Hexose Monophosphate Shunt in Rat Lens: Stimulation by Vitamin C

Shambhu D. Varma, Sandra A. Bauer, and Richard D. Richards

The metabolism of glucose through the hexose monophosphate (HMP) shunt has been studied in rat lens in vitro, in the absence and presence of ascorbic and dehydroascorbic acids. Both forms of the vitamin stimulated the utilization of glucose through the HMP shunt, the stimulatory effect of dehydroascorbate being substantially greater than that of ascorbate. The stimulatory effect of ascorbate, as well as of dehydroascorbate, was antagonized by sodium iodide, and N,N-bis (dichloroethyl)-N-nitrosourea, compounds known to inhibit glutathione reductase. N-ethylmaleimide also antagonized the stimulation. These findings, therefore, suggest that the DHA/AA redox couple acts in concert with the GSSG/GSH couple in stimulating the tissue shunt activity. Invest Ophthalmol Vis Sci 28:1164–1169, 1987

Ascorbic acid is one of the most essential nutrients for man, monkeys, guinea pigs, certain fish and birds. Although its absence from the diet of these species leads to a complex debilitating syndrome called scurvy, its normal biochemical mode of action remains largely unknown, except perhaps as a cofactor for certain monooxygenases. These enzymes, also called mixed function oxidases, catalyze various oxygen-dependent hydroxylation reactions, such as hydroxylation of the proline residues of the procollagen peptides, para hydroxyphenylpyruvic acid, tryptophan, steroids and various xenobiotics and drugs. The requirement of ascorbic acid for the mixed-function oxidation reaction is, however, not considered specific. In certain reactions, other appropriate compounds can substitute for ascorbate. The dietary requirement of ascorbic acid is also rather large in comparison to other nutrients: ascorbic acid in humans is required in amounts well within the milligram/day range. This raises the possibility that the nutrient, in addition to its possible function as a cofactor in the monooxygenase dependent hydroxylation reactions, might be required in certain nonenzymatic reactions. Its property as a potent reducing agent is well known. Its oxidized product, dehydroascorbate, is also easily reducible. The oxidized and the reduced forms of the nutrient can thus potentially facilitate electron transfer between various redox couples operative in the cell.

Mapson and Moustafa observed that in germinating pea seedlings, dehydroascorbate/ascorbate is able to facilitate electron transfer from NADPH to oxygen enzymatically, with the GSSG/GSH couple acting as the intermediate electron carrier operating between NADPH and dehydroascorbate (Fig. 1). A similar but nonenzymatic electron transfer reaction has been suggested to be operative in the lens. Results presented in this communication are in accordance with the above concepts. Ascorbate, as well as dehydroascorbate, has been found to accelerate the utilization of glucose in the rat lens through the hexose monophosphate (HMP) shunt.

Materials and Methods

The chemicals used in these experiments were procured from Sigma Chemical Company (St. Louis, MO). Radioactive glucose was obtained from New England Nuclear Medicine (Boston, MA). N,N-bis(2-chloroethyl)-N-nitrosourea (BCNU) was obtained from the University of Maryland Cancer Research Center (Baltimore, MD). The determination of the HMP shunt was accomplished by measuring the production of CO2 from 1-14C glucose by intact rat lenses incubated in Tyrode bicarbonate solution pulsed with radioactive glucose (specific activity was approximately 3.5 µC/µmole). Results have been expressed as nanomoles of CO2 produced per lens. Sodium iodide and BCNU were used as inhibitors of glutathione reductase. N-ethyl maleimide was used as an -SH blocking agent. The addition of these reagents was done after decreasing the NaCl content of the tyrode solution.

Sprague-Dawley rats weighing approximately 150 g were sacrificed by decapitation and the eyes enu-
Fig. 1. Integration of ascorbate (AA)/dehydroascorbate (DHA) with the transport of hydrogen from glucose-6-phosphate to oxygen through NADPH.

