Biochemical Pharmacology, Vol. 35, No. 4, pp. 601–606, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Press Ltd.

ENZYMATIC DEFENSE AGAINST RADIATION DAMAGE IN MICE

EFFECT OF SELENIUM AND VITAMIN E DEPLETION

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(*Received* 22 *April* 1985; *accepted* 16 *July* 1985)

Abstract—Radiation effects are mediated in part by the generation of oxygen-derived free radicals and hydrogen peroxide. Membrane polyunsaturated fatty acids are important biological targets of these toxic molecules which cause lipid peroxidation. Radiation damage to DNA is also known to result in base hydroperoxides, especially thymidine hydroperoxide. Glutathione (GSH) is known to inhibit lipid peroxidation both chemically and through its interaction with the selenium-dependent glutathione peroxidase (GSH-Px). Although cytosolic GSH-Px can metabolize organic lipid peroxides in solution, it cannot metabolize phospholipid peroxides in micelles. This may be due to the interference of phase differences between the aqueous cytosol and the membrane, or the result of steric hinderance. Recent studies have suggested the presence of a membrane-bound GSH-dependent peroxidase system. We examined the cytosolic versus membrane-associated GSH-Px, in various tissues of mice on a selenium and vitamin E deficient diet, and found significant differences among organs in the distribution of enzyme activity in these two subcellular fractions. The effect of single high-dose whole body irradiation did not appear to be related to the activity of these enzymes.

Radiation mediates its cytotoxic effects in part through the generation of oxygen-derived reactive molecules [1]. These may disrupt a wide variety of biologically critical macromolecules. Membranes are targets of radiation and may be major sites for the development of the oxygen effect. Oxygen free radicals and hydrogen peroxide (H_2O_2) have been shown to peroxidize polyunsaturated lipids in cell membranes [2]. Radiation also can result in DNA base hydroperoxides, especially thymidine hydroperoxide [3].

An elaborate system of cellular defenses has evolved in aerobic organisms to prevent and neutralize these events. Non-protein sulfhydryls, mostly reduced glutathione (GSH), act alone to scavenge free radicals, as well as being critical to the function of the enzymes glutathione peroxidase (GSH-Px) and glutathione-*S*-transferase (GST) [4]. GSH-Px is a selenium-dependent enzyme that can metabolize a variety of potentially important organic peroxides, including DNA peroxides and lipid peroxides, and has a low apparent K_m for H_2O_2 of 1 μM [5]. GSH-Px has been localized primarily to the cytosol (70%), and it has also been shown to exist in the mitochondrial matrix of rat hepatocytes [6]. Recently, membrane-associated GSH-Px activity has been described in the intermediate space of mitochondria [7] and on the membranes of erythrocytes [8]. This is of particular interest since the work of McCay *et al.* [9, 10] demonstrated the inability of the classical cytosolic GSH-Px to metabolize micellar-bound phospholipid peroxide. In our previous study [7], we found that the mitochondrial membrane-associated enzyme in mouse cardiac tissue was not affected by a 6-week selenium deficient diet in the same way as the cytosolic GSH-Px.

Previous studies have shown an apparent interaction between selenium-dependent GSH-Px and vitamin E, which is situated in the membrane lipid phase and may function in concert with GSH-Px [11].

Two other enzymes are capable of reducing peroxides. Catalase is localized to cellular peroxisomes and has a much higher K_m (1.1 M) for H_2O_2 than does GSH-Px. It is incapable of metabolizing organic peroxides [12, 13]. GST represents a family of isoenzymes that conjugate a broad spectrum of electrophilic toxins to GSH. GST has selenium-independent peroxidase activity using organic peroxides, but cannot reduce H_2O_2 [14]. The K_m for organic peroxides (for example, cumene hydroperoxide) is 6–20 times higher for GST than it is for selenium-dependent GSH-Px [15]. Despite the fact that the peroxidase specific activity of GST is only 5–10% that of GSH-Px for the same substrate, GST may comprise as much as 5% of the soluble protein in rat livers. In selenium deficiency it has been shown to be even higher [16].

