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Correlation Between Glutathione Peroxidase Activity and Selenium Concentrations in Both Plasma and Erythrocytes

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CORRELATION BETWEEN GLUTATHIONE PEROXIDASE ACTIVITY AND SELENIUM CONCENTRATIONS IN BOTH PLASMA AND ERYTHROCYTES

by

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ABSTRACT

Plasma and erythrocyte glutathione peroxidase activities and selenium concentrations were determined for 38 apparently healthy female subjects. In addition, erythrocyte lead and plasma lead and iron concentrations were determined in each subject. Enzyme activity was measured using a modification of the coupled enzyme method of Paglia and Valentine. Direct determinations of all metal concentrations were made using Polarized Zeeman Effect Flameless Atomic Absorption Spectroscopy (PZAAS). Mean $\pm$ standard deviation (SD) enzyme activities found were 78.6 $\pm$ 12.8 U/g haemoglobin (U/g Hb) and 424.6 $\pm$ 39.6 U/L for erythrocyte and plasma glutathione peroxidase, respectively. Mean selenium concentrations were 141.4 $\pm$ 14.2 µg/L and 96.3 $\pm$ 14.2 µg/L for erythrocytes and plasma, respectively. A low positive correlation between erythrocyte glutathione peroxidase activities and selenium concentrations ($r = 0.41$, $p = 0.01$) was found. Mean concentrations for the other metals did not differ significantly from literature values. Effects of age, smoking, vitamin and mineral supplementation, use of caffeinated beverages and oral contraceptives on glutathione peroxidase activity were determined and interelement relationships were investigated using the one tailed t-test and Spearman Rank Correlation Coefficients.

ACKNOWLEDGEMENTS

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Introduction

Selenium is known to be an essential cofactor for the enzyme, glutathione peroxidase. Animal experiments have shown low enzyme activity in erythrocytes of animals that are selenium deficient (1). This finding has suggested a correlation between the enzyme and its cofactor. The few human experiments investigating correlation between glutathione peroxidase activity and selenium concentrations have produced conflicting results (2-5).

The purpose of this research was to investigate the possibility of a correlation between glutathione peroxidase activity and selenium levels in both plasma and erythrocytes of healthy female subjects. We investigated effects of age, sex, smoking habits, and use of oral contraceptives, minerals, vitamins and caffeinated beverages, all of which are known to affect plasma enzyme activities. Lead levels were determined in both plasma and erythrocytes because it has been suggested that high levels of toxic metals are associated with low enzyme activities (6). In addition, iron concentrations in erythrocytes and plasma were also measured because iron deficiency has been reported to be associated with low glutathione peroxidase activity both in humans and rabbits (7, 8).

The parameters for the enzyme assay were optimized and assays for erythrocyte and plasma selenium developed. The effects of age, smoking, mineral and vitamin supple-

mentation and use of caffeinated beverages and oral contraceptives were then correlated to selenium, iron, lead and enzyme activity in both plasma and erythrocytes. Possible mineral interactions were also investigated.

HISTORICAL REVIEW

Mills and Randall (9) first described human erythrocyte enzyme, glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide to water with simultaneous oxidation of glutathione. In addition to hydrogen peroxide, the enzyme also catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols. Thus, the enzyme does not only protect cytosolic and mitochondrial compounds from hydrogen peroxide toxicity, but may protect cell membranes from lipid peroxidation. Glutathione peroxidase has a protective effect only in the presence of adequate amounts of reduced glutathione, which serves as a hydrogen donor. The reaction has been represented by the following equation:

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2H_2O$$

$$ROOH + 2GSH \xrightarrow{GSH\text{-}Px} ROH + H_2O + GSSG$$

$$2NADPH + GSSG \xrightarrow{GSH\text{-}Reductase} 2GSH + 2NADP^+$$

Reduced nicotinamide adenine diphosphonucleotide (NADPH), which functions as a coenzyme for glutathione reductase, is supplied by the hexose shunt. There it is generated during the dehydrogenation of glucose-6-phosphate and 6-phosphogluconate.

Gerald Chen and Paul Hochestein (10) designed experiments, using human erythrocytes, to evaluate the competition between glutathione peroxidase and a second hydrogen peroxide reducing enzyme, catalase, for their

common substrate. When extracellular hydrogen peroxide concentrations were maintained at an upper limit of 10^{-6}M, the oxidation of reduced glutathione was related to the major fraction of hydrogen peroxide added to the cells. At higher concentrations of hydrogen peroxide, decomposition of hydrogen peroxide by catalase became increasingly predominant. They concluded from their observations that under physiological conditions, glutathione peroxidase linked to the hexose shunt activity represented the major pathway of hydrogen peroxide metabolism in intact erythrocytes.

Glutathione peroxidase has a highly specific requirement for its donor substrate glutathione. While working with erythrocyte glutathione peroxidase, Mills (9) found little activity with other thiols such as cysteine, cysteinylglycine and ergothione. The enzyme did not catalyze the breakdown of hydrogen peroxide in the presence of o-toluidine or guaiacol even though these compounds are widely used as hydrogen donors for other peroxidases. Flohe et al. (11) observed that only glutathione, among 29 thiols studied, was the only substrate having physiological significance for the enzyme. γ-L-glutamyl-L-cysteine methylester and mercaptoacetic acid methyl ester gave more than 10% of the activity observed with reduced glutathione under similar conditions.

Glutathione peroxidase shows rather low specificity for the peroxide substrate. For a while, hydrogen peroxide was exclusively employed; later a variety of hydroperoxides were found to be decomposed by the enzyme at rates comparable to that of hydrogen peroxide. Little and O'Brien (12) following their initial work with linoleic hydroperoxide, demonstrated that cumene hydroperoxide, t-butylhydroperoxide and ethyl linolenate hydroperoxide could serve as substrates for rat liver glutathione peroxidase. Similar results were reported by Holmberg (13) for the enzyme from bovine lens. Christopherson (14) reported the reduction of thymine hydroperoxide and peroxidized DNA by rat liver glutathione peroxidase. The list of hydroperoxides that serve as substrates for glutathione peroxidase has been extended by the work of Little (15). He tested six steroid hydroperoxides and found the rate of glutathione oxidation with progesterone 17-hydroperoxide to be nearly as fast as with hydrogen peroxide. Cholesterol-25-hydroperoxide was the first hydroperoxide reported that could not be metabolized by glutathione peroxidase.

Little and O'Brien (12) also observed that unlike heme proteins, neither erythrocyte nor liver enzyme was inhibited by cyanide or azide. These substances are added to inhibit catalase in the assay for glutathione peroxidase in crude systems. Schneider and Flohe (16)

found a weak readily reversible inhibition of erythrocyte glutathione peroxidase with 2 x 10^{-4}M mercuric chloride following preincubation with 10^{-4}M glutathione (glutathione reduces the enzyme from its native form -the oxidized form). In the presence of EDTA, the same concentration of mercuric chloride inhibited the enzyme by 90%. They postulated that EDTA formed a complex with mercuric chloride that was inhibitory to the enzyme. High concentrations of multivalent anions including phosphate, sulfate and maleate caused reversible inhibition of glutathione peroxidase (17). Holmberg (13) reported that glutathione peroxidase had lower activity when assayed in phosphate than in tris-hydroxymethyl-aminomethane buffer. Flohe and Brand (17) found no significant difference in activity with various cations such as Na^+ and K^+. In fact Little and coworkers (18) found that a wide range of nucleotides such as NADPH were more inhibitory than phosphate. The inhibitory effect increased with the number of phosphate groups in the nucleotide.

In 1972 Rotruck Hoesktra and coworkers (1) at the university of Wisconsin began an investigation of glutathione peroxidase relation to the overlapping nutritional roles of selenium, vitamin E and sulfur amino acids. Rotruck's group was the first to arrive at a correct conclusion that selenium was an integral constituent of glutathione peroxidase. Looking for the metabolic cause

of hydrogen-peroxide-dependent hemolysis in selenium-deficient rats, Rotruck found that glutathione peroxidase activity was significantly decreased. In addition, if labeled ^{75}Se was administered to selenium-deficient rats, the labeled selenium was incorporated into a protein fraction that co-chromatographed with glutathione peroxidase activity. From these results, the group concluded that glutathione peroxidase was a selenium-dependent enzyme.

Major contributions to the knowledge about the chemistry and biological function of glutathione peroxidase have come from the work of Flohe and his coworkers (19) in Germany. By the early 1970's, this group of scientists had succeeded for the first time in isolating weighable quantities of purified bovine erythrocyte glutathione peroxidase. They estimated the molecular weight of the enzyme, analyzed the enzyme for subunits and selenium content by sedimentation equilibrium, electrophoresis in SDS and neutron activation, respectively. The molecular weight of each subunit was about 21,000 and that of the intact protein enzyme about 83,000. There are 4 gram atoms of selenium per mole of enzyme protein. Spectral studies showed no absorption bands in the visible region and thus indicated that the enzyme was neither a heme nor flavin protein (20).

Further evidence that glutathione peroxidase is a selenoenzyme was found by various groups of scientists.

The enzyme has been purified from human erythrocyte by Awashti et al. (21) rat liver by Nakamura et al. (22) and from human placenta by Awasthi et al. (23). Awasthi demonstrated that the human erythrocyte glutathione peroxidase had two immunologically similar isoenzymes A and B (21). Both forms were selenoproteins. Nakamura estimated the molecular weight of the rat liver glutathione peroxidase by thin layer gel filtration. They also performed enzyme subunit analysis, amino acid analysis and selenium content analysis. They found the molecular weight of the enzyme protein was about 84,000 and that there were four identical subunits per enzyme protein. The amino acid analysis showed that there were two cysteine and three methionine residues per mole of protein subunit. Selenium content analysis showed that there were four atoms of selenium per mole of enzyme. X-ray crystallographic studies on the enzyme by Ladenstein (24) showed that the four selenium atoms were located at the surface of the subunits, suggesting that the selenium atoms may be part of the catalytic site.

