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Glutathione Peroxidase Activities in Sheep and Rat Muscle and Some Effects of Selenium Deficiency

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Abstract

The activity and distribution of the selenium-containing enzyme, glutathione peroxidase, has been determined in muscle fractions in normal adult rats and sheep. Skeletal and cardiac muscle have been examined, and in both types of muscle the major proportion of the enzyme appeared in the cytosol fraction. Enzyme activity was higher in cardiac muscle than in skeletal muscle in both species, and based on total protein present in fractions, it appears that rat muscle contains more enzyme activity than sheep muscle.

In tissues from lambs born to selenium-deprived ewes the levels of enzyme were significantly depressed. Two sampling periods were selected, the first when the lambs were 2–3 weeks of age and the second at slaughter when they were 10 weeks old. Muscle, plasma and erythrocyte levels of the enzyme indicated that the most sensitive measure of selenium deficiency is likely to be that of the erythrocyte enzyme level.

Introduction

The enzyme glutathione peroxidase (EC 1.11.1.9) has been described as the first positively identified seleno-enzyme found in animal tissues (Oh *et al.* 1974). Rotruck *et al.* (1973) suggested that selenium is an integral part of the enzyme and pointed out that the enzyme might provide a useful means of defining selenium requirements, and of identifying selenium deficiency in animals and humans.

Most of the observations on glutathione peroxidase made so far have been concerned with its presence in erythrocytes and plasma (Paglia and Valentine 1967; Flohé *et al.* 1971; Hoekstra *et al.* 1973; Lie-Injo *et al.* 1973; Rotruck *et al.* 1973). Enzyme activities have been measured in liver fractions (Flohé and Schlegel 1971; Noguchi *et al.* 1973), and there is limited data available for rat muscle (Chow *et al.* 1973).

The enzyme catalyses the removal of H_2O_2 according to the following coupled reaction:

$$2\ GSH + H_2O_2 \xrightarrow{\text{glutathione peroxidase}} GSSG + H_2O$$

$$GSSG + NADPH \xrightarrow{\text{glutathione reductase (NAD(P)H)}} 2GSH + NADP^+$$

Noguchi *et al.* (1973) have shown that when chicks are fed a crystalline amino acid diet very low in selenium and vitamin E, the level of glutathione peroxidase in plasma is related directly to the dietary level of selenium. It becomes important therefore to know what the level of this enzyme is in muscle and whether there is a direct relationship between its activity and the development of dystrophic change. This is

particularly important in the ruminant where muscular dystrophy is the characteristic feature of selenium deficiency.

This paper reports glutathione peroxidase activities in fractionated muscle from normal sheep and rats, and some preliminary observations on the effects of low-selenium intakes in ewes on levels of the enzyme in their lambs.

Materials and Methods

(*a*) *Animals*

Three groups of animals provided the experimental material: firstly, mature male rats of the hooded Wistar strain from an SPF colony established in this laboratory (Godwin *et al.* 1964); secondly, normal adult Merino sheep, reared at the CSIRO Field Station, Glenthorne, S.A.; thirdly, young lambs born to ewes fed a reduced-selenium intake as from approximately 1 month prior to parturition.

As fodder for the ewes of the third group, wheaten-hay chaff having a selenium content of 0·03 μg/g dry matter was selected from several batches analysed. Twelve ewes were fed the low-selenium fodder. Three received no supplement, three received 100 mg α-tocopherol acetate (vitamin E) as an intramuscular injection weekly, three received 3 mg selenium as an oral drench of sodium selenite twice-weekly and three were given an intraruminal pellet containing 5% Se and 95% Fe and a grinder (Kuchel and Buckley 1969). The lambs did not receive any direct supplementation.

(*b*) *Sample Preparation*

Heart muscle preparations were obtained from the pooling of three rat hearts; skeletal muscle determinations were derived from pooled gastrocnemius and soleus muscles from the same three animals. Heart muscle from sheep was from the left ventricle and skeletal muscle from the adductor.

