Chemical Aspects of the Mutagenic Activity of the Ascorbic Acid Autoxidation System

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It has been proposed that the mutagenic activity associated with the ascorbic acid autoxidation system may involve hydrogen peroxide and peroxide radicals. We report here that the mutagenic effect may also partially reside in as yet unknown secondary products. The observed mutagenicity of 2,3-diketogulonic acid, one of the main oxidation products, reflects its ability to form hydrogen peroxide.

Introduction

A variety of different biological effects induced by ascorbic acid (AH_2) have been discussed in the recent past. For instance, both antimutagenic [1] and anticlastogenic [2] properties have been ascribed to it and one recent report claims selective toxicity for ascorbate-copper acting in melanoma cells [3]. Other authors [4] have suggested that the vitamin could even be employed to reduce the incidence and progression of malignant tumors in human patients. The substance is, however, also known to induce chromosomal aberrations and unscheduled DNA synthesis and is mutagenic in the Ames test [5]. Moreover, ascorbate induces sister chromatid exchanges in cultured human fibroblasts or Chinese hamster ovary cells [6], exerts a genotoxic effect in DNA and RNA phages [7], triggers a DNA repair synthesis [8] and fragments isolated calf thymus DNA [9]. Even enhancing effects of ascorbate on carcinogen activity have been reported [10].

The chemical and biological origins of these seemingly contradictory observations remain for the most part unclear. For example, while in tumorigenesis experiments ascorbate inhibition can, in principle, be explained by scavenging of carcinogen precursors [11, 12], the cause of the mutagenic effects is evidently more complicated and has been suggested to originate in the enediol reductone structure [13] or the hydrogen peroxide and free radical formed in the autoxidation [14–16].

The enhancement of the effect by metals, especially copper (II), and its inhibition by catalase strongly supports the latter proposal. The fact, however, that the range of active concentrations is narrow and can easily be missed suggests that this explanation may be oversimplified. The autoxidation of AH_2 is complex and leads to not only hydrogen peroxide and dehydroascorbic acid (A) but also, at high turnover to 2,3-diketogulonic, threonic and oxalic acids as the main, among many other reductone type products [17–19]. Previous authors appear to have paid little attention to the detailed chemistry of these systems in which we surmise, especially in incubated samples the consumption of AH_2 may be high. Additionally, since both ascorbic [20] and dehydroascorbic acids [21] react with hydrogen peroxide its yield and hence the quantitative microbiological effect of a given sample will be expected to be a sensitive function of the samples' history. Thus with the intention of delineating the species responsible for the mutagenic effect we have conducted a chemical and toxicological study of the AH_2 autoxidation system and some of its more accessible products. In designing experiments of this kind we were primarily interested in correlating the mutagenic character of the various products, as revealed by the Ames test [22], with their ability to reduce molecular oxygen to hydrogen peroxide.

Materials and Methods

All materials were of the highest purity commercially available and were used without further purification. The synthetic oxidation products 2,3-diketogulonic acid, threonic acid, reductone X and triose reductone were prepared according to methods as described in the literature [19, 23, 24].

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The incubation of AH_2-Cu^{2+} mixtures was carried out by maintaining stirred solutions at 37 °C for 3 h in open Erlenmeyer flasks. Oxygen uptake was measured after completion of the incubation and cooling to 21 °C with an Orion Research microprocessor ionalyzer 901, equipped with a Clark type electrode. For kinetic measurements the electrode was equilibrated with the solvent in a specially constructed cell (20 ml) for at least 10 min prior to initiating reaction. After achieving a stable baseline the reaction was commenced by injecting a solution of the substances to be investigated into the known volume of solvent. The electrode response was then monitored on a Hewlett-Packard recorder (model 17501 A). Hydrogen peroxide yields were determined by injecting fresh hydrogen peroxide oxido reductase (Boehringer 260000 U/ml) through a second injection arm.

