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GLUTATHIONE PEROXIDASE ACTIVITIES FROM RAT LIVER

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Summary

There are two enzymes in rat liver with glutathione peroxidase activity when cumene hydroperoxide is used as substrate. One is the selenium-requiring glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) and the other appears to be independent of dietary selenium. Activities of the two enzymes vary greatly among tissues and among animals. The molecular weight of the enzyme with selenium-independent glutathione peroxidase activity was estimated by gel filtration to be 35 000, and the subunit molecular weight was estimated by dodecyl sulfate-polyacrylamide gel electrophoresis to be 17 000. Double reciprocal plots of enzyme activity as a function of substrate concentration produced intersecting lines which are suggestive of a sequential reaction mechanism. The K_m for glutathione was 0.20 mM and the K_m for cumene hydroperoxide was 0.57 mM. The enzyme was inhibited by *N*-ethylmaleimide, but not by iodoacetic acid. Inhibition by cyanide was competitive with respect to glutathione and the K_i for cyanide was 0.95 mM. This selenium-independent glutathione peroxidase also catalyzes the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene. Along with other similarities to glutathione *S*-transferase, this suggests that the selenium-independent glutathione peroxidase and glutathione *S*-transferase activities in rat liver are of the same enzyme.

Introduction

There is an enzyme in rat liver which catalyzes the reduction of cumene hydroperoxide with glutathione as electron donor and which is distinct from the selenium-containing glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) [1,2]. This selenium-independent glutathione peroxidase uses organic hydroperoxides as substrates, but not H_2O_2; the selenoenzyme is less specific. It was recently suggested that glutathione peroxidase activity in rat testis is associated with glutathione *S*-transferase A activ-

ity [3]. The selenium-independent glutathione peroxidase activity in rat liver investigated here appears also to reside in glutathione *S*-transferase.

Polyunsaturated fatty acid hydroperoxides can cause biochemical damage by initiating further lipid peroxidation and by direct oxidation of labile biomolecules. Reduction of fatty acid hydroperoxides is an important protection mechanism in vivo. This reduction of hydroperoxides by selenium glutathione peroxidase contributes to its protective function [4]. Glutathione peroxidase activity of glutathione *S*-transferases may protect animals from lipid hydroperoxide damage also, even though such protection may not be the primary action of this closely related group of enzymes.

Since little was known about selenium-independent glutathione peroxidase activity in rat liver, the goals of this study were to purify the enzyme responsible, distinguish it from the selenium-containing enzyme and heme-containing peroxidases, and investigate its distribution, kinetics inhibition and substrate specificity.

Materials and Methods

Reagents and chromatography materials. NADPH, glutathione, glutathione reductase, cysteine, and protein standards were purchased from Sigma Chemical Co., St. Louis, Mo. Sephadex G-100 and G-150 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J.; carboxymethyl-cellulose (CM-52) was from Whatman, Clifton, N.J.; and hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif. All peroxides except H_2O_2 were obtained from Polysciences, Rydel, Penn.; H_2O_2 was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. 1-Chloro-2,4-dinitrobenzene was purchased from Matheson, Coleman and Bell, Norwood, Ohio.

Enzyme assays. A coupled assay system was used in which oxidation of glutathione (GSH) was coupled to NADPH oxidation by glutathione reductase [2]. The oxidation of NADPH was followed spectrophotometrically at 340 nm. The reaction mixture consisted of 0.25 mM GSH, 1.0 mM cumene hydroperoxide, 50 mM phosphate (pH 7.2), 0.10 mM EDTA, and an excess of glutathione reductase. Reaction rates were measured at 37°C. Enzyme units are reported as nmol NADPH oxidized per min.

A different assay system, based on the use of Ellman's reagent, was used to compare reaction rates with glutathione to rates with cysteine. Enzyme was incubated with 1.0 mM glutathione or cysteine and the reaction was initiated by addition of cumene hydroperoxide to produce a final concentration of 1.2 mM. This assay was also carried out in 50 mM phosphate, pH 7.2, and 0.10 mM EDTA. The reaction was stopped by addition of one volume of 10% trichloroacetic acid. After adjustment to pH 8.9, Ellman's reagent was added and absorbance was read at 412 nm, according to the procedure of Sedlack and Lindsay [5].

