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Journal of Nutritional Biochemistry 12 (2001) 235–241

THE JOURNAL OF Nutritional Biochemistry

Fructose triggers DNA modification and damage in an *Escherichia coli* plasmid

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Received 13 June 2000; received in revised form 22 November 2000; accepted 7 December 2000

Abstract

The nonenzymatic reaction between reducing sugars and amino groups of long-lived macromolecules results in an array of chemical modifications that may account for several physiological complications. The characteristics of the reaction are directly related to the type of the reducing sugars involved, whether aldoses or ketoses, phosphorylated or non-phosphorylated, and these in turn determine the consequences of the induced modifications. So far, most studies have been focused on the nonenzymatic reaction between glucose and proteins, while the reaction with fructose, a faster glycating agent, attracted only a minor attention. We have recently demonstrated that long-term fructose consumption induces age-related changes in collagen from skin and cortical bones faster than glucose. In the present study we provide evidence that fructose and its phosphate metabolites can modify DNA faster than glucose and its phosphate metabolites under *in vitro* conditions. Incubating the plasmid pBR322 with fructose and glucose phosphate metabolites induced DNA modifications and damage that were verified by gel electrophoresis and transformation capacity of the plasmid into an *Escherichia coli* host. The intensity of the tested sugars to modified and damage DNA after incubation for 15 days increased significantly in the following order: glucose 1-phosphate < glucose < glucose 6-phosphate < fructose 1-phosphate < fructose < fructose 6-phosphate. The data suggest that fructose should deserve more attention as a factor that may influence glycation and induce physiological complications.

Keywords: Fructose; Sugar phosphates; Plasmid DNA; Glycation

1. Introduction

The nonenzymatic reaction between the aldehyde group of reducing sugars and the amino groups of macromolecules is known as the Maillard reaction or nonenzymatic glucosylation (glycation). The early stages of the reaction result in the formation of Amadori or Heyns rearrangement products [1]. Consequences of the advanced stages of glycation of extracellular long-lived proteins are structural alterations, crosslinking and formation and deposition of fluorescent age pigments [2].

Preliminary studies with nucleic acids have demonstrated that the amino groups of DNA bases can react nonenzymatically with reducing sugars. Incubation of DNA with reducing sugars raised chromophores and fluorophores with similar spectral properties when compared with advanced glycated protein products [3,4]. Several investigations have shown that DNA structure and function are affected after incubation with the intracellularly occurring reducing sugars, glucose and glucose 6-phosphate [3,5], and the reactive glycated products formed from glyceraldehyde 3-P and lysine [6]. In addition, Lee and Cerami [7] showed that increased intracellular levels of glucose 6-phosphate increased plasmid DNA mutation in *Escherichia coli*.

In principal, all reducing sugars whether aldoses or ketoses [1] can initiate glycation in vivo. However, most studies so far have been focused on glucose, since glucose is the most abundant monosaccharide in blood and tissues, and is elevated in diabetes. However, among reducing sugars, glucose is the least reactive in initiating glycation. In contrast, the nonenzymatic reaction between fructose and amino groups (fructosylation or fructation) has not yet been a special focus of research although it has been mentioned in comparative studies including various sugars [8]. In vitro studies suggest that fructose compared to glucose is a much more potent initiator of the Maillard reaction [8–10]. Since the Maillard reaction is considered to be involved in the aging process [11], fructation may have a vital impact on

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0955-2863/01/$ – see front matter
PII: S0955-2863(00)00158-3

age-related processes. First, in some organs, such as ocular lens, kidney and peripheral nerves, fructose is synthesized from sorbitol through the polyol pathway [12]. Second, although in healthy subjects extracellular concentrations of fructose are lower than that of glucose, its higher reactivity suggest fructose as a strong candidate for fructation in vivo. In diabetic subjects however, fructose may play a greater role since concentration of fructose approach and even exceed that of glucose in ocular lenses [13] and in peripheral nerves [14]. Third, 10–20% of the hexose bound to human ocular lens proteins was found to be attached via carbon 2, indicating that the proteins had reacted with endogenous fructose [9]. Fourth, although dietary fructose has some adverse side effects, it is still advocated as a preferred sweetener for diabetic subjects [15]. Fifth, the recent increase in the dietary consumption of fructose [16] might effect the concentration of fructose, and fructose metabolites in blood and tissues. Sixth, the distinct metabolism of fructose in the liver stimulates the formation of several unique metabolites namely fructose 1-phosphate, dihydroxyacetone phosphate, glyceraldehyde and glyceraldehyde 3-phosphate [17], and it should be mentioned that several sugar phosphates display highly accelerated Maillard reactivity compared to their nonphosphate analogues [8]. Recently we have demonstrated that long-term fructose consumption compared with glucose, elevates plasma fructosamine and glycated hemoglobin levels in blood, and accelerates the aging process as assessed by several age-related variables in collagen from skin and cortical bones of male rats [18].

