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Glutathione ester delays the onset of scurvy in ascorbate-deficient guinea pigs

(buthionine sulfoximine/dehydroascorbic acid/bone/brain/liver)

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ABSTRACT **Previous studies showed that administration of ascorbate to glutathione (GSH)-deficient newborn rats and guinea pigs prevented toxicity and mortality and led to increased tissue and mitochondrial GSH levels; ascorbate thus spares GSH. In the present work, we tried to answer the converse question: Does administration of GSH spare ascorbate? Because administered GSH is not well transported into most cells, we gave GSH monoethyl ester (which is readily transported and converted into GSH intracellularly) to guinea pigs fed an ascorbate-deficient diet. We found that treatment with GSH ester significantly delays appearance of the signs of scurvy and that this treatment spares ascorbate; thus, the decrease of tissue levels of ascorbate was delayed. The findings support the conclusions that (*i*) GSH is essential for the physiological function of ascorbate because it is required *in vivo* for reduction of dehydroascorbate and (*ii*) there is metabolic redundancy and overlap of the functions of these antioxidants. The sparing effect of GSH in scurvy may be mediated through an increase in the reduction of dehydroascorbate (which would otherwise be degraded) and to antioxidant effects of GSH that are also produced by ascorbate. Other studies indicate that GSH deficiency in adult mice stimulates ascorbate synthesis in liver. During this work we found that administration of GSH itself is highly toxic to ascorbate-deficient guinea pigs when given in divided i.p. doses totaling 3.75 mmol/kg daily.**

In recent work it was shown that glutathione (GSH) deficiency in newborn rats (1–4, 30) and in guinea pigs (5), produced by administration of buthionine sulfoximine [a transition-state inhibitor of γ-glutamylcysteine synthetase (6–8)], leads to death within a few days. In newborn rats (which apparently do not synthesize ascorbate), GSH deficiency is associated with severe mitochondrial and other cell damage (30) in kidney (1, 2), lung (1, 2), liver (1, 2), brain (1–3), and lens (1, 2, 4, 9). Similar results were found in guinea pigs (10). GSH deficiency in newborn rats (2) leads to decreased tissue ascorbate levels and to increased dehydroascorbate levels. GSH deficiency in adult mice does not lead to early mortality but is associated with damage to skeletal muscle (11, 12), lung (12, 13), jejunal epithelium (14), and colon epithelium (14). The relatively decreased morbidity that accompanies GSH deficiency in adult mice may be ascribed, at least in part, to their ability to synthesize ascorbate (1). Mortality due to GSH deficiency in newborn rats (1, 2, 4) and in guinea pigs (10), as well as tissue damage in these animals and in adult mice, may be decreased or prevented by administration of GSH esters or of ascorbate (1–4, 30). Newborn rats develop cataracts when subjected to mild GSH deficiency (2, 4), and cataracts are prevented by giving GSH esters or ascorbate; interestingly, dehydroascorbate protects in this model because of the availability of moderate GSH levels (2). These experiments indicate that (*i*) ascorbate spares GSH, (*ii*) GSH and ascorbate have actions in common in the destruction of reactive oxygen species, and (*iii*) reduction of dehydroascorbate to ascorbate is a significant physiological function of GSH (2).

Because ascorbate can serve as an essential antioxidant in GSH deficiency, it is of interest to ask the converse question—i.e., can GSH function in place of ascorbate? In the present studies we used the ascorbate-deficient guinea pig model. Because administered GSH is not significantly transported into most cells, we gave GSH monoethyl ester, which is efficiently transported and split to form GSH intracellularly (15–17).

A preliminary account of these studies has appeared (18).

EXPERIMENTAL PROCEDURES

Materials. Male Hartley guinea pigs (260–300 g) were obtained from Hilltop Labs (Scottsdale, PA). The control diet was standard guinea pig chow from Purina; the ascorbate-deficient diet was from United States Biochemical. All animals had free access to water. GSH was obtained from Sigma. GSH monoethyl ester was prepared as the hydrosulfate salt (17, 19, 20, 31).