It is possible to deplete mice of vitamin E and selenium using a well established deficient diet, resulting in a marked depression of glutathione peroxidase, potentially increasing the susceptibility of normal tissue to peroxidative damage [17]. This diet has been used by others and ourselves in a number of previous studies and has been determined to differ

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only in selenium and vitamin E content [18, 19]. To examine the effect of this diet on defense against radiation damage, we compared survival after whole body irradiation of mice, as well as the response of two clinically relevant tissues, jejunum and bone marrow, in irradiated mice on the normal and depleted diets.

MATERIALS AND METHODS

Weanling male CDF1 mice from Charles River Laboratory (Portage, MI) were fed either the control diet consisting of Ralston Purina NIH rat and mouse Ration No 5108, containing 0.33 ppm of selenium and 80 I.U./kg of tocopherol, with soybean and fish oil as major sources of fat, or the Torula yeast and lard based diet deficient in selenium and vitamin E [18, 19]. Animals were maintained on either diet for 6 weeks by which time the liver cytosolic GSH-Px in those on the deficient diet had declined to less than 15% of the control animals. The mice were housed five per cage; acid water (pH 4) and food pellets were provided *ad lib*. They were kept on a 12-hr light and dark cycle, in the NIH clean conventional animal facility.

Irradiation

Four or five mice were irradiated in a 15 cm × 15 cm lucite box that was placed on a platform half-way between two opposing X-ray tubes separated by a distance of 108 cm. X-rays were generated at 235 kV, 15 mA, with a half-value layer (HVL) of 0.9 mm Cu^{2+}. The target-skin distance was 54 cm, and the dose rate was 1.25 Gy/min with equal contributions from the two tubes. All mice were irradiated to the whole body without anesthetic while breathing air.

Radiobiological assays

Jejunal crypt survival. The microcolony technique of Withers and Elkind [20] was used to score the number of regenerating crypts around a transverse histological section of jejunum 3.5 days after irradiation. Groups of at least four mice from each of the control and deficient diets were given graded single doses of X-rays to the whole body. Mice were killed by cervical dislocation, and a segment of jejunum was removed, fixed in 10% buffered formalin, processed routinely for histology, and stained with hematoxylin and eosin. The number of regenerating crypts were counted from at least three separate cross sections per mouse. The data were analyzed using the average score of four mice per dose group. The number of surviving crypts was converted to surviving cells per circumference by applying a Poisson correction for crypts regenerating from more than one stem cell. These data were then plotted as a function of dose. Lines were fitted to the geometric means of the data for each dose group using a least squares regression analysis.

Survival of bone marrow stem cells. The spleen colony assay of McCullock and Till [21] was used to assess bone marrow damage after irradiation. Groups of at least four mice from each of the dietary groups were given graded single doses of X-rays to the whole body. The animals were killed, the femoral bones were removed, and single cell suspensions of the marrow contents were prepared. Appropriate numbers of bone marrow cells were injected intravenously into groups of ten mice that had received 800 rads whole body irradiation on the previous day. The number of macroscopic spleen colonies present on the spleen surface was scored 9 days later. An average number of colonies was calculated and converted to the number of surviving bone marrow stem cells injected. These data were plotted as a function of dose for each dietary group.

In a separate experiment, mice fed either the deficient or the control diet were exposed to graded single doses of X-rays to the whole body. The survival was monitored for 30 days, and the dose resulting in 50% mortality (LD_{50}) in each of the dietary groups was calculated by a logit analysis.

Biochemical assays

All tissue samples assayed for enzymatic activity were obtained immediately upon killing the animal and were processed at 4°. The measurements were done on three mice in each dietary group. Each set of animals were litter-mates that differed from one another only with regard to the diet. The tissues of three animals were pooled for each of the three measurements of each enzyme assay performed. The gall bladders were removed, and livers were thoroughly washed, finely minced, and adjusted in a 10% (w/v) solution with a buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7.4 (MSH buffer). This was subjected to homogenization using a Polytron PU-2 homogenizer, meter setting at 5, three times for 10 sec each. An aliquot of the resulting homogenate was set aside for assay. The remaining homogenate was then subjected to centrifugation at 126,000 *g* for 1 hr. The supernatant fraction and pellet were each collected for assay.

Gut mucosa was prepared by longitudinal opening of a section of jejunum and gentle scraping off of the mucosal layer with a glass slide. This was collected in MSH buffer and subjected to homogenization three times for 5 sec. The fractions were prepared as discussed above for liver.

