IS L-GULONOLACTONE-OXIDASE THE ONLY ENZYME MISSING IN ANIMALS SUBJECT TO SCURVY?

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SUMMARY: Evidence has recently appeared implicating an unusual microsomal D-glucuronolactone reductase, which requires carbonyl reagents for activity, in the biosynthesis of ascorbic acid. It was also shown that this microsomal enzyme activity was missing in guinea pigs and primates suggesting that L-gulonolactone oxidase deficiency was not the only defect in animals subject to scurvy. However, we have shown that highly purified L-gulonolactone oxidase catalyzes the conversion of the oxime and semicarbazone of D-glucuronolactone to the corresponding ascorbic acid derivative. There is, therefore, no need to propose a second pathway to ascorbic acid, nor is there evidence for more than the one enzyme defect in scurvy-prone animals.

Following the elucidation of the biosynthetic pathway of ascorbic acid in mammals (D-glucose → D-glucuronic acid → L-gulonic acid → L-gulono-γ-lactone → L-ascorbic acid) (1, 2), it was shown that tissues of animals which are subject to scurvy (guinea pigs and primates) contain no L-gulonolactone oxidase activity (2, 3, 4). Since all of the other enzyme activities shown above were present it was concluded that the genetic defect in scurvy-prone animals was due to a deficiency of a single enzyme, L-gulonolactone oxidase.

In 1961 Chatterjee et al. (5) questioned the one-enzyme-deficiency theory for scurvy-prone animals. He reasoned that since L-gulonolactone oxidase is a microsomal enzyme, the other enzymes involved in the formation of ascorbic acid should also be microsomal. He suggested that the enzyme in cytosol which converts D-glucuronic acid to L-gulonic acid is not a sufficient source of L-gulonolactone for the biosynthesis of ascorbic acid. He thus questioned the accepted pathway for ascorbic acid biosynthesis and concluded that there must be a microsomal enzyme which converts D-glucuronolactone to L-gulonolactone,

(i.e., D-glucuronolactone → L-gulonolactone → L-ascorbic acid). He found evidence for this in washed rat liver microsomes which catalyzed the conversion of D-glucuronolactone to ascorbic acid in the presence of carbonyl reagents such as hydroxylamine or semicarbazide. Since conversion of L-gulonolactone itself to ascorbic acid did not require a carbonyl reagent he concluded that a specific, D-glucuronolactone reductase which required carbonyl reagents for activation, was present in liver microsomes and that this enzyme was involved in the biosynthesis of ascorbic acid. When guinea pig liver microsomes were examined they were found to be deficient in this carbonyl reagent requiring D-glucuronolactone reductase, leading Chatterjee to conclude that there are at least two enzymes lacking in scurvy-prone animals (5, 6).

During the purification of liver L-gulonolactone oxidase we were led to consider Chatterjee's reports. We considered that the addition of carbonyl reagents to D-glucuronolactone might produce derivatives in which the furanose ring was opened and which therefore resemble L-gulonolactone (Fig. 1). If this

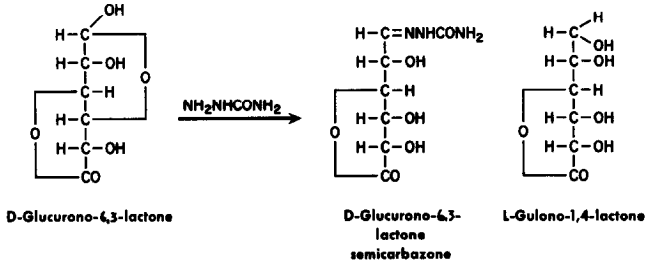

Fig. 1 Possible derivatives of D-glucuronolactone in the presence of carbonyl reagents.

were so then L-gulonolactone oxidase might itself be responsible for the oxidation of D-glucuronolactone in the presence of carbonyl reagents. With the purification of liver L-gulonolactone oxidase to homogeneity (7) it became possible to test for the conversion of D-glucuronolactone derivatives to "ascorbic acid-like" products.

