

# Gluconeogenesis from ascorbic acid: ascorbate recycling in isolated murine hepatocytes

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**Abstract** Ascorbic acid synthesis and breakdown were investigated in isolated hepatocytes prepared from fasted mice. Stimulation of gluconeogenesis by alanine or xylitol led to ascorbate synthesis. On the other hand, ascorbate or dehydroascorbate addition resulted in concentration-dependent glucose production and elevation of the pentose phosphate pathway intermediate xylulose 5-phosphate. Stimulation of ascorbate oxidation and/or the inhibition of dehydroascorbate reduction increased glucose formation. Inhibition of the pentose phosphate pathway decreased glucose production from dehydroascorbate with increased accumulation of xylulose 5-phosphate. These results suggest that ascorbate can be recycled by a novel way involving intermediates of the pentose phosphate pathway, gluconeogenesis and hexuronic acid pathway.

**Key words:** Ascorbic acid; Dehydroascorbic acid; Gluconeogenesis; Glutathione; Pentose phosphate pathway; Isolated hepatocyte (Mouse)

## 1. Introduction

Ascorbic acid is one of the most important water-soluble antioxidants [1]. In ascorbate-synthesising animals it is produced through the hexuronic pathway in liver or kidney [2]. Its oxidation produces dehydroascorbic acid, which can be converted back to ascorbate by glutathione-dependent non-enzymatic [1] or enzymatic reactions [3]. Since dehydroascorbate at physiological pH is unstable with a half-life of a few minutes, it may be rapidly metabolised through diketogulonate to 5-carbon intermediates. Although the participation of the pentose phosphate pathway in the metabolism of dehydroascorbate has been suggested [4], its final fate has not been well defined. A minor pathway of catabolism gives oxalate and 4-carbon intermediates [4].

Oxidative stress results in simultaneous consumption of the two main antioxidants, GSH and ascorbate. In the case of shortage of GSH, since reduction of dehydroascorbate to ascorbate is decreased, its degradation becomes the dominant pathway, which would worsen the antioxidant state of the cell.

The aim of the present study was to investigate the further metabolism of dehydroascorbate and the role of the pentose phosphate pathway in this process. Our results suggest that ascorbate is a gluconeogenic precursor via pentose phosphates in isolated murine hepatocytes prepared from starved mice.

This metabolic route provides substrates for ascorbate re-synthesis.

## 2. Materials and methods

### 2.1. Materials

Collagenase (type IV), glucose UV-visible enzymatic (hexokinase) determination kit, Nucleosil C<sub>18</sub> column (average particle size 5 µm, 250×4.6 mm I.D), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, yeast transketolase, ribose 5-phosphate disodium salt, cocarboxylase (thiamine pyrophosphate chloride), β-nicotinamide adenine dinucleotide sodium salt, sodium arsenate, L-buthionine-(S,R)-sulfoximine, menadione and oxythiamine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Dehydroascorbic acid was bought from Merck (Darmstadt, Germany). All other materials were of analytical grade.

### 2.2. Preparation and incubation of isolated mouse hepatocytes

CFLP male mice (25–35 g body weight, Human, Gödöllő, Hungary) fasted for 48 h were used. Isolated hepatocytes were prepared by the collagenase perfusion method as described earlier [5]. Hepatocytes (1–2×10<sup>6</sup> cells/ml) incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 2.5 mM CaCl<sub>2</sub> and 1% albumin were stirred by constant bubbling with gas (O<sub>2</sub>/CO<sub>2</sub>, 95:5 v/v%) at 37°C. Viability of the cells checked by the trypan blue exclusion test was about 85–95%.

### 2.3. Treatment of mice

The B<sub>1</sub> vitamin antagonist oxythiamine hydrochloride was dissolved in sterile nonpyrogenic physiologic saline. The pH of this solution was adjusted to 7.4 at 37°C. 20 mg/kg body weight thiamine antagonist was given intraperitoneally to 42 h starved mice in 1 ml final volume. Control groups of 42 h starved animals received 1 ml isotonic saline [6].

