PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 333–337 (1986)

Glucose-Independent Transport of Dehydroascorbic Acid in Human Erythrocytes[1] (42261)

JOHN BIANCHI AND RICHARD C. ROSE[2]

Departments of Surgery and Physiology, The Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, Pennsylvania 17033

Abstract. It has been previously reported that glucose and its structural analogs inhibit dehydroascorbic acid (DHA) transport across the membranes of nonpolar cells, which led to the suggestion that the hexose transporter mediates dehydroascorbic acid transport. The present study examines the role of the erythrocyte hexose transport system in dehydroascorbic acid uptake. We have confirmed that dehydroascorbic acid may be a ligand of the hexose transport system under certain experimental conditions. However, there is an additional pathway of dehydroascorbic acid transport that is uninfluenced by external glucose. This pathway is one of facilitated diffusion, demonstrating saturation kinetics of transport, *cis*-inhibition, and *trans*-stimulation. The K_m for the system is 412 μM. It is suggested that this previously undescribed sugar-independent transporter is the physiologically important route of DHA uptake in erythrocytes. © 1986 Society for Experimental Biology and Medicine.

Vitamin C, in the form of ascorbic acid or dehydroascorbic acid (DHA) is a micronutrient, essential to human health. One mechanism of ascorbic acid accumulation in cells is the uptake of DHA with subsequent reduction to ascorbic acid (1, 2). More rapid erythrocyte uptake of DHA than ascorbic acid seems to support this concept. It was first postulated that DHA uptake into cells may be mediated by the glucose transport system as an insulin load caused a decrease of plasma ascorbic acid levels and an increase of blood cell ascorbic acid content in canine models (3). The role of glucose on DHA transport was later studied in insulin-insensitive human erythrocytes (4). The conclusion of that study was that a hexose transporter is involved in DHA transport. Additional studies indicated that glucose inhibits DHA transport in neutrophils and fibroblasts (5).

It must be considered that D-glucose, a macronutrient present in plasma at >1000 times the DHA level, would effectively compete with DHA for the binding sites of a common transport system. Two distinct possibilities exist that might account for the uptake of both glucose and DHA into erythrocytes at rates that normally occur *in vivo*. One possibility is that a single carrier mediates the entry of both DHA and glucose, as previously suggested. To achieve meaningful rates of DHA uptake in the presence of vastly higher circulating glucose concentrations, the affinity of the hexose carrier system for DHA would have to exceed that for glucose by a large factor, i.e., 100–1000-fold. A second means by which DHA could be taken up into erythrocytes without interference by circulating glucose is if a carrier-mediated pathway exists for DHA that is distinct from the glucose transporter and uninfluenced by the presence of hexose. The present studies were designed to distinguish between these two possibilities.

Materials and Methods. *Cell preparation.* Human erythrocytes were isolated from heparinized venous blood from healthy, 12-hr-fasted adults. Blood was spun in a Beckman J21B centrifuge (rotor No. JA-21) at 850*g*, followed by removal of plasma and the buffy coat. The erythrocytes were washed three times with Ringer (in mM; NaCl, 142; KH_2PO_4, 1.5; K_2HPO_4, 4.2; $CaCl_2$, 1.2; and $MgCl_2$, 1.2; pH 7.0); Ringer and the top layer of cells were discarded after each wash.

Chemicals. All chemicals were reagent grade obtained from commercial sources. Water was deionized and glass-distilled. All buffers were filtered (0.22 μM, Millipore Corp.) prior to use. [1-^{14}C]-L-ascorbic acid and [^{3}H]-D-glucose were obtained from Amersham. Dehydroascorbic acid was prepared by Br_2 oxidation either from nonlabeled ascorbic acid or from

[1] This work was supported by USPHS Grant AM19119.

[2] To whom reprint requests should be addressed.

0037-9727/86 $1.50

Copyright © 1986 by the Society for Experimental Biology and Medicine.
All rights reserved.

[1-^{14}C]-L-ascorbic acid (6) with quality control monitored by HPLC (7). Sodium-free buffer was prepared by substituting KCl for NaCl in Ringer.

Transport measurements. Uptake was determined by tracer flux using a filtration technique (0.45 μM, nitrocellulose, Whatman). All studies were done at 20°C. A 20-μl sample of washed erythrocytes was suspended in 100 μl of phosphate-buffered Ringer with radiolabeled substrate at indicated concentrations. Uptake was stopped by dilution of the reaction mixture with 2.0 ml of ice-cold buffer, then rapid (<2 sec) filtration of the suspension. Filters were washed with an additional 2.0 ml of cold Ringer. Radioactivity on the filters was determined by liquid scintillation counting. After subtracting nonspecific retention of radioactivity by the filters, the counts per minute were expressed as picomoles of substrate. The cell number was determined in a hemacytometer and all uptake values were expressed as picomoles per 10^6 cells. Binding of DHA by the erythrocytes was determined by lysing the cells with 0.2% Triton X-100 to abolish transport. The binding represented no more than 0.23 ± 0.04 pmole DHA/10^6 cells.

