

## Heinz body anaemia in lambs with deficiencies of copper or selenium

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1. The progression of Heinz body anaemia was studied in groups of lambs of low- and high-copper status, produced through breeding or Cu supplementation, when they were transferred from improved pasture to rape (*Brassica napus* L.) in autumn. Some lambs had previously received selenium by injection. The Cu and Se supplements markedly increased superoxide dismutase (EC 1.15.1.1; SOD) and glutathione peroxidase (EC 1.11.1.9; GSHPx) activities respectively in the erythrocytes, and both supplements had elicited growth responses at pasture.

2. At the time of transfer to rape, lambs not treated with Cu had lower whole-blood haemoglobin (Hb) concentrations and a higher percentage of erythrocytes containing Heinz bodies (6.6 v. 3.7%,  $P < 0.01$ ) than Cu-treated lambs: the corresponding effects of Se treatment were similar in direction but lower in magnitude ( $P < 0.05$ ).

3. After grazing rape for 2 weeks the mean Hb concentration had fallen by 30 g/l while Heinz body count had increased from 5 to 25%. However, counts were negatively correlated with the initial values and were unaffected by the Cu and Se treatments which maintained high plasma Cu concentrations and SOD and GSHPx activities.

4. The results provide the first evidence that Cu deficiency can induce Heinz body formation and the anaemia in grazing Cu-deficient lambs may be partly haemolytic in origin. The concomitant Se deficiency added marginally to the problem but neither the separate nor combined deficiencies increased the susceptibility of lambs to brassica anaemia.

Haemolytic anaemias with Heinz body formation can arise from a number of related causes involving oxidative stress on the erythrocyte (Gordon-Smith & White, 1974), including nutritional factors such as selenium deficiency, with or without copper deficiency, and brassica feeding. In severe Se deficiency in cattle, the appearance of Heinz bodies has been attributed to a lack of the Se-containing-enzyme, glutathione peroxidase (EC 1.11.1.9; GSHPx), in the erythrocyte leading to the accumulation of toxic peroxides (Morris *et al.* 1984). When cattle low in both Se and Cu are turned out to graze lush spring pasture, they can develop a haemolytic anaemia, characterized by Heinz body formation, which responds to Cu treatment (post-parturient haemoglobinuria (PPH); Smith, 1973; Black, 1981). Haemolytic anaemia has not been directly associated with Cu deficiency *per se* but Heinz bodies and haemolysis have been reported in newborn infants with low activities of the cupro-enzyme, superoxide dismutase (EC 1.15.1.1; SOD), which protects the erythrocyte from oxidative damage caused by accumulation of superoxide (Rotilio *et al.* 1977). Ruminants consuming brassica crops develop haemolytic anaemia, because dimethyl disulphide, a rumen metabolite of S-methyl cysteine sulphoxide in the crop, irreversibly oxidizes haemoglobin (Smith, 1978). Barry *et al.* (1981) have suggested that the susceptibility of ruminants to brassica anaemia is exacerbated by a concomitant Cu deficiency, since Cu supplementation apparently enhanced the recovery from anaemia in cattle given kale (*Brassica oleracea*).

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Table 1. *Numbers in the experiment according to genotype, set and supplements*

Genotype	Set	Supplement			
		0	Copper	Selenium	Cu + Se
L	1	20	25	—	—
	2	25	28	—	—
H	1	28	32	—	—
	2	28	25	—	—
B	1	9	15	14	13
	2	3	2	3	4
W	1	8	10	9	9
	2	9	9	10	8

L, low-plasma Cu; H, high-plasma Cu; B, Scottish Blackface; W, Welsh Mountain.

In lambs on improved hill pastures, combined deficiencies of Cu and Se had been encountered, which were sufficiently severe to retard growth (Suttle *et al.* 1984). In that study, the management policy involved the transfer of lambs to a rape (*Brassica napus* L.) diet for finishing purposes and the opportunity was taken to investigate whether single or dual deficiencies of Cu and Se influenced the development of haemolytic anaemia.