Mutagenicity Testing

Mutagenicity of oxidative decomposition products of ascorbic acid was tested toward *S. typhimurium* strain TA 100. To 2 ml of molten top agar were added 0.1 ml bacterial suspension, 0.1 ml of test solution (dissolved in phosphate buffer, pH 7.0) and 0.1 ml of catalase (0.01 mg/plate), if required. The mixture was overlaid on minimal glucose agar according to the method of Ames *et al.* [22]. After incubation for 2 days colonies of histidine phototrophs were counted. Values are means of 3 assays in triplicate. The number of spontaneous revertants per plate was 98 ± 7.

Results and Discussion

Incubated ascorbic acid-copper(II) – oxygen systems

Fig. 1. shows some typical results for various AH_2-Cu^{2+} mixtures recorded immediately after incubation. The concentrations employed have been shown by Stich *et al.* [5] to be non-mutagenic (b) and mutagenic (a, c, d). Both oxygen consumption and hydrogen peroxide formation are demonstrated. The regeneration of oxygen, after catalase addition, results from the decomposition of the peroxide to oxygen and water (Eqn. (1)).

$$2\,H_2O_2 = H_2O + O_2\,. \qquad (1)$$

Several points are apparent: i) an amount of residual oxygen inversely proportional to the initial

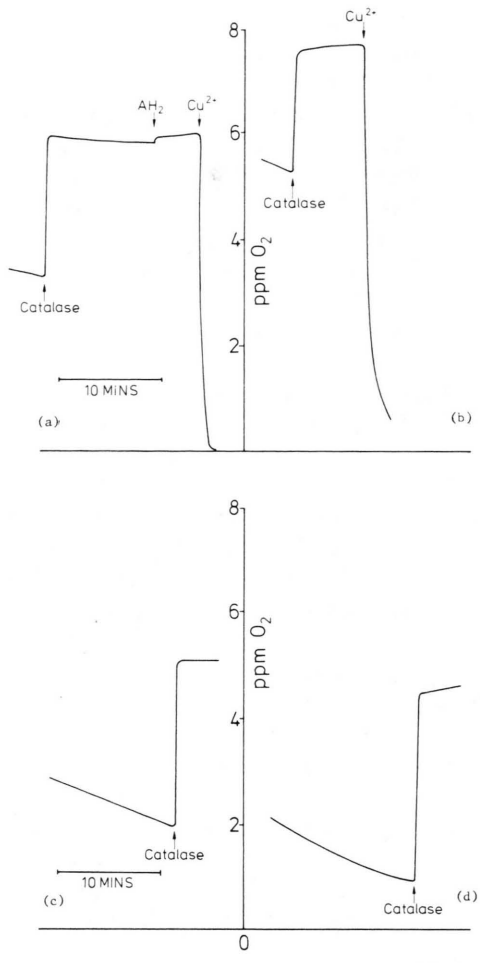

Fig. 1. The formation of hydrogen peroxide in incubated ascorbic acid – Cu^{2+} mixtures. Initial concentration of AH_2: a) 1.5×10^{-2} M, b) 5×10^{-3} M, c) 2.5×10^{-2} M, d) 3.5×10^{-2} M. AH_2/Cu^{2+} 1000/1; pH 3.2–3.6. The initial oxygen concentration was 8–8.2 ppm. The time axis is kinetically insignificant and runs from left to right.

AH_2 remains after incubation, ii) in all cases the system is still reacting, albeit slowly compared to that in the presence of fresh Cu^{2+} catalyst (Fig. 1 a and b). This slow oxygen take-up rate is apparently connected with the depletion of active Cu^{2+} catalyst since it can be accelerated by injecting a small amount of the latter, not however, by ascorbic acid (Fig. 1 a). In any case, in these experiments an excess of ascorbic acid will always be present since complete consumption of all available oxygen would have removed only $2-3 \times 10^{-5}$ M of AH_2 based on Eqn. (2).

$$AH_2 + O_2 = A + H_2O_2\,. \qquad (2)$$

It is interesting to compare the peroxide yields. Namely although the hydrogen peroxide yield (as indicated by the intensity of the oxygen peak after catalase injection) increases, as expected, with increasing initial AH_2 concentration the differences in the amounts present are too small to account for the drastic dose effect observed during Ames testing [5]. The stoichiometric yield, however, which should be unity in the absence of peroxide consuming reactions decreases from 0.93 in the case of the non-mutagenic (b) to 0.5–0.6 for the remaining solutions.

