Relationship between the Reduction of Tetrazolium Salt XTT and DNA Strand Breakage with Aminosugars

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Dihydropyrazine derivatives formed by the self-condensation reaction of D-glucosamine have the DNA breaking activity. To establish the monitoring method of the biological active dihydropyrazines, we investigated the relationship between the XTT (3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzensulfonic acid hydrate) reducibility and the DNA breaking activity of aminosugars. Aminosugar in 50 mM sodium phosphate buffer (pH 7.4) was incubated at 37 °C. At a given time, the XTT reducibility and the DNA breaking activity of the incubated aminosugar were measured. Both XTT reducibility and DNA breaking activity showed a maximum value within 1−4 h after the incubation and then gradually decreased with the incubation time. Superoxide anion was suggested to involve in both of the DNA breaking activity and the XTT reducibility by the addition of the radical scavengers into those assay mixtures. The quantity of remaining covalently closed circular DNA and the XTT reducibility of all aminosugars showed a good correlation (r = 0.825, n = 26). This means that the XTT assay is applicable for the monitoring of those biologically active products derived from aminosugars when the participation of superoxide anion in DNA scission is recognized.

Keywords: *XTT; aminosugar; DNA strand breakage; dihydropyrazine; Maillard reaction*

INTRODUCTION

Many kinds of aminosugars are naturally occurring. Among them, D-glucosamine is present in connective tissue and cartilage as proteoglycan in vivo. It is also distributed in crab and shrimp shell as a constituent of chitin or chitosan. It was found, moreover, that D-glucosamine was effective for rheumatoid arthritis and wound healing because it allowed increased production of proteoglycan (Prehm, 1984; Kim and Conrad, 1974). Actually, D-glucosamine hydrochloride and D-glucosamine sulfate are used as remedies clinically (Russell, 1998; McCarty, 1996). Aminosugars other than D-glucosamine are present as polysaccharide or mucopolysaccharide in vivo and in antibiotics (Horton and Wander, 1980; Garrod et al., 1973).

It was reported that some aminosugars cause DNA strand breakage (Kashige et al., 1994). Particularly, in the presence of Cu^{2+}, the DNA breaking activity of aminosugars increased remarkably (Watanabe et al., 1986 and 1990). Since the DNA breakage is involved in various diseases such as inflammation, mutagenesis, carcinogenesis, and aging (Sohal and Weindruch, 1996), the elucidation of the DNA breaking activity will contribute to the clarification of those biological phenomena. Yamaguchi et al. (1996) and Kashige et al. (1995) suggested that 2,5-bis(D-*arabino*-tetrahydroxybutyl)dihydropyrazine formed by the condensation of two molecules of D-glucosamine was responsible for the DNA breakage. They also found that certain carbon-centered radicals produced from the dihydropyrazine were responsible for the DNA strand scission (Yamaguchi et al., 1998). Thus, the monitoring of these biological activities of dihydropyrazines derived from D-glucosamine is important for its further application as a drug or supplement.

Recently, we proposed a novel monitoring method of the Maillard reaction based on the use of a water-soluble tetrazolium salt XTT (3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzensulfonic acid hydrate) shown in Figure 1. As XTT is reduced to the corresponding water-soluble formazan, it is suitable for the photometric assay. We applied the XTT assay to evaluate the extent of heating treatment of UHT-milk. Compared with the previously reported method for the Maillard reaction products as the quantification of lactulose (Geier and Klostermeyer, 1980), hydroxymethylfurfural (Keeney and Bassette, 1959), and furosine (Finot et al., 1981; Resmani et al., 1990), the XTT assay is a more rapid and convenient method and applicable for estimating not only the extent of thermal treatment but also the storage conditions because the XTT reducibility of the heated milk decreased depending on the storage period and temperature (Ukeda et al., 1995, 1996). During the course of that investigation, we found that dihydrofructosazine, which is generated by self-condensation of D-glucosamine, showed a high XTT reducibility (Ukeda et al., 1998). When dihydrofructosazine was oxidized, the XTT reducibility was perfectly lost. The behaviors were similar to those of the DNA breaking activity reported by Kashige et al. (1995). On the basis of this result, in the present investigation, the relationship between the XTT reducibility and the DNA breaking activity of various kinds of aminosugars

