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Preliminary communication

Formation of superoxide and *in vitro* inactivation of viruses, by hexopyranosid-3-uloses*

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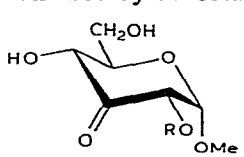
Much interest in the strand scission of nucleic acids, and inactivation of viruses, by oxygen radicals generated in the autoxidation of quinone antibiotics and reductones has recently been aroused[1]. There has been little study, however, of similar action of reducing sugar derivatives, except sugar reductones[1c,1d], and the formation of oxygen radicals, especially superoxide and hydroxyl radicals, by common reducing saccharides, including glycosiduloses, has not been examined.

It has recently been reported by the present authors that reducing sugar phosphates[2,3], aldopentoses[4], and some synthetic and natural oxidized polysaccharides[5] produce, in buffered, aqueous solutions, oxygen radicals that are able to cleave nucleic acids, and, *in vitro*, to inactivate bacteriophages. These sugar derivatives were shown to have, with oxygen, higher reactivity to yield superoxide than common aldohexoses. Such substituted dicarbonyl sugars as pyranosiduloses therefore appeared to constitute another class of moderately autoxidizable sugar derivative that may yield superoxide, and apparently be responsible for the phagocidal action of the aforementioned, oxidized polysaccharides[5].

We now report the autoxidation reaction, and the virus-inactivating action, of some methyl hexopyranosid-3-ulose derivatives.

Three 2-*O*-substituted, methyl α-D-*ribo*-hexopyranosid-3-ulose derivatives were prepared by oxidation of 2-*O*-substituted, methyl 4,6-*O*-benzylidene-α-D-glucopyranosides, followed by deacetalation[6]. Purification of the hygroscopic glycosid-3-uloses (**1**, **2**, and **3**)



1 R = COPh

2 R = $SO_2C_6H_4Me$-*p*

3 R = CH_2Ph

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was performed by column chromatography on Sephadex G 15, followed by freeze-drying. Autoxidation of the glycosiduloses was studied by determining the rate of formazan formation (ΔA_{560}/min) by superoxide ($O_2^{\cdot -}$) from Nitro Blue tetrazolium chloride (NBT) in sodium carbonate buffer (pH 10.4) at[2] 25°.

Inactivation of viruses *in vitro* was examined by the use of bacteriophage ϕX174 (a single-stranded, DNA phage) and tobacco mosaic virus (TMV). Bacteriophage ϕX174 was treated in 0.1M phosphate buffer (pH 7.0) with the glycosiduloses in the presence, or absence, of Cu^{2+} ($CuCl_2$, 10μM) for 3 h at 37°, and the survival ratio (%) was determined by the assay for plaque-forming ability on *E. coli* C_N, as described previously[3]. Tobacco mosaic virus (TMV-OM, A_{260}/A_{280} 1.16, 70 μg/mL) was similarly treated with the glycosiduloses and Cu^{2+} (mM), and the survival ratio was obtained by the typical, local-lesion assay on 6–8 half-leaves of *N. glutinosa* or *N. tabacum* c.v. Xanthi n.c.[7]

As a preliminary index of the ability of the glycosiduloses to cleave DNA, the degradation of calf thymus DNA in the presence of Cu^{2+} (100μM) was monitored by measurement of the decrease of fluorescence of DNA–ethidium bromide complex (λ_{em} 600, λ_{ex} 365 nm)[8], using a Hitachi fluorescence spectrophotometer Model 650-60. The glycosiduloses were found not to be toxic to the host bacterium and plants under the conditions examined.