In 1978, Forstrom et al. (25) identified the catalytic site of glutathione peroxidase as selenocysteine. They derivatized the seleno group in the intact ^{75}Se labeled rat liver glutathione peroxidase with either iodoacetate or ethylenimine. The products were hydrolyzed with 6N HCl and co-chromatographed with known standards of selenocysteine labeled with tritium. The ^{75}Se labeled

products co-chromatographed with the tritium labeled standards. From these experiments, they concluded that the selenium moiety at the catalytic site of the enzyme was selenocysteine.

Selenium has long been known to be one of the most toxic trace elements. In 1957 Klaus Schwarz (26) discovered that selenium was also an essential trace element of profound physiological significance in warm blooded animals. Rats which he fed on selenium deficient diet developed liver necrosis, but recovered from the disease after resupplementing the diet with Na_2SeO_3. They found that chemical treatments that released selenium irreversibly from glutathione peroxidase also inhibited the enzyme.

Animal experiments have demonstrated that there is a good correlation between dietary selenium intake and glutathione peroxidase activity in various tissue (1, 27). Animals that were maintained on selenium deficient diet showed rapid declines in tissue glutathione peroxidase activity which correlated with the development of selenium deficiency signs (28, 29). These selenium deficient animals showed rapid increase in tissue glutathione peroxidase activity upon resupplementation of diets with Na_2SeO_3.

Rudolph and Wong (30) used paired samples of maternal and cord blood to investigate the possibility of a

correlation between selenium levels and glutathione peroxidase activity in plasma and erythrocytes. Earlier, they had demonstrated that there was low glutathione peroxidase activity in the erythrocytes of neonates. They found the selenium levels in cord erythrocytes to be significantly lower than the levels in either maternal or nonpregnant females. However, they found the enzyme activity in pregnant females to be higher than the activity in nonpregnant females. They concluded that there was a correlation between erythrocyte selenium levels and erythrocyte glutathione peroxidase activity. Their findings of correlations between selenium levels and enzyme activity were confirmed by Thomson et al., (2), who reported correlation between selenium levels and enzyme activity in blood samples of New Zealand residents. On the other hand some investigators including Behne and Wolters (3), Schrauzer and White (4) and Schmidt and Heller (5) did not observe any correlation between selenium levels and enzyme activity.

Necheles and Maldonado (31) reported the first clinical case of hemolytic anemia due to glutathione peroxidase deficiency in human erythrocytes. This case was observed in an 18 year old Puerto Rican boy, who developed a severe hemolytic episode following autotransfusion after surgery. During the crisis his erythrocytes showed Heinz bodies (due to methemoglobin

formation, heme becomes detached and the residue globins precipitate out) his G-6-P-D, pyruvic kinase were normal, blood cultures were negative, Coomb's tests were negative, but his glutathione peroxidase activity was lower than normal. Both his parents' glutathione peroxidase activities were slightly lower than normal. To these investigators it looked like the parents were heterozygous glutathione peroxidase deficient while the child was homozygous glutathione peroxidase deficient.

Hopkins and Tudhope (7) assayed erythrocyte glutathione peroxidase in normal subjects and patients with various diseases. In the nine cases of iron deficiency anemia, the mean glutathione peroxidase activity per volume of erythrocytes was low. During the first 2-4 weeks of treatment with iron, erythrocyte glutathione peroxidase activity increased in parallel with hemoglobin. Enzyme activities above normal range were found in 11 cases of megaloblastic anemia due to vitamin B_{12} deficiency. These high glutathione peroxidase activities showed marked reduction within the first 2 days of specific therapy. Enzyme activity tended to be low in chronic lymphocytic and chronic myeloid leukemia. Normal erythrocyte glutathione peroxidase activity was found in polcythemia vera.

In 1973 (8) a similar study was carried out in rabbits. The investigators observed that glutathione

peroxidase activity began to fall within 10 days of the institution of iron deficient diet to the test rabbits. With reinstitution of an iron replete diet, the haemoglobin concentration began to rise and returned to normal level in 5 weeks. Glutathione peroxidase activity also increased with iron repletion, but the rate of rise lagged behind the changes in haemoglobin concentration. It took 15 weeks before the enzyme activity reached initial levels. To further study the pattern of loss and recovery of glutathione peroxidase activity, rabbit erythrocytes were separated into young and old. In normal or control rabbits there was no significant difference in glutathione peroxidase activity between reticulocyte rich erythrocytes and reticulocyte poor erythrocytes. But when the test rabbits were resupplemented with iron, young erythrocytes had higher enzyme activity than the older erythrocytes. To these scientists, it appeared that glutathione peroxidase activity was determined by the availability of iron during the development of the erythrocytes within the bone marrow.

Experiments in which animals were fed vitamin B_2 and vitamin B_6 deficient diets, showed decreased glutathione peroxidase activity (32, 33). The enzyme activity returned to normal upon resupplementing the diet with the vitamins. Both excess dietary vitamin E and vitamin E deficiency in rats have been shown to depress

glutathione peroxidase activity in the liver and plasma of the laboratory animals (34).

METHODS AND PROCEDURES

Apparatus:

The measurement of glutathione peroxidase activity was made using a Cary Model 219 recording, UV-Visible Spectrophotometer (Varian Associates, Palo Alto, CA) equipped with a thermostated cell holder. The temperature was maintained at 37°C by a circulating water bath, Model No. 2095 (Masterline Forma Scientific, Maritta, OH).

Micro quartz curvettes (Savant Supplier Industries, Inc., Hicksville, NY) with a 1 cm path length were used for the determination of glutathione peroxidase activity.

Determination of erythrocyte and plasma selenium, lead and iron were made using a Polarized Zeeman Effect Flameless Atomic Absorption Spectrophotometer (PZAAS), Model No. 180-70 (N.S.A. Hitachi, Ltd., Mountainview, CA). Automatic Eppendorf micropipets (Brinkman Industries, Westburg, NY) and Finn pipets (Finn pipette, KY, Finland) were used for all pipetting steps.

Fluorometric selenium analyses were made using a Perkin Elmer Fluorescence Spectrophotometer, Model No. 204-0011 (Hitachi Ltd., Tokyo, Japan) equipped with a Perkin Elmer 150 Xenon power supply.

Blood specimens were drawn using Trace Element Vacutainers (Becton-Dickinson, Rutherford, NJ) containing both heparin or no additive.

All glassware used was Kimas grade (Owens Illinois, Toledo, OH) and acid washed polypropylene tubes (Falcon Industries, (Oxnard, CA) were used for the metal analyses.

Measurements of pH were made using a Beckman zeromatic II Digital pH meter (Beckman Instruments, Inc., Fullerton, CA).

Haemoglobin levels were measured using a Perkin Elmer Spectrophotometer, Coleman 124 (Coleman Instruments Division, Oak Brook, ILL).

Tris-hydroxymethyl-aminomethane buffer, pH 7.6 was prepared by dissolving 0.1 moles of a primary grade tris-hydroxymethyl-aminomethane (Frederick Smith Chemical Co., Columbus, OH) in 1 liter of deionized water. To the 1 liter of the buffer was added 2 millimoles of an ACS reagent grade disodium ethylenediamintetraacetate (EDTA) (J. T. Baker Chemical Co., Phillipsburg, NJ) and 2 millimoles of practical grade sodium azide (J. T. Baker Chemical Co., Phillipsburg, NJ). This buffer was used for plasma enzyme assay. When erythrocyte glutathione peroxidase activity was assayed, a similar buffer containing 4mM sodium azide was used. Reduced glutathione 99% (Sigma Chemical Co., St. Louis, MO) was used as substrate for glutathione peroxidase; NADPH 98% (enzymatically reduced form, Sigma Chemical Co., St. Louis, MO) was used as a substrate for glutathione reductase. The glutathione peroxidase substrate, hydrogen peroxide

was prepared from a 30% hydrogen peroxide. (Frederick Smith Chemical Co., Columbus, OH). Ten microliters of 30% hydrogen peroxide were diluted to 10 ml with deionized water to give a final concentration of 8.8 mM. This solution was prepared a few minutes before use.

Lead working standard was prepared from a 1000 ppm lead atomic spectral standard (J. T. Baker Chemical Co., Phillipsburg, NJ). A 0.1 ml of this stock standard (1000 ppm) was diluted with deionized water to give a final concentration of 1 ppm lead standard (1 mg/L).

Selenium working standard was prepared from a 1000 ppm selenium atomic spectral standard (J. T. Baker Chemical Co., Phillipsburg, NJ) by diluting 0.1 ml of the stock standard (1000 ppm) with deionized water to give a final concentration of 1 ppm.

An ACS reagent grade nitric acid (J. T. Baker Chemical Co., Phillipsburg, NJ) and an ACS reagent grade, 70.9% perchloric acid (J. T. Baker Chemical Co., Phillipsburg, NJ) were used for selenium digestion. The chelating agent used for selenium assay by the fluorometric method was 2,3-diaminonaphthalene 99% (DAN) (Aldrich Chemical Co., Inc., Milwaukee, Wis.). In a 250 ml flask, 0.25 gram of DAN (weighed in a semi-dark room) was dissolved in 25 ml of concentrated hydrochloric acid (Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, NJ). The resulting solution was diluted to the mark with deionized water. Then the DAN

solution was extracted with 50 ml of 99% UV grade cyclohexane (J. T. Baker Chemical Co., Phillipsburg, NJ) using a 500 ml separatory funnel. The cyclohexane removed any interfering fluorescent materials from the DAN solution. The aqueous DAN solution was stored at 4°C in a dark bottle (stable for a week). A stabilizing solution was prepared by dissolving 0.4 moles of Na_2H_2-EDTA and 2.5 grams of hydroxylamine hydrochloride, both practical grade reagents (J. T. Baker Chemical Co., Phillipsburg, NJ) in 1 liter of deionized water. The purpose of this solution was to mask metal ion interferences. A 15% ammonia hydroxide solution was prepared by diluting 30% ammonium hydroxide, ACS reagent (J. T. Baker Chemical Co., Phillipsburg, NJ) with equal volume of deionized water.

