

THE MEMBRANE TRANSPORT OF ASCORBIC ACID *

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In 1941 Ralli and Sherry observed a diminution of the level of plasma ascorbate in dogs following the administration of insulin.^{1, 2} This effect was confirmed in diabetic animals³ and in human beings.⁴ Since neither the urinary ascorbate nor metabolic products were increased by this treatment with insulin, the effect was presumed to be a result of accelerated tissue uptake. The problem was left there until Pauling's contention that the human requirement for ascorbate may be much larger than the officially defined requirement⁵ led us to reconsider Ralli's findings.

We have formulated two hypotheses.⁶ The first proposes that the transport of ascorbate across cell membranes may be impaired by glucose. The second proposes that the transport of ascorbate in certain tissues is facilitated by insulin. If either hypothesis is valid, those species requiring exogenous ascorbate would be in double jeopardy if they were also hyperglycemic. Carbohydrate intolerance resulting from either a lack of or a resistance to insulin is common in Western man.⁷ Gore et al.⁸ have shown with electron microscopy that the vascular lesion of scurvy involves collagenous structures in the basement membranes, and this is also the site of the lesion in diabetic microangiopathy.⁹

These hypotheses, which propose that the intracellular availability of dehydroascorbate (DHA), the transportable form of vitamin C, would be impaired in certain tissues by either hyperglycemia or lack of insulin, suggest that diabetic microangiopathy, the main complication of human diabetes, may be a consequence of local ascorbate deficiency. The laboratory investigations described here deal with the first and somewhat simpler of these hypotheses: Glucose will impair the transport of dehydroascorbate into cells. The data collected show that D-glucose does inhibit the transport of dehydroascorbate into human red blood cells, a noninsulin-dependent tissue. Trials with other sugars show a hierarchy of sugars that inhibit transport, suggesting that DHA and D-glucose share a carrier mechanism.

Methods

The studies were done with human red blood cells obtained from a non-diabetic adult male. Venous blood was drawn into heparin (10 u/ml), the plasma and buffy coat were separated, and the cells were washed three times in equal volumes of 0.15 M sodium chloride. They were suspended in nine volumes of saline-phosphate buffer, pH 6.0, made by diluting 0.1 M phosphate

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buffer 1 to 10 with 0.15 M sodium chloride. The ^{14}C ascorbate (AA) used was obtained from New England Nuclear Corporation (4–10 mCi/mM). Dehydroascorbates (DHA) were prepared from this by stoichiometric oxidation with benzoquinone.¹⁰ The radioascorbic acid and radiodehydroascorbic acid were stored in frozen aliquots.

The reaction tube contained 500 μl of a mixture of buffer-saline and the substrate to which were added 500 μl of the cell suspension at time zero. All solutions were preequilibrated at 15°C and adjusted to 305 mOsm/liter. The reaction was terminated by withdrawing 200 μl of the reaction mixture with a precooled Eppendorf pipette and expelling this into five volumes of ice-cold Stein's solution containing HgCl_2 , 2.0 mM, and KI, 0.8 mM.¹¹ The cell suspension was centrifuged for 10 minutes in the cold, the supernatant was aspirated and discarded, and the cell pellet was washed again with cold Stein's solution.

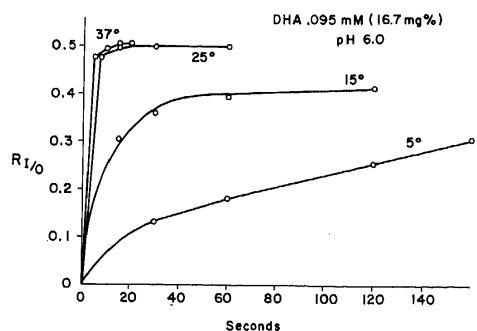


FIGURE 1. The effect of temperature on the transport of dehydroascorbate into human red blood cells. Quench-background corrected dpm of TCA extract of cells wash with cold SS. $\frac{\text{QBdpm/ml ICW}}{\text{dpm/ml ECW}} = R^{1/0}$. ICW is 0.7 of RBC volume. Steins' Stopper allows $\leq \pm 5\%$ of leakage. Transport is assumed rate-limiting process.