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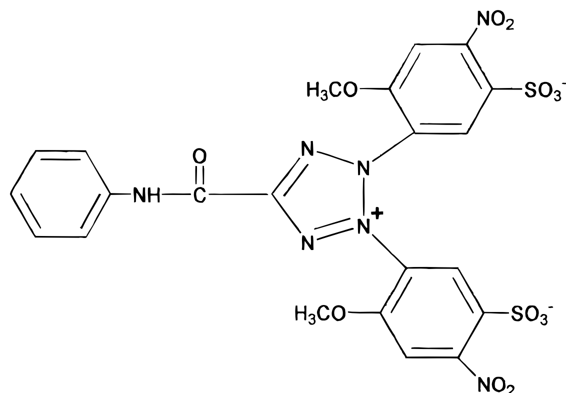

Figure 1. Structure of XTT.

was examined in detail in order to establish the monitoring method of the biologically active reaction products derived from aminosugars.

MATERIALS AND METHODS

Reagents. D-Glucosamine hydrochloride was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). D-Galactosamine hydrochloride, nitroblue tetrazolium (NBT), 1,4-diazabicyclo[2.2.2]-octane (DABCO), D-mannitol, and potassium iodide were purchased from Wako Pure Chemical Industries (Osaka, Japan). XTT, D-mannosamine hydrochloride, D-glucosamine 6-phosphate, superoxide dismutase (SOD; EC 1.15.1.1; 4000 units/mg of protein) from bovine erythrocytes, catalase (EC 1.11.1.6; 46500 units/mg of protein) from bovine liver, 4,5-dihydroxy-1,3-benzendisulfonic acid (Tiron), 2-aminoethylisothiouronium bromide (AET), and 2-mercaptoethylamine (MET) were obtained from Sigma Chemical Co. (St. Louis, MO). Milli-Q water was used for all experiments.

Sample of Aminosugar. Aminosugar (0.2 or 0.5 M) dissolved in 50 mM sodium phosphate buffer (pH 7.4) was incubated at 37 °C. After incubation for the indicated duration, the reaction mixture was used in the subsequent experiment.

XTT Assay Procedure. The assay was performed in a 96-well microtiter plate using an eight-channel adjustable volume pipet according to the method described previously (Ukeda et al., 1996). In the standard procedure, each well contained 60 μL of 0.5 mM XTT solution prepared with 0.2 M potassium phosphate buffer (pH 7.0) containing menadione at the saturated level. Then 40 μL of sample was added into the well. After it was mixed in a microplate shaker for 15 s at a speed of 500 rpm, the difference in the absorbance at 492 and 600 nm was read on a microplate reader MPR A4i (Tosoh, Tokyo, Japan) as the absorbance at 0 min. After 20 min at room temperature, the absorbance difference was read, and the increase in the absorbance difference for 20 min was recorded as the ability of sample to reduce XTT (XTT reducibility). When the XTT reducibility of a sample solution was too high, it was subjected to the assay after an appropriate dilution.

To investigate the effect of a radical scavenger for reactive oxygen species, SOD (1 mg/mL; 4000 units/mL), catalase (1 mg/mL; 46500 units /mL), Tiron, D-mannitol, potassium iodide, DABCO (100 mM), and ethanol (10%) were prepared with 0.2 M potassium phosphate buffer (pH 7.0) saturated with menadione. Ten microliters of the scavenger solution, 40 μL of sample (diluted 5-fold in this experiment), and 50 μL of XTT solution were mixed, and XTT reducibility was measured in a manner similar to that described above.