#### MATERIALS AND METHODS

The genetic backgrounds of the animals, their environment, management and Cu and Se treatments before transfer to rape have been described in detail elsewhere (Wiener *et al.* 1985; Woolliams *et al.* 1986*a*). Briefly, the animals were from two lines genetically selected over several generations for low (L) or high (H) concentrations of Cu in plasma, within an interbred Scottish Blackface (B) × Welsh Mountain (W) population, and from contemporary unselected B and W pure breeds. The grazing of the ewes and lambs was restricted up to 24 weeks of age to grass pastures that had been limed and reseeded within the previous 3 years. Lambs were divided into two sets (early and late lambing). In 1983 ('year 2'), half the lambs of both lines and both pure breeds were given Cu supplements (1 g copper oxide needles) at 6 weeks of age and half the B and W lambs were treated with Se at 12 weeks of age (weaning), giving, within B and W, a 2 × 2 design. The pasture grazed by set 2 in the 6 weeks before transfer to rape had been treated with a foliar spray of sodium molybdate solution as described elsewhere (Woolliams *et al.* 1986*b*).

At 24 weeks of age, lambs were moved from pasture to rape undersown with turnips (*Brassica rapa* L.) and ryegrass (*Lolium perenne*). The rape leaves contained 80 μmol Cu, 3.5 μmol molybdenum and 234 mmol sulphur/kg dry matter (DM), and the turnip leaves 162 μmol Cu, 3.2 μmol Mo and 234 mmol S/kg DM. Lambs were weighed and blood samples taken immediately before transfer to rape and again 2 weeks later. The samples were analysed for the concentration of Cu in plasma and haemoglobin (Hb), Heinz-Ehrlich bodies and activities of SOD and GSHPx in whole blood. Heinz bodies were counted in blood smears prepared on average 5 h after sampling, using crystal violet staining, and expressed as per cent erythrocytes containing one or more inclusion bodies. GSHPx was assayed at 37° by the method of Thompson *et al.* (1976). Details of the remaining analyses are described elsewhere (Woolliams *et al.* 1986*b*).

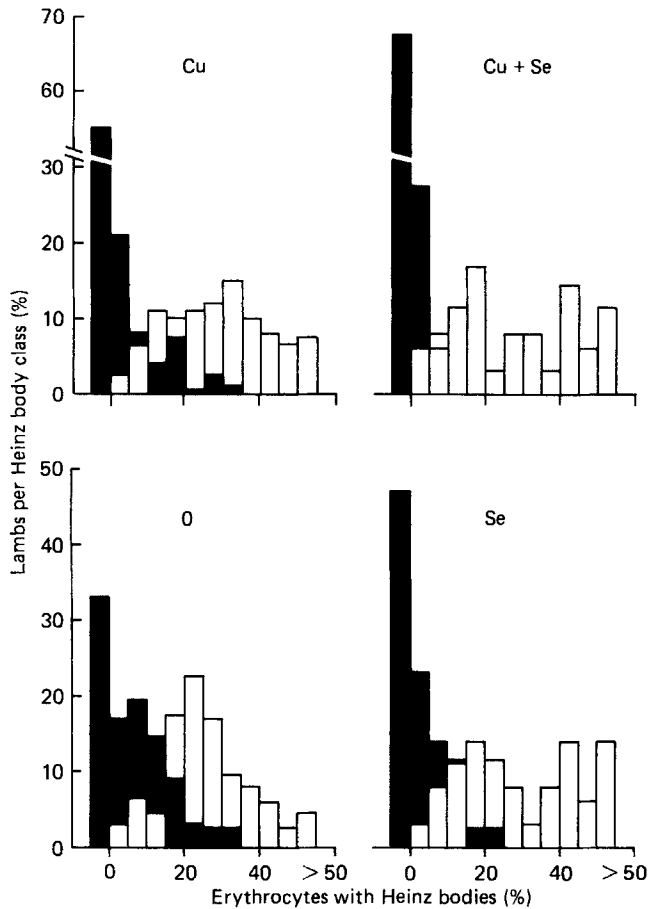


Fig. 1. Histograms showing the distribution of Heinz body counts in lambs according to copper and selenium treatment before (■) and after (□) they grazed rape (*Brassica napus* L.). Note the separate class for zero counts at the extreme left of the x axis. Number of animals per treatment group were: no treatment (0) 130, Cu 146, Se 36, Cu + Se 34. Groups 0 and Cu contained lambs selected for low- or high-plasma Cu; groups Se and Cu + Se contained only pure-bred Scottish Blackface and Welsh Mountain lambs.

