EFFECTS OF COPPER DEFICIENCY AND HYPERPHENYLALANINE ON THE DEVELOPING RAT BRAIN

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
JOSEPH ROBERT PROHASKA
1974

This is to certify that the

thesis entitled

EFFECTS OF COPPER DEFICIENCY AND

HYPERPHENYLALANINE ON THE DEVELOPING RAT BRAIN

presented by

Joseph Robert Prohaska

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

William W. Wells
Major professor

Date September 12, 1974

O-7639

ABSTRACT

EFFECTS OF COPPER DEFICIENCY
AND HYPERPHENYLALANINE ON THE
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By

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It was the initial intent of this thesis to test the possibility that high levels of phenylalanine could affect copper metabolism. Experiments producing hyperphenylalaninemic conditions in rats were carried out by feeding L-phenylalanine (5%) to male weanling rats or by subcutaneous injection of L-phenylalanine with or without DL-ρ-chlorophenylalanine to neonatal rats. When a diet adequate in copper was fed to the rats or to the dams whose offspring were injected, hyperphenylalanine induction did not affect plasma or brain copper levels, brain cytochrome c oxidase, plasma diamine oxidase, or fecal copper output. The activity of plasma diamine oxidase (ceruloplasmin) from patients with phenylketonuria, an inherited disease in which hyperphenylalaninemia is characteristic, was equivalent to age matched controls. Injections of phenylalanine to offspring from dams fed a low copper diet suggested retention of body copper based on a delayed fall in the plasma diamine oxidase level and on an increased brain copper content and cytochrome oxidase level. The hyperphenylalaninemic pups from dams fed a diet adequate in copper and the copper-deficient offspring injected with saline both developed abnormally and further studies were carried

out on the two groups, independently, as model systems for the genetic disorders Phenylketonuria and Menkes' steely-hair disease.

Since it was known that both copper deficiency and hyperphenylalaninemia resulted in hypomyelination of the central nervous system, a marker to follow this process was investigated. The myelin enriched protein 2',3'-cyclic nucleotide 3'-phosphohydrolase was employed as an indicator of myelination in the experimental models. An improved assay for this protein was devised resulting in an increased precision - a coefficient of variation of 1.7% for six trials. This was partially the result of optimizing the temperature (30°C) and the pH (6.2) for the reaction. The concentration of this protein in whole brains of three neurological mutant mice (quaking, jimpy, and myelin synthesis deficiency) was reduced five-fold when compared to littermate controls. There were no differences among these mice in plasma diamine oxidase, brain copper content, or cytochrome c oxidase activities suggesting that copper metabolism was grossly normal in these mice despite several similarities to the copper-deficient rat.

Since prior work on the effects of hyperphenylalaninemia on the developing rat brain had emphasized the reduction in myelin lipids, a study was carried out to investigate brain protein development. It was found that subcutaneous injections of L-phenylalanine and DL-ρ-chlorophenylalanine into developing rats resulted in reduced body and brain weights and a small but significant decrease in 2',3'-cyclic nucleotide 3'-phosphohydrolase. These reductions were less marked when injections were reduced by half. When animals were allowed to recover after 13 days of age, no differences were observed at 40 days of age suggesting

recovery when treatment was discontinued before hyperplasia was complete. Brain hexokinase and cytochrome c oxidase development was not changed by the injection treatments. This is consonant with the idea that the reduction in the myelin protein 2',3'-cyclic nucleotide 3'-phosphohydrolase represents less myelin and that the reduction was not a general feature of all brain proteins.

Copper deficiency was produced in developing rats by feeding a low copper diet (0.3 parts per million) to rats during gestation and lactation and providing the offspring the same diet or by preconception depletion followed by marginal supplementation during gestation. The progeny showed an appreciable decrease in body growth, a slight decrease in whole brain and cerebellar growth and a significant reduction in the concentration of 2',3'-cyclic nucleotide 3'-phosphohydrolase. Specific effects of a five-fold decrease in copper content were reductions in the metalloproteins cytochrome oxidase (to 20% of control) and superoxide dismutase (to 70% of control). A similar reduction in norepinephrine concentration was also found. Zinc levels of control and copper deficient brains were equivalent; there was some evidence of a slight reduction in iron concentration. Electron microscopic examination of the cerebral cortex revealed the presence of enlarged mitochondria from the copper-deficient animals. When isolated, these mitochondria oxidized succinate and glutamate at 70% the rate control mitochondria did; oxygen consumption in the presence of glutamate was not appreciably increased by the addition of adenosine diphosphate to the reaction. Mitochondrial difference spectra verified the large reduction in cytochrome oxidase and demonstrated slight increases in cytochromes

b, c_1, and c. Enzyme analysis of isolated mitochondria showed slight increases in succinic dehydrogenase and fumarase, and a small decrease in hexokinase. Malondialdehyde production was equivalent in controls and copper-deficient brain homogenates suggesting no abnormal lipid peroxidation. Determination of metabolites suggested that the copper-deficient brain was in a more reduced state since both lactate/pyruvate and α-glycerol-phosphate/dihydroxyacetone-phosphate ratios doubled. However, creatine-phosphate, adenosine triphosphate, and adenosine diphosphate concentrations were not different. Therefore, the tenet that hypomyelination in copper deficiency results from a decrease in cytochrome oxidase and therefore a reduction in energy production should be questioned until further investigation is performed.

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By

Joseph Robert Prohaska

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1974

ACKNOWLEDGEMENTS

The author would like to thank Professor William W. Wells for his enthusiastic suggestions, generous financial support, and unselfish flexibility. Doctors Richard Luecke, Steven Aust, Loran Bieber, Clarence Suelter and Duane Ullrey, members of my guidance committee, have my gratitude for their cooperative manner of instruction, suggestion and criticism.

I would also like to acknowledge Douglas Clark for his technical assistance and Don Musick whose penetrating discussions facilitated my thesis. This work could not have transpired without the totally unselfish encouragement of my parents and my loving wife, Carol. Lastly, I wish to acknowledge the financial help I received from the National Science Foundation.

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LIST OF ABBREVIATIONS

2',3'-cAMP	adenosine 2',3'-cyclic monophosphate
AMP, ADP, ATP	adenosine 5'-mono-, di-, or triphosphate
CNS	central nervous system
CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase
CoA	Coenzyme A
DAO	diamine oxidase
EDTA	ethylenediaminetetraacetic acid
K_m	Michaelis constant
NAD	nicotinamide adenine dinucleotide
ρ-Clphe	parachlorophenylalanine
RCR	respiratory control ratio
sc	subcutaneous
SD	standard deviation
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

ORGANIZATION

Each chapter in the thesis text is presented in a form found in biochemical journal articles. Each one includes its own abstract or summary, introduction, materials and methods, results, discussion, acknowledgements and references. Style varies somewhat depending on the particular journal for which the chapter was written. Chapter II, under its title, is presented as it was accepted for publication in the Proceedings of the Society for Experimental Biology and Medicine. Chapter III has been published under the chapter title in the Journal of Neurochemistry (1974) 23, 91-98. Chapter IV is in review, Archives of Biochemistry and Biophysics. These were all accepted or submitted under my authorship and that of W. W. Wells. For the reader's convenience, a reprint of an additional article (Analytical Biochemistry (1973) 56, 275-282.) of some interest to the thesis has been inserted as an APPENDIX. A DISCUSSION section has been included to consider material not specifically dealt with in the preceeding chapters and to offer some overview on the project's results.

APPROACH

Despite a multitude of hypotheses concerning the underlying cause of the mental retardation associated with the untreated phenylketonuric patient, no single concept seemed satisfactory. There are several superficial similarities between the phenylketonuric and the copper-deficient animal. Both are characterized by hypomyelination of the central nervous system (CNS) and had pigmentation abnormalities and behavior changes that could be rationalized by catecholamine alterations.

Since it was also known that amino acids had a high affinity for Cu^{2+} (1) and that Cu-amino acid complexes existed in serum (2), a study was initiated to determine whether the induction of hyperphenylalaninemia could result in a change in copper homeostasis. These experiments were performed with albino rats which served as models throughout the course of this research. Results indicated that under normal conditions phenylalanine did not significantly alter copper metabolism. However, both the production of hyperphenylalanine and copper deficiency resulted in abnormal development of the offspring.

It has been estimated (3) that during the peak of myelination a single oligodendroglial cell synthesizes greater than 3-fold its own weight of myelin per day. This endergonic process is, therefore, most susceptible to metabolic insults. In order to monitor myelination a marker protein, 2',3'-cyclic nucleotide 3'-phosphohydrolase, was employed during rat brain development (4). An improved assay method for this enzyme made the handling of multiple samples more feasible. In some studies the development of the cerebellum was emphasized because of its higher risk of injury in postnatal development (5).

Experiments producing hyperphenylalanine were designed to see if myelin protein levels were changed by this treatment since prior work had involved only myelin lipid constituents. Another objective was to learn if this retardation in myelination was reversible upon discontinuation of the injection treatment, since it was known that dietary restriction of phenylalanine, if applied at an early age, was effective in the amelioration of the phenylketonuric.

While this research was in progress Danks et al. (6) reported that

the genetic disorder referred to as Menkes' disease was characterized by copper deficiency. Severe neuropathology is also characteristic of this disorder. The developing rat brain was, therefore, chosen as a model for this disease. Dietary production of copper deficiency was carried out and the offspring were used to follow myelination and the development of the copper metalloenzymes, cytochrome oxidase and superoxide dismutase. Norepinephrine was measured to evaluate the status of another copper protein, dopamine-β-hydroxylase. Subsequent work revealed that the brains of copper-deficient rats had abnormal mitochondria. So further work dealing with oxygen consumption and enzyme components of isolated mitochondria was performed. These studies were accompanied by metabolite analyses in an attempt to correlate the in vitro studies of isolated mitochondria and enzyme assays with the in vivo brain energy status.

LITERATURE REVIEW

Copper-Amino Acid Interaction. In 1950 Albert (1) reported quantitative results on the stability constants of bivalent amino acids with trace metal ions. He found Cu^{2+} was bound most tightly of the eight ions studied. Fifteen years later the first reports of amino acid-copper complexes in serum appeared (2, 7). This bound copper was in equilibrium with a pool bound to serum albumin. There is good evidence that a ternary complex of albumin - Cu^{2+} - amino acids exists in vivo (8). This amino acid pool is thought to be involved in the biological transport of copper (7) and thus under pathological situations where the elevation of an amino acid occurs there may be

adverse effects (2).

2',3'-Cyclic Nucleotide 3'-Phosphohydrolase. Drummond et al. (9) first reported the activity of this protein from brain which was later (10) shown to be associated with the myelin. The use of this protein as an enzyme marker for myelin as well as a detailed summary of the many techniques available for its detection have been described before (11) (see APPENDIX). Newer methods for the detection of CNP (2',3'-cyclic nucleotide 3'-phosphohydrolase) include potentiometric analysis (12), a fluorometric assay (13) using N^6-ethenoadenosine-2',3'-cyclic monophosphate as substrate and a spectrophotometric technique (14) using 2',3'-cyclic cytosine monophosphate. CNP activity can also be detected after electrophoresis on polyacrylamide gels (15). This technique demonstrated that CNP was not one of the major myelin proteins. It is reported to have a molecular weight of approximately 100,000 (14). Its continued useage as a myelin marker is evidenced by reports on studies of spinal cord tissue cultures (16), Wallerian degeneration (17), and human brain tumors (18).

Hyperphenylalanine. The hyperphenylalaninemic state is used in experimental models to learn more about this condition in man, since it is a consequence of the disease phenylketonuria (PKU). PKU was first described by Følling (19) in 1934 and has since been characterized as an autosomal disorder resulting in the failure to convert phenylalanine to tyrosine (20), thus, creating the hyperphenylalanemic state. It has been suggested by many authors that this condition results in the neuropathological changes seen in PKU specimens. These defects are usually hypomyelination in the young

patient and progressive demyelination in the older cases (21). These conditions vary somewhat and the reader is referred to an excellent discussion on this topic (22). Another characteristic of PKU brain tissue is a reduction in the catecholamine content, dopamine and norepinephrine, (23); due to the combined effects of an inhibition of tyrosine hydroxylase and to competitive inhibition of tyrosine transport. The pigmentation abnormality in PKU can be attributed to inhibition of tyrosinase by phenylalanine (24) and to reduced substrate, tyrosine. Of the many models producing hyperphenylalaninemia, dietary induction (25) or subcutaneous injection techniques (26, 27) are the most common.

Menkes' Steely-Hair Disease. In 1962 Menkes et al. (28) first reported cases of a sex-linked recessive disorder accompanied by neural degeneration. Ten years later Danks et al. (6, 29) reported that this disease was characterized by copper deficiency due to defective intestinal absorption. It is a rare disorder and only limited biochemical studies have been made. Preliminary data based on reduced myelin lipids such as cerebroside and sulfatide (30) and on lower yields when myelin was isolated (31) suggested that the disorder was associated with hypomyelination. A recent publication (32) reported the presence of cholesterol esters in a brain specimen suggestive of active demyelination. One positive argument as to the implication of copper's role in the etiology of this disease has been strengthened by the finding of low brain copper in three cases of this disorder (33).

Copper and Human Brain Metabolism. A lack of copper can be

detrimental to the development of the CNS as in the case of Menkes' disease. An excess of copper can also be detrimental to the CNS and a genetic disease in which this occurs is known, Wilson's disease or hepatolenticular degeneration (34). This is an autosomal recessive disorder which results in brain damage due to an accumulation of copper because of an abnormal binding protein in the liver (35). Besides these genetic disorders of copper, many more subtle roles of this element in brain metabolism can be found in a cursory perusal of Chemical Abstracts. Some of these include Parkinson's disease, schizophrenia, epilepsy, reflex reactions of the spinal cord, meningeal affection and multiple sclerosis. Copper homeostasis in the mammalian system has been recently reviewed (36) and will not be covered here.

Copper Deficiency in Animal Brain. One of the earliest observations concerning the copper-deficient brain was a reduction in cytochrome A ($a + a_3$) and oxidase activity (37). This finding was later verified by Gallagher et al. (38) in the most extensive work to date on copper-deficient rats. Reduced cytochrome oxidase associated with low copper levels has been reported for other animals besides rats and also for yeast (39). This loss of cytochrome oxidase was thought to be partially responsible for the hypomyelination seen in swayback lambs (38, 40).

Gallagher et al. (40) also reported a reduced rate of phospholipid synthesis in copper-deficient rats which was due to a failure of condensation of acyl CoA with α-glycerol phosphate. Others have been unable to repeat this (41). Despite the fact that the original work (40) found normal ^{32}P labeling of brain phospholipids and that all

evidence suggests that copper deficiency has no effects on brain phospholipid distribution (42, 43), current dogma supports the notion that abnormal phospholipid synthesis is responsible for hypomyelination.

Most studies concerning the effect of copper deficiency on brain metabolism have been carried out in animals whose CNS was already fully developed. However, prior to this research it had been shown that guinea pigs (44) were severely affected by gestational deprivation of copper. Carlton and Kelly (45) had done a study on the offspring of copper-deficient rats but did not include biochemical evaluations. While this work was in progress Di Paolo et al. (43) reported findings which support the results of a portion of this thesis (46) (chapt. III) by histochemically showing less myelin in the cerebella of neonatal rats from dams fed a low copper diet.

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CHAPTER I

EFFECTS OF HYPERPHENYLALANINE ON COPPER METABOLISM

ABSTRACT

Hyperphenylalaninemia was produced by either feeding a diet 5% L-phenylalanine or by the subcutaneous injection of L-phenylalanine (L-phe) with or without DL-ρ-chlorophenylalanine (DL-ρ-Clphe). These treatments were given to rats fed either a diet adequate or deficient in copper or to offspring from dams fed these diets. This was done to study the chronic effects of an elevated amino acid on copper homeostasis. When a diet adequate in copper was supplied, treatment with L-phe or L-phe with DL-ρ-Clphe did not affect copper levels in the plasma, diamine oxidase activity, fecal copper output, brain copper concentration, or brain cytochrome c oxidase levels. Likewise, ceruloplasmin (diamine oxidase) activity of phenylketonuric patients and age matched controls were equivalent. However, injection of L-phe and DL-ρ-Clphe into copper-deficient pups delayed the large fall in diamine oxidase activity and resulted in higher brain copper and cytochrome c oxidase. Three neurological mutations of mice with faulty myelin synthesis (quaking, jimpy and myelin synthesis deficiency) were studied because of similarities to the copper-deficient rat. They were found to have greatly reduced activities of the myelin protein 2',3'-cyclic nucleotide 3'-phosphohydrolase but displayed no differences in comparison to littermates in brain copper, cytochrome c oxidase or plasma diamine oxidase. It was concluded that under normal conditions hyperphenylalanine does not influence copper homeostasis, but that both the copper-deficient and hyperphenylalaninemic rat develop abnormally.

INTRODUCTION

Plasma copper exists predominantly (over 90%) bound to the glycoprotein ceruloplasmin (1). This copper has a biological half-time of 13 hrs, a maximum tissue uptake of 40 hrs (2) and is not exchangeable *in vivo* (3). A smaller fraction of copper (less than 10%) is bound to albumin (3) has a half-time of 10 min, a maximum tissue uptake of 1 hr (2) and is rapidly exchangeable *in vivo* (4). This albumin bound fraction is in equilibrium with a third pool, an amino acid-bound fraction (5), and is thought to have a physiological role in copper transport. In a comparison of the avidity of bivalent amino acids for the ions of trace metals, Cu^{2+} complexes were found to have the highest stability constants (6).

It has been suggested that the cupruria seen in acute viral hepatitis may result from the observed aminoacidemia and aminoaciduria (7). Oral methionine loading of patients with Wilson's disease, which is characterized by high tissue levels of copper, resulted in a two-fold increase in both fecal and urinary copper output (8). Administration of histidine to man has produced hyperzincuria with an associated hypozincemia (9). Sarkar and Kruck have suggested ". . . that in patients with abnormal plasma free amino acid levels there may be abnormal patterns of copper-amino acid complexes that could have pathological consequences." (10).

Phenylketonuria is an inherited diesease of man in which plasma phenylalanine may be highly elevated. It had been previously shown that phenylalanine could interact with Cu^{2+} in reversing the inhibition of $(Na^{+}+K^{+})$-ATPase (11). Therefore, experimental hyperphenylalaninemia

was studied to learn if it could alter copper homeostasis. Since dietary treatment with cuprizone (bis(cyclohexanone) oxaldihydrazone), a potent copper chelator, resulted in a reduction in brain copper as well as a concomitant decrease in cytochrome c oxidase (12), both these parameters were measured during phenylalanine treatment. Plasma copper was also evaluated in experimental animals as well as in phenylketonurics. Although likely fortuitous, both phenylketonuria and copper deficiency are characterized by a neuropathology involving both hypomyelination and demyelination (13, 14).

Copper metabolism was also studied in three myelin-deficient mouse mutants: quaking (15), jimpy (15) and myelin synthesis deficiency (16). Di Paolo, et al. suggested an investigation of this type based on the similarity between these mice and the copper-deficient rat (17). This would also provide a useful hypomyelination model to compare with both copper deficiency and hyperphenylalaninemia.

MATERIALS AND METHODS

Materials. Male weanling or female sperm-positive rats were purchased from Holtzman Co. (Madison, WI). Myelin mutant mice of the strain C57BL/6J were obtained from the Jackson Laboratory (Bar Harbor, ME). Frozen phenylketonuric serum and plasma were the generous gifts of Drs. Thomas Egan (Univ. Chicago), Richard Allen (Univ. Michigan), and James Higgins (Michigan State Univ.). Human ceruloplasmin was the gift of Dr. M. Wickerhauser (American Red Cross Blood Res. Lab.). Horse heart cytochrome c was received from Boehringer-Mannheim

(New York, NY) and Tween-80 from Nutritional Biochemicals (Cleveland, OH). E. coli alkaline phosphatase type III-S (EC 3.1.3.1), 2',3'-cAMP, DL-ρ-chlorophenylalanine, L-phenylalanine, and ρ-phenylenediamine were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal Treatments and Diets. The composition of the basal diet has been detailed elsewhere (18) (see chapt. III) and was used as such except that in some cases the casein was not washed with EDTA. Diets containing 5% L-phenylalanine (19) were prepared by a corresponding 5% reduction in the sucrose content. Diets with both adequate and low copper content were used with or without supplemental L-phenylalanine.

Animals were maintained in stainless steel cages throughout the feeding experiments and received distilled water and food ad libitum. When hyperphenylalanine was produced by injections, the pregnant rats were fed either adequate or low copper diets beginning on the 8th day of gestation. One day prior to parturition they were placed in plastic cages bedded with wood shavings and remained there throughout lactation. Pups were randomized at birth (day 0), 8 per dam, within each dietary group.

Offspring were injected subcutaneously with a 3% solution of L-phenylalanine at a dose of 1 mg/g body wt (20) or alternatively with the same solution containing 0.3% DL-ρ-chlorophenylalanine (21). An equivalent volume of saline was injected into age matched control rats. All solutions were adjusted to pH 7.4 and were heated to 37°C before injection. Copper analysis of 100 ml aliquots of each

injection solution resulted in equivalent levels all below 0.1 μg Cu/ml.

Mice (C57BL/6J) were killed within 24 hours after arrival and included the following genotypes: quaking (qk/qk), jimpy (jp/Y) and myelin synthesis deficiency (msd/Y).

Copper Analysis. Copper was determined by atomic absorption spectroscopy (Perkin-Elmer model 303). Tissue, feces and diets were wet-ashed in HNO_3-$HClO_4$ acids and the residue was dissolved in hot 1% HCl. Standards were prepared in this solvent. Plasma samples were diluted with 2 volumes of glass distilled water (0.3 ng Cu/ml) and were measured directly. Copper standards for use in plasma measurements were prepared in 5% (v/v) glycerol. To avoid contamination, all glassware used in these experiments was soaked in HNO_3 or HCl and was thoroughly rinsed with glass distilled water. Plastic ware used for storage of samples was soaked in 1% EDTA and thoroughly rinsed.

Tissue Preparation. Whole brains were homogenized in 9 volumes of 0.32 M sucrose in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Aliquots were used to measure cytochrome c oxidase (EC 1.9.3.1) and 2',3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16b). In some cases brain tissue was stored -85°C, before homogenization. Alternatively, brains were first dryed, 80°C, to constant wt or used directly for acid digestion and copper analysis. Plasma was obtained after centrifugation, 3,000 x g for 10 min, of heparinized blood taken by heart puncture from ether anesthetized animals. Feces were collected by placing animals in stainless steel metabolic cages for two 48 hr periods. Fecal material was collected

in copper-free plastic containers and was free of dietary contamination (unlike the urine specimens).

Enzyme Assays. The assay of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) was performed as described previously (22) (see APPENDIX). Whole mouse brain was assayed without freezing of the homogenates.

Cytochrome c oxidase was measured as detailed previously (18) (see chapt. III) by following the oxidation of ferrocytochrome c at 550 nm. It was found the 1% (v/v) Tween-80 released total activity while Triton X-100 was found to be inhibitory (these observations are also true for succinic dehydrogenase activity). The activity of cytochrome c oxidase was found to decrease by as much as 1/3 when stored as a 10% (w/v) homogenate at -20°C for as little as 3 days. Conversely, tissue stored for 1 month lost less than 20% activity. For maximum precision cytochrome c solutions must be prepared fresh. In some experiments detergent treated mitochondria were incubated at 25°C for 20 min in either the presence of 5 mM L-phenylalanine or 5 mM cuprizone (bis(cyclohexanone) oxaldihydrazone) before activity was measured.

