Antioxidant enzyme activity in the muscles of calves depleted of vitamin E or selenium or both

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(*Received 11 August 1992 – Accepted 4 December 1992*)

Feeding diets depleted of vitamin E and Se to cattle can induce a disease known as nutritional degenerative myopathy. It is believed that an increased peroxidative challenge in muscle is involved in the pathogenesis of this disease. A number of species can up-regulate the activity of some antioxidant enzymes, including glutathione reductase (*EC* 1.6.4.2), glutathione transferase (*EC* 2.5.1.18), glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49), catalase (*EC* 1.11.1.6), and superoxide dismutase (*EC* 1.15.1.1), in an attempt to mitigate the effects of a peroxidative challenge. A 2 × 2 factorial study was set up to examine possible changes in the activities of these antioxidant enzymes in muscles of ruminant calves fed on diets low in either vitamin E or Se. Four groups of four calves each were fed on a basal diet of NaOH-treated barley which was supplemented with α-tocopherol or Se or both for a total of 50 weeks. Calves fed on diets depleted of vitamin E, but not those fed on diets low in Se, developed subclinical myopathy, as judged by increases in the activity of plasma creatine kinase (*EC* 2.7.3.2), and had increased muscle concentrations of two indices of lipid peroxidation, namely thiobarbituric acid-reactive substances, with and without ascorbate activation. Feeding diets depleted of vitamin E and diets low in Se both increased muscle activities of glucose-6-phosphate dehydrogenase in heart, biceps and supraspinatus. This change may have occurred in an attempt to maintain intracellular pools of reduced glutathione. No other changes in antioxidant enzyme activity were observed.

Antioxidant enzymes: Lipid peroxidation: Vitamin E: Selenium: Cattle

Nutritional degenerative myopathy (NDM) can occur in ruminant cattle deficient in vitamin E and Se (McMurray & McEldowney, 1977; Kennedy *et al.* 1987). The disease is characterized by skeletal and cardiac myonecrosis and by an increase in the activity of creatine kinase (*EC* 2.7.3.2; CK) in plasma and may result in lameness or sudden death (Allen, 1977; Kennedy *et al.* 1987). It also has been shown that myopathy can be prevented by feeding supplements of α-tocopherol and Se (McMurray & McEldowney, 1977; Kennedy *et al.* 1987).

Vitamin E and Se, through the action of the selenoprotein glutathione peroxidase (*EC* 1.11.1.9; GPX), have important antioxidant functions within cells. However, these two substances represent only part of the cellular antioxidant defence system. A range of enzymes are also known to have an antioxidant function. These include: glutathione transferase (*EC* 2.5.1.18; GSHT), glutathione reductase (*EC* 1.6.4.2; GR), glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49; G6PDH), superoxide dismutase (*EC* 1.15.1.1; SOD) and catalase (*EC* 1.11.1.6).

Several studies have shown that the activities of these antioxidant enzymes are elevated under conditions of oxidative stress in rats. However, the mechanism by which these

adaptive responses occur is unclear. Lawrence *et al.* (1978) and Mehlert & Diplock (1985) reported that the activity of GSHT was increased in vitamin E and Se deficiency. Vitamin E deficiency was shown to induce the activities of GR and G6PDH by Chow *et al.* (1973). However, other studies have shown no adaptive changes in these enzymes in the tissues of humans with low Se status (Thomson *et al.* 1988), of vitamin E-deficient rats (Masugi & Nakamura, 1976) and of vitamin E- and Se-deficient ducklings (Xu & Diplock, 1983).

The present study was undertaken to determine if calves could up-regulate the activity of any of these antioxidant enzymes as an adaptive response to subclinical NDM caused by experimentally-induced vitamin E and Se deficiency. In addition, tissue lipid peroxidation was assessed by measuring concentrations of malondialdehyde using the test for thiobarbituric acid-reactive substances (TBARS). Lipid hydroperoxides in the presence of Fe or ascorbic acid will react to produce malondialdehyde. Thus, ascorbate-induced TBARS (ATBARS) was also measured to provide an estimate of the peroxidizability of tissue lipids.

