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Effect of purines on the oxidation of ascorbic acid induced by copper

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Summary. Uric acid and other purines including 1-methyl-, 7-methyl-, and 1,7-dimethyluric acid, adenine, guanine, xanthine, hypoxanthine, purine, and the structurally similar compound allopurinol protected ascorbic acid from oxidation catalyzed by copper. If the hydrogen at either the 3 or 9 nitrogen of the uric acid was replaced by a methyl group, the compound did not protect ascorbate. 3-Ribosyluric acid, xanthosine, adenosine, and guanosine also failed to protect ascorbate. It was concluded that in order for purines to complex with copper to protect ascorbate from copper-catalyzed oxidation, the nitrogens at both positions 3 and 9 of the purine must be unsubstituted.

Key words: Ascorbic acid — Purines — Oxidation — Antioxidants — Copper

Introduction

One of the functions of ascorbic acid in biological systems is to serve as an antioxidant (Klaui and Pongracz 1981; Cort 1982). Ascorbic acid protected oxyhemoglobin from oxidation to methemoglobin by sodium nitrite, protected erythrocytes from hemolysis induced by *t*-butyl hydroperoxide, and protected unsaturated fatty acids from oxidation when water-soluble 2,2′-azobis(2-amidinopropane) dihydrochloride was used as a radical initiator (Ames et al. 1981; Smith and Nunn 1984, 1986; Niki et al. 1985). Ascorbic acid was oxidized in air and this oxidation was enhanced by low concentrations of copper and iron salts (Martell 1982). Uric acid, as well as other purines, protected ascorbate from oxidation in the presence of these metals without the uric acid being oxidized (Giri and Rao 1944; Frieden and Alles 1958; Lam et al. 1984; Sevanian et al. 1985; Davies et al. 1986). This protection was suggested to be a result of the uric acid forming complexes with these metals so that they were no longer catalysts for the oxidation of ascorbate. Chelating agents such as EDTA and diethylenetriaminepentaacetic acid also almost completely inhibited the oxidation of ascorbate (Sevanian et al. 1985; Davies et al. 1986). Reactivity of methylated urates with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is affected by the position of the methyl groups on the urate molecule (Smith et al. 1987). For appreciable activity with DPPH, uric acid must have hydrogens at N7 and either N3 or N9. Experiments reported here were conducted to ascertain possible binding sites of uric acid for copper by determining the ability of methylated urates and other purines and purine nucleosides to protect ascorbate from oxidation in the presence of copper.

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Materials and methods

Chemicals. Uric acid, 1-methyl-, 1,3-dimethyl-, 3,7-dimethyl-, and 1,3,7-trimethyluric acid were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Methyl- and 9-methyluric acid were purchased from Adams Chemical Co. (Round Lake, IL). The other methylated urates were purchased from Fluka Chemical Corp. (Ronkonkoma, NY). 3-*N*-Ribosyluric acid was prepared from washed bovine red blood cells by the method of Forrest et al. (1961). The $CuSO_4 \cdot 5H_2O$ was purchased from Baker and Adamson (New York, NY). The other purines, purine nucleosides, uracil, allopurinol, indole, 7-azaindole, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). All of the reagents were prepared in water purified with a Barnstead NANOpure II system to reduce contamination by metals.

Oxidation of ascorbate. Oxidation of ascorbate was followed using the procedure of Lam et al. (1984). Ascorbic acid (100 μM), either alone or with the protecting agent (30 μM), was incubated in 1 ml 20 mM phosphate buffer, pH 7.5, at 25° C in a Beckman model 25 spectrophotometer equipped with a recorder. After 1 min of preincubation, 10 µl of 1.0 mM copper sulfate was added to the ascorbate and absorbance at 255 nm monitored. The ascorbate solution was prepared on the day of use.

Chromatography of the urates. Either 3-methyl- or 7-methyluric acid (30 μM) was incubated either with 10 μM copper sulfate or 10 μM copper sulfate and 100 μM ascorbic acid until the ascorbate was completely oxidized. Then 25 μl of the mixture was chromatographed on an Alltech C_{18} column (25 × 4.6 mm) using a Waters Assoc. model ALC 204 liquid chromatograph equipped with a model 6000A pump, a U6K injector, a model 481 variable-wavelength detector (set at 290 nm), and a model 3390A Hewlett Packard integrator. The solvent was 40 mM sodium phosphate, pH 4.2, in 20% methanol.