The assay for glutathione *S*-transferase activity was similar to that used by Pabst et al. [6]. The reaction solution contained 50 mM phosphate, pH 7.0, 0.5 mM glutathione, and 0.5 mM 1-chloro-2,4-dinitrobenzene. 8 mM 1-chloro-2,4-dinitrobenzene in 95% ethanol was added to initiate the reaction and absorbance was followed at 340 nm.

Enzyme purification from rat liver. Five 200-g male Sprague-Dawley rats were anesthetized by intraperitoneal injection of pentobarbital. Livers were perfused with cold 0.9% saline in situ to remove blood and were then excised. The livers were homogenized in 250 mM sucrose, 50 mM Tris (pH 7.5) and centrifuged at 27 000 $\times g$ for 30 min. Then, 1.2 volumes of acetone at −20°C were added to the supernatant. The pellet resulting from 27 000 $\times g$ centrifugation for 30 min was resuspended and the final supernatant obtained after another centrifugation was applied to a Sephadex G-100 column (5 × 100 cm). Fractions with enzymatic activity were concentrated by ultrafiltration and were eluted with a 0—1.0 M gradient of NaCl from a column of CM-cellulose in 10 mM phosphate, pH 6.8. Enzymatically active fractions were concentrated, applied to a hydroxyapatite column, then eluted with a 0.01—0.40 M gradient of phosphate, pH 6.8. Finally, the resulting enzymatically active fractions were concentrated and chromatographed on a Sephadex G-150 column.

Polyacrylamide sodium dodecyl sulfate disc gel electrophoresis. The most enzymatically active fractions eluting from the Sephadex G-150 column were dialyzed against 10 mM Tris, pH 7.5 and 0.10 mM EDTA. Sodium dodecyl sulfate (SDS)-urea disc gel electrophoresis, according to the procedure of Bruening and Wu [7], was carried out in triplicate with 50-μg samples of the purified peroxidase. Bovine serum albumin, catalase, ovalbumin, chymotrypsin, and hemoglobin were electrophoresed as standards.

Estimation of molecular weight by gel filtration. A 1.6 × 100 cm column that contained Sephadex G-150 was standardized by ascending elution of thyroglobulin, bovine serum albumin, ovalbumin, chymotrypsin, ribonuclease, and cytochrome *c*.

Selenium depletion and labeling. Male Sprague-Dawley rats (100 g) were fed either a stock diet or a Torula yeast-based diet which was deficient in selenium, and given deionized water for a period of up to 5 weeks. A 30—50 μCi dose of [^{75}Se]selenite in 2 ml of water was administered by stomach tube at times of 4, 10, and 24 h, and 1, 3, and 5 weeks before the rats were killed. The selenium-glutathione peroxidase in liver cytosol was separated from selenium-independent glutathione peroxidase activity by gel filtration using Sephadex G-150.

Peroxidase distribution in other tissues. Rat kidney was homogenized and centrifuged by the same method used for liver, and the supernatant was chromatographed on a Sephadex G-150 column. Peroxidase activity was again followed using the coupled assay and cumene hydroperoxide as substrate.

Primate tissues were treated similarly. Livers, kidneys, and duodenums from freshly killed bonnet monkeys (*Macaca radiata*) were fractionated within 48 h.

The soluble fraction from a 10-ml sample of human blood was prepared by lysing the red blood cell pellet and recombining lysate with plasma.

Kinetics, inhibition, and peroxide specificity. The coupled assay described above was used to investigate the kinetics of the selenium-independent glutathione peroxidase purified from rat liver. Reaction rates were measured in duplicate at each of nine cumene hydroperoxide concentrations and at four glutathione concentrations.