Results. The possibility was first considered that DHA has a very high affinity for the hexose transport system in erythrocytes. This evaluation was accomplished by measuring the effect of nonlabeled DHA on the uptake of [^{3}H]-D-glucose present at 10 mmole/liter. Occupation of the hexose carriers by DHA should reduce the uptake rate. As seen in Table I, the presence of DHA at a concentration as high as 130 μmole/liter did not limit uptake of [^{3}H]glucose. Thus, no evidence was obtained to support the concept that DHA has a high affinity for the glucose carrier. Conditions were therefore sought to evaluate in erythrocytes the uptake of DHA that takes place on a sugar-independent transport system.

TABLE I. EFFECT OF DEHYDROASCORBIC ACID ON D-GLUCOSE UPTAKE IN ERYTHROCYTES

DHA Concn. (μmole/l)	D-Glucose uptake (μmole/10^6 cells/5 sec)
0	9.38 ± 0.25
10	9.21 ± 0.27
65	9.25 ± 0.23
130	9.63 ± 0.42

Note. Erythrocytes were suspended in the presence of tracer levels of [^{3}H]-D-glucose with nonlabeled D-glucose added to 10 mmole/liter. Incubations were at 20°C for 5 sec. Nonlabeled dehydroascorbic acid was present at the concentration indicated. Values represent the means ± SE of 15 determinations with cells from three subjects. All values are not statistically different.

TABLE II. THE UPTAKE OF DEHYDRO-L-ASCORBIC ACID AND L-ASCORBIC ACID IN ERYTHROCYTES

	65 μM dehydro-L-ascorbic acid (pmole/10^6 cells/5 sec)	65 μM L-ascorbic acid (pmole/10^6 cells/5 sec)
Control	2.03 ± 0.09	0.92 ± 0.03
22 mM D-glucose	0.88 ± 0.08*	0.67 ± 0.04*
1 mM phlorectin	1.11 ± 0.12*	0.65 ± 0.03*

Note. Washed human erythrocytes were suspended in 150 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.0), and either 65 μM [^{14}C]dehydro-L-ascorbic acid or 65 μM [^{14}C]L-ascorbic acid. Incubations were at 20°C for 5 sec. Additional incubation mixtures contained the above and either 22 mM D-glucose or 1 mM phloretin. The values listed represent the means ± SE of 10 determinations with cells from two subjects.

* $P < 0.001$ with respect to control.

Phloretin (1 mM) or D-glucose (22 mM) inhibited the uptake of both ascorbic acid and DHA (Table II). The degree of inhibition of vitamin C uptake by both compounds was similar. When the uptake of DHA into erythrocytes was measured in the presence of varying levels of D-glucose (Fig. 1), it was observed that the hexose was inhibitory when present at a physiological level of 10 mM ($P < 0.01$). Further, it was noted that 20 mM D-glucose is a maximally inhibitory concentration. Most of the following studies were performed in the presence of a maximally inhibitory concentration of hexose so that the results would indicate the properties of any glucose-independent pathways.

When 65 μM [^{14}C]DHA was added to an erythrocyte suspension in the presence of 22 mM D-glucose, rapid influx of DHA was noted with a steady state being reached by 2 min (Fig. 2). Replacement of Na^+ in the incubation buffer by K^+ did not alter the results.

In order to begin characterizing the transport system for DHA, erythrocytes were in-

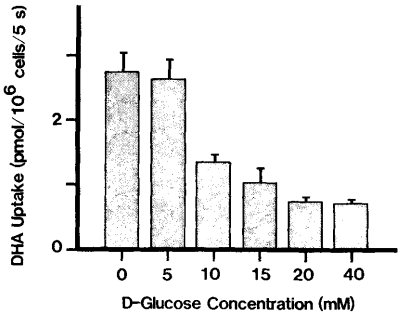

FIG. 1. The effect of D-glucose on the transport of dehydroascorbic acid (DHA). Red cells were incubated at 20°C in Ringer (pH 7.0) containing 65 μM [^{14}C]-dehydroascorbic acid and the indicated D-glucose concentrations. Bars indicate the SEM, and where not indicated are smaller than the symbols used in this and subsequent figures; n = 12.

cubated with 65 μM [^{14}C]DHA in Ringer containing 22 mM glucose, with and without the structural analog, 0.5 mM ascorbic acid. In the presence of glucose alone, DHA uptake was 0.93 ± 0.07 pmole/10^6 cells/5 sec and in the additional presence of ascorbic acid uptake was reduced to 0.58 ± 0.04 pmole/10^6 cells/5 sec. A 37 ± 4% ($P < 0.01$) *cis*-inhibition of DHA transport by ascorbic acid indicates the existence of a distinct DHA pathway. We verified by HPLC (see Materials and Methods) that our preparation did not oxidize ascorbic acid during the brief (5 sec) incubation, assuring that inhibition of [^{14}C]DHA uptake was not brought about by nonlabeled DHA derived from ascorbic acid. Others also report that erythrocytes and leukocytes maintain ascorbate in the reduced state (2, 8, 9).