MATERIALS AND METHODS

Basal diet

Barley (1 tonne batches), purchased from a local supplier, was mixed with soya-bean meal (114 kg; Dalgety Ltd, Belfast, Northern Ireland). The mixture was treated with 9·25 mol NaOH/l as described by Orskov & Greenhalgh (1977), using a purpose-built applicator (Berwyn Engineering, Chippenham, Wilts.). This treatment reduces the vitamin E content of the mixture (McMurray *et al.* 1980). This mixture was aerated by daily mixing for at least 10 d before the addition of a mineral–vitamin premix purchased from Nutrition Services International (Randalstown, Northern Ireland) which did not contain either vitamin E or Se. The α-tocopherol and Se concentrations in the basal diet used in the present study were 4·9 and 0·44 nmol/g respectively. The composition of the basal diet is shown in Table 1.

Na_2SeO_3 (Koch-Light, Haberhill) and DL-α-tocopheryl acetate (500 mg/g; F. Hoffmann-La Roche & Co., Basel, Switzerland) were added to the basal diet in the amounts indicated in Table 1 to produce four complete diets as described below.

Animal studies

The study was designed as a 2 × 2 factorial experiment, the first factor being the feeding of α-tocopherol and the second factor the feeding of Se. Level 1 of each factor corresponded to diets supplemented with these micronutrients and level 2 to diets deficient in these micronutrients. For the testing of main effects the null hypothesis was that there was no difference in the levels of factors.

Sixteen 7-month-old Friesian crossbred calves were randomly allocated to four equal groups. Calves in group A were fed on the basal diet deficient in both Se and α-tocopherol, calves in group B were fed on the basal diet supplemented with Se, calves in group C were fed on the basal diet supplemented with α-tocopherol, and calves in group D were fed on the basal diet supplemented with both micronutrients.

Each group was loose-housed in separate solid-floored houses bedded with straw. All calves were group-fed with approximately 3·5 kg diet/calf per d, according to appetite. Water was available *ad lib.* and a small amount of straw was fed daily in order to minimize the risk of ruminal tympany and other digestive upsets. Blood was collected by jugular venepuncture at approximately monthly intervals. After 50 weeks the animals were killed by an intravenous injection of pentobarbitone sodium, necropsied and tissue samples collected and stored at −70° before analysis.

Table 1. *Composition of the basal diet**

Ingredient	Amount (g/kg)
NaOH-treated barley	875
Soya-bean meal	100
Vitamin–mineral premix	25
To provide:	
Na	2·0
Ca	2·0
P	0·5
Mg	1·25
Cu	0·1
Retinoyl palmitate (mg)	8·0
Cholecalciferol (mg)	2·0
DL-α-Tocopheryl acetate (500 g/kg; mg)	400
Na_2SeO_3 (μg)	438

* DL-α-Tocopherol was added to diets C and D only and Na_2SeO_3 to diets B and D only.

Analytical techniques

Plasma, tissue and dietary α-tocopherol concentrations were determined by HPLC with fluorescence detection (McMurray & Blanchflower, 1979). Tissue and dietary Se concentrations were determined by atomic absorption spectroscopy using a Varian spectra AA10 (Varian Techtron, Pty Ltd, Mulgrave, Australia) complete with an automatic vapour generation accessory (VGA-76), essentially as described by Gelman (1985).

Whole-blood GPX activities and plasma CK activities were determined at 37° on an Hitachi 705 Autoanalyser (Hitachi, Tokyo, Japan) using test kits (Ransel and CK-NAC respectively) supplied by Randox Laboratories, Crumlin, Northern Ireland.