The effects of cyanide and azide on the activity of selenium-glutathione peroxidase and selenium-independent glutathione peroxidase activity were deter-

mined using the coupled assay. Enzyme was incubated for 10 min in 0.25 mM glutathione and increasing amounts of either NaCN or NaN_3. The reaction was initiated by addition of cumene hydroperoxide to produce a final concentration of 1.0 mM. Reaction rates with the selenium-independent glutathione peroxidase were then measured at nine cyanide concentrations (0—8.0 mM) for each of three different glutathione concentrations (0.1—0.5 mM). The cyanide was added in 50 mM phosphate, pH 7.2. These measurements were made to determine whether inhibition was competitive with respect to glutathione. Sensitivity to the sulfhydryl-alkylating agents iodoacetic acid and *N*-ethylmaleimide was tested. Selenium-glutathione peroxidase and selenium-independent glutathione peroxidase were incubated in 1.0 mM iodoacetic acid and 50 mM phosphate, pH 7.2, at 37°C. Reaction with iodoacetic acid was stopped at various time intervals by addition of nine volumes of 5 mM glutathione, after which enzyme activity was assayed. The selenium-independent enzyme was also incubated for 10 min at 37°C with *N*-ethylmaleimide at concentrations varied between 0.5 and 8.0 mM. The reaction was again stopped by dilution with nine volumes of 5 mM glutathione.

Relative reaction rates catalyzed by the selenium-independent glutathione peroxidase were measured using different peroxide substrates. The reaction was initiated by addition of peroxide to give a final concentration of 0.22 mM in the coupled assay system.

Results

Selenium depletion and labeling

Activity of the selenium-glutathione peroxidase was more than 90% decreased in liver cytosol of rats fed a selenium-deficient diet for 3 weeks following weanling age, and was barely detectable after 5 weeks. Activity of the selenium-independent glutathione peroxidase did not change significantly.

During the first 24 h after [^{75}Se]selenite administration (after 4, 10, and 24 h), ^{75}Se eluted from Sephadex G-150 with all of the detected soluble protein, suggesting mostly non-specific binding of selenium to protein. When rats were killed after longer time intervals (1, 3, and 5 weeks), ^{75}Se eluted in two peaks, one of which corresponded to selenium-glutathione peroxidase activity, and no ^{75}Se chromatographed with selenium-independent glutathione peroxidase activity.

Purification of peroxidase

The results of purification of selenium-independent glutathione peroxidase are shown in Table I. This was repeated several times with similar results. In the last two steps, the enzyme had reached highest purification. The small decrease in specific activity during the last purification step is presumed to reflect some instability to storage. SDS disc gel electrophoresis of the enzyme preparation produced only one band that stained with Coomassie Blue. Comparison with protein standards suggested a subunit molecular weight of roughly 17 000. The elution volume of selenium-independent glutathione peroxidase activity from a standardized Sephadex G-150 column corresponded to a molecular weight of 35 000. The enzyme appears to be a dimer with subunits of equal size.

TABLE I

PURIFICATION OF SELENIUM-INDEPENDENT GLUTATHIONE PEROXIDASE

Fraction	Total protein (mg)	Specific activity (nmol NADPH oxidized/ min per mg protein)	Yield (percent of total peroxidase activity)
Homogenate	12 900	72.5	100
Supernatant	4 930	185	97
Acetone precipitate	1 700	316	58
Sephadex G-100 fractions *	—	875	21
CM-cellulose fractions *	55.4	3000	18
Hydroxyapatite fractions *	11.7	3300	4.3
Sephadex G-150 fractions *	8.42	2880	2.7

* The most enzymatically active fractions were pooled.

Peroxidase distribution in other tissues

Gel filtration of liver cytosol from rats fed a stock diet showed that about 80% of the total glutathione peroxidase activity could be attributed to the selenium enzyme and 20% to the selenium-independent enzyme. The coupled assay described herein was used with cumene hydroperoxide as substrate. Selenium independent-glutathione peroxidase accounted for about 20% of the enzymatic activity in rat kidney, also. The peroxidase activities in bonnet monkey tissues differed greatly from those of the rat. Total peroxidase activity in unfractionated liver cytosol from monkey was barely detectable (compared to 200—400 units/mg protein for rat, where units are nmol NADPH oxidized per min); 51 units/mg in kidney cytosol from monkey (compared to 70—90 units/mg protein for the rat); and 48 units/mg in duodenum (compared to 90—100 units/mg in the rat). Gel filtration of monkey tissues showed that most of the glutathione peroxidase activity in kidney is due to the selenium enzyme while most of the activity measured in liver and intestine is due to the selenium-independent enzyme. Inhibition by NaCN confirmed that the main peak of activity observed upon gel filtration of monkey liver cytosol corresponded to selenium-independent glutathione peroxidase. It is not known why considerable activity was found following gel filtration when none was detected in the liver preparation applied to the column. Gel filtration of rat liver also produced enzymatic activation, but to a lesser extent.