Drabkin's reagent ($NAHCO_3$, KCN and $K_2Fe(CN)_6$), (Hycel Inc., Houston, Texas) was used for cyanmethemoglobin determinations.

Methods:

Sample Collecting and Preparation:

Specimens were collected from 42 female subjects between 20 and 75 years old. Twenty milliliters of whole venous blood was collected from these fasting individuals (10 hours, between 8 and 9 a.m.). The whole blood was collected in heparinized trace element vacutainers and in vacutainers without additives. Plasma and serum were separated from erythrocytes within 30 minutes of collecting. Serum was immediately frozen while plasma was assayed for glutathione peroxidase activity. Erythrocytes in heparinized tubes were washed three times with 0.9% sodium chloride solution. The washed cells were frozen until assay. The fasting serum was used to determine the normality of each individual subject. Blood urea nitrogen was measured in order to assess normal liver function. Cholesterol and triglyceride levels were measured to assess normal lipid metabolism and fasting glucose levels to assess normal glucose metabolism.

Glutathione Peroxidase Assay in Plasma:

The assay for glutathione peroxidase was a modification of the procedure of Paglia and Valentine (35). A 2 ml reaction mixture containing 0.05 M tris buffer (a two fold dilution of the 0.1 M tris buffer), 40 mM reduced glutathione, 4 mM NADPH and 5 IU/ml of glutathione

reductase, was prepared a few minutes before assay. Fifty microliters of this 2 ml reaction mixture, and 0.9 ml of the two-fold dilution of the 0.1 M tris buffer were incubated with 50 microliters of plasma at 37°C for five minutes. Then ten microliters of the 8.8 mM hydrogen peroxide were added to the cuvette and the mixture was mixed by inversion to start the reaction. The calculated concentration of hydrogen peroxide in the 1 ml system was 0.088 mM. For nonezymatic activity, all ingredients except plasma were added to the cuvette. The rate of reaction was measured by following the decrease in the absorbance of NADPH at 340 nm. The absorbance change ($\Delta \frac{Abs}{min}$) for the nonenzymatic activity was subtracted from the $\Delta \frac{Abs}{min}$ of the enzymatic activity in the plasma. The enzyme activity, in IU/L, was calculated as follows:

$$\frac{(\Delta \text{ Abs/min})(1 \text{ ml})(1000 \text{ ml/L})(10^6 \text{ u mole/mole})}{(0.05 \text{ ml})(6.22)(10^6 \text{ ml/moles}^{-1}\text{cm}^{-1})(1 \text{ cm path length})} \quad (1)$$

1 IU is 10^{-6} moles of substrate utilized per minute.

Glutathione peroxidase Assay in Erythrocytes:

The assay for glutathione peroxidase activity in erythrocytes was a modification of the procedure of Beutler (36). One ml of washed erythrocytes was hemolyzed with 3 ml of deionized water and left to stand for 15 minutes at room temperature, to allow complete hemolysis of erythrocytes. Then the hemolysate was

centrifuged for 20 minutes to remove erythrocyte membranes. One ml of the supernatant was diluted with 4 ml of Hycel Drabkin's reagent to convert hemoglobin to cyanmethemoglobin. The diluted hemolysate was left to stand for 20 minutes to allow complete conversion of haemoglobin to cyanmethemoglobin. A final reaction mixture, in a 1 ml reaction system, containing 0.05 M tris buffer, 2 mM reduced glutathione 0.2 mM NADPH and 0.25 IU/ml of glutathione reductase was incubated at 37°C with 20 microliters of hemolysate for five minutes. Then 10 microliters of the 8.8 mM hydrogen peroxide were added to the cuvette to start the reaction. The decrease in NADPH absorbance was monitored at 340 nm. For nonenzymatic activity, all ingredients except erythrocyte hemolysate, were added to the cuvette. The $\Delta \frac{Abs}{min}$ for nonenzymatic activity was subtracted from the $\Delta \frac{Abs}{min}$ of the enzymatic activity in erythrocytes. The enzyme activity in IU/L was calculated using equation (1). The final dilution factor for erythrocytes was 1000. Haemoglobin levels were determined in each hemolysate and glutathione peroxidase activity in IU/g haemoglobin was also reported.

Selenium Assay in Plasma by the Fluorometric Method:

Plasma selenium was assayed by the modified method of Watkinson (37). Fifty ml erlenmeyer flasks were washed

and soaked overnight in concentrated hydrochloric acid and then rinsed thoroughly with deionized water before use. Selenium standards 0, 0.05, 0.1, 0.15, 0.2, and 0.3 micrograms were prepared from a 1 ppm selenium working standard. To flasks labeled specimens, 1 ml of plasma, 5 ml of concentrated nitric acid and 3 ml of perchloric acid were added in that order. The flasks were swirled and placed on a hot plate under a perchloric hood. The flasks were heated slowly to boiling (30 minutes). Upon evolution of white fumes of perchloric acid, the flasks were removed from the hot plate and cooled. After cooling, the flasks were returned to the hot plate and heated again for 20 minutes. The flasks were swirled and digestion continued for another 10 minutes. After again cooling the flasks, 3 ml of 0.5 N hydrochloric acid were added to the flasks. The flasks were returned to the hot plate and heated for another 20 minutes. After cooling the flasks, 10 ml of Na_2H_2EDTA were added to both standards and specimens and their pH was adjusted to 1.5 using 15% ammonium hydroxide and 0.5 N hydrochloric acid. In a semi-dark room, 5 ml of DAN solution was added to both standards and specimens. The flasks were swirled and incubated at 50°C for 30 minutes. The flasks were cooled and then 6 ml of cyclohexane were added to each flask.

The flasks were then swirled for five minutes before filling them with deionized water. The cyclohexane layer was transferred to a quartz cuvette. The solution was excited at 376 nm and the emitted fluorescence measured at 518 nm. Erythrocyte selenium measurements were made using the same fluorometric method of Watkinson (37).

The fluorescence of the standards, corrected for the reagent blank, was plotted and the concentration of selenium in each specimen was read from the standard curve. Selenium concentrations are reported in micrograms/L.

Plasma Selenium Assay by the PZAAS:

Plasma Selenium was also assayed by the PZAAS method. Using reverse pipetting technique, 1 ml of a four fold dilution of plasma containing 30 microliters of a 5,000 ppm nickel standard was prepared. Standard additions of 0, 25 and 50 ppb selenium were made to plasma. Twenty microliters of the blank (containing only 150 ug Ni^{+2}/L) standard or specimen was transferred to the tube cuvette and analyzed using the parameters listed in Table 1. The standard curve was prepared by subtracting the absorbance of the endogenous selenium from that of the specimen spiked with selenium standard. The concentrations of selenium in specimens were read from a composite curve made from two standard additions assays. Selenium concentrations are reported in micrograms/L.

TABLE 1

PARAMETERS USED FOR SELENIUM ANALYSIS BY THE ZAAS

Lamp Current	=	20.0 mA	
WaveLength	=	196.0 nm	
Slit Width	=	0.4 nm	
Carrier gas	=	100 mL/min, gas interrupt mode used during atomization	
Heating Program:		Temp. (^{o}C)	Time (Sec)
Dry		60 to 90	60.0
Dry		90 to 90	30.0
Ash		1050 1050	40.0
Atom		2600 2600	7.0
Clean		2800 2800	3.0

Nitrogen carrier gas used throughout assay

Erythrocyte Selenium Assay by the PZAAS:

Selenium in erythrocytes was also quantitated by the PZAAS method. Using the reverse pipetting technique, 1 ml of a four fold dilution of erythrocytes, 25 microliters of a 10,000 ppm nickel standard were added to polypropylene tubes containing enough deionized water to give a final volume of 1.6 ml. Additions of 0, 33.3 and 66.7 ppb selenium standards were made to the hemolysate. Twenty microliters of nickel blank (25 microliters of 10,000 ppm nickel standard in 1.6 ml of deionized water) standards and specimens were transferred to a tube cuvette and analyzed using the parameters listed in Table 1. The curve was prepared by subtracting the endogenous concentration of selenium. The concentrations of selenium in specimens were read from a composite curve of two standard addition curves. Selenium concentrations are reported in micrograms/L and in micrograms/g Hb.

Lead Assay in Erythrocytes:

Lead was assayed using the PZAAS. A twenty fold dilution of erythrocytes was used for quantitating lead in erythrocytes. Using the reverse pipetting technique, 50 microliters of erythrocytes and 25 microliters of 5% nitric acid were added to polypropylene tubes containing enough deionized water to give a final volume of 1 ml. Additions of 0, 25 and 50 ppb lead standards were made to the hemolysate. Ten microliters of

reagent blank (25 microliters of 5% nitric acid in 1 ml of deionized water), standards and specimens were transferred to a cup-type cuvette and analyzed using the parameters listed in Table 2. The standard curve was prepared by subtracting the absorbance of the endogenous lead from that of the specimen spiked with lead. The concentration of lead in the specimens was read from a composite curve made from two standard addition analyses. Plasma lead assays were made in a manner similar to the erythrocyte lead assay. We used a two fold dilution of specimen. Lead concentrations are reported in micrograms/L.