### 2.4. Measurement of metabolites

Incubations were terminated by the addition of cold perchloric acid (PCA, 3% final concentration). Glucose was measured in the deproteinised neutralised supernatants by the UV-visible hexokinase method [7]. Ascorbic acid (reduced and total) was measured by HPLC following specific sample preparation as described earlier [8]. The GSH content of the suspension was determined from PCA-soluble supernatants according to [9]. The xylulose 5-phosphate level was measured in the PCA-soluble supernatants after deproteinisation and neutralisation by modified transketolase enzymatic assay using ribose 5-phosphate as other substrate beside xylulose 5-phosphate [10].

### 2.5. Miscellaneous

Cell numbers were calculated on the basis of DNA content of hepatocytes determined according to [11]. Statistical analysis was performed using Student's *t*-test.

## 3. Results

### 3.1. Glucose production from ascorbate or dehydroascorbate in isolated mouse hepatocytes

Hepatocytes prepared from 48 h starved animals are glyco-

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**Abbreviations:** BSO, buthionine sulfoximine; GSH, glutathione (reduced form).

gen depleted [12] therefore, the source of their glucose production is gluconeogenesis. Cells were incubated in the presence of various concentrations of ascorbate or dehydroascorbate for 30 min and their glucose production was measured. For comparison gluconeogenesis from alanine was also detected. Significant glucose formation from ascorbate was observed, which reached saturation at relatively low ascorbate concentrations. Dehydroascorbate-fueled glucose production showed a greater rate and saturation was reached at higher substrate concentrations. Gluconeogenesis from both substrates was surprisingly effective, its rate being comparable to that from alanine (Fig. 1). Stimulation of ascorbate oxidation to dehydroascorbate by menadione increased the rate of gluconeogenesis (Fig. 1).

### 3.2. Effect of glutathione depletion on dehydroascorbate metabolism in isolated mouse hepatocytes

Treatment of isolated hepatocytes with buthionine sulfoximine strongly reduced the intracellular glutathione pool (data not shown). As expected, this treatment resulted in diminished GSH-dependent reduction of dehydroascorbate. At the same time, increased glucose production was observed while the consumption of added dehydroascorbate remained unaltered (Table 1). BSO addition did not significantly influence gluconeogenesis from alanine (data not shown).

### 3.3. Role of the pentose phosphate pathway in gluconeogenesis from dehydroascorbate in isolated mouse hepatocytes

Addition of dehydroascorbate resulted in an increase of xylulose 5-phosphate, an intermediate of the pentose phosphate pathway in isolated hepatocytes (Table 2). Based on this observation, the possible participation of the pentose phosphate pathway in gluconeogenesis from ascorbate was supposed. This metabolic route was inhibited by *in vivo* administration of oxythiamine which is known to inhibit transketolases [6]. In cells prepared from oxythiamine-treated starved animals, the addition of dehydroascorbate resulted in lower glucose formation and simultaneously decreased consumption of added dehydroascorbate. At the same time, the accumulation of xylulose 5-phosphate was greater in oxythiamine-treated hepatocytes than in controls (Table 2). Oxythiamine treatment did not alter gluconeogenesis from alanine (data not shown).

