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Modification of DNA by reducing sugars: A possible mechanism for nucleic acid aging and age-related dysfunction in gene expression

(nonenzymatic browning/Maillard reaction)

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ABSTRACT **Reducing sugars react nonenzymatically with protein amino groups to initiate a process called nonenzymatic browning. Long-lived proteins, such as collagen and the lens crystallins, accumulate sufficient modification *in vivo* that they acquire many of the chemical properties characteristic of aged proteins. We have obtained evidence that nucleic acids also can undergo nonenzymatic modification by sugars. Incubation of DNA or nucleotides with glucose 6-phosphate (Glc-6-*P*) produces spectral changes similar to those described for nonenzymatic browning proteins. The occurrence of chemical modification was verified by measuring the transfection efficiency of viral DNA after incubation with glucose and Glc-6-*P*. A loss of transfection potential occurred that was first order with respect to time and sugar concentration. The rate of inactivation by Glc-6-*P* was 25 times that of glucose; 8 days of incubation with 150 mM Glc-6-*P* decreased transfection by 4 orders of magnitude. Glc-6-*P* also produced strand scission in a time- and concentration-dependent manner. We conclude that glucose, Glc-6-*P*, and possibly other sugars can react with DNA to produce significant structural and biological alterations. Since nucleic acids are long-lived molecules in the resting cell, the accumulation of these addition products might be a mechanism for the decreased genetic viability characteristic of the aged organism.**

Nonenzymatic browning, or the Maillard reaction, is a biochemical aging process initiated by the reaction of proteins with reducing sugars (reviewed in refs. 1 and 2). The first step of the reaction involves the nonenzymatic addition of a sugar residue, such as glucose or glucose 6-phosphate (Glc-6-*P*) to the amino group of a protein. The product of this reaction, an aldosylamine, then undergoes an Amadori rearrangement to form a 1-deoxyfructosyl adduct (3, 4). With time, these adducts are multiply dehydrated to form yellow-brown fluorescent compounds that can crosslink proteins. Maillard reaction products were first shown to occur during the aging of stored food but recently have been found in long-lived human proteins such as collagen and the lens crystallins (5, 6). It has been proposed that nonenzymatic browning contributes to the aging of tissues that are insulin independent and thus exposed to high glucose concentrations—e.g., the lens, the basement membrane of the arteries, the nerves, and connective tissue. Patients with diabetes mellitus, in fact, have an accelerated rate of nonenzymatic browning that may contribute to the earlier onset of cataracts and atherosclerosis observed in this disease (5, 7).

New insight into the biology of aging would be possible if it could be shown that a similar reaction occurs between sugars and the amino groups of nucleic acids. Because nucleic acids are long-lived molecules in the resting cell, the accumulation of stable addition products with time would affect the viability of the genetic material. Current theories of aging emphasize the concept of "error catastrophe": that functional decrements characteristic of cellular senescence are due to the progressive accumulation of unrepaired genetic lesions (8, 9). Age-dependent changes in the genetic material are well documented and include increased tumorigenesis (10), chromosomal aberration (11), DNA strand breaks (12), and decreases in DNA repair (13), replication (14), and transcription (15). Two age-related phenomena—the increased frequency of abnormal births seen with the increasing age of the mother (16) and the decreased replicative capacity of the aged fibroblast (17)—are exacerbated by the diabetic state (18, 19).

We have investigated the effect of incubating DNA with glucose and Glc-6-*P* *in vitro*. Spectroscopic changes develop in the course of incubation that indicate that DNA can also undergo nonenzymatic browning. The exposure of a single-stranded viral genome to these conditions leads to a loss of transfection potential that is first order with respect to incubation time and sugar concentration. Incubation with Glc-6-*P*, in addition, causes DNA strand scission. The hypothesis is presented that sugar–DNA adducts occur *in vivo* and contribute to biological aging.

