CITRATE DEGRADATION BY ASCORBATE AND CUPRIC OR FERROUS ION

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1. Introduction

The non-enzymatic decomposition of citrate solutions under "mild" conditions, particularly in the sunlight, was reported as early as 1859 by Liebig [1], and since by other workers, especially those concerned with pharmaceutical preparations of citrate [2, 3]. While studying the metabolism of citrate by growth cartilage, we observed that ascorbic acid stimulated $^{14}CO_2$ formation from citrate-6-^{14}C in a tissue-free system.

In this paper we show that degradation in this non-enzymatic system (Krebs-Ringer phosphate buffer pH 7.4) is enhanced by Cu^{2+} or Fe^{2+} and decreased by EDTA, homocysteine, catalase or N_2 atmosphere. We suggest that ascorbate decomposes citrate by reducing Cu^{2+} to Cu^{+} producing H_2O_2 and possibly free radicals which attack citrate and that the chelation of Cu^{2+} with citrate may make citrate more susceptible to attack. Consideration is given to possible intermediates.

2. Experimental procedures, results and discussion

In a typical experiment, 0.5 μCi citrate-6-^{14}C (0.265 μmoles equivalent to about 1×10^6 cpm) obtained from New England Nuclear Corp., Boston, Mass. and with a purity greater than 98% was incubated for 60 min in a Dubnoff apparatus with shaking (100 strokes per min) at 37° with 3 ml Krebs-Ringer phosphate buffer, pH 7.4, in an Erlenmeyer flask with a removable plastic well containing hyamine. A serum stopper was used for closure. Background lighting was not excluded. Depending on the experiment, further additions were made to a final volume of 3.2 ml. $^{14}CO_2$ formed was released from solution by the injection of 1 ml 1.2 N perchloric acid or 10% metaphosphoric acid and further incubation with shaking for 30 min. The plastic cup containing hyamine and absorbed $^{14}CO_2$ was transferred to a counting vial, 10 ml of a toluene phosphor scintillation medium added. Radioactivity was measured in a Packard Scintillation Spectrometer with an efficiency of 78–80%. Results are averages of triplicate runs.

Table 1 shows that the addition of ascorbic acid to a Krebs-Ringer phosphate solution containing citrate-6-^{14}C stimulated the formation of a volatile radioactive material, presumably $^{14}CO_2$, whereas dehydroascorbic acid was ineffective. This radioactive material was shown to be $^{14}CO_2$ by absorption in KOH, precipitation with barium, decomposition of the barium salt with HCl and absorption of the released CO_2 by hyamine. Counts observed utilizing scintillation spectrometry with this solution accounted for all of the radioactivity obtained by direct absorption of the volatile gas in hyamine. $^{14}CO_2$ formation from citrate-6-^{14}C in the presence of ascorbic acid was reduced to a very low value by prior gassing of the solution with nitrogen, suggesting the importance of molecular O_2. Homocysteine, capable of both maintaining ascorbic acid in its reduced form and also binding heavy metals, substantially decreased $^{14}CO_2$ formation. EDTA reduced $^{14}CO_2$ formation to a negligible level, suggesting the involvement of heavy metal in the decomposition of citrate. Whereas the addition of Cu^{2+} to citrate-6-^{14}C had no effect in the absence of ascorbic acid, the combination of ascorbic acid and Cu^{2+} markedly stimulated $^{14}CO_2$ formation. With the latter system, prior gassing with nitrogen or the addition of EDTA reduced $^{14}CO_2$ formation to low values.

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Table 1
Effect of various additions to citrate-6-^{14}C on $^{14}CO_2$ formation.

Additions to medium containing citrate-6-^{14}C	$^{14}CO_2$ formed (cpm)
None	130*
Ascorbic acid (0.005 M)	15,800*
Dehydroascorbic acid (0.005 M)	310
Ascorbic acid; gassed with nitrogen	900
Ascorbic acid and homocysteine (0.1 M)	1,300
Ascorbic acid and EDTA (0.003 M)	260
Ascorbic acid + Cu^{2+} (0.001M $CuSO_4$)	50,650
Ascorbic acid + Cu^{2+}, nitrogen gassed	2,950
Ascorbic acid + Cu^{2+} + EDTA	1,100
Ascorbic acid + Cu^{2+} + catalase (15 Keil units)	5,200
Cupric ion (0.001 M)	430
Cuprous ion (0.001 M CuCl)	282,200
Cuprous ion + catalase (15 Keil units)	37,300
H_2O_2 (0.015 M)	4,650
H_2O_2 + Cu^{2+}	625,000
H_2O_2 + Cu^{+}	541,000
Fe^{2+} (0.001 M)	79,800
Fe^{2+} + ascorbic acid (0.005 M)	128,000
Fe^{2+} + ascorbic acid + EDTA (0.003 M)	9,300
Fe^{2+} + ascorbic acid + EDTA (0.003 M)	9,300
Fe^{2+} (0.02 M) + H_2O_2 (0.12 M)	240,000
Fe^{2+} (0.02 M) + H_2O_2 (0.12 M) + EDTA (0.003 M)	89,800

See text for composition of the medium. Final concentrations in parentheses.