The packed cells were hemolyzed by stirring in 1 ml of distilled water, 1 ml of 10% trichloroacetic acid being added to the hemolysate. After centrifuging, the radioactivity of 1 ml of the supernatant was counted with 10 ml of Aquasol[®] ‡ solution using a channel ratio quench correction.¹²

The rate of transport of substrate was calculated as the ratio of the $\mu\text{M/l}$ of radioascorbate found in the intracellular water to the concentration in extracellular water. The use of raffinose to measure occluded extracellular water was not found necessary with the washing system used. The red cells were assumed to contain 70% water. The change of extracellular concentrations of substrate with time was not accounted for in the calculation since the intracellular space was only 3.5% of the total reaction volume.

‡ New England Nuclear Corporation scintillation mixture.

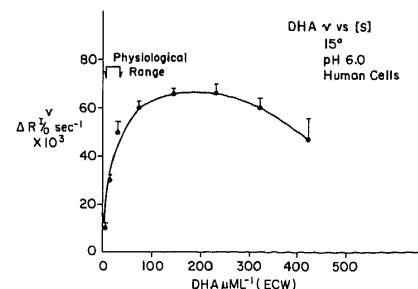


FIGURE 2. The effect of concentration of dehydroascorbate on the transport rate.

Results

The speed of the reaction made the use of 15°C a convenient reaction temperature (FIGURE 1). The uptake of DHA is much faster than that of AA, as was described by Hughes and Maton¹³ and Martin and Mecca.¹⁴ The temperature coefficient of the reaction expressed as Q_{10} , averages 2.64. The instability of DHA at physiological pH and the tendency of red cells to hemolyze at low pH led to the use of pH 6.0.

The rate of transport with increasing concentration of DHA indicates an hyperbolic kinetic relationship (FIGURE 2). However, there is evidence of substrate inhibition at concentrations of DHA above 200 μM so that the derivation of the kinetic constants by graphic analysis is of limited use (FIGURE 3).

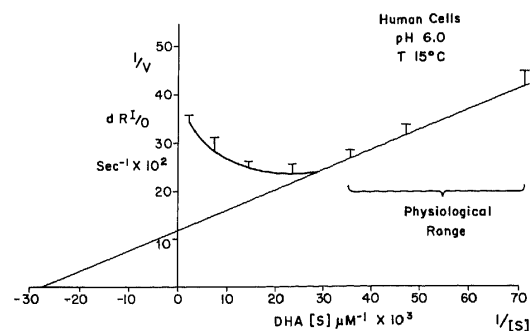


FIGURE 3. A Lineweaver-Burk plot of dehydroascorbate transport into human erythrocytes.

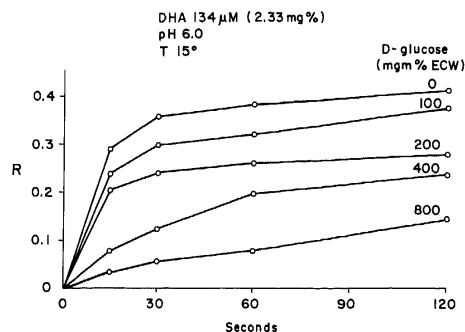


FIGURE 4. The impairment of transport of dehydroascorbate with increasing glucose concentrations.

