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Hydroxylation of Guanine in Nucleosides and DNA at the C-8 Position by Heated Glucose and Oxygen Radical-Forming Agents

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Heated glucose is mutagenic to *Salmonella typhimurium* TA 100 in the absence of S-9 mix. For identifying unknown mutagens in heated glucose (dry solid, 200°C, 20 min), reaction with isopropylideneguanosine (IPG) was followed by isolation and characterization of the mutagen–IPG adduct. Two adducts, glyoxal-IPG and 8-hydroxy-IPG, were identified in the reaction mixture by this technique.

To elucidate the mechanism of this hydroxylation reaction, we investigated the abilities of various agents to hydroxylate deoxyguanosine or guanine base in DNA. Various reducing agents, metals, asbestoses, polyphenols, aminophenols, and X-ray were effective for hydroxylation, and an oxygen radical seems to be the reactive species. For sensitive detection of 8-hydroxyguanine, a monoclonal antibody for it was prepared.

Introduction

Three potent mutagens, namely 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) were previously isolated and characterized (*1–3*) from broiled fish and/or fried beef as major mutagenic components. However, it is reasonable to speculate that there might be more unknown mutagens in broiled food, because it was often observed that a progressive and considerable loss of mutagenicity resulted during successive isolation steps. Specific activity of IQ, MeIQ, and MeIQx are quite high using a bacterial mutagenicity test. Therefore, mutagens whose specific activities in mutagenesis are low might be difficult to detect as isolated components. For this reason, new methods have been recently developed to detect and identify mutagens by analyzing their adducts with guanosine derivatives (*4*).

When this method was applied to the identification of mutagenic principles present in heated glucose as a model of cooked food, an interesting compound, the 8-hydroxy derivative of guanine, was obtained. The reaction seemed to proceed via oxygen radicals produced from heated glucose. This finding prompted us to study various oxygen radical-forming agents in the environment. In fact, it was shown that those agents are all effective for hydroxylation of deoxyguanosine as well as the guanine base residue in DNA. Formation of 8-hydroxydeoxyguanosine is a novel type of DNA modification reaction, and can be speculated to be related to mutagenesis.

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Results and Discussion

Detection and Identification of Mutagens in Heated Glucose by Analyzing Their Adducts with Guanosine Derivatives

When glucose (dry solid) is heated at 200°C for 20 min as a model reaction of cooking, it becomes mutagenic to *S. typhimurium* TA 100 as previously reported (*4,5*). A 10-mg portion of heated glucose induced approximately 150 revertants in the absence of S-9 mix (Fig. 1). We previously reported that various mutagens can be detected by analyzing their adducts after reaction with the fluorescent guanosine derivative, 2′-deoxy-2′-(2″,3″-dihydro-2″,4″-diphenyl-2″-hydroxy-3″-oxo-1″-pyrrolyl)guanosine (FG) (*4*), which serves to trap the reactive species. Therefore, this method was applied to the screening of heated glucose in order to identify the active principle(s) present. When heated glucose was

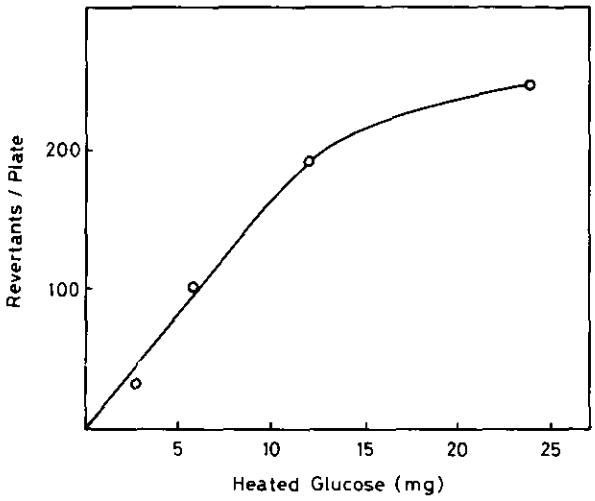
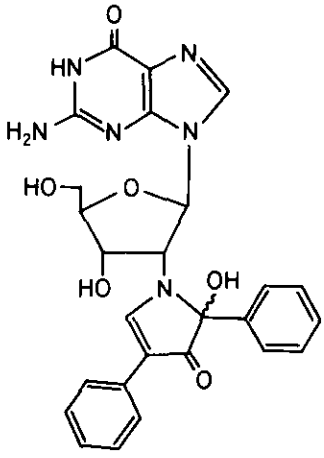


FIGURE 1. Mutagenicity of heated glucose (dry solid, 200°C, 20 min) to TA 100.