DNA Breaking Activity of Aminosugar. Covalently closed circular DNA (cccDNA) of plasmid pBR322 was isolated from *Escherichia coli* with a FlexPrep Kit (Pharmacia Biotech, Uppsala, Sweden) by a rapid alkaline extraction procedure. After preincubation at 37 °C, 5 μL of supercoiled pBR322 DNA (20 μg/mL) was mixed with 1 μL of 1 M phosphate buffer (pH 7.4), 1 μL of 125 mM EDTA (pH 7.0), and 3 μL of sample. The mixture was incubated at 37 °C for 5 min.

To investigate the effects of reactive oxygen species and radical scavengers, SOD (4 mg/mL; 16000 units/mL), catalase (4 mg/mL; 186000 units /mL), Tiron, D-mannitol, KI, DABCO, MET, AET (400 mM), and ethanol (10%) were used as a scavenger in this experiment. Five microliters of supercoiled pBR322 DNA (20 μg/mL) was mixed with 1 μL of scavenger solution, 1 μL of 1 M phosphate buffer (pH 7.4), 1 μL of 125 mM EDTA (pH 7.0), and 3 μL of sample. The mixture was incubated at 37 °C for 10 min.

After the reaction mixture was mixed with 2 μL of 0.25% bromophenol blue containing 40% sucrose, the whole mixture was subjected to 0.7% agarose gel electrophoresis. Agarose gel electrophoresis was run at 50 V for 1 h at room temperature using a Mupid-2 submarine electrophoretic apparatus (Advance Co. Tokyo, Japan). The buffer (pH 7.8) used for the electrophoresis contained 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA. After electrophoresis, the gel was stained with GelStar Nucleic Acid Stain (Takara Shuzo, Kyoto, Japan). The stained DNA bands were made visible using ultraviolet lamp and photographed with Polaroid film through a GelStar Acid Stain filter. The cccDNA changes to open circular DNA (ocDNA) when the strand is cleaved. The DNA breaking activity of aminosugar was evaluated by relative value of cccDNA to ocDNA. The relative value was calculated from the staining intensity as judged by densitometric scanning with an imaging densitometer Model GS-700 (Bio-Rad, CA).

NBT Assay Procedure. The NBT assay was performed according to the method of Ghiggeri et al. (1988). Briefly, the sample solution (100 μL) was incubated with 3.0 mL of 0.25 mM NBT prepared with 0.1 M carbonate buffer (pH 10.35) at 30 °C. The NBT reducibility was defined as the difference between the absorbance (530 nm) after 10 and 30 min of the reaction.

UV Spectroscopy. UV spectra were obtained by means of a Pharmacia Biotech Ultrospec 3000 spectrophotometer.

RESULTS AND DISCUSSION

The XTT and NBT Reducibility of Aminosugars. D-Glucosamine hydrochloride, D-galactosamine hydrochloride, D-mannosamine hydrochloride, and D-glucosamine 6-phosphate (0.5 M) were incubated for 0−24 h at 37 °C. Figure 2A indicates the time course of the XTT reducibility during the reaction. In addition, the time course of 0.2 M D-mannosamine hydrochloride is indicated in Figure 2. The XTT reducibility was recognized in all aminosugars used. A maximum of the XTT reducibility appeared within 1−4 h of the reaction after which it decreased with the time. Among the four kinds of aminosugars examined, D-mannosamine hydrochloride showed the highest XTT reducibility. The order of the XTT reducibility was as follows: D-mannosamine hydrochloride > D-galactosamine hydrochloride > D-glucosamine hydrochloride > D-glucosamine 6-phosphate. In our previous paper, it was suggested that dihydrofructosazine formed by self-condensation reaction of D-glucosamine might be involved in the generation of the XTT reducibility (Ukeda et al., 1998). It has not been elucidated, on the other hand, whether the dihydrofructosazine is generated from aminosugars other than D-glucosamine or not. Thus, we spectrophotometrically examined the formation of dihydrofructosazine in the reaction mixture of aminosugars other than D-glucosamine hydrochloride. Dihydrofructosazine gave an absorption maximum at 274 nm (Kashige et al., 1995; Horowitz, 1991). The reaction mixture of D-glucosamine 6-phosphate, D-galactosamine hydrochloride, and D-mannosamine hydrochloride incubated for 1−4 h at 37 °C exhibited the absorbance maximum at 274, 272, and 275 nm, respectively. Although those absorption maxima did not perfectly accord with the