Methods. Control guinea pigs were given chow ad libitum; ascorbate-deficient animals were maintained on an ascorbate-deficient diet for 9 or 21 days. Control animals received no treatment; animals on the ascorbate-deficient diet were injected at 8 a.m., 4 p.m., and midnight with physiological saline, GSH ester, or GSH, as indicated. GSH ester hydrosulfate and GSH were given as isosmolar solutions, adjusted to pH 7 by adding NaOH, at a dose of 1.25 mmol/kg at each injection; saline was given in an equivalent volume. Treatment with GSH was continued for only 9 days because of adverse reactions (see below). Treatment with saline and GSH ester was continued for 21 days. Animals were sacrificed by decapitation on the 10th or 22nd day at 10 a.m. The organs were perfused through the heart with ice-cold saline. The tissues were excised, rinsed with cold saline, and blotted; portions were used for isolating mitochondria (12, 21, 22), and portions were frozen in liquid nitrogen within 1 min. The frozen samples were weighed and homogenized in 5 vol of 5% (wt/vol) 5′-sulfosalicylic acid. After centrifugation (10,000 × *g*, 5 min), ascorbate, total ascorbate (ascorbate plus dehydroascorbate), and total GSH (GSH plus GSH disulfide in GSH equivalents) were determined. Ascorbate was determined by the 2,6-dichlorophenolindophenol method (23). Total ascorbate was determined after treatment with copper sulfate and 2,4-dinitrophenylhydrazine (24). GSH was determined by the GSH disulfide reductase recycling procedure

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Abbreviation: GSH, glutathione.

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(25, 26). The data are given as means (± SD) for groups of experimental (n = 4–6) and control (n = 10) animals. Radiographs were taken after immobilization of the animals by light anesthesia (ketamine). Light microscopy was done as described (2).

RESULTS

Guinea pigs given an ascorbate-deficient diet gained weight through day 14, but gained at a slower rate than the control animals, and then lost weight (Table 1, group A). The animals given GSH ester (group B) gained more weight than those of group A, and the weight gain during days 10–14 was ≈70% of the control group. Animals in group A became obviously sick after about day 17. They could not walk and moved very little, apparently immobilized by fractures of the hind legs and by swelling of the joints of the extremities, which were tender and had periosteal hematomas. Radiography showed major fractures of the femur in two animals. Animals in group A died or were sacrificed on day 21 or 22. Animals in group B (GSH ester) did not have fractures or hematomas; ≈75% of these animals were indistinguishable by general appearance from controls. Histological study showed significant loss of osteoid material from long bones in group A, whereas most animals in group B had no decrease of osteoid material (Fig. 1) or only a moderate decrease. In a separate experiment, several animals comparable to those of group B were kept for 40 days and showed no significant signs of scurvy (tender swollen joints, fractures); they exhibited some weight loss.

Animals of group C (GSH treated) exhibited decreased food intake and weight loss. They had significant hair loss and appeared moribund ≈9 days after start of treatment; they were therefore euthanized. They appeared cachectic at autopsy. Gross examination and light microscope studies did not reveal abnormalities of liver, kidney, brain, lung, spleen, heart, or adrenals. Normal amounts of osteroid material were found in bone. These animals did not show signs of scurvy.