MATERIALS AND METHODS

Spectroscopic Studies. Solutions (1 ml) were prepared that contained 10 mM deoxyribonucleotide (Sigma) and 150 mM Glc-6-*P* (Calbiochem) or glucose (Baker) in 50 mM Hepes/0.5 mM EDTA, pH 8.0, adjusted with NaOH. All solutions were sterilized by filtration through a Millex (Millipore) filter prior to incubation at 37°C in the dark. Control solutions contained sugar alone or nucleotide alone. DNA incubation mixtures contained calf thymus DNA (Sigma) at 2 mg/ml. Single-stranded DNA (ss DNA) was generated by boiling DNA for 5 min and then rapidly cooling the mixture on ice. At the end of 4 days, the solutions were diluted 1:1 with distilled H_2O and the visible and UV spectra were recorded on a Hewlett-Packard model 8450A spectrophotometer.

Fluorescence measurements were made on a Perkin-Elmer model 204 fluorescence spectrophotometer. Incubation mixtures showing absorbance changes were diluted 1:6 with distilled H_2O, and the excitation and emission maxima were determined by preliminary scanning. Final recordings were made at an excitation wavelength of 315 nm and an emission wavelength of 420 nm. Fluorescence spectra of modified nucleotides and DNA were determined by subtraction of the background fluorescence generated by Glc-6-*P* degradation (20), which occurs during prolonged incubation. DNA and nucleotides incubated without Glc-6-*P* did not exhibit measurable fluorescence. The magnitude of the quantum yield was calculated with reference to the emission at 442 nm of a standard solution of quinine (10 μM in 0.05 M H_2SO_4) excited at 315 nm (21).

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Abbreviations: Boc-lysine, *N*-α-*tert*-butoxycarbonyllysine; Glc-6-*P*, glucose 6-phosphate; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.

DNA Transfection. Bacteriophage f1 DNA was isolated by phenol extraction of concentrated phage particles. Each transfection assay mixture contained 2 μg of DNA in 0.1 ml of sterile 50 mM Hepes, pH 8.0/0.5 mM EDTA and either glucose or Glc-6-*P*. Control incubation mixtures contained either DNA or sugar alone. After incubation at 37°C in the dark, duplicate aliquots were transfected into 0.3 ml of a suspension of calcium chloride-treated *Escherichia coli* (strain K38) (22). Plaque-forming units were quantitated after an overnight growth.

DNA Electrophoresis. DNAs were electrophoresed in 1% agarose gels under alkaline conditions (30 mM NaOH) sufficient to separate linear from covalently closed DNA (23). After neutralization, the gel was stained with ethidium bromide at 1 μg/ml. For detection of Glc-6-*P* incorporation, 4 μCi of D-[1-^{14}C]glucose 6-phosphate (50 mCi/mmol; 1 Ci = 37 GBq; New England Nuclear) was incubated with 16 μg of purified f1 DNA for 2 weeks as described above. Radiochemical purity was assessed by HPLC using a C_{18} column (Waters Associates) and a mobile phase of 20 mM potassium phosphate buffer (pH 5.5) containing 2% methanol that increased linearly to 20% methanol from 0 to 20 min (24). At the end of the incubation period, 8 μg of DNA was loaded per lane and electrophoresed, and the gels were impregnated with EN^{3}HANCE (New England Nuclear). Fluorography was for 4 weeks at −80°C.

RESULTS

Although the Maillard reaction is characteristically a slow process, studies with proteins have shown that modification occurs as an integral function of time and sugar concentration (2). As a first step toward the study of DNA modification by sugars, therefore, individual nucleotides and purified DNA were incubated at 37°C with high concentrations of either glucose of Glc-6-*P*. As shown in Fig. 1, 4 days of incubation with Glc-6-*P* was sufficient to produce absorbance changes in the 300- to 400-nm range that are similar to those described for the nonenzymatic browning of proteins (6, 25). No changes were detected in the 220- to 300-nm region. The enhanced browning effect of Glc-6-*P*—in contrast to glucose, which did not show any effect under these conditions—is consistent with the observation that phosphorylated sugars glycosylate proteins more readily than nonphosphorylated ones (26) and at a rate that is a function of their reducing action (27). Of significance is the fact that negligible spectral changes occurred with TMP, the only nucleotide lacking a primary amino group, and with double-stranded DNA (ds DNA), presumably because hydrogen bonding hinders the reaction of the amino groups with Glc-6-*P*.

