THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 5, Issue of January 30, pp. 2758–2762, 1998
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Dehydroascorbate and Ascorbate Transport in Rat Liver Microsomal Vesicles*

(Received for publication, October 21, 1997)

Gábor Bánhegyi‡§¶, Paola Marcolongo‡, Ferenc Puskás§, Rosella Fulceri‡, József Mandl§, and Angelo Benedetti‡

From the ‡Istituto di Patologia Generale, Università di Siena, 53100 Siena, Italy and the §Department of Medical Chemistry, Semmelweis University of Medicine, 1444 Budapest POB 260, Hungary

Ascorbate and dehydroascorbate transport was investigated in rat liver microsomal vesicles using radiolabeled compounds and a rapid filtration method. The uptake of both compounds was time- and temperature-dependent, and saturable. Ascorbate uptake did not reach complete equilibrium, it had low affinity and high capacity. Ascorbate influx could not be inhibited by glucose, dehydroascorbate, or glucose transport inhibitors (phloretin, cytochalasin B) but it was reduced by the anion transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid and by the alkylating agent *N*-ethylmaleimide. Ascorbate uptake could be stimulated by ferric iron and could be diminished by reducing agents (dithiothreitol, reduced glutathione). In contrast, dehydroascorbate uptake exceeded the level of passive equilibrium, it had high affinity and low capacity. Glucose *cis* inhibited and *trans* stimulated the uptake. Glucose transport inhibitors were also effective. The presence of intravesicular reducing compounds increased, while extravesicular reducing environment decreased dehydroascorbate influx. Our results suggest that dehydroascorbate transport is preferred in hepatic endoplasmic reticulum and it is mediated by a GLUT-type transporter. The intravesicular reduction of dehydroascorbate leads to the accumulation of ascorbate and contributes to the low intraluminal reduced/oxidized glutathione ratio.

Ascorbate producing and utilizing pathways are connected to the endomembrane system of the cell. The final enzymatic steps of ascorbate synthesis are located in the endoplasmic reticulum of hepatocytes or kidney cells; enzymes utilizing ascorbate (prolyl-3-hydroxylase, prolyl-4-hydroxylase, and lysyl hydroxylase) or its oxidized form dehydroascorbate (protein disulfide isomerase) are characteristic proteins of the lumen (1–3). Their presence in the lumen is necessary for the post-translational modification and folding of many proteins. Since ascorbate and dehydroascorbate are charged water-soluble compounds, transporter(s) should exist for their permeation through biological membranes. Such transporters have been thoroughly investigated in plasma membrane of different cells (4–9) and in chromaffin granula (10), but the transport of ascorbate and dehydroascorbate in microsomes has not been described in detail. The aim of the present study was to detect and characterize the activity of the possible ascorbate and/or dehydroascorbate transporter(s) in the endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Microsomes—Microsomes were prepared from 24-h fasted male Sprague-Dawley rats (180–230 g) as reported (11). Microsomal fractions were resuspended in buffer A (100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS,[1] pH 7.2). The suspensions (60–80 mg of protein/ml) were rapidly frozen and maintained under liquid N_2 until used. Intactness of microsomal vesicles was checked by measuring the latency of mannose-6-phosphatase (12) and *p*-nitrophenol UDP-glucuronosyltransferase activity (13), they were greater than 95% in all the preparations employed. Microsomal protein concentrations were determined by the biuret reaction using bovine serum albumin as standard. To measure microsomal intravesicular water space, microsomes were diluted (10 mg of protein/ml) in buffer A containing $[^3H]H_2O$ (0.2 μCi/ml) or $[^3H(C)]$inulin (0.17 μCi/ml) and centrifuged (100,000 × *g*, 60 min), and the radioactivity associated with pellets was measured to enable calculation of extravesicular and intravesicular water spaces (14).