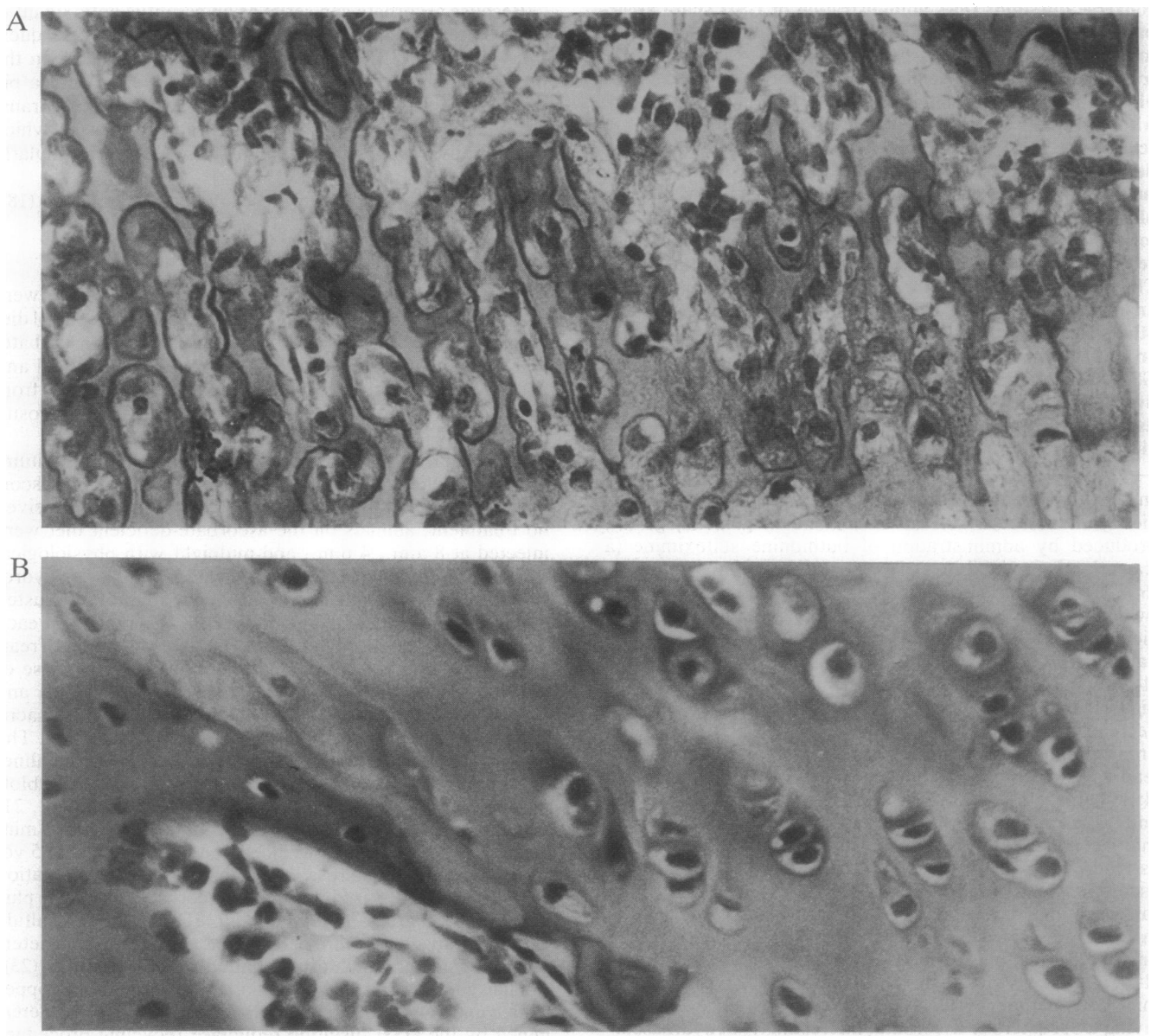

FIG. 1. Light microscopy of the osteochondral junction of the distal humerus of typical guinea pigs fed an ascorbate-deficient diet for 21 days. (*A*) Saline-treated. (*B*) GSH ester-treated. Sections were stained with hematoxylin/eosin. In *A* osteoid material (intercellular acidophilic matrix substance) markedly decreases; thus, cells appear more closely packed. Nuclei appeared denser in *A* as compared with *B*. In *A* the small amounts of osteoid material present stained with a blue hue, whereas in *B* the osteoid material stained pink. Sections from control animals were virtually identical to *B*. (X640.)

Table 1. Weight change and status of control and ascorbate-deficient guinea pigs treated with saline, GSH ester, or GSH

	Weight change, g			
		Animals on ascorbate-deficient diet		
Days of treatment	Control	Saline treated*	GSH ester treated†	GSH treated‡
1–9	+ 60 ± 5	+ 19 ± 8¶	+ 21 ± 11¶	− 71 ± 13¶‖
10–14	+ 85 ± 10	+ 19 ± 17¶	+ 58 ± 16‖	
15–21	+ 130 ± 12	− 49 ± 18¶	− 5 ± 12¶‖	

*After 21 days, these animals (group A) had periosteal hematomas, osteoporosis, and fractures; all were obviously sick.
†After 21 days, these animals (group B) had no hematomas or fractures; they had moderate osteoporosis, but they had much less osteoporosis than seen in group A members. Some animals were indistinguishable from control animals; some animals appeared slightly sick.
‡These animals (group C) had marked hair loss and died 8–10 days after start of treatment.
¶Data are statistically significantly different from controls (¶) or saline-treated (‖) animals at $P < 0.05$; control data are from normal guinea pigs of this type.

Administration of an ascorbate-deficient diet led to greatly decreased tissue ascorbate and total ascorbate levels (Table 2). Accumulation of dehydroascorbate (difference between total ascorbate and ascorbate) was substantial in liver and kidney after 21 days. The ascorbate level in brain declined greatly after 21 days. Treatment with GSH ester for 21 days led to better maintenance of initial ascorbate levels in all tissues except brain. Administration of GSH did not affect ascorbate levels significantly.

Guinea pigs given an ascorbate-deficient diet and treated with saline showed an increase in total GSH levels in the liver after 9 days as compared with control animals (Table 3); liver GSH was lower than in the control animals after 21 days (Table 4). Ascorbate-deficient animals treated with GSH ester had decreased GSH levels in the liver after 9 days (as compared with ascorbate-deficient saline-treated and control animals) and increased levels (compared with saline-treated) after 21 days. Liver GSH levels after giving GSH for 9 days were about the same as those found after giving saline. The GSH levels were somewhat increased in animals on the ascorbate-deficient diet in kidney after 9 days (Table 3) and after 21 days (Table 4). After treatment with GSH ester for 21 days, there was an increase, as compared with the saline-treated group, of the GSH levels of all tissues except brain (Table 4).

