THE DEPOSITION OF EXOGENOUS COPPER UNDER EXPERIMENTAL CONDITIONS WITH OBSERVATIONS ON ITS NEUROTOXIC AND NEPHROTOXIC PROPERTIES IN RELATION TO WILSON'S DISEASE

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Plates 70 to 76

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Hepatolenticular degeneration, Wilson's disease, is regularly characterized by notable and well known morphologic alterations in the central nervous system and liver (1). These are regularly associated with lower concentrations of copper-binding globulin, ceruloplasmin, and copper in the serum, with storage of copper in the tissues, and with increased excretion of copper in the urine (2). Although it is clear that copper metabolism is altered in hepatolenticular degeneration, the part played by the metal itself in bringing about the cellular changes remains obscure (3, 4).

Fish often take in and retain heavy metals from their environment (5, 6). The present studies make it clear that when fish are kept in water rich in ionized copper under circumstances that keep this from coagulating the mucus of the gills, the metal accumulates within the cells of many tissues in concentrations comparable to those found in Wilson's disease; also that these depositions are accompanied by cytologic alterations in the central nervous system that are notably similar to those that characterize the naturally occurring disease.

Materials and Methods

Work by others has shown that ionized copper in water in concentrations of one part in ten million usually kills fish by coagulating the mucus on the gills and thus interfering with oxygen transfer and causing asphyxiation (7, 8). In the present work, "alevaire" (oxyethylated tertiary octylphenol-formaldehyde polymer-Winthrop) was used to prevent the accumulation of coagulated mucus on the gills; under such circumstances goldfish survived many weeks in water to which concentrations of copper up to one part in 500,000 had been added. Control fish were kept in normal tap water and in water to which alevaire had been added. The experimental and control animals were observed and then killed periodically during 35 weeks. The tissues were studied by histologic and histochemical methods and quantitative analyses for copper were performed.

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Animals and Chemicals.—Goldfish, 3 to 3.5 inches in length, were procured directly from a supplier. Analytical reagent grade copper sulfate (Mallinckrodt) was used.

Aquaria.—Each aquarium contained many water plants and was mechanically aerated at all times. Twenty fish were placed in a 20 gallon tank that contained 60 liters of aged tap water, 19.5 cc. of alevaire, and 60 cc. of a 0.1 per cent solution of copper sulfate, to make a 1 to 1 million concentration. As controls, 10 goldfish were kept in each of two 10 gallon aquaria that contained 28 liters of aged tap water, to one of which 8.25 cc. of alevaire was added. All solutions were replaced weekly.

Chemical Analyses for Copper.—Tissues from the liver, kidneys, and brain of each goldfish and from the eyes and skin of six were fixed in 10 per cent formalin made with demineralized water. Analyses for copper were made upon duplicate samples by the method of Eden and Green using a DU Beckman spectrophotometer (9). The values were expressed in gamma of copper per 100 milligrams of wet tissue.

Histologic and Histochemical Procedures.—As routine, small portions of tissue from the liver, kidneys, brain, muscle, and gills of each goldfish were fixed in 10 per cent formalin. These tissues were embedded in paraffin and when sectioned at 5 μ were stained by hematoxylin and eosin and Masson's trichrome method. Selected deparaffinated sections were exposed for 1 hour to the vapors of concentrated HCl, then stained histochemically by rubeanic acid for copper (10). Portions of the central nervous system were stained by Nissl's cresyl violet method for neurons, by Weil-Loyez's method for myelin, and by Bodian's method for axis cylinders. Portions of kidney were stained by Von Kossa's method for inorganic phosphate to show calcification.

RESULTS

Observations on Goldfish in Copper-Rich Water

Goldfish kept in water to which copper sulfate and alevaire had been added regularly remained active and had normal appetites for 15 weeks. The appearance and activity of most fish were usually unaltered by their abnormal environment during the subsequent 10 weeks; however, an occasional fish abruptly developed signs of distress, usually on the 2nd or 3rd day after renewal of the water. These fish regularly became inactive, breathed from the surface of the water, and had a fluttered, gyrational, or backward swimming motion; concomitantly, most developed hemorrhages in the bases of the pectoral and pelvic fins, in the gills, and occasionally at the base of the dorsal fin and at the roots of the scales. When such fish were transferred to normal tap water, they often recovered and the hemorrhages were reabsorbed over a period of 1 or 2 days. The signs recurred and progressed to death within several days when the fish were again placed in water that contained copper sulfate and alevaire. Most fish were killed when they showed evidence of distress and developed hemorrhages; several died unexpectedly and were discarded. The fish that were kept in copper-rich water for periods longer than 25 weeks regularly consumed less than the normal amount of food, were inactive, and had marked wasting of the musculature in the paravertebral region. All fish of the experimental group were killed or had died within 35 weeks. By contrast, fish that were kept

in tap water and in water to which alevaire alone had been added remained active, had normal appetites, and did not lose perceptible amounts of weight or develop hemorrhages during the same period.

TABLE I

The Copper Content of the Organs of Fish Kept in Copper-Rich Water

Time in aquarium	Composition of water in aquaria								
	Copper and alevaire			Alevaire			Tap water		
	Copper content—gamma per 100 mg. of wet tissue*								
	Liver	Brain	Kidney	Liver	Brain	Kidney	Liver	Brain	Kidney
wks.									
6	1.01	—	0.06				—	0.00	0.53
8	4.57	0.32	—	0.66	0.00	—	1.47	0.52	0.72
10	5.05	0.57	—	1.36	0.28	—	1.78	0.64	0.61
11	6.01	2.35	—						
12	11.95	1.51	—	2.04	0.95	0.00	1.78	0.40	0.66
18	2.58	1.59	2.29	1.55	0.25	—	0.21	0.69	0.83
21	2.90	1.14	3.27	1.84	0.90	0.00			
24	4.77	—	3.02						
24	23.20	2.92	2.91				1.51	0.62	0.94
25	7.02	—	2.30	2.16	0.00	1.05			
25	3.21	13.75	7.12						
27	10.55	1.38	9.10						
32	3.71	—	—						
33	4.32	—	—						
35	5.38	2.20	1.67	1.54	—	0.47			
35	4.70	1.53	1.25				1.66	0.22	0.67

* As determined by method of Eden and Green, see text.