cleated gently. The use of animals followed directives of the ARVO Resolution on the Use of Animals in Research. The enucleated globe was incised posteriorly and the intact lens excised without trauma. It was then immediately transferred to 250 μl of the incubation medium contained in a 10-cm × 1-cm test tube with a rubber-stoppered sidearm and maintained at 37°C in an incubator. The medium was gassed with air and CO₂ to a pH of 7.0. After the lens transfer, additional reagents in 50 μl volumes were added and the test tubes capped tightly with a serum stopper. The downside of the serum stopper had a plastic cup containing a filter paper soaked with 100 μl of N hyamine hydroxide, acting as a carbon dioxide trap. At the end of the incubation, 100 μl of a 10% solution of trichloroacetic acid was injected through the sidearm and CO₂ collection in the hyamine-containing cup completed by incubating the tubes for another 30 min. The hyamine cup was then severed and transferred to a vial containing a liquid scintillation mixture (Beckman EP 10 ml + 200 μl glacial acetic acid) (Beckman Instruments, Columbia, MD) and radioactivity determined by scintillation counting. The radioactive counts obtained in presence of the lens were corrected for any nonspecific counts (fluorescence) due to the CO₂ trap. The total amount of CO₂ produced by the lens was then calculated from the specific activity data. Blank incubations were also conducted without the lens to determine the effect of hyamine hydroxide on the pH of the medium; no significant effect was observed. This was further ascertained by conducting blank incubations with medium containing NaH¹⁴CO₃ and determining the extent of CO₂ trapped by the hyamine hydroxide before and after acidification. Less than 10% of the total CO₂ was found trapped without acidification, thereby decreasing the bicarbonate content from the initial 12 mM to approximately 11 mM. The final pH of the medium was 7.9 ± 0.1. With the lens in the medium, the postincubation pH was approximately 8.25 ± 0.2.

Ascorbate was determined by titrating the incubation medium with 2,6-dichlorophenol indophenol.¹⁵ The blank consisted of the sample treated with 20 units of ascorbate oxidase. Hydrogen peroxide was tested iodometrically. To 0.25 ml of the ascorbate oxidase-treated sample 100 μl of each of the following were added: 0.1 M acetate buffer pH 3.5, 0.6 M KI, 1.7 × 10⁻³ M ammonium molybdate, and 1% starch. Peroxide was indicated by the development of a blue color. The sensitivity of the test extended to 50 μM. A catalase treated sample was used as the control.

**Results**

Table I describes the results of initial experiments conducted to ascertain the suitability of the experimental protocol for measuring the HMP shunt activity of the rat lens. One lens of the animal was incubated in medium containing 6-¹⁴C-glucose; the contralateral lens in a medium containing 1-¹⁴C-glucose. The amounts of CO₂ produced representing the 6-C

<table>
<thead>
<tr>
<th>CO₂ (nmol)</th>
<th>1-¹⁴C/6-¹⁴C</th>
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<tr>
<td>6-C¹⁴-glucose</td>
<td>0.120 ± 0.051</td>
</tr>
<tr>
<td>1-¹⁴C-glucose</td>
<td>2.71 ± 0.183</td>
</tr>
</tbody>
</table>

The lenses weighing 28 ± 2 mg were cultured in Tyrode medium. The specific activity of ¹⁴C-glucose was adjusted to 3.5 μCi/μmol. The period of incubation was 2.5 hr. The CO₂ was collected by the method described in the text. The figures are expressed as mean ± SD. The number of samples in each case was four.
The time course of CO₂ production through the shunt, in the presence and absence of ascorbate and dehydroascorbate, has been described in Figure 2. The rate of CO₂ production was enhanced in the case of the lenses incubated with ascorbate, the enhancement becoming apparent after 30 min of incubation. The amount of CO₂ produced by incubation for 60 min in the presence of ascorbate was approximately twice that produced in its absence.

Ascorbate oxidation is known to produce hydrogen peroxide. Previous studies have shown that peroxide stimulates the HMP shunt. Addition of catalase to the medium in amounts ranging from 25 to 100 units, however, did not abolish the ascorbate effect. This suggests that the observed ascorbate-induced stimulation is not due to the hydrogen peroxide that might have been produced. In addition, no peroxide was detectable in the medium. The possibility that the peroxide formed got decomposed further to water and oxygen by the excessive ascorbate cannot be entirely ruled out. The concentration of ascorbate in the medium decreased to approximately 95% of the original during incubation.

Another product of ascorbate oxidation is dehydroascorbate. It therefore appeared that the observed stimulation of the shunt by ascorbate may be initiated by DHA. In accordance with this prediction, addition of DHA to the medium stimulated the shunt very strikingly. The amount of CO₂ liberated by the lens when incubated in the presence of DHA was approximately four to ten times that in its absence (Fig. 2). It was also about four to five times greater than that produced by the lenses incubated with ascorbate.