Uptake Measurements—Liver microsomes (1 mg of protein/ml) were incubated in buffer A containing the indicated amount of ascorbate, dehydroascorbate, or glucose and their radiolabeled analogues (1, 1, and 9 μCi/ml, respectively) at 22 °C. At the indicated time intervals, samples (0.1 ml) were rapidly filtered through cellulose acetate/nitrate filter membranes (pore size 0.22 μm) and filters were washed with 1 ml of Hepes (20 mM) buffer (pH 7.2) containing 300 mM sucrose and 0.5 mM DIDS. The total radioactivity retained by filters was measured by liquid scintillation counting. In each experiment, the pore-forming agent alamethicin (Ref. 15; 0.1 mg/mg protein) was added to parallel incubates to distinguish the intravesicular and the bound radioactivity. The alamethicin-permeabilized microsomes were filtered and washed as above; that portion of radioactivity so released was regarded as intravesicular (16).

Inhibitors used in the experiments (cytochalasin B, phloretin, DIDS, and *N*-ethylmaleimide) were added to the microsomes 30 min before the uptake measurement. The putative competitive inhibitors (ascorbate, dehydroascorbate, and glucose) and $FeCl_3$ were added at the beginning of the uptake measurement. Loading of microsomes (10 mg of protein/ml) was accomplished by incubating them in the presence of the indicated compound for 30 min at 22 °C, then incubates were diluted 10-fold with buffer A containing ascorbate or dehydroascorbate.

Light-scattering Measurements—Osmotically induced changes in the size and shape of microsomal vesicles (17) after the addition of ascorbate or dehydroascorbate (12.5–12.5 mM) were monitored at 550 nm excitation and emission wavelenght by the light-scattering technique as described in detail in an earlier paper (18).

Microsomal Metabolism of Ascorbate and Dehydroascorbate—The ascorbic acid content (reduced and total) of microsomal incubates was measured by high performance liquid chromatography after specific sample preparation as described earlier (19, 20).

Materials—Ascorbate, alamethicin, DIDS, cytochalasin B, *N*-ethylmaleimide, phloretin, and D-[1-^{3}H]glucose (15.5 Ci/mmol) were obtained

* This work was supported in part by grants from Orzágos Tudományos Kutatási Alap and the Ministry of Welfare, Hungary. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a Federation of European Biochemical Societies Short-term Fellowship and an Eötvös Hungarian State Fellowship in Siena. To whom correspondence should be addressed: Dept. of Medical Chemistry, Semmelweis University of Medicine, 1444 Budapest, P. O. Box 260, Hungary. Tel./Fax: 36-1266-2615; E-mail: banhegyi@puskin.sote.hu.

