Effect of Copper on Red Cell Glutathione and Enzyme Levels in High and Low Glutathione Sheep[1] (37055)

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Among animals sheep are considered to be the most susceptible to copper toxicity (1). The common form is chronic copper poisoning which occurs under grazing conditions in parts of Australia; in sheep foodstuffs contaminated with copper, and in sheep housed for a long time and fed rations containing excessive copper supplement. Chronic copper poisoning in sheep consists of two phases: (i) copper accumulates in the liver when the animal shows no symptoms of copper toxicity; and (ii) hemolytic crisis characterized by sudden release of copper from the liver into the blood stream, resulting into hemolysis, hemoglobinemia, hemoglobinuria, and jaundice. Normally, the animals die although there are some breed differences; for example, British breeds rarely survive the hemolytic crisis, but Merino may withstand two or three crises before succumbing (1, 2). The few studies made on the biochemical changes in blood during the hemolytic crisis indicate that the most remarkable feature is the dramatic fall in blood glutathione (GSH) to levels of less than 10% of precrisis levels (3, 4).

Smith and Osburn (5) reported that three sheep in a flock of 104 had erythrocyte GSH that was less than 20% of the mean for the remaining animals. It has since been shown that GSH level in sheep red cells is controlled by a single pair of autosomal alleles, giving rise to two GSH types: (1) GSH^H, in sheep with a mean GSH value of 100 mg/100 ml red cells; and (2) GSH^h, in those having a mean of 30 mg/100 ml red cells (6, 7).

In view of these facts, it seemed desirable to determine effect of copper on GSH levels and enzyme activities of the erythrocytes of GSH^H and GSH^h sheep. This communication records such a study.

Materials and Methods. Blood was collected in heparin (for GSH studies) and ACD (for enzymic studies) from sheep of GSH^H and GSH^h types, each consisting of four animals.

a. Effect of copper on glutathione oxidation. Blood with different concentrations of copper acetate was incubated 2 hr at 37°; GSH then was estimated by the DTNB method (8).

b. Effect of copper on glutathione regeneration. GSH regeneration was measured using a modification (9) of the original techniques of Kosower, Vanderhoff and London (10). Erythrocyte GSH was oxidized with diazenedicarboxylic acid bis(*N*,*N*-dimethylamide), II (diamide) (11). Copper acetate in the final concentration of 0.5 and 1 m*M* was added to two tubes (experiment) and saline was added to a third tube (control). After standing 15 min at room temperature, dextrose in the final concentration of 0.02 ml/ml red cells was added to each tube and the tubes were incubated at 37°, so that cellular metabolism could reduce the oxidized glutathione. The GSH was measured every 5 min, and the regeneration rates were calculated by a least squares fit.

c. Effect of copper on red cell enzyme activities. These red cell enzymes were assayed for copper sensitivity: Hexokinase (HK), glucose phosphate isomerase (GPI), fructose 6-phosphate kinase (F6PK), aldolase, glycer-

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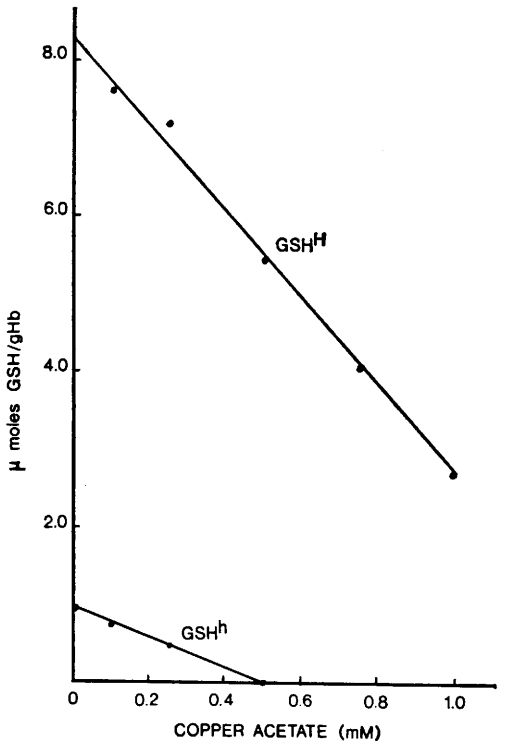

FIG. 1. Effect of copper on glutathione oxidation in GSH^H and GSH^h sheep.

aldehyde-phosphate dehydrogenase (GAPD), phosphoglyceric kinase (PGK), enolase, pyruvate kinase (PK), lactic dehydrogenase (LDH), glucose 6-phosphate dehydrogenase (G6PD), and glutathione reductase (GR). All assays were carried out at pH 7.2 by methods previously described (12). Possible effects of copper on the blank and of acetate on normal enzyme activity were determined by adding copper acetate to blank and sodium acetate to normal samples in the same concentrations in which copper acetate was used.

