To the Graduate Council:

I am submitting herewith a dissertation written by Sung-Woo Cho entitled "Studies on Aluminum Neurotoxicity." I have examined the final copy of this dissertation for form and content and recommend that it will be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

J.G. Joshi, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost and
Dean of The Graduate School

STUDIES ON ALUMINUM NEUROTOXICITY

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Sung-Woo Cho

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Dedicated To

My Wonderful Parents and Beautiful Sisters

ACKNOWLEDGMENTS

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ABSTRACT

This work reports studies of the effects of aluminum on glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in yeast, and in human, pig, and rat brains. Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme in the pentose phosphate pathway. It catalyzes the conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone.

Male Sprague-Dawley rats (10-12 weeks old) were fed with 0.1 mM $AlCl_3$ in drinking water. After one year, the animals, as well as controls (those fed with tap water), were killed by decapitation and brains were dissected and homogenized in 0.1 M Tris/HCl, pH 7.4. The 800 x g supernatants were assayed for aluminum and the activities of acetylcholine esterase (AChE), hexokinase, and glucose-6-phosphate dehydrogenase (G6PD). The concentration of aluminum in the homogenates were 40 ng and 80 ng/g wet weight, respectively. The activities of AChE were the same as in both groups but those of hexokinase and G6PD in the aluminum-fed group were about 73 % and 70 % of the control, respectively. Dialysis of the homogenates restored the activities of G6PD of the aluminum-fed group without changing those of the control group. However, the activities of hexokinase of both the aluminum-fed group and

the control group increased by dialysis, 2.7 and 2 fold, respectively. Therefore, at this elevated level, the activities of hexokinase of the aluminum-fed group were the same as those of the control.

The concentration of aluminum during enzyme assay even for the undialyzed homogenates was too low to account for the inhibition of hexokinase. It is, therefore, suggested that dialyzable inhibitor(s) for hexokinase is normally present in rat brain and that the concentration of the inhibitor may be increased further in aluminum-fed group to inhibit the utilization of glucose.

To investigate the inhibitory effects of aluminum on G6PD, the yeast enzyme was chosen because several aspects of this enzyme was well studied. Preincubation of yeast G6PD with $AlCl_3$ produced an inactive enzyme containing 1 gram atom of aluminum per mol of enzyme subunit. None of the bound aluminum was dissociated by dialysis against 10 mM Tris/HCl, pH 7.0 with or without 0.2 mM EDTA at 4 ^{o}C for 24 h. Citrate, $NADP^{+}$, EDTA, or NaF protected the enzyme against the aluminum inactivation. The aluminum-inactivated enzyme, however, was completely reactivated by citrate or NaF, and only partially by $NADP^{+}$ or malate. Modification of the histidine and lysine residues of the enzyme with diethyl pyrocarbonate and acetylsalicylic acid,

respectively, inactivated the enzyme. However, the modified enzyme still bound 1 g atom of aluminum per enzyme subunit. K_D value for G6PD-aluminum complex was calculated to be 4 uM using NaF. Circular dichroism studies showed that the binding of aluminum to the enzyme induced a decrease in α-helix and β-sheet and a increase in random coil. Therefore it is suggested that aluminum binding induces a conformational change unfavorable to enzyme activity.

Encoraged by these data, these studies were extended to G6PD from brain. This enzyme has been detected in the brain but it has not been purified. As a consequence, nothing is known about its molecular properties. Therefore a new procedure was developed for the purification of the brain G6PD. Human and pig brain homogenates were electrophoresed by PAGE and stained for G6PD activity. Five distinct bands were visible. Isozymes corresponding to two of those bands were purified from human and pig brain by conventional procedures. This included ammonium sulfate fractionation, hydroxylapatite chromatography, affinity chromatography with NADP-agarose and Blue-Sepharose CL-6B, and gel filtration with Sephadex S-300. The final preparations of isozymes were electrophoretically homogeneous, and had a molecular weight of 220,000 as determined by gel filtration and nondenaturing gradient polyacrylamide gel

electrophoresis and a subunit molecular weight of 57,000 as determined by SDS-polyacrylamide gel electrophoresis. The isozymes were $NADP^+$-specific. Km values for all isozymes were similar for $NADP^+$ (6 to 8 uM), but different for G6P (56 to 180 uM). Interestingly, only the isozyme II in human and pig brain were active with 6-phosphogluconate as a substrate (Km = 864 and 279 uM). All isozymes were inhibited by NADPH competitively with respect to $NADP^+$ (Ki = 15 to 30 uM) and noncompetitively with respect to G6P (Ki = 31 to 100 uM). NADH did not affect any of the isozymes. The inhibition by NADPH were abolished by GSSG and yeast glutathione reductase, but not by GSSG alone. ATP inhibited the isozymes competitively with respect to G6P (Ki = 3.5 to 4.2 mM) and noncompetitively with respect to $NADP^+$ (Ki = 0.9 to 1.5 mM). Palmitoyl-CoA dissociated the active tetramers into enzymatically inactive dimeric forms as determined by nondenaturing gradient polyacrylamide gel electrophoresis. HPLC peptide maps of tryptic digest and amino acid analyses of the isozymes showed extensive homologies between the isozymes. Based upon the arginine and lysine contents of the isozymes, the expected peaks for the peptides were about 67. Under the conditions in Fig. 6, at least 62 major peaks were detected. Therefore, the native enzyme appears to be composed of four identical

polypeptide chains.

Mg^{++} and Ca^{++} at 1 mM concentration activated the brain G6PD isozymes by 150 %, even though they were not necessary for the enzyme activities. The G6PD isozymes were inhibited by Zn^{++} and p-chloromercurybenzoic acid at 1 mM concentration, and inactivated by heat at 50 ^{o}C for 5 min. EDTA had no effect on the G6PD isozymes. Like yeast enzyme, aluminum inactivated G6PD activity of the human and pig brain isozyme I and isozyme II without affecting the 6-phosphogluconate dehydrogenase (6-PGD) activity of the G6PD isozyme II. The aluminum-inactivated enzyme contained 1 gram atom of aluminum per mol of enzyme subunit. Aluminum, bound to the G6PD isozymes, was not dissociated by dialysis against 10 mM Tris/HCl, pH 7.0, containing 0.2 mM EDTA for 16 h at 4 ^{o}C or. The dissociation constants for the enzyme-aluminum complex were calculated to be 2 to 4 uM. The aluminum inactivated enzyme was reactivated by NaF, citrate, and transferrin. These reagents also protected the G6PD isozymes against the aluminum inactivation.

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LIST OF ABBREVIATIONS

The abbreviations used are:

DEPC : diethyl pyrocarbonate

HEPES : 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid

EDTA : ethylenediamine tetraacetic acid

G6P : glucose-6-phosphate

G6PD : glucose-6-phosphate dehydrogenase

H6PD : hexose-6-phosphate dehydrogenase

6-PGD : 6-phosphogluconate dehydrogenase

ASA : acetylsalicylic acid

CD : circular dichroism

GSSG : reduced glutathione

GSSGrase : glutathione reductase

PCMB : p-chloromercurybenzoic acid

DTT : dithiothreitol

RNase : ribonuclease

Al : aluminum

$NADP^+$: β-nicotinamide adenine dinucleotidephosphate

PMSF : phenylmethyl sulfonylfluoride

AChE : acetylcholinesterase

INTRODUCTION

Earth crust contains 8 % aluminum. It is, therefore, one of the most common element and the most abundant metal. Aluminum is found in almost 300 different minerals, especially double silicates such as feldspars and micas and their weathering products, the clays (1). The interaction of aluminum with cellular components have aroused interest because aluminum has been implicated as a toxic element in a number of biological processes. One of the first reports of poisoning which was attributed to aluminum was in 1921 (2). Since then, aluminum toxicity has been implicated in the pathogenesis of a number of clinical disorders in patients with chronic renal failure on long-term intermittent hemodialysis treatment (3-6). The predominant disorders have been those involving either bone (osteomalacic dialysis osteodystrophy) or brain (dialysis encephalopathy) (1). An increased brain aluminum concentration has been implicated as a neurotoxic agent in the pathogenesis of Alzheimer's disease (7) and dialysis dementia (8). In brain tissue from patients with Alzheimer's disease, accumulations of aluminum have been identified within the nuclear region of a high percentage of neurons containing neurofibrillary tangles (9). It has

been proposed that the accumulation of aluminum in the brain tissue of patients with Alzheimer's disease may be a secondary phenomenon rather than an aetiological agent (10). Therefore, the accumulation of aluminum would represent a relatively non-specific aetiological factor. Two potential sources for the increased tissue content of aluminum in patients on hemodialysis have been proposed: [i] intestinal absorption from aluminum containing phosphate-binding gels, and [ii]transfer across the dialysis membrane from aluminum in the water used to prepare the dialysate (11).

The preceding discussion of aluminum toxicity underscores the growing interest in aluminum biochemistry. Much of the research into aluminum biochemistry has centered on its neurotoxicity. Even though the reaction mechanism for aluminum toxicity has not been established, many studies have produced useful informations in aluminum toxicity. In addition to chromatin binding (12), aluminum increases the permeability of the blood-brain-barrier and this may contribute to dementia or other neural disorders (13). Aluminum also induces accumulation of 10 nm filaments in human neural cells (14). The inhibition of dihydropteridine reductase would reduce the brain content of tetrahydrobiopterine, tyrosine, and neurotransmitters

(15). The neurotoxicity of aluminum alternatively may involve alterations in the major postsynaptic enzymes of cholinergic neurotransmission (16). In support of this mechanism is the observations that aluminum inhibits choline transport in erythrocytes (17) and decreases acetylcholinesterase in nerve tissue (18). Mazarguil et al. (19) investigated binding of aluminum to [Leu^5]-enkephalin using NMR spectroscopy and reported that aluminum coordinates at one binding site with Tyrosine and leucine in a tetra-coordinated structure, and with the terminal $-NH_2$ of the tyrosine residue at another binding site. How aluminum binding to enkephalins affects their neurological function is not known.

Aluminum binds to calmodulin and results in the exposure of large hydrophobic domains (20). The alterations in calmodulin conformation induced by aluminum are strongly inhibited in the presence of citrate, which can chelate aluminum. It has been suggested that loss of regulatory effects of the aluminum-calmodulin complex may be the key lesion in aluminum toxicity (21). Aluminum also binds to the iron binding proteins such as transferrin (22) and ferritin (23). Putterill and Gardner (22) reported that aluminum-induced structural changes in calmodulin were reduced by transferrin and transferrin was able to relieve

the inhibitory effect of aluminum on the activity of cAMP phosphodiesterase stimulated by Ca^{++}-calmodulin. Recently, Fleming and Joshi (23) isolated aluminum-ferritin complex from brain. Ferritin isolated from the brains of patients with Alzheimer's disease contained more aluminum and more iron than that from age-matched controls.

Aluminum has been shown to be required for the fluoride activation of the guanine nucleotide-binding subunit of adenylate cyclase (24). Activation of adenylate cyclase constitutes the first evidence of a possible essential biological function for aluminum. Aluminum has been used immunologically in Freund's adjuvant, and has been shown to activate complement and inhibit capping of immunoglobulin receptors on mouse spleen cells (25,26). Aluminum inhibits ristocetin-induced platelet aggregation (27), can be concentrated in lysosomes (28), competes with calcium for the binding sites on lactalbumins (29), increases lipid peroxidation in rat brain (30), and is transported into Chinese hamster ovary cells with a melanin carrier (31). Clearly, the biochemistry of aluminum is both extensive and varied.

It has been reported that in post-mortem cerebral tissue from the patients with Alzheimer's disease, the RNA content is reduced, suggesting either reduced transcription

or increased degradation (32,33). It has been speculated that the changes in the concentration of RNA are associated with a significant increase in alkaline RNase activity due to an abnormality in the RNase-inhibitor complex, and that the decrease in protein synthesis in the brain of the patients with Alzheimer's disease may be partly related to an RNase-inhibitor alteration that affects RNA levels and activity (33). It was reported that the RNase-inhibitor complex was dissociated by PCMB in vitro. Since the brain of the patients with Alzheimer's disease contains more amount of aluminum than that of the normal brain, it was of interest to examine the effects of aluminum on the dissociation of the RNase-inhibitor complex. Thus a better understanding of the molecular properties of the inhibitor from the brain appeared to be essential. We, therefore, started to purify and characterize the RNase inhibitor from pig brain. While this work was in process, it was reported that there were no differences in the level of RNase activity between control and Alzheimer's samples, and that the decrease in the concentration of RNA in Alzheimer's disease was due to reduced transcription but not due to the abnormality in RNase and its inhibitor complex (34). Therefore, further studies on the RNase-inhibitor complex were discontinued. We then focused on the studies on G6PD in the

brain.

It has been reported that there is an overall decrease in glucose metabolism in the patients with a dimentia of the Alzheimer's disease in comparison to the age-matched normal control (35). Johnson and Jope have reported that aluminum impairs glucose utilization and cholinergic activity in rat brain in vitro (36). Womack and Colowick (37) reported that hexokinase was inhibited by aluminum and the inhibition was due to the formation of Al-ATP complex which reduced the concentration of ATP. Karlik et al. (38) reported that aluminum bound to ATP, $NADP^+$, DNA and many other neucleotide. Since G6PD catalize the first reaction in the utilization of glucose via pentose phosphate pathway, it is of interest to examine the effects of aluminum on G6PD. Even though G6PD has been purified and characterized from various sources, the brain enzyme has not been purified. Therefore, a study of the molecular properties of G6PD from the brain and the effects of aluminum on the brain G6PD were initiated. The inhibitory effects of long-term feeding of aluminum on G6PD in rat brain are presented. Also, further studies on the inhibition of yeast and pig and human brain G6PD by aluminum are reported.

The desertation has four parts:

[1] Effect of long-term feeding of aluminum chloride on G6PD in rat brain.

[2] Inactivation of Baker's yeast G6PD by aluminum.

[3] Purification and characterization of human and pig brain G6PD isozymes and the effect of aluminum on the isozymes.

[4] Purification and partial characterization of RNase inhibitor from the pig brain.

INTRODUCTION REFERENCES

1. King, S.W., Savory, J., and Wills, M.R. (1981) CRC Crit. Rev. Clin. Lab. Sci. 14, 1-20
2. Spofforth, J. (1921) Lancet 1, 1301
3. Alfrey, A.C. (1978) Ann. Rev. Med. 29, 93
4. Parkinson, I.S., Feest, T.G., Ward, M.K., and Fawcett, R.W.P. (1979) Lancet 2, 406
5. Eliott, H.L., MacDougall, A.L., Fell, G.S., and Gardiner, P.H.E. (1978) Lancet 2, 1255
6. Alfrey, A.C., LeGendre, G., and Kaehny, W.D. (1976) N.Eng. J. Med. 294, 184
7. Crapper, D.R., Krishnan, S.S., and Dalton, A.J. (1973) Science 180, 511
8. McDermott, J.R., and Smith, A.I. (1978) Lancet 1, 901
9. Perl, D.P., and Brody, A.R. (1980) Science 208, 297-299
10. Shore, D., and Wyatt, R.J. (1983) J. Nervous Mental Diseases 171, 553-558
11. Kaehny, W.D., Hegg, A.P., and Alfrey, A.C. (1977) N. Eng. J. Med. 296, 1389
12. Lewis, P.N., Lukiw, W.J., De Boni, U., and Crapper-McLachlan, D.R. (1981) J. Neurochem. 37, 1193-1202
13. Banks, W.A., and Kastin, A.J. (1983) Lancet 2, 1227

14. De Boni, U., Seger, U., and Crapper-McLachlan, D.R. (1980) Neurotoxicol. 1, 65-81

15. Leeming, R.J., and Blair, J.A. (1979) Lancet 1, 556

16. Marquis, J. (1982) Bull. Environ. Contamin. Toxicol. 29, 43-49

17. King, R.G., Sharp, J.A., Boura, A.L.A. (1983) Biochem. Pharmacol. 32, 3611-3617

18. Kosick, K.S., Bradley, W.G., Good, P.F., Rasool, C.G., and Selkoe, D.J. (1983) J. Neuropathol. Experi. Neurol. 42, 365-375

19. Mazarquil, H., Haran, R., and Laussac, J. (1982) Biochim. Biophys. Acta 717, 465-472

20. Siegel, N., and Haug, A. (1983) Biochim. Biophys. Acta (1983) 744, 36-45

21. Suhayda, C.G., and Haug, A. (1984) Biochem. Biophys. Res. Commun. 119, 376-381

22. Putterill, J.J., and Gardner, R.C. (1988) Biochim. Biophys. Acta 964, 137-145

23. Fleming, J., and Joshi, J.G. (1987) Proc. Natl. Acad. Sci. USA 84, 7866-7870

24. Northup, J.K., Smigel, M.D., Sternweis, P.C., and Gilman, A.G. (1983) J. Biol. Chem. 258, 11369-11376

25. Morita, K., Incue, S., Murai, Y., Watanabe, K., and Shima, S. (1982) Experimentia 38, 1227-1228

26. Ramanathan, V.D., Badenoch, P., and Turk, J.L. (1979) Immunology 37, 881-888

27. Baugh, R.F., Brown, J.E., and Hougie, C. (1975) Thrombosis et Diathesis Haemorrhagica 33, 540-546

28. Galle, P. (1983) Adv. Nephrol. 12, 85-99

29. Murakami, K., and Berliner, L.J. (1983) Biochemistry 22, 3370-3374

30. Ohtawa, M., Seko, M., and Takayama, F. (1983) Chem. Pharmacol. Bull. 31, 1415-1418

31. Pajak, S., Hopwood, L.E., and Hyde, J.S. (1983) Experi. Cell Res. 149, 513-526

32. Marotta, C.A., and Sajdel-Sulkowska, E.M. (1984) Trans. Am. Soc. Neurochem. 15, 14

33. Sajdel-Sulkowska, E.M., and Marotta, C.A. (1984) Science 225, 947-948

34. Guillemette, J.G., Wong, L., Crapper-McLachlan, D.R., and Lewis, P.N. (1986) 47, 987-997

35. Benson, F. (1983) in Biological aspects of Alzheimer's disease, Cold Spring Harbor Laboratory, NY. pp 309

36. Johnson, G.V.W., and Jope, R.S. (1986) Toxicology 40, 93-102

37. Womack, F.C., and Colowick, S.P. (1979) Proc. Natl. Acad. Sci. USA 76, 5080-5084

38. Karlic, S.J., Elgavish, G.A., and Eichhorn, G.L. (1983)
J. Am. Chem. Soc. 105, 602-609

CHAPTER I

Effect of Long-Term Feeding of Aluminum Chloride on Glucose-6-Phosphate Dehydrogenase in Brain

INTRODUCTION

Aluminum is one of the most abundant elements in the earth's crust. Human ingests about 30 mg aluminum per day, mostly as a food contaminant (1). Although generally assumed to be a non-essential and non-toxic, accumulation of aluminum in the brains of patients with Alzheimer's disease (AD) (2-4) and Parkinson's disease (5,6) and of patients with dialysis dementia (7) has renewed the interest in Al toxicity. Banks and Kastin (8) have suggested that aluminum increases the permeability of the blood-brain-barrier and this may contribute to dementia or other neural disorders. Aluminum also induces accumulation of 10 nm filaments in human neural cells (9). Aluminum is a neurotoxin in experimental animals. Intracranial injections of Al salts in cats, rabbits, and dogs cause neurofibrillary degeneration (10) and impair learning and memory (11).

In most of in vitro experiments, the concentration of aluminum was very high. Under the physiological conditions, accumulation of metal ions results from prolonged exposure

at very low levels. We therefore assayed the activities of hexokinase, glucose-6-phosphate dehydrogenase (G6PD) and AChE in the extracts of brains of rats which had received 100 uM $AlCl_3$ in drinking water for 12 months. These enzymes were chosen because of the facts that, except during starvation, glucose is the sole source of energy for the brain and AChE regulates the levels of one of the most important neurotransmitter, acetylcholine. Formation of glucose-6-phosphate (G6P) catalyzed by hexokinase is the first reaction in the utilization of glucose by glycolysis and the conversion of G6P to 6-phosphoglucono-δ-lactone catalyzed by G6PD is the first reaction in the utilization of glucose via pentose phosphate pathway.

EXPERIMENTAL PROCEDURES

Materials

ATP, glucose, $NADP^+$, G6PD, G6P, acetylcholine iodide, DTNB, EDTA, HEPES, and Tris were purchased from Sigma. All reagents were of analytical grade. Fresh pig brains were obtained from the local slaughter house (Lay Packing Co. Inc., Knoxville, TN). Human brains were obtained from the autopsy unit of the University of Tennessee Memorial

Research Center, Knoxville, TN. Rats were obtained from Charles River Breeders.

Enzyme Assays

The enzymes were assayed as described below. The specific activities were expressed as milliunits/mg of protein.

< Hexokinase >

Hexokinase was assayed according to Joshi and Jagannathan (23). One unit of hexokinase was defined as the amount of enzyme which catalyzed the formation of 1 umol G6P per minute at 23 ^{o}C. The standard assay mixture contained 15 umol of glucose, 20 umol of Tris/HCl, pH 7.6, 0.13 umol of $NADP^{+}$, 0.1 umol of EDTA, G6PD (2units/ml), and 30-60 ul of the source of hexokinase in a final volume of 0.97 ml. The reaction was initiated by the addition of 30 ul of 0.3 M ATP (Sodium salt, pH 7.6).

< Acetylcholine Esterase >

AChE was assayed as described by Ellman et al.(24). The reaction was initiated by the addition of 30 ul of the source of enzyme to a reaction mixture containing 100 umol potassium phosphate buffer, pH 8.0, 0.75 umol acetylcholine iodide, and 0.4 umol DTNB in 0.1 M phosphate buffer, pH 7.0 in a final volume of 1 ml. One unit of enzyme was defined

as the amount of enzyme which produced 1 umol thiocholine per minute at 23 ^{o}C.

< Glucose-6-Phosphate Dehydrogenase >

G6PD was assayed as described by Glaser and Brown (25). One unit was defined the amount of enzyme which reduced 1 umol of $NADP^{+}$ per minute at 23 ^{o}C. The assay system contained 2 umol of G6P, 0.9 umol of $NADP^{+}$, 17.5 umol of HEPES, pH 7.5, and 30-60 ul of the source of G6PD in a final volume of 1 ml.

Measurement of Metal Ions

Concentration of Cu, Cd, Zn, and Al were measured with the Instrumentation Laboratory Atomic Absorption Spectrophotometer 551 equipped with a graphite furnace atomizer 655. Pyrolytically coated graphite tubes and pyrolysis plat-forms were used with 10 ul aliquots. Before use, the tubes and the plat-forms were conditioned by heating twice at 2800 ^{o}C. A 309.2 nm aluminum wavelengh, a 160 um slit width, and a lamp current of 8 mA were used. Background correction was used throughout. The aluminum contents were expressed as ng aluminum/g wet weight.

Protein Measurement

The concentration of protein was measured by the method of Lowry et al.(26).

Statistical Analysis

Statistical analysis and P values were obtained according to Sandler's test (27).