We conclude that only for solution (b) secondary reactions with H_2O_2 are not important. In the remaining cases reactions leading to partial removal of H_2O_2 will complicate the reactant mixture over and above that suggested by Eqn. (2). In fact, all the reacting solutions (initial parts of Fig. 1 a–d) will contain not only initial reactants and products but also the variously postulated intermediates in low stationary concentration. Newer kinetic investigations have implied different copper containing species, for example, Cu^+, Cu^{3+}, Cu_2AH as well as the free radicals $A^{\cdot}$ and $HO_x^{\cdot}$ as intermediates [24]. The fact that the only AH_2 initial concentration yielding a near stoichiometic amount of H_2O_2 is that one showing minimal mutagenic activity in the Ames test is significant and points unambiguously to at least partial mutagenic activity residing in products arising from reactions with H_2O_2. The incomplete inhibition of the mutagenic effect on addition of excess catalase, reported by Galloway and Painter [26] and Stich [27] supports this argument. A scheme such as 1 would explain the available results.

Synthetic oxidation products

In order to test the basic validity of this proposal we have tested the oxygen consumption and peroxide forming ability of the most important ascorbic acid oxidative decomposition products. Table I shows the results. Among these products immediately striking is the lack of oxygen take-up activity in the case of the first oxidation product of AH_2, dehydroascorbic acid (A). The oxygen content of solutions (2×10^{-3} M, pH 4 and 8.4) remained constant for several hours even in the presence of normally catalytic amounts of copper. Thermally degraded solutions of A, which according to Kurata *et al.* [28], give rise, under these conditions, to a complicated mixture of reductones (reductone III = 5-methyl-3,4-dihydroxytetrone and reductone B = 2,3,4-trihydroxy-2-pentenoic acid), furan derivatives (furoic acid, furfural) and C_4/C_5 fragments regains some oxygen consuming ability although the peroxide yields were too small to be measured. In contrast to A the second product, 2,3-diketogulonic acid showed a rapid pH dependent metal catalysed reaction with molecular oxygen and the highest yields of peroxide. However, in common with the other oxygen consuming reductones yields were much lower than stoichiometric and increased on addition of EDTA (Fig. 2). Seemingly, secondary reactions involving hydrogen peroxide with i) trace metals and ii) byeproducts are contributing to the losses. The final products of the oxidation, threonic and oxalic acids both showed no tendency to react with molecular oxygen.

Mutagenicity testing

The results from the *Salmonella*/mammalian-microsome mutagenicity test with dehydroascorbic, 2,3-diketogulonic and threonic acids are shown in Table II. At concentrations identical to those at which Stich *et al.* [5] observed the highest frequency of his^+ revertants with ascorbic acid – Cu(II)