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

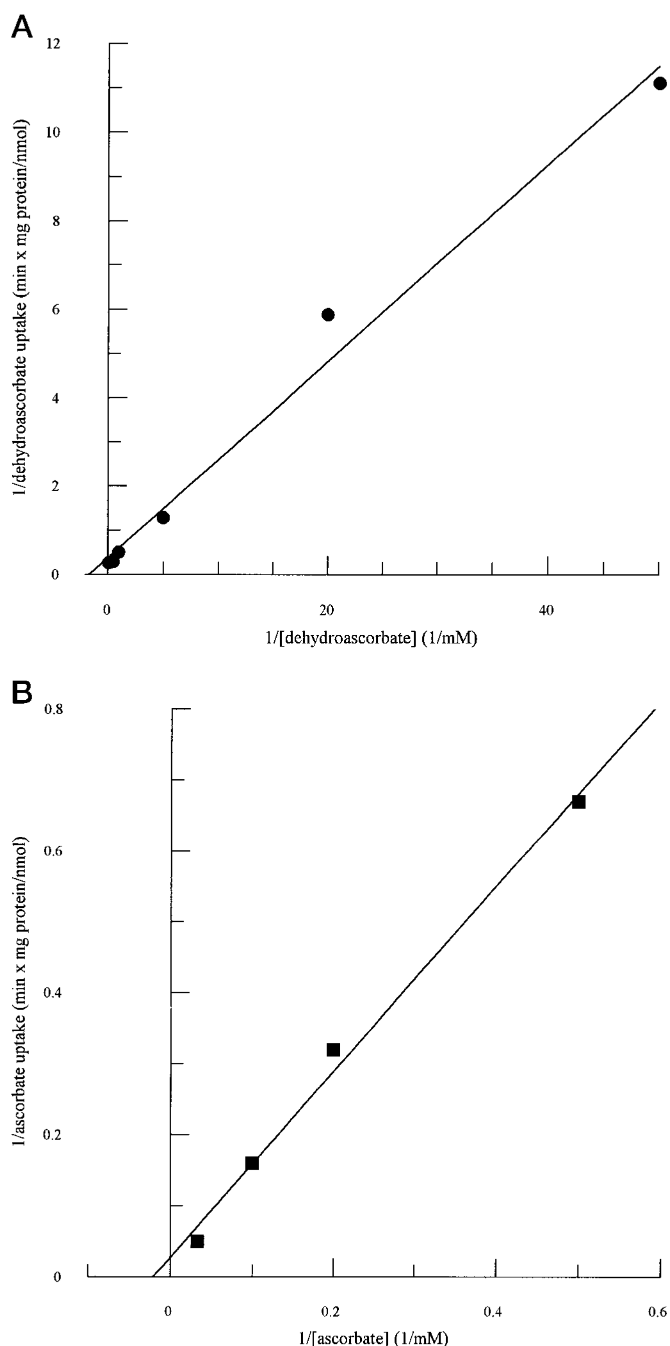

FIG. 1. **Dependence of dehydroascorbate and ascorbate uptake on ligand concentration.** Microsomes (1 mg/ml protein) were incubated in the presence of various concentrations of dehydroascorbate (*a*) and ascorbate (*b*) plus the radioactive tracer for 1 min. After the incubation, 0.1 ml of sample was filtered as described under "Experimental Procedures." Parallel samples were incubated in the presence of the pore-forming alamethicin; the radioactivity associated with these samples were regarded as binding and were subtracted from the total radioactivity associated with the microsomes. Incubations were performed at room temperature (22 °C). Means of four to ten experiments are shown on a double-reciprocal plot.

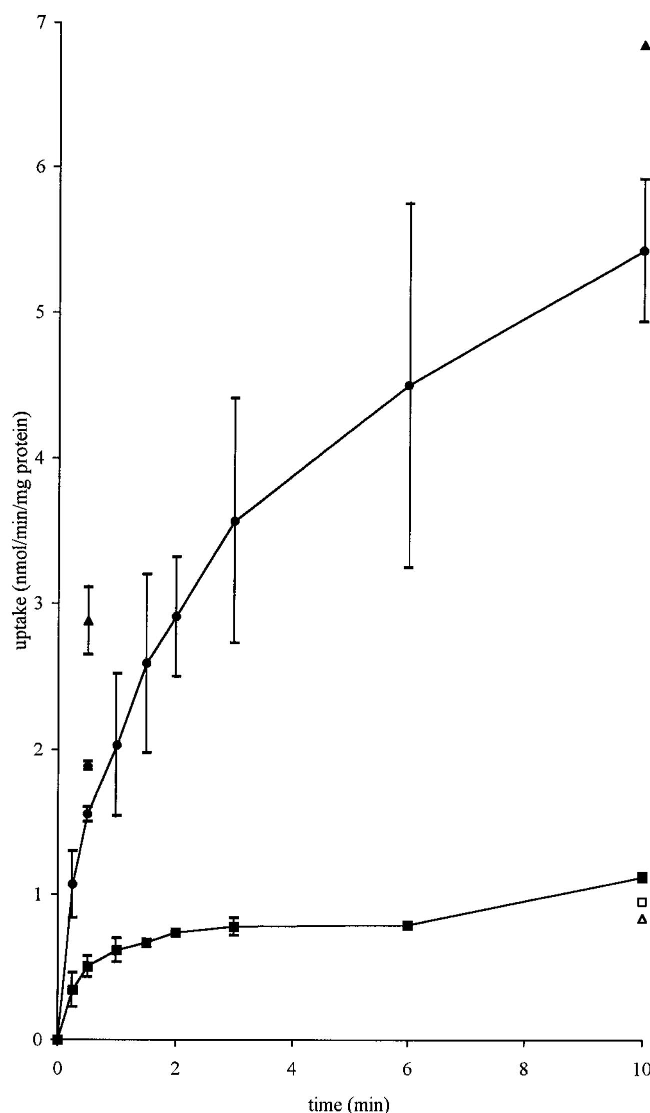

FIG. 2. **Time course of dehydroascorbate and ascorbate uptake in rat liver microsomal vesicles.** Microsomes (1 mg/ml protein) were incubated in the presence of 1 mM dehydroascorbate or ascorbate at 22 °C. The alamethicin-releasable portion of dehydroascorbate (●) or ascorbate (■) associated with microsomes is shown. The effect of reducing compounds (2 mM dithiothreitol, Δ; and 3 mM reduced glutathione, □) on ascorbate uptake is also indicated. To modify the intravesicular environment, microsomes (10 mg/ml protein) were loaded with 1 mM glutathione (▲) or 1 mM glucose (♦) by a 30-min preincubation in the presence of the indicated compound; then they were diluted 10-fold simultaneously with the addition of ascorbate or dehydroascorbate. Data are mean ± S.D. of four to ten or mean of two experiments.

