Few leukocytes.
4	208	Quadriparesis progressing to quadriplegia.	2 days		Moderate necrosis in margins of cord and brain stem. Moderate leukocytic infiltration.
5	208	Quadriplegia. Tonic and clonic seizure terminally.	2 days	Lent. nuc. 1.07 Motor cort. N.M.* Cerebellum 0.41 Spinal cord 1.82	Moderate to marked necrosis in cord. Inflammatory cells relatively sparse.
6	208	Quadriplegia. Clonic motion of left hind leg.	2 days		As above.
7	208	Quadriplegia. Generalized tonic and clonic seizure.	3 days	Lent. nuc. 0.68 Motor cort. 1.00 Cerebellum 1.30 Spinal cord 0.55	Necrosis with extensive loss of myelin. Spotty inflammation.
8	208	Quadriplegia persistent until death.	3 days		Moderate to marked necrosis and inflammation marginally in cord.
9	208	Quadriplegia (Fig. 1)	3 days		Extensive marginal necrosis in cord with little inflammation.
10	208	Quadriplegia persistent until death.	4 days		Marginal necrosis in cord contains some macrophages, but few leukocytes.
11	208	Quadriplegia. Clonic motion of hind leg.	5 days		Marked loss of myelin and axis cylinders with little inflammation.
12	208	Quadriparesis progressing to quadriplegia.	5 days		Spongy state of marginal portions of cord. Many macrophages.
13	208	Persistent quadriplegia.	7 days		Marginal necrosis about cord and brain stem. Many macrophages.
14	208	Quadriplegia.	7 days		Well defined areas of necrosis in cord and brain stem. Scant inflammation.
15	208	Quadriplegia. Clonic twitching.	8 days		Moderately large areas of necrosis in cord without inflammation.
16	104	Quadriplegia. Clonic movements of hind legs. Respiration slow.	24 hrs.		Early necrosis in peripheral zones of cervical cord. Scant inflammation.

* Not measurable by technique.

TABLE I—*Concluded*

Animal No.	Copper injected as albumin complex	Changes in living animal	Survival time	Copper content of tissues	Essential microscopic changes in the central nervous system
	γ			*γ/100 mg. wet tissue*	
17	52	Limbs rigid. Unable to stand. Sensitive to sharp noise.	3 days	Spinal cord 0.50	Moderate necrosis in cervical cord and over brain stem.
18	52	Quadriplegia with rigid muscle tone. Resistant to passive motion.	9 days		Moderately large areas of total parenchymal necrosis. Macrophages present.
19	26 (x 5)	Nil.	26 days		Nil.
20	26	Weakness of both hind limbs. Some recovering of strength.	16 days		Small focal areas of necrosis in spinal cord without inflammation.
21	0‡	Nil.	1 day	Lent. nuc. N.M.* Caud. nuc. 0.43 Motor cort. 0.44 Thalamus N.M.* Spinal cord 0.54	Nil.
22	0	Nil.	3 days	Spinal cord N.M.*	Nil.
23	0	Nil.	5 days	Spinal cord 0.14	Nil.
24–28	0	Nil.	3–5 days		Nil.

‡ Intraventricular injection of 1 cc. of a 5 per cent albumin solution for control purposes.

Sudden sounds evoked a generalized auditory-motor reflex of pronounced character in most animals.

The injection of 26 gamma of copper in 0.125 cc. of albumin solution caused no detectable neurologic deficits in one cat, even after the animal was re-injected with the same quantity of metal five times during a period of 20 days. However, another cat promptly showed paraplegia after a single similar injection, the neurologic deficit gradually regressing during the 16 days before it was killed. Two additional animals injected more rapidly with the same quantity of metal showed slowing and cessation of respiration and died during the period of anesthesia. Death occurred in a similar manner in approximately one-third of all animals injected with larger quantities of metal.

All 8 cats that were injected with 0.125 to 1 cc. of a 5 per cent solution of bovine albumin, for control purposes, appeared normal after the effects of anesthesia had disappeared.