The diamine oxidase activity of ceruloplasmin (EC 1.16.3.1) was measured by a modification of the method of Levine and Peisach (23). The reaction was run at 37°C in 0.1 M sodium acetate (pH 5.5). The commercial ρ-phenylenediamine (1,4-diaminobenzene) was purified by repeated sublimation and was employed at 4 mM final concentration. It was dissolved just prior to use and adjusted to pH 5.5 as suggested (23). To avoid interference by traces of iron,

5 μM EDTA was also employed (24). Sodium azide, a potent inhibitor of ceruloplasmin (24), was used (0.5 mM) to terminate the reaction and to provide a nonenzymatic oxidation blank when included prior to substrate addition. Routine assays were performed for 30 min with 50 μl of plasma in a 1 ml final volume. The appearance of the purple oxidation product N,N'-bis-(2,5 diaminophenyl)-ρ-quinonediimine (Bandrowski's base) (25) was followed at 533 nm. Extrapolation from the data of Rice (25) would yield a value of $\varepsilon = 11.6 \times 10^3$ for this product. Based on this constant, ceruloplasmin activity was defined as μmoles/min/liter of plasma.

Chemical Methods. Protein was measured by the method of Lowry et al. (26) using bovine serum albumin as standard; Tween-80 interference was corrected for in the appropriate cases. Powdered human ceruloplasmin was dissolved in 0.15 M NaCl (pH 7.0) and the absorbance at 280 and 610 nm taken on several samples (Gilford model 2400S). The purity was estimated to be greater than 95% based on 280/610 ratio (27). Crystalline Bandrowski's base was prepared according to Rice (25) and was found to have an equivalent melting point (237°C). The visible spectrum (Cary model 15) displayed a broad peak centered at 533 nm. Attempts at solution of this compound, in a media comparable to the assay, were not successful although the molar absorptivity did approach that reported by Rice (25).

Statistical Methods. Mean comparisons were evaluated by use of a computer program written to calculate variance ratios and statistical *t*. Student's *t*-test was used and hypothesis testing was done at two levels of α:0.05 and 0.01.

RESULTS

Since modifications to the diamine oxidase assay were introduced, conditions for validity were studied. Figures 1A and 1B show that the formation of product, as measured by an increase in absorbance at 533 nm, is proportional to the amount of plasma and to the reaction time used in the final assay, 50 µl and 30 min respectively. A plot of velocity versus substrate is shown in Figure 2. When the data were analyzed by the method of Hofstee (28), the human ceruloplasmin had a K_m of 0.5 mM for 1,4-diaminobenzene. Activity was not influenced by inclusion of 2.5 mM L-phenylalanine during the reaction.

Dietary induction of hyperphenylalanine was carried out in conjunction with both copper-adequate and copper-deficient diets in three separate experiments of similar design, Table 1. The copper contents of the diets (both control and low) varied somewhat depending on whether the casein was washed with an EDTA solution (i.e. I versus III). The animals fed the low copper diet excreted less than 1/10 the amount of fecal copper that animals fed adequate copper did, irrespective of the presence of dietary phenylalanine, exp. I. No consistent statistical differences were evident in plasma copper levels or diamine oxidase activites (DAO) when dietary phenylalanine was the sole criterion, Table 1. This is illustrated more clearly in Figure 3 which shows that a strong correlation exists between the diamine oxidase activity and the dietary copper level regardless of the presence of 5% L-phenylalanine. There were no differences in the DAO activities between phenylketonurics and age matched controls, Table 2, although the younger patients had less activity than the

adult cases.

Injections of L-phenylalanine were administered to developing rats from dams fed either adequate or low copper diets, Table 3. As can be seen, the offspring from copper adequate dams were not affected by the daily injection regimen in terms of either plasma or brain copper levels. The offspring from copper deficient dams injected with phenylalanine seemed to have higher plasma copper levels especially at age 14 days, Table 3; perhaps brain copper is also elevated at this age. Only these offspring were noticeably reduced in body wt compared to the other three groups.

When a more intensive injection sequence was carried out, including DL-ρ-chlorophenylalanine (21), pups from both control and copper-deficient dams were severely reduced in size (data not shown). Pups from copper-deficient dams but injected with saline were somewhat smaller than pups similarly injected but of dams fed a diet adequate in copper. Analysis of the plasma DAO activities of the pups from copper-deficient dams injected with either phenylalanine with or without ρ-chlorophenylalanine is displayed in Figure 4. It can be seen that in both experiments the injection of the amino acid delayed the large fall in DAO.

This suggestion of elevated plasma copper is strengthened by the data in Table 4. Injection of phe and ρ-Clphe to the copper-deficient offspring resulted in higher brain copper levels and a corresponding elevation in brain cytochrome c oxidase content. However, Table 4 also demonstrates that pups from copper-adequate dams were not affected by the injection treatment in either the

concentration of brain copper or the level of cytochrome c oxidase. Cytochrome c oxidase activity was not affected by either the presence of L-phenylalanine or cuprizone in vitro.

Plasma DAO was not affected by injections of phe with or without p-Clphe in pups from copper-sufficient dams (data not shown); however, the DAO levels were lower than the adult values reported in Table 1 in agreement with the results for human plasma, Table 2.

An evaluation of copper metabolism in plasma and brain of three neurological mutant mice deficient in myelin along with their corresponding littermates is shown in Table 5. Adult values are also listed and represent data obtained from foster mothers supplied with the suckling mice. No differences exist in DAO, brain copper, or cytochrome c oxidase concentrations between the mutants and their littermate controls. The older mice, adults and quaking mutants, do have higher copper levels than the younger suckling animals. This increase in copper content with age is also demonstrated for rats, Tables 3 and 4, even for the short period observed. When the mouse brains were analyzed for the CNS myelin associated protein 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (29) a marked reduction in activity was observed, Table 6. The littermates for the jimpy mutants had less activity than the older controls of the other mutants.

DISCUSSION

The results clearly indicate that with adequate dietary copper, phenylalanine exerts no effects on copper homeostasis. This is true for the most sensitive indicator of copper status, plasma copper, Table 1.

The linear correlation between diamine oxidase activity and dietary copper, Figure 3, has also been shown to exist between plasma copper levels and activity of DAO (30). The values for DAO, Table 2, for both control and PKU plasma lie in the accepted range for normal adult humans (25) based on a similar assay.

The fact that plasma DAO was elevated, Figure 4, by hyperphenylalaninemia in the copper deficient pups may be partially explained by the known increase in hepatic copper uptake by amino acids (31). The liver would then have an increased pool of copper for the synthesis of ceruloplasmin, i.e. DAO. Also the faster growth of the saline injected rats would deplete liver copper-stores more rapidly. It would, thus, appear that rather than remove copper from the animal, phenylalanine facilitates its retention, at least under conditions in which the copper supply is limiting, Figure 4 and Table 4.

It is perhaps not surpising that phenylalanine elevation did not reduce the copper pool because it would be a weak bivalent ligand in competition with albumin. Indeed, of those amino acids studied, the tridendate ligands histidine and cystine (cysteine) would seem to be the primary copper chelators (5, 10). This also holds true for a computed distribution (32) of amino acid-Cu^{2+} complexes

and for the ligands associated with another trace metal, zinc (33).

These experiments did suggest that the hyperphenylalaninemic or copper-deficient offspring did not develop normally. Further investigation into these separate model systems will be the subject of subsequent reports, and will utilize the enzyme marker CNP which was shown to be highly reduced in three genetic mutations, with faulty myelination.

ACKNOWLEDGEMENTS

The author wishes to thank Professor R. W. Luecke for his generosity in the use of the atomic absorption facility. The technical aid of Mr. Douglas Clark is gratefully acknowledged in the experiment in which phenylalanine and ρ-chlorophenylalanine were administered.

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TABLE 1

EFFECT OF DIETARY PHENYLALANINE ON FECAL AND PLASMA COPPER AND DIAMINE OXIDASE[a]

Exp. No.	Group	Dietary Cu (μg/g)	Fecal Cu (μg/day)	Plasma Cu (μg/ml)	Diamine Oxidase (units/l)
I	Control	16.0	142 ±19(4)	1.17±0.02[b]	26.4±1.0[b]
	L-Phe	12.3	136 ±26(4)	0.87±0.04[b]	19.1±2.3[b]
	-Cu	1.8	12.4±4.0(4)	0.30±0.08	2.6±1.9
	-Cu+L-Phe	3.2	12.3±1.6(4)	0.42±0.16	2.8(1)
II	Control	11.0	----	0.78±0.08(4)	18.1±5.2(4)
	L-Phe	12.3	----	0.88±0.01	16.7±2.3
III[c]	Control	10.5	----	0.96±0.05	18.8±2.7
	-Cu	0.93	----	0.48±0.09	0.6±0.32
	-Cu+L-Phe	1.2	----	0.67±0.34	4.6±4.0

[a]Values are means ± SD for 3 samples unless indicated (N). Male weanling rats were fed a synthetic diet containing the reported copper levels and 5% L-Phe where indicated for either 4 weeks (exp. I), 2 weeks (II) or 5 weeks (III). Copper was determined by atomic absorption and plasma diamine oxidase by its ability to oxidize 1,4-diaminobenzene as described in MATERIALS AND METHODS.

[b]Means were significantly different ($P<0.01$).

[c]Means are from 3 pools of 3 animals each.

TABLE 2

PLASMA DIAMINE OXIDASE LEVELS OF PHENYLKETONURIC PATIENTS[a]

Cases	N	Plasma L-Phe[b] (mM)	Plasma Diamine Oxidase[c] (units/l)
Adult Controls	5	----	41.5±10.2
Adult PKU	7	1.81±0.20	38.1± 6.6
Neonatal Control	1	----	22
Neonatal PKU	6	1.65±0.73	16.6± 4.6

[a]Values are means ± SD as determined on plasma and serum received from 3 sources (see Materials). Neonatal refers to cases in which the patients were less than 1 month old. Activities were measured as described in METHODS.

[b]Data was calculated from information supplied with the specimens.

[c]The normal range for adults based on a similar method is 35-65 (ref. 25).

TABLE 3

EFFECT OF INJECTIONS OF L-PHENYLALANINE ON PLASMA AND BRAIN COPPER LEVELS OF NEONATAL RATS[a]

Property	Group	Age (Days)		
		7	11	14
Plasma Copper (μg/ml)	Control	1.10	0.92	0.62
	L-Phe	1.44	1.07	0.85
	-Cu	---	0.75	0.34
	-Cu+L-Phe	0.79	0.84	0.78
Brain Copper (μg/g dry wt)	Control	7.42	8.94	10.0
	L-Phe	7.21	9.16	10.6
	-Cu	3.45	3.05	2.69
	-Cu+L-Phe	3.73	3.79	4.09[b]

[a]Values are means of duplicate determinations on pools of 4 animals each. Copper was determined by atomic absorption spectroscopy as described in METHODS. Dams were fed either adequate copper diet (control, 11 ppm) or low copper diet (-Cu, 0.44 ppm) and the offspring were injected daily with either L-phenylalanine (L-Phe) or an equivalent volume of saline beginning at 3 days of age.

[b]Only 1 brain was used.

TABLE 4

EFFECT OF INJECTIONS OF L-PHENYLALANINE AND DL-ρ-CHLOROPHENYLALANINE ON CYTOCHROME OXIDASE AND COPPER LEVELS OF NEONATAL RAT BRAIN[a]

Property	Group	Age (Days)		
		10	14	20
Cytochrome Oxidase (units/g)	Control[b]	14.3 ±0.22	15.3 ±1.29	30.7 ±3.61
	L-Phe[b]	13.8 ±1.56	14.1 ±1.31	29.7 ±1.25
	-Cu	9.97±0.99	7.91±0.49	9.44±2.13
	-Cu+L-Phe	12.1 ±0.73[c]	13.2 ±3.13[c]	19.4 ±3.06[d]
Copper (μg/g wet wt)	Control	1.56±0.24	1.72±0.22	1.97±0.34
	L-Phe	1.66±0.22	1.82±0.09	2.02±0.35
	-Cu	1.20±0.18	0.60±0.30	0.14±0.06
	-Cu+L-Phe	1.16±0.35	0.96±0.20	0.77±0.11[d]

[a]Values are means ± SD for four samples. Dams were fed either normal copper diets (control, 10 ppm) or low copper diets (-Cu, 0.53 ppm) and the offspring received twice daily injections of either saline or L-Phe DL-ρ-Clphe (L-Phe) from 6 days of age.

[b]Data taken from (34) (see chapt. II).

[c]Means are significantly different than -Cu group $P<0.05$.

[d]Means are significantly different than -Cu group $P<0.01$.

TABLE 5

BRAIN COPPER LEVELS, CYTOCHROME OXIDASE AND DIAMINE OXIDASE ACTIVITIES OF MYELIN DEFICIENT MICE[a]

Species	Age (Days)	Sex	Brain Cu (µg/g wet wt)	Cytochrome Oxidase (units/mg protein)	Diamine Oxidase (units/l)
C57BL/6J	Adult	F	4.46±0.44(3)	0.311±0.034	----
Jimpy	14-15	M	2.56±0.21	0.303±0.028	10.4 ±0.73(2)
Littermates	14-15	M	2.27±0.26(3)	0.309±0.041(3)	9.5(1)
Myelin Syn. Def.	18-22	M	2.14±0.07	0.341±0.111(5)	9.95±1.36(3)
Littermates	18-22	M	1.99±0.10(2)	0.272±0.044(5)	8.84±1.05
Quaking	64-75	F	3.60±0.16(3)	0.438±0.118(3)	11.4 ±0.57(2)
Littermates	64-75	F	3.44±0.21	0.402±0.097	10.5 ±1.31(3)

[a]Values are means ± SD for four animals unless indicated (N). Mice were of the strain C57BL/6J and of the indicated mutations. Copper was determined by atomic absorption on wet-ashed brain samples. Cytochrome oxidase was measured on whole brains spectrophotometrically as described in METHODS. Diamine oxidase was measured on plasma as described in METHODS.

TABLE 6

ACTIVITY OF 2',3'-CYCLIC AMP 3'-PHOSPHOHYDROLASE IN MYELIN DEFICIENT MICE[a]

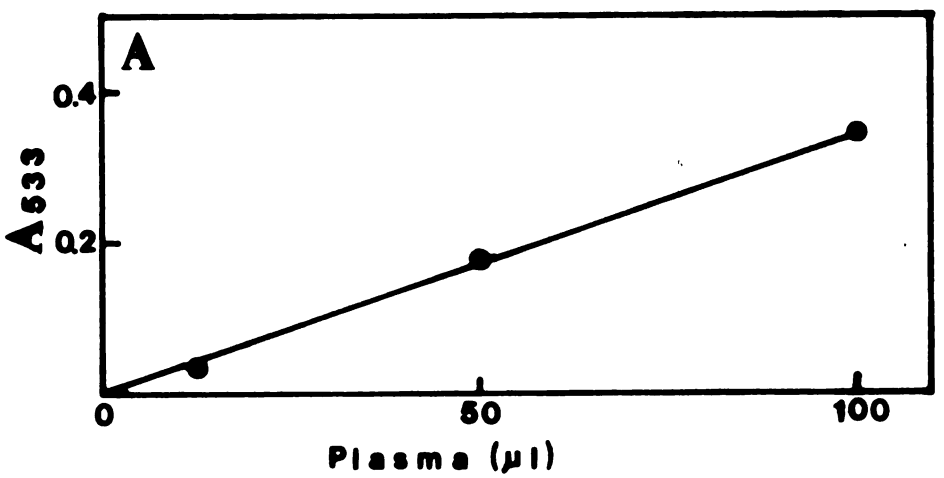
Species	Age (Days)	Activity (units/mg protein)	% Control
C57BL/6J	Adult	2.66±0.05(4)	--
Quaking	64-75	0.61±0.08(3)[b]	26
Control	64-75	2.30±0.29(4)	
Myelin Syn. Def.	18-22	0.30±0.07(4)[b]	12
Control	18-22	2.52±0.72(4)	
Jimpy	14-15	0.22±0.00(3)[b]	22
Control	14-15	0.99±0.39(3)	

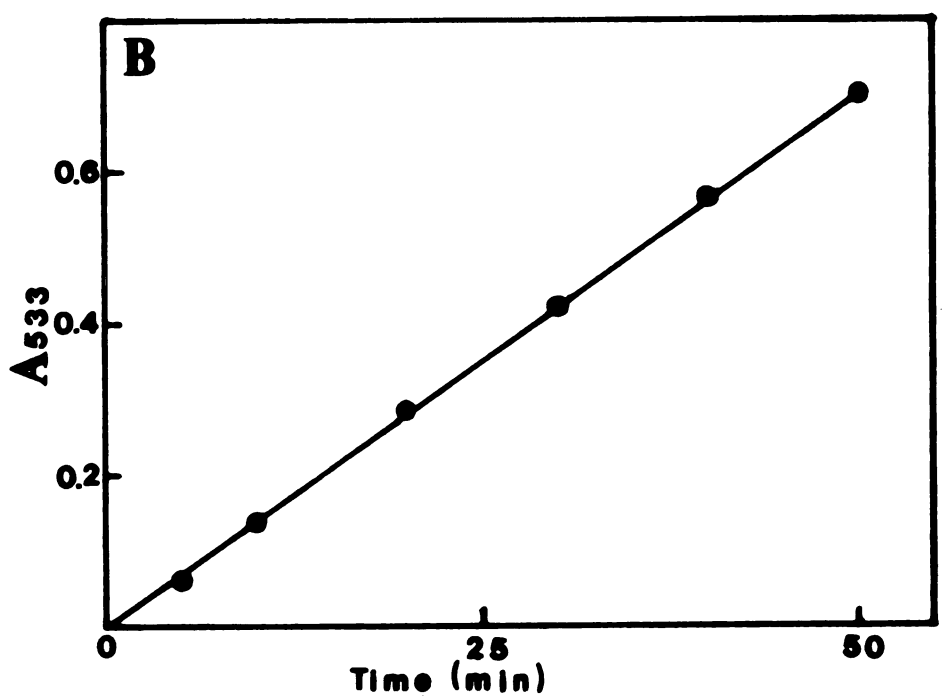
[a]Values are means ± SD for the indicated number of whole brains (N). A unit was defined as 1 μmole/min and was determined on 10% (w/v) homogenates as described under METHODS.

[b]Means are significantly different than littermate controls $P<0.01$.

Figure 1A. Formation of product with increasing plasma content, diamine oxidase activity. Varying amounts of rat plasma were incubated with 4 mM 1,4-diaminobenzene for 30 min as described in METHODS.

Figure 1B. Formation of product with increasing time, diamine oxidase activity. Reactions were carried out with 4 mM 1,4-diaminobenzene using 10 μg of human ceruloplasmin in a 1 ml final volume as described in METHODS.


A
0.4
A533
0.2
0
50
100
Plasma (µl)


B
0.6
0.4
A533
0.2
0
25
50
Time (min)

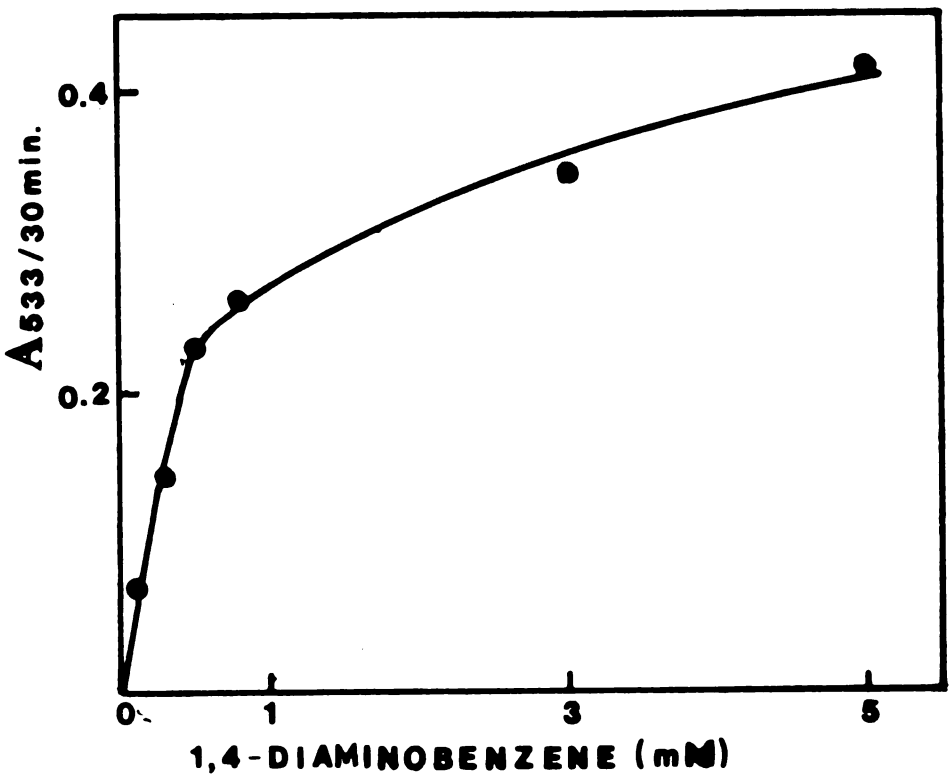


Figure 2. Velocity of the diamine oxidase reaction with increasing substrate. Reactions were carried out with 10 µg of human ceruloplasmin as described in METHODS.