Subsequent experiments were designed to investigate if the stimulation of the shunt by the oxidized and reduced forms of vitamin C is related, on the one hand, to the coupling of DHA/AA pair with the GSSG/GSH pair, and with oxygen/H₂O pair on the other hand, with oxygen serving as the final acceptor of the reducing equivalents. The level of glutathione in the tissue is maintained constant due to continued reduction of GSSG by glutathione reductase, which utilizes NADPH produced by the HMP shunt. HMP shunt measurements were therefore, conducted in the presence of sodium iodide, a known inhibitor of glutathione reductase, and NEM, a
known -SH blocking agent. As shown in Figure 3, addition of the iodide to the incubation medium in concentrations ranging from 0 to 4 mM was observed to antagonize the stimulation of the shunt by ascorbate, as well as by dehydroascorbate. BCNU, another compound known to inhibit glutathione reductase, also antagonized the HMP shunt stimulation, although the concentration of this nitrosourea had to be raised in order to show a noticeable effect (Table 2). This may be due to several competing reactions of BCNU with cellular constituents such as carbamoylation and alkylation of proteins. NEM in concentrations ranging from 0 to 4 mM was also inhibitory (Fig. 4). The inhibitory effects of iodide, BCNU and NEM, all indicate that the DHA and AA dependent stimulation of the shunt involves glucose, NADPH, GSSG/GSH, and very likely oxygen. Direct oxygen consumption measurements by the tissue, due to its small size and the microliter volumes of medium, was not feasible.

Discussion

Vitamin C is known to participate in several reactions involving electron transport. The physiological role of such reactions remains incompletely understood. Studies with germinating pea seedlings led Mapson and Moustafa to suggest that one of the functions of dehydroascorbic acid, the oxidized but biologically active form of vitamin C, may be to facilitate the transport of reducing equivalents from NADPH to oxygen through the series of reactions outlined in Figure 1. The effective operation of this series of reactions requires, in addition to glutathione reductase, dehydroascorbate reductase and ascorbate oxidase. Since the latter two are plant enzymes, the significance of these reactions in the case of animals has generally been discounted. However, the thermodynamic consideration of the various reactions involved indicates that in tissues rich in glutathione, such as the lens, the dehydroascorbate/ascorbate-mediated electron flux may be able to proceed even in the absence of dehydroascorbate reductase and ascorbate oxidase. In that event, the nutrient may participate in the regulation of the HMP shunt and tissue NADPH supply. The requirement of ascorbate oxidase may be minimized if the reduced ascorbate can diffuse out of the tissue, allowing continued reduction of dehydroascorbate. Under a hyperoxic state, where oxygen is present in several active forms, the requirement of ascorbate oxidase is further minimized. The active forms of oxygen are known to react with ascorbic acid effectively.

The above-mentioned considerations led us to examine experimentally if DHA/AA may indeed have a shunt-regulating potential. Results of in vitro studies conducted with rat lenses attest to this possibility. Ascorbate addition to the medium led to a significant increase in carbon dioxide production from 1-C of the glucose carbon skeleton. A most striking stimulation of the shunt was observed with DHA. The less striking but significant effect of ascorbate is apparently related to its poor penetration inside the cell, as well as to the possibility that DHA is the initial reactant involved.

The energetics of the various reactions involved are described in the Appendix. The potential difference of the reduction of DHA by GSH at pH 7 and 37°C is

Table 2. Effect of BCNU on CO₂ production from 1-¹⁴C-glucose

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Nanomoles of CO₂/lens</th>
<th>(N)</th>
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<tbody>
<tr>
<td>(A) BM</td>
<td>0.79 ± 0.17</td>
<td>(10)</td>
</tr>
<tr>
<td>(B) BM + BCNU (10 mM)</td>
<td>0.48 ± 0.03</td>
<td>(4)</td>
</tr>
<tr>
<td>(C) BM + Ascorbate (2.5 mM)</td>
<td>2.00 ± 0.05</td>
<td>(4)</td>
</tr>
<tr>
<td>(D) BM + DHA (2.0 mM)</td>
<td>4.32 ± 0.06</td>
<td>(4)</td>
</tr>
<tr>
<td>(E) BM + Ascorbate (2.5 mM) + BCNU (10 mM)</td>
<td>1.2 ± 0.19</td>
<td>(6)</td>
</tr>
<tr>
<td>(F) BM + DHA (2.0 mM) + BCNU (10 mM)</td>
<td>3.11 ± 0.54</td>
<td>(6)</td>
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</table>