All muscle samples were removed quickly after slaughter to ice-cold 0·15M KCl. Subsequent steps were carried out at 1–2°C. The muscle was chopped finely and incubated in a solution similar to that used by Chappell and Perry (1954), without the addition of EDTA but containing 0·05% Pronase (Koch-Light, preparation from *Streptomyces griseus*). After incubation for 5 min at 1–2°C, muscle was homogenized in a Teflon–glass homogenizer and centrifuged at 10 000 *g* for 10 min. The supernatant, containing cytosol protein and microsomes, was retained as fraction I. After further homogenization the residue was centrifuged at 700 *g* for 10 min, the nuclear debris from this slow spin being retained as fraction II and the supernatant being centrifuged at 10 000 *g* to bring down the mitochondria. Fractions III and IV were respectively the first wash from the isolation of the mitochondrial fraction and the mitochondrial pellet resuspended in 2–4 ml 0·25M sucrose.

The muscle biopsies from the lambs and muscle specimens taken from the same animals at slaughter were homogenized directly in 0·25M sucrose, treated with 1% Triton X-100 and centrifuged at 10 000 *g* for 10 min.

The preparation of blood samples for measurement of the enzyme followed the method of Noguchi *et al.* (1973). Protein determinations were carried out on all fractions using the method of Lowry *et al.* (1951). Selenium determinations were made using Watkinson's method (1966).

(*c*) *Glutathione Peroxidase Determinations*

Enzyme activities were determined by a modification of the coupled method of Paglia and Valentine (1967). The rate of glutathione oxidation by H_2O_2 was measured by following the disappearance of NADPH. Glutathione reductase (NAD(P)H) was included to maintain the presence of glutathione in the reduced form. Activities were measured in 1-ml cuvettes at 20°C using a Beckman DU spectrophotometer coupled to a Gilford multiple sample absorbance recorder, Model 2000. All readings were made at 340 nm and calculations were based on the rate of reaction over the first 4 min following the addition of H_2O_2. Final concentrations of reagents in the cuvette were: phosphate buffer (pH 7·0) 0·04M, NADPH 0·24 mM, sodium azide 0·4 mM, glutathione (freshly prepared and neutralized) 2·5 mM, EDTA 5 mM, H_2O_2 0·088 mM and glutathione reductase (NAD(P)H) (Sigma Chemical Co.) 0·3 units. Test solution (10–100 μl) was added, a base-line reading obtained and H_2O_2 added finally to initiate the reaction.

The blank contained all reagents except the test solution for which water was substituted. A slow non-enzymic oxidation of NADPH occurred in the presence of H_2O_2.

(d) Swelling Characteristics of Mitochondria

Measurement of the swelling characteristics of the mitochondria, using the method outlined by Chappell and Haarhoff (1967), and measurement of the protein content of the muscle fractions were used to indicate the integrity of the organelles and the reproducibility of the fractionation procedure. These measurements were made to allow conclusions to be drawn concerning the distribution of the enzyme within muscle cells. Substrates used in swelling experiments included potassium chloride, ammonium malate, ammonium fumarate, ammonium acetate and reduced glutathione. Liver mitochondria were included in the study as their behaviour under these conditions is well established and serves as a useful reference point.

The concentration of solutions of the first four substrates was initially 0·2M, and they were serially diluted to 0·1, 0·05, 0·025 and 0·0125M. Reduced glutathione was serially diluted from an initial concentration of 0·01M. The solutions also contained 1 μg antimycin and final concentrations of 0·33 mM EDTA and 6 mM tris–HCl, and were adjusted to pH 7·4. The change in absorbance was measured at 610 nm in 3-ml cuvettes on the addition of equal amounts of mitochondrial suspension (approximately 0·5 mg mitochondrial protein).

Table 1. Glutathione peroxidase activity in fractions of muscle in normal sheep and rats

Values are total units of enzyme activity (μmol NADPH oxidized/min at 20°C) in each of four fractions which together represent a total weight of 1 g muscle. Each value is the average for three animals ± S.E.M.

Muscle fraction	Sheep muscle		Rat muscle	
	Skeletal	Cardiac	Skeletal	Cardiac
I	0·41 ± 0·18	5·97 ± 0·69	3·90 ± 0·91	16·80 ± 1·10
II	0·65 ± 0·24	3·63 ± 0·29	0·38 ± 0·06	3·90 ± 0·22
III	0·04 ± 0·03	0·67 ± 0·11	0·30 ± 0·16	0·58 ± 0·36
IV	0·04 ± 0·01	1·35 ± 0·16	0·12 ± 0·03	3·59 ± 0·47

Table 2. Percentage distribution of protein in fractions of muscle in normal sheep and rats

