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Effect of selenium deficiency on hydroperoxide-stimulated release of glutathione from isolated perfused liver of rainbow trout (*Salmo gairdneri*)

BY J. G. BELL, J. W. ADRON AND C. B. COWEY

NERC Institute of Marine Biochemistry, St Fittick's Road, Aberdeen AB1 3RA

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1. Duplicate groups of rainbow trout (*Salmo gairdneri*) were each given partially purified diets which were either adequate or depleted in selenium for 40 weeks.

2. Although there was no significant difference in weight gain, liver Se concentration was significantly lower in fish given the deficient diet.

3. Glutathione (GSH) peroxidase (*EC* 1.11.1.9) activity was significantly reduced in liver of Se-deficient fish but a differential assay did not indicate the presence of a non-Se-dependent GSH peroxidase activity, although liver GSH S-transferase (*EC* 2.5.1.18) was significantly increased.

4. Perfusion of livers from trout given Se-adequate diets with *t*-butyl hydroperoxide (BuOOH) or hydrogen peroxide caused an increase in the rate of release of glutathione disulphide (GSSG) into the perfusate.

5. Perfusion of livers from Se-deficient trout with BuOOH or H_2O_2 did not result in any change in rate of release of GSSG into the perfusate.

6. These findings confirm the absence of any compensatory non-Se-dependent peroxidase activity in Se-depleted trout.

Selenium deficiency in mammals is known to increase the activity of the glutathione S-transferase (*EC* 2.5.1.18) group of enzymes (Lawrence *et al.* 1978). A number of these enzymes also possess peroxidase activity (the so-called Se-independent glutathione peroxidase activity; Prohaska & Ganther, 1977) with organic hydroperoxides but not with hydrogen peroxide. This activity is maintained in Se deficiency. In mammals this system is thought to protect against oxidative damage when Se-dependent glutathione (GSH) peroxidase (*EC* 1.11.1.9) is depleted during Se deficiency.

In rainbow trout (*Salmo gairdneri*) which have been depleted of Se there is no detectable Se-independent GSH peroxidase activity, although liver GSH S-transferase activity showed a marked increase (Bell *et al.* 1986*b*). Semi-purified trout liver GSH S-transferase has also been shown to inhibit the production of malondialdehyde in an in vitro microsomal lipid peroxidation system (Bell *et al.* 1984).

The present investigation was performed in order to identify further the role of GSH S-transferase in preventing lipid peroxidation in rainbow trout liver.

Perfusion of livers from control and Se-deficient rats with media containing organic hydroperoxides or H_2O_2 have clarified and confirmed the function of the Se-dependent and Se-independent GSH peroxidases in hydroperoxide metabolism. In control animals glutathione disulphide (GSSG) efflux from the liver increased in response to administration of either *t*-butyl hydroperoxide (BuOOH) or H_2O_2 used independently; by contrast, in Se-deficient animals, GSSG efflux only increased when an organic hydroperoxide was perfused (Burk *et al.* 1979). However, in studies involving isolated perfused rat heart (Xia *et al.* 1985) from Se-deficient animals, no GSSG efflux was observed on perfusion with BuOOH indicating that GSH-dependent hydroperoxide metabolism does not occur in the Se-deficient rat heart.

In the present study with rainbow trout we have studied GSH-dependent hydroperoxide metabolism in control and Se-deficient isolated perfused livers in an attempt to characterize

the antioxidant processes occurring in rainbow trout and to assess impairment due to Se deficiency. A preliminary account of this work has been given (Bell *et al.* 1986*a*).

MATERIALS AND METHODS

Animals and diets

Rainbow trout were obtained from Selcoth Fisheries, Moffat, Scotland; they had a mean weight of approximately 27 g and they were randomly distributed (thirty fish/tank) between four fibre glass tanks of diameter 1 m and depth 0·6 m, and containing 500 litres of water. The water, from Aberdeen city domestic supply, passed through an activated charcoal filter to the tanks with a total flow to each tank of 10 litres/min. The ambient temperature in the aquarium room averaged 15° and the photoperiod was 12 h light–12 h dark.