A single tissue homogenate (300 g/l) was prepared and used for the assay of all of the enzymes studied. Tissue, kept on ice, was homogenized using a Silverson emulsifier at full speed for 1 min in sodium phosphate buffer (0·01 mol/l), pH 7·0. The mixture was further disrupted with 20 strokes of a Dounce homogenizer. The resulting homogenate was then centrifuged at 100000 g_{av} at 4° for 40 min. Portions of the clear supernatant fraction were frozen at −20° until required for assay.

Tissue GPX activity was assayed as described previously. G6PDH, GSHT, GR and catalase activities were measured by the methods of Kornberg *et al.* (1955), Habig *et al.* (1974), Carlberg & Mannervik (1975) and Johansson & Hakan Borg (1988) respectively. Protein concentrations were estimated by the method of Lowry *et al.* (1951) and enzyme activities were expressed as IU per g soluble protein.

SOD concentrations were determined on the Hitachi 705 analyser using a Ransod test kit (Randox Laboratories). To permit the determination of SOD concentrations in tissue homogenates the assay was modified by the substitution of 10 μl tissue homogenate, diluted 1:10 (v/v) with sodium phosphate buffer (0·01 mol/l), pH 7·0 for the erythrocyte lysate. Results were expressed as mg total SOD per g soluble protein.

Tissue concentrations of TBARS were determined as described by Ohkawa *et al.* (1979) and ATBARS by the method of Meydani *et al.* (1988) and results expressed as mmol malondialdehyde (MDA) equivalents per g soluble protein.

Statistical analysis

A balanced factorial analysis of variance was used to assess the effects of feeding diets low in vitamin E and Se. Data are presented as means with their pooled standard errors, in accordance with the factorial design of the present experiment. Values of plasma CK activity were subjected to logarithmic transformation before analysis.

RESULTS

Plasma α-tocopherol concentrations

At the start of the experiment the mean plasma α-tocopherol concentration in all groups was 2·2 (SE 0·8) μmol/l. Plasma α-tocopherol concentrations in calves fed on diets supplemented with α-tocopherol increased steadily, reaching a plateau of between 10 and 15 μmol/l (Fig. 1(*a*)). Plasma α-tocopherol concentrations in calves fed on diets low in vitamin E fell below 2 μmol/l from week 4 until the end of the experiment. These calves had lower plasma concentrations of α-tocopherol than calves fed on diets supplemented with α-tocopherol from week 8 to the end of the experiment ($P < 0·002$).

Erythrocyte glutathione peroxidase activity

At the start of the experiment the mean erythrocyte GPX activity in all groups was 178 (SE 13) IU/g haemoglobin. Erythrocyte GPX activity in calves fed on diets supplemented with Se increased steadily, reaching a plateau of between 250 and 320 IU/g haemoglobin (Fig. 1(*b*)). Erythrocyte GPX activity in calves fed on diets low in Se fell steadily. From week 20 until the end of the experiment, the mean erythrocyte GPX activities in these calves were significantly lower than those in Se-supplemented calves ($P < 0·05$), and fell to a level of 55 (SE 7) IU/g haemoglobin at week 50.

Plasma creatine kinase activity

Calves fed on diets supplemented with α-tocopherol had plasma CK activities within the normal range (0–250 IU/l) at all times during the study. Feeding diets depleted of Se had no effect on plasma CK activity. However, calves fed on diets depleted of vitamin E had plasma CK activities which were higher than the upper limit of normality and which were significantly higher than those of calves fed on diets supplemented with α-tocopherol, from week 44 until the end of the experiment at week 50 ($P < 0·001$, Fig. 2). The spike in plasma CK activity in group A at 12 weeks was caused by a high plasma CK activity in only one animal.

Clinical signs

All animals remained clinically normal during the course of the present study.

Tissue α-tocopherol and Se concentrations

Calves fed on diets depleted of vitamin E had lower muscle concentrations of α-tocopherol than those fed on diets supplemented with α-tocopherol (Table 2). Feeding diets low in Se had no effect on tissue α-tocopherol concentrations, nor was there any interaction between factors. Similarly, calves fed on diets depleted of Se had lower tissue concentrations of Se than those of calves fed on diets supplemented with Se (Table 2). Furthermore, feeding diets low in α-tocopherol had no effect on tissue Se concentrations, nor was there any interaction between factors.