Two cats that were injected intraventricularly with 200 gamma of copper, as cupric sulfate (Mallinckrodt, analytical reagent grade) in 1 cc. of saline, manifest at the time of recovery from anesthesia paraplegia of the hind limbs and marked paresis of the forelimbs. These signs persisted until the animals were killed 4 and 6 days thereafter. The injection of 90 gamma of copper in the same form was promptly followed in another animal by weakness of the hind limbs. Animals injected with copper as cupric sulfate were generally less responsive than those that received comparable amounts of the metal as the albumin complex. Animals in groups of three injected with 300 gamma of sulfate, as ferric sulfate (Fisher Scientific Co., reagent grade) in saline and

those that received iron, in amounts up to 10 mg., as saccharated iron (proferrin-Merck, Sharp, and Dohme) appeared normal after the effects of anesthesia had disappeared.

Content of Copper in the Neural Tissues of Cats Injected with Copper-Albumin.—The results of the chemical analyses for copper are given in Table I. They make it clear that the content of metal, even in areas of maximum tissue necrosis in the spinal cord was elevated only to the heights of the normal range or slightly above that level. On the 2nd day after injection, in one animal quantities of 1.82 gamma were present in each 100 mg. of wet spinal cord tissue. In all others analyzed, the content was less than 1 gamma per 100 mg. of tissue, with a range from 0.5 to 0.98 gamma in animals injected with copper-albumin and from 0.14 to 0.54 gamma in those injected with albumin alone for control purposes.

Consistent with this finding, the histochemical preparations for copper failed to show positive staining in the neural tissues, although focal staining was present in the leptomeninges and about the superficial blood vessels in the spinal cords and brains of animals killed shortly after injection.

Morphologic Alterations in the Neural Tissues of Cats Injected with Copper.—Conspicuous morphologic lesions were regularly present and were notably similar in distribution in all cats that survived 3 hours or longer after the injection of 52 gamma or more of copper. These were circumferentially placed in the outer margins of the spinal cords, most conspicuously in the cervical segments, and in the brain stem, but also frequently present over the entire cord, and in many animals, in the pons, mid-brain, and inferior aspects of the cerebrum as well. The earliest structural changes were clearly evident within 3 hours after injections and were then characterized by swelling and sponginess of the white matter to a depth of 1 to 2 mm. Inflammatory cells were not present. Within 24 hours, there was necrosis of all parenchymal elements focally throughout the peripheral margins of the spinal cord and brain stem, with marked fragmentation of the tissues and loss of tissue architecture (Figs. 2, 3). Sparse and patchy infiltrations of leukocytes appeared, but remained largely confined to the leptomeninges, spreading only focally about the adjacent parenchymal blood vessels. As viewed in specially stained sections, the myelin sheaths within the marginal areas of the spinal cords were swollen initially and then fragmented and lost. Those in contiguous areas were generally well preserved (Fig. 4). Similarly, the axis cylinders in the peripheral regions of the spinal cords showed beading, tortuosity, and fragmentation, while those in deeper areas and those in the nerve roots were well preserved. Macrophages infiltrated the lesions and became engorged with debris. The sojourn of leukocytes was short, and only a few were present in the tissues of animals killed 4 days after injection. A small number of neurons in the anterior horns of the spinal cord showed chromatolysis, while most remained unaltered.

Similar but less extensive lesions were present in a cat that received 26

gamma of copper. None was evident in another animal that received the same quantity of metal on six occasions. No histologic changes were present in the brains or spinal cords of 8 cats that received intraventricular injections of 5 per cent albumin solution for control purposes.

The lesions were notably similar in distribution and histologic appearance in animals that received copper as cupric sulfate and in those that were injected with the metallic-albumin complex. Generally, more leukocytes were present in the former. No histologic alterations were evident in the tissues of animals injected with ferric sulfate or saccharated iron.