FG

reacted with FG and the reaction mixture was analyzed by high-performance liquid chromatography (HPLC), several adduct peaks were detected in the chromatogram (Fig. 2). The sensitivity of this method to detect mutagens is comparable with that of the bacterial mutagenicity test. Formation of the FG adduct was detected clearly by using 1 mg of heated glucose as shown in Figure 2. In order to identify the unknown mutagens in heated glucose, a more simple derivative of guanosine, isopropylideneguanosine (IPG) was used as the adduct trap (*4*), because this compound is available in large amounts and the structure determination of IPG adduct is much easier than that of the fluorescent derivatives. The prodecure for identification of mutagens by IPG is summarized in Figure 3. IPG was reacted with heated glucose at 37°C for 15 hr at neutral pH. IPG adducts were extracted from the reaction mixture

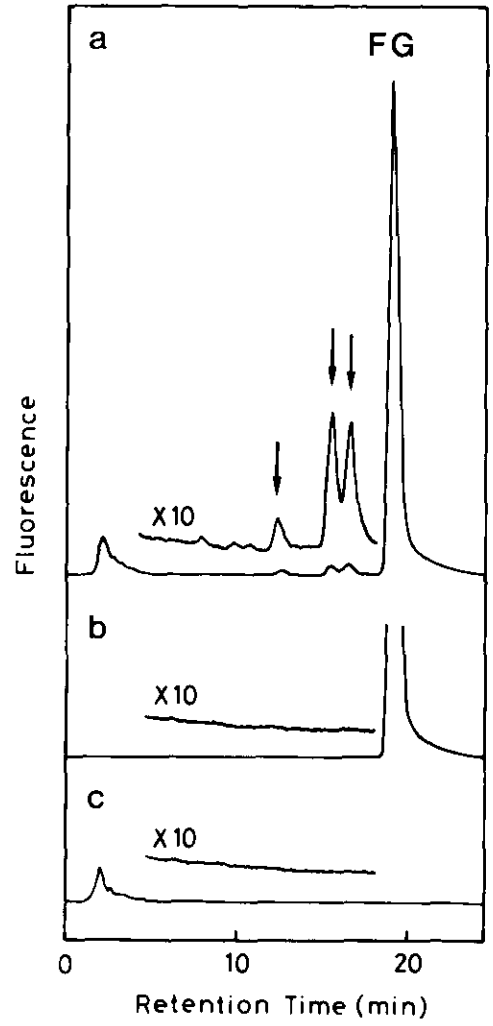


FIGURE 2. Detection of FG adduct formed between FG and heated glucose by HPLC: (*a*) reaction mixture of FG and heated glucose; (b) FG control; (c) heated glucose control. Peaks of adducts are indicated by arrows. Column: Merck Hibar column Lichrosorb RP-18 (0.4 × 25 cm), solvent: 30% aqueous methanol containing 10mM NH_4OAc (pH 5.3), flow rate: 1 mL/min, detector: JASCO FP-110 fluorescence spectrofluorometer, excitation at 365 nm, emission at 480 nm.

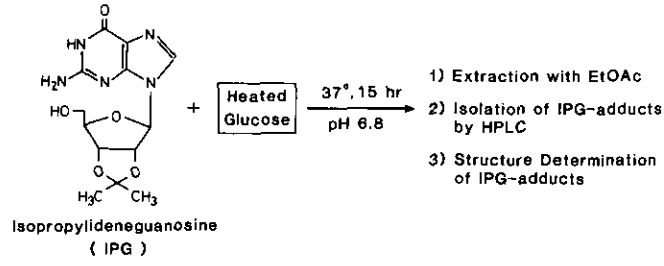


FIGURE 3. Identification of mutagens in heated glucose as their adducts with isopropylideneguanosine (IPG).