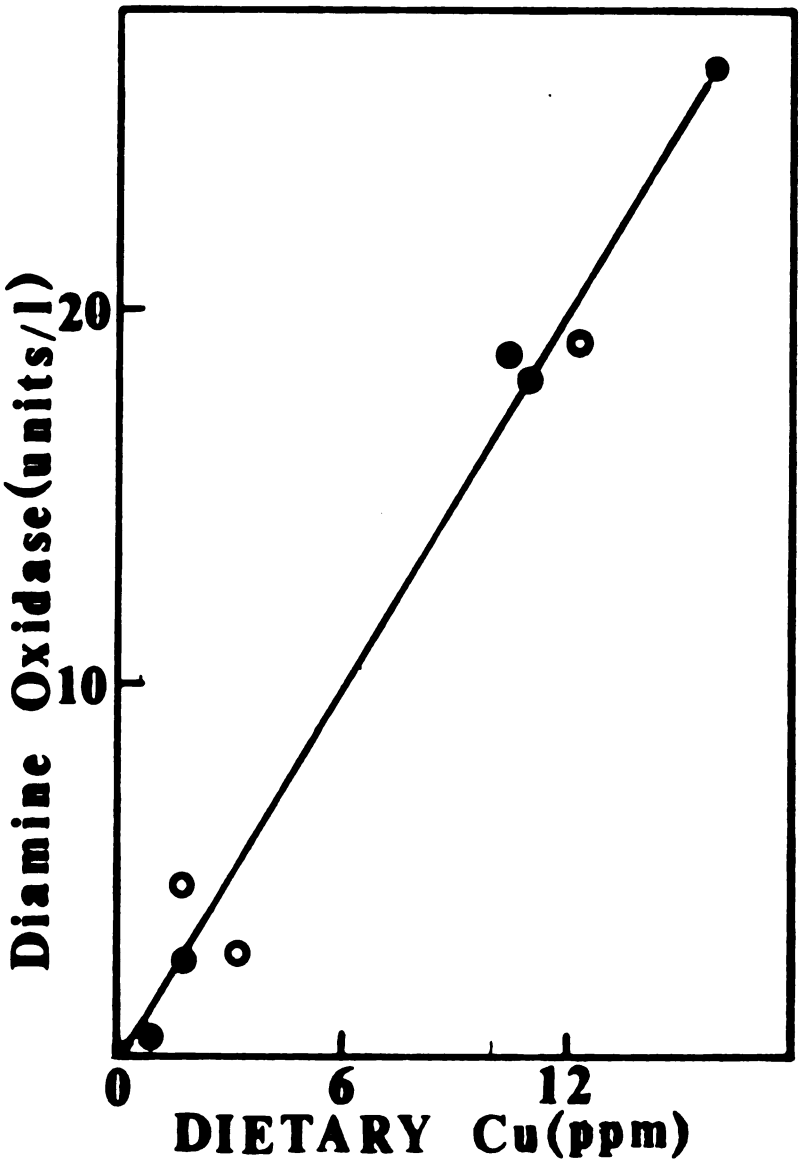
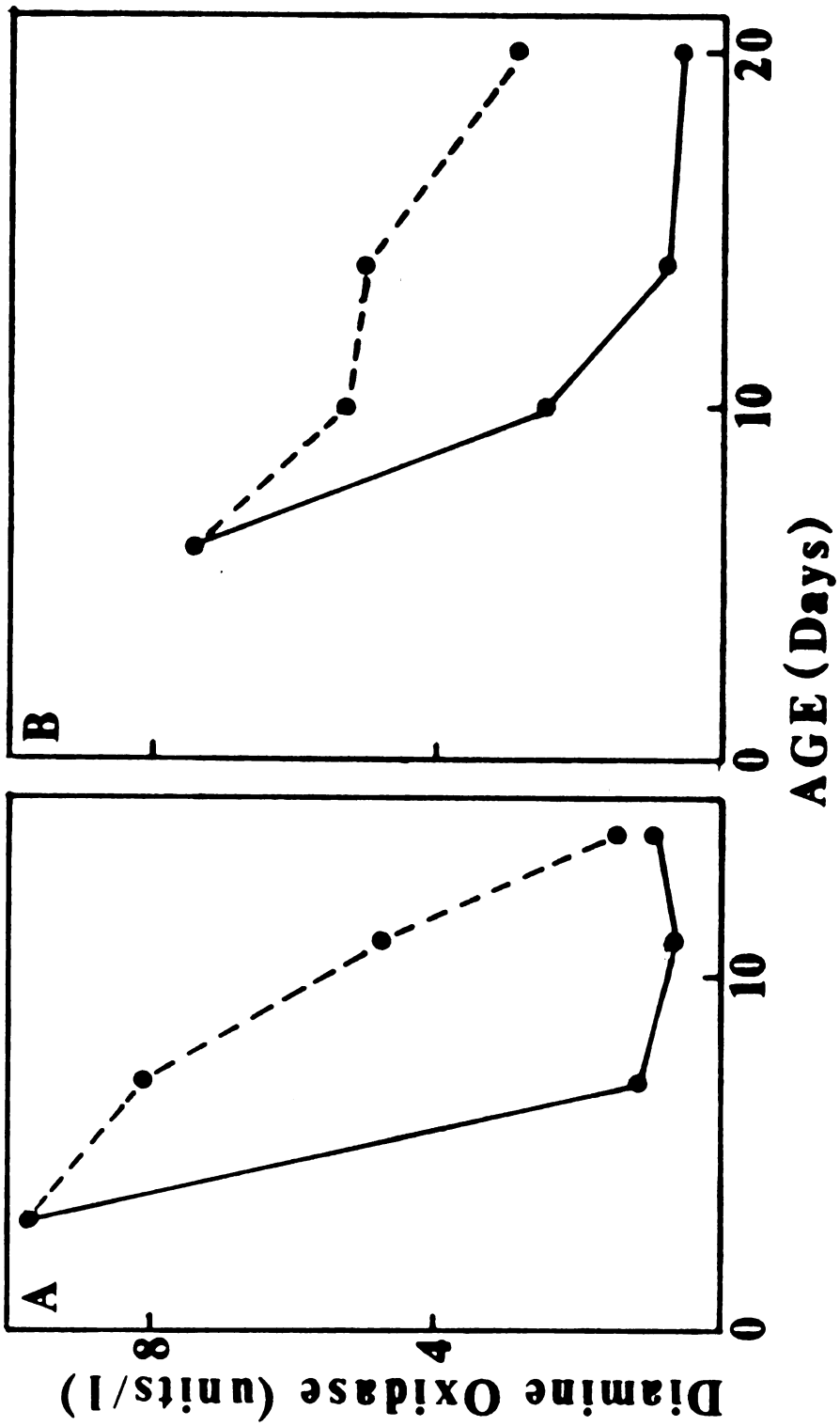


Figure 3. Relationship between dietary copper level and rat plasma diamine oxidase activity. Points are means of triplicate determinations from pools of at least 3 male rats fed synthetic diets of varying copper content (●), in some cases supplemented with 5% L-phenylalanine (○). Details of diet composition and enzyme assay can be found under METHODS.

Figure 4. Diamine oxidase activity of neonatal copper-deficient rats injected with phenylalanine or saline. Pups from dams fed a low copper diet were injected daily (A) with phenylalanine (●---●) or saline (●——●); or (B) twice daily with phenylalanine and ρ-clorophenylalanine (●---●) or saline (●——●). Details of the assay procedure and injection regimen can be found under METHODS. Plotted points are the means of triplicate assays on plasma from pools of 4 animals each.


Diamine Oxidase (units/l)
8
4
0
A
10
B
0
10
20
AGE (Days)

CHAPTER II

EFFECT OF PHENYLALANINE AND ρ-CHLOROPHENYLALANINE ADMINISTRATION ON THE DEVELOPMENT OF RAT BRAIN 2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHOHYDROLASE

INTRODUCTION

Attempts to explain the mental defect associated with the untreated phenylketonuric patient have encompassed a wide range of proposals, but as yet, a clear explanation has failed to emerge. Alvord et al. (1) first suggested that the neuropathology of phenylketonuria (PKU) was characterized by an arrest of myelination in young patients; chemical evidence (2,3) indicating lower concentrations of cholesterol, cerebrosides, and proteolipid protein supported this view. However, other theories have been suggested involving demyelination in the adult or absence of pathological changes of myelin (for a review, see (4)).

Prior work on the effects of experimental PKU on the developing rat brain have emphasized the reduction in myelin lipids (5-8). This report deals with the development of the myelin associated protein, 2',3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16b) and of the mitochondrial proteins, hexokinase (EC 2.7.1.1) and cytochrome c oxidase (EC 1.9.3.1) under conditions employing injection of both phenylalanine and ρ-chlorophenylalanine (9), since neuronal protein sythesis is disrupted by this treatment (10).

MATERIALS AND METHODS

Pregnant rats were obtained from the Holtzman Co. (Madison, WI) and were fed either a synthetic control diet described previously (11) or a commercial pellet diet purchased from Allied Mills, Inc. (Chicago, IL). DL-ρ-Chlorophenylalanine, L-phenylalanine, and L-tyrosine were the products of Sigma Chemical Co. (St. Louis, MO). 1-Nitroso-2-naphthol was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Treatment was induced in rat pups by sc injection of L-phenylalanine

(30 mg/ml, pH 7.4) at a dose of 1 mg/g body weight (12). The injection solution also contained DL-ρ-chlorophenylalanine (3 mg/ml) so that a dose of 0.1 mg/g body weight was achieved. Pups receiving 33 µl/g body weight of 0.2 M NaCl, pH 7.4, served as injection controls. In injection experiment I, dams were fed the synthetic diet and pups were injected twice daily from age 6-20 days. In injection experiments II and III, dams were fed the pellet diet and pups were injected once daily between ages 6-20 (exp. II) and ages 3-13 (exp. III). These pups were weaned at 21 days of age and were fed the pellet diet until 40 days of age. In all injection experiments, pups were randomized at 1 day of age and distributed at 8 per litter.

Pups were killed either by exsanguination (experiments I and II) or by decapitation (experiment III). In experiment I, plasma tyrosine was fluorometrically determined by the method of Ambrose et al. (13). Brain 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (14) and cytochrome c oxidase (11) were measured on tissue stored for various periods at -90°. CNP activity was stable to storage; however, loss of cytochrome c oxidase activity on storage is sufficient to permit only relative comparisons between experiments. For injection experiment III, cerebellar hexokinase was measured on crude homogenates of fresh tissue according to the procedure of Knull et al. (15). Details of these procedures including statistical methods are reported elsewhere (11). Enzyme activities were defined on a unit/g fresh weight tissue basis with one unit defined as one µmole of product formed/min.

RESULTS

During injection experiment I, measured tyrosine ratios indicated an initial rise in plasma tyrosine of the treated pups, which was followed by a drop to levels found in pups injected with saline (Table I). Pups which received no injections and whose dams were on a commercial diet had plasma tyrosine levels equivalent to the saline injected pups (Table I). Attempts to quantify plasma phenylalanine levels by the fluorometric method of McCaman and Robins (16) were unsuccessful due to interference by DL-ρ-chlorphenylalanine, which when analyzed under optimal conditions for L-phenylalanine, resulted in an increased relative fluorescence.

Twice daily injections in experiment I were accompanied by high mortality and marked differences in growth of whole body and brain (Fig. 1). In comparison (Table II), although pups from experiment II, in which injections were halved, demonstrated differences in body and brain weights even after 20 days of recovery, absolute differences between groups were less than in experiment I. When pups were allowed to recover after day 13, experiment III, only cerebellar weights were different at 40 days of age. Differences between the groups were also less reduced than in experiment I.

In experiment (I), the concentration of whole brain 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP), an enzyme enriched in CNS myelin (17) was much lower in animals receiving the L-phenylalanine DL-ρ-chloro-phenylalanine; mean differences were highly significant on days 14, 16, 18, and 20 (Fig. 2A). In experiment II in which injections were given once daily, whole brain CNP concentration was reduced at day 20 but

equivalent at day 40 following recovery (Table III). At this age, however, CNP content per brain was significantly higher in the saline injected rats (682 ± 32.4 units versus 596 ± 49.8 units ($\alpha = 0.05$)). For injection experiment III in which treatment began earlier, day 3 versus day 6, but was terminated earlier, day 13 versus day 20, CNP concentration in the cerebellum and in the whole brain less the cerebellum demonstrated a different pattern (Table III). At 7 days of age, there was a significant decrease in both cerebellar and whole brain (less cerebellum) CNP concentrations. At 20 days of age, a week after treatment, only whole brain (less cerebellum) CNP concentration was higher in the saline injection pups; at 40 days of age, no differences were measurable in either region.

Like CNP, both hexokinase and cytochrome c oxidase increased with increasing age (Fig. 2B and Table IV). However, there were no consistent differences in concentration of cytochrome c oxidase between saline injected and L-phenylalanine DL-ρ-chlorophenylalanine injected pups for either experiment I or III. Cerebellar hexokinase also failed to show a difference between groups in injection experiment III.

DISCUSSION

Experimental animal models of PKU in which administration of excess phenylalanine was used have been criticized (9) for failing to duplicate the high ratio of phenylalanine/tyrosine found in PKU patients. These authors have developed a model in which ρ-chlorophenylalanine, an <u>in vivo</u> inhibitor of rat liver phenylalanine hydroxylase (18), is administered in conjunction with phenylalanine. This

approximates the plasma phenylalanine/tyrosine ratios found in PKU (19). Since injection of either phenylalanine or tyrosine into neonatal rats produced equivalent effects on reduced body weight, brain weight, total brain lipid, and incorporation of ^{35}S-sulfate into brain (6), the model system of Andersen and Guroff (9) employing both phenylalanine and ρ-chlorophenylalanine was used in these experiments. The absence of elevated plasma tyrosine levels during the last week of injections in experiment I would imply reduction in phenylalanine hydroxylase activity. The injection of ρ-chlorophenylalanine alone causes no changes in serum tyrosine levels (20).

The chemical composition of isolated myelin from experimental PKU rat brain was reported to be normal although less myelin was present (8). These same conclusions were reached when comparing myelin from 4 PKU and 5 normal human brains (21). However, other evidence suggests that the myelin lipid from experimental PKU rat brain appears to be different, i.e., there is a reduction in unsaturated and long chain fatty acids (22). Evidence of abnormal myelin based on whole brain or white matter specimens from PKU patients also includes reductions in unsaturated and long chain fatty acids (23) and changes in proteolipid fractions (24).

The concomittant increase in 2',3'-cyclic nucleotide 3'-phosphohydrolase concentration in rat whole brains with the period of myelination (Fig 2A) agrees with the work of Olafson et al. (25). The decrease in enzyme content in treated pup brain from experiment I suggests a deficit in myelin protein concentration. Others have also suggested a decrease

in myelin levels, in studies in which pups were injected with L-phenylalanine alone, based on reduced cholesterol and galactolipid (5), sulfatide (6), or proteolipid (7). Furthermore, Clarke and Lowden (5) found lower concentrations of cholesterol and galactolipid from brains of rats 40 days of age. These animals had been injected twice daily between ages 2 to 20 days before recovery until age 40 days. In the present study, experiment II, when pups injected between ages 6 to 20 days were allowed to recover, although body and brain weight differences persisted, the concentration of the myelin protein CNP showed no differences at 40 days. However, the amount of CNP per brain was higher in the saline injected rats. When recovery was begun earlier, experiment III, body and brain weight difrerences were not observed at 40 days of age. The degree and duration of the initial insult seem to dictate whether recovery will occur. Fish and Winick (26) report that DNA synthesis continues until 17 days of age in various regions of rat brain; thus, potential for recovery would be reduced if the abuse were carried beyond this period. Geison and Waisman (27) found little change in lipid distribution in rats that were fed excess phenylalanine beginning at 3 weeks of age.

Recently, Adelman et al. (28) have reported that injections of phenylalanine to neonatal rats resulted in lesions to both cerebellar Purkinje and granule cells. Although we found no decreases in cerebellar cytochrome c oxidase or hexokinase two mitochondrial proteins presumably enriched in neuronal cells (29), neuronal cell dysfunction may exist and contribute to the reduced myelin level. The molecular events giving rise to the neuropathology of PKU, hypomyelination and demyelination, remain an area for further inquiry.

SUMMARY

Subcutaneous injections of L-phenylalanine and DL-ρ-chlorophenylalanine in developing rats resulted in reduced body weight, brain weight, and concentration of 2',3'-cyclic nucleotide 3'-phosphohydrolase -- a myelin enriched protein. These reductions were less marked when injections were reduced by half; when pups were allowed to recover after age 13 days, no differences were observed at 40 days of age. Brain hexokinase and cytochrome c oxidase development was not affected by the injection treatments.

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TABLE I. Plasma Tyrosine Ratios in Developing Rats Following Injection of L-Phenylalanine and DL-p-Chlorophenylalanine.[a]

Age (Days)	Phenylalanine p-Chlorophenylalanine Saline	Control[b] Saline
6	1.0	--
8	1.92	--
10	2.72	--
12	4.31	--
14	1.75	1.07
16	0.99	1.07
18	0.81	0.97
20	0.99	1.14
22	--	0.99

[a]Ratios were determined on mean tyrosine values of duplicate determinations on pools of at least 4 pups.

[b]Control pups refer to rats given no injections and whose dams were fed the commercial pellet diet.

TABLE II. Body, Whole Brain, and Cerebellar Weights Following Injection of L-Phenylalanine DL-p-Chlorophenylalanine[a]

Experiment No.	Age (Days)	Body Weight (g)		Brain Weight (g)		Cerebellar Weight (mg)	
		Saline	L-phe p-Clphe	Saline	L-phe p-Clphe	Saline	L-phe p-Clphe
II	13	33 ± 5.7	23 ± 3.2[b]	1.13±0.07	0.952±0.065[b]	--	--
	20	46 ± 5.1	30 ± 4.3[c]	1.35±0.06	1.08 ±0.10[c]	--	--
	40	151 ± 4.3	115 ±20.6[b]	1.59±0.05	1.37 ±0.07[c]	--	--
III	7	19.5 ± 0.74	14.2 ± 1.6[c]	0.72±0.02	0.61 ±0.03[c]	53± 3.2	41.4± 2.5[c]
	20	59.5 ± 4.4	33.2 ± 8.5[c]	1.45±0.05	1.18 ±0.11[c]	196±16.0	139 ±11.3[c]
	40	174 ±18.2	180 ±18.8	1.62±0.09	1.59 ±0.03	234±21.3	201 ±15.4[b]

[a]Values listed are means ± S.D. for 4 animals; tissue weights are on a wet weight basis. Animals were injected sc daily between 6-20 days of age (exp. II) or 3-13 days of age (exp. III), after which time they were allowed to develop without treatment. Means were compared by use of the F-variance ratio and Students' two-tailed t-test at levels of α [0.05 (significant), 0.01 (highly significant)].

[b]Means different from saline controls when tested at $\alpha = 0.05$.

[c]Means different from saline controls when tested at $\alpha = 0.01$.

TABLE III. Effect of Injections of L-Phenylalanine and DL-p-Chlorophenylalanine on Development of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase in Rat Brain[a,b]

Experiment No.	Age (Days)	Whole Brain Activity (units/g)		Cerebellar Activity (units/g)	
		Saline	L-phe p-Clphe	Saline	L-phe p-Clphe
II	13	83.5 ± 13.1	81.4 ± 11.5	--	--
	20	269 ± 22.9	217. ± 31.4[c]	--	--
	40	429 ± 9.6	436 ± 19.1	--	--
III	7	16.7 ± 0.34	15.7 ± 0.63[c]	26.0 ± 0.42	21.3 ± 3.34[c]
	20	211 ± 4.7	181 ± 13.6[d]	246 ± 14.2	234 ± 15.4
	40	396 ± 20.2	393 ± 19.9	324 ± 13.2	319 ± 22.0

[a]Whole brain less cerebella for experiment III.

[b]Values represent means ± S.D. for 4 animals and are based on fresh weight of tissue. Treatment means were compared by the F-variance ratio and Students' t-test at two levels of α [0.05 (significant); 0.01 (highly significant)].

[c]Means were different from saline controls (α = 0.05).

[d]Mean was different from saline control (α = 0.01).

TABLE IV. Effect of Injections of L-Phenylalanine and DL-p-Chlorophenylalanine on Development of Hexokinase and Cytochrome *a* Oxidase in Rat Brain, Experiment III[a]

Age (Days)	Activity (units/g)					
	Cerebellar				Whole Brain[b]	
	Hexokinase		Cytochrome *a* Oxidase		Cytochrome *a* Oxidase	
	Saline	L-phe p-Clphe	Saline	L-phe p-Clphe	Saline	L-phe p-Clphe
7	2.03±0.11	2.16±0.14	12.5±1.2	13.3±1.7	5.94± 2.0	5.07± 0.61
20	5.99±0.33	6.05±0.29	43.9±3.5	38.9±7.7	13.3 ± 3.2	16.3 ± 4.3
40	8.77±0.24	9.10±0.53	61.0±6.5	62.8±6.3	23.4 ±13.0	29.9 ±13.4

[a]Values are means ± S.D. based on four animals and wet weight of tissue.

[b]Whole brain less cerebella.

FIG. 1.--The postnatal growth (body weight (A) and whole brain weight (B)) of rats injected twice daily from six days of age with either saline (●——●) or phenylalanine and ρ-chlorophenylalanine (●---●), experiment I. Plotted points are the means of pools of pups (A) or of pools of 5 brains (B). Injection procedures can be found detailed under MATERIALS AND METHODS.

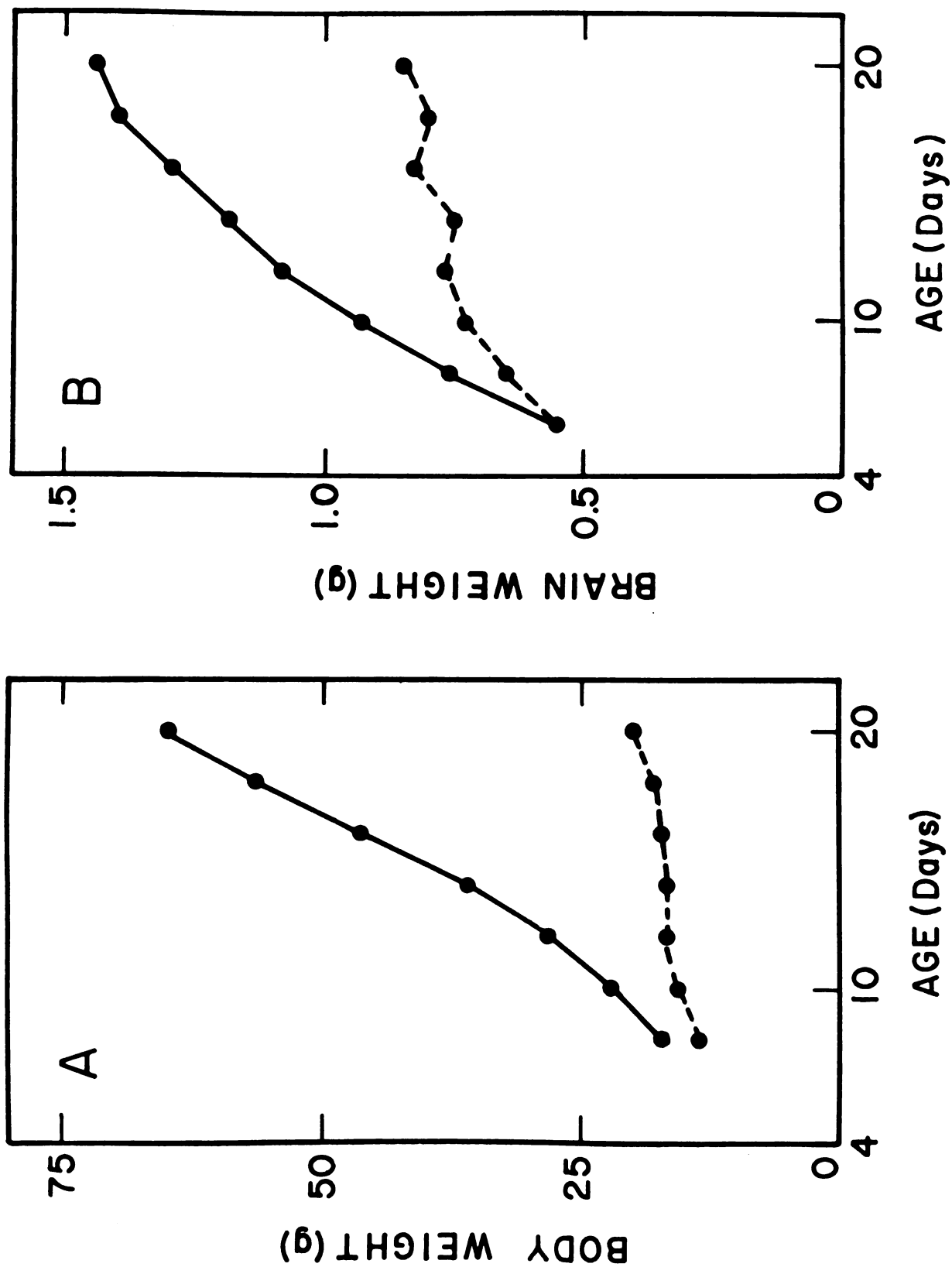

A
B
BODY WEIGHT (g)
BRAIN WEIGHT (g)
AGE (Days)
AGE (Days)
0
25
50
75
0.5
1.0
1.5
4
10
20

FIG. 2A.--The postnatal development of whole brain 2',3'-cAMP 3'-phosphohydrolase in saline injected (●——●) and in phenylalanine, ρ-chlorophenylalanine injected (●---●) rats. Injections were started at 6 days of age and were given twice daily. On alternate days, pups were selected from a random pool, decapitated, and brains were frozen (-90°) until enzyme activity was measured spectrophotometrically. Details of this injection procedure (experiment I) as well as the enzyme assay are described in MATERIALS AND METHODS. Plotted points represent means ± S.D. for four brains based on wet weight of tissue.