Effects of Copper-Albumin on the Histologic Structure of Neural Tissues in Vitro.—The histologic appearances of the spinal cord tissues incubated in vitro at 37°C. in copper-albumin solution did not differ notably from those of similar tissues exposed simultaneously to solutions of albumin alone. In each instance the white matter became porous and neurons stained hyperchromatically after incubations of 12 to 24 hours. Nevertheless, the axis cylinders and myelin sheaths were well preserved in tissues incubated in copper-albumin solutions for as long as 24 hours, being notably similar in structure and staining properties to those in tissues incubated for the same periods in a 5 per cent albumin solution.

DISCUSSION

The findings make it clear that minute quantities of copper injected either as a copper-albumin complex or as cupric sulfate, in the cerebrospinal fluid of cats resulting in diminutive increases of the metal in the neural tissues, promptly causes catastrophic disturbances in neuronal function and rapidly produces notable histologic alterations. Although hydropic swelling of the myelin sheaths was conspicuous as an initial change, necrosis of all parenchymal structures followed shortly thereafter and erased possible evidence of a selective affinity of the metal for one or another of the neural constituents. The observation that the lower motor neurons were notably well preserved made it seem likely that functional disturbances resulted largely from zones of tissue alteration that impinged upon the long corticospinal pathways. However, since neuronal degeneration characterized the lesions in patients with hepatolenticular degeneration, while necrosis of the white matter was most conspicuous in the experimental animals, it remains to be learned whether this variation arises from differences in the sensitivity of the tissues of those species or from technical aspects of the experimental procedure.

The topographic distribution of the lesions conforms closely with that autoradiographically demonstrated to be the region of maximum absorption of radioactive bovine albumin from the cerebrospinal fluid (8). This distribution was generally in accord with the distribution of copper as shown by chemical analyses, although these determinations were few and the increases were notably small and transient.

The absence of leukocytes in the initial histologic response and their appearance in only moderate numbers and in patchy distributions thereafter, suggest that the metal initiates a necrotizing, rather than an inflammatory process. Yet, the lack of activity of the copper complex upon the histologic structure of these tissues *in vitro* makes it clear that the metallic-albumin complex does not itself possess lytic properties nor does its presence in the neural tissues activate intrinsic proteolytic or lipolytic enzymes.

It seems particularly noteworthy that an albumin complex of the metal manifests so high a degree of neurotoxicity, since it is well known that in patients with deficiencies of ceruloplasmin, copper is largely transported in the serum in this molecular form. This makes it seem likely that copper itself plays a prominent role in the pathogenesis of hepatolenticular degeneration.

SUMMARY

The injection into the cerebrospinal fluid of cats of 52 to 208 gamma of copper in the form of an albumin complex or as cupric sulfate, was followed by small elevations in the content of metal in the neural tissues, but regularly and promptly produced persistent quadriplegia and conspicuous histologic changes. Smaller amounts of copper caused less, or no, neurologic manifestations or histologic alterations. The earliest lesions were essentially unaccompanied by inflammation and were initially characterized by hydropic swelling of the myelin sheaths. They progressed rapidly to focal necrosis of all parenchymal components with marked degeneration of myelin and axis cylinders in the peripheral margins of the spinal cord, brain stem, mid-brain, and cerebrum. These histologic changes did not occur in neural tissues incubated *in vitro* in solutions of the copper-albumin complex. They did not appear in animals injected intraventricularly with ferric sulfate or saccharated iron. Considered together, the findings make it clear that copper in concentrations comparable to those present in the neural tissues of patients with Wilson's disease has the property of profoundly altering neural function and causing conspicuous morphologic alterations.

