Cytotoxicity of Glucosone in the Presence of Cupric Ion

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We found that glucosone (D-*arabino*-hexosulose), a typical enediol product formed in the Maillard reaction or in γ-radiolysis of sugars, was cytotoxic on Chinese hamster lung V79 cells in the presence of cupric ion (Cu^{2+}). Several other metal ions showed no effects. The cytotoxicity was assessed by colony formation assay and measurement of cell growth rate. In both assays, catalase inhibited the cytotoxic effects. To confirm the role of H_2O_2 on the cytotoxicity, we detected H_2O_2 in the reaction mixture of glucosone and Cu^{2+}. No other metal ions except Mn^{2+} showed any effects on the formation of H_2O_2. From these results, we propose that H_2O_2 was formed after Cu^{2+}-catalyzed oxidation of glucosone and that hydroxyl radical ($\cdot OH$) formation from H_2O_2 in the Cu^{+}-catalyzed Fenton-like reaction was a possible step in the cytotoxicity of glucosone.

INTRODUCTION

Glycation of protein has been implicated recently as a mediator in cellular injury in clinical conditions related to diabetes. Glycation is well-known also as the first step of the Maillard reaction (amino-carbonyl reaction) in foods and food model systems. Therefore, secondary products of glycation should be common in these systems. Although there are many papers on the chemistry of these secondary products, their effects on mammalian cells have not yet been fully studied. To the best of our knowledge, only the metabolism of 3-deoxyglucosone administered to rats has been studied in detail (Kato et al., 1990).

It has been reported recently that glucosone (D-*arabino*-hexosulose) was formed by oxidative degradation of Amadori products, the initial products of the Maillard reaction, in the presence of metal ion (Kawakishi et al., 1991). Glucosone was also detected in calf liver extracts (Kato et al., 1970). Therefore, it is expected that glycation and the following formation of glucosone might occur in vivo.

Since glucosone is one of the main products in γ-radiolysis of D-glucose and D-fructose both in aerobic and in anaerobic conditions (Kawakishi et al., 1975, 1977; Kito et al., 1979, 1981), irradiated foods such as irradiated mango might contain glucosone (Den Drijver et al., 1970). Irradiated sugar solutions are mutagenic against *Salmonella* tester strain TA 100 (Niemand et al., 1983). Among the products of the γ-radiolysis of sugars, glucosone has been implicated as a mutagenic agent.

Concerning its biological action on mammalian cells, 2 mM glucosone is a strong inhibitor of growth and protein and DNA synthesis of in vitro cultured Ehrlich ascites tumor cells (Reiffen et al., 1981). In the study of Reiffen et al., an attempt to ascribe these effects to the inhibitory action of glucosone to hexokinase failed. So far, the mechanism of its biological effects remains unknown.

For these reasons, it is important to elucidate the biological effects of glucosone on cultured mammalian cells from a different point of view.

MATERIALS AND METHODS

Reagents. Glucosone (D-*arabino*-hexosulose) was prepared from glucose phenylosazone (Bayne, 1963). Catalase from bovine liver (thymol free) and peroxidase from horseradish were obtained from Sigma Chemical Co. (St. Louis, MO) and stored in water (1 mg/mL) at 4 °C. All other chemicals were of reagent grade and were used without further purification.

Cell Culture. V79 cells from Chinese hamster lung fibroblasts were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), unless specified otherwise. The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were detached with trypsin.

Colony Formation Assay. Cytotoxicity of glucosone in the presence of metal ion was assessed by colony formation assay as described previously (Nakayama et al., 1991). Briefly, V79 cells were seeded in 200/60-mm Petri dishes and incubated in MEM with 10% FBS. After 4 h, the cells were washed with HEPES-buffered saline (HBS) and incubated in HBS with glucosone in the presence or absence of metal ion for 30 min at 37 °C; the cells were incubated with relatively concentrated glucosone for a short time in HBS, because incubation of the cells in HBS for more than 90 min had cytotoxic effects. After culture for 5 days, the colonies were fixed with methanol and stained with Giemsa's solution. The relative survival fraction (percent) was calculated by dividing the number of colonies of the cells treated with glucosone or other reagents by the number of colonies of untreated control cells. Results are expressed as the means and SD of four separately treated cultures. The data were analyzed statistically using Student's *t*-test.

