



MAJOR-SPECIFIC STUDY & INTERN ABROAD PROGRAMS

63 Sickletown Road • West Nyack • New York • 10994 • United States of America
Ph. 1.212.931.9953 • Fx. 1.212.528.2095 • info@KEIabroad.org • www.KEIabroad.org

**Spectrophotometric Analysis of the Enzymatic Activities of Glutathione Peroxidase
and Glutathione Reductase in Ammonia Toxicity**

Summary

There is a relationship between the impairment of energy metabolism with lipid peroxidation and free radical reactivity, in liver damage. This study demonstrated that antioxidative enzymes protecting the cell and mitochondria were being compromised in ammonia toxicity related liver diseases. After an ammonium acetate injection, glutathione peroxidase and glutathione reductase's roles were observed to evaluate ammonia toxicity. Results concluded that glutathione reductase and glutathione peroxidase are involved in the prevention of ammonia toxicity.

Acknowledgment

I would like to acknowledge the help of Dr. Elena Kosenko, my professor, and Yuri Kaminsky, assistant professor, and Sacha Kaminsky, graduate student. I would like to thank the Laboratory of Regulation of Energy Supply for Physiological Functions of the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences. I would like to recognize the director of the institute, Professor Valentin Kefeli. I also would like to express my gratitude to Mr. Raymond McGraime for his guidance and support.

Introduction

Ammonia plays an important role in nitrogen circulation. It is a common natural gas, present in intracellular and extracellular fluids of all biological tissues and fluids. It is an integral part of cells, organs, and organisms. Being a natural metabolite, present as NH_3 or in the form of an ammonium ion, NH_4 , it is involved in numerous biochemical reactions dealing with intermediary metabolism.

Normal concentrations of ammonia (denoted as the sum of NH_3 and NH_4) in animal or human plasma is in the range of 20 to 50 nanomoles and 600 to 900 nanomoles in rat liver. (Kosenko et al. 1991) However, when ruminants eat excess clovers rich in protein, or humans develops liver diseases such as cirrhosis or acute liver intoxication, hyperammonemia arises and is often equivalent to liver failure. Although blood ammonia levels can reach 1-10 millimoles per liter under various pathological conditions; elevated ammonia concentrations are neurotoxic and produce convulsions, coma, and finally rapid death.

The mechanisms by which liver failure, as well as hyperammonemia, lead to a disturbance in brain functions remain obscure. Several hypotheses have been proposed to explain the mechanisms of ammonia toxicity and the role ammonia plays in pathogenesis of hepatic encephalopathy. (Bessman and Bessman, 1995) However, none of these hypotheses have been completely supported by experimental observations.

Some support has been demonstrated for the metabolic receptor mediated hypothesis. (Kosenko et al. 1991, 1993, 1994b, 1995) On the basis of the findings that were obtained on hyperammonemia rat liver and brain, it

was concluded that changes in energy metabolism were involved in the origin of ammonia-induced coma and death. (Kosenko et al. 1991, 1993) Acute intoxication of normal animals with large doses of ammonia with marked alterations in liver and brain energy metabolite. These changes specifically include, decrease adenyli-triphosphate concentrations in the liver and brain tissues. (Kosenko et al. 1991, 1993). Adenyli-triphosphate is well known to be the universal and major source of energy for biochemical reactions, muscular contractions, neurotransmission and other cellular reactions. More than ninety- percent of adenyli-triphosphate is produced by mitochondrial phosphorylation.

Studies of the oxygen uptake of the brain in hepatic coma revealed a marked diminution of oxygen utilization simultaneous with the uptake of ammonia. In hepatic coma, the oxygen utilization by the brain was about half of the control. (Bessman, 1961) The phosphorylation potential in rat brain, a characteristic of the energy status of the mitochondria under oxidative phosphorylation condition, a decrease four-fold fifteen minutes after an ammonium acetate injection in animals. Results such as this suggest that mitochondrial energy metabolism is involved in ammonia toxicity.