from Sigma. Dehydroascorbate was produced by the bromine oxidation method according to Ref. 21. L-[*carboxyl*-^{14}C]Ascorbic acid (13.7 mCi/mmol) was from Amersham, Buckinghamshire, United Kingdom. Cellulose acetate/nitrate filter membranes were from Millipore. All other chemicals were of analytical grade.

RESULTS

The uptake of dehydroascorbate and ascorbate exhibited different kinetic characteristics with V_{max} values of 3.1 and 37 nmol/min/mg protein and K_m values of 0.7 and 45 mM, respectively (Fig. 1). The time course of the uptake processes showed that dehydroascorbate uptake exceeded the level of the passive equilibrium (3.5 nmol/mg protein; calculated from the intravesicular water space of microsomal vesicles: 3.5 μl/mg protein). The uptake of ascorbate reached only one-third of the level of equilibrium within 10 min (Fig. 2) and did not reach a complete equilibrium even after 1 h incubation (data not shown). Both uptake processes were temperature-dependent (Fig. 3).

The intravesicular accumulation of radioactivity upon dehydroascorbate addition in the absence of any source of energy in the incubation medium, indicated that microsomal metabolism of dehydroascorbate and ascorbate may have affected their transport. Indeed, a slow metabolism of ascorbate (4.0 ± 1.6 nmol/min/mg protein, mean ± S.D., n = 4; predominantly oxidation) and a more evident disappearance of dehydroascor-

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

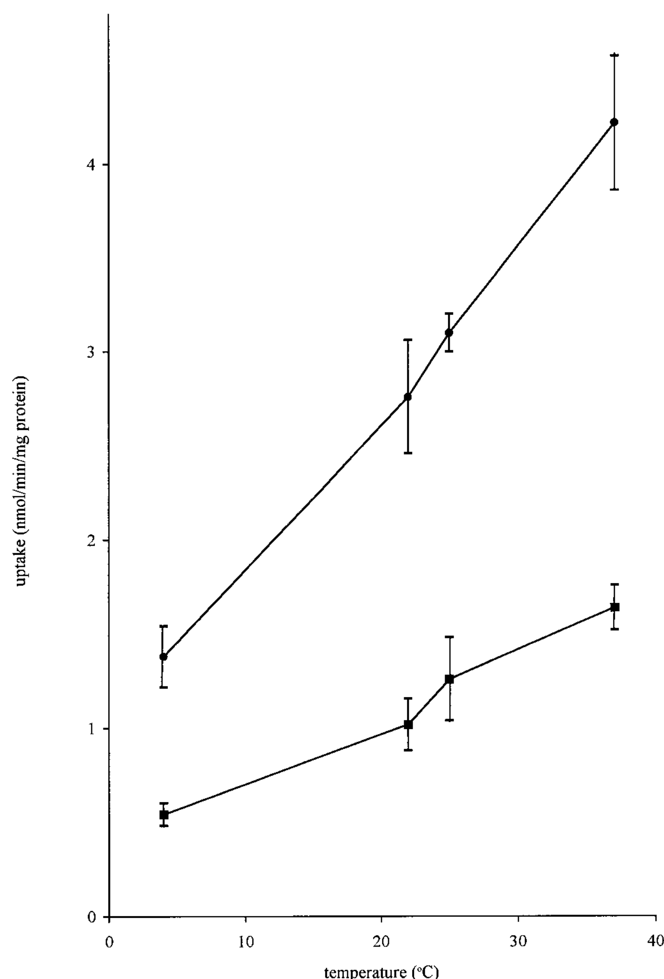

FIG. 3. **Temperature dependence of dehydroascorbate and ascorbate uptake in rat liver microsomal vesicles.** Microsomes (1 mg/ml protein) were preincubated at various temperature for 30 min. Uptake measurements were initiated by the addition of 1 mM ascorbate or dehydroascorbate (plus the radioactive tracer). After 0.5 min incubation 0.1 ml of sample was filtered as described under "Experimental Procedures." The alamethicin-releasable portion of dehydroascorbate (●) or ascorbate (■) associated with microsomes is shown. Data are expressed as mean ± S.D., n = 3–4.