Experimental techniques have been described in the text. The period of incubation was 1 hr. (N) = number of lenses studied. Values have been expressed as mean ± SD. BM = basal tyrode medium. P values between A & B, A & C, A & D, C & E and D & F are <0.001.
approximately 0.284 volts. The free energy change (ΔGo') associated with the reaction and the theoretical equilibrium constant values are -13 K cal and 1.7 × 10^9, respectively. The values of the equilibrium constant of the reaction derived from both the Gibbs and Nernst equations are identical. Based on the equilibrium constant values calculated under standard conditions, the ratio of AH_2/DHA should be approximately 1.7 × 10^8. Although the precise relationship between the steady state concentrations and equilibrium concentrations remains disputed for most reactions in the biological milieu, the equilibrium concentrations attainable under standard conditions provide a reasonable approximation of the ratios of several redox couples. In the present case, the AH_2/DHA ratio, as calculated under standard states, agrees with the fact that the DHA level in the lens is below the ordinary limits of detection, whereas AH_2 is about 3 mg/Kg wet weight of the tissue. The sequence of reactions in the direction indicated in Figure 1, therefore, appear kinetically feasible. It is also in conformity with the inhibition of the shunt stimulation by NaI and BCNU, compounds known to inhibit glutathione reductase.

Whether the rates of the nonenzymatic reduction of DHA by GSH to produce ascorbate, and the subsequent oxidation of ascorbate to DHA, are able to keep pace with the normal pace of the HMP shunt remains to be established. The physiological significance of the effect of DHA/AA on the HMP shunt is therefore not apparent at the present time, except perhaps in the attenuation of the oxidative stress to the tissue concomitant to an in situ generation of certain active species of oxygen. The latter possibility is more imminent for conditions generating excessive amounts of the active species of oxygen, such as when the animal is exposed to a hyperbaric aerobic condition. The attenuating effect of ascorbate is apparently related to its reaction with superoxide derivatives. The DHA produced in this reaction will, in turn, accelerate the shunt, stimulating the electron flow from glucose to oxygen radicals, thereby protecting the tissue against oxygen damage. Through its effect on the shunt, vitamin C may also have a modulating effect on several other NADPH-dependent reactions, such as those involved in steroid and fatty acid syntheses. Scurvy is well known to be associated with abnormalities in many aspects of lipid metabolism. The lens, however, is more resistant to ascorbate deficiency, possibly because of the ability of the ciliary body to maintain ascorbate in high concentrations in the aqueous and hence in the lens. Further studies to evaluate the role of ascorbate in the maintenance of lens metabolism and transparency are in progress.

Key words: ascorbate, dehydroascorbate, vitamin C, hexose monophosphate shunt, lens

Appendix

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔE°</th>
<th>pH 7.0</th>
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<tbody>
<tr>
<td>2 GSH → GSSG + 2H^+ + 2e</td>
<td>0.23 volts</td>
<td></td>
</tr>
<tr>
<td>DHA + 2H^+ + 2e → AH_2</td>
<td>0.054 volts</td>
<td></td>
</tr>
<tr>
<td>DHA + 2GSH → GSSG + AH_2</td>
<td>ΔE° = 0.284</td>
<td></td>
</tr>
</tbody>
</table>

\[
\Delta G^o = -nF(\Delta E^o) = -(2) 23.06 \frac{K\text{ cal}}{\text{mole °K volts}} \times 0.284 \text{ volts}
\]

\[
\Delta G^o = -RT \ln K
\]

\[
\ln K = 21.26
\]

\[
K = 1.7 \times 10^9
\]

\[
\text{in Rat Lens, Ascorbate is } 50 \text{ mg/l } = 2.8 \times 10^{-4} \text{ M (see ref. 16)}
\]

\[
[\text{AH}_2] = 3.4 \times 10^8 \text{ M}
\]

\[
[\text{DHA}] = 2.8 \times 10^{-4} \text{ M} = 0.82 \times 10^{-12} \text{ M}
\]

E° = Standard reduction potential at 37°C and pH 7.0
$\Delta E^\circ = \text{Sum of the reduction potentials of both the half reactions.}$

$\Delta G^\circ = \text{Gibbs energy of the reaction.}$

$n = \text{Number of electrons involved in the redox reaction.}$

$F = \text{Faraday, expressed in terms of K cal/mole °K volt.}$

$R = \text{Gas constant in terms of K cal/mole °K.}$

$T = \text{Absolute temperature in Kelvin.}$

$K = \text{Equilibrium constant.}$

In Rat Lens, $[\text{GSH}] = 10^{-2} \text{ M}, [\text{GSSG}] = 0.05 \times 10^{-2} \text{ M}.$

Ascorbate $= 2.8 \times 10^{-4} \text{ M}.$

Conventions, see ref. 26.

References