Bone marrow cells were obtained as described above and suspended in medium containing 10% fetal calf serum. Approximately 10^7 cells in 3 ml of this solution were layered over 6 ml of Ficoll Hypaque and centrifuged at 2000 *g* for 30 min. A band of cells that settled in the Ficoll was collected and washed with MSH buffer. An aliquot was removed to cytospin for Wright Giemsa staining, which demonstrated mononuclear cells, with few red blood cells or granulocytes. The rest of the cells were resuspended in buffer diluted 1:1 with distilled water and were homogenized as above. Light microscopy confirmed cell disruption.

Glutathione peroxidase was assayed using the technique of Paglia and Valentine [22] with hydrogen peroxide as substrate. Glutathione-*S*-transferase was measured as previously described [23] with chlorodinitrobenzene (CDNB) as substrate. Catalase measurement in the homogenates was done according to the method of Cohen *et al.* [24]. Reduced glutathione was measured according to the fluoro-

metric method of Hissin and Hilf [25]. Protein was determined by the method of Lowry *et al.* [26] using bovine serum albumin as standard. Samples were assayed from two sets of animals in triplicate.

Analytical procedure for vitamin E

Since the tocopherols in liver exist almost completely in free form, extraction of vitamin E from liver without saponification was used [27]. The extracted vitamin E was then determined by high pressure liquid chromatography using fluorescence detection. The major features of the assay include the use of the antioxidant pyrogallol to minimize oxidation during sample preparation and the use of a low u.v. wavelength for excitation of vitamin E to maximize detection sensitivity. The procedure involved a rapid cooling of the specimen by placement on a tared piece of aluminium foil on dry ice. The frozen sample was then weighed and transferred to a glass test tube containing 1.5 ml of chilled 0.5% ethanolic pyrogallol. The internal standard tocol was then added. The sample was homogenized on ice in a Polytron PU-2 homogenizer set at speed 6 for 5 sec and then sonicated in a Bronson sonifier at 40 W for 30 sec. The sample was then poured into a 15-ml screw top test tube, and 0.5 ml of distilled water and 6 ml spectral grade isooctane were added to each sample. The tube was vortexed for 30 sec and recentrifuged. The organic layer was collected and evaporated under nitrogen. The residue was redissolved in 100 μl methanol, and an aliquot was injected into a high pressure liquid chromatographic apparatus.

Separation of the vitamin E was achieved on a Waters Associates (Milford, MA) liquid chromatographic system utilizing a 10 μm RCM C18 column and a mobile phase of spectral grade methanol at a flow rate of 2.0 ml/min. Detection of vitamin E and the internal standard tocol was made on a Schoeffel 970 spectrofluorimeter using an excitation wavelength of 209 nm and the photomultiplier tube alone as the emission filter. The minimum detectable quantity in a pooled plasma sample was 1 μM. At higher concentrations of vitamin E, spectrophotometric detection at 280 nm on a Waters model 440 absorbance detector with vitamin E acetate as an internal standard was used. Quantitation of vitamin E amounts in tissue was accomplished by comparing the peak height ratio of vitamin E and the internal standard to a standard curve prepared using known vitamin E amounts carried through the assay procedure.

Statistical analysis of biochemical studies

Data are presented as mean ± standard error of the mean. The data in Table 2 were analyzed by Student's *t*-test. In Table 1, the mean enzyme specific activity is presented, along with the mean percent of control and the results of a two-sided paired "*t*-test" of the percent of control for each litter; the P values are from the test assuming the mean percent of control was zero.

RESULTS

Biochemical changes

GSH-Px activities in tissue homogenates as well as in the cytosol and membrane-associated pellets in the two dietary groups are shown in Table 1. The percent of control values in the deficient dietary group is also noted. The supernatant fraction obtained on ultracentrifugation under these conditions corresponds to the cytosol, while the pellet contains various fragment sizes of the cell membrane, the nucleus, as well as the membranes and matrices of the other subcellular organelles. Although some of the enzyme activity might be cytosolic proteins non-specifically adsorbed to membranes, the consistency of the values argues against this. Using similar techniques, McCay *et al.* [10] noted microsomal-associated GSH-Px activity, despite repeated washings.