It is understood that ammonia influences the energy metabolism of the liver and brain more than any other geographic region. Nevertheless these studies were performed at the mitochondrial level. (Katunum and Okad, 1963; McKhann and Tower, Kosenko et al., 1996) Specifically, the relation of impairment of mitochondrial function with lipid peroxidation and free radical reactivity were examined in these studies. Preliminary findings by Dr. Kosenko suggested that the enzymes involved in protecting the mitochondrial and plasma membrane from oxidative injury are degraded under ammonia loading. It was proposed that elevated ammonia levels cause oxidative stress and may play a part in early phases of ammonia-induced lipid peroxidation. More specifically, a decrease in glutathione peroxidase and glutathione reductase activities may be the reason for the oxidative damage done to the inner mitochondrial membrane, as well as to the liver, and neuronal plasma membrane, and to the erythrocyte membrane.

Glutathione peroxidase is known to play a strategic role in protection of erythrocytes from oxidative destruction and lipid peroxides in water in the presence of reduced glutathione. (GSH)



Glutathione reductase is required to reduce free glutathione disulfide (G-SS-G) to GSH, in order to maintain the intracellular GSH level and the GSH/ G_SS_G ratio, protecting SH groups of the intracellular proteins and preventing irreversible changes in the structure of the latter. This enzyme catalyzes a reduction of G-SS-G by NADPH:



The enzymes of glutathione metabolism are situated in the cytoplasm of erythrocytes, liver and brain cells, but Neubert et al. (1962) found that rat liver mitochondria showed activity in these enzymes as well.

Since no studies have been published on the effects of hyperammonemia on glutathione peroxidase and glutathione reductase in erythrocytes and blood plasma, liver and brain cytosolic fractions. Also examined were isolated liver mitochondria from rats made hyperammonemic with an acute injection of a lethal dose of ammonium acetate. Comparison of these results with those from control animals was made. Methods of isolation of red blood cells, liver and brain cytosolic and mitochondrial fractions, methods for determination of protein concentration, and enzymatic activities of animal preparations were utilized.

Materials and Methods

Animal and Experimental Conditions:

Male adult Wistar rats weighing two hundred twenty to two hundred fifty grams were used in the experiments. Since the effect of ammonia in in-vivo was being studied, rats were injected intraperitoneally with twelve millimoles per kilogram of ammonium acetate, while controls received an injection of saline solution. After fifteen minutes the subjects were killed by cervical dislocation.

The liver was then rapidly removed and perfused with ice cold isolation medium for removing blood, and homogenized in nine volumes of isolation medium using a glass dounce homogenizer with fluoroplast pestle. The isolation medium consisted of 210 mM solution of mannitol with 70 mM of sucrose, buffered with 5 mM of 4-(2-hydroxyethyl)-1-pipazinesulphonic acid (HEPES) with a pH level of 7.4, and with the addition of 1mM ethylenediaminetetraacetate (EDTA) and .5 mg of ovine serum albumin per one milliliter.

The skull was rapidly opened, and forebrain removed and the tissue was put in ice-cold isolation medium consisting of .25 molar sucrose, .5mM K-EDTA, and 10 mM Tris-HCl (pH 7.4). The brain was homogenized like the liver tissue with the only difference being the isolation medium.

Isolation of liver Mitochondria and Cytosol

Liver homogenate was centrifuged for 10 min at 800g in a refrigerator centrifuge to remove cells and nuclei: the first supernatant was centrifuged for 10 minutes at 3,300g to obtain a crude mitochondrial pellet and second supernatant. The pellet was washed twice by centrifuging at 3300g for 15 min in 5 ml of isolation medium. The sediment was suspended in .1 ml of the same medium per 1 ml of the mitochondrial pellet, and this suspension was then used as purified liver mitochondria. Mitochondrial concentration was about 20 mg of

protein per 1 ml of the suspension. The second supernatant was centrifuged at 140,000g, the pellet was decanted, and the third supernatant was used as the liver cytosolic fraction.

Isolation of Brain Mitochondria and Cytosol

The procedure for isolation of brain mitochondria was the Lai and Clark's method as described in Kosenko et al. (1996). Briefly, the chopped hemispheres were put in 2.5 ml per gram of the isolation medium and were homogenized in a Dounce homogenizer with fluoroplast pestle, a total clearance of .1 mm, for one minute. The homogenate was then diluted with the isolation medium to a final volume of 5 ml per gram of tissue and centrifuged twice at 2,000g for 3 minutes and centrifuged again 12,000g for 8 minutes to obtain the crude mitochondrial pellet. The resulting supernatant was taken to be the second supernatant. The mitochondrial pellet was suspended in 1 ml per gram of tissue of the 3% Ficoll medium, and 3 ml of this suspension was layered on to 12.5 ml of the 6% Ficoll medium and centrifuged at 12,000g for 30 minutes. The loose fluffy white top layer of the pellet was removed. The resulting brown pellet was resuspended with .5 ml per gram of the isolation medium and centrifuged at 12,000g for 10 minutes. The sediment was resuspended to make the mitochondrial concentration, which contained about 20 mg of protein per milliliter.