Fluorescence spectra were obtained for the chromophores produced by incubation of ss DNA and the nucleotides AMP, CMP, and GMP with Glc-6-*P*; excitation and emission spectra (uncorrected) are shown in Fig. 2. Excitation in the 250- to 270-nm region, where nucleotides absorb strongly, produced neither new nor significant emission. Instead, the chromophores produced had excitation maxima at 315 nm with calculated quantum yields of 2.37×10^{-2} for GMP, 1.15×10^{-2} for AMP, and 1.9×10^{-3} for CMP. AMP showed, in addition, a large second excitation maximum at 360 nm. CMP and ss DNA had smaller excitation maxima in the 330- to 350-nm region. As shown in Fig. 2, excitation at 315 nm produced emissions that ranged from 350 to 600 nm.

We next investigated the biochemical effects of glucose and Glc-6-*P* on DNA from the *E. coli* bacteriophage f1. f1 DNA is a single-stranded, covalently closed circle composed of 6,470 nucleotides (28). The properties of the DNA examined included its transfection potential into the host strain K38 and its structural integrity as analyzed by alkaline gel electrophoresis. DNA was isolated from the virus by phenol extraction and incubated with different concentrations of

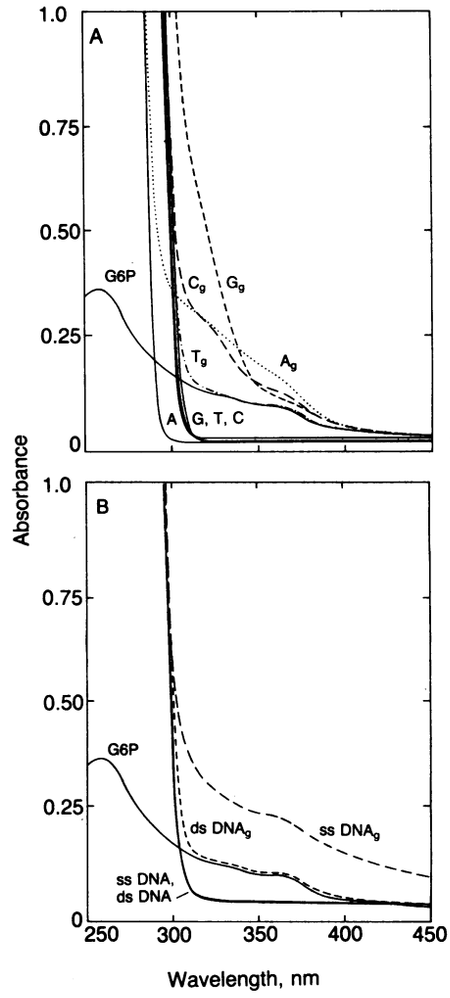

FIG. 1. UV and visible absorbance spectra of nucleotides and ss and ds DNAs after 4 days of incubation at 37°C. (*A*) Glc-6-*P* alone; A, C, G, T, nucleotides alone; A_g, C_g, G_g, T_g, nucleotides incubated with Glc-6-*P*. (*B*) Glc-6-*P* alone; ss and ds DNA; ss DNA_g and ds DNA_g, ss and ds DNA incubated with Glc-6-*P*.

sugars for 8 days at 37°C. At the end of this time, aliquots were removed and transfected into calcium chloride-treated *E. coli*.

The percentage decrease in the transfection efficiency of glucose- and Glc-6-*P*-treated DNA relative to that of DNA incubated without sugar is shown in Fig. 3*A*. DNA exposed to Glc-6-*P* showed a dramatic decrease in transfection efficiency; incubation with 150 mM Glc-6-*P* decreased the value by 4 orders of magnitude. The rate of this phenomenon was first order with respect to sugar concentration. Glucose caused a much smaller decrease in transfection efficiency; at 150 mM, efficiency was reduced to ≈33% of control.

The following experiment was carried out to test for the possibility that the carbohydrate alone, or the products of spontaneous carbohydrate degradation, could inhibit expression of the viral genome. Glucose and Glc-6-*P* (150 mM) were incubated for 8 days and equivalent amounts were added to calcium chloride-treated *E. coli* together with untreated f1 DNA. As shown in Fig. 3*A*, these conditions produced only a small (<50%) inhibition of transfection.

