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Wilson's Disease: Identification of an Abnormal Copper-Binding Protein

Abstract. *The metal-binding protein metallothionein was isolated from the livers of Wilson's disease patients and control subjects. The metals were removed from the native protein to produce the apoprotein, and copperthionein was prepared by equilibrium dialysis. Copperthionein from Wilson's disease patients had a copper-binding constant four times as great as that of the protein from control subjects. These results suggest that the alterations in copper homeostasis in Wilson's disease result from the synthesis of an abnormal metal-binding protein with an increased affinity for copper.*

Wilson's disease (hepatolenticular degeneration) is an inborn error of metabolism that affects copper homeostasis. Hepatolenticular degeneration was first described in 1912 (*1*), and the pattern of inheritance, clinical features, and pathological changes associated with the disease have been well characterized (*2*).

The genetic abnormality in Wilson's disease leads to an excessive accumulation of copper in the liver, brain, kidneys, and cornea. Other abnormalities in the symptomatic patient include low serum ceruloplasmin concentrations, elevation of serum copper not bound to ceruloplasmin, increased urinary copper, and decreased fecal copper. Although many theories have been suggested to explain the genetic defect in Wilson's disease, none has been proved.

Uzman *et al.* (*3*) first suggested that the primary defect in Wilson's disease is the synthesis of an abnormal protein with a high affinity for copper. In adult mammals, the major portion of the total hepatic copper is located in the cytosol (*4*), where the metal is bound to the enzyme superoxide dismutase and a low molecular weight protein similar to metallothionein (*5*). The experiments reported here demonstrate that hepatic metallothionein from patients with Wilson's disease has a higher copper-binding constant than does that from control patients.

Liver samples were obtained from two teenage symptomatic Wilson's disease patients (*6*) and two teenage patients with elevated hepatic copper due to biliary cirrhosis (*7*). Each sample was homogenized in 0.025*M* phosphate buffer, *p*H 7.4; 1.0 μc of ^{64}Cu was added to each homogenate; and the contents were stirred for 1 hour. The ^{64}Cu-labeled metallothionein was isolated by the method of Pulido *et al.* (*8*). Briefly, the procedure consisted of extraction in phosphate buffer followed by precipitation with ethanol and chloroform. After precipitation, the supernatants were dialyzed against distilled water and concentrated by freeze-drying. The concentrated samples were then purified by gel filtration chromatography on Sephadex G-50 (*9*), Sephadex G-75, and Bio-Gel P-10 (in that order). The [^{64}Cu]metallothionein was detected during purification by monitoring radioactivity in a gamma-well counter. Protein concentration was determined with biuret reagent.

After isolation and purification of metallothionein, the apoprotein, thionein, was prepared by dissolving 1.0 mg of metallothionein in a 1.0-ml solution that had been adjusted to *p*H 2 with 0.1*N* HCl. The acidified protein solution was chromatographed on a 2.5 by 40 cm column packed with Sephadex G-25 that had been equilibrated with 0.01*N* HCl. The purity of the apoproteins was assayed by electrophoresis in 5 percent polyacrylamide gels as described by Davis (*10*). Thionein prepared from both Wilson's disease patients and control patients migrated as a single band at *p*H 8.6 and at *p*H 6.3, which indicated a high degree of purity in the preparations.

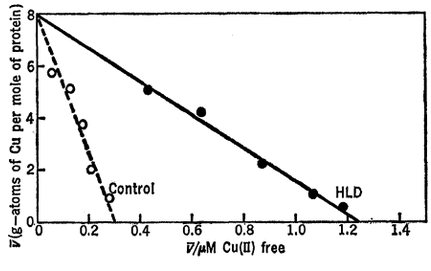

Fig. 1. Scatchard plots of equilibrium dialysis data for thionein and Cu(II). Thionein was dialyzed against various concentrations of $CuSO_4$. Least-squares regression lines are drawn through the plotted average data for controls and subjects with hepatolenticular degeneration (*HLD*).

Copperthionein was prepared from thionein by equilibrium dialysis. The apoprotein (1.0 mg) was dissolved in 10.0 ml of a solution containing 0.1*M* $KHPO_4$ (*p*H 7.4) and $CuSO_4$. The sample was placed in Visking Nojax tubing (24/32) and dialyzed against 10.0 ml of the same solution. The same procedure was repeated with several different concentrations of $CuSO_4$, and the solutions were dialyzed with continuous stirring for 48 hours at 4°C. After dialysis, the copper concentration of the solution was determined by atomic absorption spectrophotometry (*11*). The number of Cu(II) binding sites on the protein and the binding constants were determined by the method of Scatchard (*12*).

Although thionein from both groups of subjects contained the same number of copper-binding sites, the copper-binding constant for the protein from Wilson's disease patients was approximately four times as great as that from control subjects (Fig. 1). If a molecular weight of 10,000 is assumed for thionein, the copper-binding constant of the protein from Wilson's disease patients was $1.25 \times 10^6 M^{-1}$ whereas that from the control subjects was $3.0 \times 10^5 M^{-1}$; the number of copper-binding sites on the protein from both groups of subjects was calculated to be 7.9 g-atom/mole, which agrees with the number of metal-binding sites calculated in previous studies (*8*).