#### Statistical methods

The analysis included 346 lambs with complete sets of live weight and blood variables (Table 1). The results from the lines and the pure breeds were analysed separately to account for differences in Se treatment. The linear model used was of a hierarchical nature and factors for the following were fitted: selection line (with an error term given by sires within selection line); birth type, set and the selection line by set interaction (with an error term given by dams within sires); sex, Cu supplementation and the two- and three-way interactions of Cu supplement with set and selection line (with the residual mean square as the error term). For the analysis of the pure breeds, selection line and its two- and three-way interactions were replaced by breed, and additional factors for Se treatment and its interactions with breed, set and Cu supplementation were included.

Activities of SOD and GSHPx were expressed per g Hb and were transformed to a logarithmic scale. Heinz body counts were analysed using the computer package GLIM (Royal Statistical Society, 1978) with a logit link function and a linear model of fixed effects

Table 2. *Effects of copper treatment and selection for low (L) or high (H) plasma Cu on mean values for live weight and blood constituents in two sets of lambs before they grazed rape (Brassica napus L.)*

	Line†	Set†	Supplement		Statistical significance of effects				
			0	Cu	Cu	Line	Set	Cu × Line	Cu × Set
Heinz body count (%)	L	1	9.3	7.8	**	**	*	NS	NS
		2	8.2	5.9					
	H	1	7.5	4.2					
		2	5.6	1.9					
Haemoglobin (Hb) (g/l)	L	1	106	130	***	***	NS	***	NS
		2	107	128					
	H	1	136	144					
		2	137	136					
Superoxide dismutase‡ (EC 1.15.1.1) (U/g Hb)	L	1	437	628	***	**	NS	NS	NS
		2	417	617					
	H	1	575	1081					
		2	661	1479					
Plasma Cu (μmol/l)	L	1	1.9	4.5	***	***	**	**	*
		2	1.8	7.4					
	H	1	4.8	11.4					
		2	3.6	15.1					
Live wt (kg)	L	1	21.5	24.5	***	***	***	NS	NS
		2	24.0	26.9					
	H	1	25.4	25.8					
		2	28.1	28.0					

NS, not significant.

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

† For details, see p. 540 and Table 1.

‡ Geometric means derived from analyses on a logarithmic scale.

similar to that described previously incorporating a correction for extra-binomial variation (Model II of Williams (1982)).

## RESULTS

### *Before feeding on rape*

Heinz bodies were present in the erythrocytes of some lambs before they grazed rape: counts as high as 30–35% were recorded and correlated with trace element status. The histograms in Fig. 1 indicate that blood smears from the majority of unsupplemented lambs had Heinz bodies present, with counts > 5% common. By contrast, the majority of lambs treated with Cu+Se had no Heinz bodies and counts greater than 3% were rare in the remainder: each element appeared to contribute to this improvement. The results for selection lines and pure breeds are presented separately in Tables 2 and 3 respectively, because only the pure breeds were used to test the effect of Se and its interaction with Cu. The Cu supplement reduced Heinz body counts in both L and H lines (Table 2) and B and W breeds (Table 3) ( $P < 0.01$ ): in the lines the reduction was greater in H than L.

Table 3. Effects of copper and selenium supplements on mean values for live weight and blood constituents in two sets of Scottish Blackface (B) or Welsh Mountain (W) lambs before they grazed rape (*Brassica napus* L.)

