Specificity of Nucleotide Sequence in DNA Cleavage Induced by D-Glucosamine and D-Glucosamine-6-phosphate in the Presence of Cu$^{2+}$

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$^{32}$P-End-labeled restriction fragments derived from pBR322 and pUC9 DNAs were reacted with d-glucosamine or d-glucosamine-6-phosphate in the presence of Cu$^{2+}$, and, after being heated at 90°C in aqueous piperidine, the DNA products were analyzed on polyacrylamide gels for the sequence-specificity of alkali-labile cleaved sites. The intensity of oligonucleotide bands of cleaved sites was directly proportional to the concentration of aminosugars, indicating that the DNA cleavage was caused by the action of aminosugars themselves. The preferred DNA cleavage sites induced by these aminosugars were identical, both at pyrimidine-purine (5'→3') sequences, especially at thymine-guanine ones, and to some extent at pyrimidine-pyrimidine (5'→3') sequences. The 6-phosphate moiety of d-glucosamine did not affect the specificity of DNA cleavage.

In previous papers we found that D-glucosamine and its derivatives inactivated some free bacteriophages without affecting the growth of host cells and that the DNA in the virion and plasmid pBR322 were fragmented by them, especially when they were treated in the presence of Cu$^{2+}$. Aminosugars such as D-glucosamine are distributed widely in organisms, foods, and sometimes in drugs such as antitumor antibiotics, and the biological significance of aminosugars with amine-reductone structure has become of increasing interest in recent years.

This paper reports more detailed characterization of aminosugars. We have elucidated the alkali-labile sites of cleavage on the nucleotide sequences induced by D-glucosamine and D-glucosamine-6-phosphate using a conventional DNA-sequencing technique, and compared the results with those of other DNA-cleaving agents.

Materials and Methods

Chemicals and enzymes. D-Glucosamine was purchased from Wako Jun-yaku Co. and D-glucosamine-6-phosphate from Sigma Chemical Co. The restriction enzymes, Bam HI, SspI, HincII, PstI, Smal, Eco RI, and HindIII, and T4 DNA ligase were obtained from Nippon Gene Co. T4 polynucleotide kinase and bacterial alkaline phosphatase were obtained from Takara Shuzo Co. ($^{32}$P)-ATP (specific activity 3000 Ci/mmol) was purchased from Amershaim International Co.

Preparation of DNA restriction fragments as substrate. Three kinds of singly 5'-end-labeled DNA restriction fragments of defined sequence, which are 576, 575, and 577 bp long, were prepared from the chimera DNAs (pFP12 and pFP11) of pBR322 and pUC9 plasmid. To prepare pFP12, the 568 bp long Bam HI-SspI fragment of pBR322 and the ampicillin resistance-bearing 2682 bp long Bam HI-HincII fragment of pUC9 were ligated with T4 ligase. The resulting 3250-bp fragment, whose transformed host Escherichia coli C600S cells were selected on an ampicillin (20μg/ml)-containing medium, was named pFP12. pFP12 was then digested with both PstI and SmaI to produce a 579-bp fragment. To prepare pFP11, the 561 bp long PstI-SspI fragment of pBR322 and the ampicillin-resistance-bearing 2682 bp long PstI-HincII fragment of pUC9 were ligated with T4 DNA ligase. The resulting 3243-bp fragment that was selected as above was named pFP11. It was then digested with both Eco RI and HindIII to give a 585-bp fragment. Both the 579-bp and 585-bp fragments were dephosphorylated at the 5'-ends with bacterial alkaline phosphatase (0.45 units/ml), and 5'-
ends were labeled using $\gamma$-32P-ATP and T4 polynucleotide kinase (10 units/µl). The doubly end-labeled fragment of 579 bp was then digested with BamHI and the 585 bp was digested with either BamHI or PstI. The resulting singly 5'-end labeled 576, 575, and 577 bp long double-stranded linear-DNA fragments were purified by 3% polyacrylamide gel electrophoresis. The GC content of all the DNA fragments examined was about 45%.