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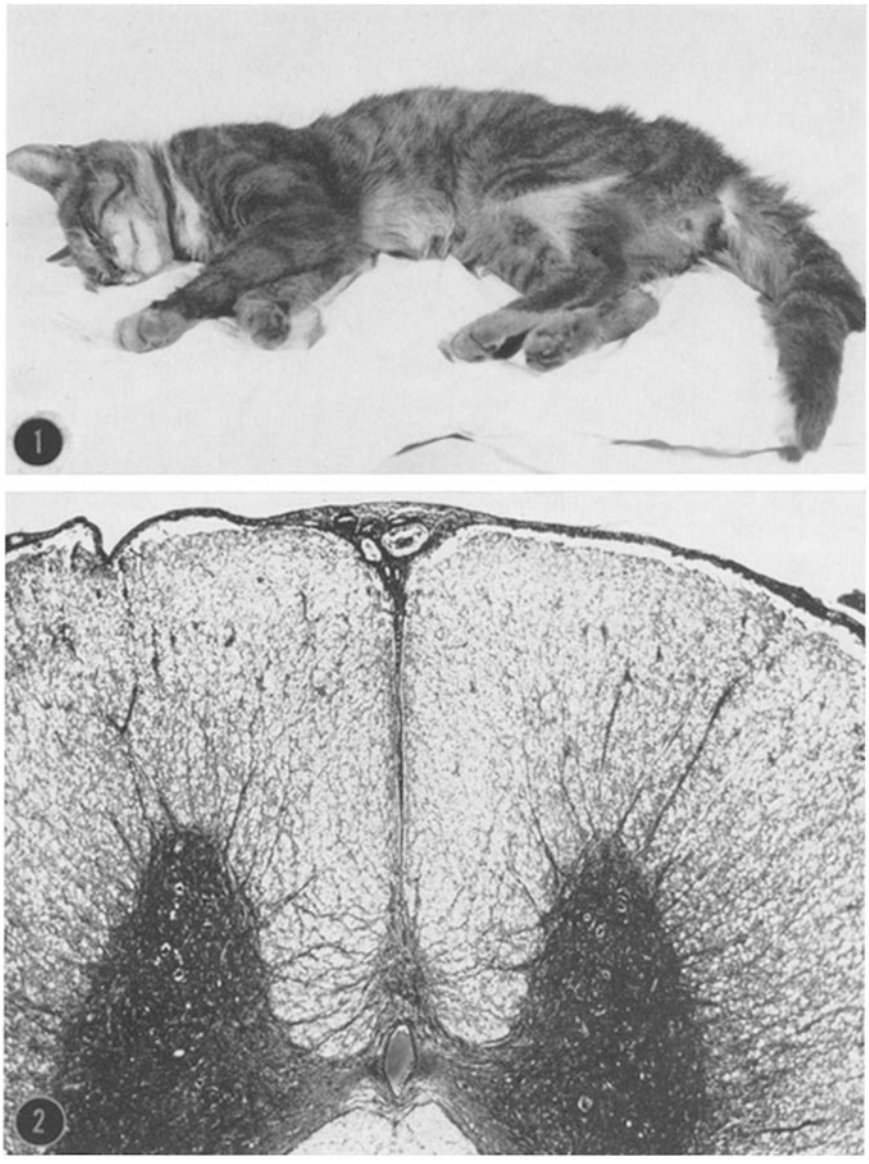
EXPLANATION OF PLATES

PLATE 103

FIG. 1. A cat received 208 gamma of copper as an albumin-copper complex in the cerebrospinal fluid. Quadriplegia developed within 3 hours and is evident 3 days thereafter. The limbs are in extension held so by tonic musculature that resists passive motion.

FIG. 2. The thoracic segment of the spinal cord of the animal in Fig. 1 shows necrosis at its peripheral margins with destruction of the corticospinal pathways evident at this low magnification as a loss of architecture and axis cylinders in the upper outer regions of the spinal cord. Bodian's stain for axis cylinders. × 25.