An abnormal protein with increased affinity for copper would explain many of the defects in copper homeostasis in hepatolenticular degeneration, especially in asymptomatic patients. In normal mammalian liver, copper enters the hepatocyte and becomes distributed throughout the cytosol and subcellular organelles where the metal is stored, incorporated into enzymes, or excreted. However, the increased binding affinity of the temporary storage protein in the hepatocyte of Wilson's disease patients probably shifts the normal equilibrium of the hepatic copper pool which results in both depressed biliary copper excretion and decreased incorporation of copper into ceruloplasmin. As the disease progresses, the binding sites on the storage protein

become saturated, and the excess metal is ingested by the hepatic lysosomes. Saturation of the hepatic copper-binding sites results in a decreased uptake of the metal with a concomitant elevation in plasma copper not bound to ceruloplasmin. Whether the increased deposition of copper in extrahepatic tissues results from the elevated nonceruloplasmin copper or from the presence of the abnormal protein in these organs is not yet known (*13*).

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Spontaneous Whole Brain Slow Potential Changes during Recovery from Experimental Neurosurgery

Abstract. *Prolonged, nonlocalized brain slow potential changes, frequently associated with cortical spreading depression, occur spontaneously during 5 days following brain implant surgery in rats. These potentials are accompanied by reductions in multiple nerve cell activity and reductions in behavioral motility. The method used in this study provides a tool for evaluating recovery from neurosurgical trauma or other brain injuries, and for testing procedures that facilitate or impede this process.*

Injury effects are unavoidable complications of neurophysiological experiments when measurement of brain functions involves implantation of various devices in the brain. As a result, brain functions are often investigated in a previously injured brain. Thus far, effects of implants on the functioning of the brain, as well as the time necessary to recover from implantation trauma, have not been extensively investigated. Brain implantations result in prolonged increases in intracranial pressure, changes in brain impedance, and nonspecific electroencephalographic (EEG) abnormalities (*1*); however, more information is needed on the nature of events during recovery from implant surgery and the effect of such events on neuropsychological and neurophysiological functions. We now report that prolonged slow potential (SP) shifts, with superimposed episodes of the cortical spreading depression (SD) described by Leao (*2*), occur spontaneously during the first 5 days of recovery from brain implant surgery. These SP brain phenomena are associated with reductions in multiple nerve cell activity (MA) as well as depression of behavioral motility. The occurrence of these episodes thus influences neurophysiological and behavioral functions and represents an uncontrolled factor in many experiments.

Table 1. Peak amplitude of SP waveforms (mean ± standard deviation).

Area	SP amplitude (mv)	
	Preceded by SD	Not preceded by SD
Front cortex	− 3.5 ± 1.17	− 3.1 ± 0.90
Back cortex	− 3.4 ± 1.18	− 3.1 ± 0.86
Hypothalamus	− 2.5 ± 1.09	− 5.4 ± 1.33

Ten male Holtzman albino rats (350 to 400 g) were prepared under Nembutal anesthesia (40 mg per kilogram of body weight, intraperitoneally) for recording SP changes from two cortical areas and one subcortical area. The potentials were recorded in reference to the occipital bone marrow. The implant procedures were similar to those reported for cats (*3*) but with the electrodes miniaturized for rats. The two intracortical d-c electrodes were located 1 mm into the left cortex 2 mm from midline, with one 3 mm anterior to bregma and the other 10 mm posterior to the first. A third d-c electrode was implanted into the area of the anterior-lateral hypothalamus on the right side 2 mm posterior to bregma, 1.8 mm lateral to midline, and 7 mm below the dura. Placements were verified by postmortem examination.

Bipolar 30-gauge Nichrome-steel electrodes, insulated except for the cut ends, were implanted into the left anterior thalamus and right lateral hypothalamus for recording MA. Bilateral 22-gauge cannulas for guiding subsequent brain penetrations were placed 2 mm anterior to the front cortex d-c electrode and inserted just into the cortex at a 30° anterior-posterior angle. Stainless steel jeweler's screws were inserted into burr holes in the skull to help anchor the entire electrode assembly. Penicillin G procaine and penicillin G benzathine (15,000 units of each) were injected intramuscularly as a prophylactic measure. After surgery, the animals were placed in a recording cage with free access to food and water.

Brain potentials were fed through a movement artifact–free cable, suspended above the cage, to the recording amplifiers. In subsequent recordings of SP and SD, the output of Grass P-17 d-c amplifiers was fed to Grass 7P1 d-c chopper-stabilized amplifiers and displayed on a Grass model 7 polygraph. Recordings of MA were made through a Grass P-15 amplifier. The output of the amplifier was filtered activity in a band between 800 and 3000 hertz half-amplitude, eliminating EEG and other frequency signals from the MA records. The activity was fed into a Grass 7P3 integrator amplifier with the integrated output continuously displayed on the polygraph paper. Behavioral movement was continuously