No peroxidase activity was detected in plasma or red blood cell lysate of either human blood or bonnet monkey blood. The same fractions prepared from rat blood had easily measured activities (for the red blood cell lysate, 30 units/mg protein; and for plasma, 1.0 unit/mg protein). Gel filtration of a preparation of human blood did reveal an activity peak that corresponded in elution volume to the selenium-glutathione peroxidase. The specific activity calculated for glutathione peroxidase in human blood was 2.4 units/mg protein after gel filtration, which was much lower than that for the rat.

Kinetic studies

Double reciprocal plots produced straight lines (Fig. 1) when the kinetic data of enzyme purified from rat liver were used. These lines intersected, in contrast

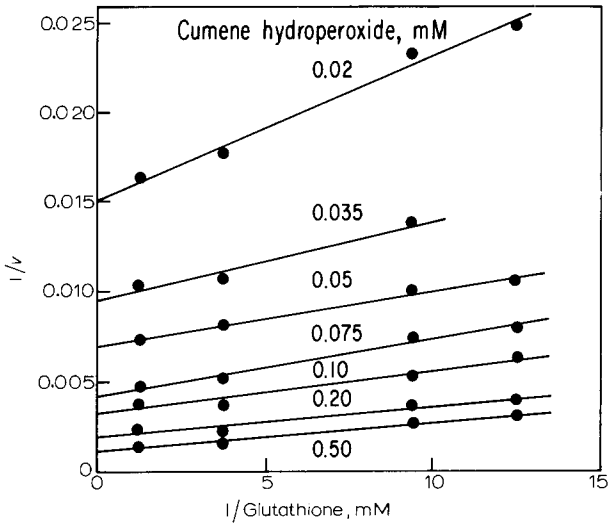

Fig. 1. Double reciprocal plot of selenium-independent glutathione peroxidase activity (v) with cumene hydroperoxide as the variable substrate. Glutathione peroxidase activity is expressed as nmol NADPH oxidized/min per 0.1 ml of enzyme solution.

to the parallel lines obtained for selenium-glutathione peroxidase [8]. Secondary plots of slopes or intercepts (Fig. 2) allowed determination of kinetic constants: the K_m for glutathione was 0.2 mM; and the K_m for cumene hydroperoxide was 0.57 mM. A sequential mechanism is suggested as opposed to the ping-pong mechanism of selenium-glutathione peroxidase [8,9].

Substrate specificity

The assay that measured loss of sulfhydryl directly was used to test the specificity of selenium-independent glutathione peroxidase for reduced gluta-

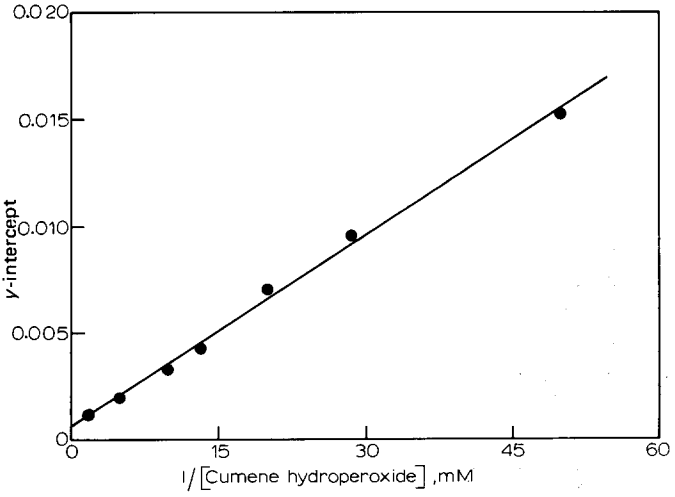

Fig. 2. Plot of y intercept from Fig. 1 vs. 1/cumene hydroperoxide concentration.