The values given are percentages ± S.E.M. and those in parenthesis give the percentage distribution of glutathione peroxidase activity in the same fractions (values derived from Table 1)

Muscle fraction	Sheep muscle		Rat muscle	
	Skeletal	Cardiac	Skeletal	Cardiac
I	34·9 ± 2·3 (35·2)	26·5 ± 1·8 (51·1)	51·4 ± 3·2 (82·8)	34·5 ± 0·6 (67·7)
II	59·1 ± 2·2 (57·4)	62·7 ± 1·5 (31·3)	42·6 ± 5·1 (8·5)	50·6 ± 1·8 (15·7)
III	4·9 ± 0·7 (3·1)	4·9 ± 0·2 (5·8)	3·5 ± 1·7 (6·0)	2·1 ± 0·3 (2·2)
IV	0·8 ± 0·1 (4·1)	5·7 ± 0·5 (11·7)	2·3 ± 0·3 (2·6)	12·7 ± 0·6 (14·4)

Results

Glutathione peroxidase activities in skeletal and heart muscle fractions in normal sheep and rats are shown in Table 1. The distribution of protein in the same fractions, together with the percentage distribution of glutathione peroxidase activity, is shown in Table 2. Triton X-100 was added to fractions II and IV to ensure the release of bound enzyme. It had been previously determined that Triton X-100 did not cause enzyme inhibition.

The behaviour of the mitochondria in various solutes showed that they were behaving normally as osmometers. The results obtained with potassium chloride and glutathione are reproduced in Fig. 1. Similar effects were found with ammonium malate and ammonium fumarate, but acetate ions were freely permeable at all concentrations. In summary, the observations indicate normal permeability characteristics and, taken together with the data in Table 2, suggest that the distribution of glutathione peroxidase activity is not altered by changes in membrane permeability of the mitochondria.

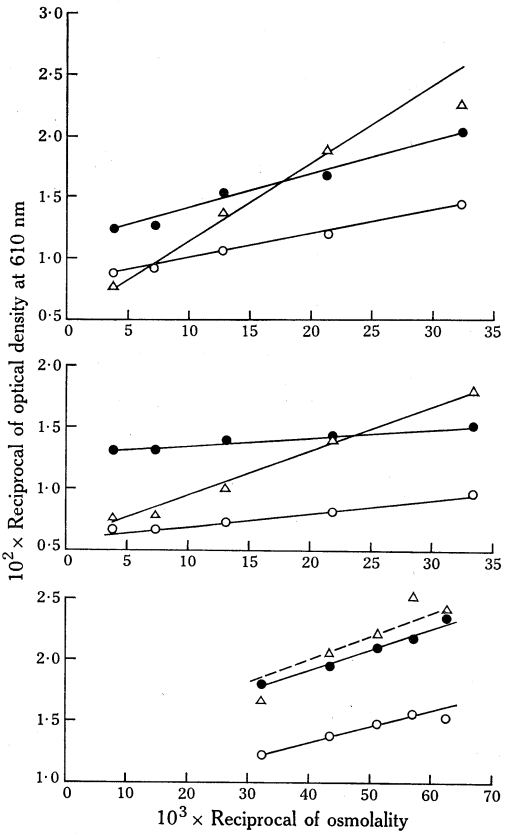

Fig. 1. Double reciprocal plots of optical density of suspension containing 100 mg against total concentration of components of the suspending medium. Composition of the medium was 1 μg antimycin, 0·33 mM EGTA and 6 mM tris–HCl, pH 7·4. Solute concentrations were serial dilutions of 0·2M solutions of KCl, ammonium malate, ammonium fumarate and ammonium acetate, and of a 0·01M solution of glutathione. Typical curves are reproduced for osmotic behaviour of (*a*) sheep mitochondria in KCl, (*b*) rat mitochondria in KCl, and (*c*) rat mitochondria in glutathione. △ Liver. O Heart. ● Muscle.

Glutathione peroxidase activity in tissues of lambs born to treated and untreated ewes, and sampled between 2–3 weeks of age, is shown in Table 3. Even at this early stage there is a highly significant reduction in erythrocyte glutathione peroxidase

activities, and this difference is reflected in muscle biopsies taken at the same time. Plasma enzyme activities show the same trend but the difference does not reach significance.

Table 3. Glutathione peroxidase activity found in muscle, haemolysed erythrocytes and plasma of 2–3-week-old lambs reared by ewes supplemented variously with selenium and vitamin E

Enzyme activities are expressed as μmol NADPH oxidized per 100 mg protein per minute ± S.E.M.