**Reaction of singly end-labeled DNA fragments with aminosugars.** The standard reaction mixture (10 µl) contained a 5'-end-[32P]-labeled DNA fragment (about 100 ng; specific activity 400 cpm/ng), 0.1 M D-glucosamine or 0.01 M D-glucosamine-6-phosphate, 1 mM CuCl2, and 50 mM Tris-HCl buffer (pH 7.2). The reaction was done for 3 hr at 37°C, and stopped by the addition of 15 µl of an ice-cold mixture that contained 0.5 M sodium acetate, 8.35 mM EDTA, and 50 µg/ml t-RNA. In this case, cupric ion concentrations of 1 mM was used, since its maximum stimulatory effects on the DNA cleavage31 and hydroxyl radicals' generation (unpublished data) were observed at this concentration, and since copper binding to plasmid pBR322 ccc-DNA was concentration-dependent and reached the highest level around this concentration (unpublished data). The reacted DNA was first precipitated with ethanol. The precipitate was dissolved in 0.3 M sodium acetate and precipitated again with ethanol, rinsed in cold ethanol, and dried. The purified DNA was then dissolved in 100 µl of 1 M piperidine and heated at 90°C for 30 min, and the solution was lyophilized twice to remove piperidine. The piperidine/heat-treated DNA was dissolved in 4 µl of loading buffer containing 10 mM NaOH, 1 mM EDTA, 80% (v/v) deionized formamide, and 0.1% bromophenol blue/xylene cyanol. After being heated at 90°C for 1 min, the solution containing single-stranded DNA was transferred to 8% polyacrylamide slab gel for sequencing analysis.

**Sequencing analysis.** The sites of DNA cleavage and the sequence of the oligonucleotides produced by aminosugars were identified by comparison with the oligonucleotides produced by the method of Maxam and Gilbert.45) The polyacrylamide gel from the preceding section was dried and autoradiographed for three days using Fuji RX film. Damage was defined as moderate or extensive based on the intensity of the autoradiograph as compared with that of the base-specific chemical reactions.

**Results**

**Reaction of DNA with aminosugars.**

Figure 1 shows a typical autoradiograph of gel electrophoresis of the 576-bp 5'-end-[32P]-labeled DNA fragment treated with 0.1 M D-glucosamine (lanes 9–12) or 0.01 M D-glucosamine-6-phosphate (lanes 5–8), together with some controls. Dense oligonucleotide bands were observed on the autoradiograph only when the DNA fragment was heated in piperidine after the reaction with aminosugars in the presence of Cu2+ (lanes 12 and 8). In the controls, where aminosugars, Cu2+ or the subsequent piperidine/heat treatment was omitted, faint random bands were observed. Figure 2 shows the effects of the concentrations of D-glucosamine (lanes 2–6) and D-glucosamine-6-phosphate (lanes 7–11) on the intensity of oligonucleotide bands. At the glucosamine concentration lower than 0.01 M, little or faint random bands as seen in the control without D-glucosamine were observed. However, at the higher concentrations, dense oligonucleotide bands were observed, and the band intensity increased in proportion to the concentration up to 0.4 M. In the case of D-glucosamine-6-phosphate, on the other hand, dense oligonucleotide bands were observed even at concentrations as low as 0.001 M, and the band intensity increased with the increasing concentration of D-glucosamine-6-phosphate up to 0.01 M. After that, it did not increase any more even if the concentrations increased, but almost faded away at 0.4 M. These results confirmed previous observations3) that D-glucosamine-6-phosphate had a greater DNA cleavage activity than D-glucosamine. An aqueous solution of D-glucosamine-6-phosphate is now found to generate larger amounts of active oxygen molecules, especially hydroxyl radicals that are directly related to the DNA cleavage activities of aminosugars, than that of D-glucosamine. In any event, it was evident that the DNA cleavage was caused by the action of these aminosugars themselves in a concentration-dependent manner.

Since the position of bands on the autoradiograph shows the cleaved sites and the band intensity shows their relative reactivity, the DNA bands in lanes 12 and 8 in Fig. 1 and those in lanes 5 and 9 in Fig. 2 were compared with those resulting from the base-specific chemical reaction by the Maxam–Gilbert meth-
DNA Cleavage by D-Glucosamine and Its Phosphate

![DNA Cleavage by D-Glucosamine and Its Phosphate](image)

**Fig. 1.** Autoradiograph of 8% Polyacrylamide Electrophoresis Gel Showing the Alkali-labile Cleaved Sites of a 576-bp DNA Fragment by D-Glucosamine and D-Glucosamine-6-phosphate.