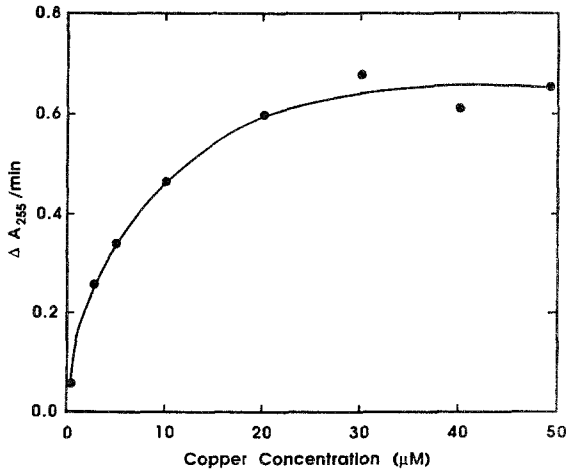

Fig. 2. Effect of concentration of copper sulfate on the initial rate of oxidation of 100 μM ascorbic acid. The rate of oxidation of ascorbic acid was measured by following the decrease in absorbance at 255 nm

Results and discussion

As reported by Lam et al. (1984), 100 μM ascorbate in phosphate buffer was slowly oxidized and the addition of 10 μM copper sulfate immediately increased the rate of oxidation of ascorbate (Fig. 1). In the presence of uric acid, the initial rate of oxidation of ascorbate was reduced both before and after addition of copper sulfate (Fig. 1). The rate of oxidation of ascorbate was directly related to the concentration of copper (Fig. 2). The effect of the concentration of uric acid, xanthine, and allopurinol on the rate of oxidiation of ascorbic acid is shown in Fig. 3. When methylated urates were added to the ascorbate, some protected the ascorbate and some offered little or no protection (Table 1). 1-Methyl-, 7-methyl-, and 1,7-dimethyluric acid protected ascorbic acid from oxidation even after addition of copper sulfate. Other urates, including 3-ribosyluric acid, failed to protect ascorbate from oxidation either before or after addition of copper sulfate. All urates that failed to protect ascorbate from oxidation had a methyl group at either or both the 3 or 9 positions. Since this could mean that either both positions 3 and 9 of uric acid must be free for it to bind cop-

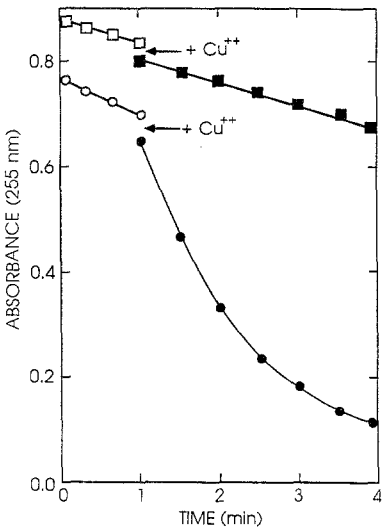

Fig. 1. Effect of copper and uric acid on the degradation of ascorbic acid. The reaction mixture contained either 100 μM ascorbic acid in 20 mM phosphate buffer, pH 7.5 (○, ●) or 100 μM ascorbic acid + 30 μM uric acid in 20 mM phosphate (□, ■). The copper (10 μM) was added after 1 min of preincubation

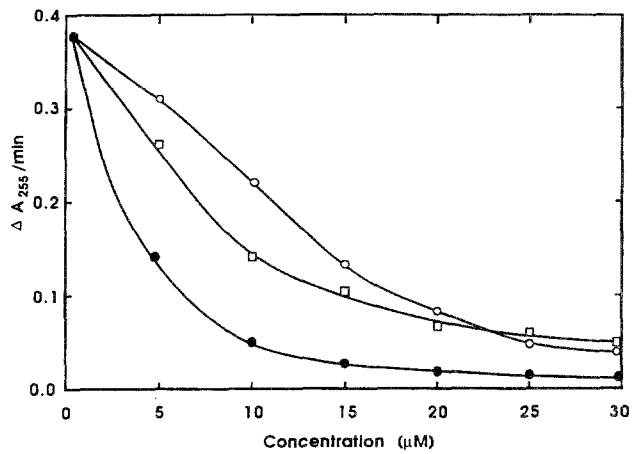

Fig. 3. Effect of concentration of allopurinol (□), uric acid (○), and xanthine (●) on the initial rate of oxidation of 100 μM ascorbic acid. The rate of oxidation of ascorbic acid was measured by following the decrease in absorbance at 255 nm