Experiments With Rat Brains

Male Sprague-Dawley rats (10-12 weeks old) were divided into 2 groups and fed with standard laboratory diet (Rodent Laboratory Chow No.5001, Purnia Mills Inc., St.Louis, MO) and tap water. The concentration of aluminum in the diet was 8.3 ug/g dry weight and that in the tap water was 2.7 ng/ml. The first group served as an age-matched control. The drinking water of the animals in the second group contained additional 100 uM $AlCl_3$. After one year, the animals were killed by decapitation and brains were dissected, rinsed with 0.9 % ice-cold saline, and homogenized in 5 volumes of 0.1 M Tris/HCl, pH 7.4. Only plastic-wares were used after completely rinsed with nitric acid followed by double deionized water. The homogenates were centrifuged at 800 x g at 4 ^{o}C for 20 minutes. Aliquots of the supernatants (crude extracts) were used for enzyme assays, protein determination, and metal ion analysis before and after dialysis against 2000 volumes of 10 mM Tris/HCl, pH 7.4 at 4 ^{o}C, three times, for a total of 20 hours.

RESULTS AND DISCUSSION

The average life span of a rat is about 30 months but the rate of growth reaches a plateau after 4 months. A 12 month period, therefore, represents about half the period of adult life. Thus all the animals were killed after 1 year rather than at intermediate intervals.

Several earlier studies have been already shown that the oral feeding of aluminum salts causes the accumulation of the metal ion various areas of the rat brain (28). Table 1 shows that the crude extracts (800 x g supernatants) of the aluminum feeding groups contains a twice amounts of aluminum compared to those of the control groups. The level of Zn and Cu remained unchanged and no Cd was detected in any of the crude extracts (Data not shown). At least part of the dietary aluminum transported to the brain without affecting the weight of body and brain or the protein content of the crude extracts.

In vivo, the synthesis and degradation of the acetylcholine is regulated by cholineacetyl transferase and acetylcholine esterase (ACE), respectively. Aluminum, at its high concentration, inhibits the synthesis of acetylcholine and activates, at low concentration, the hydrolysis of acetylcholine. To determine the in vivo

< TABLE 1 >

Effect of $AlCl_3$ Feeding on The Aluminum Content of Rat Brains. All numbers are expressed as the mean ± S.D. The aluminum contents were measured with the crude extracts after centrifuge at 800 x g for 20 min at 4 °C.

	Body wt. (g)	Total wt. of brain (g)	Protein (mg/ml)	Aluminum (ng/g wet wt)
Control (n=5)	630 ± 60	2.3 ± 0.2	10.5 ± 0.8	40 ± 6.0
Al-feeding (n=9)	625 ± 65	2.3 ± 0.2	10.2 ± 0.9	80 ± 3.0*

* Higher than the control ($P < 0.01$)

implication of these in vitro studies, the crude extracts of the brains from the two groups of rats were assayed for hexokinase, ACE, and glucose-6-phosphate dehydrogenase (G6PD). Table 2 shows that dialysis of the crude extracts does not affect the activities of ACE.

Aluminum injections into rats reduced the utilization of glucose in rat brain (30). Consistent with the observation, aluminum feeding reduced the specific activities of hexokinase and G6PD by about 27 % and 20 %, respectively. Dialysis reduced the aluminum content in crude extracts of both groups and increased the specific activities of both enzymes of the aluminum-fed group up to the levels of the control group. While dialysis had no effect on the activities of G6PD of the control group, it increased the activities of hexokinase of the control group and aluminum-fed group by 2-fold and 2.7-fold, respectively. Thus at these increased levels, the activities of hexokinase of the dialyzed crude extracts of the control group were very similar to those of the aluminum-fed group. The fact that even in the control group, only half of the total hexokinase is expressed suggests that this may be a more general phenomenon in brain. This was confirmed with the crude extracts from human and pig brain (Table 3). As shown, dialysis

< TABLE 2 >

Effect of Aluminum-Feeding on The Activities of AChE, G6PD, and hexokinase in Rat Brain. All numbers are expressed as mean ± S.D.

Condition	Specific Activities (milliunits/mg protein)					
	Before Dialysis			After Dialysis		
	AChE	Hexokinase	G6PD	AChE	Hexokinase	G6PD
Control (n=5)	62 ± 5	75 ± 3	10 ± 1	63 ± 5	143 ± 4	10 ± 1
Al-fed (n=9)	58 ± 4	58 ± 2	7 ± 1	61 ± 5	139 ± 3	10 ± 1

< TABLE 3 >

Effect of Dialysis on Hexokinase and G6PD in The 800 x g Supernatant of Pig and Human Brain homogenates. All numbers are expressed as a mean ± S.D.

Brain	Specific Activities (milliunits/mg protein)			
	Before Dialysis		After Dialysis	
	Hexokinase	G6PD	Hexokinase	G6PD
Pig (n=6)	67 ± 5	9 ± 1	121 ± 7	10 ± 1
Human (n=3)	69 ± 7	9 ± 1	100 ± 6	10 ± 1

increased the activities of hexokinase by 1.5-fold and 1.8-fold in the human and pig brain, respectively. The pig brain was used for further investigation of the dialyzable inhibitor of hexokinase. A 50 g of the pig brain was homogenized as before and 100 ml of the 800 x g supernatant was concentrated up to 50 ml using Amicon ultrafiltration cells fitted with a 3000 M.W. cut-off membrane. The filtrate served as a source of the inhibitor. The concentrated solution containing the hexokinase activity was further dialyzed as before and centrifuged at 800 x g for 15 minutes at 4 ^{o}C. The supernatant was used as a source of hexokinase. Aliquots of the filtrate were tested for their inhibitory activities. The inhibition was propotional to the amount of filtrate added to the assay medium. The activity of hexokinase decreased by 50 % when 200 ul of the enzyme containing supernatant was mixed with 200 ul of the filtrate. For preliminary characterization of the inhibitor, aliquots of the filtrate were subjected to various treatments and tested for their inhibitory effect on hexokinase activity. The treatments used were: (i) heating for 1 hour at 100 ^{o}C in a boiling water bath and centrifugation, (ii) incubation for 1 hour at 4 ^{o}C with NaOH or HCl at a final concentration of 1 N, followed by neutralization to pH 7.0, (iii) addition of trichloroacetic

acid to a final concentration of 5 %, followed by centrifugation after 1 hour and removal of the trichloroacetic acid with metal-free ether, and (iv) ashing in 10 N ultrapure HNO_3 and dissolving the ash in the original volume of 0.1 M Tris/HCl, pH 7.4. For control, equivalent aliquots of the homogenization buffer were subjected to the same 4 treatments. A 200 ul aliquot of the dialyzed homogenate was mixed with an equal volume of each of the above 4 preparations and the mixture was assayed for hexokinase activity. The values were compared with those obtained with the corresponding controls. The results showed that the inhibitory effect was abolished by the dissolved ash but not by the other 3 treatments. The inhibition produced by untreated filtrate was 52 % and that by heating, acid or alkali treatment, or trichloroacetic acid treatment was 53 %, 49 %, and 47 %, respectively.

At micromolar concentration aluminum inhibits hexokinase from yeast and brain (19,22,31). In most of our experiments, up to 60 ul of the homogenate was used for the assay in a total volume of 1 ml. Therefore the concentration of aluminum during the assay was only 36 nM (0.97 ng/ml) for the aluminum-fed groups and 18 nM (0.48 ng/ml) for the control. These concentrations are much lower than the 5 uM required to inhibit 50 % of the enzyme

activity. Therefore the concentrations of aluminum in the homogenates do not necessarily represent those observed in situ. This is particularly true for the complex organs such as brain where aluminum is known to be localized at different concentration in specific areas of the brain (28). Nevertheless, the restoration of the enzyme activity by dialysis (Table 3) is not due to the removal of aluminum alone because hexokinase from the dialyzed homogenates of either group was not inhibited by the presence of 100 nM aluminum in the assay medium. In a separate series of experiments, similar homogenates of liver were assayed for hexokinase and G6PD before and after dialysis. There were no differences in in the activities of hexokinase and G6PD before and after dialysis. Therefore the dialyzable inhibitor seems to reside in the brain.

We therefore suggest that in the brain only half of the total catalytic activity of hexokinase is normally expressed. The remainder is latent because of the presence of the dialyzable inhibitor(s). Aluminum-feeding, even at low levels, increases the concentration of the latent enzyme by increasing the concentration of the unidentified inhibitor. The 3000 M.W. cut-off filtrate obtained from the homogenates of the aluminum-fed group also inhibited the G6PD and the inhibition was propotional to the amount of

filtrate added to the assay medium. However, 500 ul of the filtrate was required to produce 30 % inhibition. Again, addition of an equivalent of aluminum present in such filtrates was too low to inhibit G6PD. It is tempting to speculate that the same inhibitor, at least in parts, regulates both hexokinase and G6PD but hexokinase is more sensitive than G6PD. Under normal circumstances, the concentration of the inhibitor is adequate to inhibit hexokinase by 50 % and aluminum-feeding increases the steady state concentration of the inhibitor to affect both enzymes.

It is noteworthy that even in the normal brain only half of the total activity of hexokinase is expressed. This situation is functionally similar to but different in mechanism from that observed for phosphoglucomutase in muscle and liver (32). In these tissues, about half of the activity of phosphoglucomutase is dominant because it is complexed with inhibitory metal ion(s) such as Zn (33). However increasing the available tissue concentration of Zn by administering Cd to rats does not elevate the concentration of the dominant enzyme activity. Although Cd injection is known to stimulate metallothionein synthesis in liver and muscle (32), it was shown that Cd injections also increased the concentration of zinc associated with the

fraction of molecular weight less than 3000. On the other hand, in vitro, hexokinase is inhibited by aluminum because the metal ion complexes with ATP in the assay medium and the ATP-aluminum complex competes with ATP (22). Increasing the cerebral aluminum concentration in aluminum-fed group seems to increase the concentration of the dialyzable inhibitor normally present in brain. Thus the observed reduction in the activity of hexokinase in the crude extracts appears to be an indirect consequence of aluminum-feeding rather than direct interaction of the metal ion itself with the protein. It is also possible that the complex of aluminum and the dialyzable inhibitor reduces the activity of hexokinase even at nmolar concentration.

The reduction of the activities in hexokinase and G6PD in aluminum-fed rat brain observed here accounts, at least in part, for the previously reported result of the reduced glucose utilization impaired by aluminum in rat brain (30). The reduction in glucose utilization would obviously exert damaging effects on cerebral metabolism and contribute to aluminum-induced neuronal disorders.

The data presented here show that in rats: [i] prolonged intake of low level of aluminum in the drinking water does not affect their growth, [ii] at least some of the ingested aluminum accumulates in the brain, [iii] the

accumulated aluminum does not affect ACE but inhibited hexokinase and G6PD, [iv] normally only half of total cerebral hexokinase is expressed but aluminum-feeding causes further reduction in the expressed activity, and [v] it is suggested that reduction is accomplished by increasing the concentration of a pre-existing dialyzable inhibitor(s).

REFERENCES

1. Alfrey, A.C., Le Gendre, G.R., and Kaehny, W.D. (1976) N. Engl. J. Med. 294, 184-188
2. Crapper, D.R., Krishnan, S.S., and Quittkat, S. (1976) Brain 99, 67-80
3. Trapp, G.A., Miner, G.D., Zimmerman, R.L., Mastri, A.R., and Heston, L.L. (1978) Biol. Psychiatry 13, 709-715
4. Shore, D., and Wyatt, R.J. (1983) J. Nerv. Ment. Dis. 171, 553-558
5. Yase, Y (1980) Neurotoxicology 1, 101-109
6. Perl, D.P., Gajduesk, D.C., Garruto, R.M., Yanagihar, R.T., and Gibbs, C.J. (1982) Science 217, 1053-1055
7. Alfrey, A.C. (1980) Neurotoxicology 1, 43-53
8. Banks, W.A., and Kastin, A.J. (1983) Lancet 2, 1227-1229
9. DeBoni, U., Seger, M., and Crapper-McLachlan, D.R. (1980) Neurotoxicology 1, 65-81
10. Boegman, R.J. and Bates, L.A. (1984) Can. J. Physiol. 62, 1010-1014
11. Bowdler, N.C., Beasley, D.S., Fritze, E.C., Goulette, A.M., Hatton, J.D., Hesion, J., Ostman, D.L., Rugg, D.J., and Schmittdiel, C.J. (1979)

Pharmacol. Biochem. Behav. 10, 505-512

19. Lai, J.C.K., Baker, A., Carlson, K.C., and Blass, J.P. (1985) Comp. Biochem. Physiol. 80 C, 291

22. Womak, F.C. and Colowick, S.P. (1979) Proc. Natl. Acad. Sci. USA, 76, 5080-5084

23. Joshi, M,D, and Jagannathan, V. (1966) Methods in Enzymol. 4, 371-375

24. Ellman, G.L., Courtney, K.D., Andres, V.,Jr., and Fetherstone,R.M. (1961) Biochem, Pharmacol. 7, 88-95

25. Glaser, L. and Brown, D.H. (1955) J. Biol. Chem. 216, 67-79

26. Lowry, O.H., Rosenbrough, N.H., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

27. Bruning, J.L., and Kintz, B.L. (1977) in Computational Handbook of Statistics, 2nd Ed. Scott, Foresman and Company, Glenview, IL pp.17

28. Salnina, P., Falkenborn, Y., Frech, W., and Cedergren, A. (1984) Fel. Chem. Toxicol. 22, 391-398

29. Marquis, J.K., and Lerrick, A.J. (1982) Biochem. Pharmacol. 31, 1437-1440

30. Johnson, G.V.W., and Jope, R.S. (1986) Toxicology 40, 93-102

31. Lai, J.C.K., and Blass, J.P. (1984) J. Neurochem. 42, 438-446

32. Fleming, J., Miller, H., and Joshi, J.G. (1985) Toxicology 36, 151-158

33. Ray, W.J.,Jr. (1967) J. Biol. Chem. 242, 3733-3744

CHAPTER II

Inactivation of Bakers' Yeast Glucose-6-Phosphate Dehydrogenase by Aluminum

INTRODUCTION

Environmental stress such as acid rain has aroused interest in aluminum toxicity (1). Although a unified hypothesis for aluminum toxicity remains to be established, aluminum affects diverse cellular functions. For example, it inhibits (i) synaptosomal uptake systems with some selectivity toward the uptake of choline, glutamate, and γ-aminobutyric acid (2-4), (ii) in vitro assembly of tubulin into microtubules (5), and (iii) mitosis in murine cells in tissue culture (6). Aluminum affects the brain microsomal protein synthesis in immature rats (7) and the activity of the regulatory component of adenylate cyclase by fluoride (8). Aluminum binds to iron storage proteins such as transferrin (9) and ferritin (10), and induces a structural and functional changes in calmodulin (11). Aluminum inhibits several enzymes such as acetylcholinesterase (12), Na^+-K^+ ATPase (2), ferroxidase (13), dihydropteridine reductase (14), and hexokinase from brain and yeast (15).

We report here that aluminum also inactivates yeast

glucose-6-phosphate dehydrogenase, the first enzyme in the pentose phosphate pathway. Inactivation of G6PD by specific reagents has shown that lysine and histidine residues were involved in the catalytic activity (16,17). We used a similar approach to determine the role of these amino acid residues in the binding of aluminum. The enzyme from yeast was chosen for these studies because it has been well studied.

EXPERIMENTAL PROCEDURES

Materials

Glucose-6-phosphate dehydrogenase from Baker's yeast (type XV), $NADP^+$, glucose-6-phosphate, EDTA, malate, sodium fluoride, acetylsalicylic acid, diethyl pyrocarbonate, and citrate were purchased from Sigma. Aluminum chloride was purchased from Fisher Scientific. Aluminum standard solution was purchased from Spex Industries, Inc.

Measurement of Aluminum

Concentration of aluminum was measured with an Instrumentation Laboratory atomic absorption spectrophotometer 551 equipped with a graphite furnace atomizer 655. Pyrolytically coated graphite tubes and pyrolytical platforms were used with 10 ul aliquots. Before

use, the tubes and platforms were conditioned by heating twice at 2800 ^{o}C. A 309.2 nm aluminum wavelength, a 160 um slit width, and a lamp current of 8 mA were used. Background correction was performed throughout.

Enzyme Assay

Standard reaction mixture contained 2 umoles of G6P, 1 umole of $NADP^{+}$, and 25 umoles of HEPES, pH 7.0. The reaction was started by the addition of enzyme. Various other reactants were added as required. The final volume of the reaction mixture was 1.0 ml. The enzyme activity was measured by following the initial rate of the formation of NADPH monitored at 340 nm at 25 ^{o}C. No Mg^{++} was added unless otherwise indicated. Protein was monitored at 220 nm or 280 nm spectrophotometrically or assayed by the method of Larson et al. (18).

Inactivation of G6PD by Aluminum and Quantitation of Aluminum Binding to G6PD

To determine the amount of aluminum bound to the enzyme in a typical experiment, 3 nmoles of enzyme was incubated at 25 ^{o}C for 20 minutes with 300 nmoles of aluminum chloride in 100 mM Tris/Hcl, pH 7.0 in a final volume of 200 ul. The completely inactivated enzyme was applied to a Sephadex G-25 column (1 x 30 cm) equilibrated with the same buffer. The protein was eluted with the same buffer and

monitored spectrophotometrically at 220 nm. The fractions containing the protein were collected and assayed for protein and aluminum. An aliquot of the protein was also dialyzed twice against 2000 volumes of 10 mM Tris/HCl, pH 7.0 at 4 ^{o}C for 24 hr with or without 0.2 mM EDTA and assayed for aluminum.

The aluminum chelator, fluoride (19), was used to remove aluminum from the enzyme-aluminum complex (E-Al). E-Al was prepared as described above and incubated with varying amount of NaF. The Al-F complex was separated from the E-Al by centrifugation in for 10 minutes at 2900 rpm in Centrifree filters (Amicon). The aluminum concentration in the filtrate was used to measure the amount of aluminum removed from E-Al by NaF.

Effect of Modification of Histidine on Aluminum Binding

Modification of histidine in G6PD with diethyl pyrocarbonate (DEPC) was performed by the method of Ovadi et al. (20). 1 mg of enzyme was incubated at 25 ^{o}C with 10 mM DEPC in 25 mM HEPES, pH 6.0 in a final volume of 1 ml. DEPC was dissolved in 95 % ethanol and the final concentration of ethanol was kept below 10 % during the incubation of the enzyme with the modifying agent. DEPC concentration was diluted at least 10-fold during the assay for enzyme activity. The formation of N-carbetoxy-histidyl

was monitored at 240 nm using the extinction coefficient of 3200 $M^{-1}cm^{-1}$ at 240 nm. After completely saturating the histidine in G6PD with DEPC, the modified enzyme was incubated with 100 fold molar excess aluminum in 25 mM HEPES, pH 7.0 for 20 minutes. Free aluminum was removed by dialysis against 10 mM HEPES, pH 7.0 at 4 ^{o}C for 24 hr, and the dialyzed enzyme was assayed for aluminum and enzyme activity.

Effect of Modification of Lysine on Aluminum Binding

Modification of lysine residues was achieved by the method of Jeffery et al. (17). 1 mg of enzyme was incubated at 25 ^{o}C with 1.5 mM acetylsalicylic acid in 10 mM Tris/ HCl, pH 8.5 in a final volume of 1 ml. After 30 min, further acetylsalicylic acid was added to a final concentration of 3 mM, and aliquots withdrawn at different times were assayed for enzyme activity. When 95 % of the enzyme activity was abolished, an equal volume of 150 mM ethanolamine acetate at pH 6.0 was added to stop the reaction. Reagents were removed by dialysis against 10 mM Tris/HCl, pH 7.0. The modified enzyme was treated with aluminum as described above (See histidine modification) and assayed for aluminum.

Circular Dichroism Study

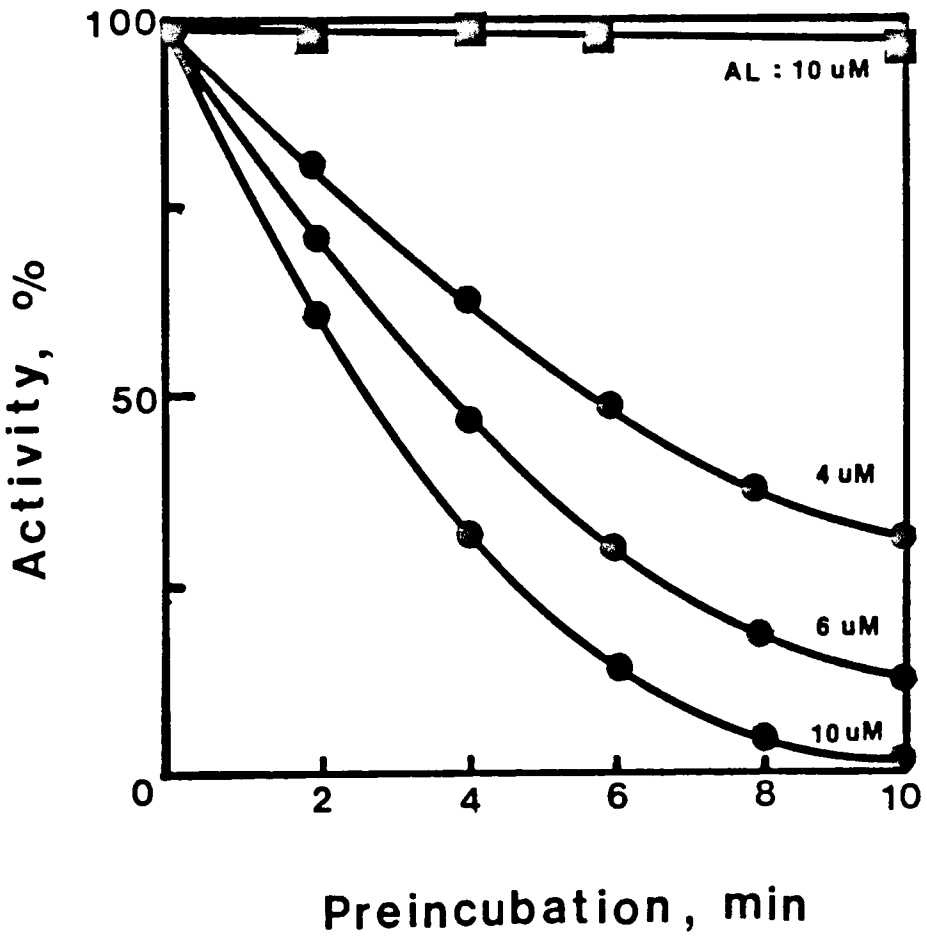
0.4 mg of enzyme was incubated with or without 0.5 mM

$AlCl_3$ in 25 mM HEPES, pH 7.0 at 25 ^oC for 30 minutes and dialyzed against the same buffer. The spectrum of the inactive enzyme-aluminum complex was compared to that of the control enzyme. CD measurements were made in a Jasco J-500 C spectrophotometer using 0.02 cm pathway cell (Hellma Scientific Suprasil quartz) at room temperature. CD results were reported in terms of [θ] (deg cm^2 $dmol^{-1}$) using a computer program modulating the spectrophotometer. CD analysis were performed by the method of Yang et al. (21) with reading from a 'smoothed' curve through 5 successive point window average assigned to middle to determine the secondary conformation

RESULTS

Inactivation of Yeast Glucose-6-Phosphate Dehydrogenase by Aluminum

Preincubation of yeast G6PD with $AlCl_3$ in 25 mM HEPES buffer, pH 7.0 at 25 ^oC produced an inactive enzyme. The loss of enzyme activity was proportional to the preincubation time and to the concentration of aluminum (Fig. 1). The inactivation was pH-dependent. A marked increase in sensitivity to aluminum was observed as the pH decreased. Above pH 8.0, aluminum did not affect the rate of the enzymatic reaction (Fig. 1). The studies described



< Fig 1 > Inactivation of G6PD by aluminum at different pH. $AlCl_3$ was added to the enzyme solution in 25 mM HEPES buffer, pH 8.0 (■) and pH 7.0 (●) at 25 °C. At indicated times, the remaining activity was assayed by addition of the reaction mixture. This diluted the concentration of aluminum 10-fold during the assay.

here were performed at pH 7.0. The inactive enzyme contained one mol of aluminum per mole of enzyme subunit and was not reactivated by exhaustive dialysis against 10 mM Tris/HCl, pH 7.0 with or without 0.2 mM EDTA (Table 4). This result indicates that there is a rather specific locus for the aluminum binding on the enzyme.