The Content of Copper in the Tissues of Goldfish Kept in Copper-Rich Water

The results of the chemical analyses, given in Table I, were obtained by the Eden and Green method performed upon duplicate samples of wet tissue. They make it clear that fish kept in copper-rich water take up this metal and accumulate it in the brain, liver, and kidneys.

The neural tissues of many goldfish in the control groups did not contain quantities of copper detectable by the method employed; others were found to have amounts regularly less than 1.0 gamma for 100 mg. of wet tissue. Only trace quantities of metal were also present in the brains of fish killed after sojourns of 8 and 10 weeks in copper-rich water. In contrast to these findings, the brains of fish killed after longer stays in copper-rich water showed notable

elevations of copper content. These regularly exceeded 1.0 gamma per 100 mg. of tissue. Similarly, the hepatic tissues of fish kept 8 weeks or longer in copper-rich water contained the metal in abundant quantities that varied in amounts up to 23.2 gamma per 100 mg. of wet tissue, whereas the same quantity of tissue from fish of the control groups regularly contained less than 2.10 gamma. Excessive quantities of copper were to be found in the kidneys of fish from copper-rich environments. The concentrations varied upwards to 9.10 gamma per 100 mg. of wet tissue as compared with amounts less than 1.10 gamma in the same quantity of renal tissue from fish of the control groups. Chemical analyses showed no notable increase in the copper contents of the eyes or skin.

The histochemical preparations clearly showed abundant accumulations of copper in the neurons of the central nervous system, in the epithelial cells of the nephrons, in the hepatic parenchymal cells, in the sarcoplasm of the skeletal muscle, and in the epithelial cells of the gills, as will be described more fully further on.

The Neurotoxic Properties of Copper for Goldfish

Coronal sections through the olfactory lobes, telencephalon, mesencephalon, metencephalon, and through the spinal cord at various levels, when stained histochemically for copper showed this metal in great abundance in the larger neurons, and principally in those of the telencephalon, mesencephalon, and anterior horn region of the spinal cord. The metal appeared in the form of fine and coarse granules haphazardly distributed throughout the cytoplasm, often in a distribution similar to that of the Nissl substance. The axon hillock and proximal segment of the axis cylinder of some neurons contained lesser amounts of copper, while the nuclei were regularly free of stainable metal (Figs. 1, 2). As has been stated, chemical analyses regularly disclosed elevations in the copper content of the brain. These varied in amount but, in general, showed only poor correlation with the quantities of metal demonstrated by histochemical methods.

Detailed cytologic studies made on the neural tissues of goldfish kept less than 12 weeks in copper-rich water failed to show notable cytologic alterations. The same was true of all fish in the control groups. When stained by cresyl violet the neural tissues of goldfish kept 18 weeks or longer in copper-rich water regularly showed varying degrees of neuronal degeneration. This was most conspicuous in the telencephalon, in the basal ganglia, and in the lower motor neurons in the anterior horns of the spinal cord. The changes were notably more marked in fish kept 25 weeks or longer in copper-rich water. As has been stated, the cytologic alterations varied in degree but were uniform in their morphologic characteristics. The altered cells regularly showed contraction of the nucleus

with condensation and hyperchromaticity of the chromatin material. Many contracted nuclei contained a single vacuole that occupied up to one-half of the nuclear area. The cytoplasm of the altered cells was condensed and deeply eosinophilic. The axis cylinders arising from these cells were tortuous and hyperchromatic (Fig. 3). In tissues prepared by Bodian's method, the axis cylinders were even more tortuous and some showed beading and fragmentation. The myelin sheaths were not structurally altered and there was no appreciable loss of myelin. The glial cells, as shown in histochemical preparations, did not contain demonstrable copper and their cytologic appearance did not differ notably from that of the controls. No inflammatory exudate was to be found in the neural tissues or in the supporting mesenchymal structures.

The Nephrotoxic Properties of Copper for Goldfish

The renal tissues of goldfish kept in copper-rich water regularly accumulated excessive amounts of this metal (Table I). The histochemical preparations regularly and clearly showed abundant quantities of stainable copper in the form of fine and coarse granules in the cytoplasm of the epithelial cells, principally those of the larger tubules. Lesser amounts of stainable metal were present in the nuclei of these cells and small quantities were to be seen in the glomerular tufts (Fig. 4).

In histological sections stained by hematoxylin and eosin, the nephrons of fish kept in copper-rich water for 21 weeks or longer regularly showed variable degrees of cytologic change. Necrosis of the epithelial cells, principally those of the larger tubules, was conspicuous in fish that were killed when they showed signs of distress and had hemorrhages in the fins. The necrosis was coagulative in appearance and was characterized by either lysis or hypereosinophilia of the cytoplasm and pyknosis or karyorrhexis of the nuclei. Many tubules were circumferentially denuded of epithelium and contained eosinophilic cellular debris (Fig. 5). Both necrotic cells attached to the tubular basement membrane as well as those free-lying in the lumen, occasionally contained fine and coarse basophilic particles. These stained positively for calcification by Von Kossa's method. In two instances the renal tissues of fish that were killed at times when hemorrhages were not present showed minimal amounts of necrosis; more frequently, they were free of necrosis and showed hyperplasia of the tubular epithelium, often with much calcification (Fig. 6). The hyperplastic epithelium, formed of columnar cells with abundant clear cytoplasm, was thrown into small papillary folds. Calcification occurred most often in the larger tubules and was confined to the tubular areas without notable deposition in the interstitium or blood vessels. The glomeruli were not notably altered. The interstitial regions and pelves were regularly free of inflammatory exudate and showed no fibrosis. The histologic sections of the renal tissue of control fish kept in normal tap water or in water to which alevaire had been added regularly showed well preserved nephrons. The tubular epithelium was orderly without necrosis or hyperplasia, the glomeruli were not structurally altered, and there was no calcification.