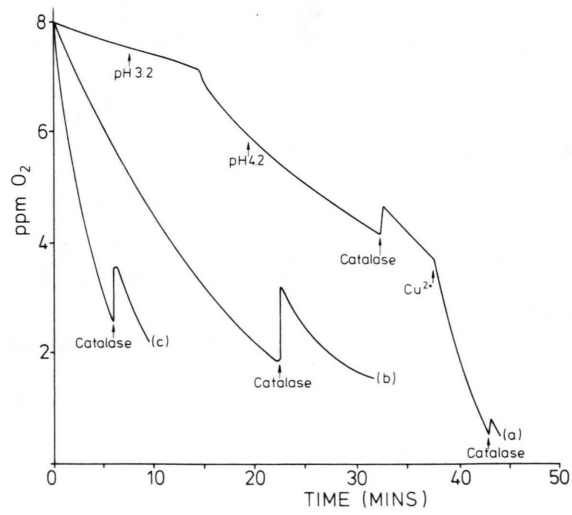

Fig. 2. Influence of substance nature and initial pH on oxygen consumption of reductones. a) reductone X (2,3,4,5-tetrahydroxy-penten(2)al(1) 2×10^{-2} M, pH 3.2. b) triose reductone (2,3-dihydroxyacrolein) 1×10^{-2} M, pH 5.3. c) as a) pH 6.2.

Table I. Oxygen consumption rate and hydrogen peroxide yield in the ascorbic acid autoxidation system.

Compound		pH	Initial Rate [ppm min^{-1}]	O_2 consumed [ppm]	O_2 recovered [ppm]	Ratio H_2O_2/O_2
I	(10^{-4} M)	9.00	0.62	2.5	0.5	0.2[c]
I	(10^{-3} M) phosphate buffer	6.88	< 0.01	n.d.	–	–
II	(2×10^{-3} M)	4.00	< 0.001	n.d.[a]	–	–
II	(2×10^{-3} M)	8.40	< 0.001	n.d.	–	–
	+ (Cu^{2+}, 10^{-5} M)	4.00	< 0.001	n.d.	–	–
II	(thermally degraded)[b]	1.30	< 0.001	n.d.	–	–
		3.70	< 0.15	< 0.1	n.d.[a]	–
		7.00	7.50	< 0.1	n.d.	–
		11.00	8.10	< 0.1	n.d.	–
III	(10^{-2} M)	5.60	2.54	4.4	0.3	0.07
	+ EDTA (5×10^{-3} M)	5.60	1.11	4.0	1.2	0.30
III	(10^{-2} M)	6.90	4.78	4.3	0.5	0.12
	+ EDTA (5×10^{-3} M)	6.90	2.40	4.3	1.6	0.37
III	(10^{-2} M)	6.80	8.50	5.2	1.1	0.21
	+ Fe^{3+} (10^{-5} M)					
IV	(2×10^{-2} M)	3.20	0.06	n.d.	–	–
IV	(2×10^{-2} M)	4.20	0.40	4.17	0.5	0.12
IV	(2×10^{-2} M)	4.20	1.04	3.30	0.3	0.04
	+ Cu^{2+} (10^{-5} M)					
V	(10^{-2} M)	5.30	0.40	6.30	1.45	0.23
V	(10^{-2} M)	5.30	0.40	n.d.	–	–
	+ Fe^{3+} (10^{-5} M)					
V	(10^{-2} M)	3.80	3.20	n.d.	–	–
	+Cu^{2+} (10^{-5} M)					
VI	(2×10^{-2} M)	5.60	< 0.001	n.d.	–	–
VII	(10^{-2} M)	2.50	< 0.001	n.d.	–	–

Abbreviations for compounds: **I**, Ascorbic acid; **II**, Dehydroascorbic acid; **III**, 2,3-diketogulonic acid; **IV**, Reductone X; **V**, Triose reductone; **VI**, Threonic acid; **VII**, Oxalic acid.

[a] Not determinable; [b] thermally degraded in 5% H_2SO_4 for 60 min. Solution investigated without product separation, [c] H_2O_2/O_2 gives the ratio of the yield of oxygen arising from catalase induced decomposition of H_2O_2 to that of oxygen consumed in the reaction. It is thus an indication of the role of H_2O_2-consuming side reactions.

Table II. Mutagenicity of oxidative decomposition products of ascorbic acid toward *S. typhimurium* strain TA 100.