Muscle indices of lipid peroxidation

Feeding calves on diets depleted of α-tocopherol resulted in increased concentrations of both TBARs and ATBARS in muscle (Table 2). Feeding calves on diets depleted of Se had no effect on either index of lipid peroxidation. With the exception of TBARS concentrations

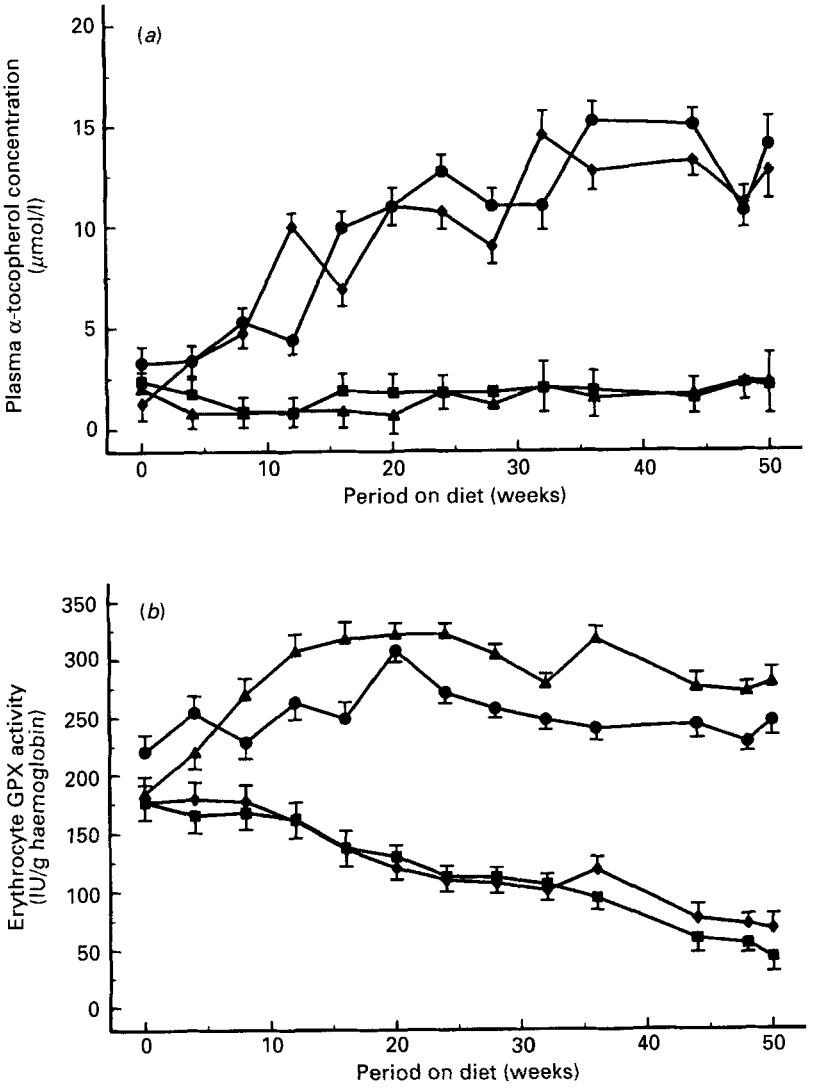

Fig. 1. (*a*) Changes in the plasma concentrations of α-tocopherol in calves fed on the four experimental diets. Plasma α-tocopherol concentrations were measured using HPLC with fluorescence detection. (*b*) Changes in the activity of glutathione peroxidase (*EC* 1.11.1.9; GPX) in the erythrocytes of the calves fed on the four experimental diets. GPX activity was measured using a Ransel test kit on an Hitachi 705 autoanalyser. Group A (vitamin E-deficient (−E), Se-deficient (−Se); ■); group B (−E, Se-supplemented (+Se); ▲); group C (vitamin E-supplemented (+E)−Se; ◆); Group D (+E+Se; ●). Values are means with their standard errors represented by vertical bars. For details of dietary composition and experimental procedures, see Table 1 and pp. 622–623.

in heart, there was no interaction between factors. Superimposition of Se depletion on α-tocopherol depletion increased the TBARS concentration in heart from 0·33 to 0·44 μmol/g soluble protein ($P < 0·01$).