In the present investigation, we have examined the effect of incubating fructose, glucose and their phosphate derivatives with a double-stranded DNA plasmid (pBR322) which contains the selectable *amp* gene that confer resistance to ampicillin on an *Escherichia coli* host. DNA modifications and damage that developed during incubation were evaluated by agarose gel electrophoresis and by measuring antibiotic resistance after transforming the treated plasmid into an *Escherichia coli* host.

2. Materials and methods

2.1. DNA treatment

The plasmid pBR322 contains the selectable *amp* gene that confers resistance to ampicillin on an *Escherichia coli* host and was used to determine the reducing sugars potential to induce lesions in double stranded DNA. Plasmid DNA was incubated in vitro with several reducing sugars for various incubation periods. At the end of each incubation period the treated plasmid samples were transformed into an *Escherichia coli* HB101 strain. Two micrograms of DNA were incubated for 3, 6, 9, 12 and 15 days, at 37°C under sterile conditions in the dark, with either fructose, fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), glucose, glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P) in 0.4 ml of buffer containing 50 mmol/L hydroxyethyl piperazine ethanesulfonic acid (HEPES, pH 8.0) and 1.0 mmol/L EDTA. The final concentration of the reducing sugars in the reaction mixture was 280 mmol/L for fructose and glucose, and 160 mmol/L for their phosphate derivatives. These levels provide an equal concentration on a weight per volume basis (approximately 50 mg/ml) for all the tested sugars. For a positive control we used hydrogen peroxide (10 μmol/L) known to produce lesions in double-strand DNA [19]. All chemicals were obtained from Sigma Chemical Company, St. Louis, MO. To exclude the possible contamination by DNA nucleases, buffer and sugar solutions were filtered through a dialysis membrane with a molecular weight cut-off of 3500 Daltons. Buffer containing DNA and EDTA (without sugar) were incubated under identical conditions, and served as the control. Following the 15-day incubation period pH levels were slightly reduced (pH 7.8).

2.2. Plasmid transformation

At the end of the incubation periods each reaction mixture was extensively dialyzed against a 50 mmol/L HEPES buffer (pH 8.0) containing 1.0 mmol/L EDTA at 4°C. The retained DNA was precipitated with 8.0 mol/L ammonium acetate in 70% ethanol [20], centrifuged at 15000 x g and resuspended in 16 μl of 10 mmol/L Tris buffer (pH 8.0) containing 1.0 mmol/L EDTA. Four microliters were transformed into 100 μl of *Escherichia coli* cells (strain HB101) treated with calcium chloride. One milliliter of L broth was added and the cells were incubated for 1 h at 37°C to allow the expression of antibiotic resistance. Ten microliters of the cell suspension were then plated on ampicillin-supplemented (100 mg/L) TY plates, and colonies were counted after an overnight incubation at 37°C.

The following procedure was conducted to test for the possibility that the presence of the reducing sugars, or the products of their spontaneous degradation, could inhibit expression of the pBR322 DNA in the host cells. At every incubation period, equivalent amount of each of the tested sugars was incubated alone in 50 mmol/L HEPES buffer (pH 8.0) containing 1.0 mmol/L EDTA, and was then added to $CaCl_2$-treated *Escherichia coli* together with untreated plasmid. Furthermore, to reject the possibility that differences in transfection rates are responsible for our results, equivalent amounts of a different type of an untreated plasmid (M13), containing the gene for β-galctosidase activity, were co-transfected with the treated pBR322 plasmid, and the expression of β-galctosidase activity was evaluated [20]. Under the above circumstances no inhibition of transfection capacity was observed.