Cell Growth. Cells in triplicate for one measuring point were plated onto dishes at a cell density of 2.5×10^4 cells/dish. After 24 h of incubation, the cells were incubated in HBS with glucosone and/or other reagents for 30 min. After culture in MEM with 10% FBS for the specified times, the cells were washed, trypsinized, and counted with a hemocytometer.

Quantification of Hydrogen Peroxide. We incubated glucosone and metal ion in HBS for 30 min at 37 °C. The amount of H_2O_2 was quantified according to the method of Guilbault et al. (1968). Briefly, nonfluorescent 3-methoxy-4-hydroxyphenylacetic acid was quantitatively converted upon oxidation by H_2O_2 to the highly fluorescent dimer (λ_{ex} = 315 nm, λ_{em} = 425 nm) in the presence of horseradish peroxidase. The lowest limit of detection for H_2O_2 was less than 0.1 μM.

RESULTS AND DISCUSSION

Although neither glucosone (10 mM) nor Cu^{2+} (10 μM) was cytotoxic in the colony formation assay, glucosone was cytotoxic in the presence of Cu^{2+} under the same conditions (Figure 1a). Since H_2O_2 was formed after Cu^{2+}-catalyzed oxidation of ascorbic acid [Dekker et al., 1940; Scheme I, according to Martell (1982)], which has an enediol structure similar to that of glucosone, H_2O_2 is expected to be formed after oxidation of glucosone through the formation of a glucosone–metal ion–oxygen complex. To elucidate the role of H_2O_2 in the cytotoxicity of glucosone, we investigated the effects of catalase. Figure 1b shows

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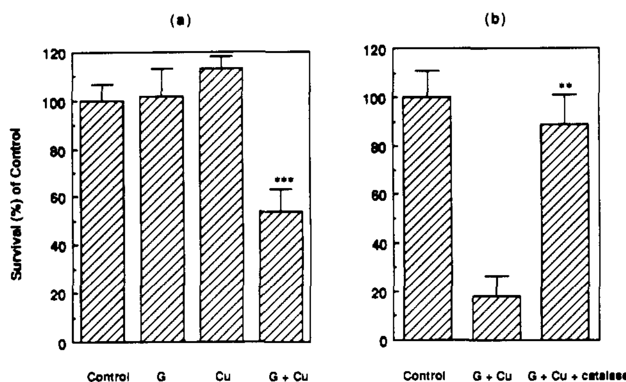
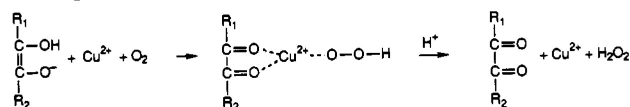


Figure 1. Cytotoxic effects of glucosone and Cu^{2+} on V79 cells assessed by colony formation assay. The cells were treated with reagents for 30 min as described under Materials and Methods. After culture for 5 days, the number of colonies was counted. The control experiments were carried out in a similar manner without treatment with glucosone and Cu^{2+}. The data were analyzed with Student's *t*-test. Glucosone is abbreviated "G". (a) Effects of glucosone (10 mM) and/or Cu^{2+} (10 μM). (***) $P < 0.001$, compared with the control. (b) Effects of catalase (500 units) on the cytotoxicity of glucosone (10 mM) and Cu^{2+} (10 μM). (**) $P < 0.01$, compared with the group treated with glucosone and Cu^{2+}.