Protection and Reactivation of G6PD by Chelators against Aluminum Inactivation

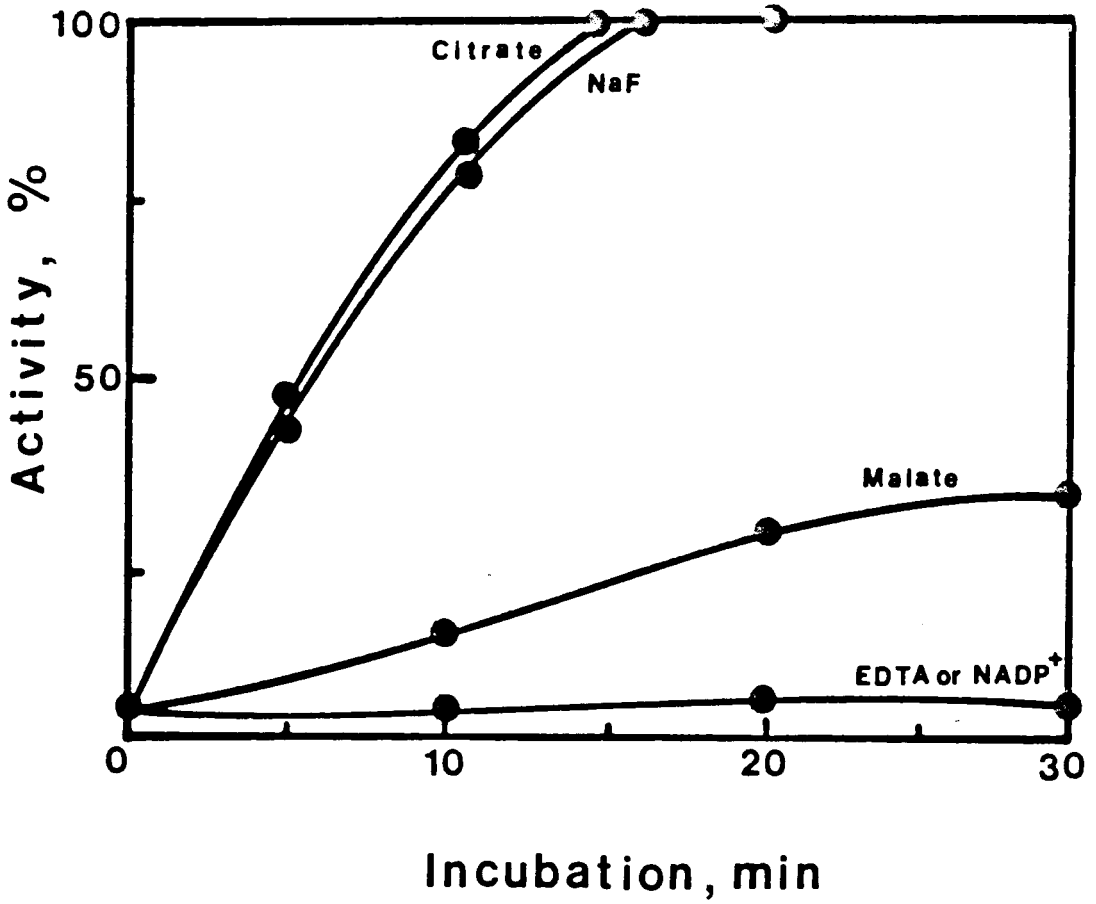
Fig. 2 shows that the inhibitory effect of aluminum is abolished by some chelators. A sample of the inactivated G6PD-aluminum complex, prepared as above, was treated with the known chelators for aluminum (15,22) such as citrate, malate, EDTA, NaF, and $NADP^+$ at final concentrations of 2 mM. At different times, aliquots were withdrawn and assayed for the activity in the standard reaction mixture. The aluminum inhibition could be fully reversed only by citrate or NaF. Full activity was restored in 15 minutes. EDTA, even at 2 mM concentration, did not reactivated the aluminum-inactivated enzyme. In a related experiment (Fig. 3), aluminum was preincubated with the chelators for various times and then tested as a source of aluminum available to inhibit the enzyme. The results showed that the rate of chelation of aluminum by malate was the slowest because a significant amount of free aluminum was retained

< Table 4 >

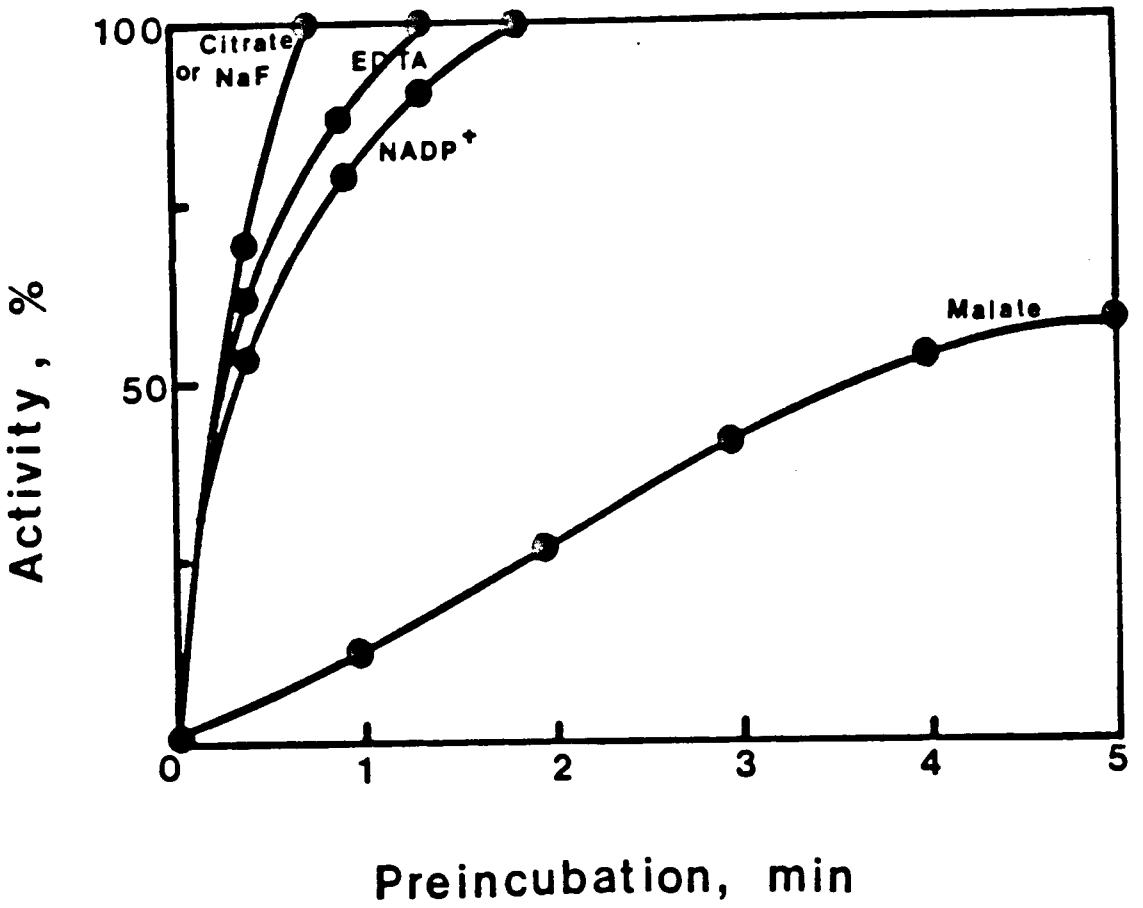
Binding of aluminum to G6PD. The enzyme (3 nmole) was incubated with 300 nmole of aluminum chloride in a total volume of 200 ul of 10 mM Tris/HCl, pH 7.0 at 25 °C for 20 minutes, and excess aluminum was removed by gel filtration on a Sephadex G-25 column (1 x 30 cm). The fractions containing protein-aluminum complex were collected and assayed for aluminum and protein. The fractions containing protein were concentrated, dialyzed against 10 mM Tris/HCl, pH 7.0 or the same buffer containing 0.2 mM EDTA, and reassayed for aluminum and protein.

Treatments	[Al]/[subunit][a]
Sephadex G-25	0.84
Dialysis against Tris/HCl	0.80
Dialysis against Tris/HCl with 0.2 mM EDTA	0.76

[a] Calculations are based on the subunit molecular weight of 50,000 (35).



< Fig 2 > Reactivation of aluminum-inactivated G6PD by several chelators. Reaction mixtures containing the enzyme and aluminum chloride in 25 mM HEPES buffer, pH 7.0 were preincubated at 25 °C for 20 minutes. Chelators were added to the reaction mixture in a final volume of 1.0 ml. Final concentration of aluminum chloride and the chelators were 50 uM and 2 mM, respectively. At different times, aliquots were withdrawn and assayed for activity.



< Fig 3 > Effect of preincubation of aluminum with the chelators on the G6PD. Preincubation mixture contained 25 umole of HEPES buffer, pH 7.0, 50 nmole of aluminum chloride, and 2 umole of the indicated effectors in a final volume of 0.9 ml and kept at 25 ^{o}C for various times. The reaction was started by the addition of enzyme and assay mixture with a final concentration of 2 mM G6P, and 1 mM $NADP^{+}$ in 25 mM HEPES buffer, pH 7.0.

in a solution to produce 50 % inhibition even after 5 minutes. By contrast, only very small amounts of free aluminum seemed to have remained in a solution in the presence of citrate, EDTA, NaF, or $NADP^+$, as shown by the full recovery of the enzyme activity after 2 minutes of incubation with these chelators.

Measurement of Dissociation Constant for The Enzyme-Aluminum Complex

Dissociation constant of aluminum for the E-Al complex was calculated to be 4×10^{-6} M by an indirect method using NaF, a known reversible chelator for aluminum (19). Accordingly,

$$E\text{-}Al \rightleftharpoons Al + E , \qquad Al_{Kd(E\text{-}Al)} = \frac{[Al][E]}{[E\text{-}Al]} \qquad [1]$$

$$F + Al \rightleftharpoons Al\text{-}F , \qquad Al_{Ka(F)} = \frac{[Al\text{-}F]}{[Al][F]} \qquad [2]$$

$$SUM : E\text{-}Al + F \rightleftharpoons E + Al\text{-}F , \qquad K_{eq} = \frac{[E][Al\text{-}F]}{[E\text{-}Al][F]} \qquad [3]$$

Thus the equilibrium expression [3] of the reaction carried out is the sum of two individual elementary reactions [1] and [2]. From these three reactions,

$$K_{eq} = \frac{[E][Al\text{-}F]}{[E\text{-}Al][F]} = Al_{Kd(E\text{-}Al)} \cdot Al_{Ka(F)} \qquad [4]$$

When half of the total aluminum is removed from the enzyme,

the concentration of enzyme and E-Al are equal. Therefore, equation [4] simplifies to :

$$\frac{[Al\text{-}F]}{[F]_{Total} - [Al\text{-}F]} = Al_{Kd(E\text{-}Al)} \cdot Al_{Ka(F)} \qquad [5]$$

Half-maximal removal of aluminum (20 uM) was achieved at 25 uM NaF (Fig. 4). We then substitute the value of $Al_{Ka(F)}$ (from Ref. 19) and $[Al\text{-}F]/([F]_{Total} - [Al\text{-}F])$, and calculated the dissociation for E-Al.

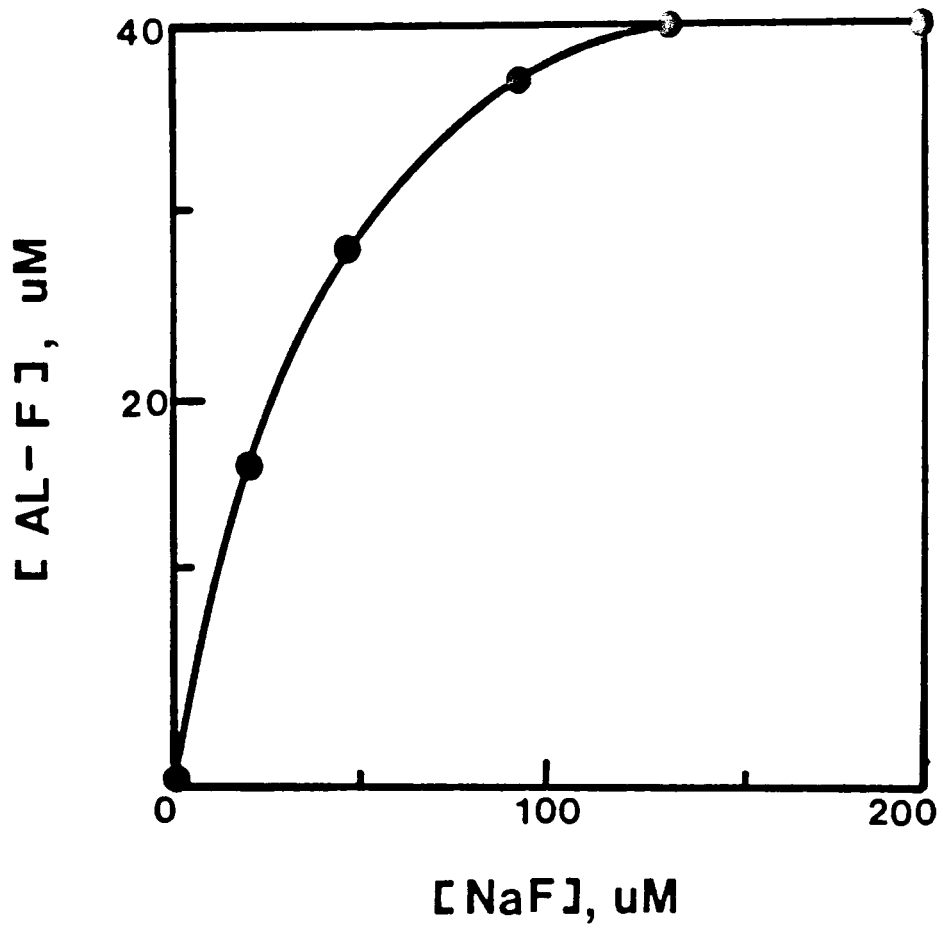
$$\frac{20 \times 10^{-6}}{25 \times 10^{-6} - 20 \times 10^{-6}} = Al_{Kd(E\text{-}Al)} \cdot (1 \times 10^{6})$$

Therefore, $Al_{Kd(E\text{-}Al)} = 4 \times 10^{-6}$ (M).

Effect of modification of G6PD with Acetylsalicylic acid or DEPC on Aluminum Binding

Consistent with the earlier observation (17), modification of one lysine residue of yeast G6PD with acetylsalicylic acid inactivated the enzyme. However, the modification did not affect the stoichiometry of the aluminum binding to the protein (Table 5). One mol of aluminum still bound per mol of the modified enzyme subunit. Therefore it appears that the reactive lysine residue is not directly involved in the aluminum binding site.

Preliminary experiments showed that the aluminum binding to the enzyme induced an spectral changes around



< Fig 4 > Removal of aluminum from enzyme-aluminum complex by NaF. The completely inactivated enzyme (protein concentration 20 uM containing 40 uM of aluminum) was prepared as described in "Experimental Procedures" and incubated with increasing levels of NaF (final concentration 200 uM) in 20 mM Tris/HCl, pH 6.5 at 25 °C for 2 hr in a 1 ml final volume. Aluminum released from the enzyme-aluminum complex was assayed as described in "Experimental Procedures."

< TABLE 5 >

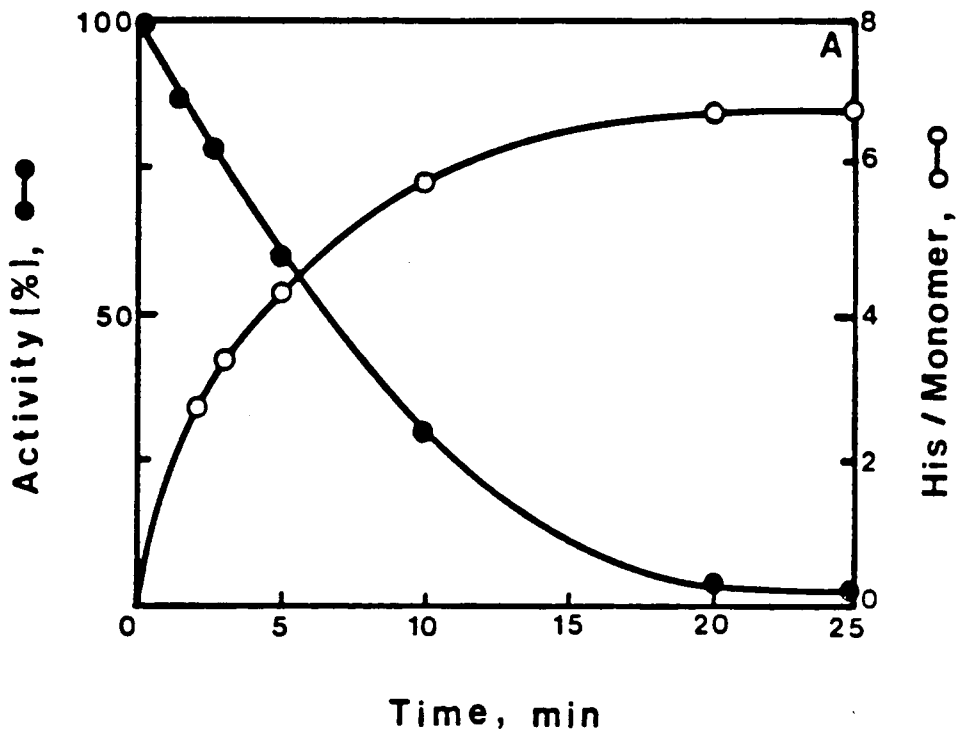
Effects of treatments of G6PD with ASA or DEPC on the enzyme activity and aluminum binding to the enzyme. The DEPC or ASA-modified enzymes were prepared as described in "Experimental Procedures", and the enzyme activities and aluminum contents were measured. The modified enzymes (protein concentration 5 uM) were separately incubated with 50 uM $AlCl_3$ and dialyzed. The enzyme activities and the contents of aluminum in the dialyzed samples were measured.

Treatments	Enzyme Activity (% of control)	[Al]/[subunit]
Enzyme (control)	100	0.07 (0)[a]
Enzyme + Aluminum	0	0.80 (1)
ASA modified enzyme	0	0.07 (0)
ASA modified enzyme + Aluminum	0	0.80 (1)
DEPC modified enzyme	0	0.07 (0)
DEPC modified enzyme + Aluminum	0	0.80 (1)
DEPC modified enzyme in the presence of G6P (2 mM) & Mg^{++} (5 mM	96	0.08 (0)
DEPC modified enzyme in the presence of G6P (2 mM) & Mg^{++} (5 mM) + Aluminum	0	0.80 (1)

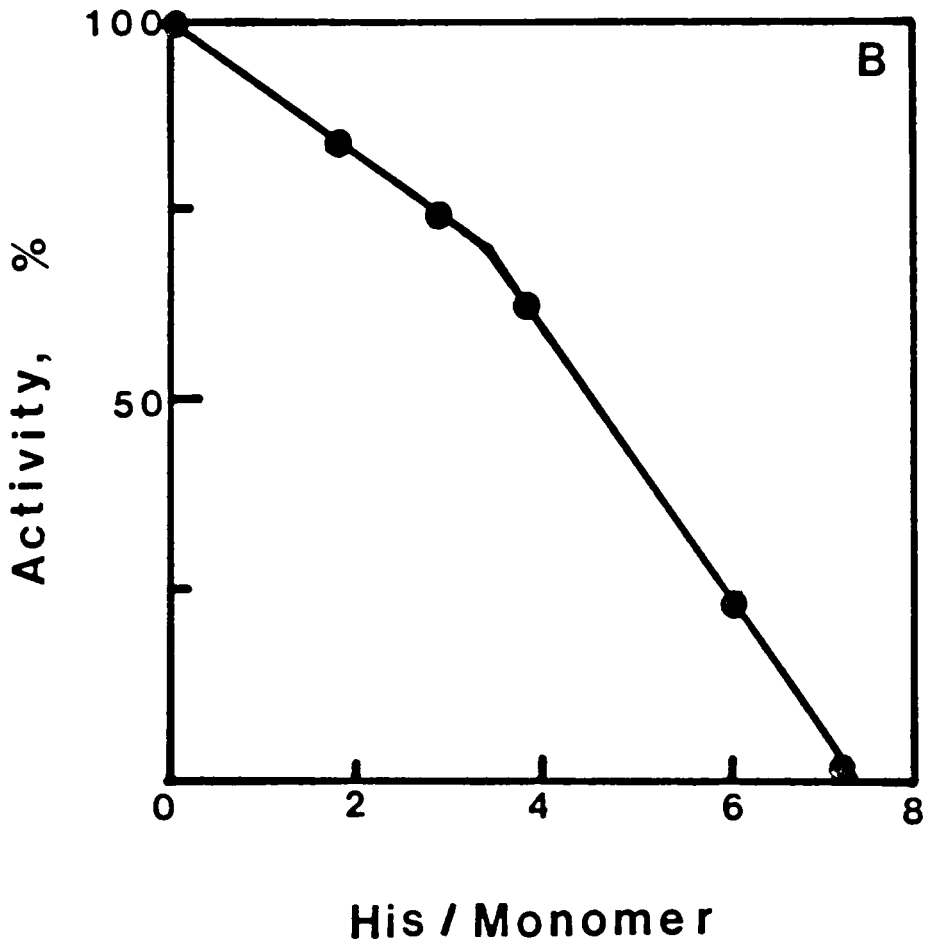
[a] The numbers in parentheses are the nearest integer values.