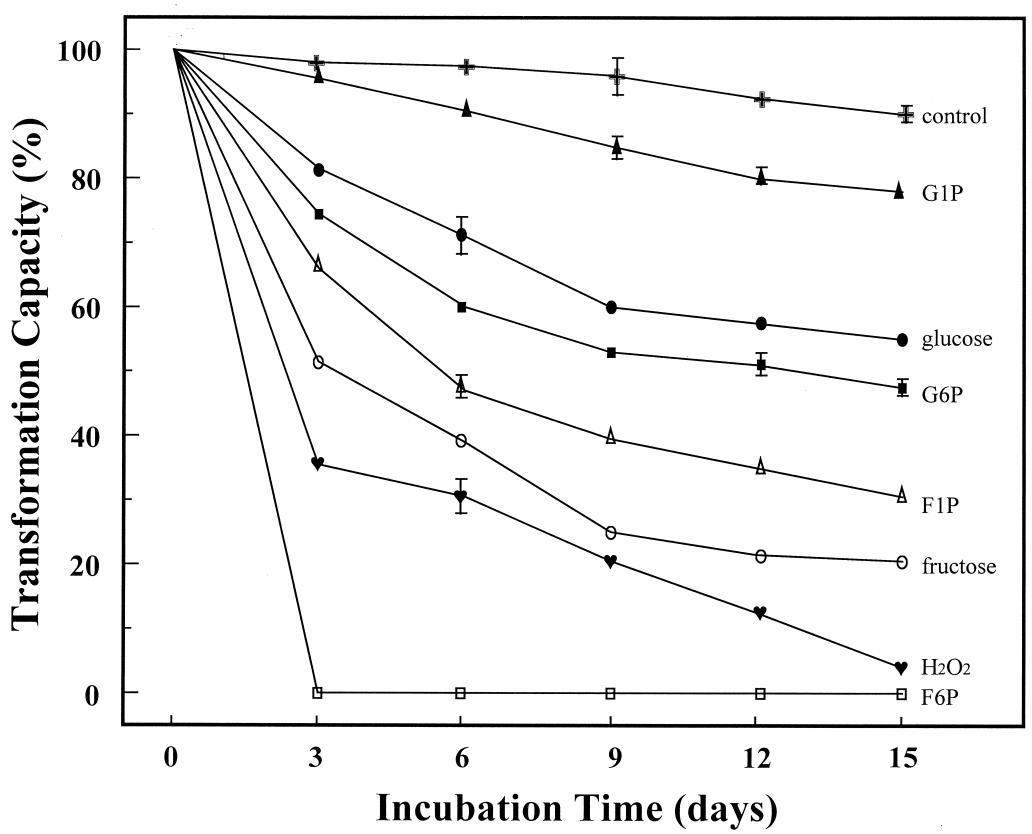

Fig. 1. Time dependent losses in pBR322 transformation capacity after incubation without sugar (Con), with 280 mmol/L fructose (Fru),160 mmol/L fructose 1-phosphate (F1P), 160 mmol/L fructose 6-phosphate (F6P), with 280 mmol/L glucose (Glu), 160 mmol/L glucose 1-phospahte (G1P), 160 mmol/L glucose 6-phosphate (G6P) and 10 μmol/L hydrogen peroxyde (H2O2). At the indicated times, 0.1 ml of $CaCl_2$-treated *E. coli* strain HB101 was transformed with 4 μl aliquot of the incubated solutions. Cells were plated onto ampicillin- supplemented TY plates and colonies were counted after overnight growth. Results are the mean $\pm$ SD counts of three plates. At each incubation period values with different superscript differ significantly ($P < 0.05$).

2.3. DNA electrophoresis

pBR322 DNA treated with reducing sugars was subjected to gel electrophoresis under conditions [20] which allow to separate the super-coiled DNA form (SCF) from the two relaxed DNA forms namely the linear form (LF) and the open circular form (OCF). DNA from the remaining sample was separated to its forms on a 1% agarose gel in 40.0 mmol/L Tris buffer (pH 8.0) containing 20.0 mmol/L glacial acetic acid and 1.0 mmol/L EDTA [20]. The gel was stained with ethidium bromide (1 mg/L) and photographed under ultraviolet light. The amount DNA in each form was determined densitometrically after scanning the negatives and processing data by the RFLP-SCAN image analysis software (Scanalytics, Billerica, MA). To evaluate the amount of total DNA left at the end of each incubating period, DNA was digested with Hind III (Sigma Chemical Company, St. Louis, MO), a restriction enzyme that cleaves pBR322 DNA at a single site and converts the SCF or OCF to the LF that migrates on an agarose gel as a function of its molecular weight [5]. A standard Hind III digest of λ DNA was introduced with samples.