bate due to its instability at neutral pH (17 ± 5 nmol/min/mg protein, mean ± S.D., n = 4) could be observed. A minor fraction of dehydroascorbate was reduced to ascorbate (0.4 ± 0.1 nmol/min/mg protein, mean ± S.D., n = 4.). In accordance with our assumption, reducing compounds (dithiothreitol, reduced glutathione) decreased ascorbate uptake, more evidently after longer incubation (Fig. 2). On the other hand, oxidation of ascorbate by ferric iron stimulated the uptake (Table I). Reduction of dehydroascorbate by extravesicular reducing compounds inhibited its uptake, while reduced glutathione present intraluminally in preloaded vesicles stimulated the influx (Fig. 2). These findings suggest that dehydroascorbate transported into the lumen could be reduced at the expense of reduced glutathione and/or protein thiols; the reduction led to the intravesicular accumulation of ascorbate.

Since GLUT transporters have been reported to mediate dehydroascorbate transport through the plasma membrane, we checked the effect of GLUT inhibitors on ascorbate and dehydroascorbate transport. Phloretin and cytochalasin B inhibited dehydroascorbate but not ascorbate uptake. On the other hand, the anion transport inhibitor DIDS and the alkylating agent N-ethylmaleimide inhibited the uptake of ascorbate more effectively (Table I). Accordingly with the effect of GLUT inhibitors, glucose *cis* inhibited dehydroascorbate uptake (Fig. 4*a*), while from the *trans* side (*i.e.* in glucose-loaded vesicles) it was stimulatory (Fig. 2) suggesting that glucose and dehydroascorbate use the same microsomal transporter. Dehydroascorbate also *cis* inhibited the microsomal glucose uptake (Fig. 4*c*), supporting this assumption.

TABLE I
Ascorbate and dehydroascorbate uptake in rat liver microsomal vesicles

Microsomes (1 mg/ml protein) were preincubated in the presence of various compounds for 30 min except for $FeCl_3$ that was given together with dehydroascorbate or ascorbate. Uptake measurements were initiated by the addition of 1 mM ascorbate or dehydroascorbate (plus the radioactive tracer). After 1 min incubation, 0.1 ml of sample was filtered as described under "Experimental Procedures." Parallel samples were incubated in the presence of the pore-forming alamethicin; the radioactivity associated with these samples were regarded as binding and were subtracted from the uptake. All preincubations and incubations were performed at room temperature (22 °C). Data are expressed as means ± S.D. (n).

Preincubation	Ascorbate	Dehydroascorbate
	uptake (nmol/min/mg protein)	
None	0.65 ± 0.15 (14)	1.89 ± 0.12 (10)
0.1 mM N-ethylmaleimide	0.47 ± 0.01 (3)	1.80 ± 0.36 (3)
5 mM N-ethylmaleimide	0.06 ± 0.06 (3)	0.92 ± 0.43 (3)
0.25 mM DIDS	0.42 ± 0.04 (3)	2.22 ± 0.18 (3)
0.25 mM phloretin	NM[a]	1.46 ± 0.11 (3)
0.25 mM cytochalasin B	0.59 ± 0.16 (6)	0.48 ± 0.07 (5)
2 mM dithiothreitol	0.70 (2)	0.88 (2)
0.03 mM $FeCl_3$	1.74 (2)	NM

[a] NM, not measured.

Ascorbate and dehydroascorbate did not influence the transport of each other (Fig. 4, *a* and *b*). Ascorbate inhibited glucose transport only at high concentrations presumably due to the shrinkage of vesicles (Fig. 4*c*), while glucose did not alter ascorbate influx (Fig. 4*b*).