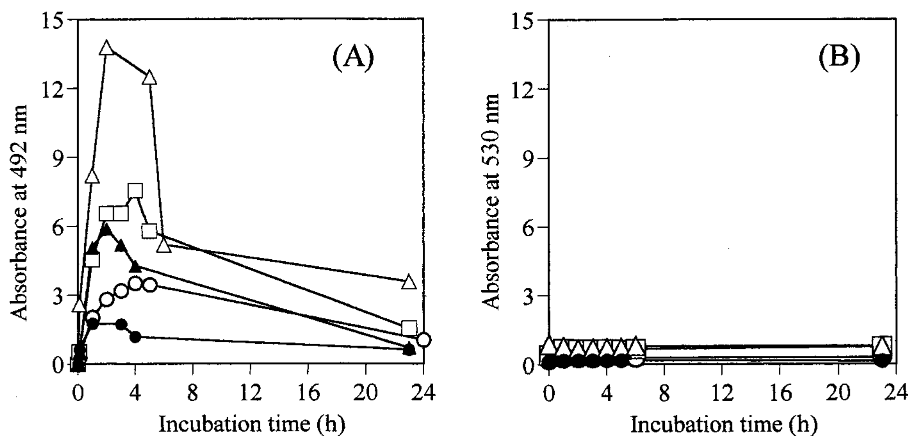

Figure 2. Time course of XTT (A; 492 nm) and NBT (B; 530 nm) reducibility of aminosugars: D-Glucosamine (○, 0.5 M), D-galactosamine (□, 0.5 M), D-mannosamine (△, 0.5 M; ▲, 0.2 M), or D-glucosamine 6-phosphate (●, 0.5 M) in 50 mM sodium phosphate buffer (pH 7.4) was incubated at 37 °C.

absorption maximum of dihydrofructosazine formed from D-glucosamine, the similarity may suggest that the analogous nitrogen-containing heterocyclic structure was formed in the incubation of those aminosugars. Since dihydrofructosazine is easily oxidized to fructosazine and other products (Fujii et al., 1966; Sumoto et al., 1991), the decrease in the XTT reducibility after the maximum can be considered to be due to the oxidation reaction.

At the same time, NBT reducibility of aminosugar was also measured. The NBT assay is known to be a method for the Amadori compound produced during the Maillard reaction (Ghiggeri et al., 1988). Aminosugars were incubated for 0−24 h at 37 °C. Figure 2B depicts the time course of the NBT reducibility. This result was obtained by the conventional method. But the final concentration of aminosugar used in the NBT assay was 1 order lower than that used in the XTT assay. Then we tried to measure the NBT reducibility using comparable concentration to the XTT assay, though the assay procedure differed from the original one. The high NBT reducibility was recognized even at 0 h. During the 24-h reaction, the change of NBT reducibility was negligibly small compared with XTT reducibility. This result indicates that the condensation product of D-glucosamine can reduce XTT while the product has little ability to reduce NBT.

It is well-known that the various kinds of radical species form during the Maillard reaction (Hayashi et al., 1981). As to the reaction of aminosugar, the generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH) from the aqueous solution of various aminosugars was reported by Kashige et al. (1991). To elucidate whether the reduction of XTT was caused by dihydropyrazine itself or reactive oxygen species generated from dihydropyrazine by the autoxidation, we investigated the additive effect of radical scavengers on the XTT reducibility of aminosugars. For this purpose, various scavengers of reactive oxygen species were added into the assay mixture of the XTT reducibility. Table 1 shows the inhibition effect by the addition of radical scavengers on the XTT reducibility of D-glucosamine hydrochloride incubated for 4 h at 37 °C. The addition of Tiron showed most effectively inhibition (92.1%), and SOD also inhibited strongly the reduction of XTT (83.8%). The scavenger, other than SOD and Tiron, was only slightly inhibitory. On the contrary, the addition of DABCO and ethanol slightly