Controls for the Glc-6-*P* inhibitory effect were also carried out. The possibility that trace metals might be present in the Glc-6-*P* solution and catalyze DNA degradation was tested by treatment with the resin Chelex 100. Similarly, the presence of any contaminating DNA nucleases was diminished by filtering the Glc-6-*P* solution through a dialysis membrane

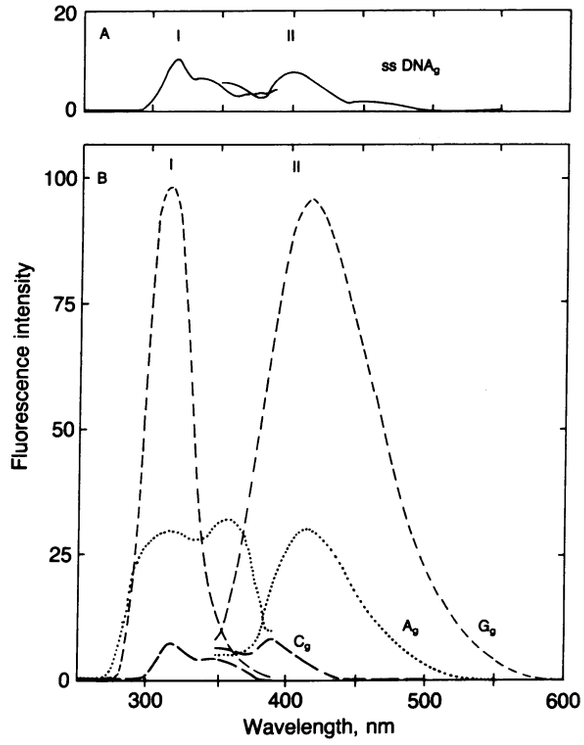

FIG. 2. Excitation (I) and emission (II) difference spectra (uncorrected) for ss DNA and Glc-6-*P*-modified nucleotides. Fluorescence intensity is expressed in arbitrary units. (*A*) ss DNA_g, Glc-6-*P* modified ss DNA. (*B*) A_g, C_g, and G_g, Glc-6-*P*-modified AMP, CMP, and GMP.

(M_r cutoff 3,500). The effects of these treatments were insignificant (Fig. 3*A*).

f1 DNA from the incubation mixtures was subjected to gel electrophoresis under conditions that separate covalently closed DNA from DNA in a linear form. Linear DNA would result from a single nick in the phosphodiester backbone of the circular DNA. Fig. 3*B* shows that there is a concentration-dependent strand scission that occurs in the course of incubation with Glc-6-*P*. With increasing amounts of Glc-6-*P* (lanes 3–6), the proportion of DNA in the circular form decreases as the amount of linear form increases. At very high Glc-6-*P* concentrations (lanes 5 and 6), the closed circular DNA disappears and increasing amounts of low molecular weight DNA fragments become visible. Control incubations without sugar (lane 2) or with glucose (lanes 7 and 8) show only a slight increase in linear DNA when compared with unincubated DNA (lane 1). A similar progression of strand scission was observed with Glc-6-*P* purified by ultrafiltration.

Electrophoresis and fluorography of f1 DNA incubated with [^{14}C]Glc-6-*P* showed that ^{14}C is incorporated both into intact closed circular DNA as well as into linear DNA (Fig. 4). Thus, the formation of a Glc-6-*P* addition product precedes the event that leads to strand breakage. Fig. 3 shows that strand scission also must occur at a rate slower than the rate of transfection inhibition. Lane 1 (Fig. 3*B*), containing 8 μg of unincubated f1 DNA, shows that 80–90% of the starting DNA is in the intact closed circular form. Incubation with 50 mM Glc-6-*P* (lane 4) decreases the amount of intact DNA by about one-half when compared with the starting material, so that the amount of intact circles is roughly equal to the amount of linear DNA present. If DNA breakage events follow a Poisson distribution, then about one-third of the original molecules should remain unnicked when the number of linear and circular molecules are equal. Under the same incubation conditions, however, transfection efficiency was decreased by more than two orders of magnitude (Fig. 3*A*). While we cannot exclude contaminating nuclease activity, it alone cannot account for the observed decrease in transfection efficiency. The initial Glc-6-*P* adduct may be