Light-scattering experiments performed at high (12.5 mM) ligand concentration revealed that ascorbate was taken up by microsomes (for details see Ref. 16) and the permeabilization of vesicles by alamethicin resulted in a further influx. By contrast, dehydroascorbate rapidly entered the vesicles and after alamethicin addition only a minor further influx could be observed (Fig. 5).

DISCUSSION

Ascorbate and dehydroascorbate uptake in the endoplasmic reticulum, similarly to plasma membrane, appears to involve different transporters. Both processes are temperature, time, and microsomal protein dependent, saturable, and inhibitable, indicating that they are mediated by membrane proteins. The uptake of dehydroascorbate is preferred, it has higher affinity and higher velocity in the physiologic range of concentrations. Moreover, ascorbate transport(er) is present only in one-third of the hepatic microsomal vesicles. Since dehydroascorbate can enter virtually all of the vesicles, intravesicular ascorbate accumulation upon dehydroascorbate reduction can occur. A similar mechanism is operative at plasma membrane level; the concerted action of these mechanisms may result in a high ascorbate concentration in the endoplasmic reticulum. The accumulated ascorbate serves as a cofactor of several intraluminal enzymes (prolyl-3-hydroxylase, prolyl-4-hydroxylase, and lysyl hydroxylase), while the reduced glutathione dependent reduction of dehydroascorbate may produce an excess of oxidized glutathione necessary for the oxidation of protein thiols and for protein folding in the lumen (22, 23).

The microsomal metabolism of ascorbate and its influx which can be inhibited by reducing compounds and stimulated by oxidizing agent indicate that the oxidation of ascorbate to dehydroascorbate helps the uptake process. It is an open question