Table 1. Effect of purines on the degradation of ascorbate induced by copper

Compound	Ascorbate degradation (ΔA_{255}/min)	
	−Cu	+Cu
None	0.037	0.380
Uric acid	0.010	0.039
1-Methyluric acid	0.011	0.045
7-Methyluric acid	0.007	0.025
1,7-Dimethyluric acid	0.020	0.026
3-Methyluric acid	0.047	0.411
9-Methyluric acid	0.044	0.429
1,3-Dimethyluric acid	0.034	0.445
1,9-Dimethyluric acid	0.051	0.424
3,7-Dimethyluric acid	0.043	0.435
3,9-Dimethyluric acid	0.043	0.401
7,9-Dimethyluric acid	0.044	0.379
1,3,7-Trimethyluric acid	0.045	0.411
1,3,9-Trimethyluric acid	0.037	0.423
3,7,9-Trimethyluric acid	0.023	0.450
1,3,7,9 Tetramethyluric acid	0.049	0.374
3-Ribosyluric acid	0.020	0.383
Adenine	0.012	0.020
Adenosine	0.050	0.350
Guanine	0.021	0.022
Guanosine	0.035	0.369
Xanthine	0.008	0.008
Xanthosine	0.051	0.386
Hypoxanthine	0.020	0.042
Purine	0.022	0.045
Allopurinol	0.023	0.048
2-Hydroxybenzimidazole	0.040	0.402
Uracil	0.054	0.330
Indole	0.038	0.362
7-Azaindole	0.042	0.424

The concentration of the ascorbic acid was 100 μM. The concentration of each of the compounds added was 30 μM while that of copper sulfate (when present) was 10 μM. Values are the mean from three experiments. The individual values differed from the mean by less than 10%

per or the presence of a methyl group at either position sterically hinders the interaction of copper with uric acid, the ability of 2-hydroxybenzimidazole to protect ascorbic acid from copper-induced oxidation was also determined (Table 1). 2-Hydroxybenzimidazole failed to protect ascorbic acid. From this it was concluded that both positions 3 and 9 are required for the binding of copper. In addition, the related nitrogen-containing compounds indole and 7-azaindole did not protect ascorbic acid (Table 1).

Purines other than uric acid, including adenine, guanine, xanthine, hypoxanthine, and purine at a concentration of 30 μM, also protected ascorbate from oxidation both in the presence and absence of copper (Table 1). These results suggest that both the 3 and 9 positions of purines must be free in order for them to protect ascorbate from oxidation either with or without addition of copper. Uracil, xanthosine, guanosine, and adenosine were ineffective at 30 μM. As reported by Frieden and Alles (1958), higher concentrations of the purine nucleosides protected ascorbic acid. Approximately ten times as much of the purine nucleoside was required to give the same degree of protection as the free base.

Chromatography of 3-methyl- and 7-methyluric acid, following incubation of each with ascorbic acid until it was completely oxidized, showed that these urates were not degraded during the incubation. Lam et al. (1984), Sevanian et al. (1985), and Davies et al. (1986) reported that although ascorbic acid was completely oxidized, uric acid was not oxidized when it was incubated with ascorbic acid and copper.

Das et al. (1987) reported that allopurinol scavenged ClO_2 and HOCl, but failed to scavenge $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals; allopurinol also failed to react with DPPH (Smith 1982). Das et al. (1987) suggested that allopurinol may protect ischemic myocardium during reperfusion by scavenging free radicals rather than by inhibiting xanthine oxidase as others have suggested. Allopurinol also protected ascorbic acid from copper-induced oxidation (Table 1, Fig. 3). This suggests that in addition to scavenging some free radicals, allopurinol also chelates copper.

Lam et al. (1984) and Davies et al. (1986) suggested that urate protects ascorbate from oxidation catalyzed by either copper or iron by forming a complex with the metal. Although both Lam et al. (1984) and Davies et al. (1986) had data which suggested the complexing of uric acid to copper and iron, the structure of the complex is not known. Davies et al. (1986) reported that urate can form a 2:1 complex with Fe(III) in which binding of the second urate was facilitated by binding of the first urate. They also reported that urate bound copper and manganese. Data reported here for methylated urates and nucleosides suggest that nitrogens at both positions 3 and 9 of the purine must be unsubstituted to interact with metal ions. de Meester and Skapski (1971; 1972) reported that copper(II) complexes of adenine can contain either two copper(II) ions, held together by four bridging adenines coordinated through N9 and N3, or three copper(II) ions held by three adenines. In the latter case, the center copper is coordinated with N3 of both adenines and each terminal copper is coordinated to one N9 atom of each adenine. Hypoxanthine formed a complex with copper in which four molecules of

hypoxanthine complexed with two atoms of copper (Sletten 1970). Each hypoxanthine coordinated to one copper through N9 and to the other copper through N3. All of these structures would require that both the N3 and N9 positions of the purines be unsubstituted for them to complex with copper.