The 6% Ficoll medium contained 6% (weight/weight) Ficoll, .24 M mannitol, .06 M sucrose, .05 mM K-EDTA, and 10 mM Tris-HCl, with a pH of 7.4

Rat brain cytosolic fraction was prepared essentially as that of rat liver except that third supernatant was obtained by centrifuging the second supernatant at 14,000g. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Preparation of Erythrocytes

To obtain erythrocytes, rat blood was taken by a heparinized pipette into equal volumes of chopped saline solution buffered with 10 mM Tris-HCl (pH 7.4) and immediately centrifuged at 1,000g for 15 minutes. Red sediment was washed twice under the same conditions and stored in the buffered saline on ice.

Determination of Enzyme Activities

To measure the enzymatic activities, mitochondria were disrupted by osmotic shock in 10 mM phosphate buffer (pH 7.4, for 10 min at 313 °K), frozen and thawed three times. Then the suspension of disrupted mitochondria was centrifuged for 20 min at 140,000g and the supernatant was used as a source of the enzymes. Erythrocytes were lysed by incubating at 313°K in 12 mM triethanolamine buffer (pH 7.5) with .02% saponin. Fifteen minutes later the mixture was centrifuged at 300 G for 15 min. and the insoluble

material was discarded. Enzymatic activity of glutathione reductase was measured by following the decrease in absorbance due to the oxidation of NADPH by oxidized glutathione. (Goldberg and Spooner, 1984)

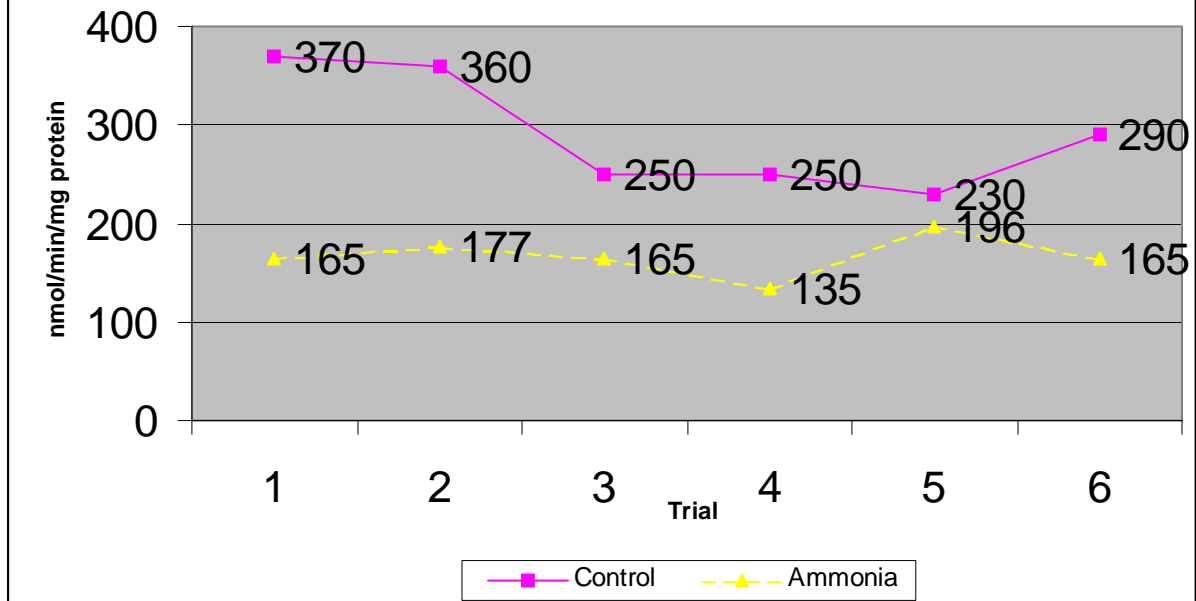
Absorption was measured by continuous monitoring by spectrophotometric analysis using the Specord UV spectrophotometer. The reaction solution consisted of .1 M phosphate buffer (pH 7.2), .5 mM EDTA, 2.2 mM G-SS-H and a variable amount of the enzymatic preparation (within 20-100 μ l) with a total volume of 2.5 ml. The reaction was initiated with an addition of .2 mM NADPH and registered by recording at 310°K and 300 nm. Five nanomolar FAD was added in the solution to determine the glutathione reductase activity in lysed erythrocytes.