Scheme I. Proposed Mechanism of H_2O_2 Formation during Cu^{2+}-Catalyzed Oxidation of Enediol



that catalase inhibited the cytotoxicity of glucosone in the presence of Cu^{2+}, indicating that H_2O_2 formed in the medium had an important role in the cytotoxic mechanism. In addition to the decrease of the number of colonies, the sizes of the colonies were also apparently smaller than those of the control, suggesting that the growth rate of cells also decreased. This being the case, we checked the number of cells after incubation of glucosone and Cu^{2+} in the cell growth assay. Figure 2b shows that the combination of glucosone and Cu^{2+} apparently decreased the growth rate, but incubation of cells with one reagent showed no effect (Figure 2a), supporting the results of the colony formation assay. The inhibition of catalase on the decrease of the growth rate was also observed (Figure 2b), confirming the participation of H_2O_2 in the cytotoxicity.

Next we tried to investigate the effects of other metal ions on the cytotoxicity and to confirm H_2O_2 formation in the medium. Table I shows that the metal ions used in our experiments, other than Cu^{2+}, did not have any effect on the cytotoxicity of glucosone. After incubation of glucosone and metal ions in HBS under the same conditions as the colony formation assay, we determined H_2O_2 concentration in the solution. The fluorescence formation was observed in the solution of glucosone with Cu^{2+} or Mn^{2+} but not with the other metal ions. Since the fluorescence value was completely removed by the addition of catalase, the value can only be due to H_2O_2 (Table I). Interestingly, Mn^{2+} participated only in the formation of H_2O_2 in the medium and had no effects on the cytotoxicity of glucosone. We suppose that, unlike Cu^{2+}, Mn^{2+} does not catalyze the Fenton-like reaction (H_2O_2 + metal$^{(n)}$ $\rightarrow$ $^{\bullet}$OH + OH^- + metal$^{(n+1)}$), which produces the hydroxyl radical ($^{\bullet}$OH) as the ultimate species causing cell lesions (Halliwell and Gutteridge, 1984; Stadtman et al., 1990). From these results, we conclude that H_2O_2 is formed in the medium in the case of glucosone and Cu^{2+} and

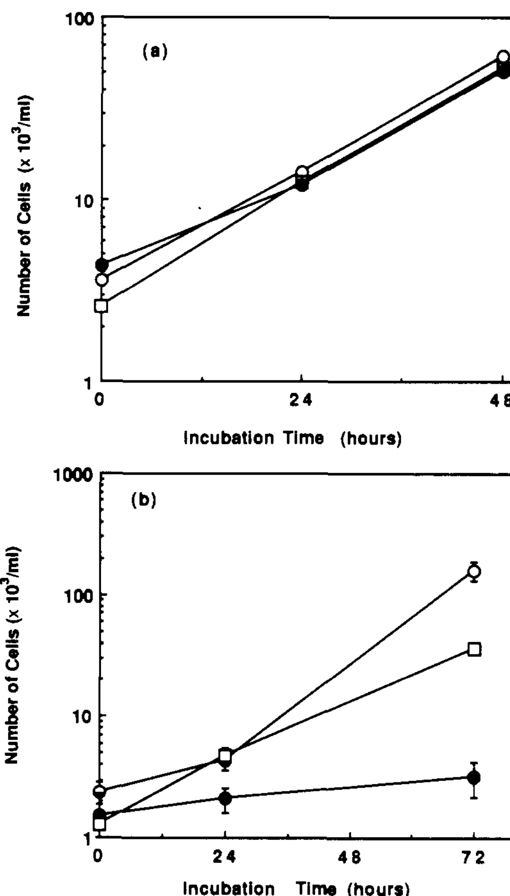


Figure 2. Cytotoxic effects of glucosone and Cu^{2+} on V79 cells assessed by cell growth rate. The cells were treated with reagents for 30 min as described under Materials and Methods. After culture for the times indicated, the number of cells was counted. The control experiments were carried out in a similar manner without treatment with glucosone and Cu^{2+}. (a) Growth rates of cells treated with glucosone or Cu^{2+}. The results are expressed as the mean of triplicate dishes. (○) Control; (●) glucosone (10 mM); (□) Cu^{2+} (10 μM). (b) Growth rates of cells treated with glucosone and Cu^{2+} in the presence or absence of catalase. The results are expressed as the mean and SD of triplicate dishes. (○) Control; (●) glucosone (10 mM) and Cu^{2+} (10 μM); (□) glucosone (10 mM) and Cu^{2+} (10 μM) in the presence of catalase (500 units).

assume the presence of H_2O_2 in the cells because it can cross cell membranes freely (Halliwell and Gutteridge, 1990).