* Comparable results were obtained in Krebs-Ringer bicarbonate buffer.

The relationship between copper-catalyzed ascorbic acid oxidation and $^{14}CO_2$ formation from citrate-6-^{14}C is shown in fig. 1. Although oxidation of ascorbic acid was complete within 15 min, $^{14}CO_2$ formation continued at a slow rate for the next 45 min. This suggested the formation of a citrate-degrading substance or substances in conjunction with the oxidation of ascorbic acid rather than a direct role of ascorbic acid oxidation. During this oxidation, Cu^{2+} is reduced to Cu^{+} followed by generation, aerobically, of hydrogen peroxide and free radicals [4–9] which subsequently degrade citrate. An analogous situation is that observed by Lieberman and Mapson [10, 11] in which a copper-

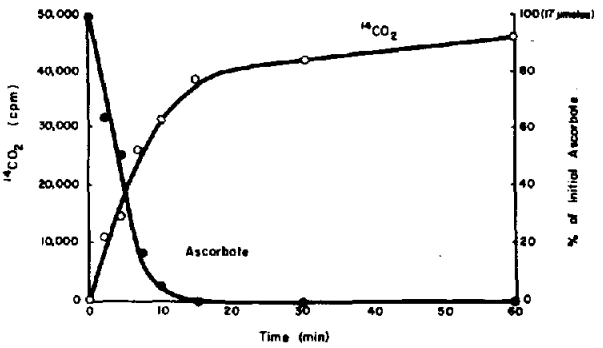

Fig. 1. Relationship between ascorbic acid breakdown and $^{14}CO_2$ formation from citrate-6-^{14}C. As described in the text, 0.5 μCi citrate-6-^{14}C in Krebs-Ringer phosphate buffer, pH 7.4 was incubated at 37° for varying periods of time in the presence of ascorbic acid (4.1 mM) and Cu^{2+} (0.73 mM). The reaction was stopped by the injection of 0.4 ml 0.1 M EDTA and 1 ml 10% metaphosphoric acid (HPO_3). Following further incubation of the flasks for absorption of $^{14}CO_2$ by hyamine, a 0.7 ml aliquot from each flask was diluted to 50 ml with 5% HPO_3 and analyzed spectrophotometrically at 245 nm for ascorbic acid. $^{14}CO_2$ was measured as described in the text.

ascorbate system, believed to generate H_2O_2, is effective in the *in vitro* formation of ethylene from methionine. Hydrogen peroxide is also thought to be implicated in other reactions involving ascorbate–O_2–cupric or ferrous ion systems: hydroxylation [6, 12–14] depolymerization of hyaluronic acid [15, 16] and catalase inhibition [17]. These reactions probably occur largely because of formation of the hydroxyl free-radical [6–8, 14] or an active metal–O_2–ascorbate complex [9]. Furthermore, Corvaja et al., using electron spin resonance spectroscopy have evidence for the formation of free-radicals containing five and six carbon atoms from citrate by hydrogen peroxide–metal systems which generate the hydroxyl free-radical [19]. As shown in table 1, Cu^{+}, which can react with H^{+} and O_2 to give H_2O_2, when added alone to citrate-6-^{14}C, was highly effective in generating $^{14}CO_2$. That hydrogen peroxide was a key substance in catalyzing citrate breakdown was indicated by the addition of catalase which markedly reduced $^{14}CO_2$ formation but H_2O_2 was less effective than ascorbic acid alone or ascorbic acid plus Cu^{2+}. However, H_2O_2 in combination with either Cu^{2+} or Cu^{+} resulted in the most extensive breakdown of citrate observed. Table 1 shows that Fe^{2+} was also effective in degrading

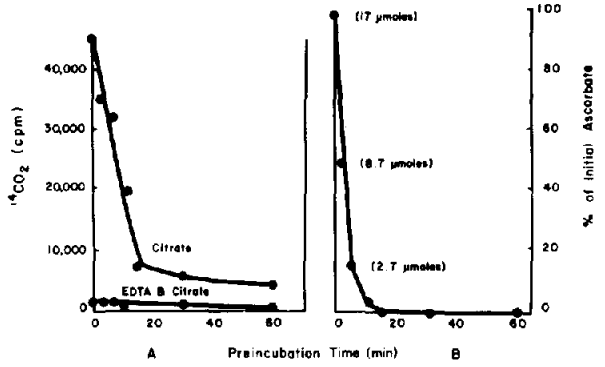

Fig. 2. (A) Effect of time of preincubation of ascorbate and Cu^{2+} on $^{14}CO_2$ formation during subsequent 1 hr incubation with citrate-6-^{14}C or EDTA and citrate-6-^{14}C. For upper curve: to 3 ml iced Krebs-Ringer phosphate buffer pH 7.4 were added 0.017 mmole ascorbate in 0.1 ml and 0.003 mmole Cu^{2+} in 1 ml. Flasks were capped with serum stoppers and incubated with shaking at 37° for varying periods up to 60 min. Then 0.4 ml H_2O followed by 0.5 μCi citrate-6-^{14}C in 0.5 ml were injected and the incubation continued for 60 min longer. For the lower curve: after preincubation of ascorbate and Cu^{2+}, 0.4 ml 0.1 M EDTA was substituted for H_2O before adding the citrate. Stopping the reaction and measurement of $^{14}CO_2$ were as stated in fig. 1.