Dehydroascorbic acid		2,3-Diketogulonic acid		2,3-Diketogulonic acid + Catalase		Threonic acid	
Conc. [M]	Number of his^+-revertants	Conc. [M]	Number of his^+-revertants	Conc. [M]	Number of his^--revertants	Conc. [M]	Number of his^--revertants
3.5×10^{-2}	102 ± 3	3.82×10^{-1}	126 ± 10	3.82×10^{-1}	93 ± 5	4.0×10^{-1}	106 ± 5
2.5×10^{-2}	106 ± 5	3.67×10^{-1}	198 ± 8	3.67×10^{-1}	95 ± 3	3.5×10^{-1}	102 ± 6
		3.36×10^{-1}	163 ± 6	3.36×10^{-1}	95 ± 5	3.0×10^{-1}	99 ± 4
		3.06×10^{-1}	146 ± 8	3.06×10^{-1}	96 ± 4		
		2.75×10^{-1}	122 ± 5	2.75×10^{-1}	84 ± 5		

mixtures, dehydroascorbic acid was not mutagenic. With 2,3-diketogulonic acid a dose-related increase in the number of his^+ revertants was noted in the narrow dose range of $2.75 \times 10^{-1} - 3.67 \times 10^{-1}$ M. Doses exceeding the latter were cytotoxic and caused a decrease in the reversion rate. In the presence of catalase (0.01 mg/plate) the mutagenic effect of 2,3-diketogulonic acid was abolished. Triose reductone has been shown to exhibit a mutagenic effect in the Ames test [14]. In the dose range of $3.0 - 4.0 \times 10^{-1}$ M corresponding to the mutagenically active concentrations of the structurally similar 2,3-diketogulonic acid, threonic acid had no mutagenic effect on *S. thyphimurium.*

Scheme 1. Chemistry and mutagenicity of the ascorbic acid – Cu^{2+} system

Reaction	Comments
1. $AH_2 \rightleftarrows AH^- + H^+$	Production of chemically active species AH^-. Since $pK_1 = 4.14$ ca. 25% will be present at the natural pH's of our solutions (3.2–3.6)
2. $AH^- + O_2 + (Cu^{++}) \xrightarrow{H^+} A + H_2O_2 + (Cu^+)$	Hydrogen peroxide generating reaction – slow at low pH in the absence of Cu^{2+}. Mild mutagenic effect will be removed by prior catalase addition.
3. $H_2O_2 + AH_2$, AH^-, A etc. $\rightarrow$ Radicals ($AH^{-\cdot}$, $HO_x^{\cdot}$) and products (reductones, hydroxy- and carboxylic acids)	Hydrogen peroxide destruction reactions. Will be favoured at the higher initial AH_2 and Cu^{2+} concentrations. Mutagenic effect will not be completely removed by prior catalase addition.

We conclude that the lack of a measurable mutagenic effect and its inability to produce H_2O_2 exclude any contribution from dehydroascorbic acid in the AH_2–Cu(II) system. In contrast, our results show that, in general, since subsequent products form H_2O_2 more or less readily a mutagenic effect deriving from this source will remain present up to high oxidative conversion. The main final products, oxalic and threonic acids, however, are neither mutagenic themselves nor did they yield H_2O_2 under our conditions. Oxalic acid can however give rise to peroxide during its metal catalysed oxidation [29]. The residual mutagenic effect, which cannot be inhibited by prior addition of catalase to the reagent solutions must therefore arise from as yet unidentified minor products and/or free radicals specific to the system (Scheme 1). The more recent work of Stich *et al.* [27] lends support to this conclusion since while catalase (0.1 mg/ml) virtually abolished the mitosis inhibiting and chromosome damaging effect of ascorbate high frequencies of chromosome aberrations were observed for some higher AH_2–Cu^{2+} mixtures. The stationary concentration of radicals will be higher and the formation of secondary products more rapid in these solutions.

We are currently investigating the free radical chemistry of these systems further. In any case, we are of the opinion that the appearance of hydrogen peroxide forming capacity throughout the oxidative decomposition of ascorbic acid should be taken into account in food processing.

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