The fish were weaned from a commercial diet to the basal diet (Se-deficient) (Bell *et al.* 1986*b*) and about 1 week later initial weight measurements were made on individual fish which had been anaesthetized with MS222 (ethyl *m*-aminobenzoate methane sulphonate; Sigma Chemical Co. Ltd, Poole, Dorset; 0·2 g/l). Fish were fed at a rate of 20 g/kg biomass per d (the diet being given four or five times per day), 6 d each week. Any food uneaten from the daily ration was weighed and recorded. Fish were weighed at 28 d intervals and the ration adjusted accordingly. The growth experiment lasted 30 weeks. Thereafter fish continued to be fed at the same rate for a further 30 weeks when they had attained a size suitable for liver perfusion, i.e. about 400 g.

The basal diet (Se-deficient) contained Torula yeast and was designed to meet the nutritional requirements of trout other than for Se ((US) National Research Council, 1981). The basal diet formulation (Table 1) and preparation were as described previously (Bell *et al.* 1986*b*). The control diet (Se-supplemented) had Se added as sodium selenite to 1 mg/kg. The basal diet contained 0·025 (SE 0·004) mg Se/kg and 0·63 (SE 0·06) g vitamin E/kg. The aquarium water contained 0·035 μg Se/l. Each of the two diets was given to duplicate groups (tanks) of fish. Feed : gain values were calculated at each weighing on a per tank basis as total diet consumed : total increase in biomass.

Liver perfusion

Rainbow trout were anaesthetized with MS222 and injected with 100 units lithium heparin (in isotonic saline (9 g sodium chloride/l)) via the caudal vein. After 15 min the fish were killed by a blow to the head. The liver was cannulated via the hepatic portal vein (Portex green luer 2FG; Portex Ltd, Hythe) then rapidly excised and mounted on a recirculating perfusion apparatus similar to that described by Hayashi & Ooshiro (1975). The average liver weight was 7·43 (SE 0·83) g. The perfusion medium, which was a glucose-free Hanks medium, pH 7·4, contained: 112 mM-NaCl, 4·2 mM-potassium chloride, 0·1 mM-ammonium sulphate, 26·2 mM-sodium bicarbonate, 1·0 mM-disodium hydrogenphosphate, 1·3 mM-calcium chloride, 1·2 mM-magnesium sulphate, 2·1 mM-lactic acid, 0·3 mM-pyruvic acid, 5 mM-HEPES, 0·17 mM-histidine, 0·07 mM-aspartic acid, 0·12 mM-glutamic acid. It was perfused at a rate of 1·3 ml/g per min and at a temperature of $20 \pm 2°$. The medium was continuously oxygenated with oxygen–carbon dioxide (95:5, v/v). After an initial 15 min perfusion where effluent was allowed to run to waste, recirculation was started and the volume of perfusion medium adjusted to 50 ml. After a further 10 min perfusion the zero time sample (0·6 ml) was taken and after 10 min the medium was made either 1 mM in BuOOH or 2 mM in H_2O_2 by addition to the media reservoir. Samples (0·6 ml) were collected post aerator every 10 min up to 90 min and were assayed for total glutathione (GSH + GSSG), hydroperoxide content and lactate dehydrogenase (*EC* 1.1.1.27) activity.

Table 1. *Composition of basal diet (g/kg dry diet)*

Torula yeast	350
Cod liver oil*	100
Vitamin mix†	28
Mineral mix‡	40
Starch	157·1
Antioxidant mix§	0·4
Amino acid mixture‖	324·5

* Super Solvitax; British Cod Liver Oils Ltd, Hull.

† Supplied (/kg diet): thiamin hydrochloride 50 mg, riboflavin 200 mg, pyridoxine hydrochloride 50 mg, nicotinic acid 750 mg, calcium pantothenate 500 mg, *myo*-inositol 2 g, biotin 5 mg, folic acid 15 mg, choline bitartrate 9 g, ascorbic acid 1 g, menaphthone 40 mg, cyanocobalamin 0·09 mg, DL-α-tocopheryl acetate 400 mg.