2.4. Statistical analysis

Statistical studies were performed using SAS/STAT Version 6.04 software, SAS Institute, Cary, NC. Data were analyzed by one-way ANOVA followed by Duncan's multiple range tests. A probability level of 0.05 was selected as the point at which differences between treatments in a given incubation period were considered significant. Data are presented as means $\pm$ SD.

3. Results

Changes in the transfection capacity (TC) of pBR322 DNA incubated with or without sugars, and expressed as percentage relative to the value obtained for each treatment at time zero is shown in Fig. 1. At each of the incubation periods the capacity of pBR322 to provide ampicillin resistance to an *Escherichia coli* host was significantly lower following incubation with the tested reducing sugars and hydrogen peroxide (except for G1P after 3 and 6 days) compared to the control (without sugar). A significant rapid loss of TC was observed following the incubation with F6P

compared to the other tested substances. Ampicillin resistance was completely lost soon after a 3-day incubation period and no transfected *Escherichia coli* host cells were observed. In comparison, hydrogen peroxide significantly lowered TC to 4% of its initial value following a 15-day incubation period. Incubation with fructose or F1P for 15 days significantly reduced TC to 20 and 33% of their initial value, respectively. In contrast, glucose and its phosphate derivatives were significantly less reactive. TC was significantly decreased to 80, 55 and 50% of the initial value following the incubation with G1P, glucose and G6P, respectively.

Exposure of pBR322 DNA to the reducing sugars induced a shift from the SCF toward the LF and OCF. The initial SCF to OCF ratio was 1.76 (Table 1) and no LF was observed in the untreated plasmid. Whereas the ratio between the SCF and OCF declined, the LF/OCF ratio increased as incubation time advances. Incubation of pBR322 with F6P for three days caused a complete DNA degradation and none of the DNA forms was observed following electrophoresis. Comparing the effect of the other tested reducing sugars revealed that in all the incubation periods the SCF/OCF ratio was significantly the lowest in the plasmid DNA treated with fructose or F1P (Table 1). Fructose-treated pBR322 completely lost its SCF after 9 days, while in the F1P-treated plasmid SCF vanished after 12 days. After 15 days we observed SCF/OCF ratios of 1.35 for the non-treated DNA and 1.09, 0.39 and 0.07 for the plasmid treated with G1P, glucose and G6P, respectively (Table 1). No SCF was observed in the DNA treated with hydrogen peroxide soon after 3 days of incubation and thereafter. The first linear forms were observed after 3 days in the hydrogen peroxide-, fructose- and F1P-treated plasmid and after 12 days in G6P-treated DNA. No LF was observed in the control or in glucose- and G1P-treated plasmid throughout the entire incubation period.

Electrophoresis of the pBR322 DNA under alkaline conditions revealed that the SCF migrates faster than the two relaxed DNA forms, while the OCF migrates slower than the LF (Fig. 2A). Electrophoresis of the treated and untreated plasmid following the digestion with Hind III revealed that the total amount of DNA in plasmid treated with either fructose, F1P, glucose, G1P, G6P or without any sugar was not changed during the 15-day incubation period (Fig. 2B). In contrast, DNA was partially degraded by hydrogen peroxide, as the intensity of its band was much weaker in contrast to the above sugars, and was completely vanished soon after a 3-day treatment period with F6P.

4. Discussion

The purpose of the present study was to investigate the biological properties of plasmid DNA following an in vitro incubation with several reducing sugars or their phosphate compounds. The amino groups of nucleic acids can serve as

Table 1
Ratios of super-coiled/open circular and linear/open circular of pBR322 DNA following 3, 6, 9, 12 and 15 days of incubation in HEPES buffer (pH 8.0) with 280 mmol/L of fructose (Fru) or glucose (Glu), 160 mmol/L fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), glucose 1-phosphate (G1P) or glucose 6-phosphate (G6P), 10 μmol/L H_2O_2 and without sugars (Con).