Plasma Iron Assay:

Iron was assayed using the PZAAS. Using the reverse pipetting technique, 50 microliters of plasma were added to polypropylene tubes containing enough deionized water to give a final volume of 1 ml. Additions of 0, 50 and 100 ppb from standards were made to the plasma. Ten microliters of standards, specimens or reagent blank (1 ml of deionized water) were transferred to a tube cuvette and analyzed for iron using the parameters listed in Table 3. The curve was prepared by subtracting the endogenous iron concentration. The concentrations of iron in specimens were read from a composite curve of two standard addition curves. Iron centrations are reported in micrograms/dl.

TABLE 2

PARAMETERS USED FOR LEAD ANALYSIS BY THE ZAAS

Lamp Current	=	7.5 mA		
WaveLength	=	283.3 nm		
Slit width	=	1.3 nm		
Carrier gas	=	100 mL/min, gas interrupt made used during atomization		
Heating Program:		Temp. (^{o}C)		Time (Sec)
Dry		80	to 120	30.0
Ash		400	to 400	30.0
Atom		2000	to 2000	7.0
Clean		2400	to 2400	3.0

Nitrogen carrier gas used throughout assay

TABLE 3

PARAMETERS USED FOR IRON ANALYSIS BY THE ZAAS

Lamp Current	=	10.0 mA	
WaveLength	=	248.3 nm	
Slit width	=	0.2 nm	
Carrier gas	=	100 mL/min, gas interrup made used during atomization	
Heating Program:		Temp. (^{o}C)	Time (Sec)
Dry		80 120	30.0
Ash		600 600	30.0
Atom		2500 2500	7.0
Clean		2800 2800	3.0

Nitrogen carrier gas used throughout analyses.

Haemoglobin Determination:

Haemoglobin measurements in the packed erythrocytes was made by using the Hycel Cyanmethemoglobin standard procedure (38).

RESULTS

Conditions under which glutathione peroxidase activity has been assayed varies between groups of investigators. In this research, the assay conditions were optimized for pH, temperature and NADPH concentration.

Figure 1 shows a plot of glutathione peroxidase activity versus pH. Plasma enzyme activity increased from 0 U/L at pH 6.0 to 386 U/L between pH 7.8 and 8.0.

A plot of enzyme activity versus temperature is shown in Figure 2. The activity increased from a value of 258 U/L at 25°C to 559 U/L at 37°C. Enzyme activities were determined at pH 7.6.

The effect of NADPH on glutathione peroxidase activity is shown in Figure 3. Maximum activity was observed at 0.10 mM NADPH (lowest concentration used) and minimum activity at 0.40 mM NADPH. Enzyme activity was stable at concentrations 0.20 mM and 0.25 mM NADPH. A significant decrease in glutathione peroxidase activity was observed at 0.40 mM NADPH. To further clarify which of the coupled enzymes (glutathione peroxidase or glutathione reductase) was being inhibited, the effect of NADPH on glutathione reductase activity was determined. Figure 4 shows a significant decrease in glutathione reductase activity at 0.30 mM NADPH. There is a greater relative decrease in glutathione reductase activity with