We have already reported that *o*-phenanthroline, a potent iron chelator that enters into the cells and renders the metal incapable of generating $^{\bullet}$OH, inhibits the cytotoxicity of H_2O_2 on V79 cells (Nakayama et al., 1991). This means iron in the cells is necessary for the toxic mechanism of H_2O_2. Conversely, Cu^{2+} chelated by *o*-phenanthroline enhances $^{\bullet}$OH formation (Que et al., 1980). To clarify the roles of metal ions in the cells, the cells were pretreated with *o*-phenanthroline for 15 min. After the medium was changed to exclude the effects of *o*-phenanthroline in the medium, the cells were incubated with glucosone and Cu^{2+}. Since this pretreatment of *o*-phenanthroline enhanced the cytotoxicity of glucosone in the presence of Cu^{2+} (Figure 3), Cu^{2+} or Cu^{+} taken into the cells probably played the catalytic role in the $^{\bullet}$OH formation instead of iron. When the cells were incubated with glucosone and Cu^{2+} in the presence of several $^{\bullet}$OH scavengers in the medium, no inhibitory effects on the cytotoxicity were observed (Figure 3). This means that $^{\bullet}$OH produced in the medium did not have any cytotoxic effects on cells. From these results, we propose $^{\bullet}$OH formation in the cells must be critical.

Table I. Cytotoxicity of Glucosone in the Presence of Various Metal Ions Assessed by Colony Formation Assay and Concentration of H_2O_2 Formed under the Same Conditions

metal ion	survival,[a] %		H_2O_2,[b] μM
	−glucosone	+glucosone	
Ca^{2+}	95 ± 15	96 ± 6	0.0
Fe^{3+}[c]	91 ± 9	98 ± 7	0.0
Fe^{2+}	91 ± 8	87 ± 3	0.0
Mg^{2+}	98 ± 21	88 ± 5	0.0
Co^{2+}	97 ± 22	85 ± 13	0.0
Mn^{2+}	94 ± 12	82 ± 6	11.2
Cu^{2+}	113 ± 5	54 ± 9***	8.8

[a] The cells were treated with each metal ion (10 μM) in the presence or absence of glucosone (10 mM) for 30 min as described under Materials and Methods. After culture for 5 days, the number of colonies was counted. The results are expressed as the mean ± SD of four separately treated cultures. The data were analyzed with Student's *t*-test, comparing the group treated with the metal ion vs the group treated with glucosone and the respective metal ion. (***) $P < 0.001$. [b] Glucosone (10 mM) was incubated with various metal ions for 30 min under the same conditions as the cell experiments. The concentration of H_2O_2 formed in the reaction mixture was quantified as described under Materials and Methods. The results are expressed as the mean of duplicate experiments. [c] Added as EDTA (final 10 μM) chelates, because Fe^{3+} is unstable in HBS in the absence of the chelator.

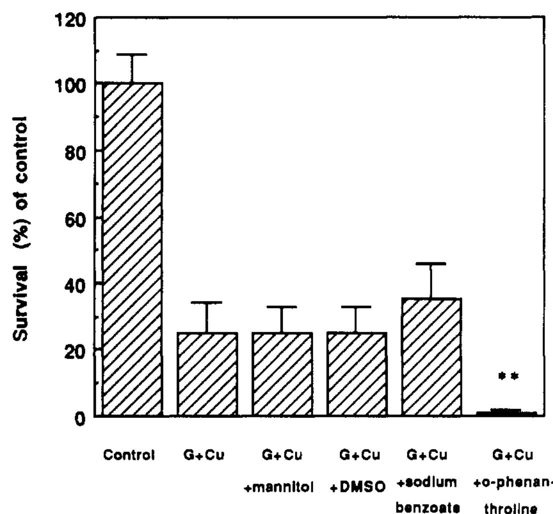
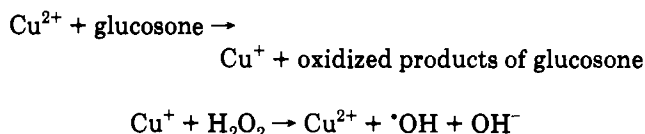