Table 3. Effects on animals fed an ascorbate-deficient diet for 9 days

	Tissue total GSH, μmol/g			
		Animals on diet		
Tissue	Control	Saline treated	GSH ester treated	GSH treated
Liver	9.02 ± 2.70	14.7 ± 2.10*	7.00 ± 1.10†	12.0 ± 3.3
Lung	2.05 ± 0.50	2.90 ± 0.85	3.28 ± 0.67	3.03 ± 0.64
Kidney	2.29 ± 0.50	3.68 ± 0.60*	3.70 ± 0.77	3.48 ± 0.76
Brain	1.64 ± 0.15	1.49 ± 0.27	1.61 ± 0.22	1.40 ± 0.21

*Data are statistically significantly different from controls (*) or saline-treated (†) animals at $P < 0.05$.

Table 4. Effects on animals fed an ascorbate-deficient diet for 21 days

	Tissue total GSH, μmol/g		
		Animals on diet	
Tissue	Control	Saline treated	GSH ester treated
Liver	8.32 ± 0.75	4.92 ± 1.45*	8.78 ± 1.41**
Lung	1.71 ± 0.49	2.19 ± 0.55	3.32 ± 0.82
Kidney	2.05 ± 0.60	3.55 ± 0.73*	4.30 ± 0.46*
Brain	1.63 ± 0.44	1.32 ± 0.11	1.23 ± 0.32

*, $P < 0.05$ vs. control; **, $P < 0.05$ vs. saline.

Mitochondrial GSH levels were substantially decreased in all tissues by giving an ascorbate-deficient diet (Table 5). Treatment with GSH ester led to higher levels than found in the saline-treated animals; these levels, except for brain, were lower than those of the control animals.

The finding of markedly decreased mitochondrial levels of GSH, in the absence of evidence for a similar decrease in total cellular GSH (Tables 3 and 4), suggests that GSH transport from cytosol into mitochondria may be defective. Mitochondria do not synthesize GSH but acquire it by transport from the cytosol (27, 28). The kinetics of mitochondrial uptake of

Table 2. Effect of GSH Ester and of GSH on tissue ascorbate levels of control and scorbutic guinea pigs

	Tissue ascorbate, μmol/g							
			Animals on ascorbate-deficient diet for 9 or 21 days*					
	Controls		Saline treated		GSH ester treated		GSH treated	
Tissue	Ascorbate	Total ascorbate	Ascorbate	Total ascorbate	Ascorbate	Total ascorbate	Ascorbate	Total ascorbate
Liver								
Control	1.91 ± 0.16	1.97 ± 0.19						
9 days			0.19 ± 0.01	0.27 ± 0.03	0.18 ± 0.05	0.19 ± 0.04	0.21 ± 0.07	0.23 ± 0.04
21 days			0.19 ± 0.01	0.35 ± 0.02	0.50 ± 0.05	0.58 ± 0.04	†	
Lung								
Control	1.46 ± 0.23	1.57 ± 0.41						
9 days			0.20 ± 0.11	0.29 ± 0.02	0.21 ± 0.08	0.30 ± 0.06	0.14 ± 0.03	0.24 ± 0.04
21 days			0.15 ± 0.04	0.17 ± 0.04	0.30 ± 0.06	0.64 ± 0.04	†	
Kidney								
Control	0.67 ± 0.06	0.69 ± 0.12						
9 days			0.17 ± 0.10	0.18 ± 0.06	0.21 ± 0.08	0.15 ± 0.01	0.13 ± 0.09	0.16 ± 0.09
21 days			0.06 ± 0.01	0.11 ± 0.03	0.32 ± 0.03	0.45 ± 0.07	†	
Brain								
Control	1.08 ± 0.06	1.09 ± 0.20						
9 days			0.75 ± 0.11	0.82 ± 0.02	0.71 ± 0.03	0.74 ± 0.10	0.70 ± 0.14	0.72 ± 0.13
21 days			0.27 ± 0.04	0.37 ± 0.04	0.25 ± 0.02	0.35 ± 0.04	†	

*These data are statistically significant from controls at $P < 0.05$.
†GSH-treated animals did not survive.