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

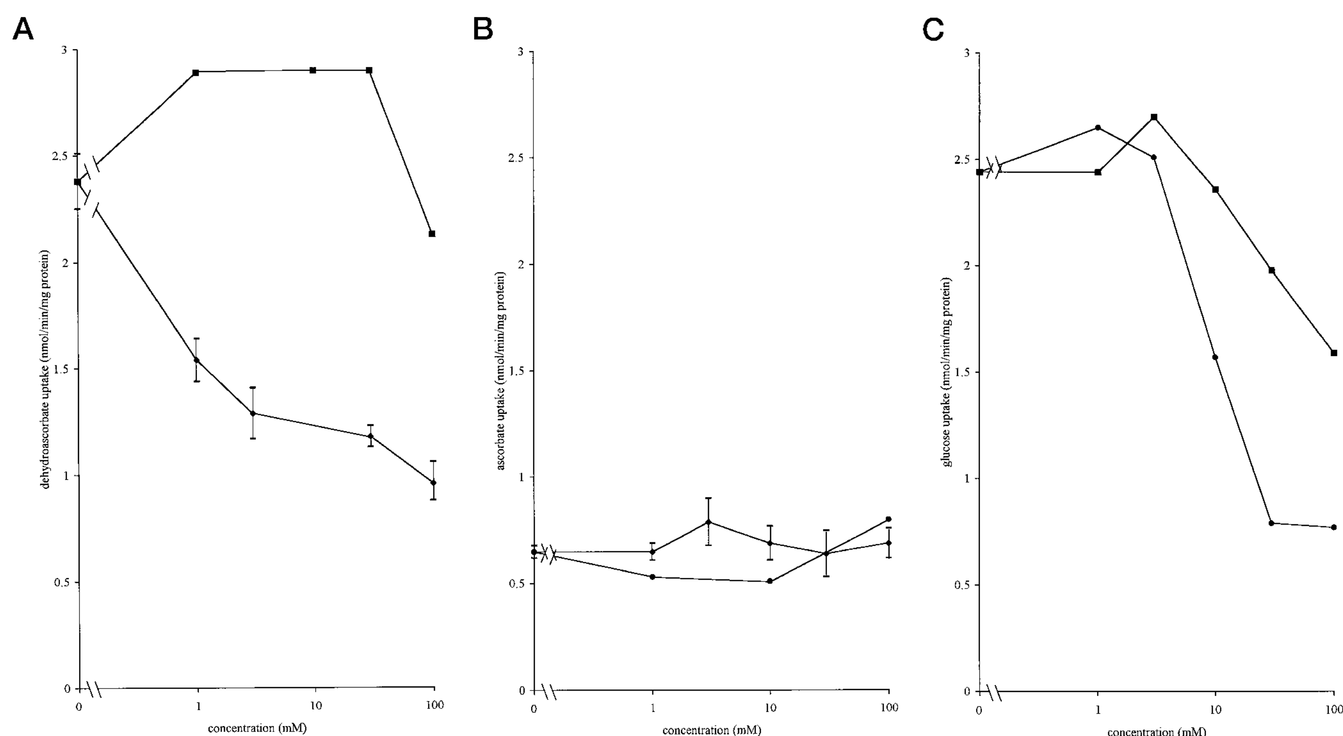

FIG. 4. **Effect of ascorbate, dehydroascorbate, and glucose on the uptake of each other in rat liver microsomal vesicles.** Transport measurements were performed at 1 mM concentration for 1 min. The alamethicin-releasable portion of dehydroascorbate (*a*), ascorbate (*b*), or glucose (*c*) associated with microsomes is shown. Ascorbate (■), dehydroascorbate (●), and glucose (♦) were added in the indicated concentrations. Data are mean ± S.D., n = 4–6, or mean of two measurements.

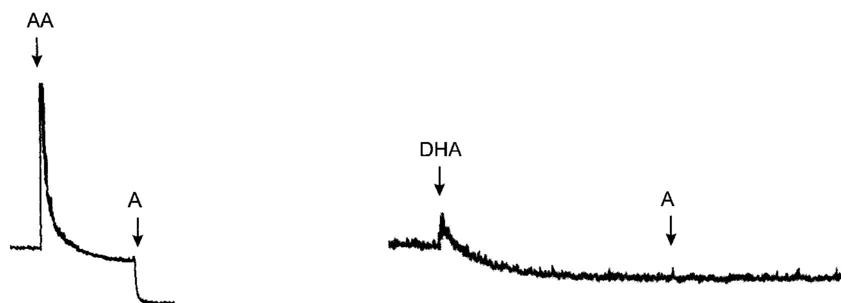

FIG. 5. **Osmotically induced changes in light-scattering intensity of rat liver microsomal vesicles caused by dehydroascorbate or ascorbate.** Light-scattering measurements were performed as described under "Experimental Procedures." Concentrated solutions (0.5 M) of dehydroascorbate (*DHA*) and ascorbate (*AA*) were added resulting in 12.5 mM final concentration. When indicated (*A*), alamethicin (0.1 mg/mg protein) was added. A typical set of experiments out of six is shown.

whether this oxidation is mediated enzymatically (by a putative ascorbate oxidase) which could generate a high local dehydroascorbate concentration.

The results indicate that glucose and dehydroascorbate share the transporter not only in the plasma membrane, but also in the endoplasmic reticulum. GLUT1, GLUT2, and GLUT4 are efficient transporters of dehydroascorbate in the plasma membrane (4). These glucose transporters may also be present in the endomembranes due to vesicular transport and recycling (24); additionally, GLUT7, a microsomal glucose transporter (25, 26) can also mediate the transport of dehydroascorbate in the endoplasmic reticulum. Therefore, dehydroascorbate can be used as a surrogate or as a competitive inhibitor in the investigation of microsomal glucose transport, which is an important question in respect of the topology and mechanism of the glucose-6-phosphatase system (27). Moreover, high glucose levels (*e.g.* in diabetes) can efficiently inhibit the dehydroascorbate transport in the endoplasmic reticulum of hepatocyte and pancreatic β-cell. This effect worsens the intracellular shortage of ascorbate due to the decreased transport through the plasma membrane (6), may contribute to the inhibition of insulin secretion (28), and leads to latent scurvy (29) and elevation of plasma dehydroascorbate level (30).