Table 1. Effect of Radical Scavengers on the Reduction of XTT by D-Glucosamine

	inhibition[a] (%)
SOD	83.8
Tiron	92.1
KI	7.9
D-mannitol	5.1
catalase	8.2
DABCO	−4.9
ethanol	−7.9

[a] Inhibition (%) = [(Abs_{492} without scavenger − Abs_{492} with scavenger)/Abs_{492} without scavenger] × 100.

accelerated the reduction of XTT. As for D-galactosamine hydrochloride, D-mannosamine hydrochloride, and D-glucosamine 6-phosphate, the reduction of XTT was strongly inhibited by the addition of SOD or Tiron (data not shown). To clarify whether the radical scavengers affect the formation of dihydrofructosazine or not, the absorption spectrum of incubated mixtures of D-glucosamine was investigated during 24-h reaction in the presence of SOD, catalase, or KI. The reaction mixture incubated in the presence of radical scavenger exhibited the absorption maximum at 274 nm that is characteristic of dihydrofructosazine. Moreover, the absorbance at 274 nm was equal to that in the absence of scavenger. This result suggested that radical scavengers did not affect the formation of dihydropyrazine but the reduction of XTT. SOD is the enzyme that catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen (Weisiger and Fridovich, 1973). Tiron is also known to be the scavenger of superoxide anion (Fridovich and Handler, 1962). These results suggested that superoxide anion mainly contributed to the reduction of XTT by aminosugars. Thus, the present XTT reducibility assay can be recognized as an indirect monitoring method of dihydropyrazine derived from aminosugars.

The DNA Breaking Activity of Aminosugars. Figure 3 depicts the DNA breaking activity of aminosugars incubated for 0−24 h at 37 °C. DNA breaking activity was examined using cccDNA, which is convertible into ocDNA by single-strand breakage on agarose gel electrophoresis. When cccDNA of plasmid pBR322 was treated with aminosugars incubated, the amount of cccDNA decreased with the reaction time and equivalent amount of ocDNA appeared, indicating that aminosugars primary induced a single-strand breakage. No double-strand breakage was induced in the cccDNA in

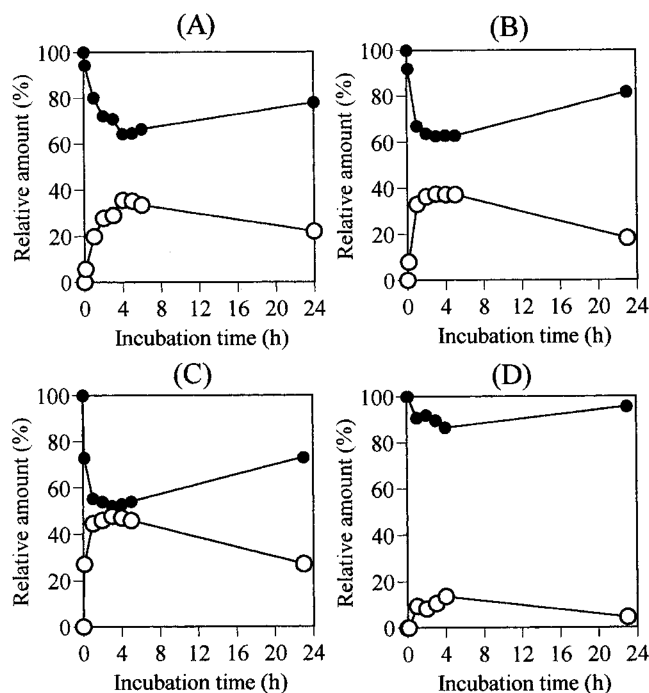

Figure 3. Time course of DNA breaking activity of D-glucosamine (A), D-galactosamine (B), D-mannosamine (C), and D-glucosamine 6-phosphate (D). D-Glucosamine, D-galactosamine, D-glucosamine 6-phosphate (0.5 M), and D-mannosamine (0.2 M) in 50 mM sodium phosphate buffer (pH 7.4) were incubated at 37 °C. The relative amount of cccDNA (●) and ocDNA (○) was plotted as a function of reaction time.