### 3.4. Ascorbate synthesis from gluconeogenic precursors

Addition of alanine, a known gluconeogenic substrate, resulted in moderate, but measurable ascorbate synthesis in isolated hepatocytes. The gluconeogenic effect of xylitol, a puta-

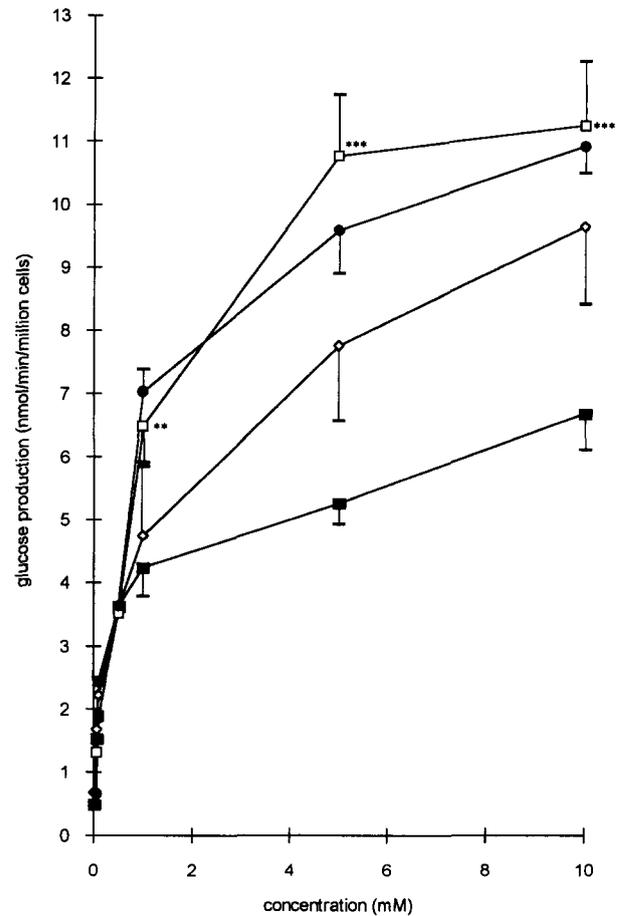


Fig. 1. Gluconeogenesis from ascorbate or dehydroascorbate in murine hepatocytes. Isolated hepatocytes prepared from 48 h starved mice were incubated in the presence of different concentrations of ascorbate (■), ascorbate+0.1 mM menadione (◇), dehydroascorbate (□) and alanine (●). Glucose production was determined after 30 min incubation. Data are given as means  $\pm$  S.E.M.,  $n=3-16$ . Statistically significant differences from corresponding values gained in the presence of ascorbate: \*\* $P < 0.02$ , \*\*\* $P < 0.01$ .

tive intermediate of ascorbate catabolism [4], was also investigated. Similarly to alanine, addition of xylitol stimulated glucose production and ascorbate resynthesis (Table 3).

## 4. Discussion

Although several papers indicate the further metabolism of dehydroascorbate, it has usually been regarded as a waste

Table 1  
Effect of glutathione depletion on dehydroascorbate metabolism

Addition	Glucose production (nmol/min per $10^6$ cells)	Conversion of dehydroascorbate to ascorbate (nmol/min per $10^6$ cells)	Consumption of added dehydroascorbate (nmol/min per $10^6$ cells)
None	0.49 $\pm$ 0.10	N.D.	N.D.
5 mM buthionine sulfoximine	0.79 $\pm$ 0.16	N.D.	N.D.
5 mM dehydroascorbate	8.35 $\pm$ 1.33 <sup>a</sup>	8.04 $\pm$ 1.81	185.97 $\pm$ 12.17
5 mM buthionine sulfoximine + 5 mM dehydroascorbate	11.64 $\pm$ 1.16 <sup>a,b</sup>	3.44 $\pm$ 0.81 <sup>b</sup>	162.05 $\pm$ 19.09

Isolated hepatocytes prepared from 48 h fasted animals were incubated in the presence or absence of 5 mM dehydroascorbate and/or 5 mM buthionine sulfoximine (BSO) for 30 min. Glucose production, dehydroascorbate consumption and its conversion to ascorbate were measured. Values are expressed as means  $\pm$  S.E.M.,  $n=4-12$ . Significant difference from the corresponding controls: <sup>a</sup> $p < 0.01$ , and between data obtained from dehydroascorbate or dehydroascorbate + BSO addition: <sup>b</sup> $p < 0.05$ ; N.D., not determined.