Table 5. Mitochondrial GSH levels after 21 days on ascorbate-deficient diet

	Total GSH, μmol of GSH per mg of protein			
		Animals on diet		
Tissue	Control	Saline treated	GSH ester treated	GSH treated (9 days)
Liver	9.39 ± 1.65	2.26 ± 0.71*	5.98 ± 1.72*,**	2.77 ± 0.51*†
Lung	6.19 ± 1.63	1.52 ± 0.10*	2.47 ± 0.42*,**	2.34 ± 0.36*†
Kidney	6.16 ± 1.49	0.98 ± 0.24*	3.51 ± 0.96*,**	ND
Brain	10.7 ± 1.08	5.75 ± 0.94*	13.8 ± 1.93**	ND

Values are means ± SD determined for tissues from groups of four to six animals. ND, not determined.
*, $P < 0.05$ vs. control; **, $P < 0.05$ vs. saline.
†After 9 days of saline treatment, mitochondrial GSH levels were 3.01 ± 0.22 ($n = 4$) in liver and 2.58 ± 0.48 ($n = 4$) in lungs of animals on the diet.

GSH have been studied in rat liver mitochondria and indicate the presence of a high-affinity transporter (28). Studies on guinea pig mitochondria would be of interest, especially because guinea pig mitochondrial GSH is poorly conserved as compared with rat liver mitochondrial GSH (5, 10). Administration of GSH ester did not increase brain levels of ascorbate (Table 2) or of GSH (Tables 3 and 4), suggesting that the ester is poorly transported across the blood–brain barrier. The marked increase of brain mitochondrial GSH (Table 5) is, therefore, of some interest, and also needs further study.

DISCUSSION

The observation that administration of GSH ester prevents or significantly delays the onset of scurvy in guinea pigs fed an ascorbate-deficient diet supports the idea that GSH and ascorbate function together as a physiologically important antioxidant system. Administration of GSH ester to ascorbate-deficient animals led to increased ascorbate and total ascorbate levels in liver, lung, and kidney, indicating a sparing effect (Table 2). GSH ester slowed the disappearance of osteoid material characteristically seen in scurvy. In addition to the well-studied effect of scurvy on hydroxylation reactions involved in collagen synthesis, recent work suggests that ascorbate-dependent hydroxylation may also be closely connected with vitamin D metabolism and formation of bone (29).

The finding of an apparent increase in the GSH levels of tissues (liver, kidney) of guinea pigs fed an ascorbate-deficient diet for 9 days (Table 3) suggests that ascorbate deficiency may provide a metabolic signal that increases GSH synthesis in the early stages of scurvy. Further studies of this phenomenon are required. It was previously found that GSH deficiency in adult mice induces an increase in ascorbate in the liver (refs. 1, 32; J.H. and O.W.G., unpublished work), followed by a decrease of ascorbate in liver, kidney, and lung (1, 32). Such an increase does not occur in newborn rats (1, 2), which do not show an increase but exhibit a decrease of tissue ascorbate after administration of buthionine sulfoximine (2). This result suggests that newborn rats (1–5 days of age), like guinea pigs, do not synthesize ascorbate to a significant extent. Presumably rats acquire ascorbate from the mother during the early neonatal period before development of an adult active ascorbate-synthesis pathway. The newborn rat appears useful as a convenient (and relatively inexpensive) model for studies on ascorbate deficiency.

The findings show that GSH continues to be synthesized in the tissues of ascorbate-deficient guinea pigs (Tables 3–5). The antiscorbutic effects of GSH ester may be ascribed to sparing effects—for example, promotion by GSH of the reduction of dehydroascorbate to ascorbate (which would decrease the rate at which the residual ascorbate and dehydroascorbate disappear in animals receiving an ascorbate-deficient diet)—and to antioxidant effects of GSH that are also performed by ascorbate. It is evident that GSH is essential for the physiological function of ascorbate and that there is metabolic redundancy and overlap of the functions of these antioxidants. The present and previous studies establish that ascorbate can spare GSH and, thus, protect against the effects of GSH deficiency and that GSH (given as an ester) can spare ascorbate and, thus, delay onset of scurvy. Although GSH and ascorbate can perform functions in common, each is likely to participate in reactions that the other cannot do efficiently. There are probably critically essential minimum cellular levels of both GSH and ascorbate.

We found that administration of GSH itself to ascorbate-deficient guinea pigs led to severe toxicity and death when given by i.p. injection at a dosage of 1.25 mmol/kg of body weight three times daily. Similar treatment of control guinea pigs did not produce toxicity, but toxicity and lethal effects were seen in control animals given twice that daily dosage of GSH (i.e., 7.5 mmol/kg per day). It thus appears that administration of high doses of GSH may be toxic, especially to animals (such as humans) that are unable to synthesize ascorbate and may have a dietary deficiency of this vitamin.

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