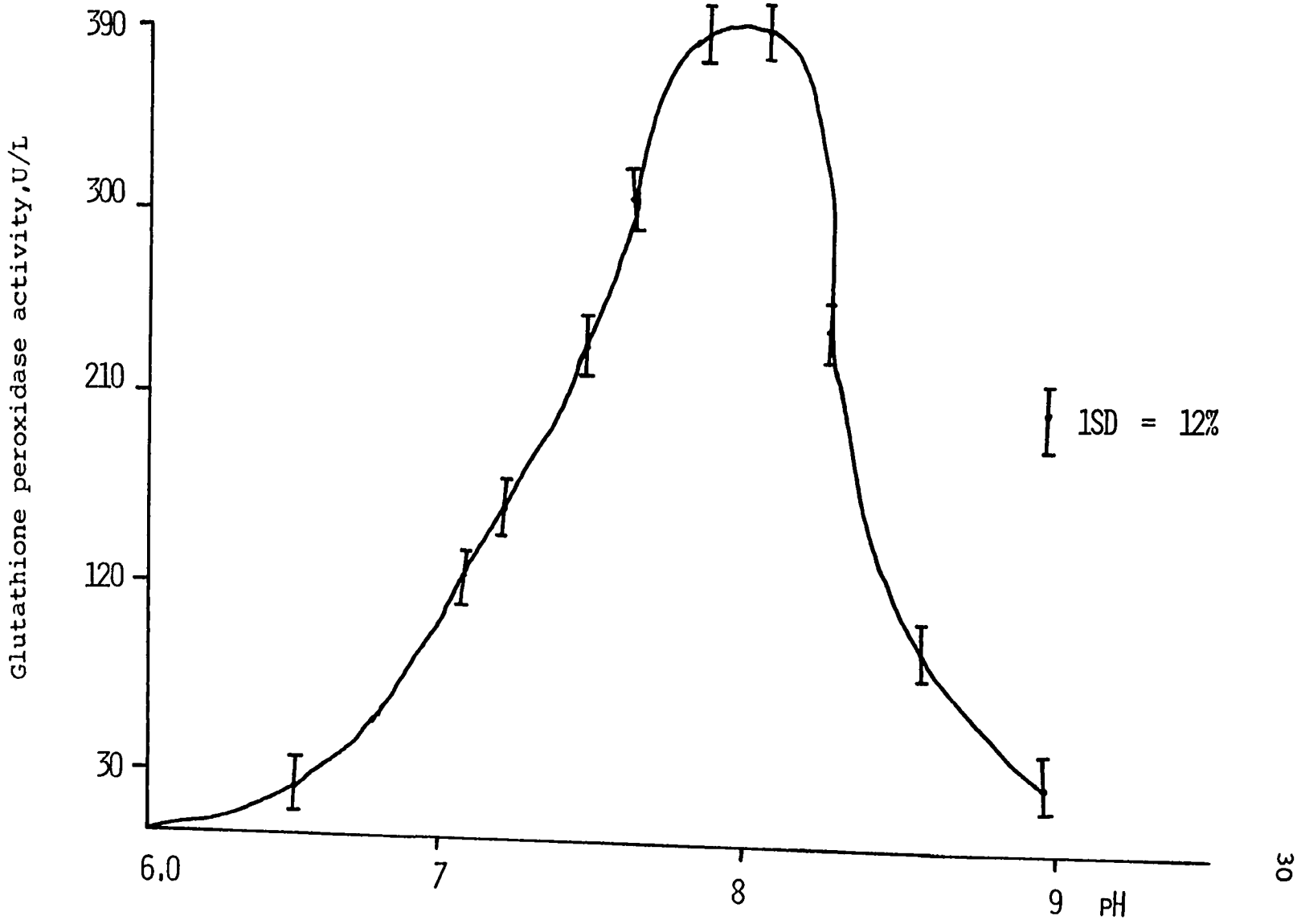

Figure 1. pH versus enzyme activity

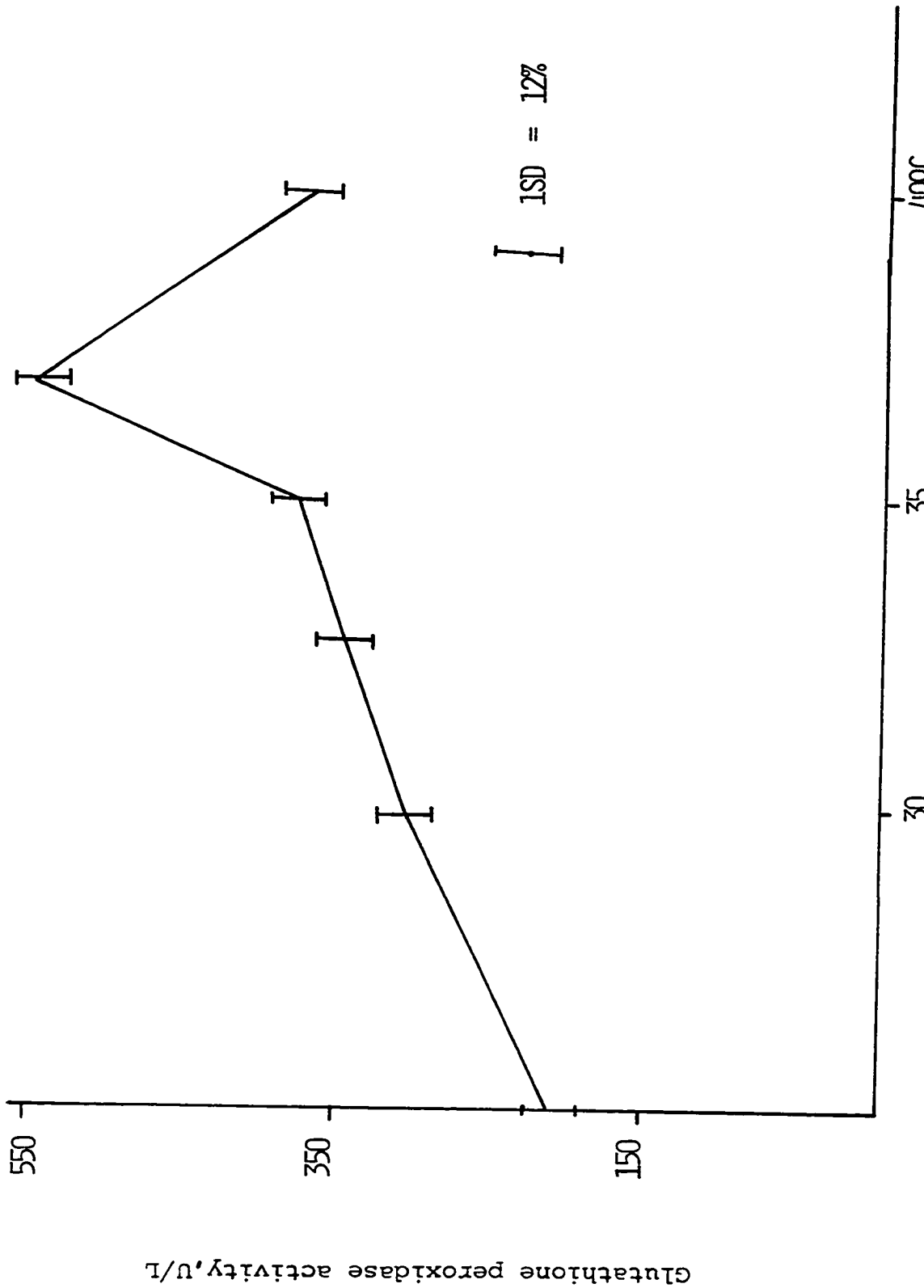

Figure 2. Temperature versus enzyme activity

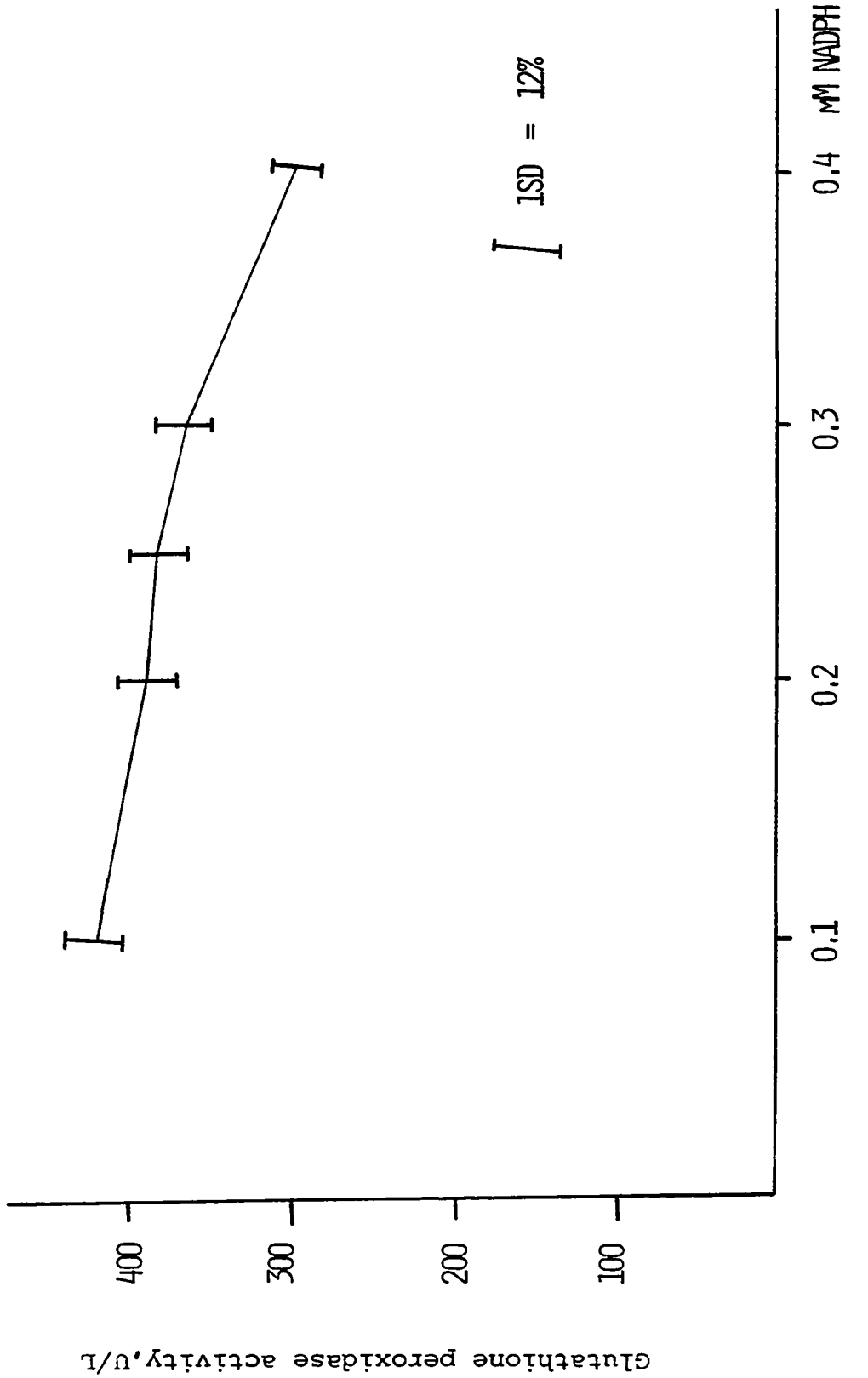

Figure 3. NADPH versus enzyme activity

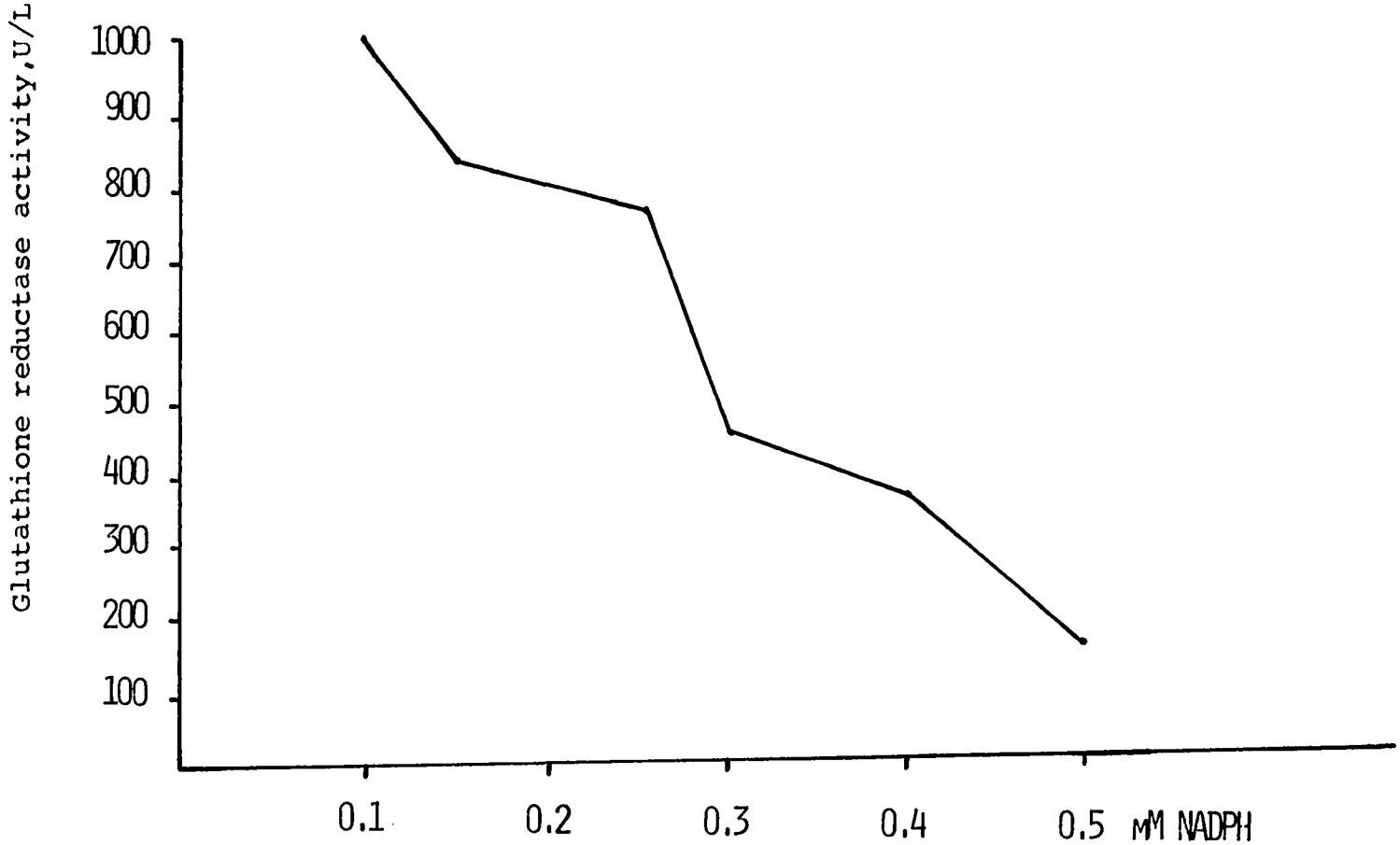

Figure 4. NADPH versus enzyme activity

increasing NADPH concentration than that seen for glutathione peroxidase activity over the same range of NADPH concentrations.

The effects of reduced glutathione on glutathione peroxidase activity was determined previously (39). Maximum activity was observed in the concentration range 1.80 mM and 2.40 mM of reduced glutathione. Above concentrations of 2.40 mM, enzyme activity began to decrease gradually.

Storage studies indicated that enzyme activity in plasma was stable for two days when kept at $4^{o}C$. After two days, glutathione peroxidase activity began to deteriorate. Frozen plasma glutathione peroxidase activity remained fairly stable for 4 days. Erythrocyte glutathione peroxidase activity remained stable for 5 days when stored at $-20^{o}C$. Results are shown in Table 4.