Muscle antioxidant enzyme activities

Feeding diets low in Se decreased GPX activities in all muscles studied whereas feeding diets low in α-tocopherol had no effect on tissue GPX activities (Table 3). Feeding diets low in Se increased G6PDH activity in heart, biceps and supraspinatus, but not in masseter.

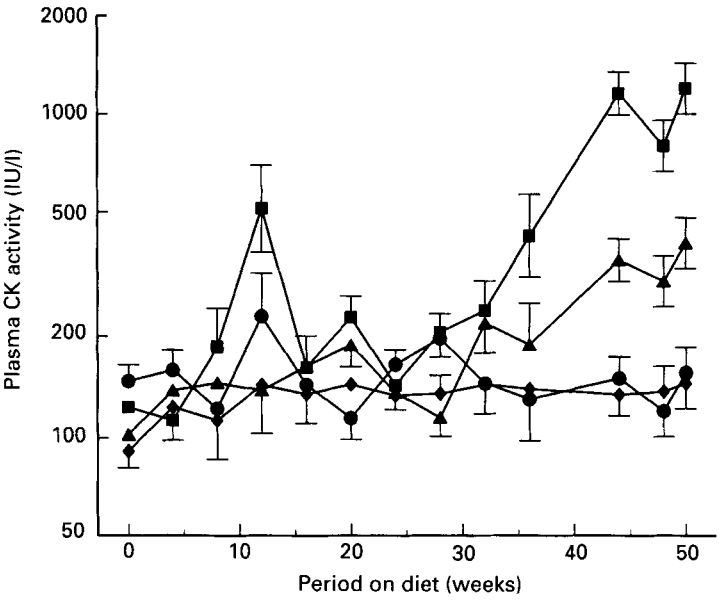

Fig. 2. Changes in the activity of creatine kinase (*EC* 2.7.3.2; CK) in the plasma of calves fed on the four experimental diets. Plasma CK activity was measured using a CK-NAC test kit on an Hitachi 705 autoanalyser. Values are geometric means with their standard errors, represented by vertical bars, and are presented on a semilogarithmic scale for clarity. Group A (vitamin E-deficient (−E), Se-deficient (−Se); ■); group B (−E, Se-supplemented (+Se); ▲); group C (vitamin E-supplemented (+E)−Se; ◆); Group D (+E+Se; ●).

Similarly, feeding diets low in α-tocopherol increased G6PDH activity in heart, biceps and supraspinatus, but not in masseter. There was no interaction between feeding diets low in Se and diets low in α-tocopherol. No other changes in antioxidant enzyme activity were observed.

DISCUSSION

Feeding diets depleted of α-tocopherol reduced plasma concentrations of α-tocopherol (Fig. 1(*a*)) to levels below the lower limit of normality used at this laboratory (2·3 μmol/l). There are few reports describing the tissue concentrations of α-tocopherol in calves with experimentally-induced subclinical nutritional myopathy. However, those presented in the present study (Table 2) were similar to those previously associated with the development of subclinical myopathy (Rice & McMurray, 1986; Kennedy *et al.* 1987).

Feeding diets depleted of Se reduced erythrocyte GPX activity (Fig. 1(*b*)) to levels similar to the lower limit of normality used at this laboratory (approximately 45 IU/g haemoglobin). However, since erythrocyte GPX activity depends on the half-life of the erythrocyte, it does not accurately reflect the current Se status of tissues during a period of Se depletion. An earlier report from this laboratory demonstrated that even higher activities of erythrocyte GPX (116 IU/g haemoglobin) were associated with subclinical myopathy in calves (Kennedy *et al.* 1987). There are few reports describing the tissue Se concentrations in calves with subclinical myopathy. However, the tissue Se concentrations (Table 2) were significantly lower in the animals fed on the Se-depleted diets and were similar to those previously associated with the development of subclinical myopathy (Rice & McMurray, 1986; Kennedy *et al.* 1987).