‡ Supplied (g/kg diet): $Ca(H_2PO_4)_2 . H_2O$ 27·6, $CaCO_3$ 2·1, $MgCO_3$ 3·6, $FeSO_4 . 7H_2O$ 1·2, KCl 2·0, NaCl 3·2, $Al_2(SO_4)_3 . 16H_2O$ 0·008, $ZnSO_4 . 7H_2O$ 0·16, $CuSO_4$ 0·04, $MnSO_4 . 4H_2O$ 0·14, KI 0·008, $CoSO_4$ 0·04.

§ Contained (g/l): 200 butylated hydroxyanisole, 60 propyl gallate and 40 citric acid dissolved in propylene glycol.

‖ Supplied (g/kg diet): lysine 32, histidine 12·2, arginine 21·6, threonine 14·6, valine 15·8, methionine 6, isoleucine 13·6, leucine 28·3, phenylalanine 13·2, tryptophan 4·3, aspartic acid 32·1, serine 15·9, glutamic acid 50, proline 12·2, glycine 14·9, alanine 21·7, tyrosine 11·3, cystine 4·8.

Analytical methods

Total glutathione (GSH+GSSG) in the perfusate was determined by the glutathione reductase-DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) recirculating assay described by Oshino & Chance (1977). GSSG was determined by measuring NADPH oxidation in the presence of glutathione reductase (NAD(P)H) (*EC* 1.6.4.2) as described by Sies & Summer (1975). Se in tissues and aquarium water was measured by the method of Hasunuma *et al.* (1982). GSH peroxidase was measured by following the rate of oxidation of NADPH at 340 nm in the coupled reaction with GSH reductase as described by Bell *et al.* (1986*b*) and GSH S-transferase activity was assayed by following the conjugation of 1-chloro-2,4-dinitro-benzene with GSH at 340 nm (Habig *et al.* 1974). Lactate dehydrogenase in the perfusate was measured by following the oxidation of NADH at 340 nm as described by Lush *et al.* (1969). The assay contained (final concentrations): 87 mM-potassium phosphate buffer, pH 7·4, 0·09 mM-sodium pyruvate and 0·15 mM-NADH. The reaction was started by addition of up to 0·2 ml of enzyme source. Perfusate hydroperoxide was measured by the method described by Cathcart *et al.* (1983).

Statistical analysis

The initial and final weight measurements on the trout in all four tanks were examined by analysis of variance as described by Bell *et al.* (1980*b*).

RESULTS

Initial and final weights of trout given the two diets are shown in Table 1 and there were no significant differences in weight gain in any of the tanks.

Se concentrations in liver were very markedly reduced in trout fed on the Se-deficient diet (Table 2) and reflecting this liver GSH peroxidase activity was similarly reduced (Table 2). The differential assay performed with either H_2O_2 or organic hydroperoxide did not provide convincing evidence for the appearance of a non-Se-dependent GSH peroxidase as a result of Se deficiency, although there was a significant increase in liver GSH S-transferase activity (Table 2).

Table 2 *Weight gain values, liver selenium and some liver enzyme activities in rainbow trout* (Salmo gairdneri) *fed on diets either supplemented or deficient in Se*

(Mean values with their standard errors)

Diet...	−Se				+Se			
	A		B		C		D	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial wt (g)	25·70	1·24	26·34	1·74	26·57	2·01	28·12	2·11
Final wt (g)	144·46	8·47	155·94	12·76	159·37	10·63	163·87	9·62
Feed : gain	1·71		1·61		1·54		1·50	
	Mean		SE		Mean		SE	
Liver Se (μg/g wet tissue)	0·122		0·019		1·321		0·258	
Liver GSH peroxidase (*EC* 1.11.1.9)								
Hydrogen peroxide substrate*	2·50		0·68		14·38		1·83	
Cumene hydroperoxide substrate†	5·05		0·85		18·60		1·72	
Liver GSH S-transferase (*EC* 2.5.1.13)‡	0·713		0·072		0·311		0·034	

A and B are duplicate tanks, C and D are duplicate tanks.
* nmol NADPH oxidized/min per mg protein, 0·25 mM-H_2O_2 substrate.
† nmol NADPH oxidized/min per mg protein, 1·5 mM-cumene hydroperoxide substrate.
‡ μmol thioester bond formed/min per mg protein.

