KINETIC ANALYSIS OF GLUTATHIONE TRANSFERASE FROM RATS EXPOSED TO SUB-LETHAL AND LETHAL CONCENTRATIONS OF LEAD ACETATE

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ABSTRACT

The effects of lethal and sublethal doses of lead acetate on the induction and kinetic characteristics of glutathione transferase (GST) isozymes in rat liver and kidney were investigated. GST isozymes induction was monitored by the ability of the induced enzyme to conjugate glutathione (GSH) with model GST substrates. Liver isozymes were purified and separated into three main isoforms using ion-exchange chromatography and the kinetic characteristics in the absence and presence of the toxic metal ions examined.

Lethal and sublethal doses of lead acetate resulted in different levels of GST induction in both liver and kidney depending on the substrates employed to assay for enzyme induction. Presence of 1 mM Sodium arsenate brought about changes in the kinetic parameters of the isozymes.

We conclude that GST isozymes are induced in rats irrespective of the lead acetate dose that was administered and that in the presence of toxic metal ions, GST isozymes responded by altering their kinetics, permitting tight binding for substrates in some isozymes and loose binding in others.

Key words: Glutathione transferase, Lead acetate, Induction, Kinetics.

INTRODUCTION.

The glutathione transferases (GST, EC 2.5.1.18) are a supergene family of dimeric enzymes that catalyse the conjugation of glutathione (GSH) to a variety of electrophiles including arene oxides, unsaturated carbonyls, organic halides and other substrates (Strange *et al.,* 2000), as a first step in their detoxication, followed by excretion of the conjugate. They are also known to bind and transport a variety of non-substrate ligands in the tissue of vertebrates (Hayes and Pulford, 1995; Flanagan and Smythe, 2011).

Based on protein and DNA sequence similarities, immunological cross-reactivity and kinetic properties, cytosolic GSTs are classified into Alpha (α), Mu (μ), Pi (π), Theta (θ), Sigma (σ), Zeta (ζ), Omega (ω), Beta (β), Phi (φ), Tau (τ) and Delta (δ) families (Sheehan *et al.,* 2001) whereas the mitochondrial form belongs to a separate Kappa (κ) class (Flanagan and Smythe, 2011).

Apart from the aforementioned functions, GST isozymes perform many other functions such as catalyzing the synthesis and transport of steroids, prostaglandins and leukotrienes (Hayes *et al.,* 2005), detoxification of product of oxidative stress and degradation of aromatic amino acids (Fernandez-Canon and Penalva, 1998; Hayes *et al.,* 2005). Some cytosolic GSTs have non-enzymatic functions in cell-signaling pathways (McIlwain *et al.,* 2006). For example, GSTP1-1 functions in regulating Jun N-terminal kinase activity (Adler *et al.,* 1999) and in binding of tumor necrosis factor receptor-associated factor 2 (TRAF2) (Flanagan and Smythe, 2011).

Reports from many laboratories have shown that GSTs can be induced in a tissue-specific manner by a wide variety of drugs and toxicants including barbiturates, reactive oxygen species, chemoprotectants and heavy metals such as lead (Hayes and Pulford, 1995; Daggett *et al*, 1998).

Lead has attracted much attraction as a pollutant due to the fact that a lot of people are exposed to it, both in the environment and through their occupation. It is toxic to hematopoietic and nervous systems, bone, and kidney (Nolan and Shaikh, 1992). High levels of lead accumulate in soft tissues where lead-induced pathological changes occur in their structure and function (Khalil-Manesh *et al.,* 1992). Specifically, lead is known to cause kidney dysfunction and impairment of liver functions (Patrick, 2006).

Recently, due to increased mining activities for

minerals such as gold, deaths resulting from exposure to lethal concentrations of lead have been reported in some parts of the developing countries. This is probably because gold ore is often closely associated with lead ore. Though lead is not a substrate for glutathione transferase, the enzyme is often induced in response to lead exposure. Much work has been done on the induction of GSTs at sub-lethal concentrations of lead in rats, but very little information is available on the induction of GSTs in animals exposed to lethal doses of lead and on the kinetics of GSTs in the presence of non-biological metals which they come in contact with under physiological condition.

In order to provide this basic information, we have exposed rats to lethal and sub-lethal doses of lead and determined the level of native GST induced in some organs and the kinetic properties of the main isoenzymes from the liver in the presence and absence of toxic metal salt.