The presence of subclinical myopathy was confirmed in calves fed on diets depleted of α-tocopherol by the demonstration of significant elevations in plasma CK activity.

https://doi.org/10.1079/BJN19930153 Published online by Cambridge University Press

Table 2. *Tissue α-tocopherol, selenium, thiobarbituric acid-reactive substances (TBARS) and ascorbate-induced TBARS concentrations in tissues of calves fed on the four experimental diets for 50 weeks**

Tissue		Experimental group A (−E−Se)	B (−E+Se)	C (+E−Se)	D (+E+Se)	SEM	Effect of low α-tocopherol	Effect of low Se
Heart	α-Tocopherol†	3·03	1·81	31·99	29·90	1·946	$P < 0·0001$	
	Se†	1·04	2·69	1·25	2·82	0·219		$P < 0·0001$
	TBARS‡	0·44	0·33	0·21	0·27	0·026	$P < 0·0002$	
	ATBARS‡	1·05	0·93	0·17	0·16	0·037	$P < 0·0001$	
Biceps	α-Tocopherol	0·68	1·33	10·82	11·85	0·837	$P < 0·0001$	
	Se	0·44	1·14	0·52	1·37	0·070		$P < 0·0001$
	TBARS	0·13	0·14	0·08	0·06	0·011	$P < 0·0002$	
	ATBARS	0·59	0·56	0·21	0·24	0·043	$P < 0·0001$	
Masseter	α-Tocopherol	2·31	2·08	22·40	27·25	2·536	$P < 0·0001$	
	Se	0·87	1·76	0·82	1·85	0·065		$P < 0·0001$
	TBARS	0·31	0·23	0·15	0·16	0·034	$P < 0·005$	
	ATBARS	1·44	1·31	0·25	0·38	0·097	$P < 0·0001$	
Supraspinatus	α-Tocopherol	0·88	1·62	14·63	16·24	0·772	$P < 0·0001$	
	Se	0·81	1·39	0·70	1·33	0·059		$P < 0·0001$
	TBARS	0·17	0·16	0·11	0·06	0·012	$P < 0·0001$	
	ATBARS	0·61	0·72	0·21	0·16	0·048	$P < 0·0001$	
Liver	α-Tocopherol	2·53	3·05	23·21	23·72	0·696	$P < 0·0001$	
Kidney	Se	11·18	15·00	12·28	15·52	0·828		$P < 0·002$

−E, +E, vitamin E-deficient and vitamin E-supplemented respectively; −Se, +Se, Se-deficient and Se-supplemented respectively.
* For details of dietary composition and experimental procedures see Table 1 and pp. 622–623.
† nmol/g soluble protein.
‡ μmol malondialdehyde equivalents/g soluble protein.

Table 3. *Antioxidant enzyme activities in muscles of calves fed on the four experimental diets for 50 weeks**