TABLE II

PEROXIDE SPECIFICITY OF SELENIUM-INDEPENDENT GLUTATHIONE PEROXIDASE

Substrate	Relative activity
Hydroperoxides	
Benzoyl	100
Cumene	16
Linoleic acid	6
tert-Butyl	0.3
H_2O_2	0
Peroxides	
Dicumene	0
Di-*tert*-butyl	0

thione. Under conditions in which activity was observed with 1.0 mM glutathione and 1.2 mM cumene hydroperoxide as substrates, the enzyme was not active when 1.0 or 2.5 mM cysteine was substituted for the glutathione. The same requirement for glutathione was then shown assaying the selenium-glutathione peroxidase. It is very unlikely that differences in pK_a values for dissociation of -SH groups of the two mercaptans could account for the difference in enzymatic reaction rates, as the pK_a in the case of cysteine is slightly lower.

The relative activity of selenium-independent glutathione peroxidase with different peroxides is shown in Table II. No activity was detected using peroxides with the structure ROOR, and among hydroperoxides, ROOH, greater reactivity of hydrophobic, especially aromatic, substrates was found.

Purified selenium-independent glutathione peroxidase catalyzed the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene. Elution of peroxidase and transferase activities from carboxymethyl-cellulose coincided.

Inhibition studies

Selenium-glutathione peroxidase was not inhibited by azide or cyanide, in agreement with the findings of other workers [10]. Selenium-independent glutathione peroxidase likewise was not inhibited by azide, but cyanide did inhibit enzymatic activity. The inhibition was competitive with respect to glutathione, as shown in Fig. 3. Plots of $1/v$ vs. cyanide concentration give straight lines at three different GSH concentrations, suggesting simple competitive inhibition according to:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]}$$

The K_i for cyanide was determined to be 0.95 mM by a replot of data.

Selenium-independent glutathione peroxidase was not inhibited by incubation with 1 mM iodoacetic acid for 10 min. In contrast, selenium-glutathione peroxidase was more than 90% inhibited when it was preincubated in glutathione to reduce the enzyme. Incubation of selenium-independent glutathione peroxidase in increasing concentrations of *N*-ethylmaleimide decreased enzymatic activity with a half-maximal inhibition at about 0.5 mM, while parallel incubations with the same concentrations of iodoacetic acid did not affect activity.

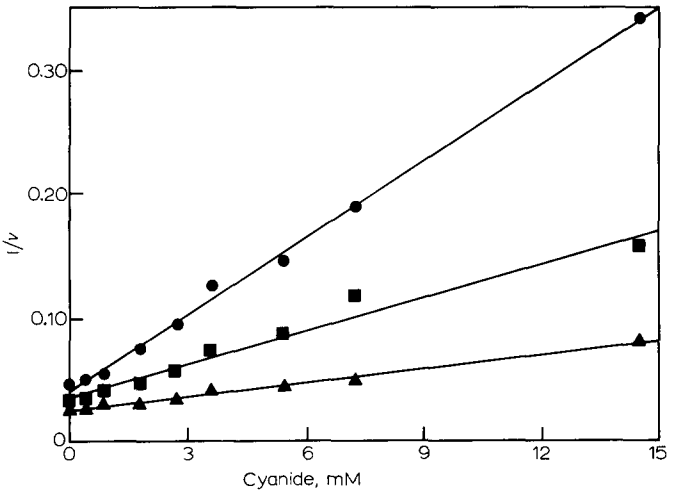

Fig. 3. Inhibition of selenium-independent glutathione peroxidase activity by cyanide. The units of v are nmol NADPH oxidized/min per 0.1 ml enzyme solution. Glutathione concentrations in mM are 0.1 (●), 0.2 (■), and 0.5 (▲).

Absorbance spectrum

The absorbance spectrum for purified selenium-independent glutathione peroxidase showed a peak at 280 nm, and no absorbance characteristic of either a heme protein or flavoprotein. The broadening of the absorbance peak around 280 nm observed by others [9] for selenium-glutathione peroxidase, and attributed to the selenium moiety, was not seen.

Discussion

The enzyme responsible for selenium-independent glutathione peroxidase activity in rat liver lacks heme and thus differs from heme peroxidase. Its mechanism differs from that of selenium-glutathione peroxidase, as indicated by kinetic data, inhibition by cyanide and resistance to inhibition by iodoacetic acid. Selenium-glutathione peroxidase shuttles between reduced and oxidized forms. In its reduced state it is alkylated by iodoacetic acid and inactivated [11]. In its oxidized state, prolonged incubation with cyanide in basic solution releases selenium and inactivates the enzyme [12]. The kinetics of selenium-independent glutathione peroxidase are more consistent with a sequential addition of substrates followed by release of products.