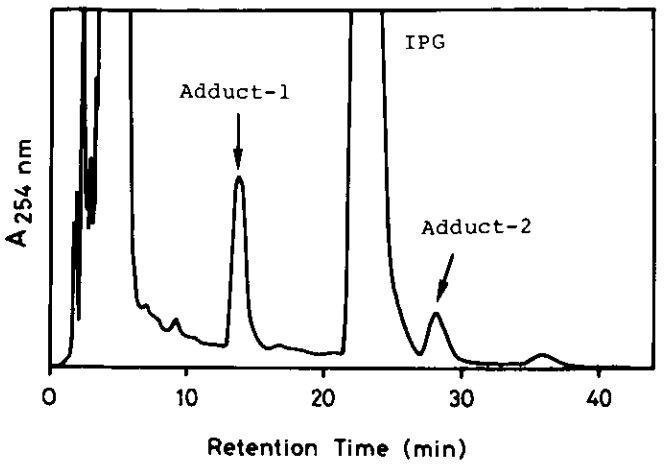
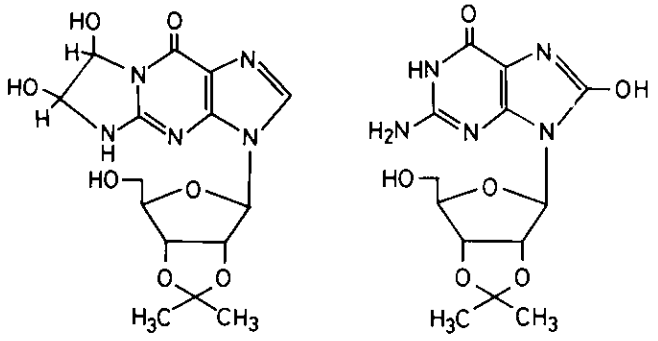


FIGURE 4. Fractionation of the reaction products of heated glucose and IPG by HPLC. Column: waters, μBondapak C_{18} (0.78 × 30 cm), solvent: 15% aqueous methanol, flow rate: 6 mL/min.



Adduct 1 Adduct 2

with ethyl acetate and isolated by HPLC. Structures of adducts thus isolated were determined by spectral measurements, and based on their structures it is possible to deduce the identity of the original mutagens. Figure 4 shows a chromatogram of the reaction mixture of IPG and heated glucose. Two adducts, adduct 1 and adduct 2, were isolated by HPLC, and their structures were determined from UV, mass, and ^{1}H-NMR spectra. The structure of adduct 1 was identified to be a cyclic adduct formed between glyoxal and IPG, and therefore, one of the mutagens in heated glucose is glyoxal.

The structure of adduct 2 was determined as 8-hydroxy-IPG. This was a rather unexpected and interesting result. Although various oxidation products of DNA components have been identified, the hydroxylation reaction at the C-8 position of guanine residue in DNA has not been reported thus far. The detection of 8-hydroxy-IPG raises the following interesting questions. What is the mechanism of this hydroxylation reaction? How is it related to mutagenesis? In the following section, we show that various compounds which produce oxygen radicals and are known to be mutagenic and/or carcinogenic are effective in hydroxylation of the deoxyguanosine residue in DNA.

8-Hydroxydeoxyguanosine may tautomerize to the 8-keto form in aqueous solution, but we tentatively describe this compound as 8-hydroxydeoxyguanosine (8-OH-dGuo) [Eq. (1)].

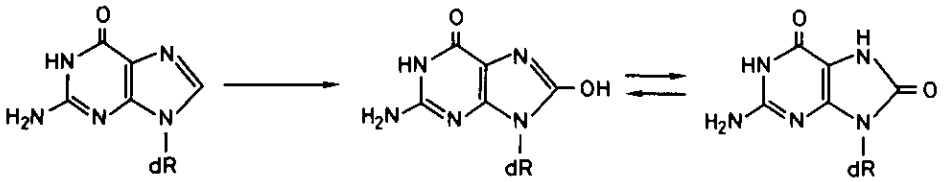
Hydroxylation of Deoxyguanosine at the C-8 Position by Various Oxygen Radical Producing Agents

When a solution of deoxyguanosine was shaken with various reducing agents at 37°C, the formation of 8-OH-dGuo was observed (*6*). Hydroxylamine, hydrazine, dihydroxymaleic acid, ascorbic acid, sodium bisulfite, and acetol were effective for the hydroxylation reaction. The yields of 8-OH-dGuo after treatments of dGuo with these agents for 1 hr are shown in Table 1. This hydroxylation reaction seems to require oxygen (O_2), because when oxygen was removed from the reaction mixture by bubbling nitrogen gas, no hydroxylation reaction was observed. An oxygen radical produced by reduction of molecular oxygen seems to be the reactive species. Among the above compounds, acetol is known

