Efficacy of the Ascorbic Acid Stereoisomers in Proline Hydroxylation *in Vitro*[1] (34232)

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The nutritional requirement for vitamin C (L-xyloascorbic acid) in the development of connective tissue has long been recognized. Part of this requirement has been ascribed to the role of ascorbate in the hydroxylation of peptidyl proline in the biosynthesis of collagen. An essential role of L-xyloascorbate in this reaction for the enzymic reaction is counter indicated by the work of Hutton *et al.* (1) who found D-araboascorbate can substitute for the natural isomer. On the other hand Priest and Bublitz (2), using a cell culture system, showed D-araboascorbate could not be substituted for vitamin C in the collagen synthesis reaction although other reducing compounds, such as pteridines were effective in promoting formation of hydroxyproline. In this study we have extended the work of Hutton *et al.* to test the efficacy of all four stereoisomers of ascorbic acid in the enzymic hydroxylation of the peptidyl proline in protocollagen.

Materials and Methods. Preparation of ascorbate stereoisomers. L-Xyloascorbic acid (vitamin C) and D-araboascorbic acid were obtained commercially. L-Araboascorbic acid and D-xyloascorbic acid were prepared by alkaline isomerization of the no. 4 carbon of the commercial products according to a modification of the method reported by Brenner *et al.* (3): 17.6 g of ascorbic acid were dissolved in 200 ml of 50% aqueous methanol and neutralized with 11.2 g of potassium hydroxide (10% excess over the dipotassium salt). Nitrogen was bubbled through the solution as it refluxed at 75° for 30 hr. The solution was then cooled and Dowex-50 H^+ was added until a stable pH 2 was attained. The resultant mixture was filtered and the filtrate was concentrated in a rotary vacuum concentrator to remove methanol, shell-frozen, and lyophilized to dryness. The mixture of ascorbate stereoisomers thus obtained was separated on the basis of their differential solubility in acetonitrile. The xylo isomer is less soluble and repeated crystallizations gave chromatographically pure material. The arabo isomer was isolated by serial extraction of the mixed solid material with acetonitrile and was further purified by fractional crystallization from acetonitrile.

The purity of all diastereoisomers was analyzed by thin-layer chromatography on silica gel–metaphosphoric acid plates developed in an acetonitrile:butyronitrile:water system (66:33:2). The R_f values of the diastereoisomers differ sufficiently (0.26 vs. 0.38) that good resolution is achieved. Analytical samples were applied and developed in streaks with reference material spotted at both sides of the plate. Sample areas were removed from the plates in strips, eluted with water–4% trichloroacetic acid solution and the ascorbate quantitatively determined by the Shaefferts–Kingsley method (4) using dinitrophenylhydrazine. All products were pure within the limits of the assay procedure, about 1%.

Proline hydroxylase assay system. The system employed was that developed by Hutton *et al.* (1, 5, 6). Chick embryos are used both to prepare a crude preparation of a hydroxylating enzyme and a tritium labeled peptidyl substrate. When these components are incubated in the presence of ascorbic acid, the peptidyl proline-3,4-^3H is hy-

[1] Supported by Contract No. DA-49-193-MD-2611, Surgeon General's Office, United States Army.

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droxylated, releasing one of the no. 4 hydrogens to the medium as water. This water, which can be collected by lyophilization and counted, is a measure of proline to hydroxyproline conversion.

The enzyme was obtained from the 105,000 *g* supernatant fraction of 8-day embryos which had been washed in 0.25 *M* sucrose, minced, and homogenized in 2.5 *M* sucrose (tissue:solvent, 1:9, w/w). To the supernatant was added 0.05 vol of 1 *M* Tris–HCl buffer, pH 7.5, and 0.3 vol of 5% aqueous streptomycin sulfate solution. The white precipitate, which formed during 15-min standing, was removed by centrifugation and the streptomycin precipitation was repeated on the supernatant. The supernatant was made 60% saturated with $(NH_4)_2SO_4$ by the addition of 0.04 *M* Tris–HCl buffer, saturated with $(NH_4)_2SO_4$ and adjusted to pH 7.5 with HCl. After standing 20 min, the resulting precipitate was removed by centrifugation, dissolved in 0.01 *M* Tris–HCl buffer, pH 7.5, and dialyzed overnight vs. two 1200-ml volumes of 0.01 *M* Tris–HCl buffer, pH 7.5. The retentate, which contained ~3 mg/ml protein when assayed by a modified Lowry determination, was frozen and then thawed as needed for hydroxylation reaction studies.

The substrate was prepared from 8-day chick embryos which were washed in a modified Krebs–Ringer buffer (7), decapitated, and finely minced. To each 4.3 g of tissue were added 9.2 ml of Krebs–Ringer buffer (adjusted to pH 7.5 with HCl); 1 ml of 0.015 *M* α,α'-dipyridyl; and 470 μCi of L-proline-3,4-^{3}H (sp act 5.26 Ci/mmole) in 0.43 ml 0.01 *M* HCl (New England Nuclear Corporation). The mixture was placed in a 37° water bath on a shaker for 80 min. After chilling on ice, the mixture was homogenized and centrifuged. The pellet was washed twice with 1 ml of 0.25 *M* sucrose, suspended in 1 vol of 0.5 *M* acetic acid corresponding to 2 ml/g of starting tissue, and stirred for 90 min to extract the substrate. Extracts were dialyzed vs. four 1200-ml volumes of water for 20 hr.