To see the effect of copper on the enzyme activities of hemolysates copper acetate in the final concentration of 0.1 m*M* was added to one of the cuvettes and the activity was determined in parallel with the blank and a normal sample. To see the effect of copper on the enzyme activities of intact cell, whole blood was incubated with copper acetate in the final concentration of 1 m*M* for 2 hr at 37°. After the end of the incubation period, cells were washed three times with cold 0.145 *M* KCl solution. A sample of blood without copper acetate, treated in the same way, was used to measure normal enzyme activity.

Results and Discussion. The effect of copper on GSH oxidation was similar in all animals of a particular GSH type. Therefore, only the results obtained on one animal from each group are presented in Fig. 1, which clearly shows that different levels of copper acetate were required to oxidize GSH in sheep of different GSH types. Copper acetate in the final concentrations of 0.5 m*M* was sufficient to oxidize all GSH in GSH^h animals, but even twice that concentration was unable to oxidize all GSH in GSH^H animals. Because initial levels of GSH differ greatly in sheep of GSH^H and GSH^h types, our results were not unexpected and are in accord with published findings (3, 4). Differences in the slopes of the curves in Fig. 1, when calculated by least squares analysis of variance, were found to be significantly different ($p < 0.05$), indicating that the two types of sheep responded differently to dissimilar doses of copper and that when the consumption of copper was relatively small, as it usually is in chronic copper poisoning, GSH^h animals might be as resistant as GSH^H.

The GSH regeneration rate proceeded linearly in blood obtained from sheep of both GSH types, and more than 90% of the original GSH was regenerated in less than 30 min. Mean GSH regeneration was 0.1890 for GSH^H sheep and 0.0862 for GSH^h sheep, the difference being nonsignificant. Adding copper acetate in the final concentration of 0.5 m*M* reduced GSH regeneration rate to 34.8% in GSH^H sheep and to 46.9% in GSH^h sheep. Increasing copper concentrations to 1 m*M* resulted in further reduction of regeneration (46.3% in GSH^H and 57.5% in GSH^h) (Table I). Copper induced inhibition of GSH rates was found to be significantly different in sheep of different GSH types. Accordingly, only results obtained with one GSH^H sheep have been presented to represent normal GSH regeneration and the effect of copper on regeneration in GSH^H and GSH^h sheep (Fig. 2).

Enzyme activities measured with copper added to hemolysates (Table II) also showed

TABLE I. Copper Induced Inhibition of GSH Regeneration in GSH^H and GSH^h Sheep.[a]

Concn of copper (mM)	GSH^H		GSH^h		Significance levels between GSH^H and GSH^h groups (t test)
	Mean	SE	Mean	SE	
0.5	34.8	4.26	46.9	3.52	NS
1.0	46.3	5.24	57.5	7.31	NS

[a] Results are expressed as percentage inhibition of control values.

no differences between the two types of animals. In both GSH^H and GSH^h sheep, some enzymes (HK, GAPD) were totally inhibited by copper while others (G6PD, LDH, GPI, Enolase, not given in the table) had similar activity with or without copper. In general F6PK, aldolase, PGK, and PK enzymes were inhibited 50 to 80%. GR was inhibited only 20% (Table II). When enzyme assays were performed on hemolysates prepared from blood incubated with copper, only HK activity was completely inhibited. Activity of aldolase, GAPD and PKG was inhibited less than 20%. PK and GR, showed activity reduced 75 to 20%, respectively, in hemolysates (Table II), did not change when copper was added to intact cells.