Selenium-independent glutathione peroxidase activity in rat liver can be attributed to glutathione *S*-transferase. Transferase and peroxidase activities co-elute from CM-cellulose. The specificity for aromatic and hydrophobic peroxides agrees with the specificity found for electrophilic substrates of glutathione *S*-transferase A [6]. The sequential reaction mechanism resembles that for glutathione *S*-transferase A at glutathione concentrations above 0.1 mM [6]. The K_m of 0.2 mM determined for glutathione by the glutathione peroxidase assay is identical with that for glutathione *S*-transferases A and B [6,13,14]. The molecular weight determined for the selenium-independent

glutathione peroxidase is similar to that of glutathione *S*-transferase and also consists of two subunits [6]. The failure of iodoacetic acid to inhibit peroxidase activity while *N*-ethylmaleimide did so may agree with findings for glutathione *S*-transferase A. Pabst et al. [6] found that while approximately four sulfhydryls per enzyme were required for activity, iodoacetic acid was completely ineffective as an inactivator of the enzyme. It was pointed out that some of the substrates of glutathione *S*-transferase A are classical sulfhydryl alkylating agents [6].

Gluthathione *S*-transferases have been shown to catalyze reactions in which glutathione participates at electrophilic sites other than carbon [14]. In reacting with nitroglycerin or organic thiocyanates, the electrophilic atom is nitrogen or sulfur, respectively. Oxidized glutathione is produced in the reaction with nitroglycerin [14]. A similar reaction, in which oxygen serves as the electrophilic atom, might account for the glutathione peroxidase activity of glutathione *S*-transferases.

When tissues are assayed for selenium-glutathione peroxidase using the aromatic substrate cumene hydroperoxide, interference from the selenium-independent enzyme can be an important problem. This problem becomes even greater when tissues from selenium-deficient animals are used. When both peroxidases are present, a separate measurement with H_2O_2 will reflect only the activity of the selenoenzyme. It should also be possible to selectively inhibit one or the other of the two enzymes by either incubation of enzyme with iodoacetic acid or by addition of cyanide.

An article by Prohaska and Ganther [15] was published as the present study was being prepared for publication. They found that glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene copurified with selenium-independent glutathione peroxidase activity from rat liver.

Differences in kinetics make it difficult to compare Michaelis constants for the two peroxidases. The selenoenzyme is not saturated with glutathione even at high concentrations of this substrate [16]. Nevertheless, the affinity of selenium-independent glutathione peroxidase for glutathione appears to be greater, and the affinity for cumene hydroperoxide less, when compared to the selenoenzyme. The apparent K_m values for glutathione (at a cumene hydroperoxide concentration of 0.20 mM) were 0.125 mM for the selenium-independent enzyme, and 1.0 mM for the selenoenzyme. The K_m of the selenoenzyme for cumene hydroperoxide can be estimated, using an equation from the work of Flöhé et al. [17], to be roughly 0.120 mM at 5 mM glutathione. This concentration of glutathione roughly corresponds to that for the liver in vivo, and may be considered saturating for the selenium-independent glutathione peroxidase.

Selenium-independent glutathione peroxidase activity might afford some protection from lipid hydroperoxides when dietary selenium levels fluctuate. Although the V as measured with cumene hydroperoxide is much lower than for the selenoenzyme [15], the selenium-independent enzyme represents a much larger fraction of soluble protein in liver. The glutathione *S*-transferases are also present in large amounts in rat liver; they have been estimated to account for 10% of the soluble protein [18].

Prohaska and Ganther [15] found that the selenium-independent enzyme accounted for over one-half of the glutathione peroxidase activity in rat testis,

which was a much larger relative amount than in liver, kidney or brain. Another study [19] showed that glutathione peroxidase activity in rat testis did not respond to selenium depletion and repletion as it did in other tissues. In view of the susceptibility of testis to heavy metal toxicity when selenium is limiting, suggesting that the selenoenzyme is a target site [20], a high level of selenium-independent glutathione peroxidase activity in the testes might be advantageous to an animal.

Acknowledgements

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