The complete assay system contained the following components in a total volume of 3 ml: enzyme, 1 ml; substrate, 1 ml; ascorbic acid, 1.25×10^{-3} *M*; ferrous ammonium sulfate, 7.5×10^{-5} *M*; Tris–HCl buffer, pH 7.5, 0.1 *M*; α-ketoglutarate, 1×10^{-5} *M*; and water, to volume. Reaction mixtures were incubated aerobically for 30 min in a 30° water bath and stopped by the addition of 0.1 vol of 50% trichloroacetic acid. The water from the reaction mixture was removed by vacuum distillation, and an aliquot was taken for counting in 15 ml of toluene–base scintillation fluid with Bio Solv solubilizer. Counting efficiencies were 25%.

TABLE I. Ability of the Four Ascorbate Stereoisomers to Stimulate Formation of ^3H_2O from Peptidyl Proline-3,4-^{3}H.

System[a]	Expt. 1 ^3H_2O (cpm)	Expt. 2 ^3H_2O (cpm)
No enzyme	125	152
No ascorbate	119	125
L-Xyloascorbate (Vitamin C)	385	440
L-Araboascorbate	395	466
D-Xyloascorbate	305	428
D-Araboascorbate	403	476

[a] Ascorbate concentrations were 1.25×10^{-3} *M*; substrate contained 1.6×10^5 cpm/ml; for complete system, see text.

Results. The results of two sets of experiments are shown in Table I and indicate that all four ascorbate stereoisomers can stimulate proline hydroxylation to the same extent. In order to determine whether contaminating traces of stereoisomers could have accounted for these results, dilution experiments were performed with three of the ascorbates at levels of 1.25×10^{-3}, 1.25×10^{-4}, and 1.25×10^{-5} *M*—corresponding to about 200, 20, and 2 μg/ml. The results given in Table II show that 1.25×10^{-3} *M* ascorbate is close to the minimum required to catalyze this reaction and that even a 10% impurity in isomer preparations could not have stimulated hydroxylations to the extent observed in our experiments.

That the reaction studied actually resulted in the synthesis of tritium-labeled hydroxyproline was demonstrated by hydrolyzing the reaction mixture in 6 *M* HCl and separating the hydroxyproline on a Dowex-50 ion exchange column. The ratio of recovered radioactivity in hydroxyproline-^{3}H to pro-

TABLE II. Ability of Varying Concentrations of Ascorbate Stereoisomers to Stimulate Formation of ^3H_2O from Peptidyl Proline-3,4-^3H.

Ascorbate isomer	Concentration (M)	^3H_2O (cpm)
L-Xylo (Vitamin C)	1.25×10^{-3}	293
	1.25×10^{-4}	97
	1.25×10^{-5}	89
D-Arabo	1.25×10^{-3}	330
	1.25×10^{-4}	124
	1.25×10^{-5}	99
D-Xylo	1.25×10^{-3}	402
	1.25×10^{-4}	126
	1.25×10^{-5}	83
Blank, no enzyme		85

line-^3H was comparable to the ratio of H_2O-^3H formed to labeled substrate added.

Discussion. The data of Tables I and II clearly show that there is no stereospecificity in the role of ascorbate as a cofactor for the hydroxylation of peptidyl proline under the conditions tested. This conclusion is consistent with the result of Hutton *et al.* (1) for D-araboascorbic acid. It is interesting to note that in L-xylo and in D-arabo ascorbate the conformation of the lactone ring is the same. That is the side chain, $-CHOHCH_2OH$, has the same stereo orientation to the ring system in both molecules. Only the optical configuration of the no. 5 carbon is reversed. Stereospecificity for the lactone ring could have occurred that did not extend to the no. 5 carbon of the side chain. That such an effect was not observed in this study suggests that ascorbate is not bound to the hydroxylating enzyme system, but that it merely serves as a very effective chemical reagent.

Priest and Bublitz (2) have shown that D-araboascorbate is not an effective substitute for ascorbate in a collagen-synthesizing cell culture. Such results are best explained by invoking a stereospecific active transport mechanism or a stereospecific oxidation–reduction cycle for ascorbate.

Summary. The two uncommon stereoisomers of ascorbic acid have been prepared by alkaline isomerization of L-xylo and D-arabo ascorbate. All four stereoisomers of ascorbic acid have been tested for their efficacy in promoting the hydroxylation of peptidyl proline using the procedure of Hutton *et al.* and showed comparable efficiency in this system. Thus no stereospecific role for ascorbate in prolyl hydroxylation is demonstrated for the hydroxylation reaction itself.

The authors thank Mrs. Dale Karr for the chromatographic analysis of the purity of the ascorbate stereoisomers.

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Received June 9, 1969. P.S.E.B.M., 1969, Vol. 132.