Stereospecific Effect of Ascorbate Isomers on *p*-Hydroxyphenylpyruvate Oxidase[1] (34726)

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Nutrition experiments with guinea pigs have shown that dietary D-araboascorbic acid (isoascorbic acid) may be able to substitute for L-xyloascorbic acid (vitamin C) but at appreciably higher concentrations (1, 2). This would indicate that there is a stereospecificity in some of the biochemical reactions involving ascorbic acid. Kutnink, *et al.* (3) have studied the efficacy of the four stereoisomers on the hydroxylation of proline, and found that all the isomers acted equally well. A number of other workers have measured the relative efficacy of L-xylo and D-araboascorbic acid as a cofactor for specific enzymic systems and failed to show an appreciable stereo effect (4, 5). Typical of this are the studies of LaDu and Greenberg (6) or Knox and Knox (7) who found no difference between L-xylo and D-araboascorbate as cofactors for *p*-hydroxyphenylpyruvate (pH PP) oxidase.

In this study, we have measured the effect of the four isomers of ascorbic acid on the enzymatic conversion of *p*-hydroxyphenylpyruvate to homogentisate at various concentrations and calculated $K'_{ascorbate}$ and V'_{max} values from the data.

Methods. Purification of p-hydroxyphenylpyruvate oxidase. pHPP oxidase was purified from frozen canine liver by the technique of LaDu and Zannoni (8). The final 55 to 75% saturated ammonium sulfate precipitate from 300 g of liver was dissolved in 200 ml of 0.01 *M* sodium phosphate buffer, pH 6.5. This solution was dialyzed three times against 1 liter of the buffer and frozen. A precipitate which occurred after dialysis and upon thawing was removed by centrifugation before assays with the enzyme. The oxidase had a specific activity of 1.92 μmoles of pHPP oxidized /ml of oxidase /10 min when analyzed using a pHPP concentration of 1.43×10^{-4} *M*, no ascorbate and a pHPP molar extinction coefficient of 9850.

Purification of p-hydroxyphenylpyruvate keto–enol tautomerase. The pHPP tautomerase was purified from fresh porcine kidneys according to the technique of Knox (9). The final precipitate from 360 g of kidney was dissolved in 120 ml of 0.57 *M* sodium borate buffer, pH 8.0, dialyzed against three 1 liter batches of the same buffer and frozen. It had a specific activity of 2.0 k units/ml, as defined by Knox (4). Two-tenths ml of the tautomerase was found to be nonlimiting in the pHPP oxidase assay.

p-hydroxyphenylpyruvate oxidase assay. pHPP oxidase activity was determined by the continuous spectrophotometric assay of Lin *et al.* (10). Two-tenths ml of the tautomerase, 0.4 ml of the oxidase, ascorbic acid solution, pHPP solution, and 0.57 *M* sodium borate buffer (pH 8.0) to give a total volume of 3.5 ml were used in the assay. pHPP and ascorbic acid were dissolved in the borate buffer. Both solutions were made fresh just prior to each determination by dissolving the solid. The tautomerase was added to the required amount of pHPP solution and the mixture allowed to equilibrate for about 5 min at room temperature before beginning the reaction by adding the oxidase. The reaction was run at 25° in a Cary 16K recording spectrophotometer. The initial slope of the reaction was determined graphically. Up to five samples were run at one time. In all cases a control of L-xyloascorbic was run si-

[1] Supported by NSF Grant No. GY 5369 and by U.S. Army, Surgeon General's Office Contract No. DA-49-193-MD-2611.

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multaneously, since the observed difference between ascorbate isomers were smaller than those due to different batches of enzyme.

Determination of Michaelis constants for the ascorbic acids. The K'_{asc} values were determined at a pHPP concentration of 1.6×10^{-5} M. Ascorbate concentrations of 1×10^{-4}, 2×10^{-4}, 4×10^{-4}, and sometimes 8×10^{-4} M were used and the K' values obtained from a Lineweaver-Burke plot. V'_{max} values were also calculated from these graphs.

Ascorbate isomers. L-Xyloascorbic acid (vitamin C) and D-araboascorbic acid were commercial materials, checked for purity by thin-layer chromatography (3). D-Xyloascorbate acid and L-araboascorbic acid were prepared by alkaline isomerization of the no. 4 carbon and are the same material reported by Kutnink *et al.* (3). All compounds were pure within the limits of the assay procedure. UV absorption spectra of the four samples in water gave nearly identical absorption maximum at 265 nm with values of 9900, and no other peaks to 190 nm.

Results and Discussion. The Michaelis constants and V'_{max} values for the four stereoisomers of ascorbic acid are given in Table I. Individual and average data are presented and compared to the L-xyloascorbate value which was obtained simultaneously. The rate curves and Lineweaver-Burke plots followed good straight lines. D-xyloascorbic acid has the lowest K'_{asc} value and hence the highest enzyme–substrate affinity. L-xyloascorbic acid (vitamin C) and L-araboascorbic acid have similar intermediate K'_{asc} values. D-Araboascorbic acid, the isoascorbic acid of commerce, has the highest K' value. The differences in K'_{asc} values given here are small, but definite. Each experiment was repeated at least three times and always against a control of L-xyloascorbic acid. In all cases the effects and relative direction of effects were the same. These small changes are not likely due to impurities or concentration differences. The direction of change also is not consistent with possible impurities (*i.e.*, L-arabo contaminated with a few percent of L-xylo and D-xylo contaminated with D-arabo).