The stability constants of copper complexes of purines are higher than those of the corresponding nucleosides substituted at N9 by either a ribosyl or a methyl group (Albert 1953; Reinert and Weiss 1969; Tauler et al. 1986). Tauler et al. (1986) concluded that copper(II) binds more strongly to adenine than to 9-methyladenine, but that zinc(II) forms a more stable complex with 9-methyladenine than it does with adenine. The difference in stability constants for copper complexes of bases and nucleosides support the current observation that the purine bases protect ascorbic acid from copper-induced oxidation at concentrations at which the corresponding nucleosides do not protect.

Xanthine and uric acid protected erythrocytes from hemolysis induced by xanthine plus xanthine oxidase (Kellogg and Fridovich 1977) and also protected erythrocytes from peroxidation induced by ascorbate and iron (Davies and Goldberg 1987). In the present study, purines such as xanthine and hypoxanthine also protected ascorbate from oxidation induced by copper by presumably binding the metal ion. However, as pointed out by Davies et al. (1986), the high concentrations of uric acid in plasma, and probably other tissues (Sajiki et al. 1980), suggest that uric acid may be one of the major low-molecular-mass metal-binding compounds in human blood. Uric acid contributes between 35–65% of the total peroxyl-radical-trapping activity of human blood plasma (Wayner et al. 1987) and has been proposed as a contributor to the increased life span and decreased age-specific cancer rates in humans (Ames et al. 1981; Cutler 1984).

Uric acid has been proposed to be an important antioxidant and free radical scavenger in the plasma and tissues of humans (Ames et al. 1981; Cutler 1984). This activity could result either from its reactivity with oxidizing agents or its ability to bind metals that catalyze oxidations. 3-Ribosyluric acid has been suggested to serve as an antioxidant in the bovine erythrocyte (Smith and Nunn 1984). Antioxidant activity of uric acid has been compared to that of 3-ribosyluric acid in a number of systems (Smith et al. 1987). Uric acid reacted with the stable free radicals DPPH and galvinoxyl at about twice the rate of ribosyluric acid (Smith et al. 1987; Smith and Hargis 1985). Although both uric acid and 3-ribosyluric acid protected linolenic and arachidonic acid from air oxidation in the presence of copper (Smith and Lawing 1983), protected erythrocytes from oxidation by *t*-butyl hydroperoxide (Smith and Nunn 1986), and protected oxyhemoglobin from oxidation to methemoglobin by sodium nitrite (Smith and Nunn 1984), in most cases, uric acid was effective at a lower concentration than 3-ribosyluric acid. Protection of ascorbate from copper-catalyzed oxidation by uric acid is the first example of an oxidation system in which uric acid, but not 3-ribosyluric acid, was an effective antioxidant. Present data indicate that 3-ribosyluric acid failed to protect ascorbate because it lacks a hydrogen at N3. Collectively, these data suggest that, if 3-ribosyluric acid is important as an antioxidant in the bovine red cell, this activity is a result of its reactivity with free radicals and oxidizing agents and not its ability to bind metals such as copper and iron.

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References

Albert A (1953) Quantitative studies of the avidity of naturally occurring substances for trace metals. Biochem J 54:646–654

Ames BN, Cathcart R, Schwiers E, Hochstein P (1981) Uric acid provides an antioxidant defense against oxidant and radical-caused aging and cancer. Proc Natl Acad Sci USA 78:6858–6862

Cort WM (1982) Antioxidant properties of ascorbic acid in foods. In: Seib PA, Tolbert BM (eds) Ascorbic acid: chemistry, metabolism, and uses. Adv Chem Ser 200:533–550

Cutler RG (1984) Urate and ascorbate: their possible roles as antioxidants in determining longevity of mammalian species. Arch Gerontol Geriatr 3:321–348

Das DK, Engelman RM, Clement R, Otani H, Prasad MR, Rao PS (1987) Role of xanthine oxidase inhibitor as free radical scavenger: a novel mechanism of action of allopurinol and oxypurinol in myocardial salvage. Biochem Biophys Res Commun 148:314–319