	Breed†	Set†	Supplement				Statistical significance of effects			
			0	Cu	Se	Cu+Se	Cu	Se	Breed	Set
Heinz body count (%)	B	1	6.8	2.9	5.9	0.6	**	NS	NS	
		2	0	0	0					
	W	1	3.8	3.9	3.8	2.0		*	NS	
		2	9.8	1.7	2.4					
Haemoglobin (Hb) (g/l)	B	1	11.5	14.5	11.7	141	***	*	NS	
		2	11.2	14.1	12.3					
	W	1	13.9	14.2	14.5	142			**	
		2	12.9	13.1	14.4					
SOD ‡ (U/g Hb)	B	1	630	1030	574	1030	***	NS	*	**
		2	650	2203	650					
	W	1	1016	1462	1054	1663				
		2	923	2748	1050					
GSHPx‡ (U/g Hb)	B	1	13.8	15.2	231.7	204.6	NS	***	NS	NS
		2	16.7	13.3	295.8					
	W	1	12.3	18.3	219.3	262.4				
		2	15.2	16.4	287.7					
Plasma Cu (µmol/l)	B	1	3.1	8.0	4.1	10.2	***	NS	*	NS
		2	2.7	13.9	3.7					
	W	1	7.0	12.9	6.8	14.0				
		2	3.8	16.1	3.6					
Live wt (kg)	B	1	27.0	30.9	27.0	31.8	***	***	***	NS
		2	30.1	32.9	30.2					
	W	1	24.1	24.5	25.3	27.5			***	NS
		2	24.3	24.5	25.6					

SOD, superoxide dismutase (EC 1.15.1.1); GSHPx, glutathione peroxidase (EC 1.11.1.9); NS, not significant.

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

‡ Geometric means derived from analyses on a logarithmic scale.

† For details, see n. 540 and Table 1.

Table 4. *Linear relations between logit (Heinz body count, %)\* and other blood constituents ignoring genotype, supplement and set grouping in lambs with low (L) and high (H) plasma copper concentrations before they grazed rape (Brassica napus L.)*

Blood constituent	Regression coefficient		Constant	
	Mean	SE	Mean	SE
Plasma Cu ( $\mu\text{mol/l}$ )	-0.108	0.023	-2.12	0.14
Haemoglobin (Hb) (g/l)	-0.0218	0.0041	0.01	0.51
log <sub>e</sub> superoxide dismutase (EC 1.15.1.1) (U/g Hb)	-0.731	0.134	1.96	0.24

\*  $\text{Log}_e p - \text{log}_e (1-p)$  where  $p$  is Heinz body count (%) / 100.

Additionally, in B and W, Se supplementation reduced Heinz body counts ( $P < 0.05$ ) but by a lesser degree than Cu ( $P < 0.01$ ) and with no interaction between the effects of the two supplements (Table 3). Whilst breed differences between B and W were not consistent, H lambs had fewer Heinz bodies than L lambs ( $P < 0.01$ ) and the magnitude of the difference was similar to that due to the Cu supplement. Set 1 lambs were generally more prone than set 2 lambs to Heinz body formation, untreated W lambs being the exception.

Untreated L and B lambs were exceedingly Cu-deficient and the significant effects of line and of the Cu supplement on plasma Cu and SOD values were the reverse of those for Heinz body counts (Tables 2 and 3); H compared with L, and Cu supplemented compared with unsupplemented lambs, had the higher concentrations ( $P < 0.01$ ). The differences in plasma Cu and SOD status between B and W were less marked but still significant ( $P < 0.05$ ).

Hb concentrations were lowest in the groups with high Heinz body counts and lowest Cu status, i.e. significantly lower in L compared with H ( $P < 0.001$ ), in B compared with W ( $P < 0.01$ ) and in unsupplemented compared with Cu-supplemented ( $P < 0.001$ ) lambs.

Within treatment groups, Heinz bodies were associated with low plasma Cu ( $P < 0.05$ ), low Hb concentration ( $P < 0.05$ ) and particularly with low log SOD activity ( $P < 0.001$ ). Ignoring treatment and genotypes, the simple regressions (2 df) for the selection lines (where Se was not a complicating factor) of Heinz bodies on plasma Cu, Hb and log SOD activity, shown in Table 4, accounted for almost as much of the residual variation as the full treatment model (10 df).