The 5'-end-[32P]-labeled DNA fragment was incubated with 0.1 M D-glucosamine (GlcN) or 0.01 M D-glucosamine-6-phosphate (GlcN-6-P) in the presence or absence of 1 mM CuCl₂ at 37°C for 3 hr in 50 mM Tris-HCl buffer (pH 7.2). After the reaction and subsequent ethanol precipitation, the DNA was treated with or without 1 M piperidine at 90°C for 30 min before sequencing analysis.

Lane 1, control (none); lane 2, piperidine/heat; lane 3, Cu²⁺; lane 4, Cu²⁺ + piperidine/heat; lane 5, GlcN-6-P; lane 6, GlcN-6-P + Cu²⁺; lane 7, GlcN-6-P + piperidine/heat; lane 8, GlcN-6-P + Cu²⁺ + piperidine/heat; lane 9, GlcN; lane 10, GlcN + Cu²⁺; lane 11, GlcN + piperidine/heat; lane 12, GlcN + Cu²⁺ + piperidine/heat; lanes GA and CT, base-specific chemical reaction at guanine and adenine and that at cytosine and thymine, respectively. The sequence is indicated on the left side of the figure.

It was shown that the alkali-labile cleavages were induced preferentially at specific sites rather than randomly and that both D-glucosamine and D-glucosamine-6-phosphate had similar base specificity in the DNA cleavage mode, although the latter had much higher DNA cleaving activity than the former.³

The experiments using two other DNA restriction fragments of defined sequence (575 bp and 577 bp) also supported the above-mentioned results.
Fig. 2. Effects of the Concentrations of D-Glucosamine and D-Glucosamine-6-phosphate on Their DNA-Cleaving Activity.

The 5'-end-[32P]-labeled 575-bp DNA fragment was incubated with D-glucosamine (GlcN; 0.001, 0.005, 0.01, 0.1 and 0.4 M) or D-glucosamine-6-phosphate (GlcN-6-P; 0.001, 0.005, 0.01, 0.1 and 0.4 M) in the presence of 1 mM Cu²⁺ at 37°C for 3 hr in 50 mM Tris-HCl buffer (pH 7.2). After the reaction, the same piperidine treatment was done before sequencing analysis. Lane 1, control (none); lane 2, 0.001 M GlcN; lane 3, 0.005 M GlcN; lane 4, 0.01 M GlcN; lane 5, 0.1 M GlcN; lane 6, 0.4 M GlcN; lane 7, 0.001 M GlcN-6-P; lane 8, 0.005 M GlcN-6-P; lane 9, 0.01 M GlcN-6-P; lane 10, 0.1 M GlcN-6-P; lane 11, 0.4 M GlcN-6-P; lanes GA and CT, base-specific chemical reaction. The sequence is indicated on the left side of the figure.

Sequence specificity of cleaved sites

Figure 3 and Table I summarize the nucleotide sequence of the three kinds of DNA fragments cleaved by D-glucosamine or D-glucosamine-6-phosphate. About 66% of the total (moderate and extensive) cleavage by D-glucosamine and 73% of that by D-glucosamine-6-phosphate were induced at the purine bases located at the 3'-side of pyrimidine residues. In the case of only extensive cleavage, 94% of the cleavage were induced at the purine-pyrimidine (5’→3’) sequence in the cases of both D-glucosamine and D-glucosamine-6-phosphate. Among the cleaved purine residues, 67-69% of them were guanine. Among the pyrimidine residues adjacent to the
DNA Cleavage by D-Glucosamine and Its Phosphate

576 bp DNA fr. (Fig. 1)

GlcN

\[ \text{GlcN-6-P} \]

5'-TCAGGTATTTGGATGAGGGGTAAGTCTGAGGCACATTGTTATTTTAAGAAAAATACAAATATTGGGTTCCGCCGACATTTCCCGA
3'-AGTCCGACAGGATCTGCTATGTAATAGCCTAAACCTTCATTCATCTCTCTTCATCCAAAATAGGCTTCTTTATTG

575 bp DNA fr.