As shown in Fig. 1, glycosiduloses exhibited different rates of NBT reduction which were much lower than that of L-ascorbic acid, but higher than those of D-fructose 6-phosphate[2] and 1-hydroxy-2-propanone (data are not shown). Of the reduction, 87 and 82% were inhibited by 10.0 and 3.3 units/mL of superoxide dismutase (SOD; EC 1.15.1.1, bovine blood), respectively, which is in agreement with the results obtained with sugar phosphates[2]. Fig. 2 shows the results of DNA degradation experiments. Decrease in the

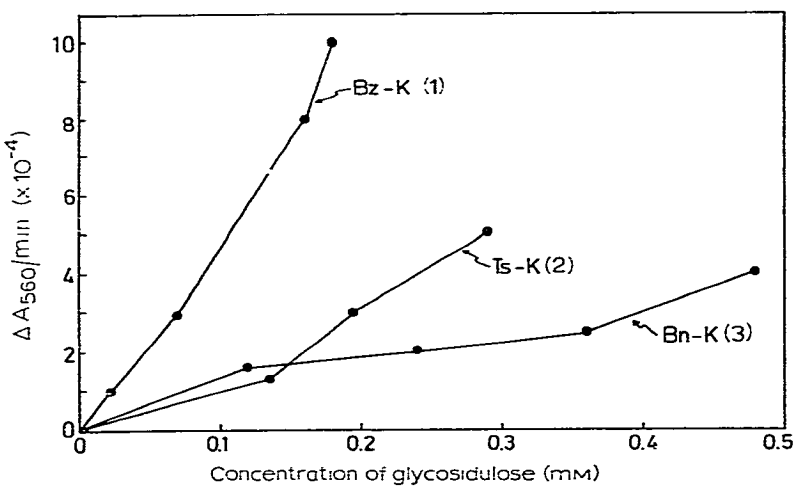

Fig. 1. Rate of reduction of NBT as a function of the concentration of glycosid-3-uloses. [The reaction rates were obtained by measuring the increase of the absorbance at 560 nm during the first 10 min of reaction. Key: Bz-K (1), methyl 2-*O*-benzoyl-α-D-*ribo*-hexopyranosid-3-ulose; Ts-K (2), methyl 2-*O*-*p*-tolylsulfonyl-α-D-*ribo*-hexopyranosid-3-ulose; and Bn-K (3), methyl 2-*O*-benzyl-α-D-*ribo*-hexopyranosid-3-ulose.]

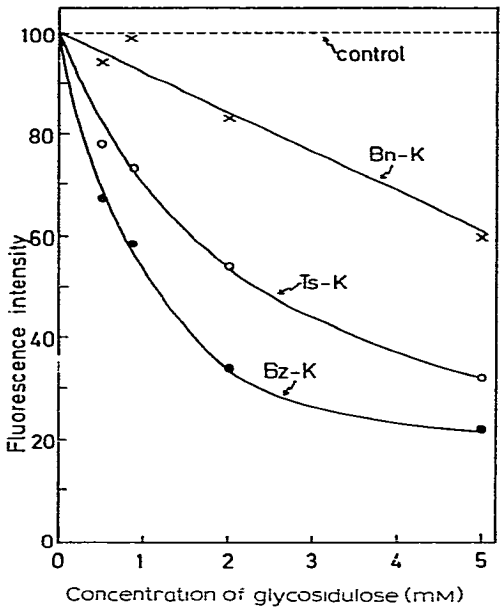

Fig. 2. Decrease of fluorescence intensity of the DNA–ethidium bromide complex as a function of the concentration of glycosidulose used for the reaction with DNA. [The reaction mixtures contained a glycosidulose and DNA (50 μg/mL) in 0.05M Tris·HCl buffer (pH 8.0), and were incubated for 20 h at 37°. An aliquot was withdrawn, diluted with the buffer, treated with ethidium bromide (1.0 μg), and the intensity of fluorescence immediately measured. A control mixture, containing no glycosidulose, was incubated for 20 h at 37° before the measurement.]

fluorescence intensity of ethidium bromide complexed with glycosidulose-treated DNA was dependent on the concentration of the glycosidulose used, and gave evidence for the depolymerization of DNA. The fluorescence intensity of the complex obtained by the treatment of **1** for 20 h corresponded to 25% of that of the intact DNA initially present. The magnitude of DNA degradation by the glycosiduloses was again higher than that caused by D-fructose 6-phosphate, and lower than that by L-ascorbic acid (data not shown). The reaction was found to be inhibited by catalase (EC 1.11.1.6, bovine liver), which gave 80% inhibition for 5 mM of **1** by 10 μg/mL of enzyme, but not by SOD (10 units/mL). No measurable degradation was observed in the absence of Cu^{2+}. These results demonstrate the ability of the glycosiduloses to generate superoxide, and to cause strand scission of DNA.