Deposition of Copper in Other Tissues of Fish

Chemical analyses showed marked elevations of copper in the liver of goldfish kept for prolonged periods in copper-rich water (Table I). These elevations equalled or exceeded those commonly present in the hepatic tissues of persons with hepatolenticular degeneration. Histochemical preparations for copper showed abundant quantities of metal. This positive staining was almost entirely confined to the nuclei of the parenchymal cells. The quantity present in individual nuclei varied markedly; some stained intensely in histochemical preparations, others as viewed by phase microscopy, contained little or no stainable metal (Fig. 7). Companion sections stained by hematoxylin and eosin and by Masson's trichrome method, showed the parenchymal cells to be well preserved, with plump, pale-staining cytoplasm which was presumably rich in glycogen. There was neither histologic evidence of necrosis nor notable increase in fibrous tissue.

The paravertebral musculature contained great quantities of stainable copper that was distributed as coarse granules in a regular periodic arrangement along the course of the myofibers (Fig. 8). The muscle fibers were atrophic and there was widening of the spaces between the muscle bundles. Necrosis was not evident and there was no fibrosis or inflammation.

The epithelial cells that covered the gills, particularly those in the sulci, contained much stainable copper (Fig. 9). Within the cartilagenous stroma, there were lesser amounts. Neither tissue showed consistent cytologic change.

DISCUSSION

The findings make it clear that goldfish assimilate copper when kept in copper-rich water and attain intracellular concentrations of this metal that are comparable to those naturally occurring in Wilson's disease, while concomitantly showing notable cytologic alterations in the central nervous system and in the kidneys.

Involvement of the lenticular nuclei has traditionally been held to be a cardinal component of Wilson's disease. However, regional assays of the central nervous system for copper content have shown marked elevations in many areas, notably in the cerebral cortex (11), and cytologic studies have made it clear that degeneration and loss of neurons with gliosis are similarly widespread. A predilection of copper for the larger neurons, rather than for a regional distribution, was apparent in the goldfish. It is possible that this finding resulted from a greater degree of stainability of the metal in these cells rather than from a selective deposition, for, in general, there was wide disparity between the quantity of copper demonstrated in the tissues by histochemical techniques and the values obtained by chemical assay. Great difficulty has also been experienced in obtaining reliable histochemical reactions in the neural

tissues of patients with Wilson's disease, although much copper is regularly shown to be present by chemical analysis (10, 11).

Contraction of nerve cells, with pyknosis, leading to cellular death and secondary gliosis characterize the neuronal response to a variety of noxious agents but in themselves are non-specific. However, in the presence of large accumulations of intracellular copper, as occurred repeatedly in the goldfish, these cytologic changes provide strong evidence of the neurotoxic properties of this metal. A clear correlation existed between the duration of exposure to copper-rich water and the presence and severity of the cytologic changes in the brain. It seems likely that time is an important factor in the neurotoxic reaction to copper.

Much consideration has been given to the pathogenesis of the renal dysfunctions in Wilson's disease that are frequently manifest as marked amino-aciduria (12) and often characterized by glycosuria, proteinuria, calcinuria, and phosphaturia (4). Assignment of these dysfunctions to a primary genetic defect has related the condition to Fanconi's syndrome and consigned the amino-aciduria to abnormal protein synthesis with resultant excessive excretion of amino acids (3). Recent observations suggest that the renal dysfunctions, formerly thought to be an independent familial trait (13), are manifest only in overt cases of Wilson's disease with disturbed copper metabolism and increased urinary excretion of this metal (4). The present studies make it clear that copper possesses notable nephrotoxic properties in goldfish. Whether these properties play a role in the renal dysfunctions of Wilson's disease remains obscure. However, in respect to this, it is noteworthy that amino-aciduria and other renal abnormalities, including conspicuous cytologic changes in the tubular epithelium, have been repeatedly observed with intoxications by other heavy metals, notably lead, mercury, cadmium, and uranium (14, 15).

The accumulation of copper in the liver of goldfish in concentrations comparable to those in Wilson's disease, has interest in the absence of notable cytologic changes in the parenchymal cells and in the absence of cirrhosis. Much attention has been given to the possible role of copper in the pathogenesis of cirrhosis in general (16) and in hepatolenticular degeneration in particular (17). Under the conditions of the present experiment, copper induced neither necrosis nor cirrhosis in the livers of goldfish.

SUMMARY

Goldfish kept in water containing ionized copper and a detergent added with the aim of decreasing coagulation of the mucus on the gills, took in and retained this metal in their brains, livers, and kidneys, in concentrations comparable to those that occur naturally in Wilson's disease, as chemical assays disclosed. Histochemical studies made it clear that much copper had accumulated

within the large neurons, principally in those of the telencephalon and anterior horn region of the spinal cord and in the tubular epithelial cells of the kidneys, the nuclei of the parenchymal cells of the liver, the sarcoplasm of the skeletal muscle, and in the epithelial covering of the gills.

The intraneuronal deposition of copper was regularly associated after a time with conspicuous cytologic changes, notably contraction and hyperchromaticity of the nerve cells with tortuosity and fragmentation of the axis cylinders and lysis and loss of neurons. The accumulation of metal in the renal epithelium was frequently accompanied by necrosis and was regularly associated with hyperplasia and calcification of the epithelial cells of the larger renal tubules in all goldfish kept for prolonged periods in copper-rich water. The deposition of copper in the liver was not accompanied by consistent cytologic changes.

The similarity of the cytologic alterations induced in the central nervous systems by copper and those that occur naturally in hepatolenticular degeneration in human beings provides evidence that copper itself plays an important role in the pathologic alterations of the brain in Wilson's disease.

