Suppressive Effect *In Vitro* of Magnesium L-Ascorbyl-2-phosphate (APM), an Ascorbic Acid Derivative, on the Impairment of DNA by Glucose-6-phosphate

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We attempted to find compounds that suppress the DNA impairment caused by D-glucose-6-phosphate (Glc-6-P) by observing the loss of ability of pBR322 to transform *Escherichia coli*, and found that APM (magnesium L-ascorbyl-2-phosphate) suppressed the loss of transformability to less than 1/100 of that in the absence of APM. When 2'-deoxyguanosine 5'-monophosphate (dGMP) was incubated in the dark with Glc-6-P and APM, changes in their absorbance patterns were observed, indicating possible suppression by APM of the interactions between dGMP and Glc-6-P. APM weakly suppressed the Maillard reaction.

The Maillard reaction¹⁾ is the nonenzymatic addition of reducing sugars to the amino groups of proteins and peptides to form yellow-brown fluorescent compounds. This reaction occurs inside organisms and impairs proteins.^{2,3)} The accumulation of proteins impaired by Maillard reaction *in vivo* may be as a cause of diabetic complications and aging.³⁾ Aminoguanidine, which inhibits the formation of the final products of the Maillard reaction called AGE (Advanced Glycosylation Endproducts)^{4–6)} is under development as a potential recessive agent against diabetes.⁵⁾

It was found that DNA is also impaired by reducing sugars both *in vitro*^{7–10)} and *in vivo*.¹¹⁾ However, there have been relatively few reports about compounds that suppress or decrease the impairments of nucleic acids by reducing sugars. We attempted to find such a compound among the L-ascorbic acid derivatives.

Materials and Methods

Detection of plasmid impairment by glucose-6-phosphate in vitro and screening of a suppressive substance by Escherichia coli transformation. Detection of the plasmid impairment by glucose-6-phosphate (Glc-6-P) *in vitro* was done by the method of Bucala *et al.*²⁾ with partial modifications as follows. As L-ascorbic acid derivatives,

sodium L-ascorbate (Wako Pure Chemicals), APM (magnesium L-ascorbyl-2-phosphate), calcium L-ascorbate, L-ascorbyl-2-sulfate, and sodium L-ascorbyl-2-phosphate (Showa Denko) were tested. Aminoguanidine hemisulfate (Sigma) was also used in comparison to the derivatives.

Plasmid pBR322 was prepared by cesium chloride density gradient centrifugation.¹²⁾ The 75 mM test compounds, 0.27 μ g of plasmid pBR322, and 150 mM D-glucose-6-phosphate monosodium salt (Sigma), were incubated in a HEPES/EDTA 50 mM/1 mM buffer (pH 8.0) at 37°C in the dark. All reagents were dissolved in autoclaved ion-exchanged distilled water to fully avoid contamination by DNA nucleases, and filtered through a presterilized 0.20 μ m cellulose acetate membrane filter (Advantec). Ten μ l of the incubation mixtures were then sampled occasionally, and used to transform competent cells of *E. coli* C600 treated with CaCl₂.¹²⁾ Cells were plated with suitable dilution on either ampicillin (50 μ g/ml) or tetracycline (15 μ g/ml) supplemented L-agar plates after an hour of expression at 37°C, and the number of transformants after overnight incubation at 37°C was counted. As the number of transformants from ampicillin-supplemented plates showed good agreement with that from tetracycline-supplemented plates, the results from ampicillin-plates are demonstrated here. Transformation capacity was expressed as the percentage of the number of transformants against that by the plasmid without treatment of Glc-6-P kept at 4°C in the dark in the same buffer. Transformation efficiency per μ g DNA was 9.3×10^6 – 3.0×10^7 throughout the experiments.

Measurement of APM by HPLC. APM stability was examined by measurement using HPLC (column: Shodex NH-pack J-411 150 mm $\times$ 4.6 mm (Showa Denko)). The

mobile phase was 0.1 M KH_2PO_4 (pH 4.2; unadjusted) with a flow rate of 0.7 ml/min at column temperature, 30°C. The elution pattern was monitored with a UV detector at 257 nm.

Spectroscopic studies of the interaction between Glc-6-P and dGMP (2'-deoxyguanosine 5'-monophosphate). The interaction of Glc-6-P with dGMP was observed by the change in absorbance spectra as follows.⁹⁾ Solutions (1 ml) containing 10 mM dGMP monosodium salt (Sigma), 150 mM Glc-6-P, and with or without 75 mM APM in a HEPES/EDTA 50 mM/0.5 mM buffer (pH 8.0) sterilized by filtration as described above, were incubated for a week at 37°C in the dark. The control solution contained Glc-6-P alone or nucleotide alone. Three hundred μl of the incubation mixtures were sampled at the 7th day of incubation, diluted 1:1 with distilled water, and their absorbance patterns between 200 nm and 450 nm were scanned and recorded with a spectrophotometer (Shimadzu UV-240).

Examination of the effects of APM on Maillard reaction of a protein. The effects of APM on the Maillard reaction of a protein (bovine serum albumin, BSA) with glucose were examined with the fluorescence spectrophotometer (F-4000, Hitachi).⁴⁾ The sample mixture was a 0.5 M sodium phosphate buffer containing 100 mM BSA (A9647, Sigma), 200 mM D-glucose, and 3 mM sodium azide as a bacteriocide. Each of the reagents, 10 mM aminoguanidine·HCl, 10 mM sodium L-ascorbate, and 10 mM APM, was added to each buffer prepared to make the total volume of the incubation mixture 3 ml. In 7 and 16 days of incubation, 440 nm fluorescence excited by 370 nm was measured using a spectrofluorimeter.