Fig. 3 shows the data for the *in vitro* inactivation of φX174 and TMV by the glycosiduloses. These were found to inactivate the two viruses at concentrations lower than those observed for the inactivation by D-fructose 6-phosphate[3]. Tobacco mosaic virus appears to be less sensitive than φX174, although response of the host cells to the viruses, the amount of virus particles used, and the kind and structural properties of the virus nucleic acids are quite different between the two inactivating reactions. The fact that methyl 2-*O*-benzoyl-α-D-glucopyranoside did not inactivate the two viruses, together with the data for inhibition experiments using radical scavengers (see notes in the legend to Fig. 3), probably exclude the possibility of a direct role of glycosidulose molecules or

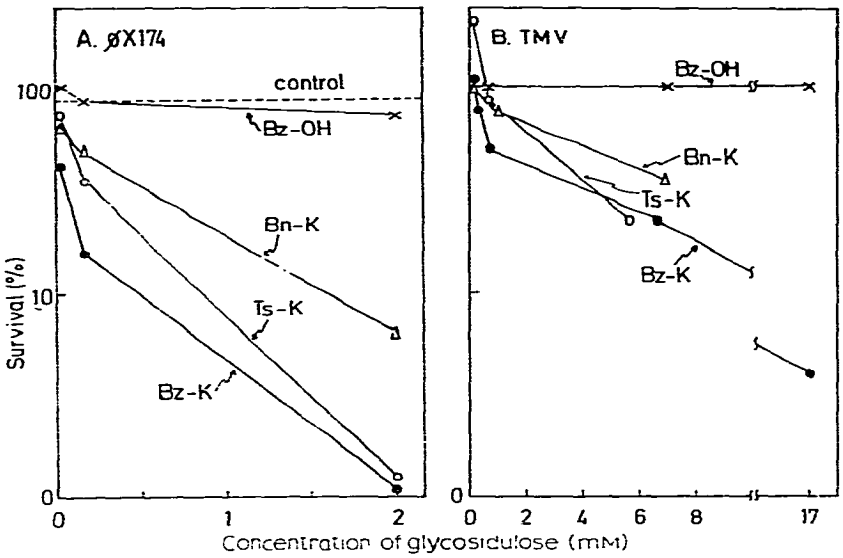

Fig. 3. Inactivation of bacteriophage ϕX174 (A) and tobacco mosaic virus (TMV) (B) by glycosiduloses. [For reaction conditions, and methods for the assay of plaque-forming ability, see text and references. Key: Bz-OH, methyl 2-*O*-benzoyl-α-D-glucopyranoside; control, a control mixture containing no glycosidulose. Of the inactivation of ϕN174 by **1**, 78% was inhibited by catalase (5 μg/mL), and 30 and 50% of inhibition by 20 and 120 units/mL of SOD were respectively observed.]

their radicals in the inactivation[2]. Acceleration of the present biological actions by cupric ion may be characteristic of sugar reductones[1c,1d] and reducing sugars[2,3]. Specific roles of metal cations, such as complexing with glycosiduloses or DNA (ref. 9) in the present system may also be suspected.

The mechanism of autoxidation of the glycosiduloses is not yet clear[10], although the intermediacy of enediol anions in the autoxidation of reducing sugars has been reported[11]. The autoxidation of α-hydroxy ketones, resulting in the formation of superoxide, may proceed through enediolate anions[12], where both the rate of enolization of ketones and the rate of univalent reduction of oxygen by enolate could be a rate-determining step. The reason for the effect of a substituent at C-2 of glycosid-3-uloses on the rate of superoxide formation and inactivation reactions is not definite; the observed order, **1** > **2** > **3**, may rather reflect the electron-withdrawing effect of the substituents, which, in turn, suggests the importance of the enolization step. However, the contribution of the electron-donating factor in the step consisting of oxidation of enolate cannot be neglected. A study using aldopentoses[4] showed that the order of the rate of enolization[13] correlates well with the order of the rate of NBT reduction and virus inactivation.

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