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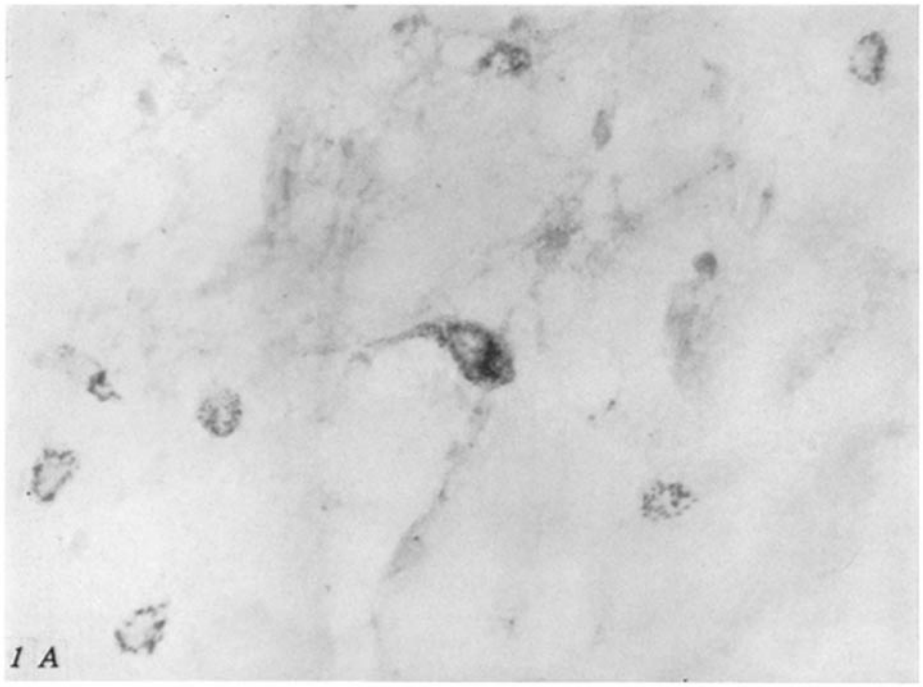
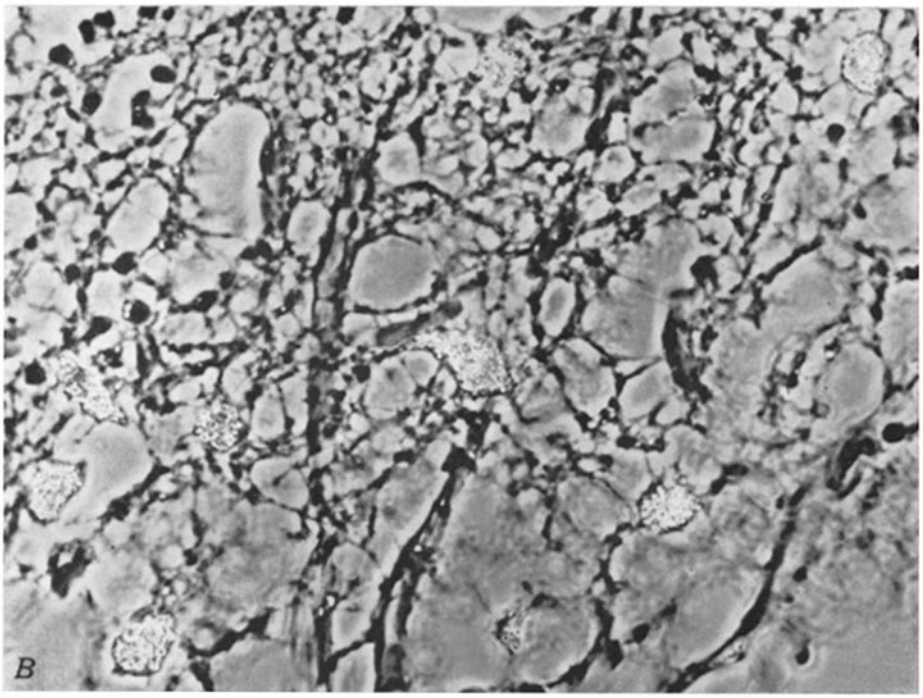
EXPLANATION OF PLATES

PLATE 70

FIG. 1. A. The telencephalon of a goldfish kept for 35 weeks in copper-rich water. The neurons contain abundant quantities of copper, appearing as dark granules in the cytoplasm and proximal segment of the axis cylinders.

B. Phase microscopy to show the tissue topography. The copper appears in bright relief. Rubeanic acid stain for copper without counterstain. × 700.