The effect of adding glucose over the range of 5.5 to 44 mM (equivalent to 100 to 800 mg%) is shown in FIGURE 4. Glucose strongly inhibits the rate of uptake of dehydroascorbate. The results with twelve other monosaccharides examined are shown in TABLE 1. These can be divided into three groups. One group, including D-mannose, D-xylose, and D-galactose, like D-glucose, strongly inhibits the red cell uptake of DHA. Somewhat less inhibitive than glucose itself is 3-O-methyl D-glucose ($p < 0.001$). A second group, which includes the

TABLE 1
EFFECT OF SUGARS ON THE RATE OF TRANSPORT OF 14 C DEHYDROASCORBATE INTO HUMAN RED BLOOD CELLS *

Added Sugars (22 mM)	Relative Transport Rate †
D-glucose	0.20
D-mannose	0.30
3-O-methyl D-glucose	0.35
D-xylose	0.35
D-galactose	0.45
L-xylose	0.65
L-arabinose	0.76
dehydro D-araboascorbic	0.76
L-sorbose	0.77
2-deoxy D-ribose	0.80
D-fructose	1.10
D-ribose	1.16
D-arabinose	1.18

* 15° C pH 6.0, 305 mOsm, 93.1 mM DHA in ECW.
† Ratio of transport rate with additive to rate without.

TABLE 2
DEHYDRO-D-ARABOASCORBATE—EFFECTS ON DHA TRANSPORT *

Additive	Concentration (mM)	Concentration (mg%)	Relative Transport Rate
D-glucose	44	800	0.16
Dehydro-D-araboascorbate	0.11	2	1.00
	0.44	8	0.90
	0.88	16	0.81
	5.5	100	0.63
	11	200	0.57
	22	400	0.53
	44	800	0.49

* Temperature 15° C, pH 6.0, human red cells, DHA 93.1 μ M.

pentoses, D-xylose, L-arabinose, and 2-deoxy-D-ribose, exerts a smaller inhibition on the transport of DHA and a third group does not impair and may accelerate transport. Oxidized isoascorbic, which is dehydro-D-araboascorbic and differs from the vitamin only in the configuration at C5, has little or no vitamin activity and has a small but definite effect on transport. It is of interest because of its wide use as a food additive. While isoascorbate does not impair the transport of DHA at the low levels attainable in serum, it does have a measurable effect at high levels (TABLE 2).

The effects of several compounds known to inhibit other transport systems are shown in TABLE 3. These are of interest because of the contrast of their

TABLE 3
EFFECT OF VARIOUS "INHIBITORS" ON THE TRANSPORT OF DEHYDRO-L-ASCORBATE INTO RED BLOOD CELLS

Compound *	Primary (Wilbrandt)		Glucose Effect
	Concentration (mM)	Inhibition	
Phloretin	0.02-0.2	50%	+
NEM	0.005-0.032	75%	+
PCMB	1.0-8.5	80%	+
2,4 DNFB	0.25-2.0	70%	+
Hg Cl ₂	5-50	50%	+
I ₂	0.2-1.7	0	+
	Secondary		
NaF	6-46	0	0
Ouabain	0.5	0	0
2,4 DNP	5-42	0	0

* 2,4 DNP=2,4 dinitrophenol, NEM=N-ethyl maleimide, PCMB=p-chloromercuribenzoate, and 2,4 DNFB=2,4 dinitrofluorobenzene.

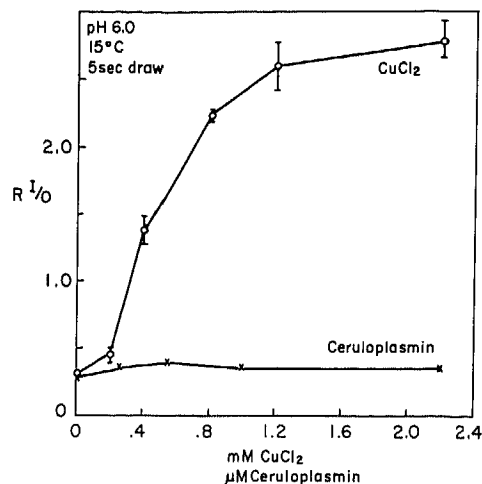


FIGURE 5. The effect of copper on the transport of dehydroascorbate into human erythrocytes.

effects on the transport of dehydroascorbate and of glucose.¹⁵ When increasing levels of cupric ion were added to the transport reaction a stimulation of transport of DHA was observed (FIGURE 5). The maximal effect at about 1.5 mM may be within the range of level of copper attainable in the blood. It is interesting that the effect of copper diminishes in aging cells (FIGURE 6). The transport rates of stored red cells for DHA also diminishes with time (FIGURE 7). We have not used cells more than three days after drawing.