Glutathione peroxidase activity was measured similar in coupled reaction with the added commercial glutathione reductase (Boehring Mannheim GmbH) (Lawrence and Burk, 1976). The reaction mixture consisted of 50 mM Potassium buffer (pH 7.0), 1mM EDTA, 1 mM NaN₃, 5 mM GSG, 0.2 mM NADPH, 1 μ l of glutathione reductase, and a 5 to 100 μ l of enzyme preparation with a total volume of 2.5 ml. The reaction was initiated with the addition of 15-mM H₂O₂.

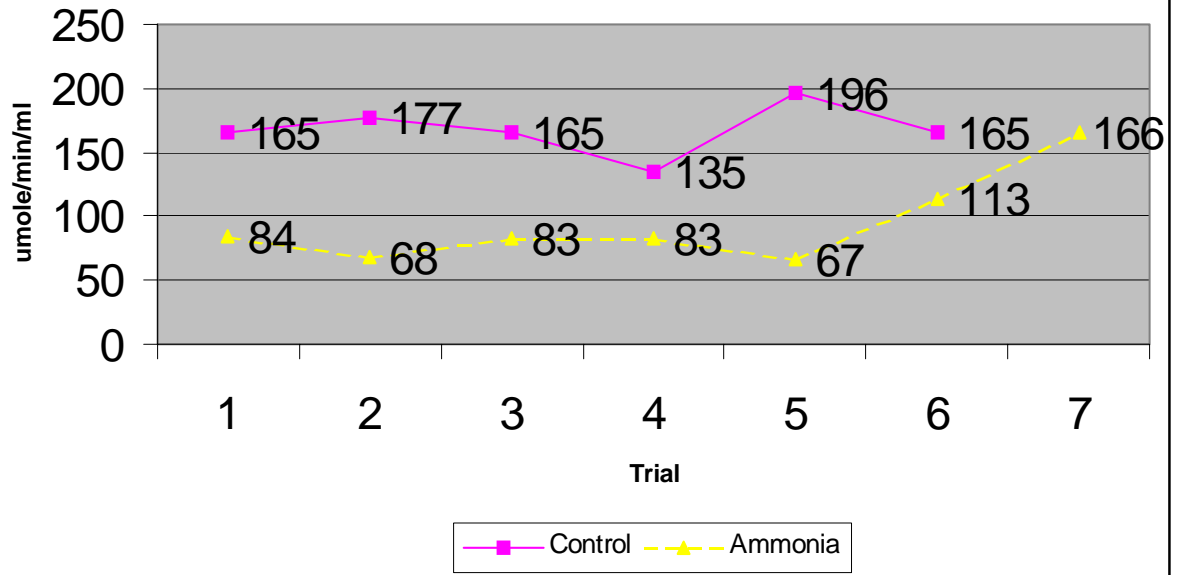
Results

The data of Table 1 shows that ammonia intoxication results in the decrease in the specific glutathione peroxidase activity of the liver and brain cytosol by 24% and 38% respectively. (Graph 1) The decrease in liver and brain mitochondria and erythrocytes 43%, 47%, and 33% respectively (P<0.05). (Graph2) Glutathione peroxidase in plasma however was not affected by ammonia injected. The glutathione reductase activity only increase in erythrocytes (by 54%) (Graph 3), but did not change in all of the other assays from the ammonia injected animals. (Graph 4). The ammonia induced decrease in glutathione peroxidase activity of the liver and brain cytosol (Table 1), correlated with the ammonia content in liver and brain tissues. (Kosenko et al., 1991,1993)