Davies KJA, Goldberg AL (1987) Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. J Biol Chem 262:8220–8226

Davies KJA, Sevanian A, Muakkassah-Kelly SF, Hochstein P (1986) Uric-acid–iron-ion complexes. A new aspect of the antioxidant functions of uric acid. Biochem J 235:747–754

deMeester P, Skapski AC (1971) Crystal structure of dichlorotetra-μ-adenine dicopper(II) chloride hexahydrate. J Chem Soc (A):2167–2169

deMeester P, Skapski AC (1972) Crystal structure of octachlorobis(adeninium)tricopper(II)tetrahydrate, a trinuclear

complex with bridging adenine. J Chem Soc Dalton Trans 2400–2404

Forrest HS, Hatfield D, Lagowski JM (1961) Uric acid riboside. Part I. Isolation and reinvestigation of the structure. J Chem Soc 963–968

Frieden E, Alles J (1958) Subtle interactions of cupric ion with nucleic acid components. J Biol Chem 230:797–804

Giri KV, Rao PS (1944) The inhibitors of enzymatic and cupric ion oxidation of vitamin C. Proc Ind Acad Sci 24:264–278

Kellogg EW, Fridovich I (1977) Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide. J Biol Chem 252:6721–6728

Klaui H, Pongracz G (1981) Ascorbic acid and derivatives as antioxidants in oils and fats. In: Counsell JN, Hornig DH (eds) Vitamin C (ascorbic acid), Applied Science Publishers, Englewood NJ, pp 139–166

Lam KW, Fong D, Lee A, Liu KMD (1984) Inhibition of ascorbate oxidation by urate. J Inorg Biochem 22:241–248

Martell AE (1982) Chelates of ascorbic acid. Formation and catalytic properties. In: Seib PA, Tolbert BM (eds) Ascorbic acid: chemistry, metabolism, and uses. Adv Chem Ser 200:153–178

Niki E, Kawakami A, Yamamoto Y, Kamiya Y (1985) Synergistic inhibition of oxidation of phosphatidylcholine liposomes in aqueous dispersions by vitamin E and vitamin C. Bull Chem Soc Jap 58:1971–1975

Reinert H, Weiss R (1969) Formation of complexes by pyrimidine derivatives. I. Formation constants of some copper(II) complexes with purines and pyrimidines. Hoppe-Seyler's Z Physiol Chem 350:1310–1320

Sajiki J, Fujishiro Y, Hirai A, Yamamoto M, Kumagai A (1980) Method for determination of uric acid in viscera. Anal Biochem 104:425–431

Sevanian A, Davies KJA, Hochstein P (1985) Conservation of vitamin C by uric acid in blood. J Free Rad Biol Med 1:117–124

Sletten E (1970) Crystallographic studies of metal-nucleotide-base complexes. II. Bis(6-hydroxypurine)copper(II) chloride trihydrate. Acta Crystallogr B26:1609–1614

Smith RC (1982) Reaction of uric acid and 3-*N*-ribosyluric acid with 1,1-diphenyl-2-picrylhydrazyl. Bioorg Chem 11:436–442

Smith RC, Hargis H (1985) Reaction of urates and other antioxidants with galvinoxyl. Microchem J 31:18–21

Smith RC, Lawing L (1983) Antioxidant activity of uric acid and 3-*N*-ribosyluric acid with unsaturated fatty acids and erythrocyte membranes. Arch Biochem Biophys 223:166–172

Smith RC, Nunn V (1984) Prevention by 3-*N*-ribosyluric acid of the oxidation of bovine hemoglobin by sodium nitrite. Arch Biochem Biophys 232:348–353

Smith RC, Nunn V (1986) Prevention by antioxidants of the hemolysis of erythrocytes of cattle, pigs, and humans treated with *t*-butyl hydroperoxide. Comp Biochem Physiol 84C:79–82

Smith RC, Reeves J, McKee ML, Daron H (1987) Reaction of methylated urates with 1,1-diphenyl-2-picrylhydrazyl. Free Rad Biol Med 3:251–257

Tauler R, Rainer MJA, Rode BM (1986) The interaction of H^+, Zn(II), and Cu(II) with adenine and 9-methyladenine. Inorg Chim Acta 123:75–82

Wayner DDM, Burton GW, Ingold KU, Barclay LRC, Locke SJ (1987) The relative contributions of vitamin E, urate, ascorbate, and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta 924:408–419

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