Results and Discussion

Detection of plasmid impairment by glucose-6-phosphate in vitro and screening of a suppressive substance by E. coli transformation

As shown in Fig. 1, when the plasmid was incubated with Glc-6-P for 53.5 hr, the transformation capacity of the plasmid decreased to 1/10000 of the original level due to the impairment of plasmid DNA by Glc-6-P. The impairment of DNA, however, was greatly suppressed by the presence of APM. APM is a magnesium salt of an L-ascorbic acid derivative in which the hydroxyl group of the C_2 position of L-ascorbic acid is stabilized by forming a phosphoester bond.

Other ascorbic acid derivatives (sodium L-ascorbate, calcium L-ascorbate, sodium L-ascorbyl-2-phosphate, and L-ascorbyl-2-sul-

Fig. 1. Suppressibility against DNA Impairment by Glc-6-P Expressed by the Loss of pBR322 Transformation Capacity with Time after Incubation with 0 mM Glc-6-P (●), 150 mM Glc-6-P (▲), 150 mM Glc-6-P and 75 mM APM (○), 150 mM Glc-6-P and 75 mM Sodium L-Ascorbyl-2-phosphate (□), 150 mM Glc-6-P and 75 mM Sodium L-ascorbate (△), and 150 mM Glc-6-P and 75 mM Aminoguanidine (■).

See the text for details.

fate) did not suppress DNA impairment by Glc-6-P. Both calcium L-ascorbate and L-ascorbyl-2-sulfate showed similar behavior to sodium L-ascorbyl-2-phosphate (data not shown). Sodium L-ascorbate enhanced the plasmid impairment, indicating DNA impairments *in vitro* by ascorbic acid.^{13,14)} Aminoguanidine also enhanced the plasmid impairment (Fig. 1).

Measurement of APM by HPLC

Examination of the APM stability by HPLC showed that neither APM concentration nor retention time changed in a month at 37°C, regardless of the presence of either Glc-6-P or the plasmid (data not shown).

Details of the mechanism of the suppressive effect by APM are not clear, and are under investigation. The specificity of APM on suppressiveness among the ascorbic acid derivatives examined suggests steric hindrance by the interaction of APM with the nucleotides against addition of Glc-6-P to nucleotides. DNA is known to be impaired by Glc-6-P in two ways.^{9,10)} One is the formation of a Glc-6-P addition product, and the other is strand breakage by oxygen radicals. As 1 mM EDTA is present in our system, by following the result that the DNA strand breakage by the sugar phosphates is inhibited with 10 μM EDTA,¹⁰⁾ the loss of transformation capacity is not

caused by the DNA strand breakage mainly by the oxygen radicals. This suggests that the suppressive effect of APM is not due to the trapping of oxygen radicals but by the inhibition of the formation of addition products.

Effects of APM on the interaction of Glc-6-P with dGMP

We investigated the effects of APM on the spectral changes produced by incubating deoxyribonucleotide and Glc-6-P *in vitro*. Among the four nucleotides of DNA monomers, dGMP showed most profound changes in absorbance, and therefore it was chosen for this model experiment for DNA modification by reducing sugars. As shown in Fig. 2, dGMP incubated with Glc-6-P had a clear increase of absorbance between 300–350 nm. Co-incubation of dGMP, Glc-6-P, and 75 mM APM showed less absorbance than that expected from the hypothesis that no reaction occurs between the dGMP-Glc-6-P mixture and APM (Fig. 2), suggesting a possible suppression of Glc-6-P modification of dGMP by their interaction with APM. Although this is the suppressive effect observed in the monomer nucleotide and Glc-6-P system, this may partly explain the reason for the clear suppressive effect in the polynucleotide pBR322 as shown in Fig. 1.

Examination of the effects of APM on Maillard reaction of a protein

As APM showed suppression of DNA impairment by reducing sugars, it seemed interesting to investigate its effects on the nonenzymatic browning of proteins. Co-incubation of BSA and glucose is accompanied by the formation of fluorescent compounds (Fig. 3). Aminoguanidine fully suppressed it. APM showed a suppressive effect which was weaker than that by aminoguanidine. Sodium L-ascorbate increased the formation of fluorescent compounds (Fig. 3).

As APM is a stable derivative of ascorbic acid with equivalent antiscorbutic activity *in vivo*, and no teratogenesis, mutagenesis, or

Fig. 2. UV and Visible Absorbance Spectra of a Nucleotide dGMP (2'-deoxyguanosine 5'-monophosphate) after 7 Days of Incubation at 37°C.

①, ②, ③, ④, ⑤, and ⑥ represent Glc-6-P, dGMP, dGMP + Glc-6-P, APM, dGMP + Glc-6-P + APM, and (dGMP + Glc-6-P) + APM (expected), respectively.

Fig. 3. Effects of AG (aminoguanidine), APM, and AsA (sodium L-ascorbate) on Advanced Glycosylation Product Formation.

"G" indicates glucose. Black and white bars denote relative fluorescence intensity after 7 and 16 days of incubation, respectively.

chronical toxicity is known,^{15–18)} the safety in long term use is recognized. Further investigation of this APM effect *in vivo* by using diabetic rats will be done.

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