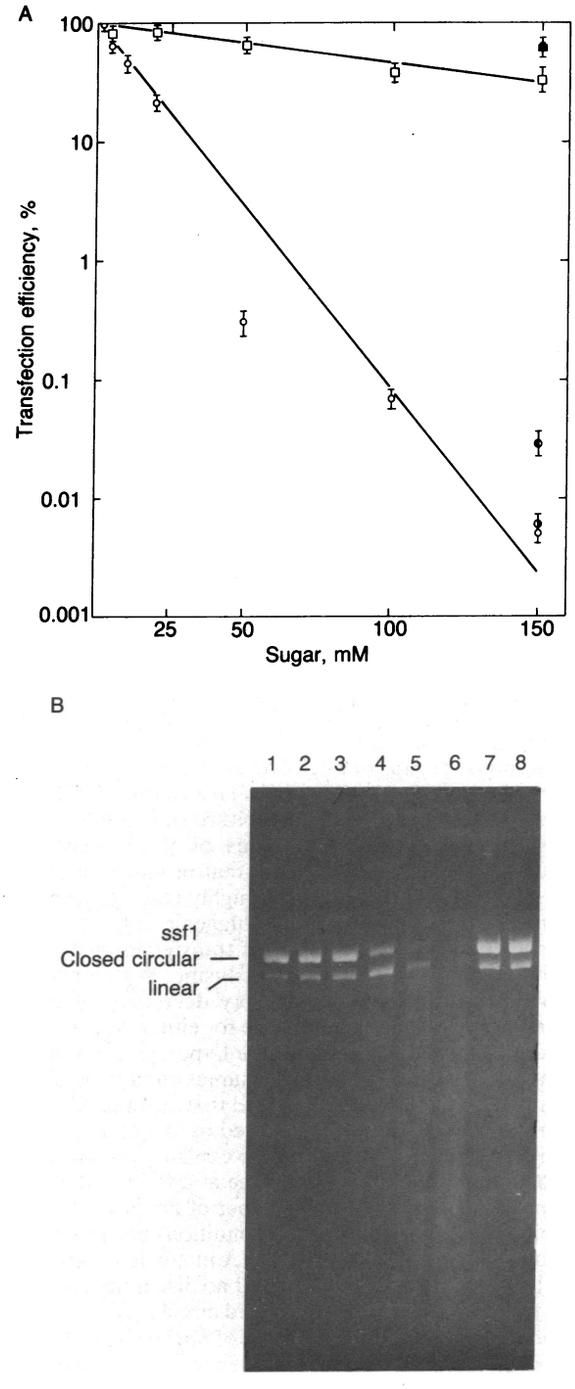

FIG. 3. (*A*) Transfection efficiency of f1 DNA after 8 days of incubation with different sugar concentrations. The percentage decrease is relative to DNA incubated for 8 days without sugar. □, Glucose; ○, Glc-6-*P*; ■ and ●, incubated sugars were added to the transfection assay with unincubated DNA; ◐, Glc-6-*P* was purified by ultrafiltration; ◑, Glc-6-*P* was first treated with Chelex 100. Results represent mean ± SEM of duplicate experiments. (*B*) Alkaline gel electrophoresis of f1 DNA that had been incubated with 10, 50, 100, or 150 mM Glc-6-*P* (lanes 3–6) or 50 or 150 mM glucose (lanes 7 and 8). Lane 1, unincubated DNA; lane 2, DNA incubated without sugar.

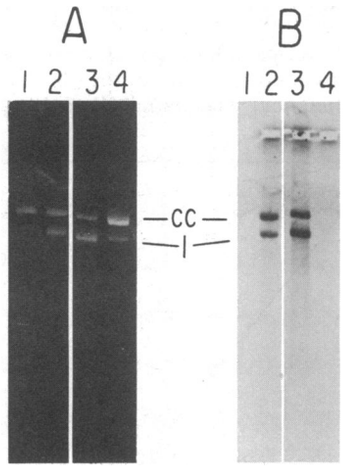

FIG. 4. Alkaline gel electrophoresis of f1 DNA that had been incubated with [^{14}C]Glc-6-*P* for 6 days (lane 2) or 14 days (lane 3). Lane 1, unincubated f1 DNA (marker); lane 4, the same f1 DNA was treated, immediately prior to electrophoresis, with [^{14}C]Glc-6-*P* that had been incubated alone for 14 days. (*A*) Ethidium bromide staining. (*B*) Fluorography. cc, Closed circular DNA; l, linear DNA.

sufficient to inhibit template activity and, with time, further reaction or rearrangement occurs that destabilizes the phosphodiester backbone and causes DNA degradation.