Pure-bred lambs not given Se had subnormal GSHPx concentrations and both Hb concentration and the activity of GSHPx were increased by the Se supplement ( $P < 0.05$  and  $P < 0.001$  respectively) (Table 3). There was, however, no relation between Heinz body count and GSHPx activity. The Se supplement did not affect plasma Cu or erythrocyte SOD activity. In selection line lambs, GSHPx concentrations were uniformly low (mean 15.6 U/g Hb) and unaffected by Cu treatment.

#### *After feeding on rape*

The mean values for live weight and blood constituents 2 weeks after grazing rape, pooled across genotype and set, are given in Table 5 and the effects of Cu and Se treatments on Heinz bodies illustrated in Fig 1. Heinz body counts increased on average from 5% before grazing rape to 25% after 2 weeks of grazing rape, while Hb values fell by approximately 30 g/l and there was no increase in live weight in any group. Unlike the situation before rape was grazed, there were no simple effects of genotype, trace element supplement or set

Table 5. Effects of copper and selenium treatments on live weight and blood constituents in lambs of various genotypes after grazing rape (*Brassica napus* L.) for 2 weeks

(Values in parentheses are the combined means for the selection lines, L+H; the remainder are means for B+W pooled between sets: significance of Cu effects for lines similar to those for B+W)

	Supplement				Statistical significance of effects for B+W†	
	0	Cu	Se	Cu+Se	Cu	Se
Heinz body count (%)	24.8 (23.7)	26.2 (25.8)	27.8	24.5	—	—
Haemoglobin (Hb) (g/l)	97 (92)	109 (104)	105	117	**	*
SOD (U/g Hb)	1069 (1057)	2021 (1492)	1293	2308	***	NS
GSHPx (U/g Hb)	13.4 (13.2)	12.4 (12.6)	305.4	276.3	NS	***
Plasma Cu (μmol/l)	3.6 (2.8)	12.3 (8.3)	5.4	13.5	***	**
Live wt (kg)	26.1 (24.0)	27.8 (25.7)	26.5	29.9	***	**

B, Scottish Blackface, W, Welsh Mountain; L, low-plasma Cu; H, high-plasma Cu; SOD, superoxide dismutase (EC 1.15.1.1); GSHPx, glutathione peroxidase (EC 1.11.1.9); NS, not significant.

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

† The effects differed significantly between line and between breeds.

on Heinz body count but there were significant three-way interactions. In B lambs, Heinz body counts were greatest when either no or both supplements were given, and, in W lambs, counts were greatest when only one supplement was given. In set 2, supplemented H lambs were most protected but in set 1 supplemented L lambs were most protected. Within treatment groups, there was no consistent association between Heinz body count and any of the biochemical measures.

A highly significant negative relation emerged between the final count ( $y$ ) and the initial Heinz body count ( $x$ ) when the adjusted treatments means for set 1 were used in which  $y = 39.6 - 1.63x$  ( $r = 0.78$ ; 10 df), but the relation was not significant in set 2.

Activities of SOD had increased in all groups after 2 weeks on rape, but GSHPx increased only in Se-supplemented lambs (compare Table 4 with Tables 2 and 3). Plasma Cu generally showed little change but there was a significant increase in Cu in Se-treated lambs after feeding on rape ( $P < 0.01$ ), particularly in those not given a Cu supplement. With this exception, the effects of line, breed and supplements on variables, other than Heinz body counts, measured after 2 weeks on rape, were similar to those found before grazing rape.

#### DISCUSSION

Heinz-body counts were unexpectedly as high (up to 35%) in the erythrocytes of some lambs before, as in most lambs after they grazed rape. These signs of oxidative damage to the Hb molecule were more prevalent in Cu-deficient and to a lesser extent in Se-deficient lambs than in lambs supplemented with Cu or Se, but even dual deficiency did not exacerbate the Heinz body anaemia caused by grazing rape. A model of the principal oxidant stresses and antioxidant defences within the erythrocyte (Fig. 2), based on recognized pathways (Babior, 1981; Jaffe, 1981), assists the explanation of the complex interactions which may be involved. The model indicates two vulnerable sites, the Hb molecule and the erythrocyte membrane; two sources of stress (exercise, and haemolysins from the rape diet); and three linked protective mechanisms involving SOD, GSHPx and reduced glutathione (GSH).