220 nm. In order to determine whether this was due to a formation of histidine-aluminum complex, diethyl pyrocarbonate (DEPC) was used as a group specific reagent. DEPC specifically reacts with histidine residues in proteins at pH 6.0 to yield an ethoxycarbonyl derivative with a characteristic absorption maximum at 240 nm (23). The result showed the loss of the enzyme activity paralleled the binding of DEPC until 7 mol of DEPC were bound to the enzyme (Fig. 5). Quantitation from the spectral changes at 240 nm showed that the native enzyme and that denatured in 8 M urea contained 7 and 14 histidine residues, respectively (Fig. 6 and Table 6). Domschke et al. (24) showed that G6P and Mg^{++} protected the histidine residue of G6PD from C. utilis against photooxidation. As shown in Fig. 7, DEPC did not inactivate the yeast G6PD in the presence of 2 mM G6P and 5 mM Mg^{++}, and permitted modification of 6 rather than 7 histidine residues (Table 6). Thus, G6P and Mg^{++} protected the catalytically essential histidine residue against DEPC induced inactivation, but either the presence or the absence of these reagents did not protect the enzyme against aluminum inactivation (Table 5).

In a separate experiment, the enzyme was preincubated with 5 mM dithiothreitol (DTT) in 25 mM HEPES, pH 7.0 at 25 ^{o}C



< Fig 5 > Time course of DEPC incorporation into G6PD and inactivation of the enzyme. Enzyme (10 nmole) was incubated at 25 °C with 7 mM DEPC in 25mM HEPES, pH 6.0. At indicated times, aliquots were withdrawn and diluted with a assay mixture for the enzyme activity or with the same buffer for the DEPC-induced spectral changes as described in " Experimental Procedures."



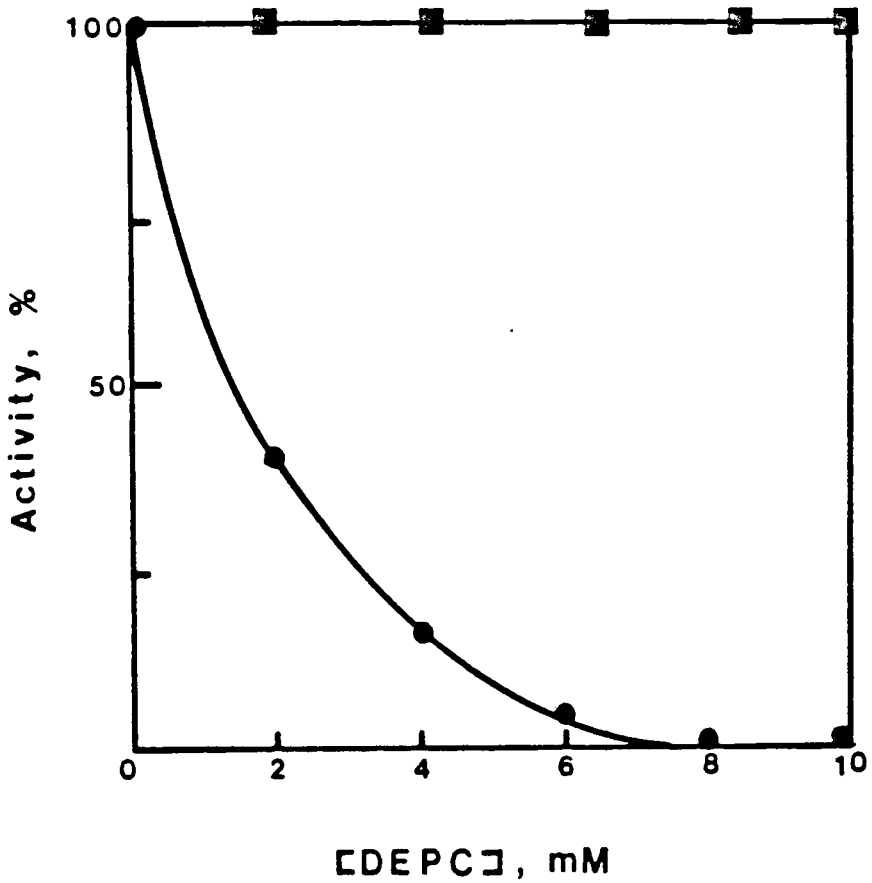
< Fig 6 > Stoichiometry of DEPC incorporation and G6PD inactivation. The inactivation of G6PD is plotted as a function of moles of DEPC incorporated per mol of enzyme subunit. This is a replot of the data shown in Fig 5.

< TABLE 6 >

Labeling of histidine residues of native and denatured G6PD with DEPC. The labeled histidine residues were quantified from the absorbance change at 240 nm as described in "Experimental Procedures." Denaturation of the protein was performed by treating the enzyme with 8 M urea in 25 mM HEPES, pH 6.0 for 30 minutes at 25 ^{o}C.

Treatments	[His]/[Subunit]
Native enzyme	7.1 (7)[a]
Denatured enzyme	14.2 (14)
Native enzyme premixed with 2 mM G6P and 5 mM Mg^{++}	5.8 (6)

[a] The numbers in parentheses are the nearest integer values.

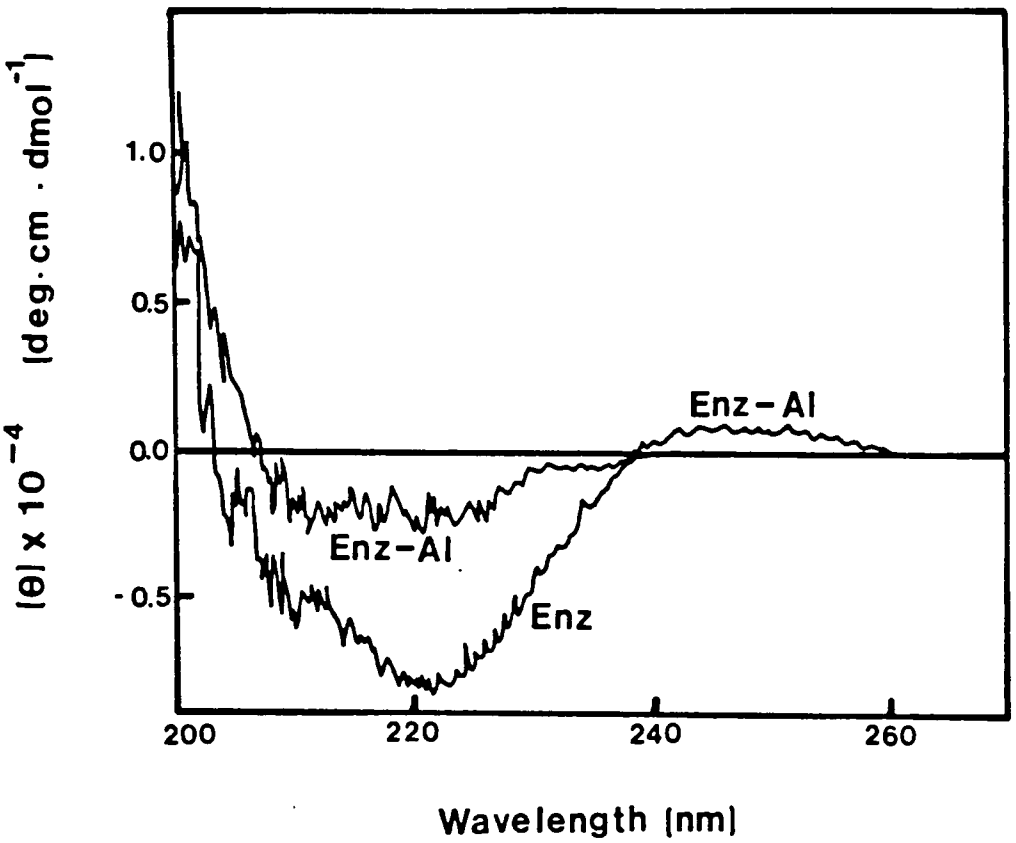


< Fig 7 > Protection of G6PD by G6P and Mg^{++} against DEPC induced inactivation. The enzyme (10 nmole) was treated with increasing concentrations of DEPC in the presence (■) and absence (●) of 2 mM G6P and 5 mM Mg^{++} for 20 minutes at 25 °C in 25 mM HEPES, pH 6.0, and assayed for enzyme activity.

for 15 minutes with or without 50 uM aluminum and the resulting activities were compared with the corresponding controls. The results showed that DTT did not affect the activity of the enzyme or the inactivation by aluminum. Taken together, these data confirm the presence of lysine and histidine, residues at the catalytic site of G6PD (17,24), but excludes them, as well as cysteine residues, from being directly involved in the aluminum-induced inactivation. These data suggest that the aluminum binding site is distinct from the catalytic site, and the inactivation of G6PD by aluminum might be due to the conformational changes induced by the metal ion.

Circular-Dichroism Study of Aluminum Induced Structural Changes

The results of the circular dichroism studies on G6PD-aluminum complex are shown in Fig. 8. As seen, the binding of aluminum to the enzyme induced a reduction in the ordered configuration (α-helix and β-pleated sheet) and an increase in random coil. Spectral signals at 200-250 nm range showed that the conformation of the aluminum-free enzyme was 30 % α-helix, 30 % β-pleated sheet, and 40 % random coil, whereas that of the aluminum-enzyme complex was 20 % α-helix, 15 % β-pleated sheet, and 65 % random coil. Large negative peaks were apparent at 208 and 222 nm,



< Fig 8 > Circular dichroism spectra of G6PD and G6PD-aluminum complex at pH 7 0. (See Text for detail).

respectively. Since random and β-conformations approach to zero at 208 nm, the signal at 208 nm is due to α-helix conformation. Increase in the β turn conformation may be due to the relaxed helical configuration since signal for 3_{10} helix was similar to that of β turn.

DISCUSSION

The results presented here clearly establish the sensitivity of Bakers' yeast G6PD to a low level of aluminum. The sensitivity of the yeast enzyme to aluminum suggests that the inhibition of this enzyme may be significant in aluminum toxicity. The inhibition is more pronounced at acidic pH, and this is consistent with the profound solubility of aluminum at low pH. The suggestion that aluminum functions only at acidic pH (25), therefore, may be a general characteristics of the reactions involving this metal ion.

It should be noted that aluminum concentrations presented in this study are those of aluminum added to the solution and the calculated Kd value of 4×10^{-6} is based on the total concentration of aluminum. The same is true for numerous studies reported by others (14,15,26,27). Solubility of aluminum salts is very high at acidic pH and very low at physiological pH (25). This would explain why

aluminum is better inhibitor of G6PD at acidic pH. The free Al^{3+} concentration rather than the much greater $Al(OH)_4^-$ concentration will be therefore the relevant quantity in assessing ligation and in determining stability constants for such association (25). Therefore, it is highly possible that the aluminum concentration, at which inhibitory effects on enzymatic reaction occur, will actually be much less than those reported in this study as well as in previous studies (9,13-15,27).

The importance of the order of addition of the reagents to produce the observed effects of aluminum on G6PD and the reactivation of the aluminum-inactivated enzyme by citrate or NaF, but not by EDTA, malate, or $NADP^+$ (Fig. 2 & 3) are similar to those observed for hexokinase (15).

The protection of G6PD by $NADP^+$ against aluminum inactivation is of particular interest. A very similar protection was reported for the aluminum binding to RNA polymerase (26) in which preincubation of the RNA polymerase with 0.5 mM aluminum decreased the rate of incorporation of nucleotides by 50 % but when aluminum was premixed with the nucleotides or with the template, there was no inhibition until the aluminum concentration exceeded 2 mM.

Modification of histidine of the G6PD from C. utilis by

photooxidation (24), lysine of the enzyme from L. mesenteroides by pyridoxal-5-phosphate (28), or lysine of the yeast enzyme by acetylsalicylic acid (17) produced inactive enzyme. We chose acetylsalicylic acid and DEPC to modify lysine and histidine residues, respectively, because of the specificity of DEPC (20) and successful application of acetylsalicylic acid to the yeast enzyme (17). The results in this study confirms the role for histidine and lysine residues at the active site but exclude them, as well as the sulfhydryl groups, from being involved in aluminum binding. The possibility of the aluminum binding to the carboxyl groups was not explored. It appears likely that the inactivation may be due to conformational changes induced by aluminum binding to the carboxyl or hydroxyl groups. The results of circular dichroism studies support this possibility. This result shown in Fig. 8 is very similar to that observed for structural changes induced by aluminum in calmodulin (29), showing that Al-calmodulin is a more random, open polypeptide than the structure of Ca-calmodulin based on their EPR resonance studies. Also, aluminum-induced structural changes in calmodulin decrease helical content, increase random coiling, and increase hydrophobic surface expression (11).

The protection of the G6PD by citrate against aluminum

binding is also very similar to previous observation (30), showing that at a molar ratio of 10:1 for [citrate]/ [calmodulin], citrate can prevent aluminum binding to calmodulin as determined by fluorescence and circular dichroism spectroscopy.

Environmental pollution by soluble aluminum has elevated aluminum from a 'harmless' to a 'toxic' metal ion. Indeed, aluminum is one of the major toxicant to plants and animals (1,31). The present report shows that the concentration of aluminum required to produce 50 % inhibition of yeast G6PD is similar to that reported for inhibiting hexokinase from brain (15,32). The resulting reduction in glucose utilization is unfavorable for normal growth of yeast. Such effects are likely to be more damaging to brain, a tissue known to accunulate aluminum. Together with the previous reports on the inhibition of pig brain G6PD by aluminum (33) and aluminum-induced reduction in glucose utilization in rat brain in vitro (34), the results presented here increase the significance of aluminum toxicity in regulation of energy metabolism.

REFERENCES

1. Godbold, D.L., Fritz, E., Huttermann, A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 3888-3892
2. Lai, J.C.K., Guest, J.F., Leung,T.K.C., Lim,L., & Davison, A.N. (1980) Biochem. Pharmacol. 29, 141-146
3. Wong, P.C.L., Lai, J.C.K., Lim, L., & Davison, A.N. (1981) J. Inorganic Biochem. 14, 253-260
4. Lai, J.C.K., Lim, L., & Davison, A.N. (1982) J. Inorganic Biochem. 17, 215-225
5. Macdonald, T.L., Humphreys, W.G., & Martin, R.B. (1987) Science 236, 138-186
6. Jones, T.R., Anthonetti, D.L., & Reid, T.W. (1986) J. Cell. Biochem. 30, 31-39
7. Magour, S., & Maser, H. (1981) Biochem. Soc. Trans. 9, 100-101
8. Northup, J.K., Smigel, M.D., Sternweis, P.C., & Gilman, A.G. (1983) J. Biol. Chem. 258, 11369-11376
9. Cochran, M., Coates, J., & Neoh, S. (1984) FEBS. Lett. 176, 129-132
10. Fleming, J, & Joshi, J.G. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7866-7870
11. Siegel, N., & Haug, A. (1983) Biochim. Biophys. Acta 774, 36-45

12. Marquis, J.K., & Lerric, A.J. (1982) Biochem. Pharm. 31, 1437-1440

13. Huber, C.T., & Frieden, E. (1970) J. Biol. Chem. 245, 3979-3984

14. Altmann, P., Al-Salihi, F., & Butter, K. (1987) N. Engl. J. Med. 317, 80-84

15. Womack, F.C., & Colowick, S.P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5080-5084

16. Kuby, S.A., Wu, J.T., & Roy, R.N. (1976) Biochemistry 15, 1975-1987

17. Jeffery, J., Hobbs, L., & Jornvall, H. (1985) Biochemistry 24, 666-671

18. Larson, E., Howlett, B., & Jagendorf, A. (1986) Anal. Biochem. 155, 243-248

19. Brosset, C., & Orring, J (1943) Sven. Kem. Tidskr. 55, 101-117

20. Ovadi, J., Libor, S., & Elodi, P (1967) Acta. Biochim. Biophys. Acad. Sci. Hung. 2, 455-458

21. Yang, J.T., Wu, C-S.C., & Martinez, H.M. (1986) Methods Enzymol. 130, 208-269

22. Karlik, S.J., Elgavish, G.A., & Eichhorn, G.L. (1983) J. Am. Chem. Soc. 105, 602-609

23. Pradel, L.A., & Kassab, R (1968) Biochim. Biophys. Acta 167, 317-325

24. Domashke, W., Engel, H.J., & Domagk, G.F. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1117-1120

25. Macdonald, T.L., & Martin, R.B. (1988) Trends in Biol. Sci. 13, 15-19

26. Crapper-McLanchlan, D.R., Farnell, B., Galin, H., Karlik, S., Eichhorn, G., & De Boni, U. (1983) in Biological Aspects of Metal and Metal-Related Disease pp. 209-218 Raven Press, New York

27. Lange, A.J., Arion, W.J., Burchel, A. & Burchell, B. (1986) J. Biol. Chem. 261, 101-107

28. Haghighi, B., Flynn, T.G., & Levy, H.R. (1982) Biochemistry 21, 6415-6420

29. Siegel, N., Coughlin, R., & Haug, A. (1983) Biochem. Biophys. Res. Commun. 115, 512-517

30. Suhayda, C.G., & Haug, A. (1984) Biochem. Biophys. Res. Commun. 119, 376-381

31. Havas, M., & Likens, G.E. (1985) Can. J. Zool. 63, 1114-1119

32. Lai, C.K.L., & Blass, J.P. (1984) J. Neurochem. 42, 438-446

33. Cho, S.W., & Joshi, J.G. (1988) FASEB J. 2, A1778, Abs. 8609

34. Johnson, G.V.W., and Jope, R.S. (1986) Toxicology 40, 93-102

35. Kawaguchi, A., & Bloch, K. (1974) J. Biol. Chem. 249, 5793-5800

CHAPTER III

Inhibitory effects of aluminum on Glucose-6-Phosphate Dehydrogenase Isozymes Purified from Human and Pig Brain.

INTRODUCTION

Except during starvation, glucose is virtually the sole energy source for a normal mature brain. It use up to 60 % of the total glucose (1) and 40 % of the total oxygen consumed by the body (2). Metabolically, brain is one of the most active tissues. Hexokinase catalyzes the first committed step in glycolysis and glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step in pentose phosphate pathway. Both enzymes are under various types of metabolic regulation. The brain lacks stores of fuel and glucose-6-phosphatase and. Therefore, the energy demand of the brain depends on a well regulated delivery of glucose and its metabolism by hexokinase and G6PD.

Brains of the patients with Alzheimer's disease have decreased glucose metabolism than the age-matched control (3). Johnson and jope (4) reported that aluminum impaired glucose utilization in the rat brain. One report proposed oxidative stress as a contributing factor in Alzheimer's disease and suggested a role for brain G6PD

in this process (5). Although G6PD from diverse source has been purified (6) and G6PD activity in the brain has been detected (7), the brain enzyme has not been purified and nothing is known about its molecular properties.

Clearly, an alteration in the activity of this enzyme in the brain would profoundly affect the neuronal metabolism. We, therefore, initiated a study of G6PD in the brain. We here report the isolation and characterization of G6PD isozymes of human and pig brain and inhibitory effects of aluminum on the isozymes are described.

EXPERIMENTAL PROCEDURES

Materials

Human brains were obtained from the autopsy unit of The University of Tennessee Memorial Research Center, Knoxville, TN, and pig brains were obtained from Lay Packing Co. Inc., Knoxville, TN. Acrylamide, bisacrylamide, and ammonium persulfate were obtained from Bio-Rad. HEPES, PMS, MTT, G6P, $NADP^+$, EDTA, PMSF, NADP-agarose, Blue-Sepharose CL-6B, NADPH, palmitoyl-CoA, GSSG, glutathione reductase, sucrose, $MgCl_2$, $CaCl_2$, DTT, 6-phosphogluconate, glucose, NAD^+, and galactose-6-phosphate were purchased from Sigma. The molecular weight

standard proteins and Sephadex S-300 were obtained from Pharmacia. Hydroxylapatite was purchased from Cal-Biochem. Aluminum was purchase from Fisher and aluminum standard solution was purchased from Spex Industries.

Enzyme Assay

Activity of G6PD was measured spectrophotometrically (8). The reaction mixture contained 2 umol of G6P, 1 umol of $NADP^+$ in 25 umol HEPES, pH 8.0 and the source of enzyme in a final volume of 1 ml. One unit was defined as the amount of enzyme which reduced 1 umol of $NADP^+$ per 1 minute at 23 ^oC. Protein concentration was determined by the method of Lowry et al. (9).

Purification of Glucose-6-Phosphate Dehydrogenase Isozymes from Human and Pig Brain

Homogenization. All procedures were performed at 0-4 ^oC and all buffer solutions contained 1 mM EDTA and 5 mM DTT unless otherwise indicated. The brains were homogenized with a Waring Blender set on high for 90 s twice in 4 volumes (w/v) of 10 mM Tris, pH 8.0 containing 1 mM EDTA, 5 mM dithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF) (0.1 mg/ml). PMSF was dissolved in isopropyl alcohol at a concentration of 20 mg/ml and the isopropyl alcohol was dissolved in double-deionized water immediately before homogenization. The slurry was filtered through 4 layers of

cheesecloth and centrifuged at 25,400 x g for 30 min. The supernatant was retained and the pellet was discarded.

Ammonium Sulfate Fractionation. Solid ammonium sulfate (209 g/liter) was slowly added into the supernatant to form a 35 % saturation. The mixture was stirred for 1 hr and centrifuged at 25,400 x g for 30 min. The supernatant was brought to 60 % saturation with solid ammonium sulfate (164 g/liter). After 1 h, the mixture was centrifuged at 25,400 x g for 30 min. The pellet was dissolved in 10 mM Tris/HCl, pH 8.0 and dialyzed 4 times for 5 h with 5 liters of the same buffer

NADP-Agarose Affinity Chromatography. The dialyzed sample was passed through an NADP-agarose column (1 x 5 cm) which had previously been equilibrated with 10 mM Tris/HCl, pH 8.0. The column was washed with the same buffer, and the enzyme was eluted with 0.2 mM $NADP^+$ in the same buffer. The enzyme was dialyzed 3 times for 5 h each with 2 liters of 10 mM potassium phosphate, pH 7.0 and used for next step.

Hydroxylapatite Chromatography. The G6PD preparation, from the NADP-agarose column, was passed through an hydroxylapatite column (2 x 30 cm) equilibrated with 10 mM potassium phosphate, pH 7.0. The column was washed with the same buffer and the isozyme I and II were eluted with a step gradient (50 mM and 200 mM potassium phosphate, pH

7.0, respectively). From this step, the two isozymes were separately purified.

Gel Filtration. The isozymes, obtained as above, were precipitated with ammonium sulfate (60 % saturation) and centrifuged at 25.400 x g for 1 h. The precipitate was dissolved in 10 mM Tris/HCl, pH 8.0 and passed through an Sephadex S-300 column (3 x 150 cm) equilibrated with 10 mM Tris/HCl, pH 8.0 containing 0.15 M NaCl. The enzyme was eluted with the same buffer.

Blue Sepharose CL-6B Affinity Chromatography. The active fractions from the above step were pooled and passed through an Blue Sepharose CL-6B column equilibrated with 10 mM Tris/HCl, pH 8.0. The column was washed with the same buffer and the enzyme was eluted by 0.2 mM $NADP^+$ in the same buffer. The enzyme was concentrated with ammonium sulfate as above and dialyzed against 10 mM Tris/HCl, pH 8.0 containing 0.1 M NaCl, 5 mM DTT, and 50 % glycerol and stored at - 20 ^oC.