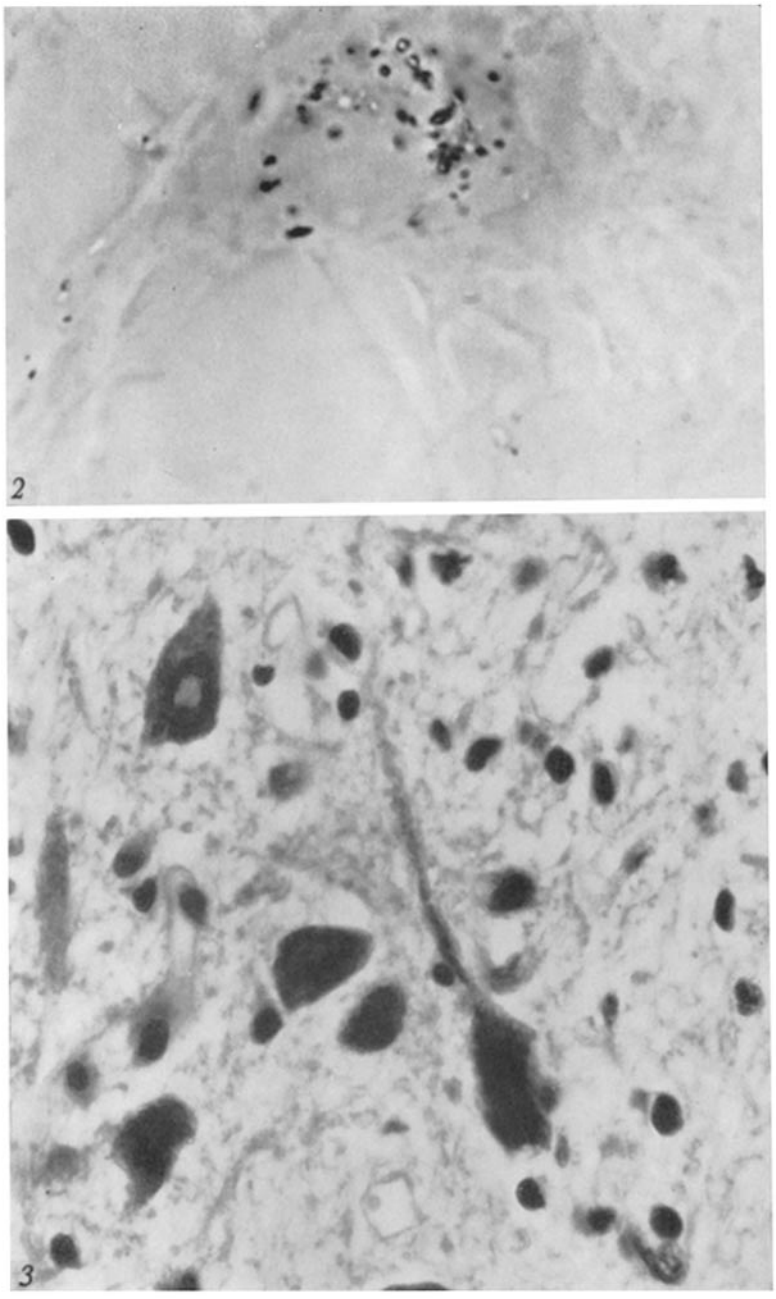


(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 71

FIG. 2. A neuron in the anterior horn region of the thoracic segment of the spinal cord of a goldfish kept for 35 weeks in copper-rich water. Abundant quantities of copper are present in the cytoplasm of the nerve cell. Rubeanic acid stain for copper without counterstain. × 2,700.

FIG. 3. Neurons in the telencephalon of a fish kept for 35 weeks in water containing ionized copper show conspicuous degeneration. The large nerve cell is contracted and hyperchromatic and its axis cylinder stains intensely and is tortuous. A large vacuole is present in the nucleus of another shrunken nerve cell. Nissl's cresyl violet stain. × 760.

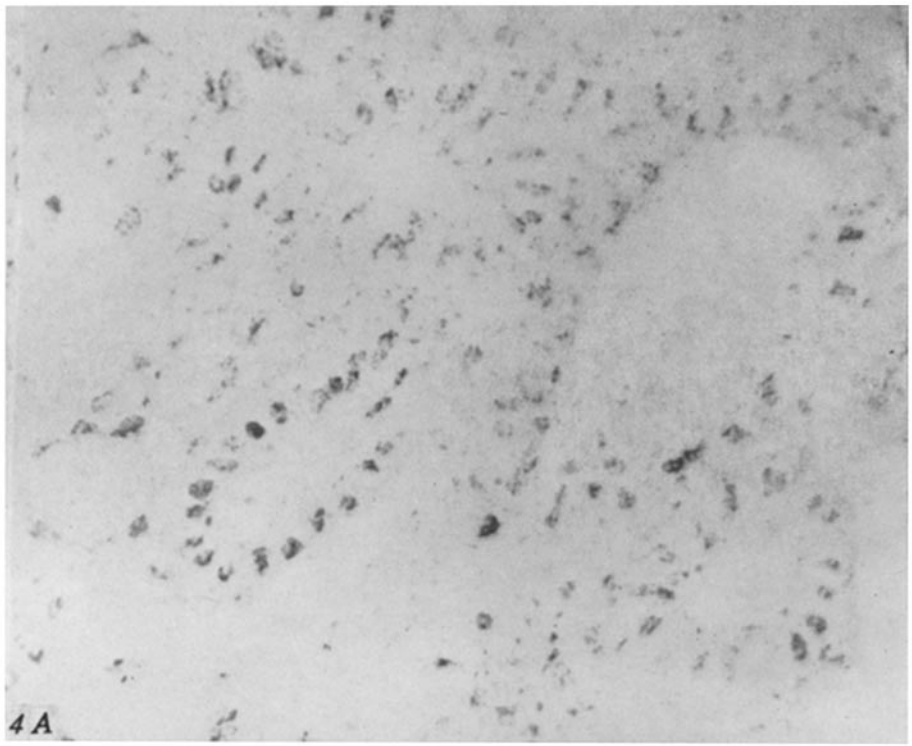


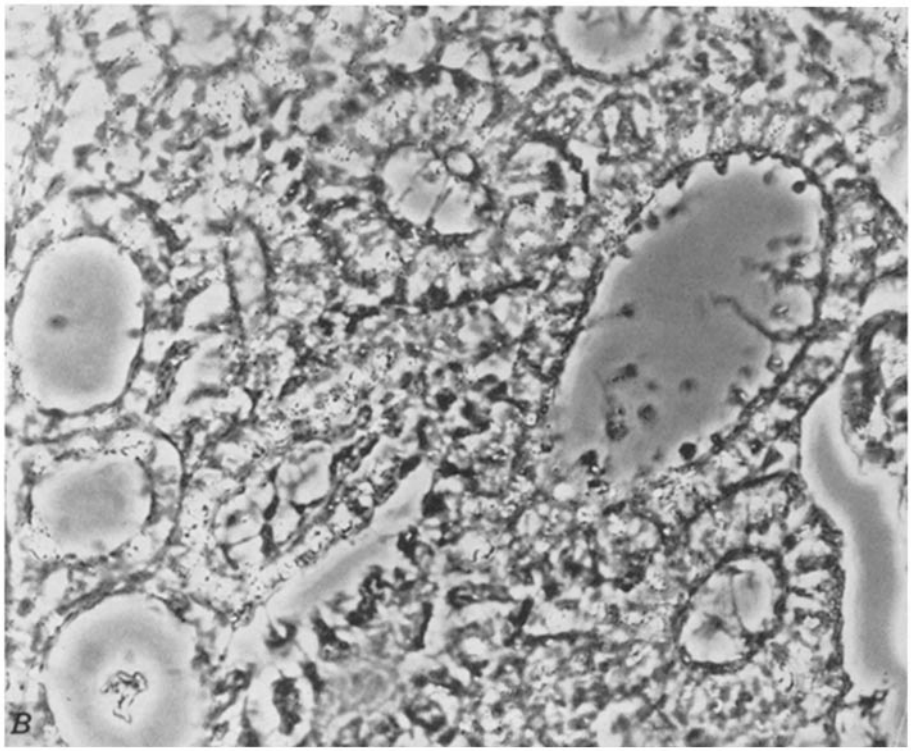
(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 72

FIG. 4. A. The epithelial cells of the renal tubules of a goldfish kept for 28 weeks in copper-rich water contain large quantities of metal, appearing as dark granules within the cytoplasm and nuclei, principally of the cells of the larger tubules.