MATERIALS AND METHODS

L-1,4 Gulonolactone was purchased from Nutritional Biochemicals Corporation, D-Glucuronolactone from Eastman Organic Chemicals. (^{14}C)Semicarbazide hydrochloride (4.8 mCi/mmole) was obtained from ICN Pharmaceuticals. D-Glucuronolactone (^{14}C)semicarbazone and D-glucuronolactone oxime were synthesized by Dr. H. Gurien, Chemical Research Department, Hoffmann-La Roche Inc., by the method described by Dutta Gupta et al. (8). Melting points, C,H,N analysis, as well as ultraviolet, infrared and mass spectral data were compatible with the postulated structures and comparable to those obtained by them. The specific activity of D-glucuronolactone (^{14}C)semicarbazone was 1.4 x 10^8 cpm/mmole.

L-Gulonolactone oxidase was purified from rat and goat liver microsomes as previously described (7) except that Biogel A 1.5 m (Bio-Rad Laboratories) (2.8 x 35 cm) was used instead of the Sephadex G 150 column.

L-Gulonolactone oxidase activity was assayed as previously described (7) except that the ascorbic acid product was measured by the method of Zannoni et al. (9). Enzymatic conversion of the D-glucuronolactone derivatives to the "ascorbic acid"-like product was measured by a modification of the method of Chatterjee (10). D-Glucuronolactone semicarbazone (3.6 mM) or D-glucuronolactone oxime (4.9 mM) were incubated with enzyme for 15 min at 37°C in a volume of 1.0 ml containing 25 mM potassium phosphate buffer pH 7.5 and 0.5 mM EDTA. The reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid, protein was removed by centrifugation and the supernatant was assayed for ascorbic acid (9). The enzymatic reaction was linear with time for 15 min and proportional to enzyme concentration. Sodium pyrophosphate and potassium cyanide, which were included in the method described by Chatterjee (10), were omitted since they did not significantly affect the rate of the reaction.

Thin layer chromatograms were visualized by a modification of the ascorbic acid assay of Zannoni et al. (9). The spray reagent consisted of 10.0 ml 5% trichloroacetic acid, 0.4 ml 85% ortho-phosphoric acid, 1.0 ml 10% α,α'-dipyridyl (in ethanol), and 0.4 ml 10% aqueous ferric chloride. Plates were sprayed thoroughly (5.0 ml/20 x 20 cm plate) and allowed to dry in air for 30 min. The lower limit of detection was 1 μg ascorbic acid/cm^2. Reducing agents such as NADPH, glutathione and homocysteine do not give a positive reaction.

Protein concentration was assayed by the method of Lowry et al. (11) except in purified samples where assays were carried out with the fluorescamine method after alkaline hydrolysis (12). Bovine serum albumin was used as a standard in both methods.

RESULTS AND DISCUSSION

It was found that when hydroxylamine or semicarbazide was included in the reaction mixture, D-glucuronolactone was indeed converted to "ascorbic acid" as measured by the method of Zannoni et al. (9). To test this further the oxime and semicarbazone derivatives of D-glucuronolactone were prepared and tested for activity with highly purified L-gulonolactone oxidase from both rat and goat liver. Both compounds were found to be converted to "ascorbic acid" as