Ewe treatment	No. of samples	Enzyme activity		
		Muscle biopsy	Erythrocytes	Plasma
No Se supplement[A]	6	1·46 ± 0·23	16·3 ± 2·8	0·29 ± 0·07
Se supplemented[B]	6	2·33 ± 0·23	37·4 ± 2·8	0·48 ± 0·07
Significance		$P = 0·05$	$P < 0·001$	n.s.

[A] This group includes lambs from ewes treated with vitamin E.
[B] Both orally-drenched and Se pellet-treated ewes.

When lambs from the same group were slaughtered at 10 weeks of age erythrocyte glutathione peroxidase activity was markedly different between the groups derived from treated and untreated ewes. There were also significant differences between groups for enzyme levels in both left and right ventricular muscle. These differences are shown in Table 4.

Table 4. Glutathione peroxidase activities in cardiac muscle homogenates and erythrocytes from 10-week-old lambs born to ewes on low-selenium intakes and supplemented variously with selenium and vitamin E

Enzyme activities are expressed as μmol NADPH oxidized per 100 mg protein per minute ± S.E.M.

Ewe treatment	No. of animals	Enzyme activity		
		Left ventricle	Right ventricle	Haemolysate
No Se supplement[A]	6	1·16 ± 0·30	1·58 ± 0·33	6·91 ± 0·79
Se supplemented[B]	6	4·61 ± 0·53	3·76 ± 0·46	17·15 ± 1·76

[A] This group includes lambs from ewes treated with vitamin E.
[B] Ewes either orally drenched or treated with Se pellets.

Selenium analysis of milk and blood samples from the ewes and their lambs (at different stages) confirmed that the lowered level of glutathione peroxidase encountered in various tissues was paralleled by the level of selenium. These values for selenium appear in Table 5. Blood selenium levels in the ewes were still normal at this stage since transfer to the low-selenium fodder occurred approximately 1 month before parturition.

Post-mortem examination showed the presence of mild but characteristic lesions of white muscle disease on the endocardial surface of the hearts of lambs born to ewes not supplemented with either vitamin E or selenium. These lesions are indicated by arrows in Fig. 2.

Table 5. Selenium concentrations of blood and milk of ewes and of blood of the corresponding lambs at three stages of development

The ewes were fed low-selenium fodder, some were supplemented with selenium either by oral drench or intraruminal pellet. There were six animals per treatment group

Animals	Sample	Se concn (μg/ml) $\pm$ S.E.M.	
		No Se supplement	Se supplemented
Ewes			
2 weeks	Blood	0·125 $\pm$ 0·006	0·178 $\pm$ 0·011
post-partum	Milk	0·005 $\pm$ 0·0003	0·010 $\pm$ 0·0008
Lambs			
At birth	Blood	0·093 $\pm$ 0·006	0·181 $\pm$ 0·011
2–3 weeks	Blood	0·069 $\pm$ 0·007	0·143 $\pm$ 0·010
10 weeks	Blood	0·038 $\pm$ 0·002	0·100 $\pm$ 0·012