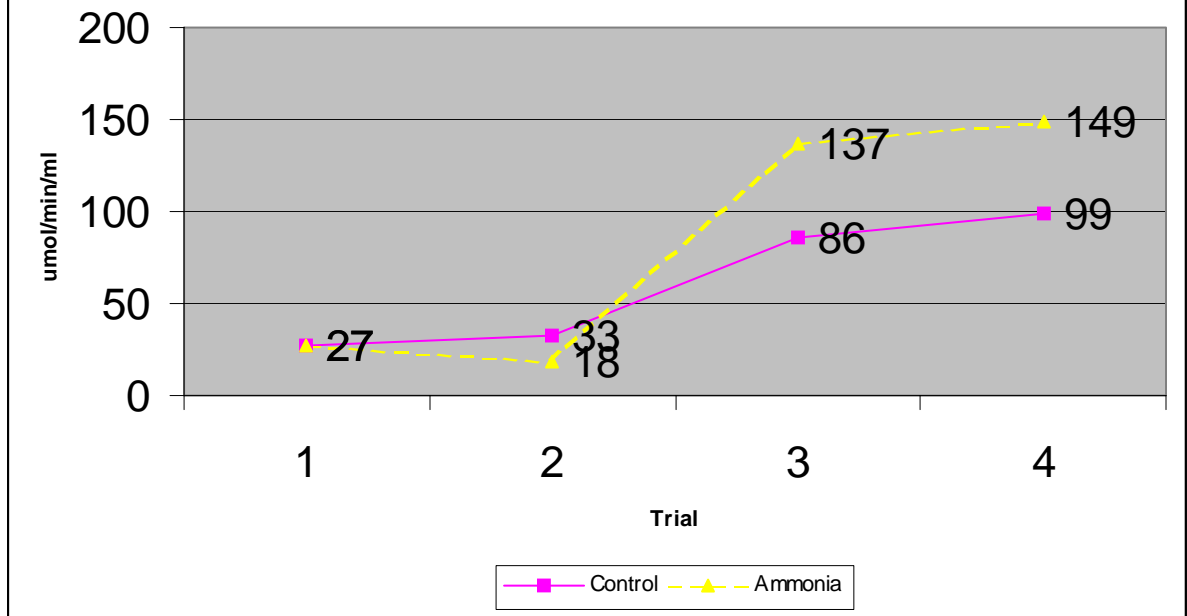
Graph 1. Spectrophotometric Analysis of Glutathione Peroxidase Activity in nmol/min/mg of Protein of Control Mitochondria and Mitochondria Ammonia at a Wavelength of 340 nm at Room Temperature.



Graph 2. Spectrophotometric Analysis of Glutathione Peroxidase Activity in umoles/min/ml of Control Erythrocytes and Erythrocytes Ammonia at a Wavelength of 340 nm at 25 C



Graph 3. Spectrophotometric Analysis of Glutathione Reductase Activity in $\mu\text{mole}/\text{min}/\text{ml}$ of Control Erythrocytes and Erythrocytes Ammonia at a Wavelength of 340 nm at 37C



Graph 4. Spectrophotometric Analysis of Glutathione Reductase Activity in nmol/min/mg protein of control Mitochondria and Mitochondria Ammonia at a Wavelength of 340 nm at 37C