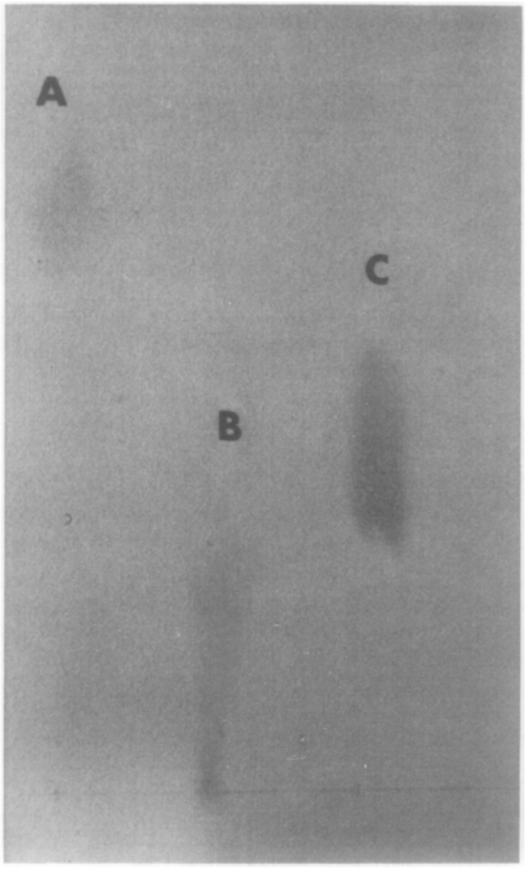

Fig. 2 Thin layer chromatography of ascorbic acid-like products. A. Product from D-glucuronolactone oxime, B. Product from D-glucuronolactone (^{14}C) semicarbazone, C. authentic ascorbic acid.

Reactions with goat gulonolactone oxidase were run as described in Materials and Methods for 30 min. Protein was removed by addition of 5.0 ml acetone at -20° for 45 min., and centrifugation for 30 min at 15,000 xg. The supernatants were rotoevaporated to remove acetone, and lypholyzed. Samples were dissolved in 0.05 ml water and spotted (0.06 µmole product/spot) on I.T.L.C. Type SA plates (Gelman Instruments). Chromatograms were developed in a solvent system of Acetone 50: Ethyl Acetate 40: H_2O 9.8 and visualized as described in Materials and Methods. Chromatograms of reactions with radioactive substrate were cut into 1.0 cm squares, placed in vials with 15.0 ml Instabrey (Yorktown Research) and counted by liquid scintillation.

assayed by the method employed (9). However, when the products were subjected to thin layer chromatography, they were shown to be different from ascorbic acid (Fig. 2). To help determine the nature of the "ascorbic acid" product formed,

the semicarbazone of D-glucuronolactone labelled in the semicarbazone moiety was treated with goat liver L-gulonolactone oxidase which had been purified almost to homogeneity. The ascorbic acid-like product isolated on thin layer chromatography was shown to be radioactive. This indicated that pure L-gulonolactone oxidase catalyzes the conversion of D-glucuronolactone semicarbazone to the semicarbazone of ascorbic acid. The carbonyl derivatives of D-glucuronolactone were found to be good substrates of L-gulonolactone oxidase. With rat enzyme, the semicarbazone had a K_m of 0.57 mM and a V_{max} of 451 nmoles/min/mg protein; for the oxime the K_m was 0.64 mM and the V_{max} 465. These compare to a K_m of 0.07 mM and a V_{max} of 500 for L-gulonolactone.[1] Comparable values were obtained with the goat enzyme. Furthermore, L-gulonolactone was shown to effectively inhibit the oxidation of the D-glucuronolactone derivatives. L-Gulonolactone (6.25 mM) inhibited the reaction with D-glucuronolactone (^{14}C) semicarbazone (4.02 mM) more than 50%.

The possibility remained that a carbonyl reagent activated D-glucuronolactone reductase was present in intact microsomes, but was removed during the purification procedure. To test for this possibility, the enzyme was carried through the purification and activity was assayed at each step using L-gulonolactone, D-glucuronolactone semicarbazone or D-glucuronolactone oxime as substrate. As shown in Table I the ratios of the three activities were not significantly altered over a 70 fold range of purification, starting with intact goat liver microsomes. The 3 activities also co-purified over a 120 fold purification during the preparation of rat L-gulonolactone oxidase.

It must be concluded that the conversion of D-glucuronolactone to "ascorbic

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[1]Homocysteine (4.9 mM) was included in the kinetic experiments to prevent autoxidation of ascorbic acid and its derivatives. After correcting the rate of reaction with gulonolactone for autoxidation of ascorbic acid, a 2.5 fold increase in activity was found. The apparent K_m, however, was unchanged. Cysteine (4.9 mM) also caused a 2.5 fold increase in activity. Dithiothreitol (2.2 mM) led to a 5.0 fold increase in gulonolactone oxidase activity, while glutathione (5 mM) had no effect on the rate of gulonolactone oxidase after correcting for autoxidation. In future studies dithiothreitol (2.2 mM) will be added to enzyme incubations.