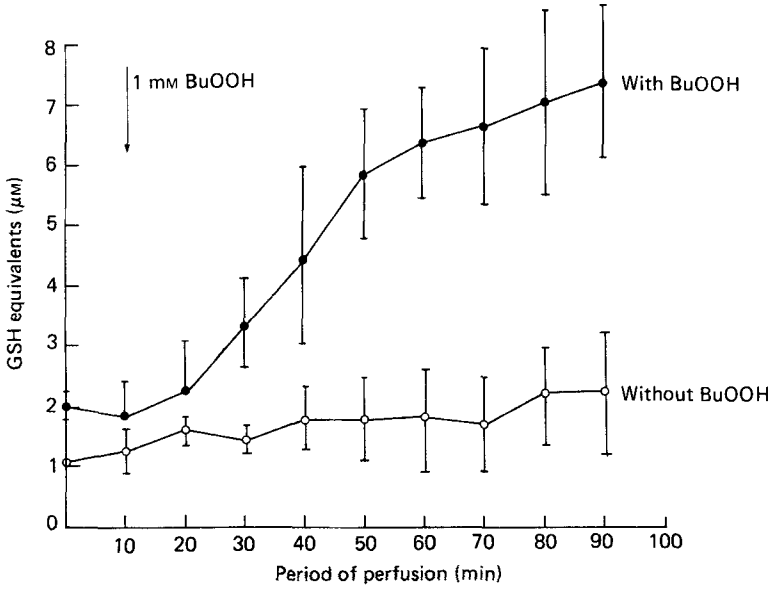

Fig. 1. Glutathione disulphide release (glutathione (GSH) equivalents) from isolated perfused liver of rainbow trout (*Salmo gairdneri*) fed on a control diet in the presence (●——●) or absence (○——○) of 1 mM-*t*-butyl hydroperoxide (BuOOH). Points are mean values, with their standard errors represented by vertical bars, from three livers.

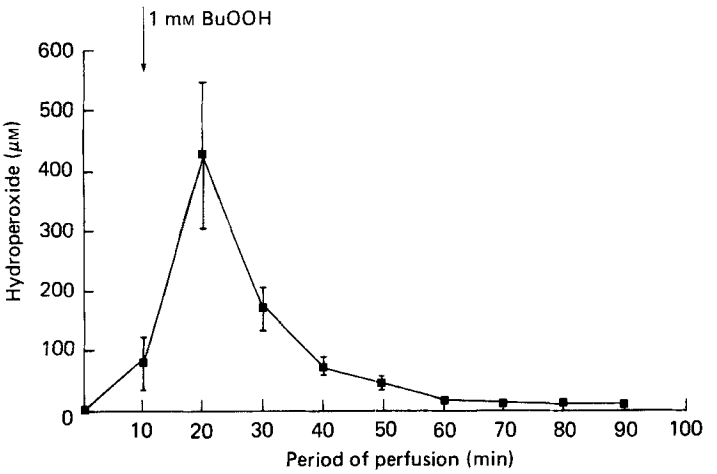

Fig. 2. Uptake of *t*-butyl hydroperoxide (BuOOH) by isolated perfused liver of rainbow trout (*Salmo gairdneri*) fed on a control diet. Points are mean values, with their standard errors represented by vertical bars, from three livers.