MATERIALS AND METHODS

Materials

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenezene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(paranitrophenoxy) propane (EPNP), para-nitrophenyl acetate (NPA), 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl), and glutathione agarose (GSH-agarose) were obtained from Sigma Chemical Company, St Loius, Mo, U.S.A. All other reagents used were of analytical grade and were obtained from reputable chemical suppliers. Five weeks old albino rats were purchased from the animal house of the Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife.

METHODS

Treatment of Animals

The animals were given free access to standard rat feed and water and were acclimatized for one week. Four rats were housed per cage and administered with 30, 70, 150, 300, and 600 mg/kg body weight lead acetate solution via the intraperitoneal route, taking into consideration the LD_{50} for the intraperitoneal dose of lead acetate in rats (Masci and Bongarzone, 1995). The control was administered with equivalent amount of distilled water. After 24 h, the animals were sacrificed by cervical dislocation.

Preparation of Liver or Kidney Homogenate

Freshly-excised liver or kidney were homogenized in 10 mM Tris-HCl buffer pH 7.8, containing 10 mM EDTA, 1 mM EGTA, 5 mM Mercaptoethanol and 10 % (v/v) glycerol to obtain 10 % (w/v) homogenate. The homogenate was centrifuged at 20,000 x g for 1 h at 4°C. The supernatant obtained was filtered through cheese cloth to remove the floating lipid. The filtrate was stored at -20°C as crude extract of glutathione transferase.

Routine GST Activity Measurement and Protein Concentration Determination

The GST activity was measured routinely following the method of Habig *et al.* (1974). The increase in absorbance following enzymatic conjugation of GSH with CDNB forming S-2,4-dinitrophenylglutathione was monitored in a spectrophotometer at 340 nm together with a suitable control. A unit of the enzyme is equal to 1 μmole of the product per minute taking the extinction coefficient as 9.6 $mM^{-1}cm^{-1}$. The assay buffer was 0.1 M phosphate buffer pH 6.5. The specific activity was defined as the enzyme unit per milligram of protein.

Protein concentration was measured routinely according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

Substrate Utilization Characteristics

The profile of the substrate utilization characteristics of the isoenzymes present in the supernatant fractions of liver and kidney of each animal group was determined using a set of model GST substrates. These include 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), para-nitrophenyl acetate (NPA), 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl) and also 1-chloro-2,4-dinitrobenezene (CDNB), the substrate used routinely. Conjugation of GSH with NPA and NBD-Cl as electrophilic substrates was determined by the method of Keen and Jakoby (1978), and Ricci *et al.* (1994) respectively.

Conjugation of GSH with CDNB was as described earlier while conjugation with DCNB and EPNP was also according to Habig *et al.* (1974). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1μmole of product per minute under the standard assay conditions. The data obtained were analysed using Dunn's multiple comparisons test.

Enzyme Purification and Separation

Homogenate obtained from the liver of group III animals (administered 70 mg/kg body weight of lead acetate) was purified using a combination of DEAE-Sephacel ion exchange and GSH-agarose affinity chromatography. The purified GST was separated on CM-Sepharose ion-exchange column into isozymes as shown in figure 3.

Determination of Kinetic Parameters.

The apparent kinetic parameters $K_{m\ app}$ and V_{max} of the purified GST isozymes were determined by measuring GST activity at a fixed GSH concentration (1 mM) and varying concentration of CDNB (0.1-1.0 mM) and at fixed CDNB concentration (1 mM) and varying concentration of GSH (0.1-1.0 mM GSH). The data obtained were analysed using Graph Pad Prism 5 non-linear regression analysis software. Due to insolubility of lead salts in the assay medium, the effect of heavy metal salt on kinetic parameters was determined using sodium arsenate ($NaAsO_4$) as model heavy metal, which was included in each of the assay mixtures. The concentration was kept at 1 mM.

Statistical Analysis

Statistical significance was performed using Graph Pad Instat (KrusKal-wallis test and Dunn's multiple comparisons test). Differences were considered significant when $P< 0.05$.

RESULTS AND DISCUSSION

Lead Administration and Measurement of GST Induction

To avoid the possibility of having a false profile of GST isoforms induction, we have used a broad range of model substrates in this study. The profile obtained for the liver and kidney supernatant extracts are shown in Figures 1 and 2 respectively.