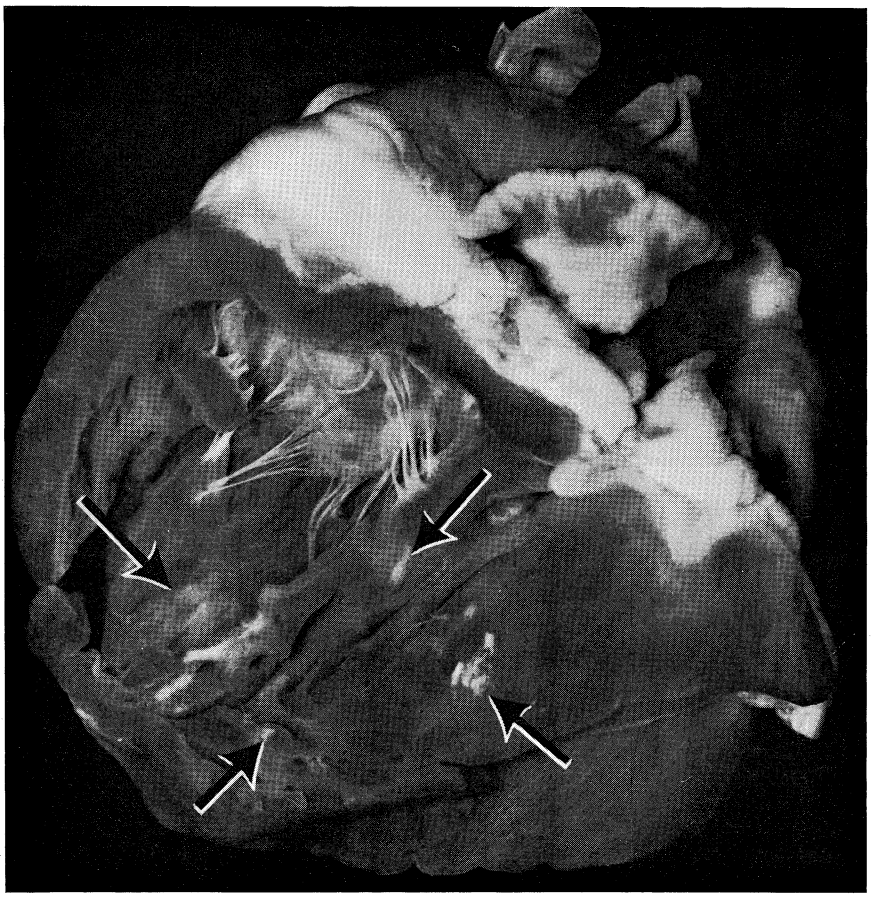

Fig. 2. Endocardial surface of heart of lamb slaughtered at 10 weeks of age, after having been reared by a dam fed a low-selenium intake as from approximately 1 month before parturition. Note the small but characteristic lesions of white muscle disease present (arrows).

Discussion

The results reported in this paper indicate the distribution of glutathione peroxidase in normal muscle from sheep and rats and show the effects of early deprivation of selenium in lambs. Administration of selenium to the dam, either as an oral drench of small amounts of sodium selenite or by the provision of an intraruminal selenium pellet prevents the decrease in glutathione peroxidase activity in both erythrocytes and skeletal muscle.

The results show that the method of Paglia and Valentine (1967) for the determination of glutathione peroxidase in erythrocytes can be suitably adapted to muscle preparations and to the determination of the distribution of the enzyme within the cell. In rats most of the enzyme appears to be in the soluble fraction in both cardiac and skeletal muscle. A relatively higher amount (15·7% compared with 8·5%) is found in the mitochondrial fraction in heart muscle compared with skeletal muscle.

On the other hand, although similar percentages are found in sheep heart and skeletal muscle, the activity of the enzyme in the nuclear debris (fraction II) in the sheep is very much higher for both heart and skeletal muscle (see Fig. 1). In sheep heart, as in rat heart, the largest percentage of activity is in the cytosol fraction.

Considering the activities of the enzyme in the various fractions, as shown in Table 1, there appears to be much more enzyme in heart muscle than in skeletal muscle in both rat and sheep.

From the values obtained from lamb tissues (Table 3) it appears that the selenium status of the ewe is very quickly reflected in glutathione peroxidase activities in the corresponding lamb. Vitamin E failed to influence the amount of activity whereas selenium supplementation of the ewes, either by oral drench or intraruminal selenium pellet, led to a marked rise in enzyme activity.

For lambs born to selenium-supplemented ewes, when glutathione peroxidase activity in the erythrocytes of lambs 2–3 weeks old (Table 3) is compared to that of lambs 10 weeks old (Table 4) it is seen that the enzyme levels are lower at 10 weeks. This is presumably due to the decreasing amount of selenium received by the lambs (Table 5) since their only supplementation was that derived from their dams. The presence of white muscle disease plaques in the hearts of lambs born to unsupplemented ewes (Fig. 2) was indicative of the dystrophogenic nature of the fodder. Normally on such fodder lambs would need to receive additional selenium after birth to maintain a normal selenium status.

These studies show that, in the tissues examined, the level of glutathione peroxidase in erythrocytes appears to be the most sensitive indicator of impending dystrophic change. Enzyme activity does decline in muscle and at the stage when mild lesions are present in the heart there are significant differences in the amount of enzyme present in both left and right ventricle in tissue removed from lambs born to selenium-supplemented or unsupplemented ewes. These differences were found in lambs weaned and then slaughtered at 10 weeks of age, not having had any direct supplementation.

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