Figure 3. Effects of ·OH scavengers and *o*-phenanthroline on the cytotoxicity of glucosone and Cu^{2+}. The cells were treated with glucosone (10 mM) and Cu^{2+} (10 μM) in the presence or absence of ·OH scavenger (final 100 μM). In the case of *o*-phenanthroline, this reagent (final 25 μM) was added to HBS and incubated for 15 min. The medium was changed before treatment with glucosone and Cu^{2+}. The control experiments were carried out in a similar manner without treatment of any reagents. (**) $P < 0.01$, compared with the group treated with glucosone and Cu^{2+}.

Factors other than H_2O_2 and Cu^{2+} are also necessary for cytotoxic effects, because no enhancing effects of Cu^{2+} on the cytotoxicity of H_2O_2 were observed when the concentrations of Cu^{2+} and H_2O_2 were adjusted to those in the reaction mixture of glucosone and Cu^{2+} (Figure 4). We suppose that glucosone reduced Cu^{2+} to Cu^+ and then ·OH was produced in the cells by the Fenton-like reaction as follows:



$$Cu^{2+} + \text{glucosone} \rightarrow Cu^+ + \text{oxidized products of glucosone}$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^-$$

Inhibition by catalase of the mutagenicity of the Maillard reaction products from D-glucose–amino acid mixtures has been observed by Kim et al. (1991). This suggests that H_2O_2 formation in the Maillard reaction products is common and has important roles in the biological effects. We suppose trace metal ions and enediol products such as glucosone have dual roles on H_2O_2 and ·OH formation as described in this paper.

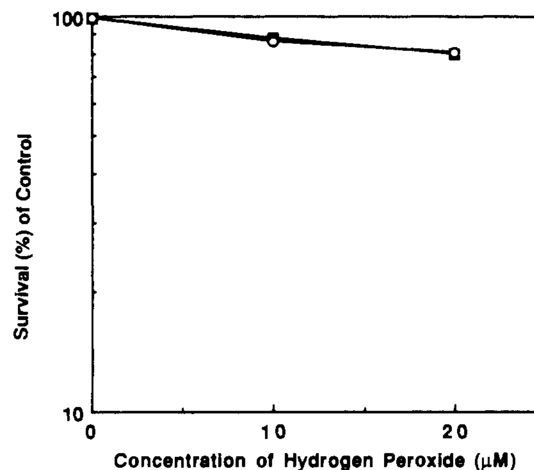


Figure 4. Cytotoxic effects of H_2O_2 and Cu^{2+} on V79 cells assessed by colony formation assay. The cells were treated with H_2O_2 (10 or 20 μM) in the presence or absence of Cu^{2+} (10 μM) for 30 min as described under Materials and Methods; 50 or 100 μL of 1 mM H_2O_2 solution was added to 5 mL of HBS and gently mixed. The control experiments were carried out in a similar manner without treatment with H_2O_2 and/or Cu^{2+}. The results are expressed as the mean of four separately treated cultures. (■) H_2O_2; (○) H_2O_2 plus Cu^{2+}.

To assess long-term effects of glucosone in foods on human and animals, it is necessary to quantify the amounts of glucosone in foods (especially in irradiated foods containing much sugar) and to investigate various biological effects by incubating mammalian cells with lower concentrations of glucosone and for longer periods of time than in the case of this study. We are conducting such experiments at present.

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Registry No. Cu^{2+}, 7440-50-8; Mn^{2+}, 7439-96-5; H_2O_2, 7722-84-1; glucosone, 26345-59-5.