Subcellular Fractionation of G6PD in Pig Brain

Fresh pig brain (50 g) was homogenized in 0.32 M sucrose and fractionated by differential centrifugation by the method of Lai and Blass (10). Nuclear, mitochondrial, microsomal, and cytosolic fractions were assayed for G6PD and hexokinase.

Gel Electrophoresis Polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis were performed according to the method of Davis (11). The subunit molecular weight was determined on SDS-polyacrylamide gel according to the method of Weber and Osborn (12). Nondenaturing gradient gel electrophoresis was performed by the method of Kuonen (13) and the molecular weight of the enzyme was determined according to the method of Bryan (14).

Enzyme Activity Staining for Gels Activity staining for gel electrophoresis was performed by the method of Nakatsuji and Miwa (15) except that ammediol/glycine (41 mM ammediol and 40 mM glycine), pH 9.4 for upper buffer and ammediol/HCl (63 mM ammediol and 50 mM HCl), pH 8.2 for lower buffer were used. The G6PD specific staining solution contained 1.6 mM G6P, 1.6 mM $NADP^+$, 0.14 mM phenazine methosulfate, 0.56 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, and 1 mM $MgCl_2$ in 100 mM Tris/HCl, pH 8.0.

Peptide Map of Tryptic Digest

Approximately 300 ug of protein in distilled water was denatured with 1 volume of cold 20 % trichloroacetic acid, centrifuged at 5000 x g for 5 minutes. The precipitated protein pellet was washed with 10 % ethanol and water. The

pellet was dissolved in 50 ul of 0.1 M NH_4HCO_3, pH 8.5 containing 0.1 mM $CaCl_2$. An aliquot of L-tosylamido-2-phenylethyl chloromethyl keton-trypsin (Worthington) was added to a final ratio of 1:100 (trypsin/protein, w/w). The mixture was incubated at 37 ^oC for 1 h, and another aliquot of trypsin (1:100, w/w) was added. The sample was then digested overnight at 37 ^oC. Peptide analysis was performed with Vydac C-18 RPS column (10 um) equipped with Waters 510 HPLC system with 0 to 60 % CH_3CN containing 0.1 % trifluoroacetic acid.

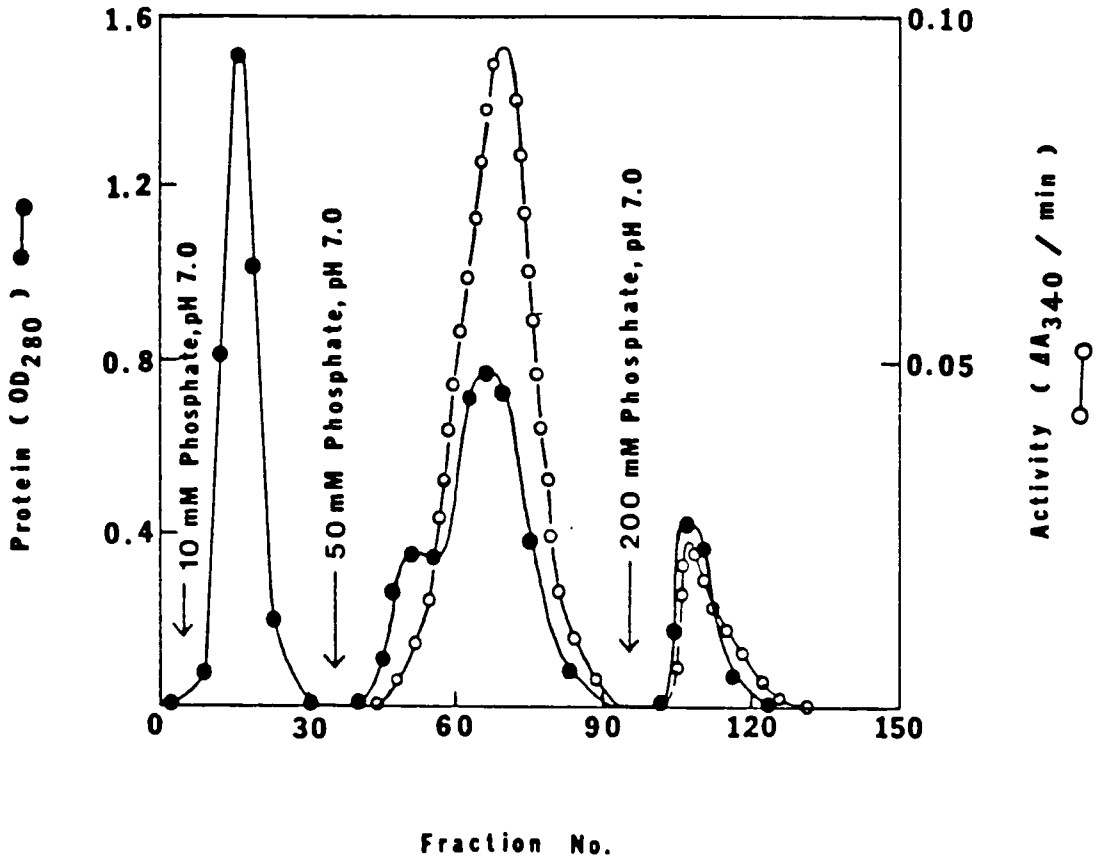
Amino Acid Analysis

Hydrolysis was routinely carried out at 110 ^{o} for 21 h in 6 N HCl and 0.01 M β-mercaptoethanol in glass tubes sealed under vacuum. The analyses were performed on a Beckman 121-M amino acid analyzer with a single column system.

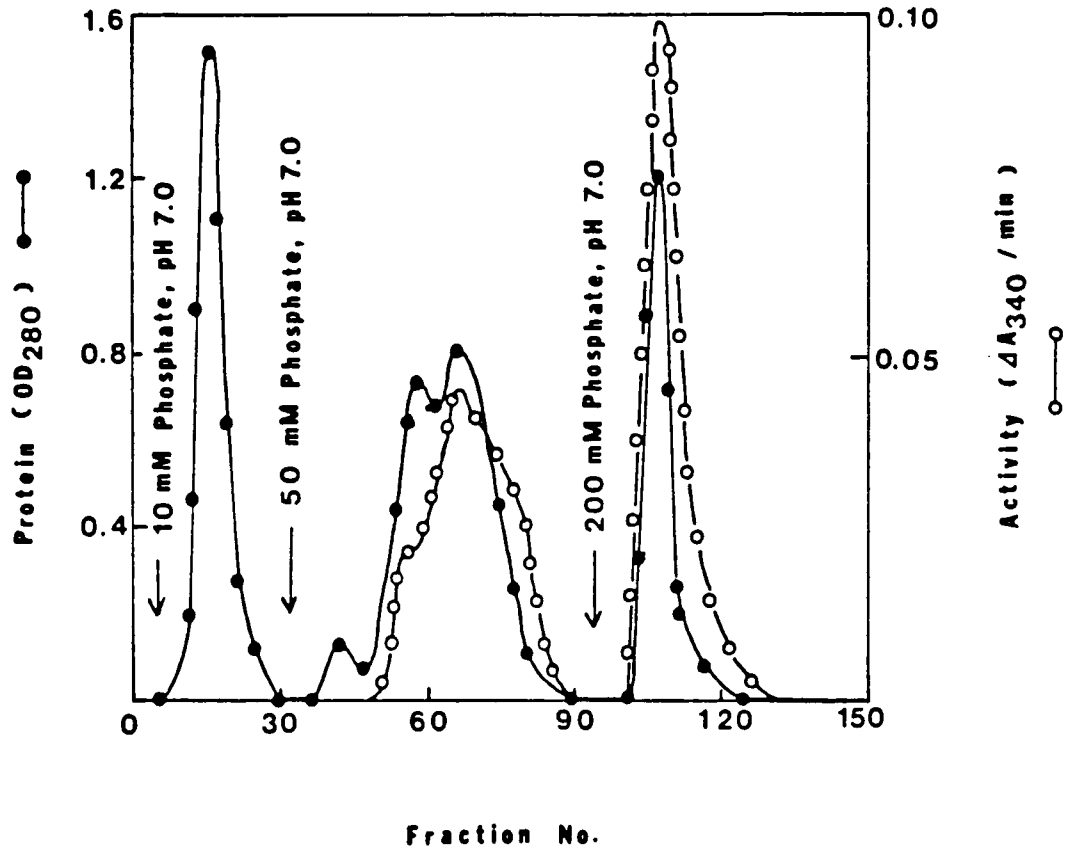
RESULTS

Localization of G6PD Isozymes in Pig Brain

Consistent with the elution pattern from hydroxylapatite column (Fig. 9 and 10), the pig brain contained more isozyme II than isozyme I. This proportion was reversed in human brain (Table 7 and 8). To determine whether the two isozymes originated from different subcellular fractions,



< Fig 9 > Separation of human brain G6PD isozymes on hydroxylapatite column. The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After loading the samples, the column was washed with the same buffer and the isozymes were separately eluted by step gradient of 50 mM and 200 mM potassium phosphate buffer, pH 7.0 at the points indicated as arrows.



< Fig 10 > Separation of pig brain G6PD isozymes on hydroxylapatite column. All conditions are the same as Fig 9.

< Table 7 >

Purification of glucose-6-phosphate dehydrogenase isozymes from human brain.

	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Yield (%)
Crude extract	22,425	897	0.04	100
35 - 60 % $(NH_4)_2SO_4$	13,705	1,031	0.08	115
NADP-Agarose	20	583	29	65
Hydroxylapatite				
Isozyme I	9	449	50	50
Isozyme II	3	47	16	5
Sephadex S-300				
Isozyme I	4	432	108	48
Isozyme II	1.5	35	23	4
Blue Sepharose CL-6B				
Isozyme I	1.8	378	210	42
Isozyme II	0.5	26	52	3

< Table 8 >

Purification of glucose-6-phosphate dehydrogenase isozymes from pig brain.

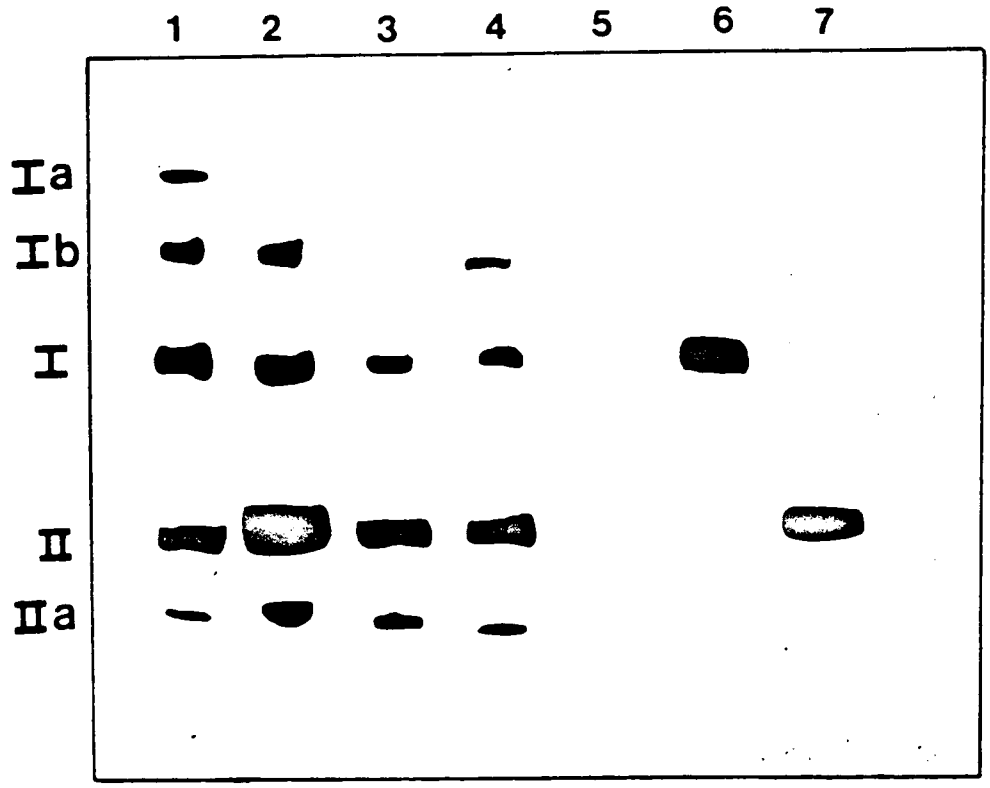
	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Yield (%)
Crude extract	57,566	1,727	0.03	100
35 - 60 % $(NH_4)_2SO_4$	32,400	2,106	0.07	122
NADP-Agarose	43	1,174	27	68
Hydroxylapatite				
Isozyme I	6	104	17	6
Isozyme II	15	829	55	48
Sephadex S-300				
Isozyme I	4	92	23	5
Isozyme II	7	725	104	42
Blue Sepharose CL-6B				
Isozyme I	1	50	50	3
Isozyme II	3	604	201	35

fresh pig brain was fractionated by differential centrifugation as described in "Experimental Procedures." The distribution of G6PD activity was 72 % in cytosol, 24 % in mitochondria, and 4 % in microsome. The mitochondrial and cytosolic fractions were chromatographed on hydroxylapatite column as before. Two active peaks emerged for mitochondrial and cytosolic fractions and relative proportions of the two peaks in both fractions were similar to those shown in Fig. 10. The existence of both isozymes in mitochondria as well as cytosol was confirmed by activity staining for G6PD on polyacrylamide gel of the cytosolic and mitochondrial fractions (Fig. 11). Therefore, the purification described under the "Experimental Procedures" was performed on the entire homogenates without separating the mitochondrial and cytosolic fractions.

Characterization of Pure G6PD Isozymes in Human and Pig Brain

Table 9 summarizes various properties of human and pig brain G6PD isozymes.

Molecular Weight. Gel filtration studies showed that both isozymes from human and pig brain had a molecular weight of about 220,000 (Fig. 12), which dissociated into a single 57,000 molecular weight subunit upon SDS-polyacrylamide gel electrophoresis (Fig. 13). Therefore each isozyme is a



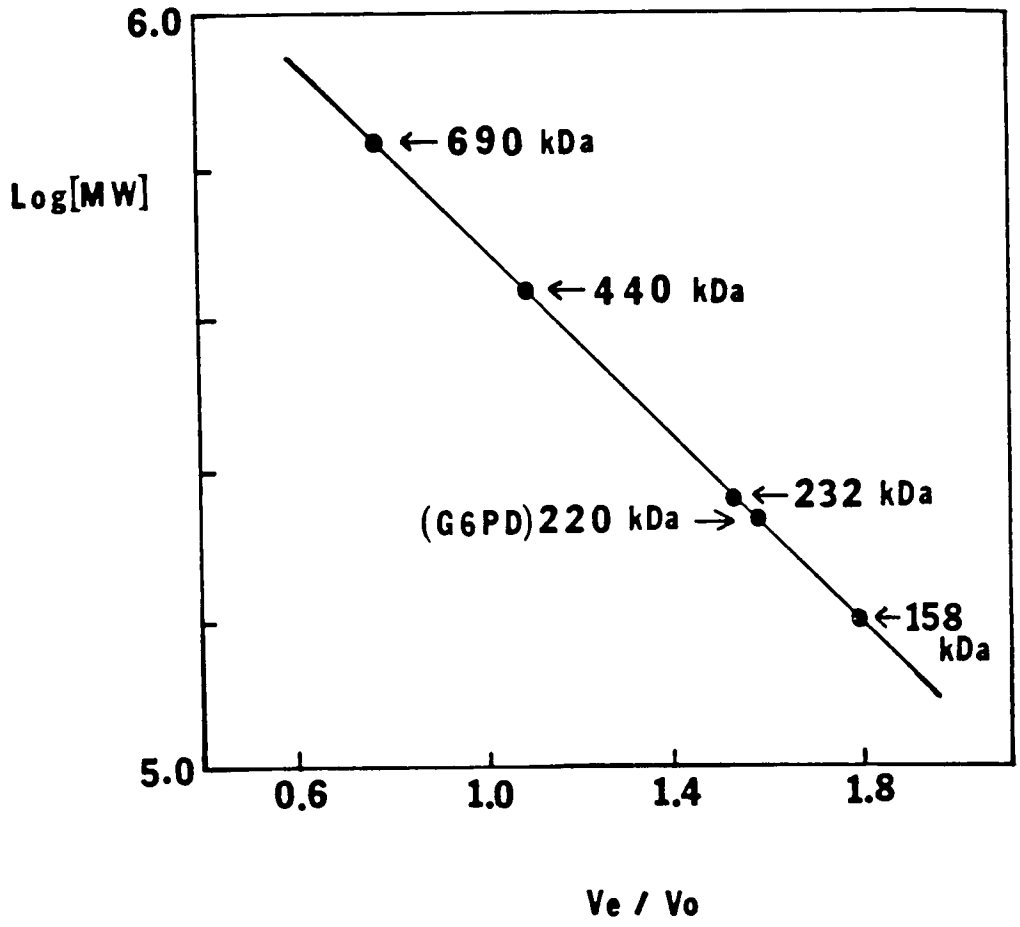
< Fig 11 > Distribution of G6PD isozymes in pig brain subcellular fractions. Gel electrophoresis on 7 % polyacrylamide was performed with 4 % stacking gel at 4 °C as described in "Experimental Procedures." After electrophoresis, the gel was stained for activities of G6PD by incubating the gel in the dark with reaction mixtures. Lane (1) homogenates, (2) after NADP-agarose affinity column, (3) crude mitochondrial fraction, (4) crude cytosolic fraction, (5) homogenization buffer (100 mM Tris/HCl, pH 7.4, containing 0.32 M sucrose), (6) purified G6PD isozyme I, and (7) purified G6PD isozyme II.

< Table 9 >

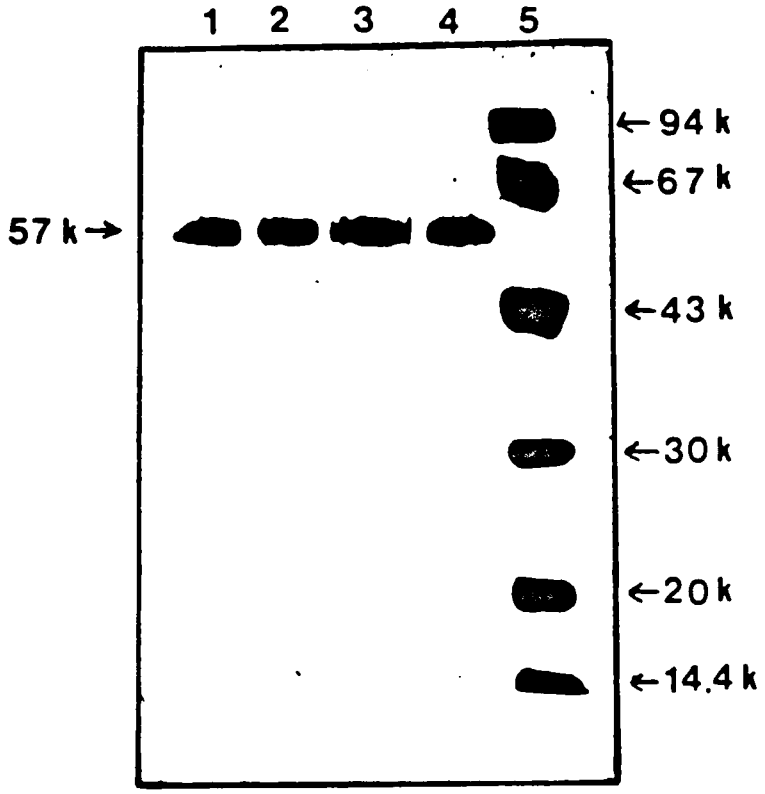
Properties of glucose-6-phosphate dehydrogenase isozymes.

	Human Brain Isozymes		Pig Brain Isozymes	
	I	II	I	II
Molecular Weight (kDa)				
Intact enzyme (tetramer)	220	220	220	220
Palmitoyl CoA-treated enzyme (dimer)	110	110	110	110
SDS-treated enzyme (monomer)	57	57	57	57
Km				
$NADP^+$ (uM)	7	8	6	8
NAD^+ (mM)	--	--	--	--
Glucose-6-P (uM)	61	180	167	56
Galactose-6-P (mM)	47	31	46	51
Glucose (mM)	--	--	--	--
6-Phosphogluconate (uM)	--	864	--	279
Ki				
NADPH (uM)				
$NADP^+$(*)	30	18	15	25
G6P(**)	100	40	31	90
ATP (mM)				
$NADP^+$(**)	3.5	3.8	4.2	3.6
G6P(*)	1.3	0.9	1.2	1.5
Effects of(***)				
Palmitoyl-CoA (40 uM)	50	28	40	43
NADH (0.1 mM)	100	98	99	100
PCMB (1 mM)	72	75	70	70
EDTA (1 mM)	95	96	93	97
Ca^{++} (1 mM)	140	142	148	138
Mg^{++} (1 mM)	160	155	162	160
Ca^{++} & Mg^{++} (1 mM)	170	160	168	165
Cu^{++} (1 mM)	100	100	100	100
Zn^{++} (1 mM)	50	50	40	45
Heat (50 ^{o}C, 5 min)	33	35	28	34

(*) Competitive inhibition
(**) Noncompetitive inhibition
(***) Activity expressed in percentage of untreated control.



< Fig 12 > Estimation of molecular weight of human and pig brain G6PD isozymes by gel filtration with Sephadex S-300. The enzyme was applied to a column (3 x 150 cm) was equilibrated with 10 mM Tris/HCl, pH 8.0 and eluted with the same buffer.



< Fig 13 > Estimation of subunit molecular weight of human and pig brain G6PD isozymes on SDS-polyacrylamide gel electrophoresis. Gel electrophoresis on 10 % polyacrylamide was performed with 4 % stacking gel. After electrophoresis, the gels were fixed, stained with Coomassie blue G-250 in methanol:acetic acid:water (5:1:5). Lane 1 and 2 are human brain G6PD isozymes I and II, respectively, lane 3 and 4 are pig brain G6PD isozymes I and II, respectively, and lane 5 is molecular weight marker proteins.

tetramer.