Table 1. Glutathione peroxidase specific activity in homogenate, cytosol, and membrane-associated fractions in animals on control versus selenium and vitamin E deficient diets

	Glutathione peroxidase specific activity (nmoles/min/mg protein)					
	Liver		Gut mucosa		Bone marrow	
	Mean specific activity	Mean% control	Mean specific activity	Mean % control	Mean specific activity	Mean % control
Homogenate						
Control	7.7 ± 1.0		55.6 ± 10.0		14.4 ± 1.4	
-Se,-E	2.8 ± 0.8*	36	12.2 ± 3.7*	26	17.3 ± 6.4	124
Cytosol						
Control	45.9 ± 14.1		91.4 ± 1.6		10.6 ± 1.6	
-Se,-E	7.4 ± 4.1†	14	4.7 ± 1.2†	5	8.4 ± 1.6	80
Membrane bound						
Control	3.7 ± 1.3		20.9 ± 6.3		11.5 ± 5.5	
-Se,-E	6.2 ± 1.3	192	5.2 ± 0.9*	33	10.2 ± 4.1	95

The mean percent of the control value measured in the dietary depleted animals is noted. The P values result from the test of mean percent control = 0. Values are mean ± S.E.; N = 3.

* $P < 0.05$.

† $P < 0.005$.

Table 2. Cytosolic *S*-transferase specific activity and homogenate catalase, GSH, and vitamin E in animals on control versus selenium and vitamin E deficient diets

	GSH-Transferase (nmoles/min/ mg protein)	Catalase (μM/min/ mg protein)	GSH (μg/g protein)	Vitamin E (M/100 mg tissue wet weight)
Liver				
Control	1808.1 ± 201.2	63.5 ± 24.2	1693.3 ± 83.3	2.6×10^{-5}
-Se,-E	2830.1 ± 313.5*	47.1 ± 9.7	1583.3 ± 104.1	2.9×10^{-6}*
Gut mucosa				
Control	295.37 ± 89.76	62.7 ± 18.5	615.0 ± 84.9	8.4×10^{-5}
-Se,-E	311.04 ± 67.03	69.2 ± 19.7	569.5 ± 36.1	2.8×10^{-5}*
Bone marrow mononuclear cells				
Control	8.67 ± 3.06			
-Se,-E	10.50 ± 5.76			

Results are mean ± S.E.; N = 3.
* $P < 0.05$.

Consistent with previous studies, the liver cytosolic GSH-Px declined dramatically on the selenium and vitamin E depleted diet, as did the gut cytosolic enzyme. On the other hand, of the three tissues examined, only the gut and liver homogenate and gut membrane-associated GSH-Px activity declined. The bone marrow mononuclear cells appeared to be quite resistant to the deficient diet. The apparent selenium-independence of the membrane-associated GSH-Px in the bone marrow and liver led to preliminary studies on longer-term diets. After 6 months on the deficient diet, GSH-Px specific activity in the pellet decreased to 35 and 85% of control in bone marrow and liver respectively. This suggests that the GSH-Px associated with membranes is, in fact, selenium-dependent, but may represent a very slowly turning over pool of the enzyme. We have not yet studied the radiobiology of these longer-term depleted mice. It is of note that experiments using rat liver microsomes to study the effects of selenium and vitamin E dietary depletion on lipid peroxidation employed a 14-week deficient diet [28].

Vitamin E levels decreased by 88% in the liver and by more than 66% in the gut mucosa (Table 2). We were unable to detect vitamin E in bone marrow mononuclear preparation from an additional fifteen animals (2 mg protein).

Neither GSH concentration nor catalase activity was affected by the diet (Table 2). These could not be measured in the limited bone marrow mononuclear cell specimens. As has been reported previously in male rats [14], the liver cytosolic GST was increased significantly by selenium deficiency. To determine whether this elevation contributes to peroxidase activity, one set of samples was assayed for peroxidase activity using the organic peroxide cumene hydroperoxide, metabolized by both GSH-Px and GST. The specific activity using this substrate was not significantly different in the deficient sample compared to control. Thus while selenium deficiency caused a marked drop in cytosolic GSH-Px, there was no significant change in the ability of this tissue to metabolize organic peroxidases due to compensatory changes in GST and in the membrane-bound GSH-Px. This change in GST was not observed in the other tissues examined.

Table 3. Results of the CFU (colony forming unit) assay performed including the mean number of spleen nodules counted (± S.E.M.)