Precision studies for analyses of glutathione peroxidase activity and selenium assays in both plasma and erythrocytes are shown in Table 5. The following formulas were used to calculate standard deviations and coefficients of variation (40):

$$SD = \sqrt{\frac{\text{Sum } (X - \bar{X}_1)^2}{\text{replicates x runs } -1}} \qquad CV = \frac{SD}{\bar{X}_1} \qquad (2)$$

where $\bar{X}_1$ is the grand mean and X is the value of each replicate. Erythrocyte and plasma enzyme activity with-in run precision studies were determined from 10 and 13

TABLE 4

Duration of storage	Storage conditions, plasma		Storage conditions erythrocytes
	U/L		
	4°C	-20°C	-20°C
Initial	463	463	23, 160
1 day	463	-	23, 160
2 days	463	-	23, 160
3 days	-	-	23, 160
4 days	232	308	23, 160
5 days	150	193	23, 160
7 days	0	39	-
14 days	-	0	-

TABLE 5

PRECISION STUDY OF PLASMA - ERYTHROCYTE SELENIUM AND GLUTATHIONE PEROXIDASE ACTIVITY

	No. of runs	$\bar{X}$, µg/L	SD, µg/L	CV
Within-run:				
Erythrocyte Selenium	10	149.9	8.3	5.6
Plasma Selenium	12	97.3	6.2	6.4
Between-run:				
Erythrocyte Selenium	32	145.7	10.3	7.1
Plasma Selenium	21	89.2	6.6	7.4
		$\bar{X}$, U/L	SD, U/L	CV
Within-run:				
Erythrocyte enzyme	10	23,160	2,980	12.8
Plasma enzyme	13	312.5	25.2	8.1

replicate values, respectively. Within-run precision for the erythrocyte and plasma selenium PZAAS assays was determined using 10 and 12 replicate values, respectively. Within-run precision was found to be 149.9 $\pm$ 8.3 µg Se/L of erythrocytes and 97.3 $\pm$ 6.2 µg Se/L of plasma. In order to determine between-run precision for the assays, both hemolysate and plasma specimens were analyzed for selenium on five consecutive days. Thirty-two erythrocyte selenium replicates were made and the precision found to be 145.7 $\pm$ 10.3 µg/L. For plasma, 21 replicate determinations yielded a mean $\pm$ standard deviation of 89.2 $\pm$ 6.6 µg Se/L.

A comparison study was made between selenium concentrations as determined by the fluorometric method and by the PZAAS. Mean selenium concentration in pooled plasma was 97.3 $\pm$ 6.2 µg/L (n = 12) using PZAAS. Replicate fluorometric analyses on the same pool yielded a higher value 103.4 $\pm$ 6.6 ug/L (n = 9). However, when plasma from eight subjects was analyzed, the PZAAS results tended to be higher. Results are shown in Table 6. Fluorometric analysis of hemolysate yielded a mean of 153.2 $\pm$ 7.3 µg/L (n = 10). PZAAS determination yielded 149.9 $\pm$ 8.3 µg/L.

Statistical parameters and correlation coefficients were determined using Statistical Package for the Social Sciences Program (SPSS) (41). Means of glutathione

TABLE 6

COMPARISON OF FLUOROMETRIC AND PZAA PLASMA SELENIUM ANALYSES

	Selenium Concentrations, µg/L				
Subject No.	Fluorometric	PZAA	Subject No.	Fluorometric	PZAA
1	78	80	5	78	84
2	82	87	6	90	97
3	90	86	7	90	95
4	90	94	8	82	80

peroxidase activity, selenium, lead, iron and haemoglobin concentrations for plasma and erythrocytes are shown in Table 7.

Table 8 shows the effects of age and smoking on mean glutathione peroxidase activity, selenium and lead concentrations. As indicated in the table, smokers had a significantly higher mean lead concentration compared to nonsmokers. From the data, mean glutathione peroxidase activity and selenium concentration do not appear to be affected by smoking, and age does not appear to affect mean erythrocyte glutathione peroxidase activity, selenium or lead concentrations.

Data in Table 9 shows the effect of vitamin and mineral supplementation and use of oral contraceptives on mean erythrocyte glutathione peroxidase activity, selenium and lead concentrations. As indicated in the table, the six subjects that were taking both vitamins and minerals had a significantly higher mean selenium concentration than the other subjects. The erythrocyte mean lead concentration determined in our research, was not affected by minerals and vitamin supplementation. Oral contraceptives users had slightly higher mean enzyme activity and slightly higher mean selenium concentration than noncontraceptive users. There was no difference in mean lead concentration of oral contraceptive users versus mean lead concentration of those not using oral contraceptives.

TABLE 7

GLUTATHIONE PEROXIDASE ACTIVITY AND TRACE ELEMENT CONCENTRATIONS IN BOTH PLASMA AND ERYTHROCYTES

	N	$\bar{X}$	SD	Range
Erythrocyte enzyme activity	38	22,274 U/L	3,068 U/L	15,440 - 27,000 U/L
Enzyme activity/g HB	38	78.6 U/gHb	12.8 U/g Hb	51-106 U/gHb
Plasma enzyme activity	38	424.6 U/L	39.6 U/L	306-502 U/L
Erythrocyte selenium	38	141.4 µg/L	14.2 µg/L	120-167 µg/L
Selenium/g Hb	38	0.49 µg/g Hb	0.7 µg/g Hb	0.37-0.68 µg/L
Plasma selenium	38	96.3 µg/L	14.2 µg/L	72-128 µg/L
Erythrocyte lead	38	142.3 µg/L	32.2 µg/L	100-212 µg/L
Plasma lead	38	14.2 µg/L	3.9 µg/L	4.4-22 µg/L
Plasma iron	38	100.2 µg/L	19.4 µg/L	52-132 µg/L
Haemoglobin	38	28.9 µg/L	2.4 µg/L	20-33 µg/L

N = no. of subjects, $\bar{X}$ = mean, SD = standard deviation, Hb = haemoglobin.

TABLE 8

EFFECTS OF AGE AND SMOKING ON ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM AND LEAD CONCENTRATIONS

		Erythrocyte			
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Pb/L	
Entire population	38	22,274 ± 3,068	141.4 ± 14.2	142.3 ± 32.2	
Age:					
20-29	22	22,601 ± 3,488	142.8 ± 16.6	148.6 ± 27.1	
30-39	11	21,569 ± 2,835	141.9 ± 10.0	121.3 ± 33.0	
40-49	1	23,160 ± 0.0	131.0 ± 0.0	124.0 ± 0.0	
Over 50	4	22,190 ± 1,120	135.5 ± 12.4	169.5 ± 32.0	
Smokers	5	23,380 ± 1,727	135.0 ± 10.6	183.0 ± 28.2	a
Nonsmokers	28	22,166 ± 3,425	143.2 ± 15.1	135.6 ± 28.1	a
Former smokers	5	22,768 ± 2,119	137.6 ± 11.5	138.8 ± 32.6	a

N = no. of subjects, a = one tailed t-test, t = 2.40, p = 0.01

TABLE 9

EFFECTS OF VITAMIN AND MINERAL SUPPLEMENTATION AND USE OF ORAL CONTRACEPTIVES ON ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM AND LEAD CONCENTRATIONS

		Erythrocyte		
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Pb/L
Entire population	38	22,274 $\pm$ 3,068	141.4 $\pm$ 14.2	142.3 $\pm$ 32.2
Minerals and vitamins	6	23,153 $\pm$ 2,118	150.6 $\pm$ 18.4 a	133.8 $\pm$ 35.0
No minerals	11	21,051 $\pm$ 3,062	140.1 $\pm$ 13.6	146.4 $\pm$ 32.6
No minerals no vitamins	21	22,663 $\pm$ 3,223	137.4 $\pm$ 12.3 a	142.6 $\pm$ 32.4
Oral contraceptives				
Yes	4	23,492 $\pm$ 5,798	147.8 $\pm$ 13.4	129.5 $\pm$ 24.2
No	34	22,131 $\pm$ 2,701	140.7 $\pm$ 14.4	143.8 $\pm$ 33.0

N = no. of subjects, a = one tailed t-test, t = 1.74, p = 0.05

Effects of consumption of caffeinated beverages on mean erythrocyte glutathione peroxidase activity, selenium and lead concentrations are shown in Table 10. Tea drinkers tended to have higher mean enzyme activity and higher mean selenium concentration compared to nontea drinkers.

Table 11 shows the effects of age and smoking on mean plasma glutathione peroxidase activity, selenium iron and lead concentrations. No significant difference in mean enzyme activity, selenium, iron and lead concentrations was observed due to age and smoking.

Slightly higher mean glutathione peroxidase activity, selenium, lead and iron concentrations were found in plasma of the six subjects that were taking both vitamins and minerals compared to that of other subjects. This data is shown in Table 12.

The effects of the use of caffeinated beverages on mean plasma glutathione peroxidase activity, selenium, iron and lead concentrations are shown in Table 13. As indicated in the table, tea drinkers had a significantly lower mean plasma iron concentration compared to that of other subjects.