Ceruloplasmin supplying up to 0.2 mM copper ion could not be shown to stimulate transport *in vitro*. The presence of copper ion also increases the total amount of DHA transported, so that a net concentration of ascorbate occurs, amounting to 3–5 times the concentration in the extracellular water. This is an exceptional behavior for facilitated diffusion to accomplish a concentration gradient. This effect of copper persists in the presence of glucose so that the ascorbate is accumulated in the cell even in the presence of 44 mM glucose.

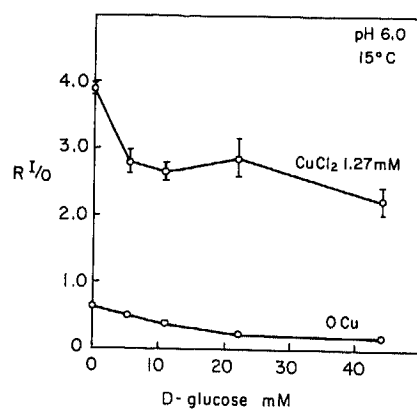


FIGURE 6. The impairment by glucose of transport of dehydroascorbate into erythrocytes in the presence and absence of copper.

However, the glucose does measurably slow transport even in the presence of copper. Since radioascorbate once incorporated in the cell does not readily leak out, the reduction of DHA to AA inside the cell may act as a trapping mechanism to account for the concentration gradient. Conversion of oxyhemoglobin to carboxyhemoglobin did not influence the effect of copper.

Discussion

The structure-activity relationships of monosaccharides to facilitated transport of glucose have been extensively studied.¹⁶ The hierarchy of potency for competition with transport of D-glucose is D-galactose = D-mannose > L-arabinose = D-xylose > D-fructose = L-sorbose, which resembles the relationship described here for DHA transport. That similarity suggests that these saccharides use a common transport mechanism. However, we have seen two differences. The DHA system is much less sensitive to PCMB and to mercury than is the glucose system, and the ability of human cells to transport DHA diminishes

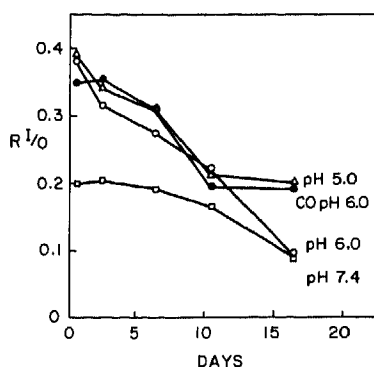


FIGURE 7. The effect of aging of erythrocytes at several pH levels on the transport of dehydroascorbate.

with aging of the cells *in vitro*. Previous transport studies with sugars have often been done with overaged blood bank cells. The mechanism of this impairment of DHA transport with cell age is unknown. It is not changed by converting the hemoglobin to carboxyhemoglobin. The effects of copper suggest that the limiting reaction in transport of DHA may be the disposition of intracellular DHA rather than the membrane translocation.

Whatever the mechanism, these findings have implications for the function of vitamin C. Species that require dietary vitamin C must also have an appropriate mechanism for transporting this into cells. The present data suggest that hyperglycemia, of whatever cause, may compromise the intracellular supply of vitamin C. While several sugars have such an effect, only glucose impairs at levels that are often found in the blood.

The histopathologic manifestations of deficiency produced by locally-impaired transport may be different from those produced by a deficient extracellular supply of vitamin C. Thus, the lesions of impaired transport may not be those of classic scurvy. This would be a more probable consequence if it were shown that different cell types have differing transport facility for DHA.