Incubation time (days)	Super-coiled/Open Circular						Linear/Open Circular					
	0	3	6	9	12	15	0	3	6	9	12	15
Con	1.76 ± 0.08	1.67 ± 0.09^{a}	1.52 ± 0.12^{a}	1.51 ± 0.08^{a}	1.45 ± 0.15^{a}	1.35 ± 0.12^{a}	0	0^{c}	0^{d}	0^{d}	0^{e}	0^{e}
H_2O_2	1.76 ± 0.08	0^{f}	0^{f}	0^{e}	0^{e}	0^{e}	0	0.145 ± 0.013^{a}	0.317 ± 0.033^{a}	0.683 ± 0.041^{a}	1.041 ± 0.089^{a}	1.503 ± 0.139^{a}
Fru	1.76 ± 0.08	0.22 ± 0.08^{e}	0.07 ± 0.03^{ed}	0^{e}	0^{e}	0^{e}	0	0.010 ± 0.003^{b}	0.027 ± 0.004^{b}	0.032 ± 0.006^{b}	0.108 ± 0.015^{b}	0.140 ± 0.022^{b}
F1P	1.76 ± 0.08	0.26 ± 0.09^{e}	0.13 ± 0.04^{d}	0.04 ± 0.02^{d}	0^{e}	0^{e}	0	0.010 ± 0.003^{b}	0.017 ± 0.003^{c}	0.020 ± 0.007^{bc}	0.075 ± 0.017^{c}	0.084 ± 0.019^{c}
F6P	1.76 ± 0.08	ND	ND	ND	ND	ND	0	ND	ND	ND	ND	ND
Glu	1.76 ± 0.08	1.15 ± 0.13^{c}	0.68 ± 0.15^{b}	0.51 ± 0.11^{b}	0.44 ± 0.10^{c}	0.39 ± 0.11^{c}	0	0^{c}	0^{d}	0^{d}	0^{e}	0^{e}
G1P	1.76 ± 0.08	1.51 ± 0.14^{ab}	1.46 ± 0.17^{a}	1.41 ± 0.19^{a}	1.25 ± 0.19^{ab}	1.09 ± 0.13^{b}	0	0^{c}	0^{d}	0^{d}	0^{e}	0^{e}
G6P	1.76 ± 0.08	0.84 ± 0.15^{d}	0.32 ± 0.11^{c}	0.20 ± 0.05^{c}	0.18 ± 0.05^{d}	0.07 ± 0.03^{d}	0	0^{c}	0^{d}	0^{d}	0.015 ± 0.004^{d}	0.044 ± 0.011^{d}

Values are means ± SD, n = 3. Values in a column with different superscript differ significantly ($P < 0.05$).
ND—Non detectable.