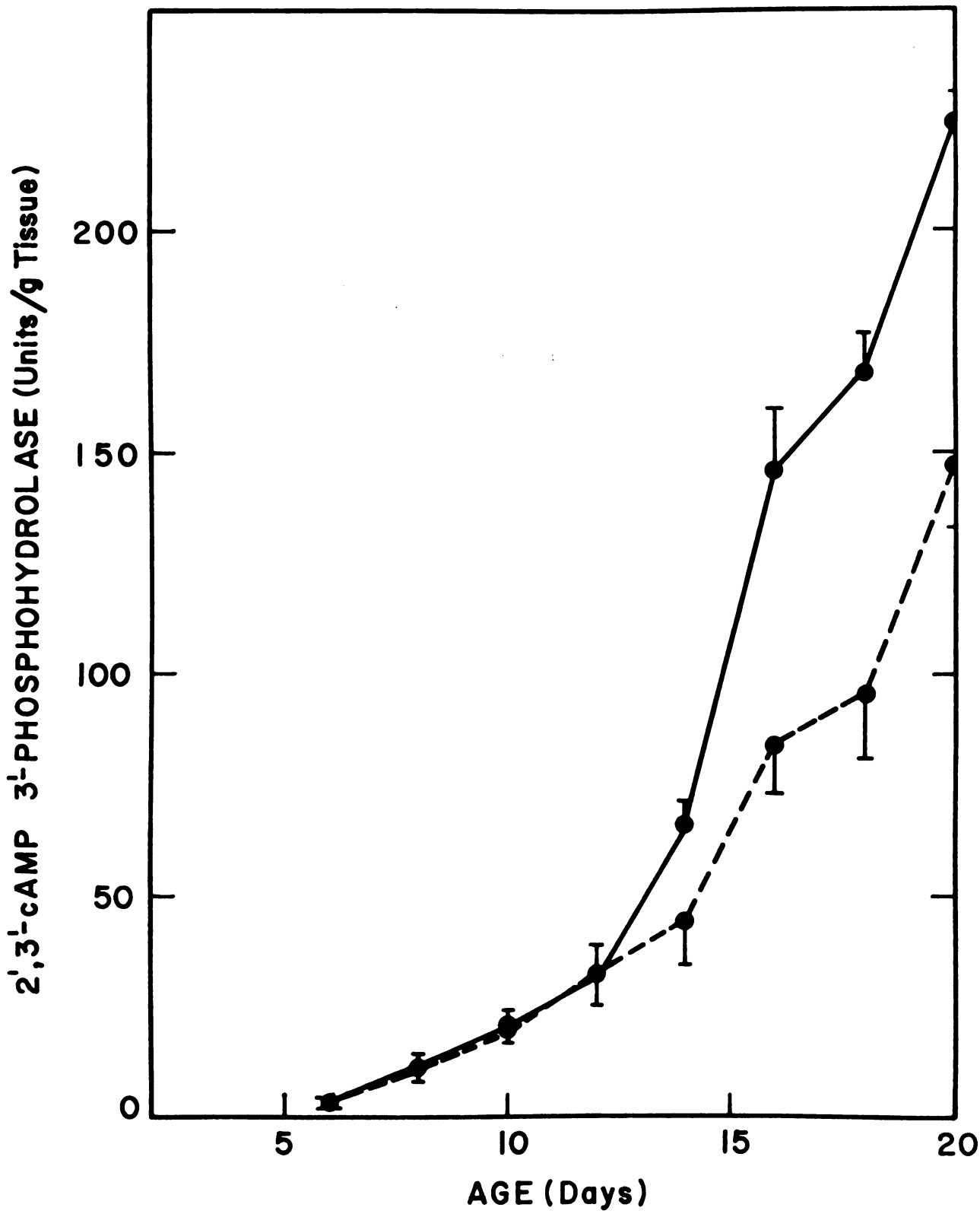
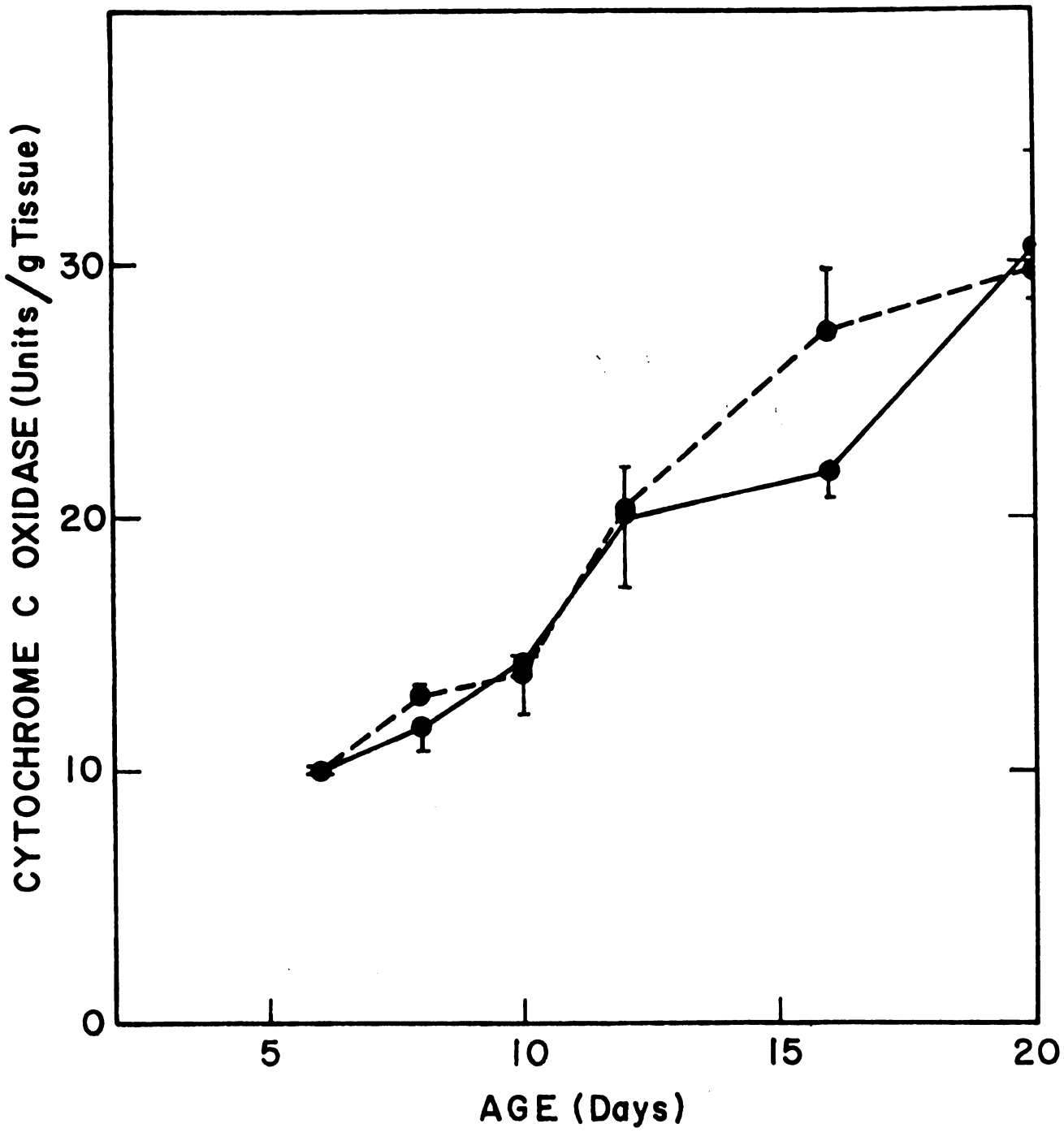

2',3'-cAMP 3'-PHOSPHOHYDROLASE (Units/g Tissue)
200
150
100
50
0
5
10
15
20
AGE (Days)

FIG. 2B.--The postnatal development of whole brain cytochrome c oxidase in saline injected (●——●) and in phenylalanine, p-chlorophenylalanine injected (●---●) rats. Injections were started at 6 days of age and were given twice daily. On alternate days, pups were selected from a random pool, decapitated, and brains were frozen (-90°) until enzyme activity was measured spectrophotometrically. Details of this injection procedure (experiment I) as well as the enzyme assay are described in MATERIALS AND METHODS. Plotted points represent means ± S.D. for four brains based on wet weight of tissue.


CYTOCHROME C OXIDASE (Units/g Tissue)
0
10
20
30
5
10
15
20
AGE (Days)

CHAPTER III

COPPER DEFICIENCY IN THE DEVELOPING RAT BRAIN: A POSSIBLE MODEL FOR MENKES' STEELY-HAIR DISEASE

ABSTRACT

In comparison to controls, copper-deficient suckling rats showed an appreciable decrease in body growth, a slight decrease in whole brain and cerebellar growth, and a highly significant decrease in myelination based on the activity of cerebellar 2',3'-cyclic nucleotide 3'-phosphohydrolase -- a myelin enriched protein. Specific effects of a five-fold reduction in the copper content of brain were seen in a drastic decrease in cerebellar cytochrome c oxidase and smaller but significant drops in cerebellar superoxide dismutase and brain norepinephrine concentration. These observations are discussed with respect to the neuropathology and biochemistry of Menkes' steely-hair disease, a sex-linked recessive disorder in humans characterized by copper deficiency.

INTRODUCTION

Copper was first shown to be an essential nutrient by its role in hemoglobin synthesis (HART et al., 1928); its absolute function in iron metabolism is still not known. This same uncertainty exists concerning the role of copper in brain metabolism. Copper deficiency in grazing animals is responsible for the condition termed "enzootic neonatal ataxia", reviewed by UNDERWOOD (1971). Copper deficiency resulting in neurological abnormalities in neonates has been experimentally produced in guinea pigs (EVERSON et al., 1967) and in rats (CARLTON and KELLEY, 1969). In developing animals, lesions vary with species and with the degree of deficiency and include neuronal necrosis in lambs (FELL et al., 1965), missing cerebellar folia with widespread appearance of delayed myelination in guinea pigs (EVERSON et al., 1968)

and focal appearance of rarefied, spongy, edematous, necrotic neural tissue in rats (CARLTON and KELLY, 1969). White matter neuropathology is reviewed elsewhere (HOWELL, 1970).

Dietary copper deficiency is rare in human infants (SHIELDS et al., 1960); however, documented reports have appeared (AL-RASHID and SPANGLER, 1971; KARPEL and PEDEN, 1972). MENKES et al. (1962) first reported cases of a sex-linked recessive disorder characterized by growth retardation and cerebral and cerebellar degeneration. This disease referred to as "Menkes' Kinky Hair Disease", "Trichopoliodystrophy", or "Menkes' Steely-Hair Disease" has been shown (DANKS et al., 1972a) to result in copper deficiency due to defective intestinal absorption.

Experimental dietary copper deficiency was studied in an attempt to explain the neuropathology of Menkes' disease using the neonatal rat brain as a model. The cerebellum, because of postnatal interneuron and neuroglial proliferation, is most susceptible to undernutrition (CHASE, 1973). It was, therefore, selected for studying copper metalloprotein development and myelination. The remainder of the brain was utilized for measuring copper content and norepinephrine levels, the latter to evaluate the status of dopamine-β-hydroxylase (EC 1.14.2.1), a copper protein (FRIEDMAN and KAUFMAN, 1965) found in brain (UDENFRIEND and CREVELING, 1959).

MATERIALS AND METHODS

Materials. A copper reference solution (1000 μg/ml) was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Horse heart cytochrome c was obtained from Boehringer-Mannheim Corp. (New York, NY). DL-(7-^{14}C)

norepinephrine (44 μCi/μmole) was purchased from New England Nuclear Corp. (Boston, MA). Vitamin free casein, Tween 80, Alphacel, and vitamin fortification mixture were the products of Nutritional Biochemicals (Cleveland, OH). Sephadex G-25 was bought from Pharmacia (Piscataway, NJ). Xanthine, xanthine oxidase (EC 1.2.3.2), L-norepinephrine·HCl, maleic acid, tris, 2',3'-cAMP, and E. coli alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma Chemical Co. (St. Louis, MO). Special copper free salt mix (modified Phillips-Hart) was prepared by General Biochemicals (Chagrin Falls, OH). Rats were obtained from the Holtzman Co. (Madison, WI).

Animal Care and Diets. Adult rats which were used had been maintained ad libitum on Lab-Blox pellets, Allied Mills, Inc. (Chicago, IL) (20 μg Cu/g) and tap water. The experimental basal diet composition (% wt.) was as follows: sucrose (63.9), casein (25), corn oil (5), alphacel (1.3), vitamin mix (1), choline chloride (0.1), salt mix (3.7) fortified with $CuSO_4 \cdot 5\ H_2O$ and/or ZnO). Cu levels of 18 μg/g in the final control diet and 0.3 μg/g in the low copper diet were measured by atomic absorption spectroscopy (Perkin-Elmer model 303). Zinc oxide was added to both diets to give a final content of 46 μg Zn per g diet. The casein used in the low copper diet was washed three times with EDTA (5 g/Kg), rinsed five times with thrice distilled water (0.3 ng Cu/ml), dried (40°C), and ground in a coffee grinder. Both diets contained adequate amounts of all the known vitamins. Animals were fed ad libitum and given thrice distilled H_2O to drink. No anorexia or difference in food consumption were observed.

In the control experiment, 12 commercially bred rats at 8 days of

gestation were administered the basal control diet and housed in individual plastic cages with wood shavings as bedding. One day after birth, the pups were randomized, divided, and maintained at 8/mother. At weaning, pups were continued on the basal diet fed their dams.

In the low copper experiment, designed after CARLTON and KELLEY (1969), 18 female weanling rats were housed in individual stainless steel cages and fed the low copper diet for nine weeks at which time they were divided into groups of three and placed with a male breeder for 72 hours. Rats were returned to stainless steel cages for 20 days and fed a basal diet which contained 2 μg Cu/g in order to prevent fetal resorption (HALL and HOWELL, 1969). Rats were then transferred to individual plastic cages and returned to the low copper diet (0.3 μg Cu/g). As in the control experiment, pups were randomized, divided at 8 per litter, and weaned on the diet fed their dams.

Tissue Preparation. At indicated postnatal ages, pups of the specified treatment group were randomized and four animals chosen as samples. The animals were weighed and killed by decapitation; the cerebellum was separated from the remainder of the brain, both tissues were bisected along the anterior-posterior midline. Half the cerebellum was used for enzyme analysis; the other half was frozen on solid CO_2 and kept at -90°C until norepinephrine extractions were performed. Cerebellar tissue was homogenized in 9 volumes of 0.32 M sucrose containing 1 mM 2-mercaptoethanol (12 strokes) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Aliquots were frozen overnight, -20°C, before analysis of 2',3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16b) and cytochrome c:O_2 oxidoreductase (EC 1.9.3.1) (cytochrome

c oxidase) were performed. Another aliquot was centrifuged for 15 minutes (4°C) at 40,000 x g; the resultant supernatant was used to measure the activity of superoxide dismutase.

Cytochrome c Oxidase. The thawed 10% (w/v) cerebellar homogenates were appropriately diluted in 0.32 M sucrose and made 0.9% (v/v) in Tween-80. The oxidation of reduced cytochrome c was followed at 550 nm, 25°C, in 0.1 M pH 7.0 potassium phosphate buffer (SMITH, 1955). Cytochrome c was reduced with excess sodium dithionite ($Na_2S_2O_4$) and separated from the reducing agent on a Sephadex G-25 column equilibrated with 0.1 M pH 7.0 potassium phosphate. The reaction mix which initially contained 50 μM reduced cytochrome c was initiated with detergent treated tissue. Linear rates were used to calculate initial velocities using $\varepsilon = 19.6 \times 10^3$ (YONETANI, 1965); a unit is defined as the transformation of 1 μmole of cytochrome c reduced to oxidized/min. All enzyme assays were carried out using a Gilford model 2400-S spectrophotometer.

Superoxide Dismutase. The copper-zinc metalloprotein first named cerebrocuprein I (PORTER and FOLCH, 1957) was measured by its ability to inhibit the reduction of ferricytochrome c by the xanthine oxidase reaction (McCORD and FRIDOVICH, 1969). The cerebellar supernatant was used as the source for the cytoplasmic superoxide dismutase and rates were corrected for substrate independent cytochrome c reductase activity. The assay procedure and definition of units were taken without modification from McCORD and FRIDOVICH (1969). A unit was defined as that amount of original tissue which caused a 50% inhibition of the rate of reduction of ferricytochrome c in a final volume of 0.5 ml.

2',3'-Cyclic Nucleotide 3'-Phosphohydrolase. Activity of thawed

cerebellar homogenates was determined by the method of PROHASKA et al. (1973). 2',3'-cAMP was used as substrate and the product 2'-AMP was measured as inorganic phosphate after treatment with bacterial alkaline phosphatase. One unit of activity was that amount of enzyme which produced 1 μmole of 2'-AMP/min.

Copper Analysis. To avoid contamination, all glassware used in this study was soaked in HNO_3 for 24 h and rinsed with thrice distilled water. Tissue was wet-ashed in HNO_3-$HClO_4$ and the residue dissolved in 1% (v/v) HCl before determination of Cu by atomic absorption spectroscopy.

Norepinephrine. Frozen acerebellar hemisections from controls (stored 5-7 weeks) and low copper fed (stored 12-14 weeks) rats were age matched and analyzed simultaneously. The extraction procedure in acetone-formic acid was that of KARIYA and APRISON (1969) and aliquots of the 0.45 ml aqueous phase were used directly to measure recovery by counting the ^{14}C-norepinephrine and to derivatize norepinephrine for fluorometric analysis (LAVERTY and TAYLOR, 1968). Recovery of norepinephrine in the extraction procedure averaged 72%. Extraction solvents were prepared according to FLEMING et al. (1965).

Statistics. Treatment means were compared using the F-variance ratio and the two-tailed Student's t-test by means of a computer program. Statistical significance was tested at two levels of α:0.05 (significant), 0.01 (highly significant).

RESULTS

Behavioral and Growth Effects. During the 9 week copper depletion period, the growing females appeared to be hyperactive especially during feeding and weighing periods. Growth became highly variant during the terminal 2 weeks of feeding and 3 animals died. Subsequent to parturition, 3 more animals succumbed; gross symptoms observed during dissection included cardiac hypertrophy, soft bones, and loss of surface vascularity on the cerebral hemispheres.

The exterior appearance of the copper deficient pups was not markedly different from the control pups except the hair coat appeared thinner and unkempt. There was a significant difference in body growth of the two groups (Table 1), and a large variation among copper deficient animals, especially after weaning. Animals severely affected became sluggish, anorexic, and hyperventilated.

Brain Development. The retarded growth pattern was seen for both the whole brain and the cerebellum (Table 1). However, because of a large variance among the copper deficient pups and the "sparing" effect on brain versus body weight, the differences for brain and cerebellar weights were statistically different only at day 22. The retarded growth pattern in the cerebellum was further evidenced by the reduced concentration (units/g tissue) of 2',3'-cyclic nucleotide 3'-phosphohydrolase (Fig. 1), an enzyme enriched in CNS myelin (KURIHARA and TSUKADA, 1967). Both groups showed a marked increase in enzyme content concomittant with the period of myelinization. However, mean differences were highly significant at all four time points compared. Dietary copper level did affect brain maturation based on this myelin marker enzyme.

Development of Copper Dependent Systems. In order to attribute differences in brain metabolism between control and copper deficient pups directly to copper, it is necessary to show a difference in copper content between groups. This is clearly demonstrated (Fig. 2) in the developmental pattern of copper accumulation. There is a highly significant difference between means at days 11, 16, 22, and 28. The low copper group maintained a basal level which appears to be invariant.

Copper Metalloproteins. Evidence that lower copper content can affect copper dependent enzymes is shown by the marked reduction of cytochrome c oxidase in the copper deficient cerebellum (Fig. 3). This Cu requiring protein (WAINIO et al., 1959) must be highly responsive to tissue levels of copper. At all points compared, mean differences were highly significant (Fig. 3).

Another copper metalloprotein, superoxide dismutase, was affected by chronic depletion of copper (Fig. 4). A significant difference was seen at day 22 and a highly significant difference at day 28, yet the dismutase levels were less responsive to copper depletion than were those of cytochrome c oxidase. The developmental pattern of superoxide dismutase has not previously been reported.

A reduction in norepinephrine content of the brain could be associated with a decrease in dopamine-β-hydroxylase. Fig. 5 shows the difference in mean norepinephrine levels between control and copper deficient rat brains. Control values for whole brain less cerebellum were significantly higher at days 22 and 28 and differences were highly significant at day 16. As with most of the other properties investigated, copper deficient animals had a larger sample variance. Experimental

variation was reduced by running daily norepinephrine standards and using an independent method for estimation of recovery (radioactive tracer).

Adult Rats. Female rats maintained on a commercial diet were used to compare with the developmental study (Table 2). Most properties of the adults examined in this study are higher than those of either the copper deficient or control rats at 28 days. For the control animals, copper and norepinephrine were at 2/3 adult levels and cytochrome c oxidase and 2',3'-cyclic nucleotide 3'-phosphohydrolase at 3/4 adult values. Cerebellar superoxide dismutase decreased slightly in adult animals, but maintained a level (Table 2) higher than in the copper deficient group.

DISCUSSION

Copper appears to be regionally concentrated in human brain, being higher in gray matter than white, with centers such as the locus ceruleus and substantia nigra having especially high copper levels (WARREN et al., 1960). The subcellular content of copper is partitioned with half in the crude mitochondrial fraction, 30% in the supernatant, and the remaining fifth divided between nuclear and microsomal fractions (MATSUBA and TAKAHASHI, 1970). This pattern of distribution remains unaffected in the copper deficient hepatocyte (EVANS, 1973). The accumulation of copper with time has been reported for human brain by SCHROEDER et al. (1966). Feeding of a copper deficient diet to 150 g rats for as long as 200 days reduced brain Cu by only 40% (OWEN, 1971; SMITH and FIELD, 1973). A larger reduction of copper was seen in this

developmental study (Fig. 2); a comparable reduction has been communicated by DI PAOLO and NEWBERNE (1972). DANKS et al. (1972a) have reported a brain copper value of 4.2 µg/g dry weight for a single six-month old case. This value is low when compared to adult values of 29.1 for cerebral cortex or 11.4 for cerebral white matter (WARREN et al., 1960). However, RESKE-NIELSON et al. (1973) have reported no differences in copper content between a case of Menkes' disease and two 4- to 6-month old controls. Two formalin-fixed specimens from the temporal and parieto-occipital lobes of each of the three brains gave values ranging between 1.6-2.0 µg/g wet weight. These values are lower than those reported by WARREN et al. (1960) for comparable regions even when corrected for age (SCHROEDER et al., 1966).

The histological appearance of focal necrosis in the CNS of copper deficient young rats was reported (CARLTON and KELLY, 1969; DI PAOLO and NEWBERNE, 1972); contrary results have also been published (FRICK and LAMPL, 1953; HALL and HOWELL, 1973). It seems the severity of the deficiency and the duration of the experiment are most critical. Localized neuronal degeneration has been reported in Menkes' disease (MENKES et al., 1962; AGUILAR et al., 1966).

The slower growth rate exhibited in experimental copper deficiency and in males with Menkes' disease (MENKES et al., 1962) is only one area in which similarities exist. Biochemical evidence for reduced myelin in the CNS of Menkes' disease patients is based on low sulfatide (O'BRIEN and SAMPSON, 1966), myelin and proteolipid protein concentrations (FRENCH et al., 1972). A decrease in sulfatide and cholesterol content of copper deficient rat brains (DI PAOLO and NEWBERNE, 1972) along with

the reduction in cerebellar 2',3'-cyclic nucleotide 3'-phosphohydrolase (Fig. 1), an enzyme which follows myelination in the rat brain (OLAFSON et al., 1969), support the hypothesis that a delay in myelination is present in experimental copper deficiency. The observations that undernutrition can delay myelination (DOBBINS, 1963) and that copper deficiency can retard developmental increases in specific proteins (MOFFITT and MURPHY, 1973) have been reported.

The gross reduction (Fig. 3) in cerebellar cytochrome c oxidase in copper deficient neonates substantiates the work of GALLAGHER et al. (1956a) where rat brain cytochrome c oxidase was lower in rats fed a low copper diet. Later work (HOWELL and DAVISON, 1959; MILLS and WILLIAMS, 1962) demonstrated lower enzyme activity in the brains of copper deficient lambs. The diminished level of brain cytochrome $a + a_3$ in a case of Menkes' disease (FRENCH et al., 1972) demonstrates another similarity between the neurological aspect of the human disease and the copper deficient neonatal rat. The significance of this reduction in cytochrome c oxidase remains an enigma.