B. The topography as shown by phase microscopy. Rubeanic acid stain without counterstain. × 750.



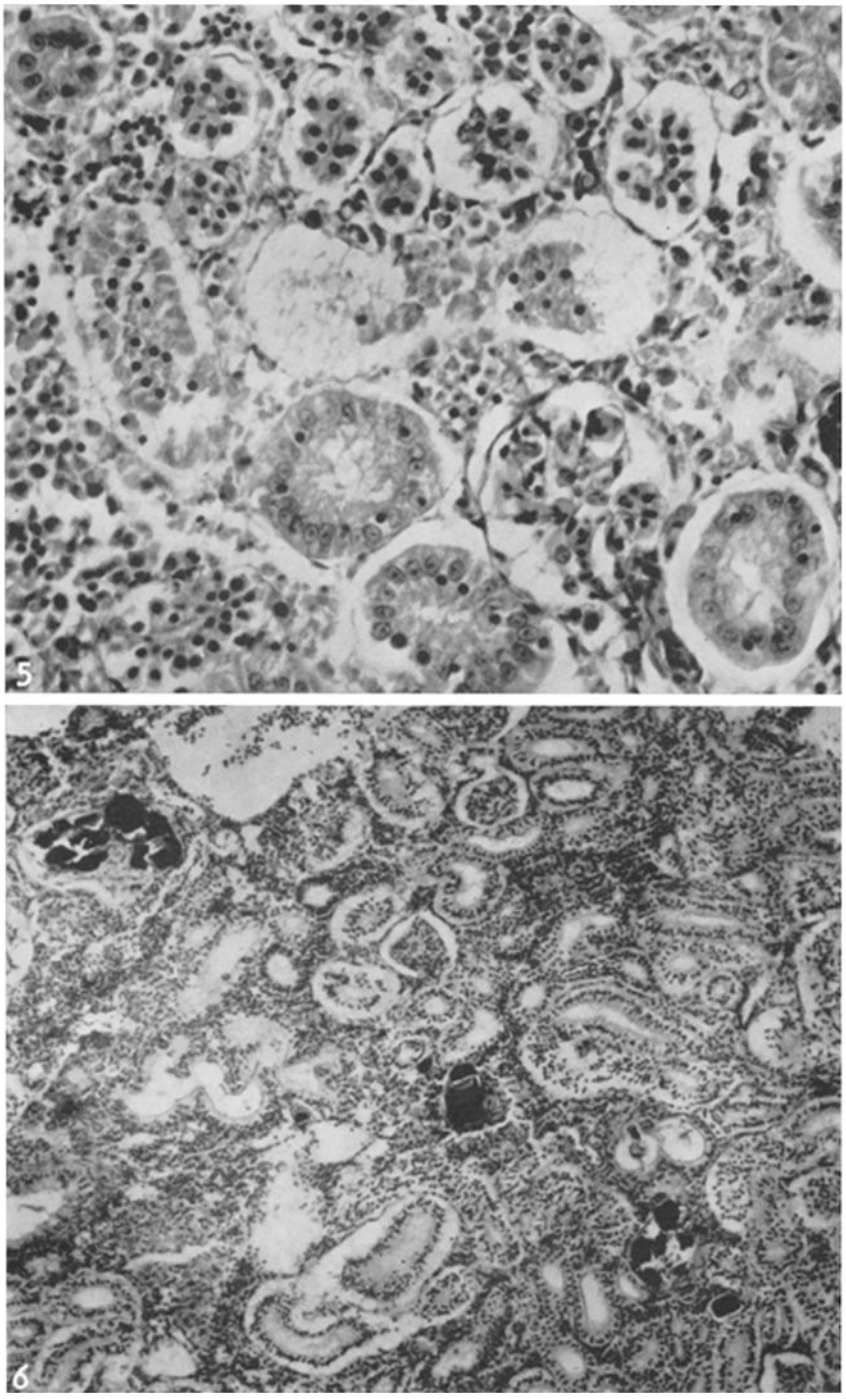


(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 73

FIG. 5. Marked necrosis of the renal tubular epithelium of a goldfish after 25 weeks in water containing ionized copper. The epithelial cells of a large renal tubule show marked lysis of the cytoplasm and pyknosis of the nuclei with desquamation. The glomerulus and epithelium of the smaller tubules are better preserved. Hematoxylin and eosin. × 455.

FIG. 6. The kidney of a goldfish kept for 24 weeks in copper-rich water shows irregular epithelial hyperplasia and calcification. The epithelial cells of the larger tubules have abundant clear cytoplasm and impinge upon the lumen. Hematoxylin and eosin stain. × 180.