a given condition. All of the tested aminosugars showed the DNA breaking activity. In particular, D-mannosamine hydrochloride showed a high DNA breaking activity. When the concentration of D-mannosamine hydrochloride is 0.5 M, the DNA scission appeared to level off at about 50% of ocDNA from immediately after the beginning reaction to 6 h (data not shown). Since we wanted to know the time course of DNA breaking activity of aminosugars, the DNA breaking activity in lower concentration, that is 0.2 M, was also investigated. The order of the activity was as follows: D-mannosamine hydrochloride (47.7% of cleavage at 3 h; 0.2 M) > D-galactosamine hydrochloride (37.2% of cleavage at 4 h) > D-glucosamine hydrochloride (35.6% of cleavage at 4 h) > D-glucosamine 6-phosphate (13.5% of cleavage at 4 h). This order of activity agreed with that reported by Kashige et al. (1994). Moreover, it was in accord with the order of the extent of those XTT reducibilities as described above. Kashige et al. (1994) pointed out that the difference in the DNA breaking activity between those aminosugars might be due to difference in the ratio of acyclic form to cyclic form characteristic in their aqueous solutions. The DNA breaking activity increased over 4 h and then gradually decreased with the incubation time. This tendency of the DNA breaking activity also agreed with that of the XTT reducibility.

The participation of reactive oxygen species and general radicals in DNA strand scission by the aminosugars was investigated (Table 2). The DNA strand scission was strongly inhibited by Tiron, whereas SOD was inclined to promote DNA breakage. The stimulation of the DNA breaking activity of aminosugars by addition of SOD was also reported by Yamaguchi et al. (1996). It was assumed that the use of Cu, Zn−SOD was responsible for this phenomenon, because Cu^{2+} is known to accelerate the autoxidation of some reducing substances in their aqueous, oxygen-containing solutions to produce reactive oxygen species and the other radicals, and consequently, the autoxidation of dihydropyrazine was stimulated by Cu^{2+} (Kono, 1978). Catalase also inhibited the DNA strand scission extensively. Scavengers of hydroxyl radical, such as KI and D-mannitol, partially inhibited. DABCO, singlet oxygen-quencher, did not affect the DNA breakage so much. These results suggested that reactive oxygen species, such as O_2^-, H_2O_2, and hydroxyl radical, were responsible for the DNA strand breakage; in particular, the participation of O_2^- and H_2O_2 was significant. The DNA strand breakage by aminosugar was also inhibited by MET, AET, and ethanol, which are well-known to scavenge not only reactive oxygen species, but also general radical structure (•R). Yamaguchi et al. (1996 and 1998) reported that certain carbon-centered radicals produced from the dihydropyrazine were responsible for the DNA breaking activity. Thus, it was presumed that the extensive inhibition by addition of MET or AET was due to scavenging of carbon-centered radicals. These results suggested that carbon-centered radicals, besides reactive oxygen species, were also responsible for the DNA strand breakage.

Table 2. Effect of Radical Scavengers on the DNA Breaking Activity of Aminosugars

	inhibition[a] (%)			
scavenger	GlcN	GalN	ManN	GlcN 6-P
SOD	1.9	−4.7	−18.1	5.4
Tiron	65.3	85.8	75.0	59.9
KI	27.8	39.7	36.2	55.7
D-mannitol	27.2	27.1	35.1	20.2
catalase	46.0	74.7	76.6	95.4
DABCO	15.6	29.3	14.1	3.8
ethanol	29.7	39.5	27.7	46.2
MET	55.3	70.0	57.9	65.3
AET	64.4	64.2	76.8	110.4