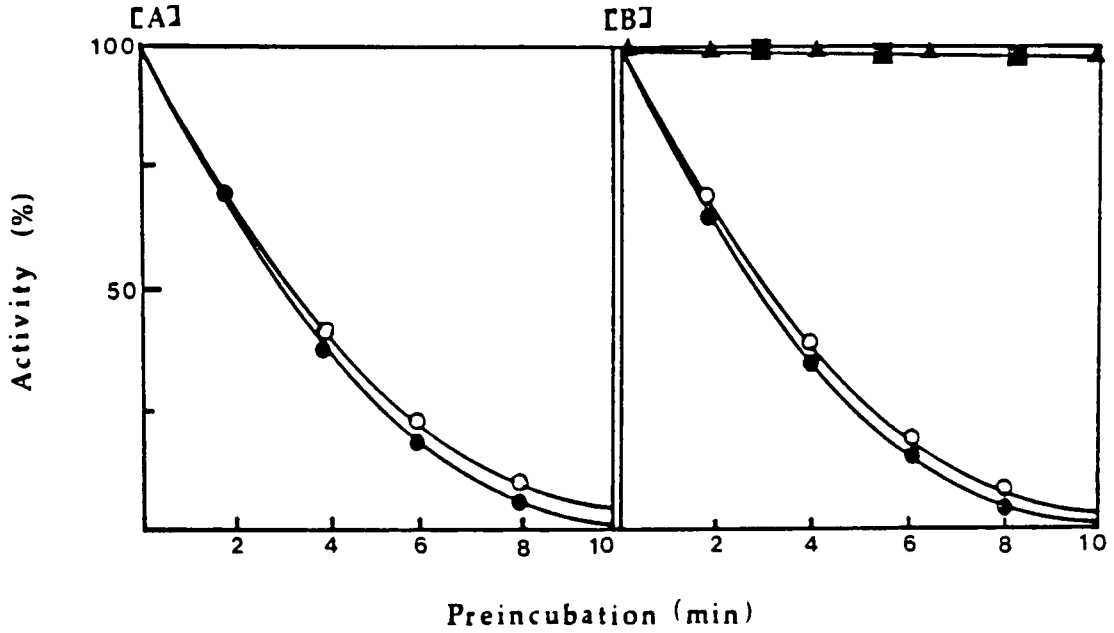
Substrate Specificity of The G6PD Isozymes. Brain G6PD isozymes are specific for $NADP^+$ with very similar Km values (6 to 8 uM). However, the Km values for G6P vary from about 60 uM for human isozyme I and pig isozyme II to about 180 for human isozyme II and pig isozyme I. The G6PD isozymes have much less affinity for galactose-6-phosphate (Km = 30 to 51 mM). None of the G6PD isozymes have an affinity for glucose or NAD^+. This is different from hexose-6-phosphate dehydrogenase (H6PD), which has an affinity for both glucose and NAD^+ as well as for glucose-6-phosphate and $NADP^+$ (16-18). An especially noteworthy and unexpected observation is that the 6-phosphogluconate, the product of G6PD reaction, was also a substrate for isozyme II. The Km values were 864 uM and 279 uM for human and pig enzyme, respectively, which is about 5 times higher than that for glucose-6-phosphate. The specific activities of the isozyme II with 6-phosphogluconate were 4 times less than those with G6P (14 and 48 unit per mg of protein for human and pig brain isozyme). No such activity could be detected for human or pig brain isozyme I.

Effect of Metal Ions and Heat on G6PD Isozymes. None of the isozymes required additional metal ion for activity, and 1 mM EDTA had no effect on the enzyme activity. However, 1 mM

Ca^{++} or Mg^{++}, independently or together, activated the isozymes. Mg^{++} was a slightly better activator (160 %) than Ca^{++} (140 %). 1 mM Cu^{++} had no effect but 1 mM Zn^{++} or 1 mM PCMB inactivated the isozymes by 50 %. Heating of the protein (0.2 mg) at 50 ^{o}C for 5 min in 10 mM Tris/HCl, pH 8.0 inactivated all the isozymes by about 70 %.

Preincubation of the isozymes with aluminum inactivated the enzymes (Fig. 14). The inactivation was time dependent and complete under the conditions in Fig. 14. However, the 6-PGD activity of the isozyme II was insensitive to aluminum (Fig. 14). Therefore all of the inhibitory effects of aluminum presented here are on the G6PD activity of the enzyme.

When 3 nmole of the enzymes were incubated with 100 fold molar excess amounts of aluminum, one mol of aluminum was bound per mol of the enzyme sununit (Table 10). None of the protein-bound aluminum was dissociated by dialysis or gel filtration as shown in Table 10. The dissociation constants for G6PD-aluminum complexes were calculated to be 4 uM for human isozyme I, 2 uM for pig isozyme I and Human isozyme II, and 3 uM for pig isozyme II (Table 11), using NaF, a well known aluminum chelator (Fig. 15), as described in Chapter II.



< Fig 14 > Effects of aluminum on human and pig brain G6PD isozymes. The isozymes were incubated with 5 uM aluminum chloride in 25 mM HEPES, pH 7.0 at 23 °C. At different times, an aliquot was withdrawn and assayed for enzyme activities. All other assay conditions were the same as described in "Materials and Methods." The results are expressed as a percentage of the controls.
(A) G6PD activities of the human (o) and pig brain (●) isozyme I. (B) G6PD activities of the human (●) and pig brain (o) isozyme II. 6-PGD activities of pig and human brain isozyme II are shown with squares and triangles, respectively.

< TABLE 10 >

Binding of aluminum to G6PD isozymes in human and pig brain.

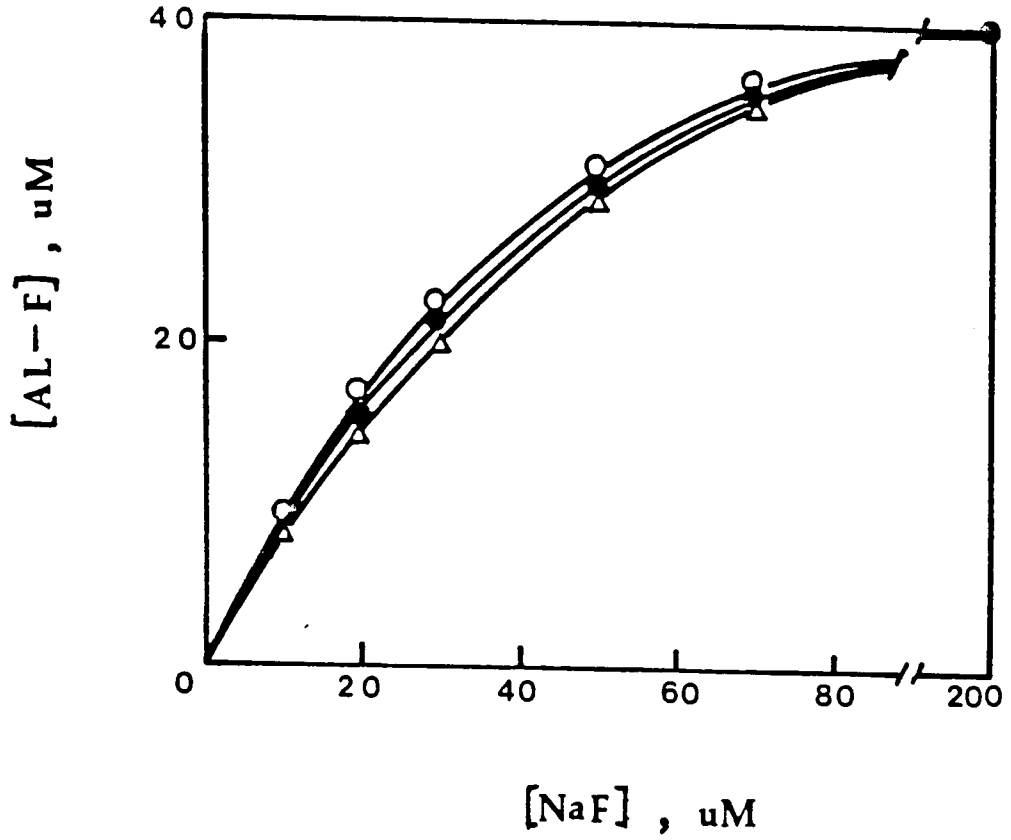
Treatments	[Al]/[subunit]*			
	Human		Pig	
	I	II	I	II
Sephadex G-25	0.9	0.8	1.0	0.9
Dialysis against 0.2 mM EDTA in 10 mM Tris/HCl, pH 7.0	0.9	0.8	0.9	0.8

* Calculated based on the subunit molecular weight of 57,000.

< TABLE 11 >

Dissociation constants for G6PD-aluminum complex

	Isozyme I		Isozyme II	
	Human	Pig	Human	Pig
$Al_{Kd(E-Al)}$ (uM)	4	2	2	3



< Fig 15 > Removal of aluminum from enzyme-aluminum complex by NaF. The completely inactivated enzyme-aluminum complex (10 uM of protein containing 40 uM of aluminum) was prepared as described in Chapter II and incubated with increasing levels of NaF (up to 200 uM) in a final volume of 0.1 ml with 20 mM Tris/HCl, pH 7.0 at 23 ^{o}C for 2 h. Open circle indicates human brain isozyme I and closed circle indicates pig brain isozyme II. Open triangle indicates human brain isozyme II or pig brain isozyme I.

The inhibitory effect of aluminum was abolished by some chelators (Table 12). An aliquot of G6PD-aluminum complex was mixed with the chelators such as citrate, NaF, malate, EDTA, $NADP^+$, apoferritin, and apotransferrin. After 20 minutes, aliquots were withdrawn and assayed for enzyme activity in the standard reaction mixture. The enzyme activity was recorvered by citrate, NaF, and apotransferrin, but not by malate, $NADP^+$, EDTA, and apoferritin (Table 12). In a related experiment, aluminum was first preincubated with the chelators for 20 minutes and tested as a source of aluminum available to inhibit the enzyme (Table 13). The results showed that the rate of chelation of aluminum by malate was the slowest because a significant amount of free aluminum was retained in a solution to produce 50 % inhibition even after 20 minutes of incubation, By contrast, only a small amount of free aluminum seemed to have remained in the solution in the presence of citrate, EDTA, NaF, and apotransferrin as shown by the recovery of the full enzyme activity after 20 minutes of incubation. Therefore, the order of addition of reagents determined the effects of the chelators to protect the G6PD isozymes against aluminum inactivation. This difference is especially striking in the case of EDTA and $NADP^+$ which are poor reactivators under the condition in

< Table 12 >

Reactivation of aluminum-inactivated G6PD isozymes. G6PD isozymes (2 nmole) were incubated with 50 nmole $AlCl_3$ in 50 mM HEPES for 30 min at 23 ^{o}C, dialyzed, and incubated in the same buffer for 5 hours at 4 ^{o}C with 10 nmoles of apotransferrin or apoferritin, or for 20 minutes at 23 ^{o}C with 2 umole of the other effectors. The results are expressed as a percentage of the untreated control.

Treatments	Activity (%)			
	Human		Pig	
	I	II	I	II
(1) Enzyme	100	100	100	100
(2) Enzyme + Al	4	3	5	2
(3) (2) + Citrate	94	93	92	94
(4) (2) + NaF	92	93	90	93
(5) (2) + $NADP^{+}$	6	4	5	4
(6) (2) + Malate	3	5	4	3
(7) (2) + EDTA	5	6	5	4
(8) (2) + Mg^{++} and Ca^{++}	5	6	5	4
(9) (2) + Apotransferrin	82	87	88	85
(10) (2) + Apoferritin	34	36	33	31

< Table 13 >

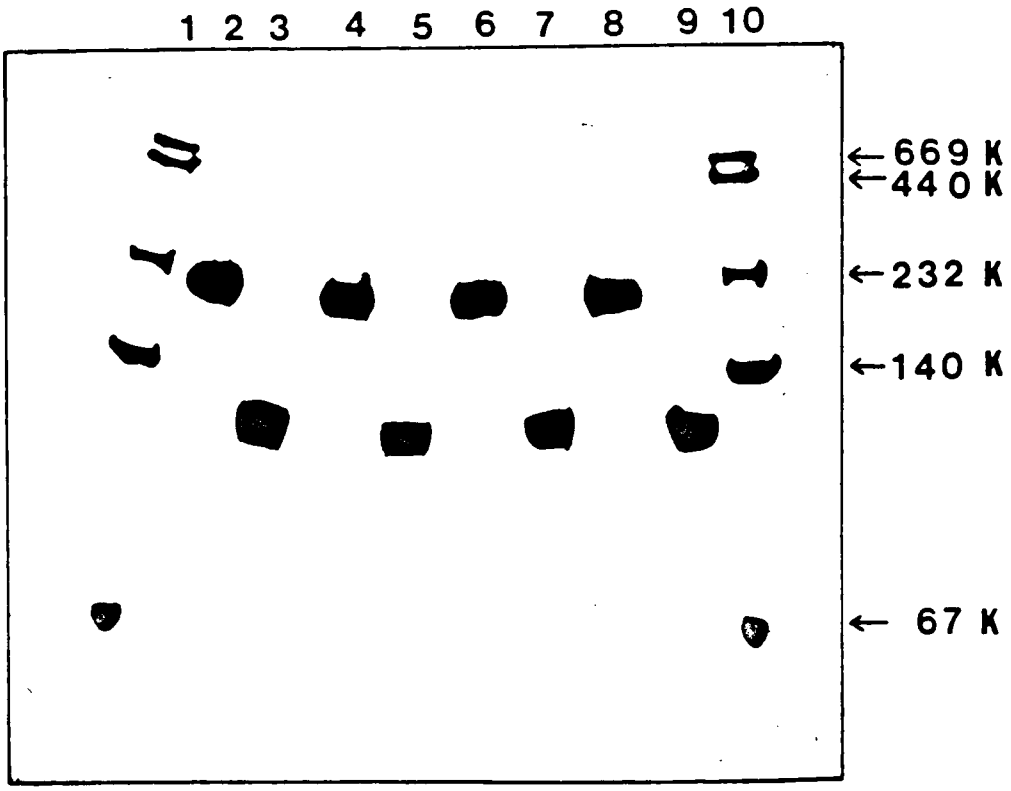
Protection of the G6PD isozymes in human and pig brain by several substances from aluminum inactivation.
Preincubation mixtures contained 25 umole of HEPES, pH 7.0, 5 nmole of aluminum chloride, and 200 nmole of the indicated chelators (except apotransferrin and apoferritin, 20 nmole) and kept at 23 ^{o}C for 20 minutes. The reaction was started by the addition of enzyme and assay mixture containing 2 umole G6P and 1 umole $NADP^{+}$ in 25 umole of HEPES, pH 7.0. The results are expressed as a percentage of the untreated control.

Treatments	Activity (%)			
	Human		Pig	
	I	II	I	II
Control (Enzyme only)	100	100	100	100
(Buffer + Al) + Enzyme	4	3	5	2
(Citrate + Al) + Enzyme	96	98	98	98
(NaF + Al) + Enzyme	98	97	96	97
($NADP^{+}$ + Al) + Enzyme	90	93	92	91
(Malate + Al) + Enzyme	48	55	47	50
(EDTA + Al) + Enzyme	98	99	97	98
(Mg^{++} + Ca^{++} + Al) + Enzyme	5	7	4	5
(Apotransferrin + Al) + Enzyme	95	105	98	99
(Apoferritin + Al) + Enzyme	65	62	61	63

Table 12. Even though Mg^{++} and Ca^{++}, independently or together, activated the isozymes, they did not protect or reverse the inactivation of the isozymes by aluminum.

Inhibition of G6PD Isozymes by ATP. ATP has been reported to be a regulator of G6PD (19-22). As shown in Table 9, the brain G6PD isozymes were inhibited by ATP. The inhibition was competitive with respect to G6P (Ki = 3.5 to 4.2 mM) and noncompetitive with respect to $NADP^{+}$ (Ki = 0.9 to 1.5 mM). The inhibition was more prominent at pH 6.5 than at pH 8.0, and ATP was a much poorer inhibitor of the brain G6PD isozymes when assayed in the presence of 5 mg Mg^{++} than assayed in its absence (data not shown).

Dissociation of Tetrameric Enzymes into Dimeric forms by Palmitoyl-CoA. In 10 mM Tris/HCL, pH 8.0, both isozymes from either source were inactivated by palmitoyl-CoA. In 5 minutes, up to 70 % of the activity was abolished at 40 uM palmitoyl-CoA and 90 uM palmitoyl-CoA abolished all of the activity. Molecular weights of the palmitoyl-CoA inactivated isozymes were 110,000 as determined by nondenaturing linear gradient gel electrophoresis (Fig. 16). Therefore, the palmitoyl-CoA induced inactivation was due to the dissociation of an active tetramer into an inactive dimer. These data are similar to those obtained for G6PD from rat liver (8) and yeast (23).



< Fig 16 > Dissociation of tetrameric G6PD isozymes in human and pig brain into dimers by palmitoyl-CoA. Nondenaturing gel electrophoresis was performed on 5 to 20 % linear gradient polyacrylamide with 4 % stacking gel at 150 voltage for 16 hr, and the gel was stained with Coomassie blue dye as indicated in Fig. 13. Each isozymes were incubated with 4 mM palmitoyl-CoA in 0.5 M Tris/HCl, pH 6.8 for 30 min at 23 ^{o}C before applied to the gel. Lane (1) and (10) are molecular weight marker proteins, (2) and (4) are intact human brain G6PD isozymes I and II, respectively, (6) and (8) are intact pig brain isozymes I and II, respectively, (3) and (5) are palmitoyl-CoA treated human brain isozymes I and II, respectively, and (7) and (9) are palmitoyl-CoA treated pig brain isozymes I and II, respectively.

Inhibition of G6PD Isozymes by NADPH and De-inhibition by GSSG and Glutathione Reductase. NADPH inhibited the brain G6PD isozymes competitively with respect to $NADP^+$ (Ki = 15 to 30 uM) and noncompetitively with respect to G6P (Ki = 31 to 100 uM) (Table 9). Neither NAD^+ or NADH affected any of the brain enzymes. The direct influence of yeast glutathione reductase on de-inhibition of the brain G6PD isozymes by GSSG is shown in Table 14. The inhibition was abolished by the addition of yeast glutathione reductase and GSSG. However the inhibition of the G6PD isozymes by NADPH was not overcome by GSSG when assayed alone, indicating that the regulation of the brain G6PD isozymes in vitro by GSSG may be directly mediated by reoxidation of NADPH catalyzed by glutathione reductase.

Amino Acid Analysis and Composition Divergence

The amino acid composition of the brain G6PD isozymes in human and pig brain (Table 15) shows considerable homologies between the G6PD isozymes. However, some differences were apparent. The largest difference was in the range of glutamic acid contents from 52 in the human brain isozyme II and pig brain isozyme I to 63 in pig brain isozyme II. For more objective comparison of the amino acid compositions between the isozymes, the composition divergence (D) was calculated by the method of Harris and

< Table 14 >

Effect of glutathione reductase on the reactivation of glucose-6-phosphate dehydrogenase isozymes by GSSG. Enzyme was added to a reaction mixtures containing 50 mM Tris/HCl, pH 7.4, 3 mM $MgCl_2$, 20 uM $NADP^+$, and 1.5 mM G6P. Other additions include 70 uM NADPH, 100 uM GSSG, and glutathione reductase as requested.

Treatments			Brain G6PD Isozymes			
			Pig		Human	
			I	II	I	II
G6PD	NADPH (-)	GSSG (-)	100	100	100	100
		GSSG (+)	99	100	98	99
	NADPH (+)	GSSG (-)	25	25	31	28
		GSSG (+)	24	25	33	29
G6PD + GSSGrase	NADPH (-)	GSSG (-)	98	99	98	102
		GSSG (+)	105	100	98	100
	NADPH (+)	GSSG (-)	26	25	27	26
		GSSG (+)	125	130	118	124

< Table 15 >

Comparison of amino acid composition of human and pig brain glucose-6-phosphate dehydrogenase isozymes.

Amino Acid Composition	Residues / Subunit molecule			
	Human		Pig	
	I	II	I	II
Asx	62.8	57.6	62.2	58.0
Thr	21.7	24.1	23.2	23.4
Ser	25.3	27.6	26.2	26.7
Glx	55.2	51.8	52.4	63.0
Pro	29.5	27.7	29.2	30.5
Gly	33.7	37.5	37.0	37.0
Ala	28.7	34.4	32.5	32.3
Val	32.9	36.1	36.4	35.7
Met	9.6	10.6	9.6	9.2
Ile	25.9	22.8	24.6	22.5
Leu	47.2	45.8	48.7	45.2
Tyr	21.4	23.4	21.8	22.3
Phe	29.9	32.0	29.2	28.7
Lys	43.7	42.4	44.4	39.1
His	9.4	8.8	8.6	8.4
Arg	23.4	22.3	22.6	22.8

(1) All numbers are expressed as mean values of three separate analyses.
(2) Data have been calculated based on a subunit molecular weight of 57,000.

Teller (24). Composition divergence is defined as

$$D = [\Sigma (X_{i,A} - X_{i,B})^2]^{1/2}$$

where $X_{i,A}$ is the mole fraction of amino acid i in protein A and $X_{i,B}$, the same amino acid in protein B. This value is a measure of the divergence of amino acid composition. Table 16 shows the results of such calculations. The lowest composition divergence was seen between human G6PD isozyme II and pig G6PD isozyme I, and the highest value between pig G6PD isozyme I and pig G6PD isozyme II. The values obtained from these comparisons were very similar, indicating extensive homologies between the isozymes.

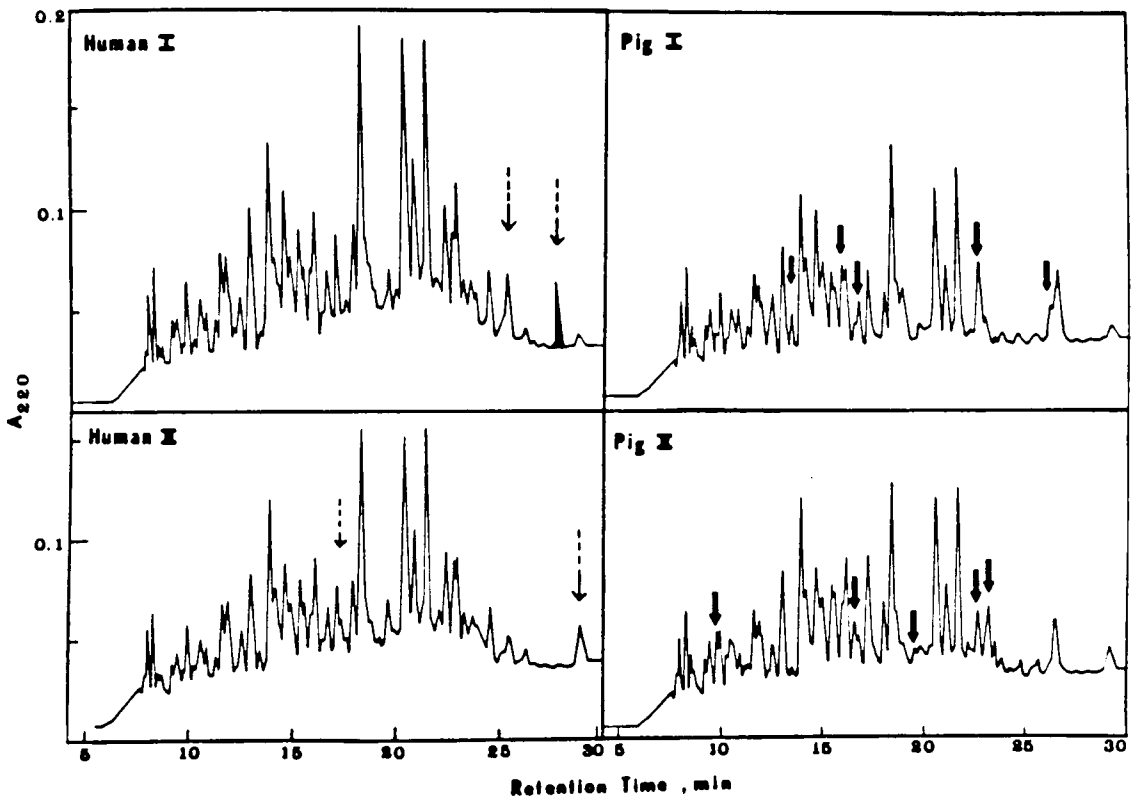
Peptide Map of Tryptic Digest of Two Forms of Brain G6PD Isozymes

Fig. 17 compares the finger print maps of the tryptic digest of the proteins. The peptides were separated by HPLC as described in "Experimental Procedures." Extensive homologies in the hydrophilic regions and some differences in the hydrophobic regions are especially noteworthy. The dashed arrows indicate dissimilarities between human G6PD isozyme I and II. The dark arrows indicate dissimilarities between pig G6PD isozymes I and II.