The occurrence of *trans*-stimulation of DHA transport was investigated by preloading erythrocytes with 0.5 mM ascorbic acid and 0.25 mM thiourea (an ascorbate antioxidant). The hexose carrier was blocked with the nonmetabolizable glucose analog, 3-*O*-methyl-D-glucose. As is shown in Fig. 3, the presence of an in-to-out ascorbic acid gradient caused a 105 ± 5% ($P < 0.01$) increase in the 5-sec uptake of 65 μM [^{14}C]DHA.

When initial uptake of [^{14}C]DHA was determined with respect to external DHA concentration, it was observed that the transport system is saturable. A double reciprocal plot of the data is presented in Fig. 4. The calculated K_m for this glucose-independent transport system is 412 ± 20 μM and V_{max} = 14.0 ± 1.2 pmole/10^6 cells/5 sec (n = 9).

The effect of external glucose concentration on the uptake of 65 and 130 μM [^{14}C]DHA was investigated. The Dixon plot analysis of this data is presented in Fig. 5. The K_i calculated from these lines is 11.1 mM D-glucose, approximately the physiological blood level.

Discussion. The present studies confirmed that dehydroascorbic acid is a ligand of the erythrocyte hexose transporter under condi-

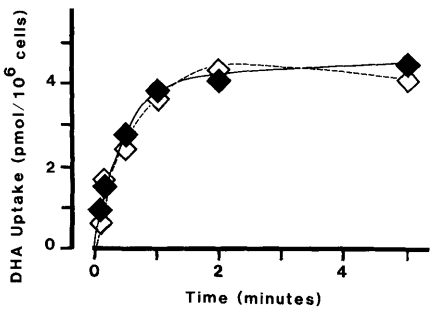

FIG. 2. The time course of DHA uptake into human erythrocytes in the presence of D-glucose. Red cells were suspended with 22 mM D-glucose and 65 μM [^{14}C]DHA in Ringer (◇), or KCl medium (◆); n = 9.

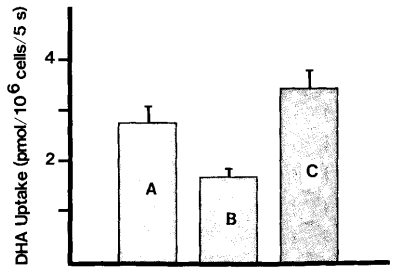

FIG. 3. The effect of preloading erythrocytes with ascorbic acid on the 5-sec uptake of DHA in the presence of a glucose analog. (A) Control erythrocytes were preequilibrated at 20°C in Ringer for 1 hr and then suspended in Ringer that contained 65 μM [^{14}C]DHA. Other erythrocytes (B) were similarly preequilibrated and had 22 mM 3-*O*-methyl-D-glucose present in the incubation mixture. Other erythrocytes (C) were preequilibrated in Ringer that contained 0.5 mM ascorbic acid and 0.25 mM thiourea (an ascorbate antioxidant). These erythrocytes were then incubated as in (B); n = 9.

tions of no- or low-sugar concentrations. These results agree with those previously reported by Mann and Newton (4). However, a condition of such low-glucose concentration in blood is unlikely to develop *in vivo*. Thus, DHA transport on the carrier would likely be restricted unless the carrier has a much higher affinity for DHA than for glucose. Evidence in favor of this possibility was not obtained.

Additional experimentation was designed to make the hexose carrier unavailable for DHA transport by including glucose at the minimal level (20 m*M*) that provides total inhibition. Although this level of glucose cannot be withstood *in vivo* without metabolic consequences, there is no indication that glucose has a direct negative effect on the system of current interest, i.e., the glucose-independent DHA pathway. A specific carrier-mediated mechanism is indicated in that DHA uptake is further inhibited by the presence of an analog in the external medium. Also, an imposed ascorbic acid gradient across the red cell membrane (in > out) results in a doubled rate of DHA uptake. Finally, the transport system that functions in the presence of high concentrations of glucose is saturable as shown in Fig. 4.