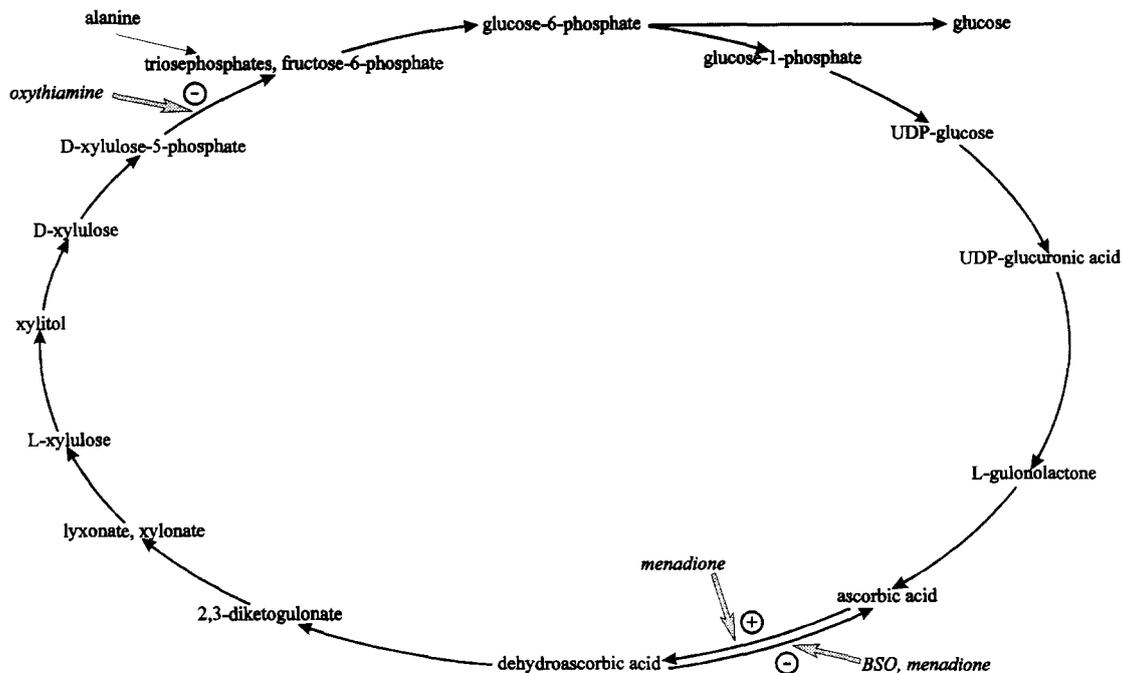


Fig. 2. Ascorbate cycle in mouse hepatocytes. Ascorbate oxidised to dehydroascorbate can be recycled by a novel, glutathione-independent way; intermediates of pentose phosphate pathway, the gluconeogenic sequence and hexuronic acid pathway are involved in the proposed cycle. The treatments and additions used in the experiments are also indicated.

product of ascorbate-consuming processes. In this study we show that besides the reduction of dehydroascorbate to ascorbate, an alternative pathway also exists, which provides the possibility for ascorbate resynthesis in a roundabout way.

It is suggested that glucose formation from ascorbate occurs via dehydroascorbate. Gluconeogenesis from ascorbate is stimulated in the presence of an oxidative agent, menadione (Fig. 1). GSH depletion caused by BSO inhibits the reduction of dehydroascorbate to ascorbate and results in enhanced glucose production (Table 1).