REFERENCES

1. Rowling, P. J. E., and Freedman, R. B. (1993) in *Subcellular Biochemistry: Endoplasmic Reticulum* (Borgese, N., and Harris, J. R., eds) Vol. 21, pp. 41–80, Plenum Press, New York
2. Levine, M. (1986) *N. Engl. J. Med.* **314,** 892–902
3. Bánhegyi, G., Braun, L., Csala, M., Puskás, F., and Mandl, J. (1997) *Free Radical Biol. & Med.* **23,** 793–803
4. Goldenberg, H., and Schweinzer, E. (1994) *J. Bioenerg. Biomembr.* **26,** 359–367
5. Vera, J. C., Rivas, C. I., Fischbarg, J., and Golde, D. W. (1993) *Nature* **364,** 79–82
6. Ngkeekwong, F. C., and Ng, L. L. (1997) *Biochem. J.* **324,** 225–230
7. Washko, P., and Levine, M. (1992) *J. Biol. Chem.* **267,** 23568–23574
8. Vera, J. C., Rivas, C. I., Velásquez, F. V., Zhang, R. H., Concha, I. I., and Golde, D. W. (1995) *J. Biol. Chem.* **270,** 23706–23712
9. Welch, R. W., Wang, Y., Crossman, A. Jr., Park, J. B., Kirk, K. L., and Levine, M. (1995) *J. Biol. Chem.* **270,** 12584–12592
10. Njus, D., and Kelley, P. M. (1993) *Biochim. Biophys. Acta* **1144,** 235–248
11. Henne, V., and Söling, H. D. (1986) *FEBS Lett.* **202,** 267–273
12. Burchell, A., Hume, R., and Burchell, B. (1988) *Clin. Chim. Acta* **173,** 183–192
13. Fulceri, R., Bánhegyi, G., Gamberucci, A., Giunti, R., Mandl, J., and Benedetti, A. (1994) *Arch. Biochem. Biophys.* **309,** 43–46

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

14. Marcolongo, P., Fulceri, R., Giunti, R., Burchell, A., and Benedetti, A. (1996) *Biochem. Biophys. Res. Commun.* **219,** 916–922
15. Ojcius, D. M., and Young, J. D.-E. (1991) *Trends Biochem. Sci.* **16,** 225–229
16. Bánhegyi, G., Marcolongo, P., Fulceri, R., Hinds, C., Burchell, A., and Benedetti, A. (1997) *J. Biol. Chem.* **272,** 13584–13590
17. Meissner, G. (1988) *Methods Enzymol.* **157,** 417–437
18. Fulceri, R., Bellomo, G., Gamberucci, A., Scott, H. M., Burchell, A., and Benedetti, A. (1992) *Biochem. J.* **286,** 813–817
19. Harapanhalli, R. S., Howell, R. W., and Rao, D. V. (1993) *J. Chromatogr.* **614,** 233- 243
20. Braun, L., Puskás, F., Csala, M., Győrffy, E., Garzó, T., Mandl, J., and Bánhegyi, G. (1996) *FEBS Lett.* **390,** 183–186
21. Del Bello, B., Maellaro, E., Sugherini, L., Santucci, A., Comporti, M., and Casini, A. F. (1994) *Biochem. J.* **304,** 385–390
22. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502
23. Gething, M. J., and Sambrook, J. (1992) *Nature* **355,** 33–45
24. Gould, G. W., and Holman, G. D. (1993) *Biochem. J.* **295,** 329–341
25. Waddell, I. D., Scott, H., Grant, A., and Burchell, A. (1991) *Biochem. J.* **275,** 363–367
26. Waddell, I. D., Zomerschoe, A. G., Voice, M. W., and Burchell, A. (1992) *Biochem. J.* **286,** 173–177
27. Chen, Y. T., and Burchell, A. (1995) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 935–965, McGraw-Hill, New York
28. Bergsten, P., Moura, A. S., Atwater, I., and Levine, M. (1994) *J. Biol. Chem.* **269,** 1041–1045
29. Price, K. D., Price, C. S., and Reynolds, R. D. (1996) *Med. Hypotheses* **46,** 119–129
30. Som, S., Basu, S., Mukherjee, D., Deb, S., Choudhury, P. R., Mukherjee, S., Chatterjee, S. N., and Chatterjee, I. B. (1981) *Metabolism* **30,** 572–577

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

Dehydroascorbate and Ascorbate Transport in Rat Liver Microsomal Vesicles
Gábor Bánhegyi, Paola Marcolongo, Ferenc Puskás, Rosella Fulceri, József Mandl and Angelo Benedetti

J. Biol. Chem. 1998, 273:2758-2762.
doi: 10.1074/jbc.273.5.2758

Access the most updated version of this article at http://www.jbc.org/content/273/5/2758

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 12 of which can be accessed free at
http://www.jbc.org/content/273/5/2758.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 25, 2018