Table 14 shows the effects of age and smoking on mean erythrocyte glutathione peroxidase activity (U/g haemoglobin), selenium (μg/g haemoglobin) and haemoglobin. Erythrocyte enzyme activity and selenium per gram of haemoglobin were calculated because glutathione

TABLE 10

EFFECTS OF CONSUMPTION OF CAFFEINATED BEVERAGES ON ERYTHROCYTE GLUTATHIONE PEROXIDASE, ACTIVITY, SELENIUM AND LEAD CONCENTRATIONS

		Erythrocyte		
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Pb/L
Entire population	38	22,274 ± 3,068	141.4 ± 14.2	142.3 ± 32.2
Beverages:				
None	10	21,606 ± 3,496	137.6 ± 17.3	153.7 ± 23.6
Tea	7	24,441 ± 1,923	150.2 ± 13.4	147.8 ± 31.6
Coffee	12	22,188 ± 1,752	137.3 ± 12.4	143.9 ± 38.1
Both	9	21,444 ± 4,163	144.3 ± 11.5	123.2 ± 28.5

TABLE 11

EFFECTS OF AGE AND SMOKING ON PLASMA GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM, IRON AND LEAD CONCENTRATIONS

		Erythrocyte			
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Fe/L	$\bar{X}$,µg Pb/L
Entire population	38	424.6 ± 39.6	96.3 ± 14.2	100.2 ± 19.4	14.2 ± 3.9
Age:					
20-29	22	424.2 ± 37.8	98.6 ± 16.6	98.8 ± 19.4	14.6 ± 4.6
30-39	11	415.1 ± 44.2	93.2 ± 10.4	105.2 ± 22.8	13.6 ± 2.7
40-49	1	424.0 ± 0.0	85.0 ± 0.0	84.0 ± 0.0	10.4 ± 0.0
Over 50	4	453.2 ± 37.3	95.0 ± 0.0	97.5 ± 9.6	15.1 ± 2.4
Smokers	5	439.8 ± 44.2	95.2 ± 14.2	95.8 ± 31.4	12.6 ± 28.2
Nonsmokers	28	424.6 ± 39.9	95.6 ± 15.2	102.0 ± 15.6	15.2 ± 3.2
Former smokers	5	409.0 ± 34.4	101.4 ± 7.9	94.2 ± 28.2	10.2 ± 3.9

TABLE 12

EFFECTS OF MINERAL AND VITAMINS SUPPLEMENTATION AND USE OF ORAL CONTRACEPTIVES ON PLASMA GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM, IRON AND LEAD CONCENTRATIONS

		Plasma			
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Fe/L	$\bar{X}$,µg Pb/L
Entire population	38	424.6 ± 39.6	96.3 ± 14.2	100.2 ± 19.4	14.2 ± 3.9
Minerals and vitamins	6	430.0 ± 29.0	98.8 ± 13.3	108.6 ± 14.7	15.4 ± 3.2
No minerals	11	424.2 ± 30.0	100.0 ± 14.8	99.5 ± 14.5	14.2 ± 4.2
No minerals no vitamins	21	423.1 ± 47.2	93.7 ± 14.2	98.1 ± 22.7	14.0 ± 4.1
Oral contraceptives					
Yes	4	414.8 ± 36.8	90.0 ± 6.1	109.0 ± 21.8	14.1 ± 6.6
No	34	425.8 ± 40.2	97.1 ± 14.7	99.1 ± 19.2	14.2 ± 3.6

TABLE 13

EFFECTS OF CONSUMPTION OF CAFFEINATED BEVERAGES ON PLASMA GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM, IRON AND LEAD CONCENTRATIONS

		Plasma			
	N	$\bar{X}$,U/L	$\bar{X}$,µg Se/L	$\bar{X}$,µg Fe/L	$\bar{X}$,µg Pb/L
Entire population	38	424.6 ± 39.6	96.3 ± 14.2	100.2 ± 19.4	14.2 ± 3.9
Beverages:					
None	10	424.1 ± 48.4	99.6 ± 21.1	106.5 ± 13.7 a	13.2 ± 4.9
Tea	7	424.4 ± 48.4	98.7 ± 10.4	83.4 ± 22.1 a	15.6 ± 4.3
Coffee	12	418.9 ± 34.0	95.8 ± 10.2	105.6 ± 17.3 a	13.4 ± 3.2
Both	9	438.8 ± 42.3	91.6 ± 12.8	109.0 ± 20.6 a	15.4 ± 3.3

TABLE 14

EFFECTS OF AGE AND SMOKING ON ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM, AND HAEMOGLOBIN CONCENTRATIONS

		Erythrocyte		
	N	$\bar{X}$,U/g Hb	$\bar{X}$,µg Se/g Hb	$\bar{X}$, Hb g/dl
Entire population	38	78.6 ± 12.8	0.49 ± 0.7	28.0 ± 2.3
Age:				
20-29	22	79.8 ± 12.8	0.49 ± 0.7	29.2 ± 1.8
30-39	11	75.2 ± 14.7	0.49 ± 0.7	29.0 ± 3.1
40-49	1	92.0 ± 0.0	0.52 ± 0.0	25.0 ± 0.0
Over 50	4	78.0 ± 8.6	0.48 ± 0.8	28.4 ± 2.3
Smokers	5	82.4 ± 16.2	0.50 ± 1.2	27.8 ± 4.8
Nonsmokers	28	76.7 ± 11.8	0.50 ± 0.7	29.2 ± 1.8
Former smokers	5	85.8 ± 15.3	0.48 ± 0.4	28.6 ± 1.2

Hb = haemoglobin

peroxidase activity and selenium concentrations may be regulated by the amount of erythrocyte haemoglobin requiring protection, rather than the cell volume. The age of subjects and smoking history did not seem to affect mean enzyme activity, selenium or haemoglobin concentrations.

The effects of minerals and vitamin supplementation and use of oral contraceptives on mean erythrocyte glutathione peroxidase activity (U/g haemoglobin), selenium (μg Se/g haemoglobin) and haemoglobin concentrations are shown in Table 15. The six individuals that were taking both minerals and vitamins had a significantly higher mean enzyme activity, as indicated in Table 15 compared to the other subjects.

Table 16 shows the effects of the use of caffeinated beverages on enzyme activity (U/g haemoglobin), selenium (μg Se/g haemoglobin) and haemoglobin concentrations. There was a significant difference between mean enzyme activity of tea drinkers and nontea drinkers as indicated in Table 16. Tea drinkers had a higher mean enzyme activity than the other subjects. Mean selenium concentration in tea drinkers was slightly higher than that in nontea drinkers.

A plot of glutathione peroxidase activity (U/g haemoglobin) versus selenium concentrations (μg/g haemoglobin) for the 38 subjects is shown in Figure 5. The graph shows a linear regression line with the

TABLE 15

EFFECTS OF MINERAL AND VITAMIN SUPPLEMENTATION AND USE OF ORAL CONTRACEPTIVES ON GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM AND HAEMOGLOBIN CONCENTRATIONS

		Erythrocyte		
	N	$\bar{X}$,U/g Hb	$\bar{X}$,µg Se/g Hb	$\bar{X}$,Hb g/dl
Entire population	38	78.6 ± 12.8	0.49 ± 0.7	28.0 ± 2.3
Minerals and vitamins	6	86.2 ± 4.5 a	0.53 ± 1.2	28.4 ± 1.4
No minerals	11	73.7 ± 10.6 a	0.50 ± 0.7	28.4 ± 1.8
No minerals no vitamins	21	79.1 ± 14.6 a	0.48 ± 0.7	29.4 ± 2.7
Oral contraceptives				
Yes	4	80.5 ± 20.4	0.50 ± 0.4	29.4 ± 1.6
No	34	79.4 ± 12.2	0.49 ± 0.9	28.9 ± 2.4

a = one tailed t-test, t = 2.64, p = 0.01

TABLE 16

EFFECTS OF CONSUMPTION OF CAFFEINATED BEVERAGES ON ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY, SELENIUM AND HAEMOGLOBIN CONCENTRATIONS

		Erythrocyte		
	N	$\bar{X}$,U/g Hb	$\bar{X}$,ug Se/g Hb	$\bar{X}$,Hb g/dl
Entire population	38	78.6 ± 12.8	0.49 ± 0.7	28.0 ± 2.3
Beverages:				
None	10	73.3 ± 9.3 a	0.48 ± 0.8	29.2 ± 2.4
Tea	7	88.6 ± 11.4 a	0.52 ± 0.5	29.2 ± 0.8
Coffee	12	76.5 ± 9.9 a	0.47 ± 0.5	29.1 ± 2.0
Both	9	79.8 ± 17.4	0.52 ± 0.8	28.3 ± 3.4