The original suggestion (GALLAGHER et al., 1956b) that reduced phospholipid synthesis in the copper deficient rat was directly attributable to a failure in the process of condensation of acyl CoA with α-glycerophosphate has been questioned (NEWBERNE and DI PAOLO, 1971). Later work in a similar study (GALLAGHER and REEVE, 1971a) has shown that the addition of ATP can reverse the in vitro reduction in liver mitochondrial phospholipid synthesis. However, ^{32}P incorporation into brain phospholipids (GALLAGHER et al., 1956b) was not reduced in copper deficiency; no changes in the total amount or composition of mature rat brain phospholipids

was observed (GALLAGHER and REEVE, 1971b). An increase of brain phosphatidylcholine in the copper deficient neonate (DI PAOLO and NEWBERNE, 1972) indicates the presence of immature myelin which was also observed by FRENCH et al. (1972) in a Menkes' disease patient. Equating a reduction in cytochrome c oxidase with reduced phospholipid synthesis, although suggested to be causally related in liver mitochondria (GALLAGHER and REEVE, 1971a), seems unjustified for neural tissue.

Brain superoxide dismutase was first detected enzymatically by McCORD and FRIDOVICH (1969) and has recently been shown immunochemically (HARTZ et al., 1973) to be high in cerebral gray matter. One might suggest that a decrease in superoxide dismutase (Fig. 4) in copper deficiency may have a deleterious effect on neural tissue, giving rise to an increase in lipid peroxidation. O'BRIEN and SAMPSON (1966) reported a decrease in docosahexaenoic acid content in phosphatidyl ethanolamine and phosphatidyl serine and suggested an increase in the peroxidation of unsaturated lipids in patients with Menkes' disease. FRENCH et al. (1972) report a diminution of unsaturated fatty acids in white matter, and an increase in hydrogen peroxide hemolysis of erythrocytes from a patient. Superoxide dismutase has been shown to play a role in protecting erythrocytes against peroxidative hemolysis (FEE and TEITELBAUM, 1972); the role of superoxide in lipid peroxidation has also been reported (PEDERSON and AUST, 1973).

The increase in rat brain norepinephrine (Fig. 5) during postnatal development has been shown previously (AGRAWAL et al., 1966). The lower concentration of norepinephrine in copper deficient animals does not appear to be solely due to retarded growth, since when expressed

on a μg/g tissue basis, norepinephrine levels of severely undernourished rats were actually elevated (SHOEMAKER and WURTMAN, 1971). We feel the difference in norepinephrine concentration could be due to copper deficiency and not to the difference in length of tissue storage. Whether the hyperactivity seen in the copper deficient dams or the tremors and loss of aggressiveness (DI PAOLO and NEWBERNE, 1972) or extreme fright and convulsive seizures (CARLTON and KELLY, 1969) are related to depletion of norepinephrine in the CNS or are equated with the lethargy or myoclonic epilepsy of Menkes' disease (DANKS et al., 1972b) is a point for further investigation. As reviewed by RASUL and HOWELL (1973), the change in monoamines (a rise in dopamine and a reduction in norepinephrine) following sodium diethyldithiocarbamate treatment, a chelator of copper and inhibitor of dopamine-β-hydroxylase, accompanies neuronal degeneration.

The similarities between Menkes' disease and the copper deficient rat include slow growth, abnormal behavior, a decrease in myelin, a reduction in cytochrome c oxidase, and presumably less brain copper. Until further evidence is presented on reduced brain copper in Menkes' disease, the explanation for aberrant behavior, neuronal damage, increeased peroxidation of lipids based on changes in the copper metalloenzymes (cytochrome c oxidase, superoxide dismutase, and dopamine-β-hydroxylase) is still speculation. As DANKS (1972a) pointed out, the brain damage may be the result of arterial disease caused by the derangement of elastin (O'DELL et al., 1961).

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TABLE 1.--EFFECT OF A LOW COPPER DIET UPON BODY, WHOLE BRAIN, AND CEREBELLUM GROWTH IN THE DEVELOPING RAT

Age (Days)	Body Weight (g)		Significance*	Brain Weight (g)		Significance*	Cerebellar Weight (mg)		Significance*
	Control	Low Copper		Control	Low Copper		Control	Low Copper	
-3	--	--	--	0.271 ± 0.012	--	--	--	--	--
2	8.3†	7.8†	--	0.327 ± 0.008	0.303 ± 0.017‡	ns	--	--	--
6	16.0 ± 0.82	--	--	0.613 ± 0.006	--	--	39.6 + 0.74	--	--
11	28.8 ± 5.32	20.5 ± 8.74	ns	1.03 ± 0.070	0.870 ± 0.171	ns	99.5 ± 16.2	86.6 ± 22.8	ns
16	44.0 ± 1.15	27.2 ± 8.34	.05	1.31 ± 0.030	1.14 ± 0.181	ns	162.7 ± 9.8	132.4 ± 33.3	ns
22	66.5 ± 5.80	43.0 ± 5.03	.01	1.47 ± 0.029	1.29 ± 0.083	.01	196.3 ± 6.7	172.3 ± 7.2	.01
28	100.0 ± 5.35	60.2 ± 14.4	.01	1.54 ± 0.037	1.51 ± 0.082	ns	209.7 ± 9.1	191.0 ± 17.7	ns

Values listed represent the means ± S.D. of 4 animals. Tissue values are reported based on wet weight.

* Means were compared at 2 levels of α [0.05 (significant), 0.01 (highly significant)] by means of the F-variance ratio and the Student's two-tailed t-test; ns refers to means not significantly different.

† Values are means of pools of unequal sizes.

‡ Mean ± S.D. for 3 animals.

TABLE 2.--ADULT RAT VALUES FOR BRAIN AND CEREBELLAR WEIGHTS: BRAIN COPPER AND NOREPINEPHRINE CONTENT: CEREBELLAR CYTOCHROME C OXIDASE, 2',3'-cAMP 3'-PHOSPHOHYDROLASE, AND SUPEROXIDE DISMUTASE ACTIVITIES*

Property	Mean ± S.D.	No. of Animals
Brain Weight (g)	1.91 ± 0.102	8
Cerebellum Weight (mg)	266 ± 22.7	8
2',3'-cAMP 3'-Phosphohydrolase (units/g tissue)	362 ± 20.5	8
Copper Content (μg/g tissue)†	3.09 ± 0.384	8
Cytochrome *a* Oxidase (units/g tissue)	94.0 ± 3.76	8
Superoxide Dismutase (units/g tissue) x 10^{-3}	2.36 ± 0.164	8
Norepinephrine Content† (nmoles/g tissue)	3.05 ± 0.521	6

After decapitation of ether anesthetized 300 g female rats, brains were rapidly removed, bisected on ice, and weighed. Cerebella were homogenized and used for spectrophotometric enzyme analysis. The bisected remainder was divided for copper quantification by atomic absorption and for norepinephrine determination by fluorometric analysis. Details of the procedure and definitions of enzyme units are presented in detail in the text under METHODS.

* Values are expressed on a tissue wet weight basis.

† Whole brain less cerebella.

FIG. 1.--The postnatal development of cerebellar 2',3'-cAMP 3'-phosphohydrolase in control (●——●) and copper deficient (0---0) rats. At the indicated times, four pups from each group were decapitated, cerebella separated, homogenized, and after storage overnight (-20°C), enzyme activity was measured spectrophotometrically as described in METHODS. Plotted points represent means ± S.D. for fresh tissue.

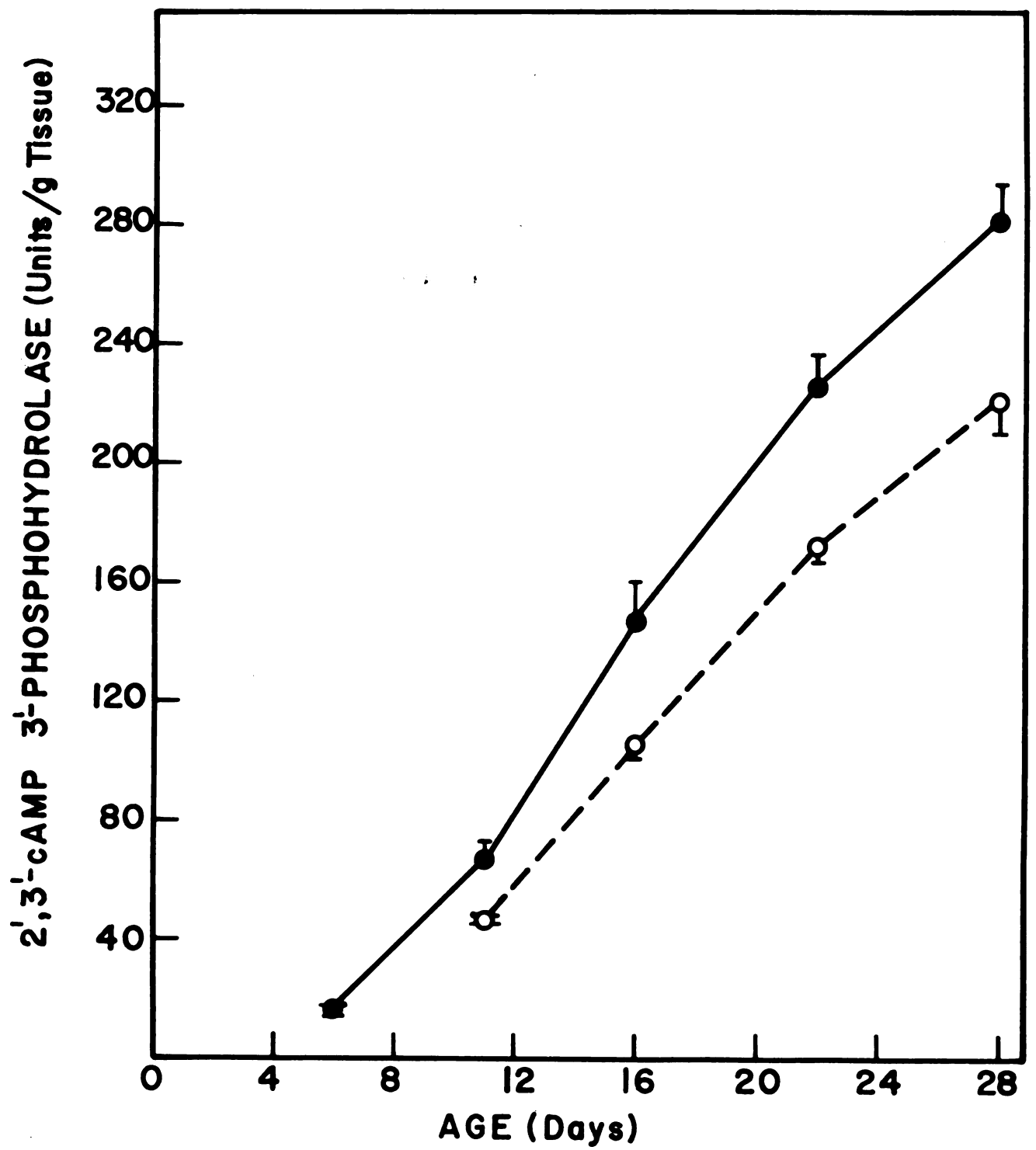

2',3'-cAMP 3'-PHOSPHOHYDROLASE (Units/g Tissue)
320
280
240
200
160
120
80
40
0
4
8
12
16
20
24
28
AGE (Days)

FIG. 2.--Concentration of copper in fresh tissue in developing control (●——●) and copper deficient (0---0) whole rat brains minus cerebella. At the indicated days (6, 11, 16, 22, 28), acerebellar hemisections were wet-ashed and copper was measured by atomic absorption spectroscopy on four individual samples. For the control pups at days (-3,2), 4 pools of at least 2 whole brains each were measured; the copper deficient value at day 2 is from 3 samples. All plotted points are means ± S.D.

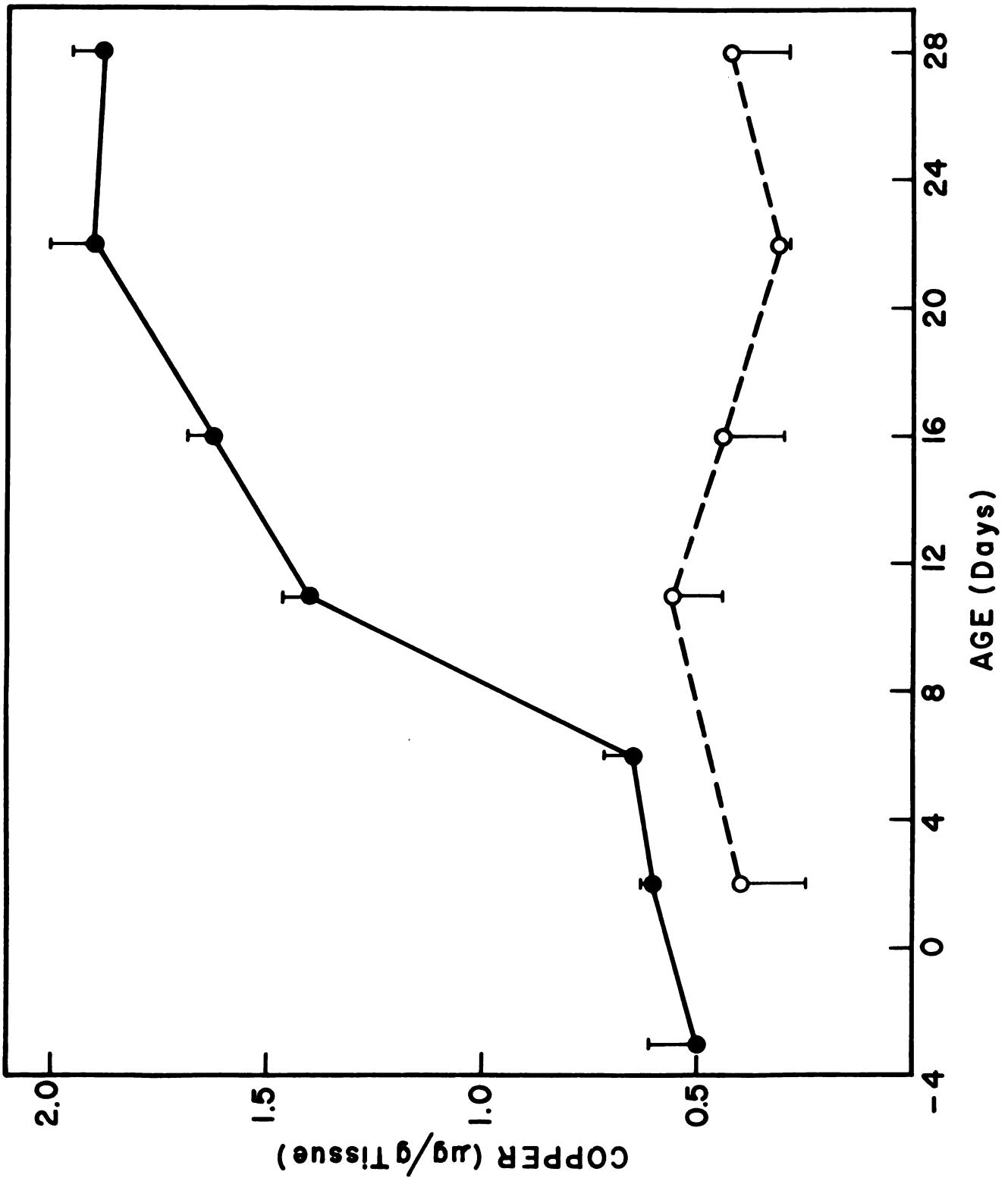

COPPER (µg/g Tissue)
2.0
1.5
1.0
0.5
-4
0
4
8
12
16
20
24
28
AGE (Days)

FIG. 3.--The postnatal development of cerebellar cytochrome c oxidase in control (●——●) and copper deficient (0---0) rats. At the indicated ages, four pups from each group were decapitated, cerebella separated, homogenized, and after storage overnight (-20°C), activity was measured on detergent treated homogenates by following the decrease in reduced cytochrome c at 550 nm as detailed under METHODS. Values shown are of four cerebella ± S.D. for fresh tissue.

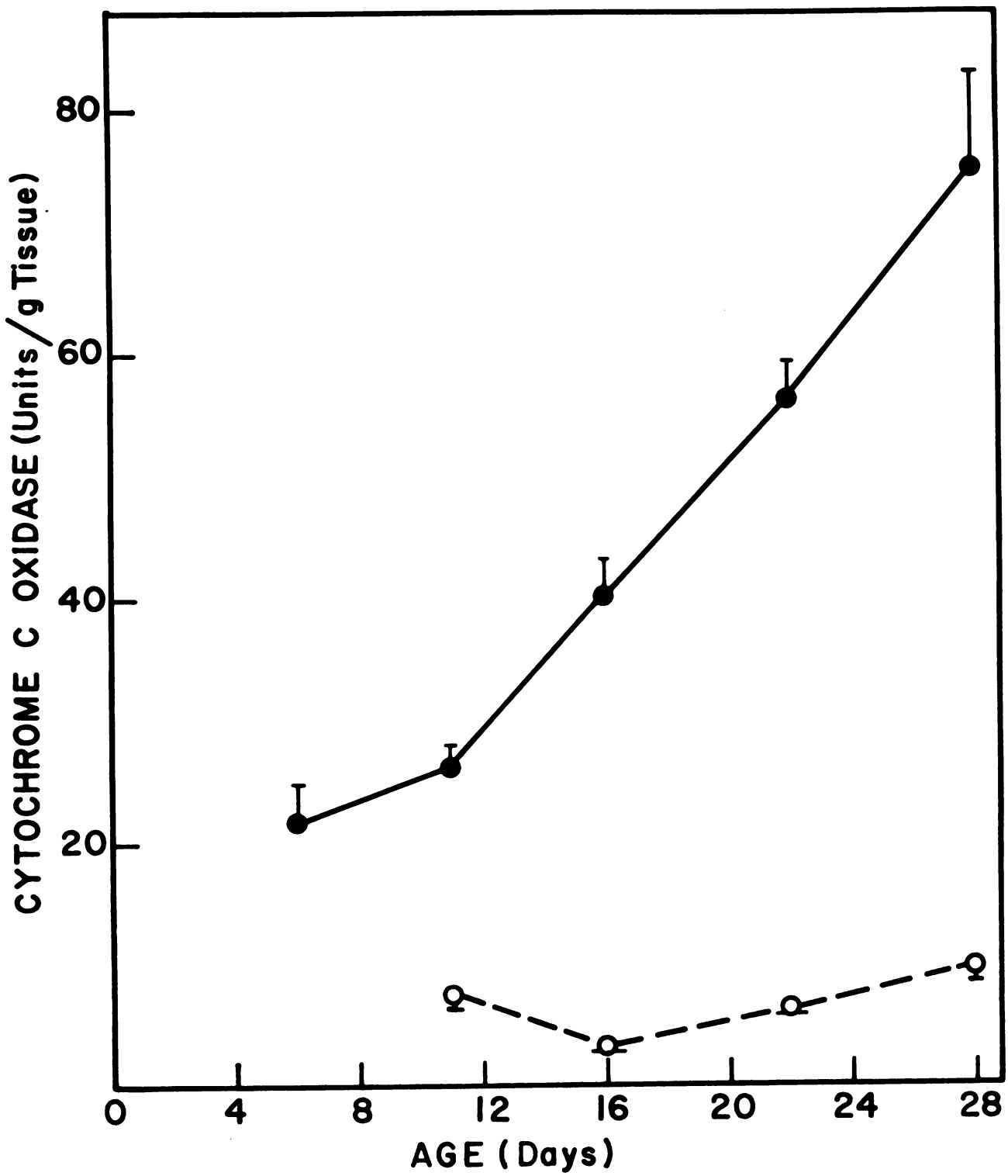

CYTOCHROME C OXIDASE (Units/g Tissue)
80
60
40
20
0
4
8
12
16
20
24
28
AGE (Days)

FIG. 4.--The postnatal development of cerebellar superoxide dismutase in control (●——●) and copper deficient (0---0) rats. At specified times, four pups were killed by decapitation, cerebella separated, homogenized, and centrifuged (40,000 x g, 15 min.). Activity was measured in the supernatant spectrophotometrically by its ability to inhibit the xanthine oxidase linked reduction of ferricytochrome c as described under METHODS. Values are means of four cerebella ± S.D. based on fresh tissue weight.

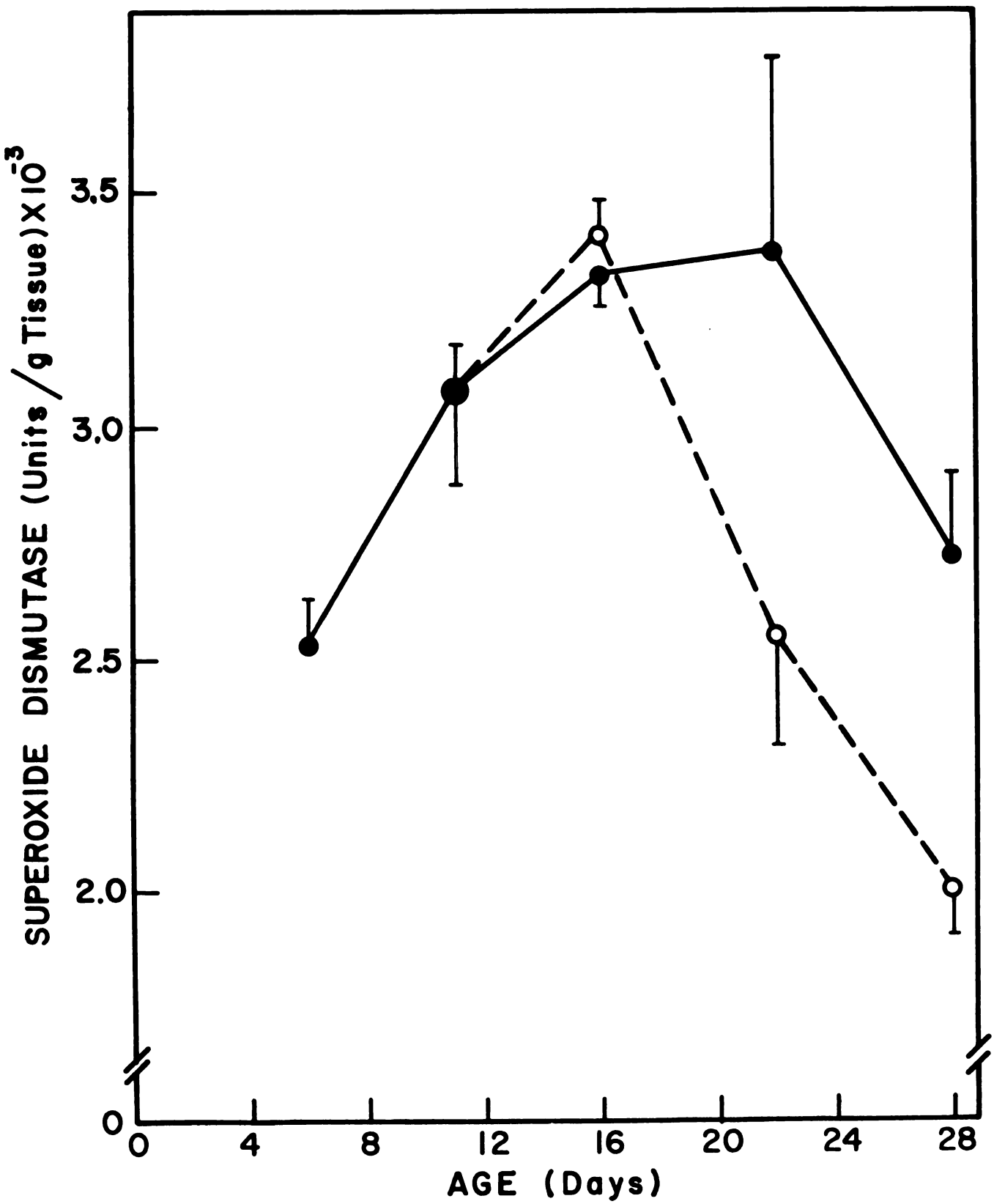
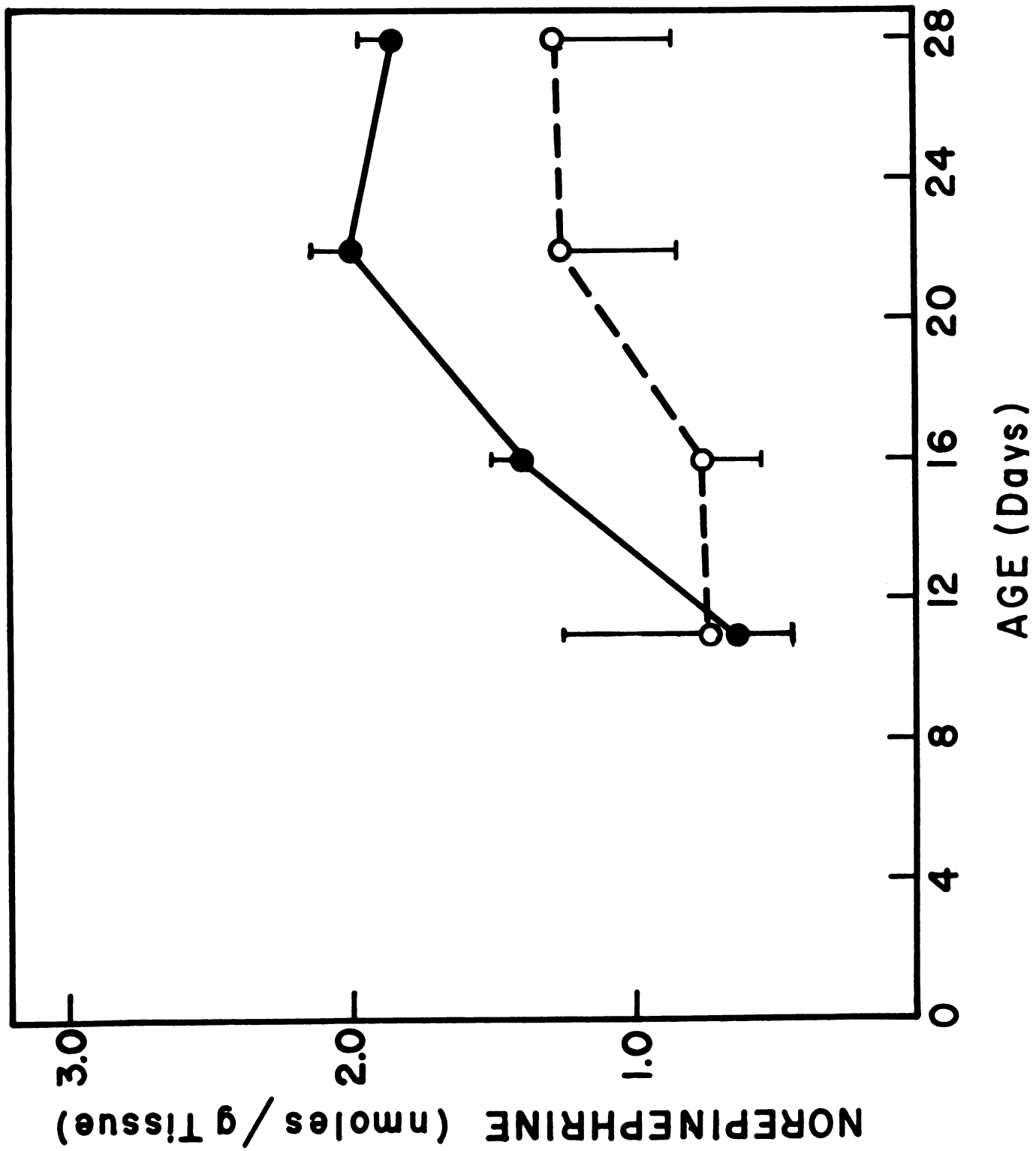