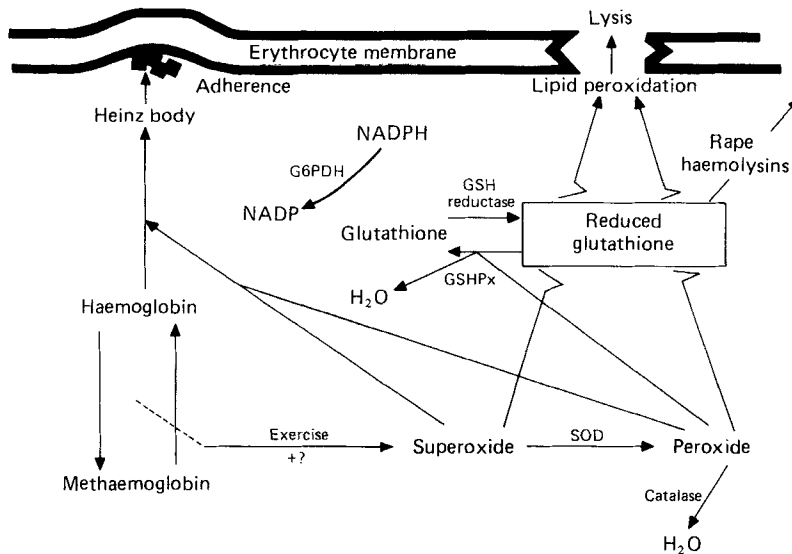


Fig. 2. Schematic representation of oxidant and antioxidant sources and pathways in the erythrocyte and their influence on membrane stability in sheep before and after grazing brassicas. Naturally low activities of glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49; G6PDH) may make the species particularly vulnerable to Heinz body formation in the presence of the low superoxide dismutase (*EC* 1.15.1.1; SOD) and glutathione peroxidase (*EC* 1.11.1.9; GSHPx) activities found in copper- and selenium-deficient lambs. When grazing brassicas, haemolysins may overwhelm the influence of these enzymes giving rise to excessive Heinz body formation unaffected by Cu and Se status.

#### *Cu deficiency*

We propose that Cu deficiency led to the degradation of Hb by superoxide or its by-products since Heinz body counts were highest in the most Cu-deficient lambs (unsupplemented L, Table 2) and negatively related to SOD activity in the erythrocytes before grazing rape (Table 4). Such associations have not been reported before in studies of Cu deficiency: they raise the possibility that in our lambs the anaemia of Cu deficiency was partly due to the accelerated removal of erythrocytes from the circulation and that the reported increased accumulation of iron in liver (Woolliams *et al.* 1986*b*) did not simply reflect an impairment of Fe metabolism.

The unique appearance of Heinz bodies in these Cu-deficient lambs may be related to the experimental conditions. The task of gathering, identifying, sorting, weighing and bleeding each set of 200 lambs involved moving them up to 3 km from the improved hill pasture to holding pens where they were kept and intermittently disturbed for 24 h before sampling. The potential for oxidative damage to Hb, mediated through superoxide anions, would probably be enhanced by exercise through the increased flux of oxygen and production of methaemoglobin within the erythrocyte and oxidants from 'external' sources (Jackson, 1987) (Fig. 2). It is also noteworthy that Heinz bodies occur in infants with low SOD activities at birth (Rotilio *et al.* 1977), a time of abrupt change in respiratory function. Alternatively, the induction of Heinz bodies may be species-dependent. The model (Fig. 2) shows the role of glucose 6-phosphate dehydrogenase (*EC* 1.1.1.49; G6PDH) in regenerating GSH and sheep erythrocytes possess relatively little of this enzyme (Budtz-Olsen *et al.* 1963).