The human red cell is known to have a unique persistence of fast, "fetal-type" monosaccharide transport.¹⁷

Since the renal threshold for AA is low,¹⁸ correction of impaired transport by raising plasma levels of AA will be ineffectual. The effect of increasing the level of DHA in overcoming the inhibition of glucose was small in these trials because DHA is itself inhibitory at high concentrations. Furthermore, DHA is a potent pharmacologic substance¹⁹ and cannot safely be given in quantity. The observation that copper ion will accelerate transport *in vitro* offers another approach to facilitating the transport of DHA since some drugs, e.g., phenylhydantoin, will markedly increase the level of serum copper in human beings.²⁰

Summary

A system for measuring the rate of transport of dehydroascorbate into human red blood cells shows Michaelis-Menten type kinetics with substrate inhibition at levels above 150 μ M DHA. The addition of sugars impairs this transport in the diminishing hierarchy D-glucose, D-mannose, D-xylose, D-galactose, L-lyxose, D-araboascorbate, L-sorbose and 2-deoxy-D-ribose. The effect of glucose on transport of ascorbate is marked at physiological levels. Transport of DHA is accelerated by copper ion and allows dehydroascorbate to move against a concentration gradient. The evidence supports the hypotheses proposing that hyperglycemia will impair the intracellular availability of vitamin C.

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DISCUSSION

DR. WAGNER: Have you looked at the effect of sucrose?

DR. MANN: No, but we have looked at fructose.

DR. H. SPRINCE: It is surprising that alcohol has a hypoglycemic effect.

DR. MANN: It does have a hypoglycemic effect, but I suppose that was for different reasons than transport.

DR. W. B. SMITH: How did you preserve the red cells when they were 48 hours old?

DR. MANN: The cells were drawn, washed, and stored in a phosphate-saline buffer.

DR. SMITH: You can store them, but they leak within 24 hours of storage.

DR. MANN: Maybe this is the reason for this aging effect. I think it is unfortunate that much of the glucose transport work has been performed with overaged cells, which surely must be leaky. But, we see no real effect in the first 3-4 days of storage of washed cells at 5° C.

DR. N. R. STEVENSON (*Rutgers Medical School, Piscataway, N.J.*): I agree with you completely on your questioning of your third hypothesis, and I question whether you can even speak of transport of dehydroascorbic acid per se, considering that conversion does occur within the cell. Second, there is a difference between the membranes of various tissue organs in their translocation capacity for L-ascorbic acid vs. dehydroascorbic acid. The gut, for instance, does not move dehydroascorbic acid to any degree, whereas red blood cells and polymorphonuclear leukocytes take it up.

DR. MANN: We are talking about the movement of the material from the extra- to the intracellular compartment. So, we are talking about transport. The point is that I'm not sure that the conversion of dehydroascorbate to ascorbate inside the cell is, indeed, the limiting factor in this phenomenon. Other tissues, for example, the retina, have an energy-dependent transport of dehydroascorbic. I am speaking here of human red blood cells.

DR. C. W. M. WILSON: We looked at this in the white cells and also in

the red cells, but in the white cells, we have shown that as people age, the saturation limit for ascorbic acid absorption diminishes.

DR. A. E. KITABCHI: What percentage of total ascorbic acid in human plasma and in white blood cells is dehydroascorbic acid?

DR. MANN: It's perhaps of the order of 10%. If the total ascorbate level is of the order of 0.8–1.2 mg%, there will be less than 0.1 mg% of dehydroascorbic acid.

DR. KITABCHI: Do you have data to support the thesis that dehydroascorbic acid is transported rather than stuck on the membrane?

DR. MANN: These cells are drawn and washed, and then hemolyzed, deproteinized, and the supernatant discarded, so I would concede that the dehydroascorbate may be in or on the membrane.