We have also extended the lead administration to lethal doses because very few reports are available on the induction of GST in animals at this concentration. There was induction of GST even at lethal doses of lead in both the kidney and liver the pattern of induction were however not the same. None of the isoforms induced in both organs could utilize EPNP as substrates. There was minimal DCNB conjugation by liver enzymes and no detectable activity with kidney crude supernatant. When CDNB was used as substrate, there was approximately a two-fold increase in GST activity at 30 mg/kg body weight of lead which gradually decreased to the level seen in the control at 600 mg/kg body weight in the liver. For DCNB, the induction increased gradually to 2.0 fold at 70 mg/kg body weight of lead and decreased gradually with a pattern similar to that of CDNB. Para nitrophenyl acetate, when used as substrate showed approximately a 2-fold induction at 30 and 70 mg/kg body weight each, which decreased to 1.3 fold at 600 mg/kg body weight. When NBD-Cl was used as substrate, there was induction at all the doses of lead administered but the highest induction (1.4-fold) was obtained at 300 mg/kg body weight of lead.

The same pattern of induction was observed in the kidney except that at lethal concentrations of lead (300 and 600 mg/kg body weight), GST induction was higher than that of the liver for all the substrates tested.

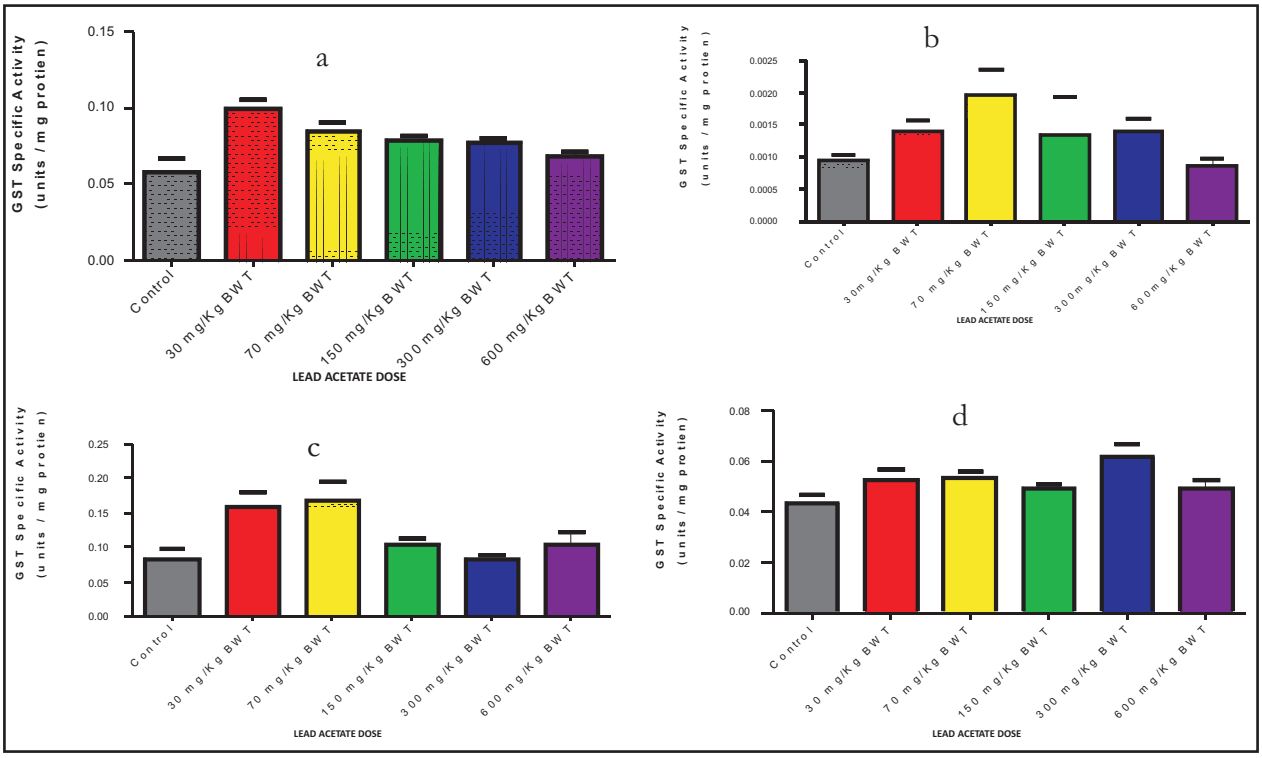

Figure 1 Profile of GST induction in the liver of rat subjected to lethal and sublethal concentrations of lead.