a = one tailed t-test, t = 1.8, p = 0.05

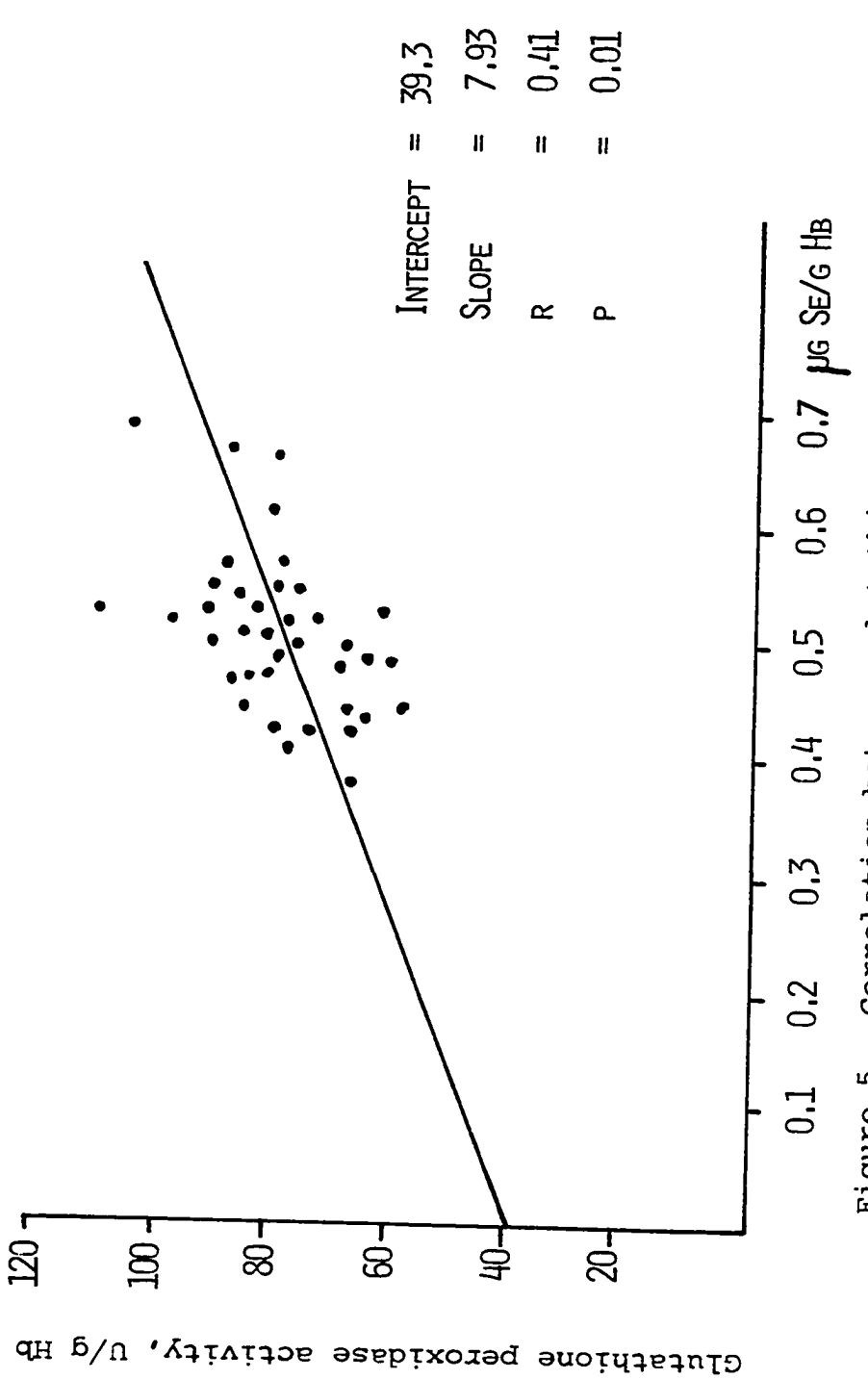

Figure 5. Correlation between glutathione peroxidase and Selenium

equation of Y = 7.93 x +39.3. The Spearman Rank Correlation Coefficient for this data was 0.41.

Finally, Table 17 shows Spearman Rank Correlation Coefficients for the parameters investigated. The Spearman Rank Correlation Coefficients were calculated rather than Pearson Correlation Coefficients in order to minimize the effects of "outlying values".

TABLE 17

SPEARMAN CORRELATION COEFFICIENTS

RBC GSH-Px, U/L	r = .0687	Plasma GSH-Px, U/L	r = .1648
vs	n = 38	vs	n = 38
RBC Selenium, µg/L	p = .341	Plasma Iron, µg/dL	p = .161
RBC GSH-Px, U/L	r = .0753	RBC GSH-Px, µg/g Hb	r = .3946
vs	n = 38	vs vs	n = 38
RBC Lead, µg/L	p = .327	Plasma Iron, µg/dL	p = .007
RBC GSH-Px, U/L	r = -.1337	RBC GSH-Px, U/g Hb	r = -.2885
vs	n = 38	vs	n = 38
Plasma Iron, µg/dL	p = .212	Hb, g/dL	p = .040
Plasma GSH-Px, U/L	r = .1984	RBC GSH-Px, U/g Hb	r = .4074
vs	n = 38	vs	n = 38
Plasma Selenium, µg/L	p = .116	Selenium, µg/g Hb	p = .006

TABLE 17 CONTINUED

Plasma GSH-Px, U/L	r = .2166	Plasma Iron, µg/dl	r = .3509
vs	n = 38	vs	n = 38
Plasma Lead, µg/L	p = .096	Hb, g/dL	p = .015
Plasma Iron, µg/dL	r = .1763	Hb, g/dL	r = -0.1714
vs	n = 38	vs	n = 38
Selenium, µg/g Hb	p = .145	RBC Selenium µg/L	p = 0.152

GSH-Px = Glutathione peroxidase

Hb = Haemoglobin

DISCUSSION

Maximum activity for glutathione peroxidase was observed between pH 7.8 and 8.0. However, pH 7.6, which gave greater enzyme activity than the usually cited pH 7.0, was used for assay. Since maximum activity for the enzyme was observed at temperature 37°C, all enzyme activities were measured at this temperature. NADPH appears to inhibit glutathione reductase activity rather than glutathione peroxidase activity. From figure 4, it is obvious that glutathione reductase is inhibited by NADPH concentrations. In our reaction mixture, we had excess glutathione reductase to overcome the inhibitory effect of NADPH. A concentration of 0.2 mM NADPH was used to assay glutathione peroxidase activity in both plasma and erythrocytes. A concentration of 2.0 mM reduced glutathione was used in this research because maximum activity for glutathione peroxidase was found between 1.80 mM and 2.40 mM reduced glutathione (39).

A reasonable agreement between selenium concentrations determined fluorometrically and by the PZAAS was observed. The PZAAS method was chosen because selenium could be determined directly in plasma or erythrocytes, while the fluorometric method required a time-consuming HNO_3-$HClO_4$ digestion followed by extraction and chelation with a light-sensitive indicator (2,3-diaminonaphthalene).

Although frozen plasma and erythrocyte glutathione

peroxidase activities were found to be stable for four and five days, respectively, enzyme assays in plasma were made on the day of collection. Erythrocyte enzyme assays were made within a day.

The six subjects that were taking both minerals and vitamins had a significantly higher mean erythrocyte selenium concentration compared to the other subjects. These six subjects had also a significantly higher mean erythrocyte enzyme activity (U/g haemoglobin) compared to that of other subjects. However, we did not determine actual vitamin concentrations in the blood of our subjects. Therefore we cannot explain the cause of high enzyme activities and high selenium concentrations in the blood of the six subjects.

Smokers had a significantly higher mean erythrocyte lead concentration than that of nonsmokers. This is in agreement with the findings reported in literature (42). Our plasma lead concentrations were variable, possibly because specimens are easily contaminated by exogenous lead. Plasma lead concentrations in some subjects were as low as 4.0 µg/L and in others as high as 22 ug/L. Lead in the heparin could have contaminated these specimens. It has been reported in literature that heparin lead should bind to plasma proteins and thus, should not be transferred to erythrocytes (43). Since we separated the

erythrocytes from plasma within an hour of collection, it is unlikely that much of the heparin lead would be transferred to the erythrocytes.

Tea drinkers tended to have comparably higher mean enzyme activity and selenium concentrations in both erythrocytes and plasma than nontea drinkers. On the other hand, tea drinkers had significantly lower plasma iron concentrations than the other subjects. The seven tea drinkers in our study were different individuals from the six subjects reported to be taking both minerals and vitamins.

The four subjects that were using oral contraceptives, had slightly higher erythrocyte enzyme activity than the other subjects. Capel et. al. (44) found significantly high erythrocyte glutathione peroxidase activity in women who had been using oral contraceptives for more than seven months. In our case, we do not know the duration of the contraceptive usage by the four subjects. We found higher erythrocyte enzyme activity in three of the four subjects using oral contraceptives (88, 85, 98 and 51 U/g haemoglobin). These subjects had erythrocyte selenium concentrations of 0.47, 0.50, 0.56 and 0.48 µg Se/g haemoglobin, respectively.

We found a rough correlation between erythrocyte glutathione peroxidase activity in U/g haemoglobin and erythrocyte selenium concentrations in µg/g haemoglobin

($r = 0.41$, $p = 0.01$). No correlation was observed between erythrocyte enzyme activity and selenium concentrations when only cellular volume was considered (enzyme activity, U/L vs µg Se/L of cells). It may be that cellular concentration of glutathione peroxidase is regulated by the amount of cellular haemoglobin. Lane et al (45) also found a correlation between erythrocyte enzyme activity and selenium concentrations when expressed per gram of haemoglobin (Pearson Correlation Coefficient = 0.67, $p = 0.01$). Their mean erythrocyte glutathione peroxidase activity and selenium concentrations for nonpregnant females were 41.0 ± 12.5 U/g Hb (pH = 7.0, 0.02 M phosphate buffer, no temperature given) and 0.68 ± 0.39 µg Se/g Hb (fluorometric method). Capel et al. (44) found selenium concentrations of 0.55 ± 0.11 µg Se/g Hb and 0.50 ± 0.13 µg/g Hb in 24 nonpregnant females and contraceptive users, respectively. Our results were 78.6 ± 12.8 U/g Hb for mean erythrocyte enzyme activity and 0.49 ± 0.07 µg Se/g Hb for mean erythrocyte selenium concentration. Our results differ from those of Lane et al. However, they assayed their enzyme activity under different conditions. Our selenium concentrations for erythrocytes agree well with those of Capel et al.

Plasma iron correlated with haemoglobin as would be expected. No correlation between plasma iron concentrations and erythrocyte glutathione peroxidase activity/volume of cells was found. However, plasma iron

correlated inversely with enzyme activity/g Hb. Since the seven tea drinkers had significantly higher mean erythrocyte glutathione peroxidase activity coupled with low mean plasma iron concentration, they may account for this apparent correlation. Their mean haemoglobin concentrations were normal. The reason for this inverse relationship is not immediately apparent.

There was no correlation observed between plasma enzyme activity and plasma selenium concentration. This is in agreement with the findings of Behne and Wolters (3).

It has been shown by Beutler and Matsumoto (46) that certain individuals such as U. S. Jewish population, Orientals and those with U. S. Mediterranean origin have a tendency to have decreased erythrocyte glutathione peroxidase activity. Two U. S. Chinese subjects and one U. S. Indian subject (not included in calculated mean enzyme or selenium concentrations) had erythrocyte enzyme activity of 61, 61 and 51 U/g Hb and selenium concentrations of 149, 143 and 149 µg/L, respectively. Thus, these results agree with their findings. The extent of Mediterranean and Jewish heritage in this group is not known and may have obscurred the correlation between erythrocyte selenium and enzyme activities.

From our studies, we observed that there are many factors which may affect both erythrocyte and plasma

glutathione peroxidase activities and selenium concentrations. These factors tend to obscure the relationship between selenium nutrition and enzyme activity. Until the effects of ethnic, nutritional and hormonal factors are better defined, activity of glutathione peroxidase should not be equated to selenium status.

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