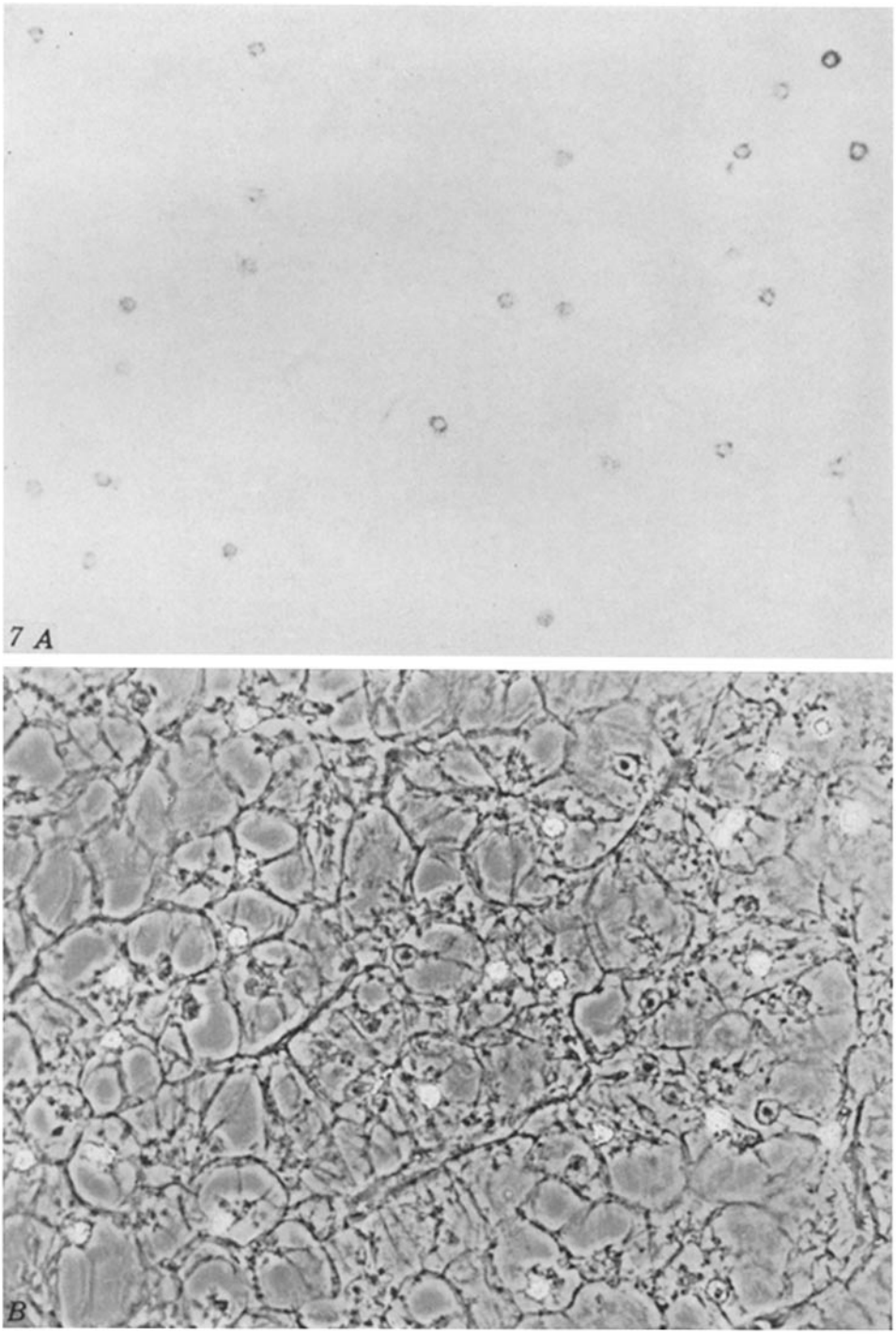


(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 74

FIG. 7. A. A goldfish kept for 27 weeks in copper-rich water shows abundant copper in the nuclei of the hepatic parenchymal cells.

B. Phase microscopy to show the tissue topography. The copper content of the parenchymal cell nuclei is variable; some are intensely stained; others contain little or no stainable metal. Rubeanic acid stain without counterstain. × 550.