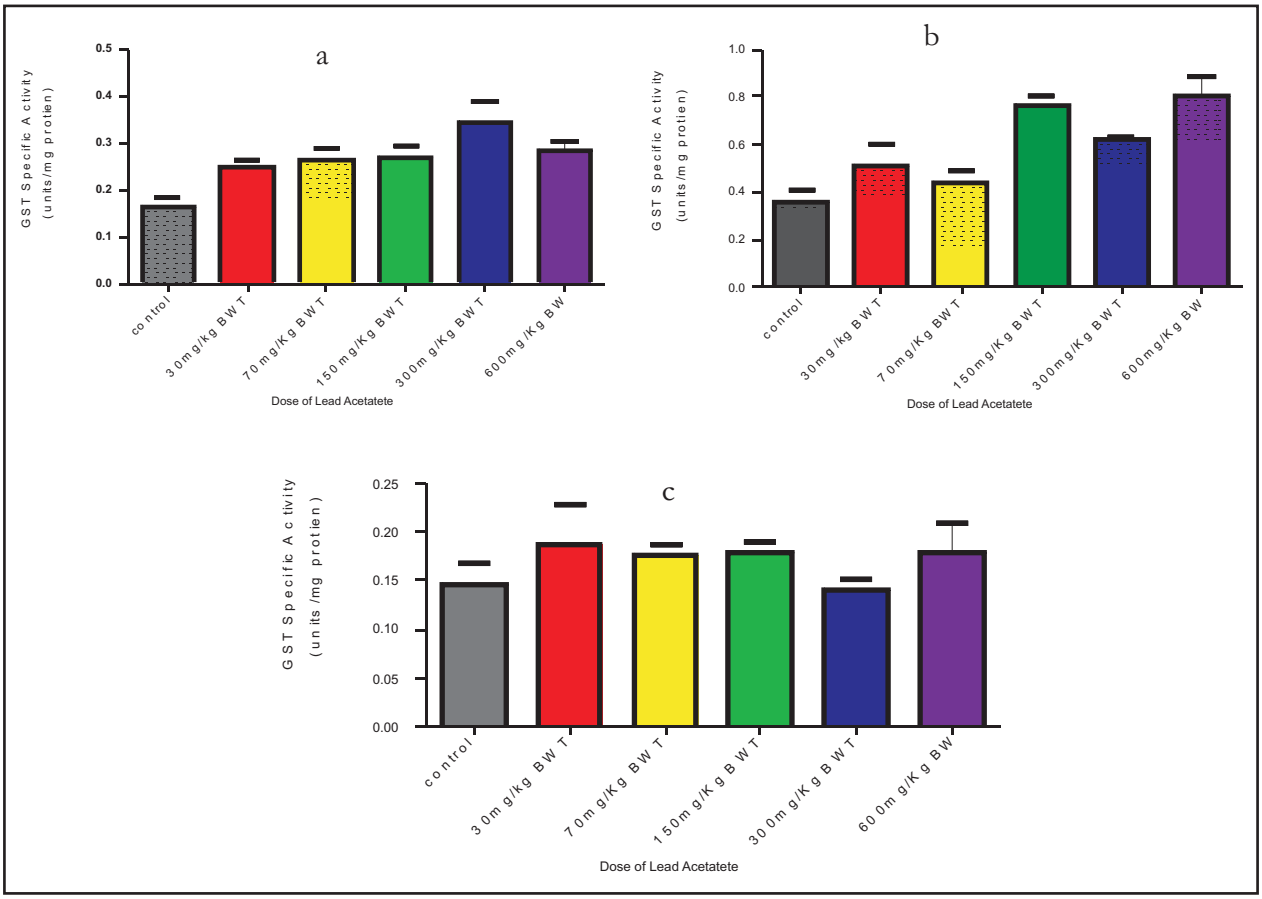

Figure 2 Profile of GST induction in the kidney of rat subjected to lethal and sub-lethal doses of lead acetate.

GST Separation into Isoforms

The induced GST in the supernatant extract of the liver of group III animals (administered with 70mg/kg body weight of lead) was separated into three main isoforms named A, B and C in reverse order (Figure 3).