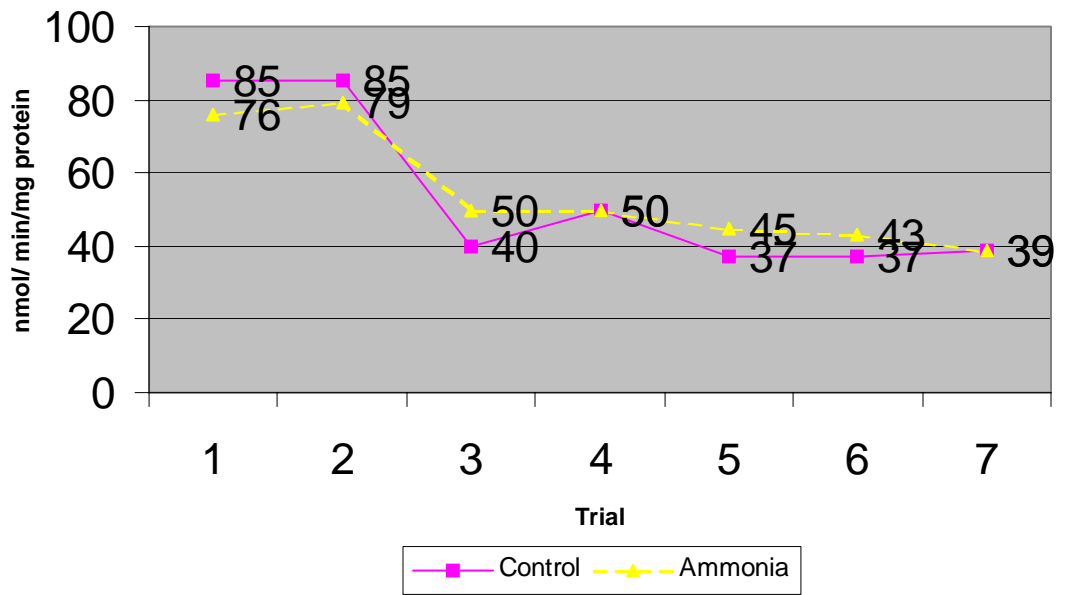


Table 1. Mean Value Activities of Glutathione Peroxidase and Glutathione Reductase in cytsolic fractions of the liver and brain, liver and brain mitochondria, and erythrocytes and plasma from rats made hyperammonemic with an injection of ammonium acetate.

Preparation	Control	Ammonium Acetate
<i>Glutathione Peroxidase</i>		
Liver cytosol(nmol/min/mg protein)	1370 ± 160	1040 ± 100 *
Brain cytosol(nmol/min/mg protein)	130 ± 30	82 ± 9 *
Liver mitochondria (nmol/min/mg protein)	292 ± 22	167 ± 9 *
Brain mitochondria(nmol/min/mg protein)	30 ± 3.5	16 ± 0.5 *
Erythrocytes (umol/min/ml of cells)	142 ± 24	95 ± 14 *
Plasma (nmol/min/ml)	5330 ± 227	5690 ± 630
<i>Glutathione Reductase</i>		
Liver cytosol(nmol/min/mg protein)	109 ± 10	103 ± 6
Brain cytosol(nmol/min/mg protein)	45 ± 7	41 ± 5
Liver mitochondria (nmol/min/mg protein)	53 ± 8	55 ± 7
Brain mitochondria(nmol/min/mg protein)	19 ± 2	14 ± 2
Erythrocytes (umol/min/ml of cells)	93 ± 3	143 ± 6 *
Plasma (nmol/min/ml)	38 ± 5	38 ± 4
Results are a mean and SE for seven to sixteen measurements on prepartes from two rats.		
* P<0,05 as compared to control		

Discussion

The decrease in glutathione peroxidase activity appeared to be a systematic effect of ammonia intoxication, observed in the liver cytosol and mitochondria, brain cytosol and mitochondria, and in the erythrocytes. The absence of such an effect in the plasma can be explained by the relative weak effect of the ammonia in vivo on the cellular and mitochondrial membranes, resulting in little or no leakage of the enzyme from cells into plasma.

Simulations of peroxidation of unsaturated fatty acids of mitochondrial and erythrocyte membranes, and an accompanied increase in non-specific membrane permeability, decrease in membrane enzyme activities and the leakage of matrix or cytosolic components. Products of lipid peroxidation are known to inactivate glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, cytochrome oxidase and other enzymes of the mitochondria citric acid cycle and respiratory chain and of the cytoplasmic glycolysis. (Vladimirov and Archakov, 1972) These effects were observed in hyperammonemic conditions (Kosenko et al., 1996; Ratnakumari and Murthy, 1986)

Glutathione peroxidase, as well as catalase and superoxide dismutase, proved to be potent inhibitors of lipid peroxidation in the inner mitochondrial membrane, and most probably the cellular membrane. The decrease in enzymatic activity is evidence of the weakening of the inhibition of lipid peroxidation, and for the deterioration of membrane stability in mitochondria and red blood cells. The lowering of glutathione peroxidase activity allows oxidizing agents such as hydrogen peroxide and ammonia to be more effective in their nature.

Conclusion

It can be concluded that glutathione peroxidase and glutathione reductase are important components of the molecular mechanisms of ammonia toxicity. Experimentation concluded that the enzymatic activity of glutathione peroxidase decreases in all samples from the hyperammonemic rat as compared to the control. This is evidence that the membranes are being subjected to the effects of oxidizing agents. The enzymes are not completing their function in the presence of ammonia. Ammonia is inhibiting the activity of the antioxidative enzyme to protect the mitochondrial or cellular membranes from lipid peroxidases and free radicals.