The time-dependent decrease in transfection efficiency of f1 DNA after incubation with 25 mM Glc-6-*P* or glucose is shown in Fig. 5. Inhibition was also first order with respect to incubation time. Fig. 5, in addition, shows the inhibition kinetics for these sugars in the presence of 5 mM *N*-α-*tert*-butoxycarbonyl (Boc)-lysine. The ε-amino group of lysine should be far more reactive toward reducing sugars than the primary amino groups of nucleotides, which, because of tautomerization, possess partial secondary character. The presence of lysine in the incubation mixture should thus accelerate the Maillard process. Studies of the nonenzymatic browning of proteins have shown that products of the later stages of the Maillard process are highly reactive, can cross-link proteins, and may even be mutagenic (29, 30).

As shown in Fig. 5*A*, the rate of transfection inhibition is initially less in the presence of Boc-lysine. With longer incubation times, transfection efficiency decreases, reaching a rate that is more rapid than the rate for either glucose or Glc-6-*P* alone. This pattern leads to the hypothesis that the amino group of the lysine initially consumes enough of the sugar to inhibit its reaction with DNA and that, with time, reactive Maillard intermediates are generated by the reaction of Glc-6-*P* with Boc-lysine. An alternative explanation is that lysine promotes the rate of chain breakage at apurinic sites, as has been previously shown for a number of amines (31). This is unlikely under our experimental conditions because gel electrophoresis at various times of DNA incubated with Glc-6-*P* or with Glc-6-*P*/Boc-lysine showed no discernible difference in the progressive decrease of closed circular DNA (Fig. 5*B*).

Products formed by the reaction of nucleic acids with simple alkylating agents often vary depending on whether the polynucleotide is in a double- or a single-stranded form. This occurs in cases in which modification affects amino positions that are hydrogen bonded in the double-stranded molecule (32). These sites are less reactive, although some reaction at these sites will occur as a result of thermal denaturation (33). f1 exists as a ds DNA during its replicative phase. Purified Rf1 (f1, replicative form) was incubated with 150 mM Glc-6-*P* under the conditions described previously for single-stranded f1. Transfection analysis of Rf1 modified by incubation with Glc-6-*P* for 1 and 3 weeks showed inhibitory effects of 0.5 and 4 orders of magnitude, respectively. Gel