< Table 16 >

Composition divergence (D) of human and pig brain glucose-6-phosphate dehydrogenase isozymes. Composition divergence was calculated based on amino acid composition shown in Table 11 (See text for detail).

	Human (I)	Human (II)	Pig (I)	Pig (II)
Human (I)	--	0.023	0.015	0.025
Human (II)		--	0.014	0.025
Pig (I)			--	0.026
Pig (II)				--



< Fig 17 > Comparison of peptide map of tryptic digest of human and pig brain G6PD isozymes. For conditions, see text.

DISCUSSION

G6PD activity staining for the polyacrylamide gel electrophoresis of mammalian tissue homogenates reveals several isoforms of the enzyme (26-32). All have a subunit molecular weight between 60 to 67 kDa (30-32). It has been suggested that some of the isoforms are molecular weight isomers varing in the number of subunits on the basis of the results of gel filtration (30) and gel electrophoresis (31). Our data establishes that both G6PD isozyme I and II from human and pig brain are tetramers but not not molecular weight isomers. All five bands were visualized using G6P as a substrate. The activities of the remaining three peaks (Ia,Ib, and IIa in Fig. 11) may be due to the molecular weight isomers or the nonspecific hexose-6-phosphate dehydrogenase. This possibility remains to be established.

The specific activities and the relative amounts of the two isozymes varied with the species. The human brain contains more of isozyme I and four times higher specific activity than the isozyme II. The opposite results were obtained for the pig brain. Such differences in the specific activities of G6PD have been reported with liver and erythrocyte enzyme (8,30,33-39). Since 1 mM Mg^{++}

activated the activities for all four G6PD preparations by about 160 %, the specific activities for isozyme I and II shown in Table 1 and 2 would be 336 and 83 units/mg of protein for human brain isozyme I and II, respectively, and 80 and 322 for pig brain isozyme I and II, respectively.

The extensive homologies between the peptide maps of tryptic digest (Fig. 17) and the similar heat stability (Table 9) suggest that the intra and inter species differences in the relative concentrations and specific activities of the isozymes are not the isolation artifacts but may represent the in vivo situation.

Hydroxylapatite chromatography of the mitochondrial and cytosolic fractions from fresh pig brain yielded the two G6PD peaks and their relative proportions were similar to those observed in Fig. 10. Taken together, the results in Fig. 10 and 11 suggest that both mitochondria and cytosol contain more than two G6PD isozymes. Only two of them were purified to homogeneity. These pure isozymes I and II are $NADP^+$ specific and prefer G6P as a substrate (Table 9). The remaining G6PD-like activity (bands Ia, Ia, IIa in Fig. 11) may be due to a nonspecific hexose-6-phosphate dehydrogenase which is active with various substrates and NAD^+ as well as G6P and $NADP^+$. But this possibility remains to be established.

One of the interesting results is the activity of isozyme II with 6-phosphogluconate. Only isozyme II from both sources appears to be active with 6-phosphogluconate as a substrate. This may be metabolically significant, especially if it is specifically localized in the neuronal tissue.

The intracellular localization of G6PD is yet uncertain. While the hexose-6-phosphate dehydrogenase is reported to be restricted to microsomes (7,27,28), the G6PD is reported to be localized in mitochondria and in cytosol (39-45). The techniques for the detection of G6PD also gives conflicting results; differential centrifugation shows equal distribution of G6PD between mitochondria and cytosol but histochemical studies show its presence only in mitochondria (43).

Regardless of the source, activity of G6PD from diverse origin is under various stringent metabolic control. In this respect, brain isozyme I and II from both sources are very similar (Table 9).

Table 15 and 16 compares the amino acid composition between the G6PD isozymes. As seen, they are very similar. The D values obtained in the cross comparison of the human and pig brain G6PD isozymes show very high homologies between the isozymes. In all these calculations, the values

for cysteine and tryptophan were neglected. As pointed out by Marchalonis and Weltman (25), these two amino acids constitute only a small fraction of most proteins, and thus the effects of these amino acids on the parameter (D) would be insignificant. Consistent with these data, the peptide maps of the tryptic digest of the proteins show that the G6PD isozymes from human and pig brain are very similar (Fig. 17). Most of the differences reside in the hydrophobic peptides (retention time after 20 minutes). Further work is essential to characterize the unique peptide in human isozyme I (shaded dark in Fig.17). Based on the arginine and lysine contents, the expected peaks for the peptides were 67. Under the conditions in Fig. 17, at least 60 major peaks were detected. therefore, the native enzyme appears to be composed of four identical polypeptide chains. It is likely that the G6PD isozymes are homologous proteins derived from a common ancestral gene.

In vivo, multienzyme complexes such as pyruvate dehydrogenase complex (1) or polypeptides with multienzyme function as observed in fatty acid synthetase (1) permit more efficient utilization of the products of the preceding reaction. In view of this, the unexpected finding of the 6-PGD activity, observed exclusively in the isozyme II from human and pig brain, is of particular

interest. Brain is the most specialized organ. The utilization of glucose in the brain is nonuniform and is determined by the physiological need in respond to a given stimulus (46). It is tempting to speculate that the isozyme II, because of its bifunctional activity, permits more efficient utilization of glucose and therefore, may be localized in specific loci in the brain.

Transferrin binds 2 mol of aluminum per mol of protein and competitive equilibrium binding studies show that the aluminum and iron binding sites are identical (47). Consistent with these findings, Putterill and Gardner (48) reported that transferrin relieved the inhibitory effect of aluminum on the activity of cAMP-dependent phosphodiesterase stimulated by Ca^{++}-calmodulin and aluminum-induced structural changes in calmodulin were reduced by transferrin. The reactivation or protection of the G6PD isozymes by transferrin against aluminum inactivation presented here may be physiologically significant. Transferrin, as well as ferritin, is one of the constituents of the neuronal tissue and their ability to bind aluminum (47) may expand role for transferrin in metal toxicity including aluminum toxicity in the brains.

The protection of the G6PD by citrate against aluminum inactivation is also very similar to previous observation

reported by Suhayda and Haug (49), showing that at a molar ratio of 10:1 for [citrate]/[calmodulin], citrate can prevent aluminum binding to calmodulin as determined by fluorescence and circular dichroism spectroscopy.

The results presented here clearly establish the sensitivity of the G6PD isozymes in human and pig brain to a low level of aluminum and increase the significance of aluminum toxicity in degrading energy metabolism.

REFERENCES

1. Stryer, L. (1981) Biochemistry, 2 nd Ed. Freeman and Co.
2. Ames, B.N. (1983) Science 221, 1256-1264
3. Benson, F. (1983) in Biological Aspects of Alzheimer's Disease, Cold Spring Harbor Laboratory, NY, pp 309
4. Jojnson, G.V.W., and Jope, R.S. (1986) Toxicology 40, 93
5. Martin, R.N., Harper, C.G., Stokes, G.B., and Masters, C.L. (1986) J. Neurochem. 46, 1042-1045
6. Levy, H.R. (1979) Adv. Enzymol. 48, 97-192
7. Levy, H.R. (1961) Biochem. Biophys. Res. Commun. 6, 49-53
8. Dao, M.L., Watson, J.J., Delaney, R., and Johnson, B.C. (1979) J. Biol. Chem. 254, 9441-9447
9. Lowry, O.H., Roseburgh, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
10. Lai, J.C.K., and Blass, J.P. (1984) J. Neurochem. 42, 438-446
11. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427
12. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

13. Kuonen, D.R., Roberts, P.J., and Cottingham, I.R. (1986) Anal. Biochem. 153, 221-226

14. Bryan, J.K. (1977) Anal. Biochem. 78, 513-519

15. Nakatsuji, T., and Miwa, S. (1981) Anal. Biochem. 112, 52-54

16. Oak, K., Takahashi, T., and Hori, S.H. (1981) Biochim. Biophys. Acta 662, 318-325

17. Takahashi, T., and Hori, S.H. (1978) Biochim. Biophys. Acta 524, 262-276

18. Ropson, I.J., and Powers, D.A. (1988) J. Biol. Chem. 263, 11693-11703

19. Yoshida, A. (1973) Science 179, 532-537

20. Wessels, G., and Buddecke, E. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 569-575

21. Veloso, D., Guynn, R.W., Oskarsson, M., and Veech, R.L. (1973) 248, 4811-4819

22. Avigad, G. (1966) Proc. Natl. Acad. Sci. USA. 56, 1543-1547

23. Kawaguchi, A., and Bloch, K. (1974) J. Biol. Chem. 249, 5793-5800

24. Harris, C.E., and Teller, D.C. (1973) J. Theol. Biol. 38, 347-362

25. Marchalonis, J.J., and Weltman, J.K. (1971) Comp. Biochem. Physiol. 38B, 609

26. Hori, S.H., and Matsui, S. (1967) J. Histochem. Cytochem. 15, 530-534

27. Hori, S.H., and Matsui, S. (1968) J. Histochem. Cytochem. 16, 62-63

28. Hori, S.H., and Noda, S. (1971) J. Histochem. Cytochem. 19, 299-303

29. Hori, S.H., and Yonezawa, S. (1972) J. Histochem. Cytochem. 20, 804-810

30. Winberry, L., and Holten, D. (1977) J. Biol. Chem. 252, 7796-7801

31. Holten, D. (1972) Biochim. Biophys. Acta 268, 4-12

32. Kanji, M.I., Toews, M.L., and Crapper, W.R. (1976) J. Biol. Chem. 251, 2255-2257

33. Hizi, A., and Yagil, G. (1974) Eur. J. Biochem. 45, 189-200

34. Kelley, D.S., Watson, J.J., Mack,D.O., and Johnson, B.C. (1975) Nutr. Rep. Intl. 12, 121-135

35. Matsuda, T., and Yugari, Y. (1967) J. Biochem. 61, 535-540

36. Cohen, P., and Rosemeyer, M.A. (1969) Eur. J. Biochem. 8, 1-17

37. Yoshida, A. (1969) J. Biol. Chem. 241, 4966-4976

38. Yoshida, A. (1967) Biochem. Genetics 1, 81-99

39. Watanabe, A., and Taketa, K. (1973)
Arch. Biochem. Biophys. 158, 43-52
40. Bagdasarian, G., and Hulanicka, D. (1965)
Biochim. Biophys. Acta 99, 367-369
41. Barquer, N.Z., and McLean, P. (1972) Biochem. Biophys.
Res. Commun. 46, 167-174
42. Barquer, N.Z., and Sochor, M., and McLean, P. (1972)
Biochem. Biophys. Res. Commun. 47, 218-226
43. Razumovskaya, N.I., Pleskov, V.M., and Perova, T.L.
(1970) Biochemistry 35, 162-167
44. Yamada, K., and Shimazono, N. (1961)
Biochim. Biophys. Acta 54, 205-206
45. Zaheer, N., Tewari, K.K., and Krishnan, P.S. (1967)
Arch. Biochem. Biophys. 120, 22-34
46. Sokoloff, L (1981) Neurosci. Res. Prog. Bull. 19, 159
47. Putterill, J.J., and Gardner, R.C. (1988) Biochim.
Biophys. Acta 964, 137
48. Cochran, M., Coates, J., and Neoh, S. (1984) FEBES 176,
129
49. Suhayda, C.G., and Haug, A. (1984) 119, 376

CHAPTER IV

Ribonuclease Inhibitor from Pig Brain: Purification, Characterization, and Direct Spectrophotometric Assay

INTRODUCTION

The alkaline RNase of brain homogenates is primarily localized in the soluble fraction (1) and higher activities are present in neuronal rather than in glial nuclei (2). RNase activity is reduced by the presence of a bound inhibitor, a 50 to 60-kDa protein, which is present in the cytosolic fraction of eukaryotic cells (3-5).

The mechanism of interaction between RNase inhibitor and intracellular RNase has been implicated ina number of important cellular processes including RNA degradation (6) and protein biosynthesis (7). Recent reports indicate that in post-mortem cerebral tissue from Alzheimer's disease patients the RNA content is reduced, suggesting either reduced transcription or increased degradation of RNA (8). It has been suggested that the changes in the RNA concentration are associated with a significant increase in alkaline RNase activity as a result of an abnormal RNase-inhibitor complex (9). Furthermore the observed decrease of protein synthesis in brains from the patients of

Alzheimer's disease may be partly related to an enzyme-inhibitor alteration that affects RNA levels and activity (9).

A better understanding of the molecular properties of the RNase inhibitor from brain appears essential. We present here a purification and a preliminary characterization of the RNase inhibitor from pig brain. Also described is the development of a simplified spectrophotometric method to assay the inhibitor. Unlike the previously reported discontinuous assay method (10), the present method is sensitive enough to be used with crude extract or with 35 to 60 % ammonium sulfate precipitate, as well as with pure inhibitor.

EXPERIMENTAL PROCEDURES

Materials

RNase A and RNase B from bovine pancreas, RNase T1 from Aspergillus oryzae, RNase U1 from Ustilago sphaerogena, RNA from Bakers' yeast, and RNase inhibitor from human placenta were purchased from Sigma.

Assay for RNase and for its inhibitor using RNA as a substrate

RNase activity on yeast RNA was determined by a modified procedure of the spectrophotometric method of Kunitz (11).

A 1 ml quartz cuvette (1 cm path length) contained 0.7 ml of 10 mM of HEPES, pH 8.0, 0.1 ml of water containing 40 ug of RNA, and water to give a final volume of 1 ml after RNase was added. The reaction was initiated by the addition of the 10 ng of RNase. The increase in absorbance at 260 nm was recorded using a Beckman DU-7 spectrophotometer at an absorption scale of 0.0000 to 0.1000. The amount of activity observed varied with the type of buffer used. Although the RNase was the most active in potassium phosphate buffer, the reaction rates were linear only during the first one minute. All subsequent assays were performed in 10 mM HEPES, pH 8.0 at 25 ^{o}C, for which initial reaction velocities were linear at least for the first three minutes and were also directly proportional to the RNase concentration.

Inhibitory potency of pig brain RNase inhibitor during its purification steps and of the commercially available RNase inhibitor from human placenta were quantified by the ability to inhibit the hydrolysis of RNA by 10 ng of RNase. One unit of inhibitor was defined as the amount required to inhibit the activity of 5 ng of RNase A by 50 %. The assay reaction was initiated by addition of a mixture of 10 ng of RNase and up to 10 ul of the source of RNase inhibitor. The mixture was preincubated in 10 mM HEPES, pH 8.0 for 2

minutes at 25 ^{o}C in a final volume of 0.2 ml. The amount of inhibitor was determined from the percentage inhibition of the 10 ng of RNase contained in the assay mixture. The inhibition of RNase at different concentrations of inhibitor was linear with respect to the inhibitor concentration in the assay medium. Thus, an aliquot of the inhibitor solution resulting in 25 to 75 % inhibition, served to quantify the concentration of inhibitor in the sample by linear interpolation.

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed using the method of Weber and Osborn (12).

Protein determination

Protein concentration was determined according to Lowry et al.(13), with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

Amino acid analysis

Hydrolysis was routinely carried out at 110 ^{o}C for 21 h in 6 N HCl and dimethylsulfoxide in glass tubes sealed under vacuum. The analyses were performed on a Beckman 121-M amino acid analyzer with a single column system. Tryptophan was determined in samples treated with 3 N mercaptoethane sulfonic acid for 21 hr at 110 ^{o}C.

RESULTS AND DISCUSSION

Purification of The RNase Inhibitor

Unless otherwise indicated, all operations were performed at 2-4 ^{o}C and in the presence of 5 mM DTT and 1 mM EDTA. RNase inhibitor from pig brain was purified by the method of Takahashi et al.(14) with some modification. Fresh pig brains, obtained from a local slaughter house, were homogenized in a Waring blender for two cycles of 90 seconds each in 2 volumes of 20 mM Tris/HCl, pH 7.5 containing 5 mM DTT, 1 mM EDTA, and 0.25 M sucrose. The homogenate was centrifuged at 16,000 x g for 30 minutes and the supernatant, containing the RNase inhibitor, was brought to 35 % saturation with solid ammonium sulfate (20.9 g/100 ml). The suspension was gently stirred for one hour and centrifuged at 16,000 x g for 30 minutes. The supernatant was brought to 60 % saturation with solid ammonium sulfate (16.4 g/100 ml), left standing for one hour, and then centrifuged at 16,000 x g for 30 minutes. The precipitate was dissolved in a minimal volume of 20 mM Tris/HCl, pH 7.5, stirred for 30 minutes, and then dialyzed twice for 4 hours against 50 volumes of the same buffer.

The dialyzed fraction was then centrifuged at 48,000 x g for 1 hour. The supernatant was applied to a DEAE-cellulose

column (40 x 4 cm) equilibrated with 20 mM Tris/HCl, pH 7.5. After loading, the column was washed with this buffer containing 0.15 M NaCl until the unadsorbed protein was eluted. The adsorbed proteins were eluted with 600 ml of a linear gradient of increasing salt concentration from 0.15 M to 0.3 M NaCl in 20 mM Tris/HCl, pH 7.5.

The fractions containing inhibitor were pooled and dialyzed twice for 6 hours against 50 volumes of 45 mM potassium phosphate buffer, pH 6.4. The dialyzed fraction was applied to a hydroxylapatite column equilibrated with 45 mM potassium phosphate buffer, pH 6.4, containing 0.15 M NaCl. After washing with the same buffer, the bound protein was eluted with 300 ml of a linear gradient from 45 mM to 150 mM potassium phosphate buffer, pH 6.4 containing 0.15 M NaCl.

The fractions containing the inhibitor were pooled and brought to 60 % saturation with solid ammonium sulfate (39.0 g/100 ml). The precipitate was dissolved in 45 mM potassium phosphate, pH 6.4 containing 0.15 M NaCl, centrifuged, and the supernatant was applied to a gel filtration column (Sephadex G-100, 1.5 x 100 cm) equilibrated with 45 mM potassium phosphate, pH 6.4. The column was eluted with the same buffer. The fractions containing the inhibitor were pooled and the protein was

precipitated with ammonium sulfate as before. The precipitate was dissolved and the above gel filtration was repeated. The active fractions were pooled, dialyzed for 20 hours at 4 ^{o}C against 45 mM potassium phosphate, pH 6.5 containing 1 mM EDTA, 5 mM DTT, 0.15 M NaCl, and stored at -15 ^{o}C in the same buffer containing 15 % (v/v) glycerol.

The purification of the inhibitor (Table 17) was monitored by the method presented here.

Molecular Weight

The molecular weight of the native RNase inhibitor estimated by gel filtration was about 50,000 (Fig. 18). The molecular weight of the inhibitor complexed with RNase A (Mr. 13,700) was about 62,000, as estimated by gel filtration. This value is consistent with a stoichiometry of 1:1 for the binding of inhibitor to RNase A. The final preparation of the inhibitor was 91 % pure in native gel electrophoresis as determined by microdensitometer (Fig. 19).