The effect of copper on the activities of red cell enzymes apparently has not been studied in any of the animal species. Deiss, Lee and Cartwright (13) reported that in human red cells GR was sensitive to lower concentrations of copper than was G6PD and Boulard, Blume and Beutler (14) recently showed that adding as low as 15 μM copper (as copper sulfate) to hemolysates or incubating blood having that much copper inhibits F6PD, PGK, PG, PK, and G6PD. Surprisingly they observed no inhibition of GR activity. We were unable to show any degree of inhibition of any enzyme by adding 15 μM concentration of copper (either as sulfate or acetate) to hemolysates or by adding 100 μM to intact cells.

During chronic copper poisoning in sheep, elevated levels of copper are found only in liver, there being no change in the copper content of any other tissue or fluid (1). Before the onset of hemolytic episode, the copper content of the blood rises markedly resulting in lowered erythrocyte GSH and eventually in hemolysis, because GSH is believed to be essential for cell integrity and survival (15). Although GSH^h animals have much lower levels of GSH in their red cells than do GSH^H animals, apparently GSH^h animals are not necessarily any more sensitive to small doses of copper than are GSH^H animals suffering from chronic copper toxicity (Fig. 1). Also, results presented in Tables I and II show that copper induced inhibition of glutathione regeneration and enzyme activities are similar in sheep of both GSH types. Although chronic copper toxicity is not uncommon in parts of Australia (2), these Merinos are relatively more resistant to that form of copper poisoning than are other breeds of sheep (1, 2), and they have the highest frequency

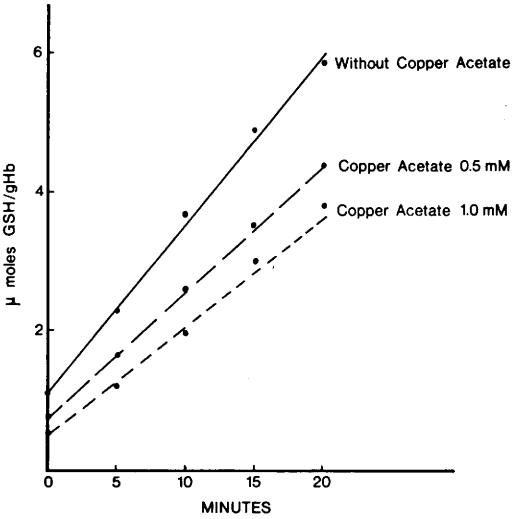

FIG. 2. Effect of copper (0.5 and 1.0 mM) on the glutathione regeneration rate. The results for the four GSH^H sheep and four GSH^h sheep were similar; those for sheep No. 1021 (GSH^H) are presented. The regeneration rate is expressed as μmoles GSH/min/g hemoglobin.

TABLE II. Copper Induced Inhibition of Some Erythrocyte Enzymes in GSH^H and GSH^h Sheep.[a]

Enzyme	GSH^H			GSH^h		
	Activity without Cu^{2+}	Activity with Cu^{2+} (0·1 m*M*)	% Inhibition	Activity without Cu^{2+}	Activity with Cu^{2+} (0·1 m*M*)	% Inhibition
HK	0.64	0.00	100	0.40	0.00	100
F6PK	1.42	0.51	65	1.39	0.52	63
Aldolase	1.15	0.46	59	1.54	0.51	65
GAPD	56.11	0.00	100	56.3	0.00	100
PGK	49.2	20.6	57	41.4	20.4	50
PK	2.63	0.80	69	2.58	0.57	77
GR	2.61	2.18	17	2.53	1.90	25

[a] Mean of four GSH^H and four GSH^h animals. Values are expressed as μmoles/min/g Hb.

for the gene responsible for GSH^h type (7). All such evidences suggest that GSH^h animals may have some adaptive mechanism(s) to compensate for the inherited low levels of GSH in their blood thus, protecting their red cells from oxidative damage and that under natural conditions a balanced polymorphism based on the effects of environment or some other selection pressure is operating.

Summary. Effects of copper on red cell glutathione and enzyme levels were investigated in sheep of high- and low-glutathione types. No significant differences were observed in copper induced inhibition of glutathione regeneration or erythrocyte enzyme activities in the two groups of sheep. These results, together with those showing the effect of copper on GSH oxidation suggest that low-glutathione sheep should not be more sensitive to small doses of copper, commonly encountered in chronic copper poisoning in sheep than are high-glutathione sheep.

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