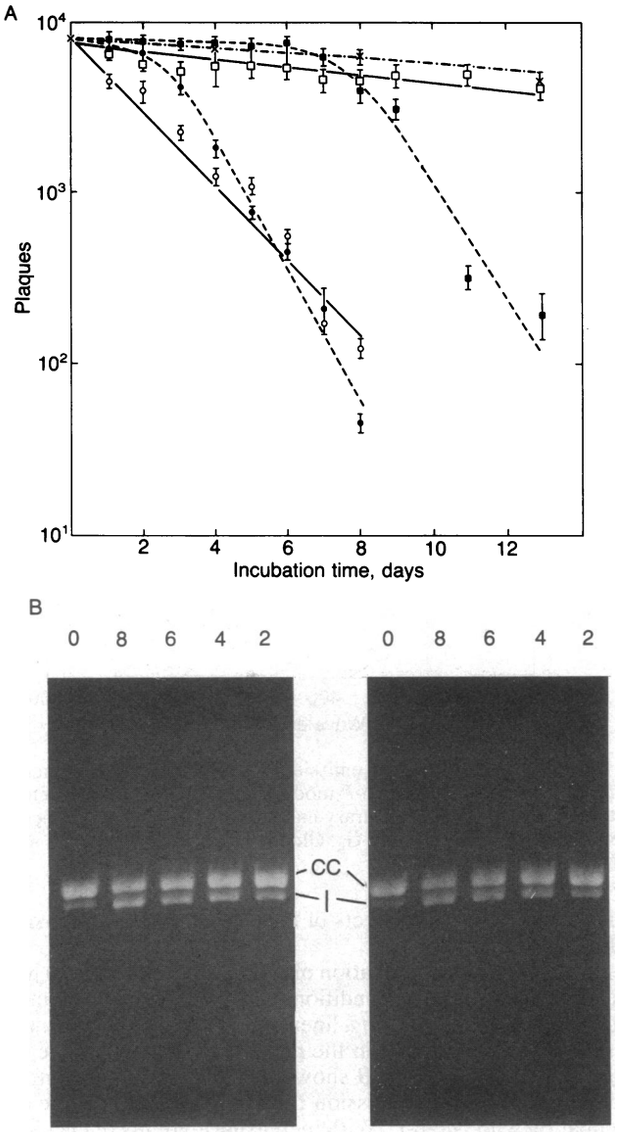

FIG. 5. (*A*) Rate of f1 DNA inactivation by 25 mM Glc-6-*P* (○), 25 mM glucose (□), 25 mM Glc-6-*P*/5 mM Boc-lysine (●), and 25 mM glucose/5 mM Boc-lysine (■). ×, Control DNA incubated with Boc-lysine alone. (*B*) Alkaline gel electrophoresis of DNA incubated with 25 mM Glc-6-*P* (*Left*) or 25 mM Glc-6-*P*/5 mM Boc-lysine (*Right*). DNA was incubated for 0, 2, 4, 6, or 8 days. cc, Closed circular DNA; l, linear DNA.

electrophoresis of samples incubated for 3 weeks showed substantial (>90%) chain breakage (data not shown).

DISCUSSION

We have investigated the chemical and biological properties of DNA incubated with either glucose or Glc-6-*P*. During incubation, absorbance and fluorescence changes develop that suggest that DNA can be glycosylated and undergo nonenzymatic browning. Spectral changes were not observed with TMP, which lacks a primary amino group, nor with ds DNA, in which hydrogen bonding may hinder the amino groups from reacting. Incubation of single-stranded viral DNA with these sugars caused a time- and concentration-dependent decrease of transfection efficiency. Inactivation by Glc-6-*P* was dramatic, 8 days of incubation with 150 mM sugar decreased transfection efficiency by 4 orders of magnitude. The rate of inhibition by Glc-6-*P* was 25 times that by glucose. Glc-6-*P* also was observed to induce strand scission

in a time- and concentration-dependent manner. Although the possibility of a contaminating nuclease cannot be eliminated completely, it is clear that the phenomenon that inhibits transfection occurs at a rate that is much more rapid than DNA breakage.

In terms of biological activity, ds DNA was substantially less reactive toward Glc-6-*P*. Transfection kinetics as they relate to chemical modification, however, cannot be directly compared between ds and ss DNA. The *E. coli* host possesses enzymatic mechanisms that can correct a chain break when present in a duplex molecule (reviewed in ref. 34). A similar lesion in single-stranded f1 is lethal.

We propose that Glc-6-*P*, and glucose at a much slower rate, forms adducts with the primary amino groups of bases that inhibit template function. With time, these adducts undergo chemical rearrangement that, in a manner analogous to several alkylating agents (35), can labilize the glycosidic bond between the purine and the deoxyribose. This leads to depurination and, after still more time, β-elimination and strand scission.

Evaluating the significance of our findings to cellular-physiology must be done with caution. Glc-6-*P* is present at reasonably high concentration, for example, 250 μM, in the mammalian cerebral cortex (36). The observation that an intracellular metabolite can modify DNA suggests a mechanism for the accumulation of genetic lesions that may lead to cellular senescence. The occurrence of free amines within the cell might further contribute to DNA damage by nonenzymatic browning, as our results with the simultaneous incubation of Boc-lysine suggest. The occurrence of reactive Maillard intermediates with nuclear proteins may account for the increased crosslinking of proteins to DNA that has been found in the chromatin of aged organisms (37). Alternatively, basic proteins, which are bound to at least 50% of the mammalian genome (38), may play a protective shielding role. Another important consideration would be the occurrence of enzymatic mechanisms that could repair DNA modified by Maillard products. Depurination, if indeed the result of Maillard product damage, may be repaired by excision repair (34).

In conclusion, we have shown that Glc-6-*P*, glucose, and possibly other reducing sugars can react with DNA *in vitro* to produce marked biological and structural alterations. Accumulation of these addition products might be a mechanism for the decreased genetic viability and increased tumorigenesis observed in the aged organism.

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