#### *Se deficiency*

The increased incidence of Heinz bodies in Se-deficient lambs is consistent with their appearance in Se-deficient cattle (Morris *et al.* 1984) and is probably attributable to a



deficiency of the only known mammalian Se-containing enzyme, GSHPx. The lack of correlation between GSHPx and Heinz body count within groups in the present study may simply have reflected the uniformly high and low Se status achieved by the treatments. The lesser effect of Se than Cu deficiency on Heinz body formation may reflect differences in the severity of impact of the respective deficiencies: it is possible for example that the presence of an alternative, catalase (*EC* 1.11.1.6)-dependent pathway for the metabolism of peroxide (Fig. 2) compensates in part for the effects of reduced GSHPx activity.

#### *Concurrent Cu and Se deficiencies*

The additive nature of the effects of Cu and Se deficiencies on Heinz body formation is consistent with the model which shows GSHPx completing the detoxification of superoxide. The model is in turn consistent with the aetiology of post-parturient haemoglobinuria in dairy cows in which combined deficiencies of Cu and Se and the stress associated with the onset of grazing in spring are all implicated (Black, 1981).

#### *Rape feeding*

The reduction in Hb after only 14 d grazing rape was accompanied by an increase in SOD, suggesting that the rape haemolysins induced the removal (by lysis or sequestration in the spleen) of the erythrocytes with lower than average enzyme activities. In the surviving and recently released erythrocytes, there was no evidence that Cu deficiency made the cell more susceptible to oxidative stress. The lack of effect of either Cu or Se deficiency on susceptibility to brassica anaemia is contrary to the suggestions of Barry *et al.* (1981) and may have been due to the destruction of GSH, the main means of averting oxidant stress, by the rape haemolysins, thus creating an obstacle to the linked Cu- and Se-dependent antioxidant mechanisms. On the latter point, it should be noted that enhanced GSHPx activity requires an increase in G6PDH activity in order to avoid substrate (GSH) depletion.

Our observation of a direct effect of Cu deficiency on Heinz body formation affords an alternative explanation for the apparent interaction between Cu status and brassica feeding reported by Barry *et al.* (1981). Their animals had normal blood Cu concentrations and were not anaemic when they began grazing kale: the fact that their Cu-treated and untreated cattle showed similar increases in Heinz body counts after 2 weeks is therefore not surprising. However, the slower return to zero Heinz body counts in their untreated cattle may have reflected the formation of new Heinz bodies as a direct consequence of a developing Cu deficiency, sufficiently severe to retard growth concomitantly, rather than failure to adapt to the challenge from kale haemolysins as they suggested.

#### *Genetic effects*

In previous papers (Woolliams *et al.* 1986*a, b*), differences between the pure breeds, B and W, in characteristics associated with Cu deficiency were attributed in part to genetic differences in Cu metabolism since selection for high (H) or low (L) plasma Cu alone within an inbred B and W cross produced a divergence in each characteristic. L and B showed the greater susceptibility to swayback, infection, anaemia and growth retardation and were more responsive to Cu supplementation than H and W. However, in the present study Heinz bodies were usually less prevalent in B than in W, though more prevalent in L than in H, while H showed a greater improvement than L in Heinz body counts when supplemented. The first inconsistency may indicate differences between B and W in erythrocyte susceptibility to oxidative damage or to the extent of oxidative stress, i.e. factors which were unrelated to Cu status but which concomitantly influenced Heinz body formation. For example the breeds differ in Hb types (Wiener *et al.* 1974) and this

may affect susceptibility to oxidant stress (Thompson, 1977). The second inconsistency may reflect the waning effect in the L line of the Cu treatment given 18 weeks earlier, since the Cu-treated L lambs had become hypocupraemic again before they grazed rape.

In summary, Cu deficiency and to a lesser extent Se deficiency, predisposed the erythrocytes of lambs to oxidative damage as judged by Heinz body formation. The deficiencies did not increase susceptibility of the erythrocyte to the haemolytic factors derived from an ingested brassica crop. The stress of muscular exercise may have been an important factor in the haematological expression of the trace element dysfunction.

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