X-ray dose (Gy)	Number of spleens	Mean number of nodules	Number of cells injected/mouse	Mean CFU	Surviving fraction
Control					
0	7	24.1 ± 2.5	10^5	24.1 ± 2.5	100
2.5	10	15.7 ± 4.3	1.9×10^6	0.82 ± 0.23	3.43 ± 0.94
3.5	10	6.6 ± 2.5	3.2×10^6	0.20 ± 0.08	0.85 ± 0.32
4.5	5	4.4 ± 2.5	3.2×10^6	0.14 ± 0.77	0.56 ± 0.32
5.5	10	1.2 ± 1.8	8×10^6	0.02 ± 0.02	0.06 ± 0.09
6.5	2	0.5 ± 0.7	10^7	0.01 ± 0.01	0.02 ± 0.03
-Se,-E					
0	5	17 ± 5.2	10^5	17 ± 5.2	100
2.5	6	3.2 ± 2.3	0.5×10^6	0.63 ± 0.464	3.72 ± 2.7
3.5	9	2.1 ± 1.4	10^6	0.21 ± 0.14	1.23 ± 0.82
4.5	5	0.6 ± 0.9	2.5×10^6	0.02 ± 0.04	0.14 ± 0.20
5.5	4	0.3 ± 0.5	6.8×10^6	0.003 ± 0.007	0.02 ± 0.04

Bioassay

Bone marrow sensitivity was examined by assessing the ability of stem cells from depleted or normal mice to form colony units in the spleen of lethally irradiated normal diet recipient mice. The mean number of colonies formed was not different in the two dietary groups after any dose of irradiation. When the fraction of surviving bone marrow cells was plotted against the dose of radiation for the control and depleted mice, the two sets of data could be fitted by a single line. The slopes of the two lines when calculated separately were very close, with a D_o of approximately 0.80 Gy. The data for this experiment are in Table 3.

The second assay for damage to the hematopoetic system, survival at 30 days after whole body irradiation, also showed no effect of selenium and vitamin E deficiency. The $LD_{50/30}$ values were 6.6 Gy for the control group (95% confidence limits, range 6.03 to 7.21 Gy) and 5.81 Gy for the depleted mice (95% confidence, 5.34 to 6.33 Gy). The difference between these two values is not significant. These data demonstrate that the dietary depletion did not enhance the response of the bone marrow to radiation when assessed by either the $LD_{50/30}$ or the clonogenic assay.

The effect of dietary depletion on the gut crypt cell survival after irradiation is shown in Fig. 1. The D_o values for these curves were 89 rads and 92 rads for the control and deficient diet groups respectively. Thus, there was no statistical difference in the slopes. For each irradiation dose the mean number of crypts counted per jejunal section was significantly smaller in the deficient group ($P < 0.05$), resulting in a statistically significant shift of this curve to the left. Light microscopic evaluation of sections of the gut mucosa prior to irradiation did not demonstrate any detectable difference between the two dietary groups.

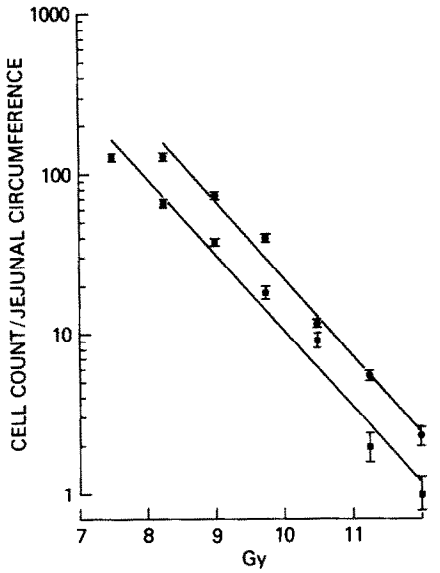

Fig. 1. Jejunal crypt cells survival to single doses of irradiation in vitamin E and selenium depleted mice (■) or in control mice (●). Both lines were fitted by unweighted linear regression. Bars represent 95% confidence limit.

DISCUSSION

Cellular defenses against oxygen-derived radicals and H_2O_2 include both chemical and enzymatic mechanisms. Glutathione has been shown to affect radiation sensitivity in some experimental systems on the basis of its ability to scavenge radiation-induced radicals as well as restore radiation-damaged molecules by hydrogen donation [4]. Catalase has been reported in one study to provide radiation protection and in another to detoxify enzymatically generated H_2O_2 [29, 33].