GlcN

\[ \text{GlcN-6-P} \]

5'-CTGCTTCTGGGATGAGGGGTAAGTCTGAGGCACATTGTTATTTTAAGAAAAATACAAATATTGGGTTCCGCCGACATTTCCCGA
3'-GACCACAGGTGGCAGAGGGGTAAGTCTGCTATGTAATAGCCTAAACCTTCATTCATCTCTTCATCCAAAATAGGCTTCTTTATTG

577 bp DNA fr.

GlcN

\[ \text{GlcN-6-P} \]

5'-CGCAGTGGGATGAGGGGTAAGTCTGAGGCACATTGTTATTTTAAGAAAAATACAAATATTGGGTTCCGCCGACATTTCCCGA
3'-GCCGACAGGATCTGCTATGTAATAGCCTAAACCTTCATTCATCTCTTCATCCAAAATAGGCTTCTTTATTG

576 bp DNA fr. (Fig. 2)

GlcN

\[ \text{GlcN-6-P} \]

5'-TCAGGTATTTGGATGAGGGGTAAGTCTGAGGCACATTGTTATTTTAAGAAAAATACAAATATTGGGTTCCGCCGACATTTCCCGA
3'-AGTCCGACAGGATCTGCTATGTAATAGCCTAAACCTTCATTCATCTCTTCATCCAAAATAGGCTTCTTTATTG

Fig. 3. Nucleotide Sequence Modes on 5'-End-[32P]-labeled 576, 575, and 577-bp DNA Fragments Cleaved by D-Glucosamine and D-Glucosamine-6-phosphate in the Presence of Cu₂⁺.

The sites of DNA cleavage and the relative intensity of dark bands, moderate or extensive, judged from their autoradiographs, are indicated by arrowhead marks and their size. GlcN, D-glucosamine; GlcN-6-P, D-glucosamine-6-phosphate.

5'-side of the cleaved purines, 59–62% of them were thymine.

On the other hand, 19% of the total cleavage by D-glucosamine and 23% of that by D-glucosamine-6-phosphate were induced at pyrimidine-pyrimidine (5'-3') sequence. Both
purine-purine (5'→3') and purine-pyrimidine (5'→3') sequences were rather resistant to the induction of cleavage.

**Discussion**

Recently, an increasing number of substances have been studied for their DNA-cleaving activity in connection with mutation, cancer, aging, and the like. Some of them, especially in the presence of heavy-metal ions, have been found to cause alkali-labile cleavage to DNA bases which can be cleaved by subsequent piperidine/heat treatment. The mechanism is mediated through the generation of active oxygen radicals.

In this paper, both D-glucosamine and D-glucosamine-6-phosphate were shown to cause, particularly in the presence of Cu²⁺, cleavage to 575-577 bp linear-DNA fragments in a concentration-dependent manner. On the other hand, D-glucosamine and D-glucosamine-6-phosphate even without Cu²⁺ were previously shown to cause direct single-strand scission of the 4363 bp pBR 322 ccc-DNA resulting in a nicked circular form. This discrepancy is not unusual, because, as Ueda et al. reported in a paper with autoxidized lipids, the difference between the results would be a matter of the resolution afforded by the two techniques: agarose gel electrophoretic analysis using a long ccc-DNA, which is very sensitive to random cleavage, and sequencing analysis using a short linear-DNA.

Both D-glucosamine and D-glucosamine-6-phosphate induced alkali-labile cleavage in the presence of Cu²⁺ preferentially at purine residues in the dinucleotide sequence of pyrimidine-purine (5'→3'). However, it is not certain at the present time if these aminosugars themselves may form a complex with DNA at specific nucleotide moieties before inducing such a DNA cleavage. The present sequence specificity and the nature of the DNA-cleaving reaction resembled those of D-fructose-6-phosphate, D-isoglucoasamine, and autoxidized methyl linolenate all in the presence of Cu²⁺, but not with NaBH₄-reduced mitomycin C, bleomycin-Mn, Fe-CoO₂ complexes with 2-mercaptoethanol and ascorbic acid-Cu²⁺. Of interest was the remarkable resemblance of the nucleotide sequence-specificity for DNA cleavage between D-glucosamine and D-glucosamine-6-phosphate, although the latter had higher DNA-cleaving activity than the former. It appears, therefore, to indicate that the 6-phosphate moiety does not contribute to the base specificity of D-glucosamine in the DNA-cleaving reaction.

**References**

8) S. Nanjou, S. Fujii, J. Morita, K. Ueda and T.