SUPEROXIDE DISMUTASE (Units /g Tissue) X 10⁻³
3.5
3.0
2.5
2.0
0
0
4
8
12
16
20
24
28
AGE (Days)

FIG. 5.--Concentration of norepinephrine in developing control (●——●) and copper deficient (0---0) whole rat brains minus cerebella. At the indicated ages, four pups of each group were decapitated and the acerebellar hemisections were frozen on solid CO_2 and stored (-90°C) for varying lengths of time. Norepinephrine was extracted with acetone-formic acid and measured fluorometrically as detailed under METHODS. Individual sample recoveries were carried out with (^{14}C)-norepinephrine. Values are the means of four brains ± S.D. based on fresh tissue weight.


NOREPINEPHRINE (nmoles / g Tissue)
3.0
2.0
1.0
0
4
8
12
16
20
24
28
AGE (Days)

CHAPTER IV

COPPER DEFICIENCY IN THE DEVELOPING RAT BRAIN: EVIDENCE FOR ABNORMAL MITOCHONDRIA

ABSTRACT

Copper deficiency was produced in developing rats by feeding a low copper diet to rats during gestation and lactation and providing the offspring the same diet. The progeny developed similar to those of an earlier model based on preconception depletion followed by marginal supplementation during gestation. Copper levels were greatly reduced in the brain, iron levels were slightly depressed, and no differences in zinc content were found. Electron microscopic examination of brain tissue revealed the presence of enlarged mitochondria from copper-deficient animals. Isolated mitochondria from copper-deficient rats showed a 30% reduction in the rate of both succinate and glutamate oxidation, and for glutamate, the respiratory control ratio (RCR) was decreased by 60%. Difference spectra displayed a four-fold reduction in cytochrome a + a_3 and slight increases in cytochrome b, c_1, and c. Enzyme analysis of isolated mitochondria revealed a five-fold decrease in cytochrome oxidase, slight increases in succinic dehydrogenase and fumarase, and small decreases in hexokinase and monoamine oxidase. No difference in peroxidation of brain lipids was evident. Determination of metabolites from fast frozen tissue suggested that the copper-deficient brain was in a more reduced state based on a doubling of both the lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P ratios. Creatine-P, ATP, and ADP levels were not different.

INTRODUCTION

Copper deficiency in the neonate is accompanied by neurological manifestations and can occur in domestic animals such as sheep, can be produced in the laboratory using guinea pigs or rats, and may occur in human infants due to either a nutritional oversight or the genetic disorder Menkes' steely-hair disease. Biochemically, the deficient rat brain accumulates only one-fifth the normal copper content (1,2) and contains lower concentrations of myelin (2, 3) and cytochrome c oxidase than controls (2). The classical work of Cohen and Elvehjem (4), 40 years ago, demonstrated that copper was essential for both oxidase activity and the A component of cytochrome. This observation was further verified by the work of Gallagher et al. (5, 6) who included studies on isolated mitochondria. They found no differences in oxidative phosphorylation of liver mitochondria nor in the ability of brain mitochondria to oxidize pyruvate. Wohlrab and Jacobs (7) concluded that interchain electron transport played a role in copper-deficient liver mitochondria. Goodman et al. (8) found normal mitochondrial function in both liver and heart mitochondria from copper-deficient rats although morphological changes were seen.

Since abnormal mitochondria from the cerebellar Purkinje cells of a young boy with Menkes' disease had also been reported (9) as well as abnormal liver mitochondrial function (10), brain mitochondria from copper-deficient rats were studied as an extension of the model for Menkes' steely-hair disease (2). Measurement of energy related compounds was also performed to correlate morphology with metabolic status.

MATERIALS AND METHODS

Pyridine and adenine nucleotides, cytochrome c (type III), 2,6-dichlorophenolindophenol, 2-amino-2-methyl-1-propanol, 2-thiobarbituric acid, and glutamic-pyruvic transaminase (EC 2.6.1.2) were obtained from Sigma. Other enzymes for metabolite determinations were purchased from Boehringer-Mannheim. Malondialdehyde bis-(dimethyl acetal) and 2,6-di-tert-butyl-4-methyl-phenol were products of Aldrich. Kynuramine was obtained from Regis and Tween-80 from Nutritional Biochemicals. Sperm positive female rats were purchased from Holtzman.

Animal care and diets. The composition of the basal diet was described previously (2) and contained either 10 μg Cu/g in the control diet or 0.3 μg Cu/g in the low copper diet as determined by atomic absorption spectroscopy (Perkin-Elmer model 303). The experimental design was modified from a preconception copper depletion period of 9 weeks followed by marginal supplementation during gestation (2) to one in which females, sperm positive for 1 day, were fed the low copper diet throughout gestation. Other details of randomization, tissue sampling, and statistical treatments by the *t*-test were described previously (2).

Enzyme assays. Most enzyme determinations were performed at 25°C in a Gilford 2400-S spectrophotometer. Cytochrome c oxidase (EC 1.9.3.1) was determined on crude cerebella or on isolated mitochondria after treatment with 1% (v/v) Tween-80 as described previously (2). Superoxide dismutase (EC 1.15.1.1) was measured by the method of

McCord and Fridovich (11) as detailed before (2). The myelin associated protein 2',3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16b) was assayed on crude cerebella as reported previously (12, 2). Succinic dehydrogenase (EC 1.3.99.1) was measured on cerebellar homogenates or mitochondrial preparations after treatment with 1% (v/v) Tween-80. The method of Earl and Korner was employed (13) initiating the reaction with potassium succinate. A value of $\varepsilon = 2.19 \times 10^4$ (14) for dichlorophenolindophenol (DCPIP) was used for calculation of units. Mitochondrial preparations were assayed for hexokinase (EC 2.7.1.1) (15) after treatment with Triton X-100 0.5% (v/v). Monoamine oxidase (EC 1.4.3.4) was assayed after treatment with Triton X-100 by following the loss of kynuramine ($\varepsilon = 3.94 \times 10^3$) at 360 nm, 30°C, pH 8.0, by the method of Weissbach et al. (16). Fumarase (EC 4.2.1.2) was assayed by the method of Racker (17) after treatment of the mitochondria with Triton X-100, 0.5% (v/v) using a value of $\varepsilon = 2.22 \times 10^3$ for sodium fumarate at 240 nm for calculations. All enzyme units (except superoxide dismutase) were expressed as μmoles/min and were either based on a g of fresh tissue or a mg of mitochondrial protein.

Electron microscopy. Thin slices of the right cerebral cortex were fixed for 2-1/2 hrs in 5% (v/v) glutaraldehyde in 0.1 M potassium phosphate (pH 7.2), postfixed 1-1/2 hrs in buffered 1% OsO_4 and dehydrated in graded aqueous ethanol prior to embedding in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips EM 300 microscope.

Mitochondrial studies. Mitochondria were isolated in 0.3 M mannitol, 0.1 mM EDTA (pH 7.4) from pools of 2 brains, excluding the medulla, by combining F_1 and F_2 fractions as detailed by Ozawa et al. (18). Oxidative phosphorylation was carried out by the methods of Milstein et al. (19) except substrate concentrations were 4 mM. Oxygen consumption was monitored using a Yellow Springs Instrument Model 53 oxygen monitor at 25°C. Calculations of respiratory control ratios (RCR) and ADP/O ratios were made according to the methods of Estabrook (20). The estimation of the contents of cytochromes a + a_3, b, c_1, and c were made following the calculations of Williams (21). The isolated mitochondria were treated with deoxycholate and the oxidized ($K_3Fe(CN)_6$) versus reduced (sodium ascorbate + $Na_2S_2O_4$) difference spectrum of ca. 1 mg of protein in 0.25 ml (1 cm cuvettes) was taken with a Cary Model 15 spectrophotometer between 500-630 nm.

Metabolite and metal analysis. Tissue used for energy metabolite determination was obtained from animals which were immersed into liquid N_2 and stored at -85°C until the brains were chipped out, powdered, and extracted as described previously (22). The neutralized extracts were analyzed spectrophotometrically for creatine phosphate, ATP, ADP, AMP, and lactate, and fluorometrically for pyruvate, α-glycerol phosphate, and dihydroxyacetone phosphate by the methods of Lowry and Passonneau (23). The fluorometric measurement of brain norepinephrine was as described previously (2). Copper analysis as well as zinc and iron determinations were performed using single element cathodes and atomic absorption spectroscopy (2). Protein was measured by the procedure of Lowry et al. (24) using bovine serum albumin as standard.

Lipid peroxidations. Evidence for peroxidation was taken as the formation of malondialdehyde as judged by reaction with 2-thiobarbituric acid according to the method of Pederson (25). To a 0.5 ml aliquot is added 1.0 ml containing 0.375% 2-thiobarbituric acid, 15% trichloroacetic acid, both (w/v), in 0.25 M HCl to which had been added just prior to use 0.01 vol of 2,6-ditertbutyl-4-methylphenol in C_2H_5OH to prevent further peroxidation (26). The mixture is heated (95°C) for 15 min, cooled, centrifuged at 1000 x g, and the absorbance determined at 535 nm. Using 9.6 nmoles of malondialdehyde bis-(dimethylacetal) as standard through the above procedure, 1.0 A is obtained. Whole brains were homogenized in 4 vols of 0.15 M KCl, 0.05 M Tris (pH 7.4), in a Potter-Elvehjem homogenizer. Aliquots were used to study autooxidation of lipids in a shaking water bath (37°C) buffered with 0.1 M Tris (pH 7.4) or metal catalyzed peroxidation at 23°C containing 2 mM ADP and 0.12 mM $Fe(NH_4)_2(SO_4)_2$ (pH 7.5).

RESULTS

Induced copper deficiency. Two equivalent experiments were designed, each consisting of twenty sperm positive females divided equally and fed either control or copper deficient diets from the second day of the presumed gestation. Offspring were randomized within each group within 24 hrs of birth and thereafter at weekly intervals to maintain a constant litter size. Evidence that copper depletion had occurred during gestation was suggested by the fact that the copper content (μg Cu/g body wt) of day old pups was 2.95 ± 0.06

in controls and 0.60 ± 0.25 in the deficient group (mean ± SD of 4 pools of 2 pups each). Offspring from the first experiment were used to compare with a previous model (2) in which copper deficiency prior to the lactation period was induced by a preconception depletion period and marginal gestational copper supplementation. The data in Table I demonstrate that the present model system produced equivalent results with the former (2). A five-fold reduction in copper concentration was accompanied by reductions in the copper proteins cytochrome c oxidase and superoxide dismutase, by reductions in norepinephrine and in the myelin protein 2',3'-cyclic nucleotide 3'-phosphohydrolase; body, brain, and cerebellar weights were also reduced as reported previously (2).

Several 28-day old rats of each group were fed a commercial pellet diet (15 μg Cu/g) for four weeks and the results of this repletion period are shown in Table I. Body weight of the deficient group (data not shown) did not reach that of the controls.

Mitochondrial morphology. When the copper deficient animals were 30 days old, severe signs of deficiency were apparent (anorexia and hyperventilation). Electron microscopic examination of 2 brains from each group revealed the presence of enlarged mitochondria in the cerebral cortex (Fig. 1). Although mitochondria of normal appearance were also present, about half appeared to be abnormal in shape and less electron dense. There appeared to be fewer mitochondria in the observed fields from the copper deficient preparations, but no quantitative estimate was made; no other cytological differences were noted.

Mitochondrial function. Because of the abnormal appearance of the mitochondria from copper deficient brain, Fig. 1, and the alteration in two mitochondrial proteins (an increase in succinic dehydrogenase and a decrease in cytochrome c oxidase, Table I), offspring from the second experiment were used to investigate mitochondria from developing rat brain. Mitochondrial oxygen consumption was measured at 2 ages using both succinate and glutamate as substrates, Table II. Statistical differences were evaluated although only 2 preparations were used for each mean; differences between the two groups are present at both ages. Mitochondria from copper-deficient rats are reduced (30%) in their ability to oxidize both substrates; however, they appear to be more normal in their ability to use succinate as opposed to glutamate (Table II) when comparing respiratory control ratios. Each preparation for the oxygen uptake studies was run separately and completed within 1-1/2 hrs from the time of decapitation.

Figure 2 shows a representative mitochondrial difference spectrum from a control and a copper-deficient brain. The striking reduction of the cytochrome a + a_3 peak at 605 nm is quantitatively shown in Table III. At both ages, the cytochrome a + a_3 content was significantly reduced (72 and 80%) while cytochromes b, c_1, and c showed a slight increase.

Table III also shows the specific activities of five enzymes associated with brain mitochondria: hexokinase and monoamine oxidase -- the outer membrane, succinic dehydrogenase and cytochrome c oxidase -- the inner membrane, fumarase -- the matrix. A significant decrease

in cytochrome c oxidase is apparent at both ages with only slight changes in the other enzymes. Both hexokinase and monoamine oxidase may be slightly reduced and both succinic dehydrogenase and fumarase slightly increased. These enzyme changes were also quantitatively seen in crude homogenates (data not presented), suggesting that the mitochondria were not drastically altered during isolation.

The data for 30-day old animals were calculated on a per cent control basis and are shown in Fig. 3. There is good agreement in the reduction in cytochrome oxidase measured enzymatically or calculated from difference spectra (20 and 25% of control).

Energy metabolites. Since it is essential to the measurement of high energy phosphates that tissue is of a size that can be frozen rapidly, the copper-deficient rat presented a problem because it is smaller than an age matched control. Weight matched animals were, therefore, included as a third control group for this study, Table IV. Metabolite levels are reported in Table IV as well as lactate/pyruvate and α-glycerol phosphate/dihydroxyacetone phosphate ratios to evaluate the cytoplasmic NAD^+/NADH levels. No differences in the levels of ATP, ADP, or creatine-P were found between any of the groups. AMP was statistically different in the weight matched control group from either age matched control or copper-deficient groups. Examination of the glycolytic intermediates, however, revealed significant differences between the copper-deficient group and both control groups. Large increases in lactate and α-glycerol phosphate accompanied by small increases in pyruvate and dihydroxyacetone-phosphate resulted in a 2- to 3-fold increase in the

lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P ratios when compared with control groups, Table IV. When compared with the weight matched controls, the larger controls (age matched) had moderate increases in lactate, α-glycerol-P and dihydroxyacetone-P reflected in small increases in lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P.

Trace element levels. Brains from five-week old rats were grossly divided into three brain regions: the cerebellum, the cerebral cortex, and the remainder (brainstem + cerebral peduncle); and the tissues were rinsed with thrice distilled water to remove surface blood. Since blood Fe was not determined nor the contamination by blood in each region determined, values for Fe are not corrected and, thus, the differences in brain Fe seen in Table V between control and copper-deficient rats are only suggestive. However, the large reduction in brain copper in all three regions, Table V, agrees with that of the whole brain less cerebella, Table I. No differences in brain zinc due to copper deficiency were noted. The reduction in brain copper was equivalent in all three regions; however, 2 of the 3 samples from the cerebral cortex were much lower than the mean reflects.

Lipid peroxidation. Four-week old rats were used to investigate the possibility that the enlarged mitochondria seen at 30 days of age, Fig. 1, could be due, in part, to lipid peroxidative swelling. Table VI shows the production of malondialdehyde in both groups with and without ADP-Fe; no differences were observed. The inclusion of the antioxidant 2,6-ditert-butyl-4-methylphenol (0.9 mM) during incubation completely prevented malondialdehyde formation. Zero time values for

malondialdehyde were low, variable, and no differences existed between groups and were thus treated as tissue blanks rather than as true endogenous malondialdehyde levels. Addition of 5 μM Cu^{2+} to the incubation, an amount equivalent to the total copper in the control contributed by the tissue, produced no dramatic difference in malondialdehyde production, Table VI.

DISCUSSION

Tissue response to low copper levels results in variable changes to the morphology of the mitochondria. Figure 1 clearly shows that brain mitochondria from copper-deficient rats are enlarged and abnormal in shape. Others have shown that the number of mitochondria increase in cardiac tissue without an appreciable size change (8, 27), while the liver may produce some mitochondria ten times the normal size (27). This same response is seen in mouse tissue after feeding cuprizone (biscyclohexanone oxaldihydrazone), a copper chelator (28). Enlarged mitochondria were also reported in copper-deficient rat erythroblasts (27).

Aberrant mitochondrial function during copper deficiency is also variable. Oxygen consumption in the control mitochondria, Table II, agrees with that reported by others (18, 19) for uptake ADP/O ratios and respiratory control ratios (RCR). However, the mitochondria from Cu deficient brains did show a definite reduction in glutamate oxidation, although no differences in tissue glutamate dehydrogenase activity were seen (unpublished data). The copper deficient liver mitochondria studied by Wohlrab and Jacobs (7) displayed small

differences when succinate was used, in agreement with our results, but no differences with glutamate. Goodman et al. (8) found no differences with either substrate for either liver or heart mitochondria between controls and copper-deficient rats. Mice fed cuprizone had normal heart mitochondrial function, but the greatly enlarged liver mitochondria were markedly reduced in all aspects of oxidative phosphorylation (28). The moderate reduction in both succinate and glutamate oxidation (30%) with a much larger reduction in cytochrome oxidase (80%) supports the observations of others (5, 7, 8).

The mitochondrial difference spectrum, Fig. 2, from copper deficient rat brain is in agreement with those reported for copper-deficient rat liver (29) and heart (8, 30) and also with that reported from the brain of a boy who died of Menkes' steely-hair disease (31). All contain a striking reduction in the cytochrome a + a_3 peak (605 nm) while the remaining spectrum is quite normal. Chemical removal of copper from purified cytochrome oxidase does not appreciably alter its spectrum (32) suggesting absence of the apoenzyme or heme A in copper-deficient mitochondria since the spectrum is changed. The difference spectrum from iron deficient mitochondria is normal (8), although the mitochondria are enlarged.

The concentrations of cytochromes from the control rat brain agree reasonably well with those reported by Williams (33) using a similar method. Wohlrab and Jacobs (29) reported no difference in cytochrome b or c_1 levels in copper deficient liver mitochondria but a decrease in cytochrome c. Gubler et al. (34) first reported an

increase in cytochrome c from copper deficient swine heart and this was confirmed by Dallman (30), who also reported that the absorption peak between 550-650 nm was equal to or slightly greater than that from control heart mitochondria. The increase in cytochromes b, c_1, and c reported in Table III tend to support this observation for brain mitochondria.

A moderate reduction in cytochrome oxidase activity of copper deficient rat brain was reported (4, 5) when deficiency was begun after lactation. A much more striking depletion (to 20-25% of control) is found in the developing brain ((2) and Tables I and III). The slight increase in succinic dehydrogenase activity, Tables I and III, has been detected histochemically from brains of copper-deficient rats (35) and in mouse brain from animals fed cuprizone (36). Monoamine oxidase (MAO), Table III, was found not to change in the copper deficient mitochondria. Chou et al. reported no change in MAO of copper deficient chicken brain (37) while O'Dell et al. (38) reported values slightly higher in copper deficient newborn lambs. Hexokinase and fumarase levels in copper-deficient brain have not been reported before.

The major difference between the copper deficient and control mitochondrion is an increased size and a reduced amount of cytochrome a + a_3 (oxidase). The results of Fig. 3 and Table III taken as a whole (for individual differences in mitochondrial components are slight) would seem to suggest that the mitochondria from copper-deficient brains have an increase in inner membrane components and perhaps matrix and a decrease in the outer membrane.

This could arise from individual mitochondria increasing in volume disproportionate to the surface area, by assuming a spherical symmetry (Fig. 1), or from the fusion of mitochondria of different sizes (radii). Either case could explain the enlarged mitochondria seen in Fig. 1 and the change in surface area/volume implied from Fig. 3 and Table III.

There does appear to be some correlation between the abnormal functioning of the copper deficient mitochondria in vitro and the energy metabolism of the brain in vivo. The increased lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P ratios in the copper-deficient brain probably reflect lower NAD^+/NADH ratios, Table IV. However, no differences in creatine-P, ATP, or ADP between groups suggest that high energy phosphate metabolism was grossly normal. Values for ATP and creatine-P are lower than those reported (39) when more rapid freezing of the tissue is possible. Since creatine-P has not dropped below 2 mM (Table IV), the ATP concentration seems to be lower than that predicted from prior studies (40). Perhaps the elevated lactate and α-glycerol phosphate levels reflect a reduced capacity of copper-deficient mitochondria to oxidase NADH as suggested by the impaired glutamate utilization.