5-Carbon intermediates of ascorbate catabolism have been presumed to enter the pentose phosphate pathway [4]. The increase in xylulose 5-phosphate levels in hepatocytes after dehydroascorbate addition or the diminished glucose production from added dehydroascorbate and the higher xylulose 5-phosphate content in hepatocytes where the non-oxidative branch of the pentose phosphate route is blocked clearly support this suggestion (Table 2). Stimulated gluconeogenesis from xylitol also supports this view (Table 3). L-Pentoses

can be converted to D-pentoses through xylitol, thus it may be an important intermediate of dehydroascorbate metabolism. The intermediates of the pentose phosphate pathway finally can enter the gluconeogenic sequence yielding glucose 6-phosphate and glucose. Glucose 6-phosphate is a precursor of ascorbate synthesis in the hexuronic pathway. Both alanine, as gluconeogenic precursor, and xylitol were able to stimulate ascorbate production in isolated hepatocytes (Table 3). These results indicate that besides the classic GSH-dependent ascorbate recycling another – GSH-independent – pathway also exists in ascorbate-synthesising cells. In hepatocytes ascorbate can be regenerated from dehydroascorbate in a cycle through the intermediates of the pentose phosphate pathway, gluconeogenesis and the hexuronic acid pathway (Fig. 2).

This novel pathway of the ascorbate cycle may be especially important during starvation when major – glycogenolysis-dependent [12] – ascorbate synthesis and its exogenous supply are missing or in oxidative stress which favours dehy-

Table 2  
Effect of inhibition of pentose phosphate pathway on gluconeogenesis from dehydroascorbate in isolated mouse hepatocytes

Treatment	Addition	Glucose production (nmol/min per 10 <sup>6</sup> cells)	Consumption of added dehydroascorbate (nmol/min per 10 <sup>6</sup> cells)	Xylulose 5-phosphate content (pmol/10 <sup>6</sup> cells)
Isotonic saline	none	0.76 ± 0.22	N.D.	21.98 ± 10.11
	5 mM dehydroascorbate	13.33 ± 1.53 <sup>b</sup>	185.97 ± 12.18	184.66 ± 26.17 <sup>b</sup>
Oxythiamine	none	1.01 ± 0.25	N.D.	162.06 ± 35.09 <sup>a</sup>
	5 mM dehydroascorbate	4.87 ± 0.43 <sup>b,c</sup>	93.48 ± 13.57 <sup>b,c</sup>	537.16 ± 13.61 <sup>b,c</sup>

42 h starved mice received a single intraperitoneal injection of 20 mg/kg body weight oxythiamine, a known inhibitor of transketolases, in 1 ml final volume. Control animals received 1 ml of isotonic saline. 6 h later mice were sacrificed and hepatocytes were prepared from their liver. Glucose production, consumption of added dehydroascorbate and xylulose 5-phosphate content were determined in both groups after 30 min of incubation as detailed in Section 2. Values are given as means ± S.E.M.,  $n = 3-12$ . Statistically significant difference from the oxythiamine untreated controls <sup>a</sup> $p < 0.02$ , <sup>b</sup> $p < 0.01$ , and in the presence of dehydroascorbate between values gained from oxythiamine treated or untreated animals <sup>c</sup> $p < 0.01$ ; N.D., not determined.

Table 3  
Ascorbate synthesis from gluconeogenic precursors

Addition	Glucose production (nmol/min per 10 <sup>6</sup> cells)	Ascorbate production (pmol/min per 10 <sup>6</sup> cells)
None	1.01 ± 0.09	-1.79 ± 0.63
10 mM alanine	16.24 ± 3.22 <sup>a</sup>	1.45 ± 0.43 <sup>a</sup>
10 mM xylitol	15.96 ± 1.63 <sup>a</sup>	1.57 ± 0.45 <sup>a</sup>

Glycogen-depleted isolated hepatocytes were incubated in the presence of alanine or xylitol for 30 min. Glucose and ascorbate production were measured. Data are means ± S.E.M., *n* = 4. Significant difference from the corresponding controls: <sup>a</sup>*p* < 0.01.

droascorbate formation directly and by GSH depletion. This cycle saves dehydroascorbate for glucose production or ascorbate resynthesis.

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