Glutathione peroxidase catalyses the oxidation of GSH(reduced) to GSSG (oxidized) at the expense of hydrogen peroxide. The reaction common in the liver, heart, lung, and brain is not specific to GSH as a hydrogen donor, but to any peroxide even hydrogen peroxide. Ammonia is obstructing the enzyme's active site, preventing it from reacting with peroxides. As the amount of peroxides increases, it exceeds the capacity of the enzyme to generate NADPH, which in turn causes the GSH/GSSG ratio to fall and glutathione peroxidase activity to stop working. Once glutathione peroxidase activity stops the hemolysis (destruction of red blood cells), anemia and jaundice occur. The metabolism of peroxides, especially hydroperoxides, is

dependent on GSH. Thus glutathione peroxidase activity plays an important role in protecting mitochondrial and cellular membranes from oxidative damage.

References Cited

1. Bessman, S.P. Ammonia and coma. In: Jordi Folch-Pi, Clinical Pathology of the
2. Nervous System. Oxford, Pergamon, 1961, 70-376.
3. Bessman, S.P., Bessman, A. N. The cerebral uptake and peripheral uptake of
4. ammonia in liver disease with a hypothesis for the mechanism of hepatic coma.
5. Journal of Clinical Investigations. 1995, 34, 622-628.
6. Flohe, L. and R. Zimmerman. GSH-induced high amplitude swelling of
7. mitochondria. In: Flohe, L., Benohr H. Ch., Sies H. Waller H.D., Wendel A., eds. Glutathion. Stuttgart, Georg Tieme Publications, 1974, 245-259.
8. Goldendberg D. M. and Spooner R. J., Glutathione reductase. In: Bergmeyer H.U.,
9. ed. Methods of Enzymatic Analysis, Volume III. New York, Wiley, 1984, 259-
10. 265.
11. Katunuma N., Okada M. Respiratory inhibition of the TCA cycle and control of
12. glutamic acid synthesis by ammonia in rat liver mitochondria.. Biochem. Biophys. Res. Community. 1963, 12:252-256.
13. Kosenko, E., Kaminsky, Y., Minan, M. D., Grisolia S., Felipo, V., High ammonia
14. levels decrease brain acetylcholinesterase. B. Mol. Chem. Pathol. 1994a, 22:177-184.
15. Kosenko, E., Kaminsky, Y., Minan, M. D., Marcaida G., Grisolia S., Felipo, V., Brain
16. ATP depletion induced by acute ammonia intoxication in rats mediate by the activation of the
- NMDA receptor and Na⁺, K⁺- ATPase. Journal of Neurochemistry. 1994b, 63:2172-2178.
17. Kosenko, E., Kaminsky, Y., Korneed, V. N., Lukyanova L. D. Protective effects of
18. the blockators of M- and N-cholinoreceptors in acute ammonia intoxication. Bul.Exp. Biol. 1995,
- 70:489-492 (in Russian)
19. Kosenko E. Felipo, V., Minana, M.D., Grisolia, S., Kaminsky Y., Effects of acute
20. hyperammonemia on rat brain mitochondria. Biochem. Biophys. acta 1996 (in press)
21. Lawrence, R. A., and Burk, R.F. Glutathione peroxidase activity in selenium-
22. deficient rat liver. Biochem. Biophys. Res. Commun. 1976. 71:952-958.
23. Lowry, O. H., Rosenbrough N.J., Farr, A.L., Randall, R.J. Protein measurment with

24. the Folin phenol reagent. J. Biol. Chem. 1951, 193:265-275.
25. McKhann, G.M., Tower, D.E. Ammonia toxicity and cerebral oxidative metabolism.
26. Am. J. Physiol. 1961, 200:420-424.
27. Neubert D., Wojtczak, A. B. and Lehninger A. L. Purification and enzymatic
28. identity of mitochondrial concentration factors I and II. PROC, Natl. Acad. Sci. (Wash) 1962, 48:1651-1658.
29. Ratnakumari, L. and Murthy C.R. K. (1989) Activities of pyruvate dehydrogenase,
30. enzymes of citric acid cycle and aminotransferases in subcellular fractions of cerebral cortex in normal and hyperammonemic rats. Neurochem. Res. 14, 221-228.
31. Vladimorv Y.A. and Arahakov A. I. Lipid peroxidation in Biological Membranes.
32. Moscow, Nauka, 1972. (In Russian)