The concentrations of copper, zinc, and iron in control brain, Table V, agree with those of Kofod (41) for rats of similar age. Feeding excess zinc (4000 ppm) to lactating rats resulted in lower brain copper and iron, but no change in brain zinc in the offspring (42). This agrees with the pattern seen in Table V when low copper diets were used to induce Cu deficiency. The reduction in brain Fe

may have some relevance to the observation that copper deficient brains have less norepinephrine, Table I (2, 38), and dopamine (38). The rate limiting enzyme in their synthesis, tyrosine hydroxylase (EC 1.14.3.2), requires Fe^{2+} for activity (43). The enzyme catabolizing them, monoamine oxidase, is unchanged. An alternate suggestion for lower norepinephrine based on a reduction of dopamine-β-hydroxylase (EC 1.14.2.1), a copper enzyme, was suggested (2). These studies are currently under investigation, and conclusions are still speculative.

In the brains of Menkes' steely-hair disease patients, there was a reduction in unsaturated fatty acids reported (31, 44), and a defect in antioxidant metabolism was suggested. Contrary results have been recently reported (45). However, an autofluorescent pigment, suggestive of lipid peroxidation, has been reported by two authors (44, 46) in the cerebellar Purkinje cells. Since superoxide dismutase activity is lower in copper deficient rat brains, Table I and (2), and has been shown to protect against lipid peroxidation in special systems (47-50), experiments on lipid peroxidation from copper deficient brains were performed, Table VI. These experiments do not appear to provide an explanation for the "aging" effect reported (5) to occur in copper deficient rat liver mitochondria or for the enlarged mitochondria from copper deficient brain (Fig. 1).

From the preliminary results in Table I, it appears as though the recovery of brain cytochrome oxidase in copper repleted rats is a slow process. Dallman (30) first studied this process of recovery and concluded that it was governed by the synthesis of new mitochondrial material. Since the turnover of brain mitochondria is

on the order of three weeks (51), full recovery would be expected to be slow. Based on body and brain weight, Table I, it would appear the animals are permanently stunted by copper deficiency when carried out to 4 weeks of age.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Steven D. Aust for his suggestions in the studies on lipid peroxidation, and Mrs. June Mack for assistance with the electron microscopic work.

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TABLE I

RAT BRAIN DEVELOPMENT-RECOVERY FROM PERINATAL COPPER DEFICIENCY[a]

Property	Group	Age		
		14 days	21 days	56 days
Brain Weight (g)	+ Cu	1.24 ± 0.03	1.43 ± 0.10	1.88 ± 0.09
	- Cu	1.09 ± 0.12	1.23 ± 0.04[b]	1.69 ± 0.08[c]
Cerebellum Weight (mg)	+ Cu	143 ± 4.7	186 ± 18.5	281 ± 19.4
	- Cu	120 ± 17.0[c]	159 ± 7.0[c]	238 ± 20.3[c]
Copper (μg/g)	+ Cu	1.58 ± 0.05	2.02 ± 0.04	2.32 ± 0.20
	- Cu	0.54 ± 0.08[b]	0.43 ± 0.08[b]	1.08 ± 0.02[b]
Norepinephrine (nmoles/g)	+ Cu	1.44 ± 0.22	2.13 ± 0.07	2.92 ± 0.19
	- Cu	0.95 ± 0.12[b]	1.53 ± 0.11[b]	3.40 ± 0.29[c]
2',3'-cAMP-3'-Phospho-hydrolase (units/g)	+ Cu	128 ± 5.0	264 ± 5.0	374 ± 16.5
	- Cu	115 ± 7.4[c]	228 ± 15.9[b]	367 ± 7.8
Succinic Dehydrogenase (units/g)	+ Cu	0.588 ± 0.040	0.901 ± 0.034	0.965 ± 0.097
	- Cu	0.562 ± 0.057	0.999 ± 0.045[c]	1.01 ± 0.083
Cytochrome *c* Oxidase (units/g)	+ Cu	28.0 ± 2.4	58.9 ± 4.0	42.1 ± 4.17
	- Cu	13.0 ± 1.8[b]	14.3 ± 2.4[b]	33.2 ± 2.89[c]
Superoxide Dismutase (units/g) x 10^{-3}	+ Cu	3.37 ± 0.24	2.93 ± 0.13	2.04 ± 0.10
	- Cu	3.34 ± 0.17	2.06 ± 0.08[b]	1.96 ± 0.22

[a]Values are means ± SD of four animals expressed on fresh weight basis. Copper deficiency was induced during gestation and until 28 days postnatally prior to four weeks of recovery on a commercial pellet diet (56 days of age). Enzyme activities were determined on cerebella, the remainder of the brain was bisected and used for copper and norepinephrine determinations.

[b]Means significantly different $p < 0.01$.

[c]Means significantly different $p < 0.05$.

TABLE II

COPPER DEFICIENCY-BRAIN MITOCHONDRIAL OXYGEN CONSUMPTION[a]

Substrate	Property	Age			
		23 Days		29 Days	
		+ Cu	- Cu	+ Cu	- Cu
Succinate	State 3	118 ±4.9	91.6 ±8.8	115 ±15.6	78.6 ±0.0
	State 4	41.1 ±6.1	37.4 ±4.9	31.8 ± 6.7	29.5 ±0.4
	RCR[b]	2.94±0.56	2.49±0.57	3.74± 1.28	2.66±0.04
	ADP/O	1.68±0.01	1.55±0.13	1.69± 0.06	1.62±0.04
Glutamate	State 3	51.9 ±0.3	34.4 ±0.3**	42.8 ± 3.6	30.3 ±1.6*
	State 4	13.9 ±0.7	21.0 ±2.0*	11.6 ± 0.2	22.6 ±1.8*
	RCR[b]	3.73±0.24	1.64±0.18*	3.67± 0.23	1.34±0.04**
	ADP/O[c]	2.95±0.07	---	2.44± 0.26	---

[a]Values are means ± SD of 2 pools of 2 brains each from either control (+ Cu) or copper deficient (- Cu) offspring. Oxygen consumption in the presence (state 3) or absence (state 4) of ADP is expressed as natoms oxygen/min/mg protein. The substrates employed were 4 mM potassium salts. Details of the procedure are described in Methods. Means were significantly different [*$p < 0.05$, ** $p < 0.01$].

[b]Respiratory control ratio.

[c]Low RCR prevented calculation of ADP/O ratios for - Cu preparations.

TABLE III

COPPER DEFICIENCY-BRAIN MITOCHONDRIAL ENZYMES AND CYTOCHROMES[a]

Component	Age			
	21 Days		30 Days	
	+ Cu	- Cu	+ Cu	- Cu
Cytochromes (nmoles/mg protein)				
b	0.168±0.003	0.172±0.002	0.258±0.035	0.289±0.023
c_1	0.127±0.001	0.132±0.020	0.112±0.010	0.131±0.008[b]
c	0.174±0.002	0.184±0.007	0.230±0.020	0.234±0.025
$a + a_3$	0.292±0.008	0.112±0.110	0.436±0.044	0.107±0.005[c]
Activities (units/mg protein)				
Cytochrome oxidase	2.12 ±0.05	0.60 ±0.13[c]	2.26 ±0.23	0.46 ±0.01[c]
Succinic dehydrogenase x 10^2	2.58 ±0.4	2.88 ±0.2	2.52 ±0.18	2.83 ±0.14
Fumarase x 10	8.32 ±0.04	8.83 ±0.07[b]	9.41 ±1.02	9.48 ±0.14
Hexokinase x 10	2.55 ±0.07	2.34 ±0.14	2.90 ±0.39	2.65 ±0.11
Monoamine oxidase x 10^3	---	---	4.02 ±0.18	3.73 ±0.27

[a]Values represent means ± SD for pools of 2 brains each with 2 pools at 21 days and 4 pools at 30 days. Mitochondria were isolated and the cytochrome content determined from the difference spectra (reduced versus oxidized). Enzyme activity was determined on the same mitochondrial preparations. One unit is defined as 1 μmole/min.

[b]Means significantly different $p < 0.05$.

[c]Means significantly different $p < 0.01$.

TABLE IV

COPPER DEFICIENCY-BRAIN ENERGY METABOLITES[a]

Property	Dietary Group[b]		
	Control - I	Control - II	-Copper - III
Age (Days)	14	20	20
Body Weight (g)	26.0 ±5.8 (4)	45.5 ±3.8	25.8 ±6.2
Metabolite (μmoles/g tissue)			
Creatine Phosphate	2.72 ±0.38	2.23 ±0.54	2.13 ±0.57
ATP	1.57 ±0.59	1.65 ±0.43	1.58 ±0.32
ADP	0.40 ±0.08	0.52 ±0.28	0.44 ±0.08
AMP	0.18 ±0.01*,†	0.30 ±0.06	0.29 ±0.07
Lactate	1.20 ±0.46*,††	2.05 ±0.21†	6.07 ±1.52
Pyruvate	0.101 ±0.012††	0.110 ±0.012††	0.167 ±0.020
α-Glycerol-P	0.046 ±0.008**,††	0.110 ±0.006†	0.191 ±0.037
Dihydroxyacetone-P	0.0116±0.0018**	0.0202±0.0021 (3)	0.0188±0.0066
Ratio			
Lactate/Pyruvate	12.2 ±6.0††	19.0 ±3.7††	35.9 ±5.5
α-Glycerol-P/Dihydroxy-acetone-P	4.0 ±0.6*,†	5.6 ±0.6† (3)	10.8 ±3.1

[a]Animals were immersed in liquid N_2 and were stored at -85°C until brains were powdered over solid CO_2. Metabolites were determined in $HClO_4$ extracts by either spectrophotometric or fluorometric procedures described in Methods. Values are means ± SD for 3 animals in control-I or 4 animals for control-II or -copper-III unless indicated (N).

[b]Mean comparisons of the weight matched control-I group were different from the age matched control-II group (*$p < 0.05$, ** $p < 0.01$). Mean comparisons of either control group I or II were different from -copper-III group († $p < 0.05$, †† $p < 0.01$).

TABLE V

COPPER DEFICIENCY-BRAIN COPPER, ZINC, AND IRON LEVELS[a]

Element (μg/g)	Group	Brain Region		
		Cerebral Cortex	Cerebellum	Brain Stem-Peduncle
Copper	+ Cu	1.88 ± 0.13	2.10 ± 0.24	1.95 ± 0.11
	- Cu	0.36 ± 0.12[b]	0.49 ± 0.05[b]	0.45 ± 0.00[b]
Zinc	+ Cu	15.7 ± 1.05	15.3 ± 0.91	12.3 ± 0.73
	- Cu	13.8 ± 0.60	15.4 ± 0.20	13.1 ± 1.48
Iron	+ Cu	12.0 ± 0.94	17.8 ± 2.51	17.1 ± 0.58
	- Cu	8.4 ± 0.59[b]	20.9 ± 2.10	11.0 ± 2.21[b]

[a]Values are means ± SD of 3 pools of 35-day old rat brains (control rats (+ Cu) 2/pool or copper deficient rats (- Cu) 4/pool). Tissue was wet-ashed and the metal content determined by atomic absorption spectroscopy as described in Methods. Tissue values are uncorrected for blood contamination and expressed on a fresh weight basis.

[b]Means significantly different $p < 0.01$.

TABLE VI

COPPER DEFICIENCY-BRAIN LIPID PEROXIDATION[a]

Additions	Time (min.)	No. (Animals)	Control	Copper Deficient
			(nmoles malondialdehyde/g tissue)	
None	40	6	64.5 ± 22.8	58.5 ± 26.1
None	80	6	170 ± 55	140 ± 38
Cu^{2+} (5 μM)	40	3	---	68.4 ± 15.0
Cu^{2+} (5 μM)	80	3	---	177 ± 40
ADP·Fe[b]	5	4	134 ± 6	136 ± 5
ADP·Fe[b]	10	4	202 ± 8	210 ± 12

[a]Values are means ± SD for the indicated number of 28-day old rats. Incubations were carried out in 1 ml containing 0.1 M Tris (pH 7.4), 80 mg brain tissue in 0.4 ml of 0.15 M KCl, 0.05 M Tris (pH 7.4) at 37°C with shaking. At the indicated times, aliquots were removed and the content of malondialdehyde was determined colorimetrically as described in Methods.

[b]Incubations were carried out as above except that ADP·Fe was included (ADP, 2 mM, Fe^{2+}, 0.12 mM) and the reaction run at room temperature (*ca.* 23°C).

Fig. 1. Electron micrographs of 30-day old rat cerebral cortex from control and copper-deficient animals. Control brain containing 1.98 µg Cu/g (a) and 2.16 µg (b) have much smaller mitochondria than copper-deficient specimens (c) 0.46 µg or (d) 0.54 µg. All photos are at the same magnification; the scale line in (c) = 1 µm. A thin tissue slice was fixed and stained as described in Methods; the remaining portion of the brain was digested and the Cu determined by atomic absorption.

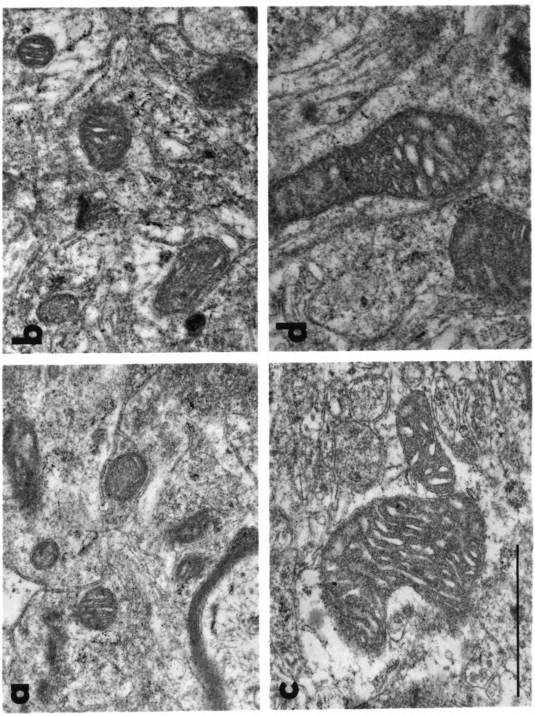

b
d
a
c

Fig. 2. Difference spectra of reduced (ascorbate + $Na_2S_2O_4$) versus oxidized ($K_3Fe(CN)_6$) rat brain mitochondria in the presence of deoxycholate. Mitochondria were isolated as described in Methods and either 1.41 mg of protein from control brain (———) or 1.38 mg from copper deficient brain (-----) in 0.25 ml final volume were used to obtain the above spectra.

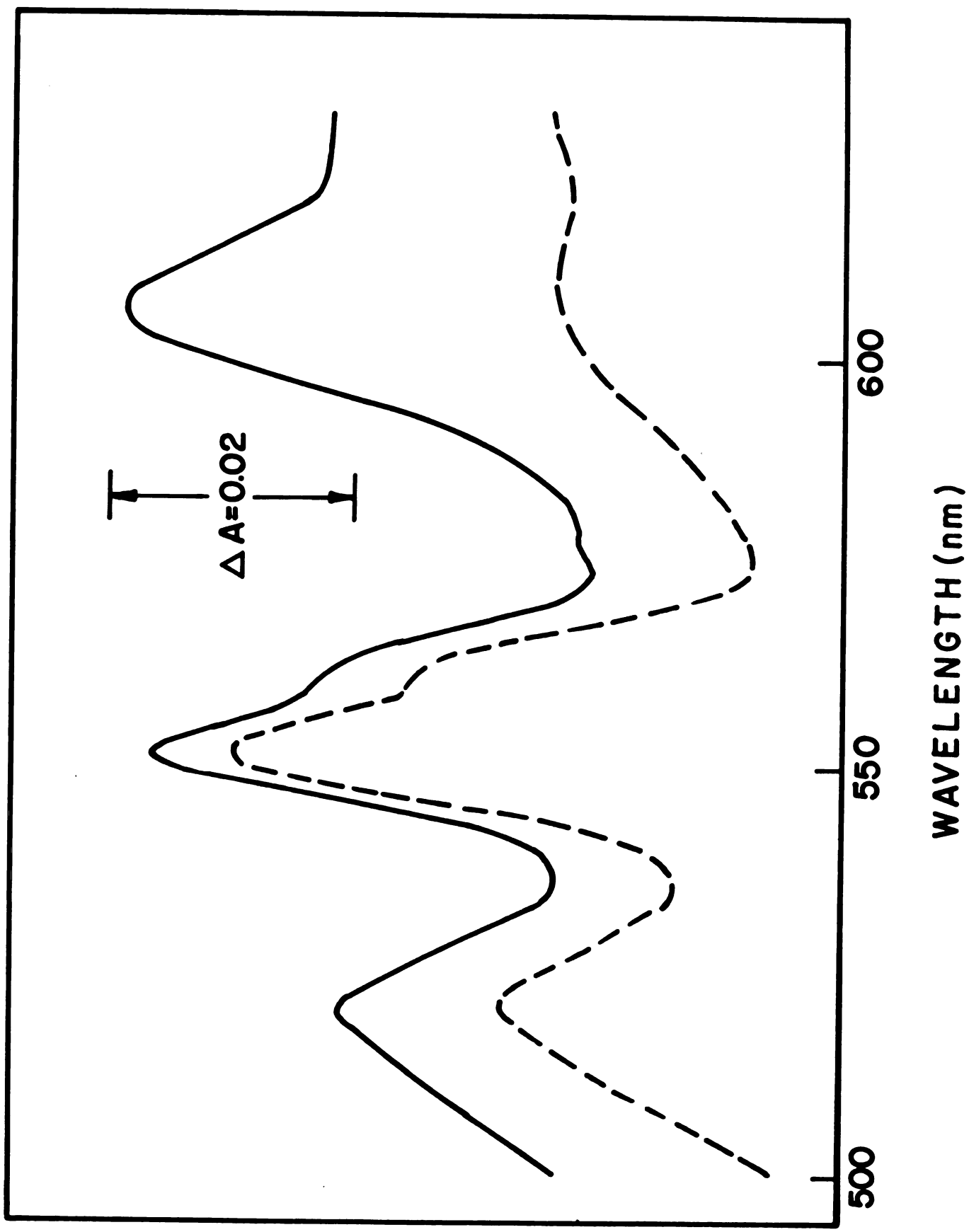
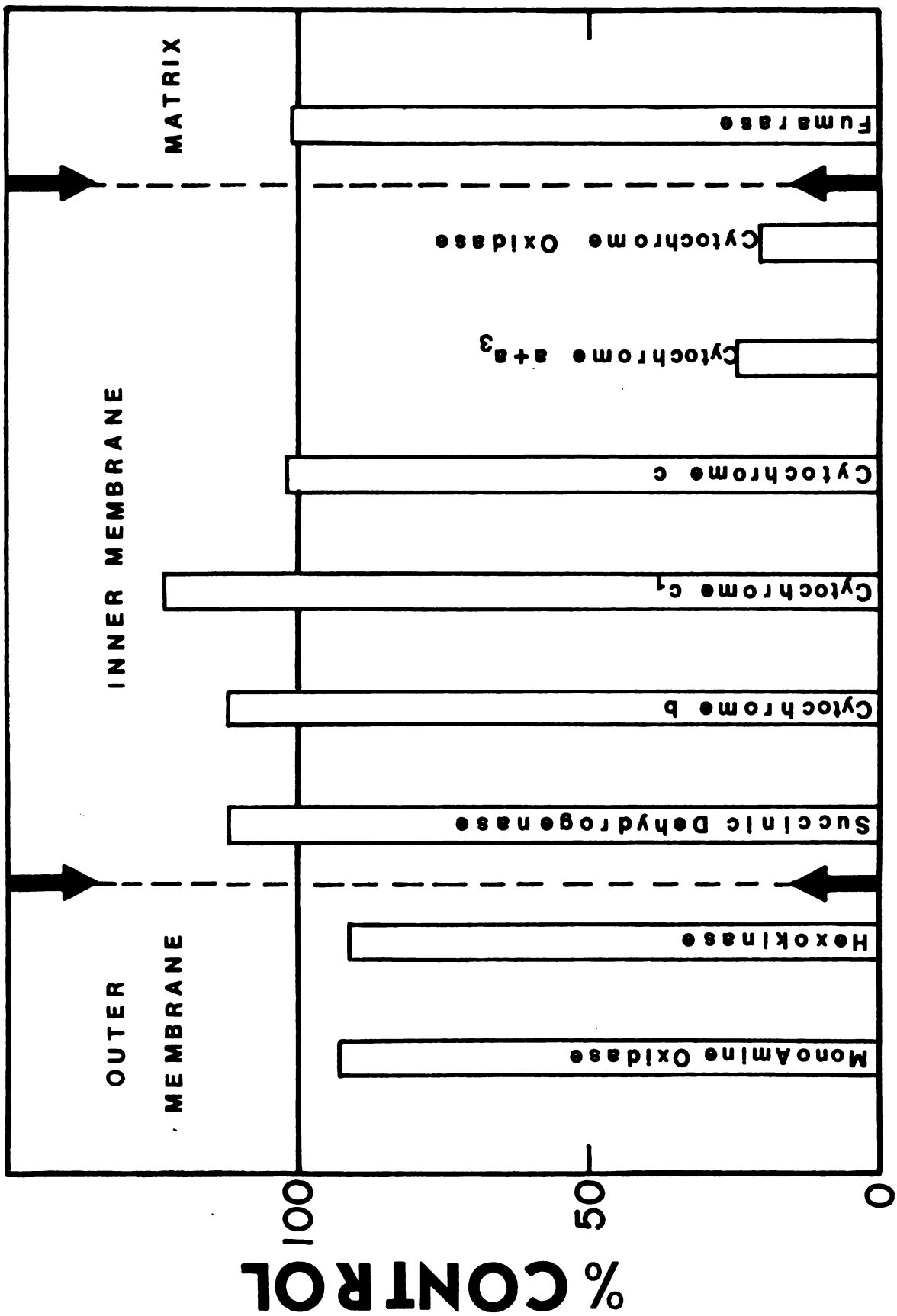

ΔA=0.02
500
550
600
WAVELENGTH (nm)

Fig. 3. Copper deficient rat brain mitochondrial components from 30-day old rats as a per cent of control rat data. Percentage was based on data expressed per mg mitochondrial protein. Locations of the individual components within mitochondria are indicated. Details of the procedures can be found in Methods and individual data can be found in Table III.


% CONTROL
100
50
0
OUTER MEMBRANE
INNER MEMBRANE
MATRIX
MonoAmine Oxidase
Hexokinase
Succinic Dehydrogenase
Cytochrome b
Cytochrome c_1
Cytochrome c
Cytochrome $a+a_3$
Cytochrome Oxidase
Fumarase

DISCUSSION

". . .copper is not a star in the drama of the mind and it cannot provide us with the kind of insight that an Ibsen play provides into human nature. . ." (1).

2',3'-Cyclic Nucleotide 3'-Phosphohydrolase. Although the enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) has been detected in nonmyelin associated membranes its usefulness as a CNS myelin marker should not be disregarded. The residual activities found in other membranes may be of embryonic importance but are of no quantitative significance. When myelin was isolated from whole rat brain by the method of Norton and Poduslo (2) the specific activity (μmoles/min/mg) of CNP increased 7 fold to 27.3. This agrees well with that reported for human white matter-20.3 (3). A composite of the CNP activities of rat cerebella under various treatments is shown in Table 1. All treatments produced significant depressions in body and brain wt, however only copper deficiency reduced CNP concentration in the development models. Di Paolo et al. (4) have recently shown that there was less myelin in the cerebella of newborn copper-deficient rats by histochemical methods. Table 1 also shows that when weanling rats are fed a low copper diet, CNP is not changed although brain copper is reduced by 40% (unpublished data). This is consonant with the suggestion that copper deprivation is most adverse during brain development. When the zinc deficient (5) or hyperphenylalaninemic (6) insults are more severe the CNP content is reduced accordingly. The cerebellar CNP from the hyperthyroid offspring is greater than the saline injected controls ($P<0.05$,

1-tailed t-test) suggesting an acceleration of myelination (7) and another case for the usefulness of CNP. However, none of the experimental treatments produced the profound decreases seen in the myelin mutant mice and, thus, the consequences of this slight reduction in CNP observed in copper deficiency (8) remain open to speculation.

Copper Deficiency. The developing rat brain provides a useful model in which to study the adverse effects of copper deprivation on brain maturation. This lack of tissue copper may be of etiological significance in Menkes' steely-hair disease.