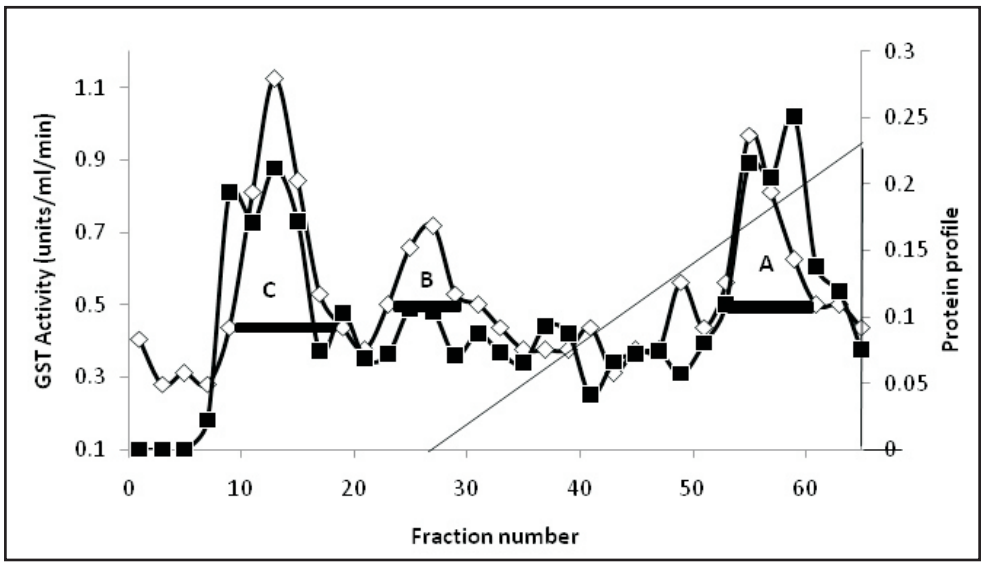

Figure 3: Isoenzyme Separation on CM-Sepharose Ion Exchange Column

Kinetic Characteristics

The summary of the apparent kinetic parameters obtained for each of the purified isoforms in the absence and presence of metal ions is shown in Table 1a and b respectively.

Parts of the toxic effects of lead in the liver or kidney have been previously suggested to involve GSH depletion which consequently provokes oxidative stress elements leading to GST induction (Ercal *et al.,* 2001). There is however scanty information on the contribution of the induced GST to the overall detoxication process of the metal ions. Specifically, the kinetics of the GST upon direct interaction with heavy metal ions is not known. As a result of limited water solubility, even at low concentrations of lead acetate in GST assay medium, we have used sodium arsenate as a model heavy metal ion to investigate this. The results showed that different isoforms responded differently in the presence of metal ions. In the absence of metal ions, the kinetic parameters were similar to the values previously reported in literature; Meyer, (1993). However, whereas the presence of arsenate salts caused a 2.3 and 1.6 fold increase in apparent K_m^{CDNB} and V_{max} respectively for GST isoenzyme C, no effect was observed on K_m^{GSH}. There was no change in apparent K_m^{CDNB} or V_{max} for isoenzyme B, but a 2- fold decrease in apparent K_m^{GSH}. For isoenzyme A, there was a 2-fold increase in apparent K_m^{CDNB} and a corresponding 2-fold decrease in apparent K_m^{GSH}.

To some extent, decrease in K_m signifies more affinity for the substrate whereas an increase in K_m indicates loose binding of substrate with enzyme. It follows that in the presence of toxic metals such as arsenate, some isoforms of GST can adjust their kinetics to allow for a tighter binding in some cases and loose binding in another instance. When substrates are tightly bound to enzymes, it may be disadvantageous in the sense that the turnover may be compromised since substrates are not quickly released for another round of catalysis. However, unlike for some enzymes, the tighter binding appears to correspond to a higher velocity of reaction in the case of some isoforms of GST in this study.

In conclusion, this study has shown that there was an increase in total GST protein in both the liver and kidney following lethal and sub-lethal administration of lead acetate. Furthermore, in the presence of metal ions, the kinetics of the GST isoenzymes are altered permitting loose binding in some and tighter binding in others. The tight binding of the substrate however did not lead to a decrease in turnover number.