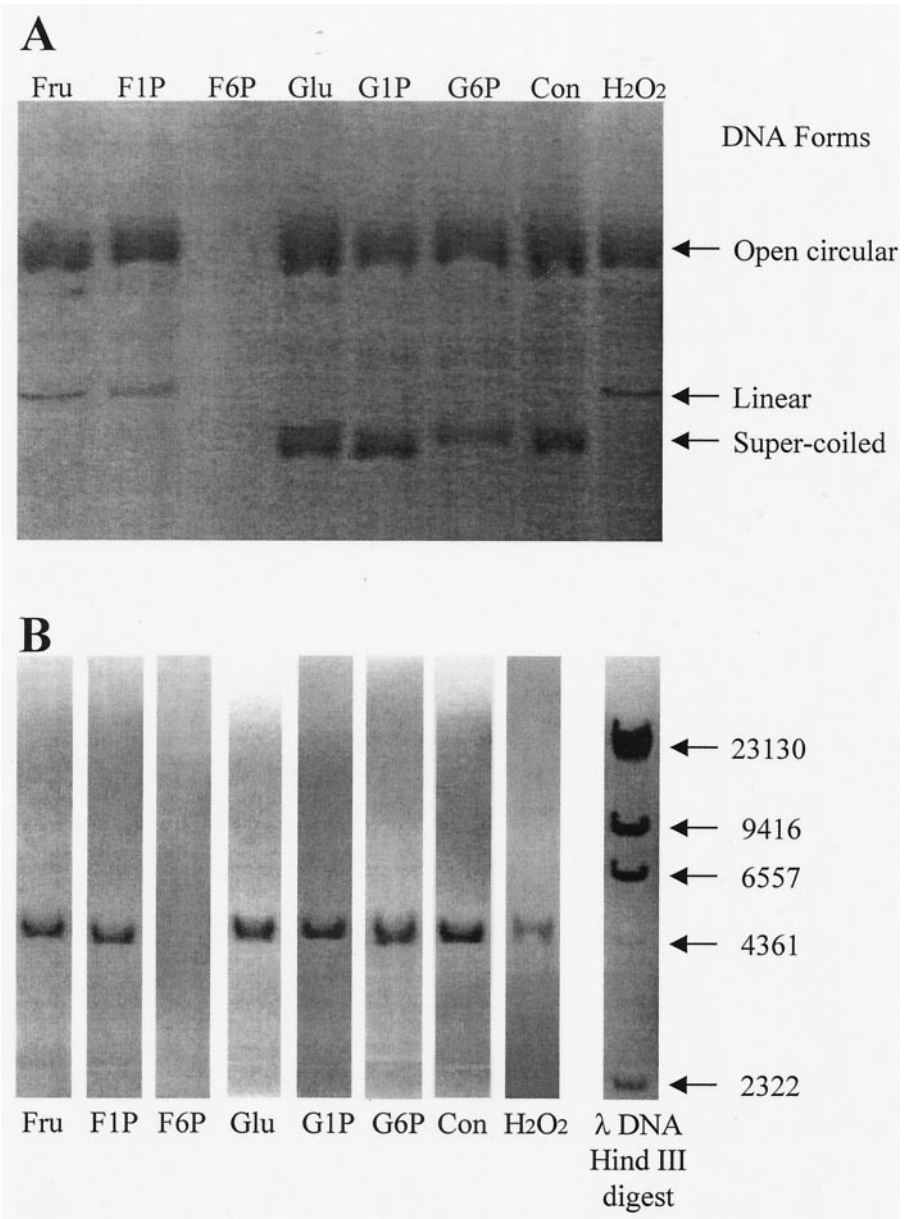

Fig. 2. Agarose gel electrophoresis of pBR322 DNA incubated in HEPES buffer (pH 8.0) either without sugars (Con) or with 280 mmol/L of fructose (Fru) or glucose (Glu), 160 mmol/L of fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), glucose 1-phosphate (G1P) or glucose 6-phosphate (G6P), and 10 μmol/L hydrogen peroxide (H2O2). **A**: following a 9-day incubation period. **B**: after incubation for 15-days and a 12-hr digestion period with Hind III at 37°C.

substrates for modification by reducing sugars, leading to spectral changes similar to those described for the nonenzymatic browning of proteins [3,4]. The hastened rate of DNA modifications and transfection inhibition observed with F6P compared to fructose, or with G6P compared to glucose is consistent with the observation that several sugar phosphates react more readily than their non-phosphate analogs [6,8,21]. The fact that G1P reacted less than glucose is probably due the loss of its reducing capacity after substitution the active aldehyde residue on carbon 1 with a non-reducing phosphate group. In contrast, fructose and F1P showed approximately the same activity since in both sugars the reactive carbonyl group is present on carbon 2, and retains the reducing power even after phosphorylation of carbon 1. The decrease in the plasmid transfection efficiency was about 100% higher with fructose- than glucose-treated DNA. However, when in vitro nonenzymatic glucosylation of proteins is involved, fructose is 7.5 to 10-fold faster then glucose [9,10]. This is probably due to the highly reactive Heyns compounds obtained from ketones which are converted faster to advanced glycated products compared to Amadori products of aldoses [10]. It should be mentioned however that beside direct glycation, oxidation processes might also be involved in the modification of DNA and possibly explain the similar effects of F6P and hydrogen peroxide on pBR322. According to Morita and Kashimure

[4] three steps are suggested for the formation of free radicals during glycation: a) reducing sugars cause transition metal-catalyzed autoxidation via enediol anion intermediates, generating oxygen-derived free radical and dicarbonyl compounds, b) Amadori or Heyns products can enolize and thereby reduce molecular oxygen, yielding oxygen radicals and dicarbonyl compounds, c) autoxidation of dicarbonyl compounds generated from Amadori or Heyns products. Although it is not clear which step of oxygen radical generation is more important, our observations confirm the superior properties of fructose compared to glucose in reacting with amino groups of double strand DNA.