At present, there is a controversy as to whether Menkes' disease is one characterized by hypomyelination (9) or demyelination (10). French et al. (9) found a reduction in long chain unsaturated fatty acids while Lou et al. (10) reported no differences in fatty acid distribution of white matter lipids. The relatively few documented cases of this disorder prevent firm conclusions. It would seem worthwhile to isolate and chemically characterize the myelin because if synthesized incorrectly (dysmyelination) it could lead to demyelination. Another genetic disease, phenylketonuria, is characterized by hypomyelination in the young followed by progressive demyelination (11). However, recent use of the hyperphenylalaninemic rat model found (12) that the myelin had a reduction in long chain fatty acids as well as a decrease in unsaturated fatty acids suggesting dysmyelination. It is not clear whether copper deficiency could also lead to dysmyelination. The mechanism for such a reduction in long chain and unsaturated fatty acids remains an enigma.

Preliminary attempts to correlate the reduction of superoxide

dismutase (8) with an aberrant pattern of lipid peroxidation (i.e. a reduction in unsaturated fatty acids) have failed to provide an answer. Perhaps an anabolic approach based on the altered NAD^+/NADH ratio may provide some insight. However, the failure to demonstrate a difference in energy metabolism in copper-deficient brain weakens this hypothesis.

The most striking difference associated with the five-fold decrease in brain copper content was the large reduction in cytochrome oxidase (8). This decrease could be demonstrated both enzymatically and spectrally. However, this large decrease (80%) was accompanied by only a 30% reduction in the ability of the mitochondria to oxidize substrates (succinate and glutamate). Of several possibilities for this observation, three seem most likely: 1) cytochrome oxidase is not rate limiting; 2) interchain electron transport contributes to the respiratory reaction of copper-deficient mitochondria (13); 3) there may be induction of an additional oxidase similar to that reported for copper-deficient yeast (14).

The reduction in the spectral peak at 605 nm (cytochromes a + a_3) is an unresolved observation. Current evidence would suggest that copper is somehow involved in the synthesis of the protoporphyrin moiety of heme A and/or in its combination with the apo-oxidase. This supposition is based on the fact that the levels of the other cytochromes are normal, thus, suggesting no abnormality in the sequence porphyrin → heme → cytochrome. Also, in yeast, copper deficiency has no effect either qualitatively or quantitatively on synthesis of the protein subunits of cytochrome oxidase (14, 15).

Not all of the copper in brain is firmly attached to functional proteins (16). The loss of copper and the concomittant reduction in metalloenzyme activity varies between different proteins (8). This would suggest that the brain has several copper ligands of varying affinity for Cu. When weanling rats were fed a low copper diet for 6 weeks their plasma copper level fell to 14% of control however their cerebellar cytochrome c oxidase activity remained unchanged (108% of control). In a similar experiment of five weeks, cerebral cortex activity was also unchanged (112% of control) but brain copper concentration had fallen to 61% of control. Again this points out that the developing brain, which has not accumulated its full copper reserves, is more susceptible to metal deprivation be it dietary or genetically produced.

"The outstanding problem in phenylketonuria is no longer the effect of the abnormal metabolism on the brain, but the nature of the alterations induced in the developing brain." (17).

Hyperphenylalanine. The discovery by Johnson and Shah (12) that the myelin isolated from hyperphenylalaninemic rats had altered levels of long chain and unsaturated fatty acids suggests that the membrane may be unstable and could explain why demyelination occurs in older PKU patients. However, a concise metabolic explanation for these facts is not available despite a generous supply of published hypotheses.

One widely accepted concept is that phenylalanine through its

inhibition of brain pyruvate kinase and phenylpyruvic acid by its inhibition of brain hexokinase (18) would decrease brain glycolysis and result in a decrease in energy production. I found that L-phenylalanine did inhibit rat brain pyruvate kinase (19) but 2 mM phenylpyruvate had no effect on either chick or rat brain hexokinase. Although Miller et al. (20) were able to verify the inhibition of pyruvate kinase in an in vivo study in which fast frozen tissue was analyzed, they found no differences in ATP or creatine phosphate. The physiological consequences of the pyruvate kinase inhibition, therefore, remain uncertain.

In addition to hexokinase, phenylpyruvate has been reported to inhibit at least seven other enzymes (21). The lowest K_i reported was for inhibition of fatty acid synthetase (0.25 mM) (21). These authors suggest that this inhibition could explain the defective myelination characteristic of PKU. There are several reasons why conclusions, like the preceding, must be delayed. Phenylpyruvate has never been detected in brain tissue of PKU patients or of experimental animals. In a study of 39 PKU patients (32), plasma phenylpyruvate averaged only 0.044 mM. Brain phenylpyruvate has been postulated to arise via transamination of phenylalanine with 2-oxoglutarate (23), it could then be enzymatically reduced to phenyllactate by lactic dehydrogenase. In a study in which rats were given intraperitoneal injections of phenylalanine the resultant level of brain phenyllactate, the product of phenylpyruvate reduction, was 2.6 μM (24). Using the kinetic constants (K_m and Vmax) reported by Land and Clark (21) for fatty acid synthetase from 14-day-old rat brain and assuming that the

concentration of acetyl-CoA is equivalent to the K_m value, 7.6 μM, enzyme velocity was calculated for various concentrations of the competitive inhibitor, phenylpyruvate. Determination of this velocity for a value of phenylpyruvate equal to 25 μM, ten times the level of phenyllactate found in rat brain (24), results in a 5% reduction from that predicted without inhibitor present.

The molecular mechanism that explains the hypomyelination in PKU is not available. Although the myelin protein 2',3'-cyclic nucleotide 3'-phosphohydrolase was reduced in rats treated with phenylalanine and ρ-chlorophenylalanine, two mitochondrial proteins, cytochrome c oxidase and hexokinase, were not affected (6) (see chapt. II). Recently Nordyke and Roach (25) have found that two other mitochondrial proteins, succinate dehydrogenase and glutamate dehydrogenase, were unaffected by this treatment. They also reported that treatment terminated at 21 days of age followed by 3 weeks of recovery still resulted in lower brain weights of experimental animals (26) in agreement with prior studies (6) (see chapt. II). The developing brain seems to be irreversibly damaged during the hyperphenylalaninemic state in agreement with most reported cases of untreated phenylketonuria.

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TABLE 1

ACTIVITY OF 2',3'-CYLIC AMP 3'-PHOSPHOHYDROLASE IN DEVELOPING RAT CEREBELLA FOLLOWING EXPERIMENTAL TREATMENTS[a]

Treatment	Body Wt. (g)	Brain Wt. (g)	Cerebellar Activity (units/g wet wt.)
Control	63.2± 4.0	1.37±0.05	224±10.5
Pair-Fed	31.4± 1.4*	1.25±0.02*	226± 6.6
Zinc Def.[b]	27.9± 4.6*	1.22±0.06*	214±19.7
Control	62.2± 6.3	1.43±0.10	226±10.1
Copper Def.[c]	36.7±10.4*	1.23±0.04*	172± 5.9*
Saline Inj.	59.5± 4.4	1.45±0.05	246±14.2
L-Phe DL-ρ-Clphe Inj.[d]	33.2± 8.5*	1.18±0.11*	234±15.4
Thyroxine Inj.	47.5± 5.4*	1.23±0.06*	262± 7.1
Control	350 ±35.6	---	402±13.1
Copper Def.[e]	323 ± 7.5	---	389± 7.3

[a]Values are means ± SD for four animals age 21 days unless noted. Means were tested by Student's t-test (treatments were different from controls * $P<0.01$). Enzyme activity was determined on thawed 10% (w/v) homogenates after freezing overnight as described in APPENDIX.

[b]Data is taken from (5) in which lactational zinc deficiency was carried out and offspring from ad libitum fed (control) and pair-fed dams were compared with zinc deficient offspring.

[c]Data is taken from (8) in which gestational-lactational copper deficiency was induced and the offspring compared.

[d]Saline and L-phe DL-ρ-Clphe injected offspring data is taken from (6). Concurrently additional pups were given 4 subcutaneous injections of 10 μg of thyroxine in saline on days 3, 6, 9 and 12 of age (7); saline injected rats served as controls.

[e]Data is from 9 week old male rats who had consumed a copper deficient diet (0.3 ppm) for 6 weeks.

APPENDIX

ANALYTICAL BIOCHEMISTRY 56, 275–282 (1973)

Improved Rapidity and Precision in the Determination of Brain 2′,3′-Cyclic Nucleotide 3′-Phosphohydrolase[1]

A rapid and precise method for the determination of brain 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) activity has been developed. Total brain homogenates were treated with deoxycholate, and CNP activity was measured as inorganic phosphate (phosphomolybdic acid, 410 nm) released from the product, 2′-AMP, by alkaline phosphatase. Measurements were carried out under optimal conditions of temperature (30°C) and pH (6.2) using the whole brain of the rat, chicken, and quaking mouse. The entire assay was applicable to multiple samples and could be completed in less than 1 hr.

Since Drummond *et al.* (1) reported the presence of a diesterase which hydrolyzes 2′,3′-cyclic nucleotides in the central nervous system (CNS), many reports have appeared further characterizing this activity. The proposition that 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) (EC 3.1.4.1) is associated with the myelin sheath of the CNS was made by Kurihara and Tsukada (2) in 1967. Recent suggestions (3) and evidence (4, 5) that CNP may also be found in the oligiodendroglial cell plasma membrane strengthen the hypothesis for location of CNP in CNS myelin.

The activity of CNP has been used to follow CNS myelination in the developing chick brain and spinal cord (6), as well as in the newborn rat brain (7). Because of its association with myelin, CNP has been used as a marker during isolation of CNS myelin or to estimate myelin contamination of other subcellular fractions (8–11). Another application of CNP levels has been the studies involving the neurological mutant mice, jimpy (jp/y) and quaking (qk/qk), both of which have subnormal myelin levels (12). A depression in CNP levels in the brains of jimpy mutants corresponding to reduced myelin levels was shown by several researchers (3, 13–15). Analogous studies on the quaking mutant have also been reported (7, 14). Recent uses of CNP measurement include a study of bovine peripheral nerve myelin (16), drug induced synthesis of CNP in the sciatic nerve of the chick embryo (17), spinal cord CNP in hypothyroid rats (18), and CNP in the chick optic tectum following deafferentation (19).

[1] This work was supported by Grant AM 10209, U. S. Public Health Service. Michigan Agricultural Experiment Station Journal Article No. 6260.

This diversity of application led to a variety of analytical techniques which either lack sensitivity, are time-consuming, or both. Because of these technical difficulties, results are often conflicting or noncomparable. The optimization of CNP detection, both for precision and speed of analysis, is the subject of this report.

MATERIALS

Female quaking mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and albino rats from Holtzman Co., Madison, Wisconsin. Day-old male Leghorn chicks were the generous gift of the McPherson Hatchery, Ionia, Michigan.

2′,3′-cAMP, 2′-AMP, and *E. coli* alkaline phosphatase type III-S were purchased from Sigma Chemical Co., St. Louis, Mo. Triton X-100 was the product of Rohm-Haas, Inc., Philadelphia, Pa. Other chemicals were analytical reagent grade and were used without further purification. Entire reactions were carried out in nitric acid-washed, 10 mm × 75 mm Pyrex test tubes.

METHODS

Preparation of tissue homogenates. Whole brains, including medulla oblongata to the level of the posterior cerebellum, were rapidly removed, weighed, and homogenized (20 full strokes) in 9 vol of 0.32 M sucrose in a Potter–Elvehjem homogenizer with Teflon pestle. The solubilization procedure was that of Kurihara *et al.* (13) and consisted of adding 0.1 ml of 0.2 M Tris–HCl, pH 7.5, to 0.2 ml of the 10% (w/v) homogenate, then adding 0.2 ml of a 1% (w/v) sodium deoxycholate solution. After 10 min at 0–4°C, thrice distilled water was added and homogenization was repeated, five strokes, so that protein was of an appropriate dilution (*ca.*, 0.1 mg/ml).

Final CNP assay conditions. Velocities were measured which were both linear with respect to time and protein concentration. In 0.2 ml final volume, 5–25 μg of protein were added to 7.5 mM 2′,3′-cAMP, 50 mM Tris–maleate buffer, pH 6.2. The substrate initiated reaction was carried out at 30°C for 10 min. A sample containing substrate and buffer without protein served as a reagent blank. The substrate level which was selected, 7.5 mM, was based on reported procedures (1, 2) and the high cost of 2′,3′-cAMP. The reproducibility of 2′,3′-cAMP concentration was verified spectrophotometrically by the content of adenosine, pH 2.0, and 257 nm, and by estimation of total cyclic phosphate. The reaction was terminated by placing the 10 mm × 75 mm Pyrex tubes in a boiling water bath for 30 sec. The mixture was returned to the 30°C water bath where 0.1 ml of 0.3 M Tris–HCl containing 21 mM $MgCl_2$, pH 9.0, was added along with 60 μg (0.72 units) of *E. coli* alkaline phosphatase (EC 3.1.3.1), re-

sulting in a final pH of 8.5. It was determined that after boiling and adjusting the pH, no detectable CNP activity remained. The alkaline phosphatase reaction was capable of completely hydrolyzing 1.5 μmoles of 2′-AMP in 20 min at 30°C.

Chemical analyses. The inorganic phosphate which was liberated was conveniently determined in the same test tube directly as the phosphomolybdic acid complex (20) and the absorbance measured at 410 nm in a Gilford Model 2000 spectrophotometer. The micromethod of Berenblum and Chain (21) was employed as modified by Martin and Doty (22) using isobutanol:benzene to extract the yellow chromophore. To the 0.3 ml reaction mixture, 1.2 ml of isobutanol:benzene (1:1, v/v) and 1.2 ml of 1.5% (w/v) $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ in 0.5 N H_2SO_4 were added and the solution vigorously shaken (Vortex) for 20 sec before centrifugation (Sorvall GLC-1) at 2200 rpm for 5 min. The yellow upper layer was read against isobutanol:benzene. Standard phosphate carried through this procedure generates a linear relation, 0.745 μmoles/absorbance unit. Protein was measured by the method of Lowry *et al.* (23) using bovine serum albumin as a standard.

One unit of enzyme activity is that amount which produces 1 μmole of 2′-AMP from 2′,3′-cAMP/min under the conditions described. Specific activity is expressed as units/mg protein.

RESULTS

Adult rat brain of either sex was used as the source of enzyme in studies attempting to optimize reaction conditions. Pretreatment of the homoge-

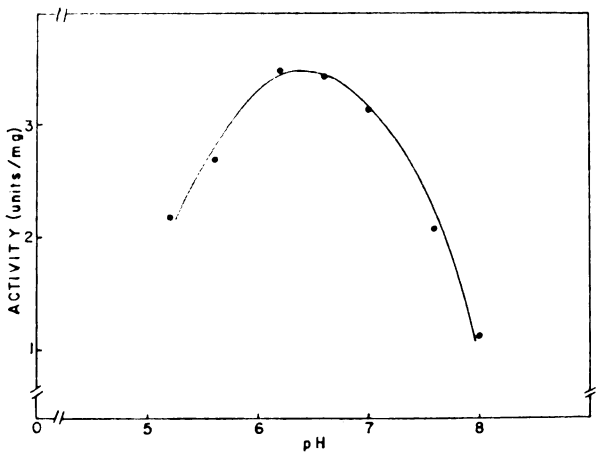

FIG. 1. Effect of pH on adult rat brain CNP activity. Assay conditions were as described under Methods. The buffer used was 50 mM Tris–maleate.

nate was the first point of study, and although absolute values varied because of animal age, tissue storage, and degree of sonication, the highest and most consistent values were always achieved when using the 1% deoxycholate treatment (13) followed in order of effectiveness by Triton X-100, sonication, and no pretreatment.

In order to eliminate buffer effects (1) and to determine an accurate pH profile, one buffer, Tris–maleate, was employed over the entire pH range (Fig. 1). An optimum can be seen for the range 6.1–6.7. CNP displayed a temperature optimum over the range 25–32°C (Fig. 2). Specific activity of CNP at 37°C was 90% of that at 30°C. The K_m of 2′,3′-cAMP for crude brain homogenate protein was determined to be 6.0 mM from the slope of the Hofstee plot (24) (Fig. 3).

Since the final form of the present CNP assay was related to the method of Kurihara *et al.* (2, 13), a direct comparison was made by us using both techniques on three adult rat brains. The mean specific activity and standard deviation obtained by our method was 3.52 ± 0.193, while the paper chromatographic technique (2, 13) gave 3.29 ± 0.126.

In order to explore the usefulness of our method to that of published procedures, CNP was determined on the whole brains of adult rats (2 mo of age or older), 14-day old chicks maintained on a commercial mash diet for 2 wk, and 2-mo old female quaking mice with their littermate controls. Table 1 shows the results of our determinations along with reported or calculated data from other authors.

Since the entire reaction sequence can be performed in the same test

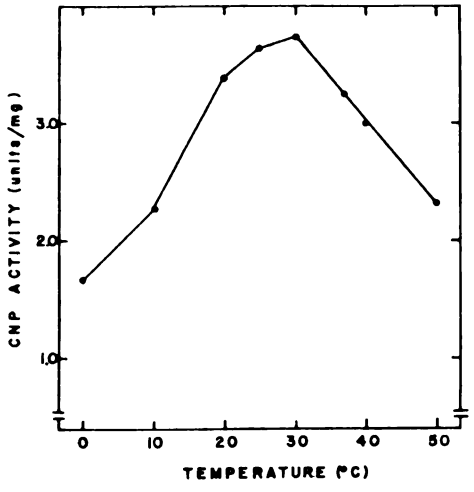

FIG. 2. Effect of temperature on adult rat brain CNP activity. Assay conditions were as described under Methods.

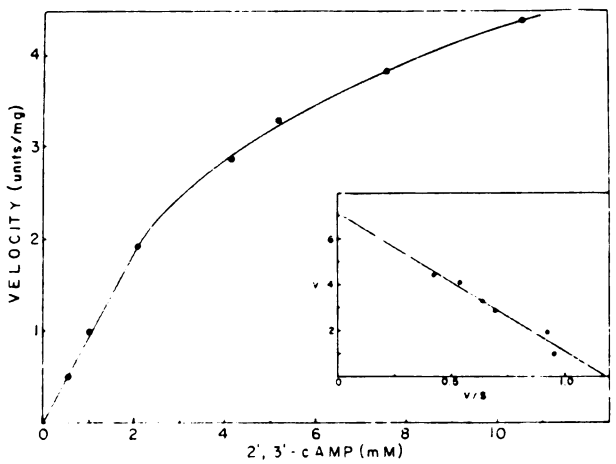

FIG. 3. Effect of 2',3'-cAMP concentration on the activity of adult rat brain CNP. The inset shows the results plotted by the Hofstee (24) method. Assay conditions were as described under Methods.

tube, our method provides a precise estimate of CNP activity—a coefficient of variation ($s/\bar{x} \cdot 100$) of 1.7% for six trials.

DISCUSSION

Table 2 is a composite of current techniques used for CNP measurement. The majority of methods separate 2',3'-cAMP from 2'-AMP chromatographically (2, 4, 5). This necessitates an increase in sampling error due to numerous transfers and elution; in addition, hours are required for the development of the chromatograms. Since total reaction time can be as low as 30 min (10 for the reaction and 20 for phosphate cleavage) and no transfer of reaction materials need take place, the speed of the present method can be appreciated when compared to the ascending paper chromatographic techniques more commonly used.

Olafson *et al.* (7) have developed an assay in which the use of alkaline phosphatase to liberate inorganic phosphate increases the speed of analysis. However, a transfer step after precipitation with TCA introduces sampling error. In addition, this method operates at pH 7.5, beyond the optimal range (see Fig. 1). No detergent pretreatment of homogenates and a substrate concentration of 4 mM further reduce the sensitivity of CNP measurement. A comparison of quaking mice values (% control, Table 1) shows a lack of agreement with our method (53.8% vs 26.5%). Absolute values deviate even further.

All current methods for measuring CNP operate at fixed substrate,

TABLE 1
Comparison of Whole Brain 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Activities

Species	Age	Pretreatment	pH	Activity[a] (units/mg protein)	% Control	Reference
Quaking mouse	60–75 days	Deoxycholate	6.2	0.61 ± 0.049 (3)	26.5	Present method
Control littermate	60–75 days	Deoxycholate	6.2	2.30 ± 0.147 (4)		
Quaking mouse	60 days	Deoxycholate	6.2	0.9	23.1	14[b]
Control littermate	60 days	Deoxycholate	6.2	3.9		
Quaking mouse	Adult	None	7.5	0.14	53.8	7
Control littermate	Adult	None	7.5	0.26		
Chicken	14 days	Deoxycholate	6.2	1.68 ± 0.082 (4)	—	Present method
Chicken	14 days	Sonication	6.2	1.6	—	6
Rat	Beyond 60 days	Deoxycholate	6.2	3.5 ± 0.08 (4)	—	Present method
Rat	22 days	Sonication	6.2	3.1 ± 0.11 (6)	—	10
Rat	Adult	Deoxycholate	6.2	3.3	—	5
Rat	Adult	Deoxycholate	6.2	4.1	—	11

[a] Values are specific activities ± standard error of the mean for the indicated number of replicates.
[b] Tissue homogenates used after being frozen at −15°C (see text).

TABLE 2
Comparison of Current Procedures for Measuring 2′,3′-Cyclic Nucleotide 3′-Phosphohydrolase

Tissue pretreatment[a]	2′,3′-cAMP	pH	T	2′-AMP quantification	Reference
	(mM)		(°C)		
None	7.5	7.5	30	Paper electrophoresis, A_{260}	1
None	4.0	7.5	30	Alkaline phosphatase, P_i[e]	7, 9, 15
Sonication	7.5	6.2	37	Paper chromatography, A_{260}	2, 6, 10, 18
Sonication[b]	7.5	6.2	37	Paper chromatography, A_{260}	17
Sonication[c]	7.5	6.2	37	Paper chromatography, A_{260}	19
Triton X-100	7.5	6.2	37	Paper chromatography, A_{260}	4, 16
Deoxycholate	7.5	6.2	37	Paper chromatography, A_{260}	11, 13, 14
Deoxycholate[d]	7.5	6.2	37	Paper chromatography, A_{260}	3
Deoxycholate	7.5	6.2	37	Thin-layer chromatography, A_{254}	5, 11
Deoxycholate	4.0	7.5	30	Alkaline phosphatase, P_i[e]	15

[a] Unless otherwise stated, the method of sonication was 150 W for 10 min; deoxycholate or Triton X-100 pretreatment was 0.4% detergent, 40 mM Tris-HCl, 0°C for 10 min.

[b] No power value listed, 10 min.

[c] 70 W for 1 min.

[d] 20–25°C for 5 min.

[e] Inorganic phosphate measured as reduced phosphomolybdic acid, A_{720}.

rather than saturating substrate. A K_m of 6.0 mM agrees well with that of Zanetta *et al.* who reported a K_m of 5 mM for various subcellular fractions of rat brain (5). Olafson *et al.* (7) have reported a value of 2 mM for bovine brain. As other workers have noted (1, 7), the high K_m of CNP for 2′,3′-cAMP suggests that its activity toward this substrate may not be significant *in vivo*, and its physiological role remains unknown.

From Table 1, it can be seen that our method agrees well with those which pretreat with deoxycholate, operate at pH 6.2, and use 7.5 mM 2′,3′-cAMP. The CNP values of quaking mutant (% control) in our method and that of Kurihara *et al.* (14) agree well with that reported by Druse and Hogan (25) for the isolated myelin of the quaking mutant (20–26% control). The difference in specific activities may be due to an activation upon freezing and thawing the 10% homogenates.[2] We have found that 30°C gave higher values than 37°C, but temperature was not as critical a parameter as pH (Figs. 1, 2). The precision gained by carrying out the reaction in a single vessel and the rapid handling of multiple samples makes this method particularly desirable for measuring CNP

[2] Unpublished observation.

as a myelin marker and for investigations of myelination under normal and pathological situations.

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Received February 23, 1973; accepted July 3, 1973