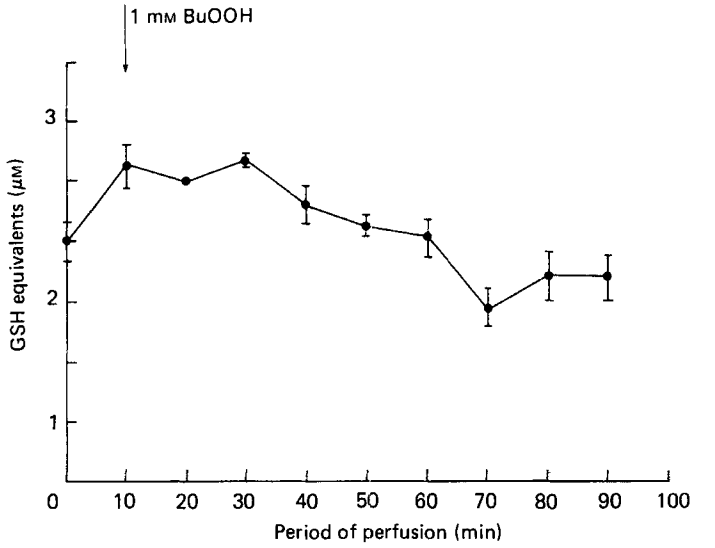

Fig. 3. Glutathione disulphide release (glutathione (GSH) equivalents) from isolated perfused liver of rainbow trout (*Salmo gairdneri*) fed on a selenium-deficient diet: effect of addition of *t*-butyl hydroperoxide (BuOOH) to the perfusion medium. Points are mean values, with their standard errors represented by vertical bars, from three livers.

Fig. 1 shows the effect of infusing BuOOH into a control liver. Although the assay employed measured 'GSH equivalents' (i.e. total GSH) the nature of GSH released on perfusion of hydroperoxide was found to be entirely as the disulphide. After a short lag-period release of GSSG into the perfusate proceeded at a steady rate for 50 min, thereafter there was some reduction in rate of GSSG release. The slowing down of GSSG release occurred when approximately 95% of the BuOOH had been removed from the perfusion medium (see Fig. 2). No major cell damage occurred over this period since lactate dehydrogenase measurements indicated only a small rise in this activity over the 90 min

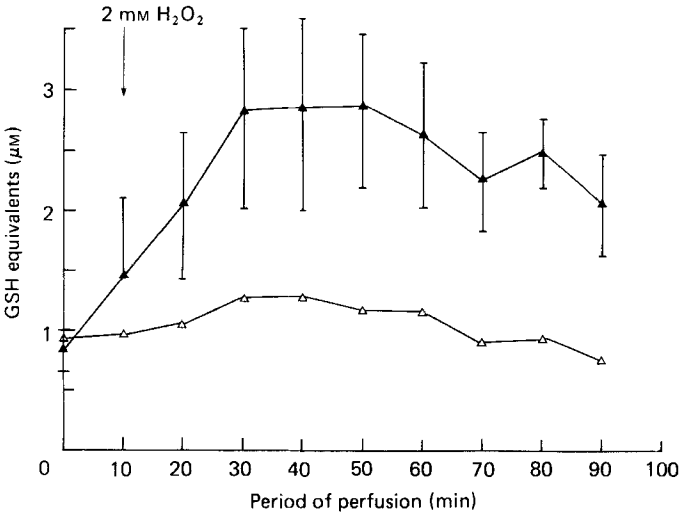

Fig. 4. Glutathione disulphide release (glutathione (GSH) equivalents) from isolated perfused liver of rainbow trout (*Salmo gairdneri*) fed on a control diet (▲——▲) or a selenium-deficient diet (△——△) when hydrogen peroxide was added to the perfusion medium. In the control fish, points are mean values, with their standard errors represented by vertical bars, from three livers. In Se-deficient fish points are mean values from two livers. The third Se-deficient liver showed high initial lactate dehydrogenase (*EC* 1.1.1.27) release which increased fourfold over 90 min. The values from this experiment were not used here.

period (results not shown). Release of GSSG from a control liver perfused in the absence of BuOOH was very low (Fig. 1).