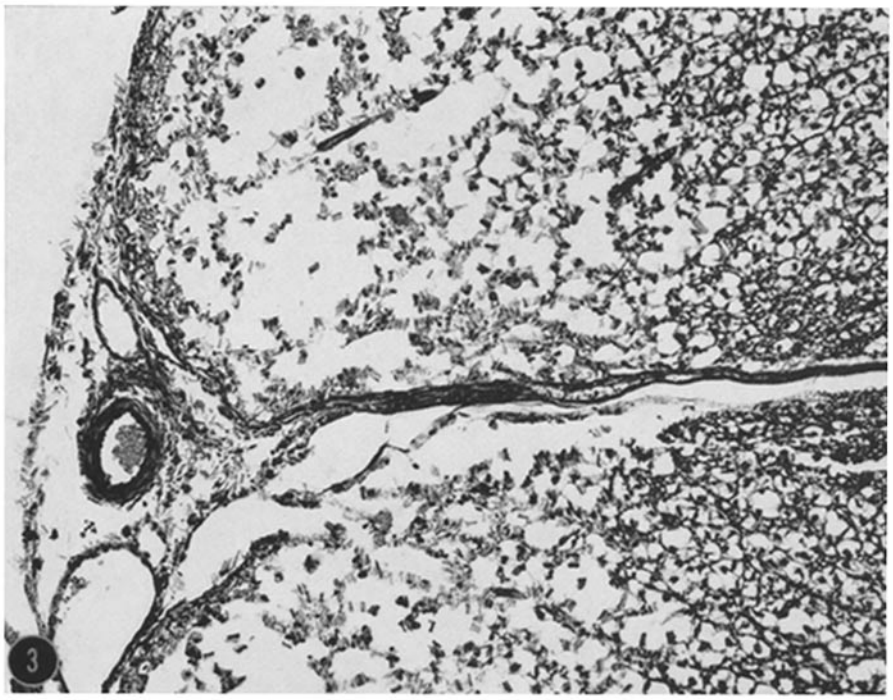


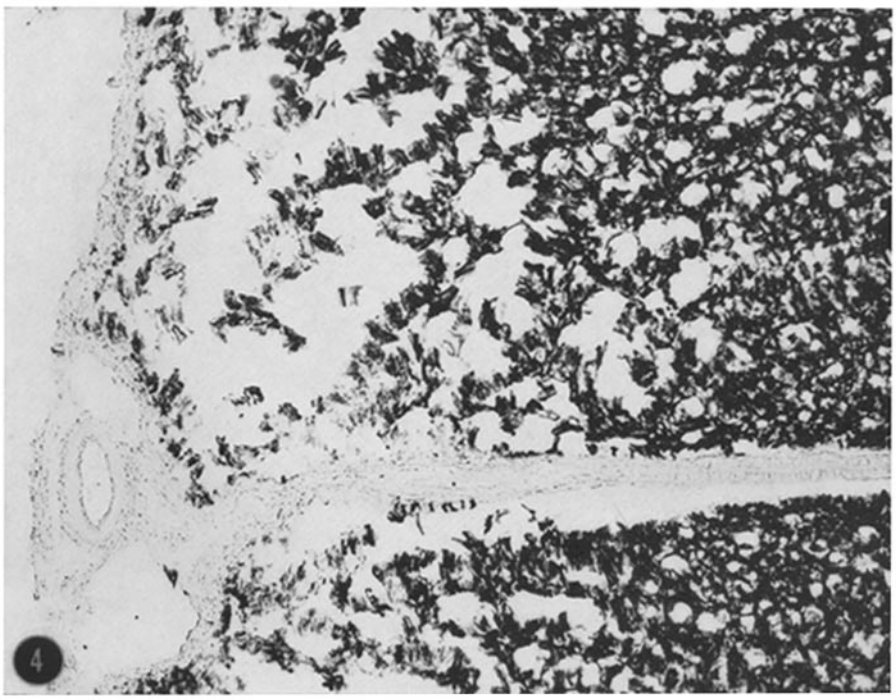
(Vogel and Evans: Alterations produced by copper)

PLATE 104

FIG. 3. Higher magnification of the same lesion shows necrosis of all neural constituents in the periphery of the spinal cord. The area is largely devoid of inflammation. Hematoxylin and eosin stain. × 260.

FIG. 4. The myelin sheaths in the same lesion are fragmented while those in contiguous areas are well preserved. Weil-Loyez stain for myelin. × 260.





(Vogel and Evans: Alterations produced by copper)