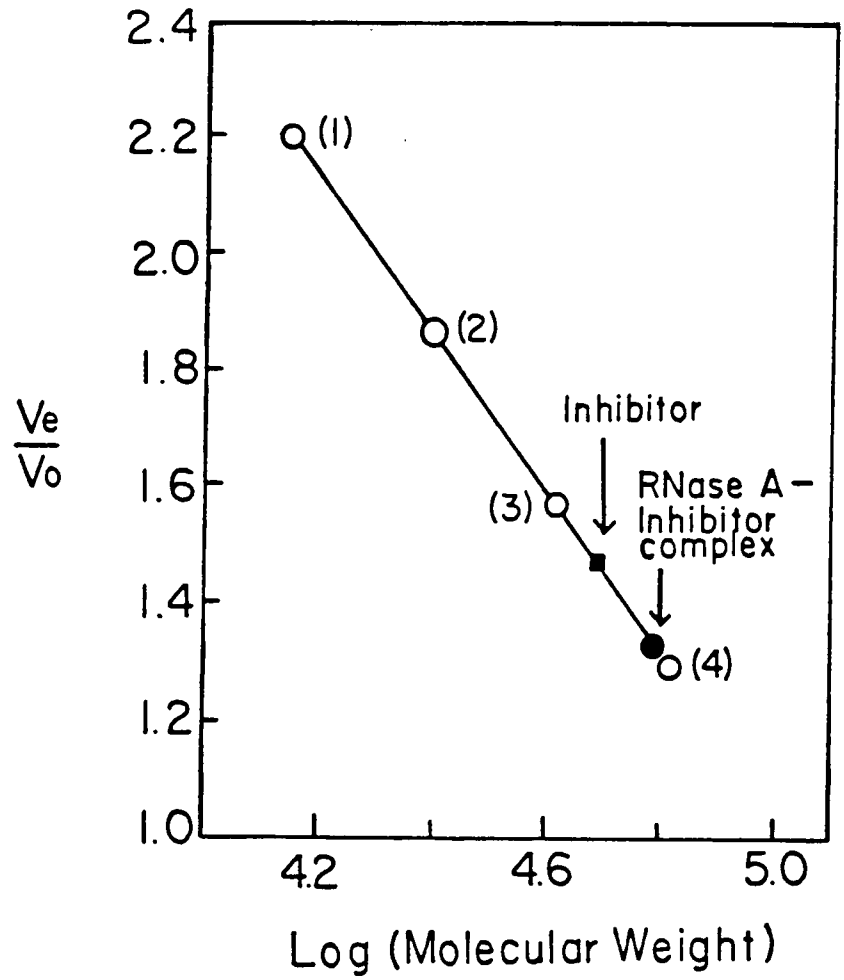
Kinetics of Inhibition

The inhibition of RNase A by the inhibitor in 10 mM HEPES, pH 8.0 is noncompetitive and has a K_i for the inhibitor of 1 nM, as determined by the double reciprocal plot, 1/v against 1/s (Fig. 20). The noncompetitive nature of the inhibitor was expected because increasing

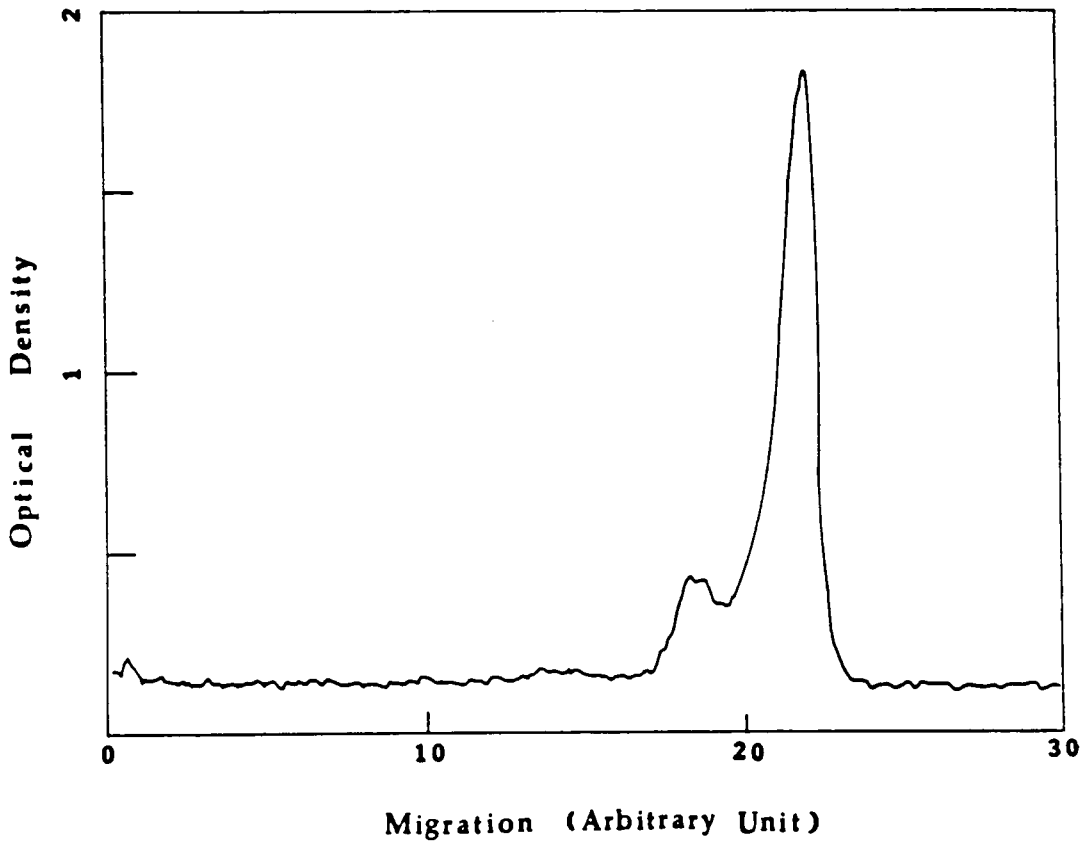
< TABLE 17 >

Purification of RNase inhibitor from pig brain

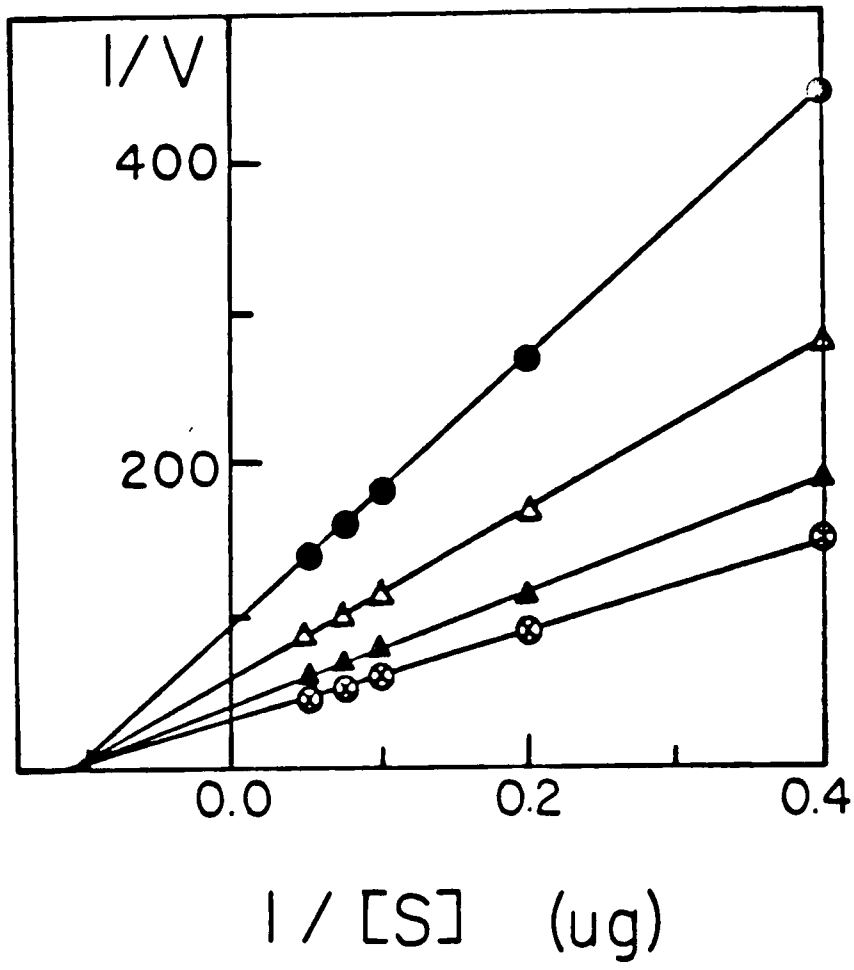
Step	Protein (mg)	Inhibitor (units)	Specific activity (units/mg)	Yield (%)
Crude extract	24,000	5,520	0.2	100
35 to 60 % Ammonium sulfate	5,400	4,030	0.8	73
DEAE-cellulose	211.4	3,256	15	59
Hydroxylapatite	60.1	2,704	45	49
Sephadex G-100 (1st)	9.2	1,656	180	30
Sephadex G-100 (2nd)	3.6	1,104	307	20



< Fig 18 > Estimation of the molecular weight of the RNase inhibitor from pig brain by gel filtration on a Sephadex G-100 column. The column was eluted with 45 mM potassium phosphate, pH 6.5 in 1 mM EDTA, 5 mM DTT, 15 % (v/v) glycerol, and 0.15 M NaCl at 4 °C. The column was calibrated with the following protein standards: (1) RNase A (Mr. 13,700), (2) α-chymotrypsin (Mr. 25,000), (3) ovalbumin (Mr. 43,000), and (4) bovine serum albumin (Mr. 67,000).



< Fig 19 > Native polyacrylamide gel electrophoresis of the inhibitor. Gel electrophoresis in 7 % polyacrylamide (1.5 mm thick) was performed with a 4 % stacking gel. After electrophoresis, the gels were fixed, stained with Coomassie blue in methanol:acetic acid:water (5:1:5), destained, and scanned by microdensitometer.



< Fig 20 > The kinetics of inhibition of RNase A by pig brain RNase inhibitor. The data are presented in a double reciprocal plot, 1/v against 1/s. Activity determinations were made in the presence of 0 ng (o--o), 25 ng (▲--▲), 50 ng (Δ--Δ), and 100 ng (●--●) of the inhibitor. PCMB-inactivated species of the inhibitor (100 ng) had no effect on the kinetics of inhibition of RNase A (x--x). All other assay conditions were as described under " Experimental Procedures."

concentrations of inhibitor produced proportionally higher percentage of inactive enzyme. When the enzyme-inhibitor complex was treated with 1mM PCMB, the inhibitor was completely inactivated. The inactivation of the inhibitor by 1 mM PCMB brought about the dissociation of the complex. Gel filtration of the PCMB treated enzyme-inhibitor complex with an elution buffer containing 1 mM PCMB led to recovery of all the RNase activity as a Mr. = 13,700 species (Data not shown). The inhibitor isolated from pig brain, as well as, from human placenta inhibited RNase A and RNase B, but did not inhibit RNase T_1 and RNase U_1.

Amino Acid Analysis

Table 18 shows the amino acid composition of the pig brain inhibitor and compares it with that of the human placental inhibitor reported earlier (3). A comparison of acidic, basic, aromatic, hydrophobic, and polar amino acid residues is also included. As seen, the total amino acid composition of the two inhibitors is significantly different. It should be mentioned that the amino acid analysis of the brain inhibitor was performed with 91 % pure inhibitor. Nevertheless, the difference in the cysteine content of the two proteins is particularly striking.

< TABLE 18 >

Amino acid composition of RNase inhibitor from pig brain

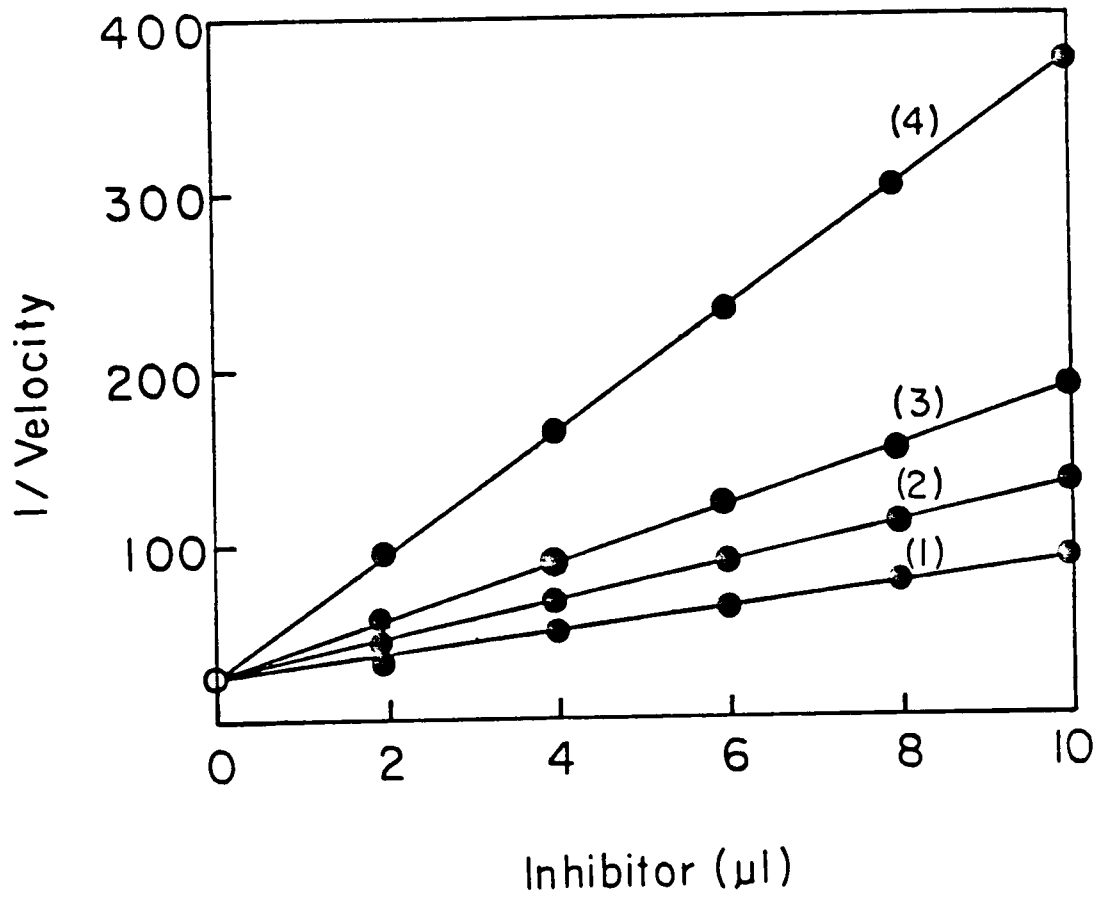
Amino acid	Residues/molecule	
	Pig brain (a)	Human placenta (b)
CYA	5	30
ASP	37	47
THR	32	16
SER	41	45
GLU	85	64
PRO	47	17
GLY	33	36
ALA	44	34
VAL	25	24
MET	10	2-3
ILE	14	12
LEU	34	85
TYR	11	4
PHE	12	6
HIS	8	6
LYS	28	17
TRP	2	5
ARG	13	23
Total	481	475
Aromatic	25	15
Basic	41	40
Acidic	122	111
Polar	106	97
Hydrophobic	174	175

(a) Mean analyses of three separate preparations: the residue ratios were calculated for a molecular weight of 50,000.
(b) From reference (3).

Application of The Direct Assay Method For Quantitation of The RNase Inhibitor

In 1946, Kuintz first developed an assay method based on the decrease in optical density at 300 nm at pH 5.0 during the action of RNase on RNA (11). However, the optimum pH for RNase A, as well as its inhibitor (3), is between pH 7 and 8. We have used the rate of increase in absorbance at 260 nm at pH 8.0 to monitor the RNase activity. All assays were conducted in 10 mM HEPES, pH 8.0 at 25 ^{o}C, in which the increase in absorbance at 260 nm was linear for the first three minutes and was proportional to the concentration of up to 25 ng of RNase A, 25 ng of RNase B, 600 ng of RNase T1, and 200 ng of RNase U1.

This direct assay method was also used to quantify the commercially available RNase inhibitor from human placenta and to follow the purification of the RNase inhibitor from pig brain (Fig. 21). The cyclic 2',3'-CMP assay for the inhibitor was not sufficiently sensitive to be used with crude extracts and 35 to 60 % ammonium sulfate precipitates during purification (10). The direct assay method presented here, however, was sensitive enough to detect and quantify RNase inhibitor in the above solutions, as well as the purified inhibitor. In each case, the reaction rates were linear with time and with the concentration of inhibitor



< Fig 21 > Dixon plot of the influence of the amount of RNase inhibitor on the inhibition of RNase A. The activity was assayed at pH 8.0 as described under " Experimental Procedures". The lines drawn through the points were calculated from a least squares fit of the data. (1) crude extracts, (2) 35 to 60 % ammonium sulfate precipitate, (3) purified inhibitor from pig brain, and (4) human placental inhibitor (from Sigma).

(data not shown). The results were reproducible within 5 % of the margin of error.

Blackburn (10) defined 1 unit of inhibitor as that amount required to inhibit by 50 % the activity of 5 ng of RNase A with 2',3'-cCMP as a substrate. 1 ug of RNase A increased the absorbance at 286 nm by 0.0146 absorbance unit per minute at pH 6.5 and 50 % inhibition of the 1 ug of RNase A was produced by 200 units of RNase inhibitor (10). In the assay method presented here, 5 ng of RNase A gives an increase in absorbance at 260 nm of 0.014 absorbance units per min at pH 8.0, while 50 % inhibition of the 5 ng of RNase A is produced by 5 units of human placental RNase inhibitor. Therefore, this method is 200 times more sensitive with respect to RNase A and 40 times more sensitive for detection of RNase inhibitor than the 2',3'-cCMP assay method.

During this work was in process, it was reported that there were no differences in the level of RNase activity between control and Alzheimer's samples, and that the decrease in the concentration of RNA in Alzheimer's disease was due to reduced transcription but not due to the abnormality in RNase and its inhibitor complex (15). Therefore, no further studies were continued. However, it is hoped that the availability of the method for the

purification of the inhibitor and a simple and sensitive method for its assay presented here would aid in our understanding of the role for the inhibitor in the neuronal metabolism.

REFERENCES

1. Roth, J.S. (1967) Methods Cancer Res. 3, 151-243
2. Ittel, M.E., and Mandel, P. (1979) J. Neurochem. 33, 521-525
3. Blackburn, P., Wilson, G., and Moore, S. (1977) J. Biol. Chem. 252, 5904-5910
4. Roth, J.S. (1956) Biochim. Biophys. Acta 21, 34-43
5. Roth, J.S. (1962) Biochim. Biophys. Acta 61, 903-915
6. Kraft, N., and Shortman, K. (1970) Biochim. Biophys. Acta 217, 164-175
7. Kraft, N. (1962) Biochim. Biophys. Acta 61, 50-55
8. Mann, D.M.A., Neary, D., Yates, P.O., Lincoln, J., Snowden, J.S., and Stanworth, P. (1981) J. Neurol. Neurosurg. Psychiatry 44, 97-102
9. Sajdel-Sulkowska, E.M., and Marotta, C.A. (1984) Science 225, 947-949
10. Blackburn, P. (1979) J. Biol. Chem. 254, 12484-12487
11. Kunitz, M. (1946) J. Biol. Chem. 64, 563-568
12. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

14. Takahashi, Y., Mase, K., and Suzuki, Y. (1970)
J. Neurochem. 17, 1433-1440

15. Guillemette, J.G., Wong, L., Crapper-McLachlan, D.R.,
and Lewis, P.N. (1986) 47, 987-997

CHAPTER V

GENERAL CONCLUSION

This dissertation has dealt with the inhibitory effects of aluminum on glucose-6-phosphate dehydrogenase (G6PD) from yeast, rat brain, pig brain, and human brain. Aluminum has been known as a toxic material in various biological process. We specifically elected to investigate the effect of aluminum on brain G6PD because accumulation of aluminum in the brains of patients with Alzheimer's disease has been reported. The other researchers reported that there was an overall decrease in glucose metabolism in the patients with a dementia of the Alzheimer's disease and aluminum impairs glucose utilization and cholinergic activity in rat brain in vitro.

We, therefore, assayed the activities of hexokinase, G6PD, and acetylcholinesterase in the extracts of brain of rats which had received 100 uM $AlCl_3$ in drinking water for 12 months. The average life span of a rat is about 30 months but the rate of growth reaches a plateau after 4 months. A 12 month period, therefore, represents about half the period of adult life. The result shows that the crude extracts (800 x g supernatants) of the aluminum feeding

groups contains a twice amounts of aluminum compared to those of the control groups. Aluminum feeding reduced the specific activities of hexokinase and G6PD by about 27 % and 20 %, respectively.

The fact that even in the control group, only half of the total hexokinase is expressed suggests that this may be a more general phenomenon in brain. In most of our experiments, the concentration of aluminum during the assay was much lower than the 5 uM required to inhibit 50 % of the enzyme activity. Therefore the concentrations of aluminum in the homogenates do not necessarily represent those observed in situ. This is particularly true for the complex organs such as brain where aluminum is known to be localized at different concentration in specific areas of the brain.

It is noteworthy that even in the normal brain only half of the total activity of hexokinase is expressed. This situation is functionally similar to but different in mechanism from that observed for phosphoglucomutase in muscle and liver.

The reduction in glucose utilization would obviously exert damaging effects on cerebral metabolism and contribute to aluminum-induced neuronal disorders.

The reaction mechanism of the inactivation of G6PD in rat brain by aluminum was further studied with yeast G6PD because of its commercial availability. The results clearly establish the sensitivity of Bakers' yeast G6PD to a low level of aluminum with a stoichiometry of 1:1. The sensitivity of the yeast enzyme to aluminum suggests that the inhibition of this enzyme may be significant in aluminum toxicity. The inhibition is more pronounced at acidic pH, and this is consistent with the profound solubility of aluminum at low pH. This result is consistent with the previous reports indicating that acid rain increased the aluminum toxicity in plant kingdom.

The protection of G6PD by $NADP^+$ against aluminum inactivation is of particular interest. A very similar protection was reported for the aluminum binding to RNA polymerase. The protection of the G6PD by citrate against aluminum binding is also very similar to previous observation showing that at a molar ratio of 10:1 for [citrate]/[calmodulin], citrate can prevent aluminum binding to calmodulin.

The results in this study confirms the role for histidine and lysine residues at the active site but

exclude them, as well as the sulfhydryl groups, from being involved in aluminum binding. The possibility of the aluminum binding to the carboxyl groups was not explored. It appears likely that the inactivation may be due to conformational changes induced by aluminum binding to the carboxyl or hydroxyl groups. The results of circular dichroism studies support this possibility. This result shown in this work is very similar to that observed for structural changes induced by aluminum in calmodulin.

Environmental pollution by soluble aluminum has elevated aluminum from a 'harmless' to a 'toxic' metal ion. Indeed, aluminum is one of the major toxicant to plants and animals. The present report shows that the concentration of aluminum required to produce 50 % inhibition of yeast G6PD is similar to that reported for inhibiting hexokinase from brain. The resulting reduction in glucose utilization is unfavorable for normal growth of yeast. Such effects are likely to be more damaging to brain, a tissue known to accumulate aluminum. Together with the previous reports of aluminum-induced reduction in glucose utilization in rat brain, the results presented here increase the significance of aluminum toxicity in regulation of energy metabolism.

The effect of aluminum on brain G6PD was further studied with human and pig brain enzymes. Two forms of G6PD isozymes were purified from human and pig brain. The specific activities and the relative amounts of the two isozymes varied with the species. The human brain contains more of isozyme I and four times higher specific activity than the isozyme II. The extensive homologies between the isozymes are shown in peptide maps of tryptic digest and amino acid compositions. It is likely that the G6PD isozymes are homologous proteins derived from a common ancestral gene.

One of the interesting results is the activity of isozyme II with 6-phosphogluconate. Only isozyme II from both sources appears to be active with 6-phosphogluconate as a substrate. This may be metabolically significant, especially if it is specifically localized in the neuronal tissue. In vivo, multienzyme complexes such as pyruvate dehydrogenase complex or polypeptides with multienzyme function as observed in fatty acid synthetase permit more efficient utilization of the products of the preceding reaction. In view of this, the unexpected finding of the 6-PGD activity, observed exclusively in the isozyme II from human and pig brain, is of particular interest. Brain is

the most specialized organ. The utilization of glucose in the brain is nonuniform and is determined by the physiological need in respond to a given stimulus. It is tempting to speculate that the isozyme II, because of its bifunctional activity, permits more efficient utilization of glucose and therefore, may be localized in specific loci in the brain.

Aluminum inactivates the G6PD activity of the human and pig brain isozyme I without affecting the 6-PGD activity of the isozyme II of the both sources. One mol of aluminum binds per mol of enzyme subunit. The aluminum inactivated enzyme was reactivated by several chelators such as citrate, NaF, and transferrin. Transferrin binds 2 mol of aluminum per mol of protein and competitive equilibrium binding studies show that the aluminum and iron binding sites are identical. Consistent with these findings, it has been reported that transferrin relieved the inhibitory effect of aluminum on the activity of cAMP-dependent phosphodiesterase stimulated by Ca^{++}-calmodulin and aluminum-induced structural changes in calmodulin were reduced by transferrin. The reactivation or protection of the G6PD isozymes by transferrin against aluminum inactivation presented here may be physiologically

significant. Transferrin, as well as ferritin, is one of the constituents of the neuronal tissue and their ability to bind aluminum may expand role for transferrin in metal toxicity including aluminum toxicity in the brains.

The results presented here clearly establish the sensitivity of the G6PD isozymes in human and pig brain to a low level of aluminum and increase the significance of aluminum toxicity in degrading energy metabolism.

VITA

Sung-Woo Cho was born in Seoul, Korea on May 7, 1959. He attended elementary schools in that city and was graduated from Bae-Myung High School, Seoul, Korea in 1978. He entered Yon Sei University, Seoul, Korea, in March 1979. After serving in the Korean Army, he re-entered Yon Sei University in March 1982, and he received a Bachelor of Science degree in Biochemistry in February 1984.

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