V'_{max} values vary less than K'_{asc} values, increasing slightly as K'_{asc} increases. Although there is a definite trend here, it is doubtful how significant this change is.

A kinetic model for the pHPP oxidase reaction has not yet been developed (11, 12). We made studies on most of the variables in this system and obtained complex results. In general pHPP concentrations about one-tenth those of ascorbate were required to provide straight Lineweaver-Burke plots. The determination of a K_{pHPP} for these conditions is not reported because the data were

TABLE I. K'_{asc} and V'_{max} Values for *p*-Hydroxyphenylpyruvic Acid Oxidase at a pHPP Concentration of $1.6 \times 10^{-5} M$.

Compound	Data for two analyses			Simultaneous L-xyloascorbate value	
	V'_{max}	K'_{asc}		V'_{max}	K'_{asc}
D-Xyloascorbate	0.22	1.8		0.25	3.5
	0.25	3.1		0.27	4.3
Av	0.24	2.5		0.26	3.9
L-Araboascorbate	0.26	2.9		0.26	2.9
	0.27	4.6		0.27	4.1
Av	0.27	3.8		0.27	3.5
D-Araboascorbate	0.26	6.2		0.27	3.2
	0.27	6.9		0.29	5.9
Av	0.27	6.7		0.28	4.5
L-Xyloascorbate			Av	0.27	4.0

not consistent and did not follow simple Michaelis kinetics under the conditions used. However, the rate of oxidation of pHPP as a function of pHPP concentration did not change significantly using different ascorbate isomers.

The K'_{asc} values were found to vary with enzyme concentration. Increasing the enzyme concentration decreased the K' values. At very high enzyme concentrations K'_{asc} values could not be obtained using ascorbate concentration of 10^{-3} to 10^{-4} M. Direct comparison of the K'_{asc} values in Table I with those reported by Wood and Zannoni (11) is not possible as our values have not been extrapolated to zero enzyme and infinite substrate concentrations. However, it would appear that the values for L-xyloascorbate would be comparable.

D-Xylo and D-araboascorbate differ only in that the alkyl side chain of the ascorbate are on opposite sides of the lactone ring: The K'_{asc} values also show the maximum difference for this pair. The difference between K'_{asc} for the two isomer pairs in which only the configuration of the no. 5 carbon is changed are comparable but in opposite directions. We do not see a relationship between stereo structure and function from these data. The data presented here indicate a stereo selectivity both for the configuration of the nos. 4 and 5 carbons, although that for the no. 5 carbon is the lesser.

The levels of ascorbate used in these experiments are comparable to average physiological levels in mammals. Small effects on *in vivo* rates for this enzyme should exist between L-xylo and D-araboascorbate. These effects should be overcome by a smaller increase in isoascorbate levels than the 5- to 20-fold increases observed in nutrition requirement studies. It appears that this enzyme system is not the site of observed nutritional abnormalities noted when isoascorbate is substituted for vitamin C.

Summary. The kinetic effect of the four stereoisomers of ascorbic acid upon the activity of *p*-hydroxyphenylpyruvate oxidase was studied. Average Michaelis constants for the four stereoisomers, obtained at a *p*-hydroxyphenylpyruvate concentration of 1.6×10^{-5} M, were: D-xyloascorbate, 2.5×10^{-5} M; L-araboascorbate 3.8×10^{-5} M; and L-xyloascorbate, 4.0×10^{-5} M; and D-araboascorbate, 6.7×10^{-5} M. V'_{max} values were very similar. The small changes in K'_{acs} demonstrate an ascorbate stereospecificity in the pHPP oxidase reaction, but it does not appear large enough to explain known nutritional effects.

The authors thank Dr. Frank Sullivan of Fitzsimons General Hospital for supplying the canine livers and Dr. E. M. Baker for helpful discussions. Porcine kidneys were kindly supplied by Cudahy Packing Plant, Denver, Colorado.

1. Fabinek, J., and Herp, A., Proc. Soc. Exp. Biol. Med. **125**, 462 (1967).

2. Reiff, J. S., and Free, A. H., J. Agr. Food Chem. **7**, 55 (1959).

3. Kutnink, M. A., Tolbert, B. M., Richmond, V. L., and Baker, E. M., Proc. Soc. Exp. Biol. Med. **132**, 440 (1969).

4. Hutton, J. J., Tappel, A. L., and Udenfriend, S., Arch. Biochem. Biophys. **118**, 231 (1967).

5. Levin, E. Y., Levenberg, B., and Kaufman, S., J. Biol. Chem. **235**, 2080 (1960).

6. LaDu, B. N., and Greenberg, D. M., Science **117**, 111 (1953).

7. Knox, W. E., and Knox, M. L., Biochem. J. **49**, 686 (1951).

8. LaDu, B. N., and Zannoni, V. G., J. Biol. Chem. **219**, 273 (1956).

9. Knox, W. E., *in* "Methods in Enzymology" (S. Colowick and N. O. Kaplan, eds.), Vol. 2, pp. 288. Academic Press, New York (1955).

10. Lin, E., Pitt, B. M., Civen, M., and Knox, W. E., J. Biol. Chem. **233**, 668 (1958).

11. Wood, A., and Zannoni, V. G., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **28**, 408 (1969) (Abstract).

12. LaDu, B. N., and Zannoni, V. G., Ann. N. Y. Acad. Sci. **92**, 175 (1961).

Received Sept. 22, 1969. P.S.E.B.M., 1970, Vol. 134.