TABLE I

Comparison of Activities of Various Substrates

During Purification of Goat L-Gulonolactone Oxidase

	L-Gulonolactone		D-Glucuronolactone Semicarbazone		D-Glucuronolactone Oxime	
	Specific Activity[a]	Purification[b]	Specific Activity	Purification	Specific Activity	Purification
Microsomes	6.75	-	6.63	-	6.02	-
Tryptic digestion	10.79	1.6	8.52	1.3	8.21	1.4
Tween 20	9.30	1.4	11.48	1.7	7.22	1.2
$(NH_4)_2SO_4$	18.73	2.8	17.25	2.6	14.19	2.4
Bio-gel A 1.5 m	42.91	6.4	39.80	6.0	37.64	6.3
DEAE Sephadex	201.35	29.8	212.99	32.1	208.27	34.6
Hydroxyl-apatite	463.28	68.6	503.12	75.9	407.1	67.6

[a]Specific activity was determined as described in Materials and Methods, and is expressed as nmoles/min/mg protein.

[b]Purification relative to microsomes.

acid" reported by Chatterjee (5) is not indicative of another microsomal enzyme in the ascorbic acid pathway. Rather, L-gulonolactone oxidase itself is capable of oxidizing the semicarbazone and oxime of D-glucuronolactone to their corresponding ascorbic acid derivative. We feel that the D-glucuronic acid reductase activity in the cytosol is more than adequate to account for the formation of L-gulonolactone in the liver. Localization of consecutive enzymes in a metabolic pathway in different cell compartments is not unusual. It occurs, for instance, in the conjugation of D-glucuronic acid with drugs. Activation of glucuronic acid to uridine diphosphate glucuronic acid occurs in the supernatant

fraction of liver, whereas conjugation of the latter with the acceptor drug is catalyzed by a microsomal enzyme (13). We conclude, therefore, that the only deficiency in scurvy-prone animals is in L-gulonolactone oxidase. Initial immunologic studies indicate that in guinea pigs and apparently in monkeys too, the defect is due to a failure in the expression of the gene for L-gulonolactone oxidase and not to the formation of an aberrant enzyme (14).

REFERENCES

1. ul Hassan, M., and Lehninger, A. L. (1956) J. Biol. Chem. 223, 123-138.
2. Burns, J. J. (1967) in Metabolic Pathways, Ed. Greenberg, D. M. (Academic Press, New York, 3rd Ed., Vol 1. 394-411.
3. Burns, J. J. (1957) Nature 180, 553.
4. Grollman, A. P., and Lehninger, A. L. (1957) Arch. Biochem. Biophys. 69, 458-467.
5. Chatterjee, I. B., Kar, N. C., Ghosh, N. C., and Guha, B. C. (1961) Nature 192, 163-164.
6. Dutta Gupta, S., Choudhury, P. K., and Chatterjee, I. B. (1973) Internat. J. Biochem. 4, 309-413.
7. Nishikimi, M., and Udenfriend, S. (in press) Arch. Biochem. Biophys.
8. Dutta Gupta, S., Sen Gupta, C., Ray Chaudhuri, C., and Chatterjee, I. B. (1970) Anal. Biochem. 38, 46-55.
9. Zannoni, V., Lynch, M., Goldstein, S., and Sato, P. (1974) Biochem. Med. 11, 41-48.
10. Chatterjee, I. B. (1970) in Methods in Enzymology, Eds. Colowick, S. P. and Kaplan, N. O. 18 (A) 28-34, Academic Press, New York, N. Y.
11. Lowry, H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
12. Nakai, N., Lai, C. Y., and Horecker, B. L. (1974) Anal. Biochem. 58, 563-570.
13. Mandel, H. G. (1971) Fundamentals of Drug Metabolism and Disposition, Eds. La Du, B. N., Mandel, H. G. and Way, E. L., Waverly Press, Inc., Baltimore, Md. 149-186.
14. Nishikimi, M., and Udenfriend, S. (in press) Proc. Nat. Acad. Sci.