Considering the biological activity of the treated plasmid, we observed that the intensity of the tested reducing sugars to modified pBR322 and to reduce its transformation capacity into an *E. coli* host cell increased in the following order: G1P < glucose < G6P < F1P = fructose < F6P. Bucala et al. [3] demonstrated that the inhibition of transfection occurs in a much faster rate than DNA strand-scission, and that double stranded DNA is less susceptible toward reducing sugars than a single-strand viral genome.

At this point it should be emphasized that concluding from our in vitro observations in a prokaryotic host into the physiology of a mammalian cell must be done with great caution. The present study attempts to prove the superior deleterious properties of fructose and its phosphate metabolites on DNA as compared to glucose. Fructose, compared to glucose, is present at relative low concentrations in the plasma of healthy subjects. Blood fructose concentration is low when no fructose is being absorbed, and maximal concentrations of 2.2 mmol/L have been recorded soon after a fructose meal [22]. Yet, no attempt was made to investigate the influence of increasing dietary fructose consumption on the concentration of its reactive metabolites in tissues, which could in turn potentiate the Maillard reaction. Glyceraldehyde and glyceraldehyde 3-phosphate (GA3P), among the major metabolites of fructose, are most reactive glycation agents acting several times faster than equimolar amounts of glucose or fructose [8,21]. Glycation products of GA3P and lysine, in vitro, produced base modification and apurinic/apyrimidinic sites in double-stranded DNA in addition to strand-breaks [6]. Furthermore, in several diabetic tissues such as ocular lens and peripheral nerves, where the sorbitol pathway is active, the concentration of fructose often approaches and sometimes exceeds those of glucose, and the concentration of fructose metabolites could also increase as fructose concentration elevates. Recently, Lal et al. [23] showed that a novel phosphomonoester, fructose-3-phosphate, is produced in the diabetic lens. This fructose metabolite is a potent glycating agent and a potential precursor of 3-deoxyglucosone. Thus, the rational of studying the influence of sugars in levels beyond their cellular physiological concentration was to minimize the time necessary to achieve a measurable effect. These levels were previously used by several investigators [3–5] and are acceptable for this type of in vitro studies. It is therefore suggested that in vivo fructation in some mammalian tissues is a probable event and may contribute to nonenzymatic browning and DNA damage. The observation that intracellular metabolite can modify DNA suggests a mechanism for the accumulation of glycated DNA that may lead to site-specific damage [24]. In addition, free amines within the cell and proteins bound to DNA might also be glycated and induce further DNA damage [3,4]. On the other hand, basic proteins bound to the mammalian genome, may play a protective role. Another crucial point would be the presence of enzymatic mechanism that could repair glycated-modified DNA [25].

The potential role of fructation in vivo, can be put in perspective when one considered two major points in fructose metabolism; a) fructose metabolism is unique in that it bypasses the need of insulin and the phosphofructokinase regulation step, and enters glycolysis or gluconeogenesis at the triphosphate level, b**)** the relative activities of two unique hepatic key enzymes in the fructose metabolic pathway i.e. fructokinase that phosphorylates fructose to F1P, and aldolase B that splits F1P to glyceraldehyde and dihydroxyacetone phosphate [22]. It seems that this metabolic pathway have evolved in mammals to metabolize fructose as efficient as possible, and to keep fructose concentrations to a minimum. In this regard it would be interesting to determine whether individuals with essential fructosuria, lacking fructokinase, or with hereditary fructose intolerance, lacking aldolase B, exhibit any signs of DNA damage compare to healthy subjects.

In conclusion, we have demonstrated that F6P, fructose, and F1P can react with DNA in vitro to produce biological alterations. Studies aimed to further elucidate the possible role of fructose and its metabolites, at physiological range concentrations, under in vivo conditions, on possible induction of DNA damage in higher biological systems (eukaryote and mammalian cells) are in progress.

Acknowledgment

Supported in part by The Israel Minister of Health (Grant # 4134), and by the fund for the promotion of research at the Technion (Grant # 080–475).

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