This glucose-independent system for DHA transport in human erythrocytes is postulated to be facilitated diffusion in that it demonstrates the characteristics of saturation, *cis*-inhibition and *trans*-stimulation (counterflow). Further, it is noted that physiological levels of D-glucose limit DHA transport on the hexose transporter but that high levels of glucose do not interfere with transport of [^{14}C]DHA through the alternate pathway. Thus, the relevant system *in vivo* may be this previously undescribed glucose-independent pathway. It must be acknowledged that it was necessary to conduct the present studies at concentrations of DHA and glucose somewhat higher than seen *in vivo*. Nonetheless, there is no compelling reason to anticipate that the transport pathway demonstrated would be unavailable to DHA present at a lower concentration or would be blocked by glucose present at physiological levels.

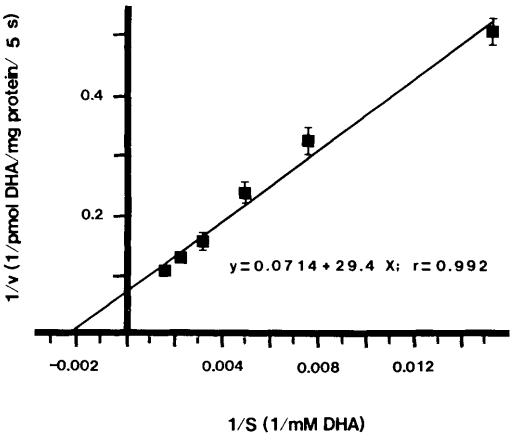

FIG. 4. The effect of external DHA concentration on uptake values. Presented is a double reciprocal plot of data from 5 sec erythrocyte incubations in Ringer that contained D-glucose at 22 m*M* and DHA at 0.065–0.52 m*M*. The apparent kinetic constants are K_m = 412 ± 20 μM and V_{max} = 14.0 ± 1.2 pmole/10^6 cells-5 sec (n = 9).

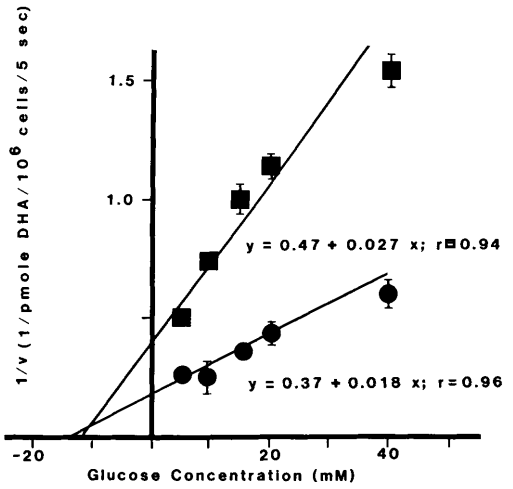

FIG. 5. Dixon plot analysis of glucose inhibition of DHA uptake. Single reciprocal plots relating DHA uptake to external glucose concentration. Erythrocytes were incubated 5 sec in Ringer containing various levels of glucose (5–40 m*M*) and [^{14}C]DHA at either 65 μM (■) or 130 μM (●); n = 11. The calculated K_i = 11.1 m*M*.

1. Hughes RE, Maton C. The passage of vitamin C across the erythrocyte membrane. Brit J Haematol **14**:247–252, 1968.
2. Bigley R, Stankova L. Uptake and reduction of oxidized and reduced ascorbate by human leukocytes. J Exp Med **139**:1084–1092, 1974.
3. Sherry S, Ralli EP. Further studies of the effects of

insulin on the metabolism of vitamin C. J Clin Invest **27**:217–225, 1948.
4. Mann GV, Newton P. The membrane transport of ascorbic acid. Ann NY Acad Sci **258**:243–252, 1975.
5. Bigley R, Wirth M, Layman D, Riddle M, Stankova L. Interaction between glucose and dehydroascorbate transport in human neutrophils and fibroblasts. Diabetes **32**:545–548, 1983.
6. Hughes RE. The use of homocysteine in the estimation of dehydroascorbic acid. Biochem J **64**:203–208, 1956.
7. Bianchi J, Rose RC. The chromatographic separation and radiochemical quantitation of ascorbic acid, dehydroascorbic acid and diketogulonic acid. J Micronutr Anal **1**:3–11, 1985.
8. Hornig D, Weiser H, Weber F, Wiss O. Uptake and release of [I-^{14}C]ascorbic acid and [I^{14}C]-dehydroascorbic acid by leukocytes of guinea pigs. Clin Chim Acta **32**:33–39, 1971.
9. Hornig D, Weber F, Wiss O. Uptake and release of [I-^{14}C]ascorbic acid and [I-^{14}C]dehydroascorbic acid by erythrocytes and guinea pigs. Clin Chim Acta **31**: 25–35, 1971.

Received June 11, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted October 31, 1985.