The Relationship between the XTT Reducibility and the DNA Breaking Activity of Aminosugars. As described above, a similar behavior was recognized between the XTT reducibility and the DNA breaking activity, and the participation of reactive oxygen species, especially O_2^-, to both activities was observed. To clarify the relationship between the XTT reducibility and the DNA breaking activity of aminosugars, the relative amount of remaining cccDNA (%) was plotted against the XTT reducibility that the incubated aminosugars (including all kinds of aminosugars used in the present investigation) showed. As the DNA breaking activity of D-mannosamine hydrochloride (0.5 M) was too high to examine the relationship between the XTT reducibility and the DNA breaking activity, we selected the behavior of 0.2 M. It can be seen from Figure 4 that there is a significant correlation ($p < 0.001$) with a regression equation as follows: $y = -0.126x + 12.4$ ($r = 0.825$, $n = 26$), where x and y represent the relative amount of cccDNA (%) and the XTT reducibility, respectively. Watanabe et al. (1986) examined the relationship between the generation of O_2^- and DNA strand breakage by aminosugars using the NBT assay in order to clarify the participation of O_2^- for DNA strand scission. As mentioned above, the NBT assay is known to be a method for the Amadori compound. However, the reduction of NBT occurred not only with the Amadori product but also with the melanoidin produced during the Maillard reaction (Ghiggeri et al., 1988; Syrovy, 1994). On the other hand, it is also known to be an assay method for O_2^- (Beauchamp

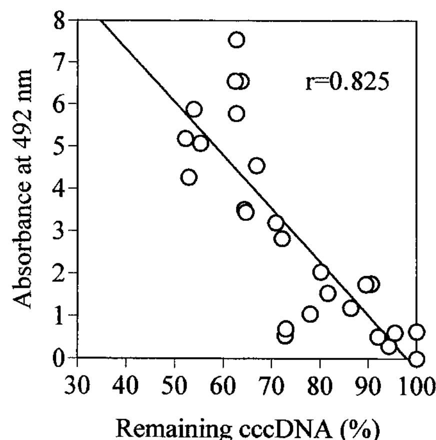

Figure 4. Relationship between XTT reducibility and DNA breaking activity of aminosugars. D-Glucosamine, D-galactosamine, D-glucosamine 6-phosphate (0.5 M), and D-mannosamine (0.2 M) in 50 mM sodium phosphate buffer (pH 7.4) were incubated at 37 °C.

and Fridvich, 1971). They mentioned that there was a good relationship between the DNA breaking activity and the NBT reducibility. However, a linear relationship between them was not recognized (they plotted in a logarithmic scale), and there was an exception showing a large deviation from the relationship (D-glucosamine 6-phosphate). On the contrary, the XTT reducibility was linearly related to the DNA breaking activity for all aminosugars including D-glucosamine 6-phosphate. It can be presumed that the difference between the XTT reducibility and the NBT reducibility might be attributed to the difference of the selectivity for superoxide anion. These results suggested that the XTT assay is more suitable for the monitoring of those biologically active reaction products derived from aminosugar than the NBT assay, when the participation of superoxide anion in DNA scission is recognized.

CONCLUSION

In the present investigation, the relationship between the XTT reducibility and the DNA breaking activity of various kinds of aminosugars was examined. The XTT reducibility and the DNA breaking activity were recognized in all aminosugars used. The order of the XTT reducibility and DNA breaking activity agreed with each other, that is, D-mannosamine hydrochloride > D-galactosamine hydrochloride > D-glucosamine hydrochloride > D-glucosamine 6-phosphate. Moreover, the time course of the DNA breaking activity was similar to that of the XTT reducibility. The superoxide anion mainly contributed to the reduction of XTT by aminosugars. In the case of the DNA strand breakage, reactive oxygen species, such as O_2^-, H_2O_2, and hydroxyl radical, were responsible especially the participation of O_2^- and H_2O_2 was significant.

Since a similar behavior was recognized between the XTT reducibility and the DNA breaking activity, and the participation of reactive oxygen species to the both activities was observed, the relationship between the XTT reducibility and the DNA breaking activity of aminosugars was examined. There was a significant correlation ($r = 0.825$, $n = 26$, $p < 0.001$) between them. This means that the XTT assay is suitable for monitoring of those biologically active reaction products derived from aminosugars, when the participation of superoxide anion in DNA scission is recognized.

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