Table 1

A

GST Isozymes	V_{max} Units/mg protein	$K_{m\ app}^{GSH}$ (mM)	$K_{m\ app}^{CDNB}$ (mM)
A	.....	0.220 ± 0.054	0.166 ± 0.057
B	0.147 ± 0.009	0.191± 0.033	0.188 ± 0.041
C	0.059 ± 0.003	0.133 ± 0.019	0.313 ± 0 .059

B

GST Isozymes	V_{max} Units/mg protein	$K_{m\ app}^{GSH}$ (mM)	$K_{m\ app}^{CDNB}$ (mM)
A	0.095 ± 0.008	0.120 ± 0.025	0.264 ± 0.079
B	0.124 ± 0.007	0.096 ± 0.007	0.163 ± 0.033
C	0.095 ± 0.004	0.131 ± 0.022	0.713 ± 0 .066

4.0 REFERENCES

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosrio, L., Tew, K.D., Pinucs, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J. and Ronia, Z 1999. Regulation of JNK signalling by GSTp. *EMBO J.* 18: 1321-1334.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protien utilizing the principle of protien-dye binding. *Ana.l Biochem.* 72: 248-254.

Daggett, D. A., Oberley, T. D., Nelson, S. A., Wright, L. S., Kornguth, S. E., and Siegel, F. L. 1998. Effects of lead on rat kidney and liver: GST expression and oxidative stress. *Toxicology* 128: 191–206.

Ercal, N., Gurer-Orhan, H., and Aykin-Burns, N. (2001). Toxic metals and oxidative stress. Part 1. Mechanisms involved in metal-induced oxidative damage. *Curr Top Med Chem.* 1: 529-539.

Fernandez-Canon, J. M. and Penalva, M. A. (1998). Characterization of a fungal maleylacetoacetate isomerase gene and identification of its human homologue. *J. Biol. Chem.* 273: 329–337.

Flanagan, J. U. and Smythe, M. L. 2011. Sigma-class glutathione transferases. *Drug Metabolism Reviews* 43(2): 194–214

Habig, W. H., Pabst, M. J. and Jacoby, W. D. 1974. Glutathione-S-Transfereases the first step in mercapturic acid formation. *J. Biol. Chem.* 249: 7130-7139.

Hayes, J. D. and Pulford, D. J. 1995. The glutathione S-transferase supergene family: Regulation of GST and the contributions of the isoenzymes to cancer chemoprotection and drug resistance. *CRC Crit. Rev. Biochem. Mol. Biol.* 30: 445-600.

Hayes, J. D., Flanagan, J. U. and Jowsey, I. R. 2005. Glutathione transferases. *Annu. Rev. Pharmacol Toxicol.* 45: 51-88.

Khalil-Manesh, F., Gonick, H. C, Cohen, A. H., Alinovi, R., Bergamaschi, E.,Mutti, A., and Rosen, V. J. (1992). Experimental model of lead nephropathy.I. Continuous high-dose lead administration. *Kidney Int.* 41: 1192-1203.

Keen, J. H. and Jakoby, W. B. 1978. Glutathione transferases. Catalysis of nucleophilic reactions of glutathione. *J. Biol. Chem.* 253: 5654–5657.

Masci, O., and Bongarzone, R. 1995. Toxicity of Lead. In N. Castellino, P. Castellino, and N.

Sannolo (Eds). *Inorganic Lead Exposure: Metabolism and Intoxication*. Lewis, Boca Raton, FL. pp. 203-213.

McIlwain, C. C., Townsend, D. M., Tew, K. D. (2006). Glutathione S-transferase polymorphisms: cancer incidence and therapy. *Oncogene*. 25:1639–1648.

Meyer, D. J. 1993. Significance of an unusually low K_m for glutathione in glutathione transferases of the Alpha-class, Mu-class and Pi-class. *Xenobiotica*. 23: 823-834.

Nolan, C. V., and Shaikh, Z. A. 1992. Lead nephrotoxicity and associated disorders: Biochemical mechanisms. *Toxicology*. 73: 127-146.

Patrick, L. 2006. Lead Toxicity Part II: The Role of Free Radical Damage and the Use of Antioxidants in the Pathology and Treatment of Lead Toxicity. *Alternative Medicine Review*. 11 (2) :114-128.

Ricci, G., Caccuri, M. A., Bello, M. L., Pastore, A., Piemonte, F. and Federici, G. 1994. Colorimetric and Fluorometric assays of glutathione transferase based on 7-chloro-4-nitrobenzo-2-oxa-1 3-diazole. *Anal. Biochem*. 218: 463–465.

Sheehan, D., Meade, G., Foley, V. M. and Dowd, C. A. 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J*. 360: 1-16.

Strange, R. C., Jones, P. W. and Fryer, A. A 2000. Glutathione S-transferase: genetics and role in toxicology. *Toxicology Letters* 112–113: 357–363