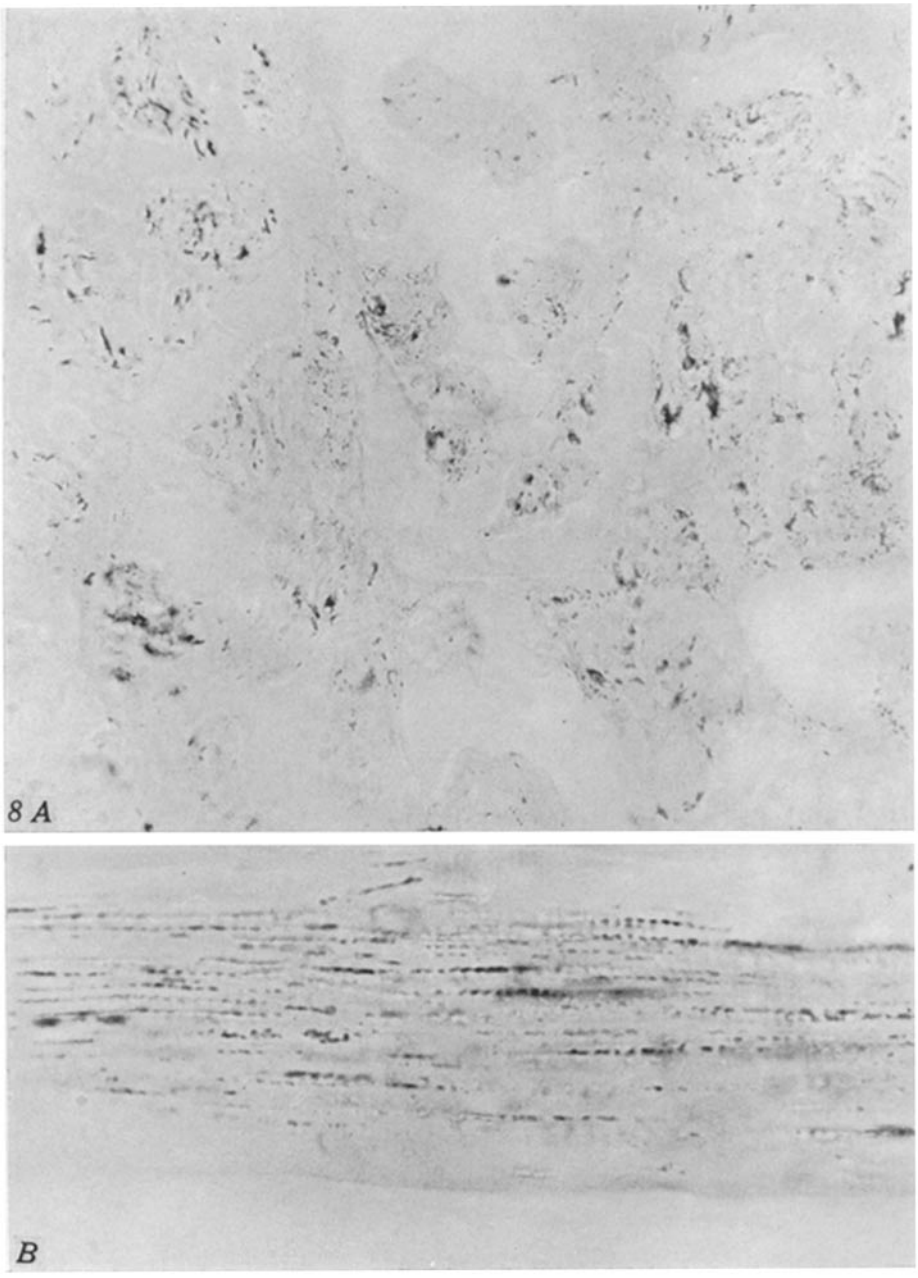


(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 75

FIG. 8. A. The paravertebral muscle of a goldfish kept for 35 weeks in copper-rich water. The muscle fibers contain abundant quantities of metal and are atrophic. × 550.

B. Longitudinal section of a muscle fiber shows much copper in a periodic arrangement. Rubeanic acid stain for copper without counterstain. × 1200.

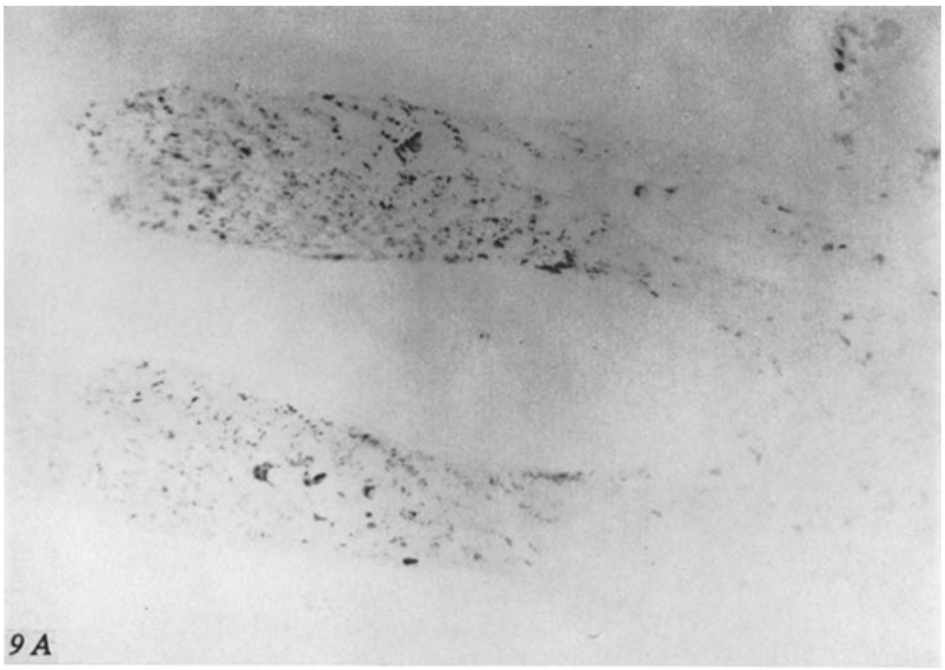


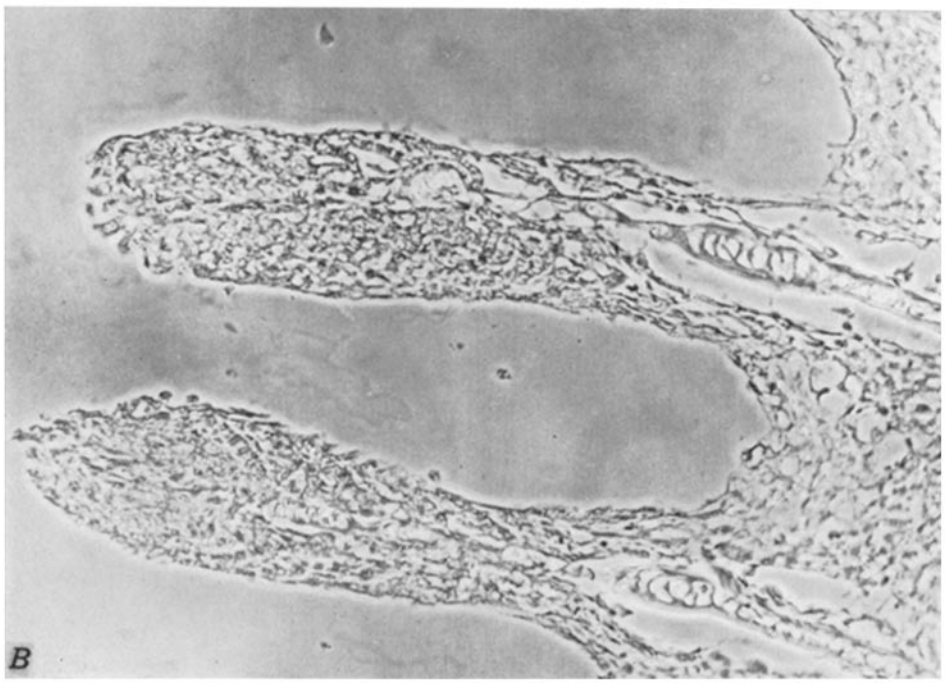
(Vogel: Neurotoxic and nephrotoxic properties of copper)

PLATE 76

FIG. 9. A. There is much copper in the epithelial cells that cover the gills and lesser quantities are present in the fibrous and cartilagenous stroma. The tissues are from a goldfish kept for 25 weeks in water that contained ionized copper.

B. The topography of the tissues is shown by phase microscopy. Rubeanic acid stain for copper without counterstain. × 160.





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