Tissue	Enzyme†	Experimental Group A (−E−Se)	B (−E+Se)	C (+E−Se)	D (+E+Se)	SEM	Effect of low α-tocopherol	Effect of low Se
Heart	GPX	253	691	306	560	42·3		$P < 0·001$
	G6PDH	6·77	4·58	4·67	3·11	0·435	$P < 0·001$	$P < 0·001$
	GR	3·06	2·42	3·10	4·94	0·706		
	GSHT	52·3	45·1	52·2	47·2	5·52		
	Catalase	0·90	0·63	0·66	0·63	0·144		
	SOD	1·27	1·05	0·96	1·20	0·064		
Biceps	GPX	42	91	34	67	6·6		$P < 0·001$
	G6PDH	3·02	0·88	0·74	0·82	0·438	$P < 0·05$	$P < 0·05$
	GR	1·16	1·32	1·82	0·97	0·323		
	GSHT	23·3	31·8	23·3	25·2	2·41		
	Catalase	0·39	0·49	0·46	0·57	0·059		
	SOD	0·85	0·74	0·76	0·91	0·062		
Masseter	GPX	145	308	179	283	24·0		$P < 0·01$
	G6PDH	4·34	2·57	3·39	3·11	0·208		
	GR	3·71	3·77	5·18	3·12	1·179		
	GSHT	53·4	66·0	66·9	55·5	6·94		
	Catalase	0·72	0·74	0·54	0·57	0·082		
	SOD	0·87	0·72	0·83	0·85	0·061		
Supraspinatus	GPX	41	125	52	118	20·7		$P < 0·001$
	G6PDH	1·72	0·91	3·18	0·30	0·586	$P < 0·005$	$P < 0·001$
	GR	1·93	2·55	3·45	2·23	0·443		
	GSHT	46·4	51·5	49·3	44·3	3·87		
	Catalase	0·58	1·00	0·97	1·27	0·265		
	SOD	0·92	0·80	1·08	1·06	0·077		

GPX, glutathione peroxidase (*EC* 1.11.1.9); G6PDH, glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49); GR, glutathione reductase (*EC* 1.6.4.2); SOD, superoxide dismutase (*EC* 1.15.1.1); −E, +E, vitamin E-deficient and vitamin E-supplemented respectively: −Se, +Se, Se-deficient and Se-supplemented respectively.

* For details of dietary composition and experimental procedures see Table 1 and pp. 622–623.

† IU/g soluble protein for all enzymes, except SOD for which units are g total SOD/g soluble protein.

Feeding diets depleted of vitamin E and diets depleted of Se produced increases in the activity of G6PDH in heart, biceps and supraspinatus. The activity of this enzyme in masseter was unchanged. An earlier report from this laboratory showed that feeding diets depleted of Se to calves for 21 weeks had no effect on tissue G6PDH activity (Walsh *et al.* 1991), but showed broadly similar effects of feeding diets depleted of α-tocopherol. The difference between the two studies is probably accounted for by the difference in the length of the two experiments; 28 weeks in the earlier study and 50 weeks in the present study.

We have shown (Walsh *et al.* 1992) that calves fed on diets depleted of α-tocopherol have elevated tissue concentrations of 4-hydroxynonenal, one of the most toxic endproducts of lipid peroxidation (Esterbauer *et al.* 1991). Removal and detoxification of 4-hydroxynonenal occurs by conjugation with GSH, a reaction catalysed by GSHT (Alin *et al.* 1985). The conversion of glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate by G6PDH is the major metabolic source of NADPH, which is required for the maintenance of intracellular GSH concentrations. Thus, the increased activity of G6PDH in calves depleted of α-tocopherol, reported in the present study, may represent an attempt to maintain the intracellular GSH concentrations required for the effective removal of 4-hydroxynonenal.

The activities of the other antioxidant enzymes (GR, GSHT, SOD and catalase) were unaffected by feeding diets depleted of either vitamin E or Se. This finding is in agreement with similar studies in ducklings (Xu & Diplock, 1983) and rats (Masugi & Nakamura, 1976) but disagrees with the results of other studies in rats (Chow *et al.* 1973; Lawrence *et al.* 1978).

In conclusion, we have shown that cattle do not respond to a peroxidative challenge, caused by vitamin E and Se deficiency, by increasing the activity of a wide range of antioxidant enzymes. The activity of only one enzyme, G6PDH, was increased in heart, biceps and supraspinatus of animals depleted of vitamin E and/or Se. It is possible that G6PDH activity was up-regulated to meet a probably increased tissue requirement for GSH which accompanies a peroxidative challenge.

The DL-α-tocopheryl acetate was a kind gift from F. Hoffmann-La Roche & Co., Basel, Switzerland.

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Printed in Great Britain