Since the levels of peroxides generated by irradiation are likely to be too low for catalase, and organic peroxides inevitably will be generated, it seems reasonable that GSH-Px should play an important role in radiation protection. The extact interaction of vitamin E with selenium and/or GSH-Px is not established. Diplock *et al.* [31] have suggested that the vitamin helps maintain the enzyme-bound selenium in a reduced state. Vitamin E itself has been demonstrated to inhibit X-ray-induced lipid peroxidation in human erythrocytes [32]. It may perform this function in concert with GSH-Px by hydrogen donation to a lipid peroxy radical yielding a peroxide which can be a substrate for the enzyme. More recently, selenium has been shown to spare vitamin E, and it has been suggested that the antioxidant effect of selenium is, at least in part, mediated via this effect on vitamin E [28].

The effect of dietary deficiency of both selenium and vitamin E has been shown to enhance the cardiac toxicity induced by doxorubicin, an antitumour drug that greatly enhances cellular H_2O_2 production [18, 30]. In a previous study, we have demonstrated a significant effect of this double deficiency on survival after irradiation in the same mouse strain [18]. In that study the time that the animals were on the deficient diet was variable, including some animals that had been on the diet for several months. Subsequent to that study, we became aware of the continued gradual decline in membrane-associated GSH-Px over time. This study was designed to test the effect of the deficient diet under more defined conditions. The time chosen may well have been too short. As noted, in studies of lipid peroxidation using rat liver microsomes, the selenium deficiency diet was maintained for 14 weeks [28].

The present study showed no effect of 6 weeks of vitamin E and selenium depletion on the radiation response of bone marrow as assessed by survival and the ability to form colonies in spleen following irradiation. The GSH-Px in the bone marrow was also not affected significantly by the dietary depletion. On the other hand, all three preparations of the gut mucosa were affected dramatically by the diet. This was associated with a significant reduction in the radiation dose required for a given level of injury, although the slope did not change. One possible interpretation is that the crypt cell population was decreased before radiation by the diet, so that the radiation-induced death of fewer cells would affect the crypt count in these mice. With increasing radiation doses the slope of the cell survival curves was unchanged, indicating a similar radiosensitivity. The combined diet and radiation effect is, therefore,

additive. This is similar to the findings of Phillips *et al.* [34] in gut mucosa where adriamycin or actinomycin D was administered prior to radiation. It is of particular interest given the fact that adriamycin dramatically depletes some tissues of GSH-Px [18]. A similar effect has also been seen with the combination of nitrogen mustard and radiation [35].

The microcolony assay system similarly does not allow assessment of the shoulder of the curve, which represents the cellular capacity to repair sublethal damage. An isolated effect on the shoulder was seen by Hellman and Hannor [36] in the bone marrow cells pretreated with adriamycin. Whatever the interpretation of the radiation effect seen in the present study, it is clear that, though statistically significant, it does not represent a dramatic biologic effect. If the membrane-associated GSH-Px does, in fact, have biologic importance, however, it might be present in great abundance and thus require even more profound depletion before the physiologic effect is dramatic.

The demonstrated inability of cytosolic GSH-Px or cytosolic GST to metabolize micellar phospholipids [9, 10] has fueled the search for a membrane-associated GSH-dependent detoxification system. McCay described GSH-Px activity in washed microsomes of rat liver. Burk *et al.* [37] have suggested that this may have been a spurious finding related to the use of the coupled GSH-Px assay. Hill and Burk [28] have described a microsomal GSH-dependent system with properties of an enzyme that inhibits chemically induced lipid peroxidation. This system appeared to be membrane associated. As noted previously, membrane-associated GSH-Px has been described in at least two systems [7, 8]. The mitochondrial membrane-associated GSH-Px was not as sensitive to short-term selenium deficiency as was the cytosolic enzyme. The membrane-associated GSH-Px activity seen in the present study was similarly resistant to this diet in some tissues (bone marrow, liver), while it appeared to be quite sensitive in others (gut mucosa). While the tests of its effect on radiation sensitivity used in this study may not be optimal to definitively answer the question, the membrane-associated GSH-Px does not appear to significantly affect this.

Acknowledgements—The authors gratefully acknowledge the assistance of Dr. Margaret Wesley in the statistical analysis.

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