When livers from Se-deficient trout were perfused with BuOOH no increase in GSSG release was observed (Fig. 3), although the initial levels of GSSG in the perfusion medium appeared greater than in control fish (Fig. 1). BuOOH was taken up by Se-deficient liver (results not shown) with a profile similar to that seen in control fish (Fig. 2) but this was not followed by a GSH-dependent metabolism of BuOOH as occurred in the control liver.

When H_2O_2 was added to the medium perfusing control livers there was an increased release of GSSG into the perfusate (Fig. 4) but this efflux levelled off after about 30 min. Infusion of H_2O_2, as with BuOOH, failed to cause a release of GSSG when Se-deficient livers were used.

Discussion

The presence of non-Se-dependent GSH peroxidase activity has been confirmed in the tissues of a number of different species (Lawrence & Burk, 1978). This activity has been attributed to one or more of the GSH S-transferase enzymes identified in rat liver (Meyer *et al.* 1985). In the present study Se-deficient rainbow trout showed an increased hepatic GSH S-transferase activity similar to that in Se-deficient rats (Lawrence *et al.* 1978), although this was not reflected in an increased GSH peroxidase activity as measured by differential assay. The ability of Se-deficient rat liver to undergo GSH-dependent metabolism of organic hydroperoxides has been confirmed in studies with isolated perfused preparations (Sies & Summer, 1975; Burk *et al.* 1978). In perfused liver of Se-deficient rainbow trout, however, no release of GSSG into the perfusing medium occurred when either H_2O_2 or BuOOH were added to the system. This result, which contrasts with that in normal control trout, confirms the enzyme findings in that no compensatory increase of non-Se-dependent

GSH peroxidase activity is present when GSH peroxidase activity is reduced due to Se deficiency.

Although the infusion of H_2O_2 into control livers produced an increased GSSG release into the perfusate the duration of release was shorter and the maximum concentration reached was lower than that with BuOOH (Fig. 4). A similar effect was noted by Sies & Summer (1975) and was thought to be due to the intervention of catalase (*EC* 1.11.1.6) acting to remove H_2O_2, so preventing its reduction by GSH peroxidase.

In some Se-deficient livers, particularly those perfused with BuOOH, a relatively high resting state release of GSSG was observed. This effect had been described previously in rat liver (Burk *et al.* 1978) and had been suggested as an indication of a higher incipient rate of lipid peroxidation occurring in Se-deficient liver (Nishiki *et al.* 1976).

The differences between trout and mammalian GSH S-transferases that affect their ability to act as a GSH-peroxidase are not clear. Possibly the Se-deficiency in trout is not severe enough to induce an increased production of the relevant GSH S-transferase. This may be due to the ability of fish to remove small amounts of Se from the water thus alleviating dietary Se deficiency. The GSH S-transferases of rainbow trout liver have not been as clearly characterized as those from rat but a number of differences have been shown to exist (Nimmo & Clapp, 1979; Nimmo *et al.* 1981) which could infer differing abilities to metabolize hydroperoxides. Indeed of the three GSH S-transferases shown to be induced by Se deficiency in rat liver (Mehlert & Diplock, 1985) only one has been found in trout liver (Nimmo *et al.* 1981).

If GSH S-transferase(s) are able to conjugate hydroperoxides (or intermediates involved in their formation) as they do other xenobiotics (Spearman *et al.* 1985) this would explain the capacity of these enzymes to inhibit malondialdehyde formation in the in vitro microsomal peroxidation system. Further investigations involving the GSH S-transferases and Se-deficiency may help to elucidate the role of GSH-metabolizing enzymes in the antioxidant protective mechanisms present in salmonid fishes.

These experiments help to verify previous observations with Se-deficient rainbow trout, namely that although hepatic GSH S-transferase activity increases in Se deficiency and although this will inhibit lipid peroxidation (as for example in in vitro systems) it does not do so by conventional peroxidase activity.

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