(B) Ascorbate loss during preincubation with Cu^{2+}. Conditions were the same as in fig. 2A except that at the end of each preincubation period there was no addition of citrate nor further incubation. Instead, flasks were injected with 0.4 ml EDTA and 1 ml 10% HPO_3 and analyzed for ascorbic acid. Each point on all curves represents the average of triplicate runs.

citrate and more so in the presence of ascorbate. The latter system simulates Udenfriend's system for hydroxylating aromatic amino acids [12]; however, whereas the hydroxylating system for aromatic amino acids is enhanced by EDTA, EDTA has a retarding effect on citrate degradation. Another system for hydroxylating aromatic amino acids, Fenton's reagent consisting of Fe^{2+} and H_2O_2, was also highly effective in degrading citrate. Again, whereas the addition of EDTA to Fenton's reagent enhances its hydroxylation of aromatic amino acids [19], EDTA retards citrate degradation by Fenton's reagent.

The degradative action of Cu^{2+} plus ascorbate on citrate was not specific for L-ascorbic acid since on an equimolar basis and in the presence of Cu^{2+}, L-ascorbate, D-glucoascorbate, D-isoascorbate, and hydroquinone were about equally effective. Ferrous ascorbate (without Cu^{2+}) was about twice as effective as L-ascorbate with Cu^{2+} in decarboxylating citrate.

The enhanced degradative effect of hydrogen peroxide when in combination with cupric or cuprous ion suggested a double role for Cu^{2+} in the ascorbate system: (1) to become reduced to Cu^{+} which reacts with H^{+} and O_2 to form H_2O_2 probably creating a system generating hydroxyl or other free-radicals that will attack citrate and (2) to catalyze the reaction between citrate and H_2O_2 possibly forming a more readily oxidizable copper complex with citrate [20, 21]. This postulated dual role of Cu^{2+} is supported by the results of an experiment in which ascorbate and Cu^{2+} were preincubated for varying periods up to 60 min, then citrate-6-^{14}C added and the mixture incubated 60 min longer. As shown in fig. 2A, upper curve, the longer the preincubation time of ascorbate and Cu^{2+} before adding citrate-6-^{14}C, the less $^{14}CO_2$ is formed, suggesting a transitory nature of the citrate degrading agent, presumably H_2O_2 or a free-radical formed during preincubation. If EDTA is added immediately before citrate but after preincubation of Cu^{2+} and ascorbate, then essentially no decarboxylation occurs as the lower curve shows. Now if one considers a preincubation time of 20 min, when ascorbate has become completely oxidized (fig. 2B), and when there is still significant $^{14}CO_2$ formation from citrate in the absence of EDTA (fig. 2A, upper curve), then it appears that the inhibition by EDTA of CO_2 formation from citrate added to this preincubated mixture of ascorbate and Cu^{2+} could not have been due to interference with ascorbate oxidation, formation of cuprous ion and generation of H_2O_2 and free-radicals. Here, EDTA must be interfering with a second role of Cu^{2+}, namely preventing Cu^{2+} from chelating with citrate and making it more vulnerable to attack by H_2O_2 or a free-radical.

To disclose the nature of intermediates arising from citrate breakdown, media in which citrate-6-^{14}C had given rise to $^{14}CO_2$ in several experiments were pooled, desalted with Dowex 21 which was eluted with formic acid. To the acid eluate were added unlabeled aconitic, succinic, fumaric, α-ketoglutaric, malic and citric acids. Two dimensional chromatography on this mixture resulted in the detection of radioactivity only in the citric acid fraction.

However, solutions of unlabeled citrate degraded by cuprous ion gave hydrazone precipitates upon ad-

dition of 2,4-dinitrophenylhydrazine reagent. When dissolved and chromatographed on Whatman 3 mm paper using the solvent system of El Hawary and Thompson [22], the precipitated material gave rise to spots corresponding to the dinitrophenylhydrazones of acetone dicarboxylic acid, acetoacetic acid, and acetone. That the latter two compounds are artifacts in the formation and chromatography of acetone dicarboxylic acid dinitrophenylhydrazone is suggested by the fact that all qualitative tests for acetone and acetoacetic acid on our degraded citrate solutions were negative. Furthermore acetone dicarboxylic acid and its decarboxylation product acetoacetic acid are highly unstable compounds. Similarly, Datta et al. [23] studying the decomposition of alkaline citrate–manganese solutions suggested that acetone dicarboxylate could arise as a result of oxidative decarboxylation and demonstrated this by paper chromatography of solvent-extracted keto-acid dinitrophenylhydrazones. They also found the instability of acetone dicarboxylic acid to be a complicating factor.

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