Table 1. Hydroxylation of dGuo by reducing agents.[a]

Compounds	Yield of 8-OH-dGuo, %
Hydroxylamine	4.59
Hydrazine	3.48
Dihydroxymaleic acid	2.70
Ascorbic acid	1.65
Sodium bisulfite	0.58
Acetol	0.23

[a] 1.3 mM dGuo, 10mM reducing agent, 0.1 M phosphate buffer (pH 6.8), 2 mL, 37°C, 1 hr.



Deoxyguanosine 8-Hydroxydeoxyguanosine (1)

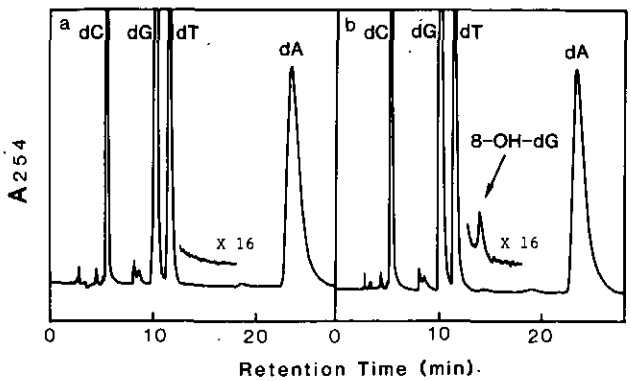


FIGURE 5. Detection of 8-hydroxydeoxyguanosine (8-OH-dG) in DNA treated with catechol, H_2O_2 and Fe^{3+}: (a) control, (b) treated with catechol, H_2O_2 and Fe^{3+}

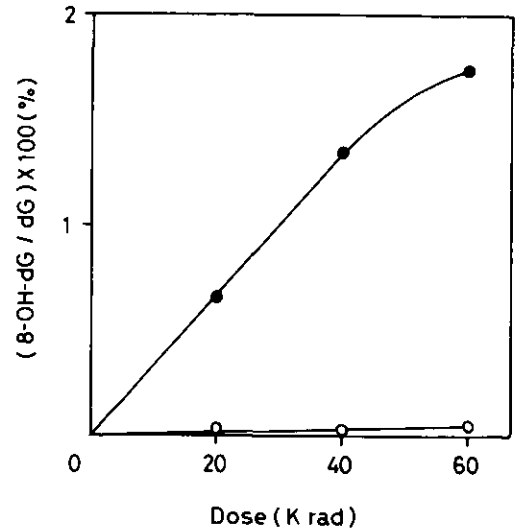
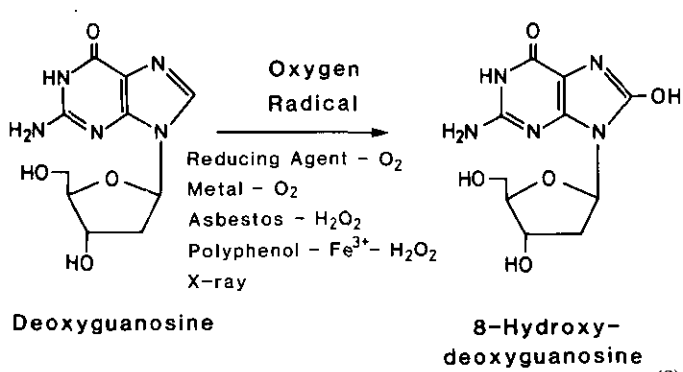


FIGURE 6. Hydroxylation of guanine residue in DNA plotted as a function of X-ray dose. (●) no EtOH, (○) with EtOH.

Table 2. Hydroxylation of dGuo by polyphenols (aminophenols) in the presence of H_2O_2 and Fe^{3+}.[a]

Coumpounds	Yield of 8-OH-dGuo, %
Catechol	7.7
Hydroquinone	15.0
Pyrogallol	7.5
Chlorogenic acid	7.2
Quercetin	1.3
3-Hydroxyanthranilic acid	2.0
3-Hydroxykynurenine	1.1
H_2O_2 + Fe^{3+} (Control)	

[a] 1.3 mM dGuo, 1 mM polyphenol, 5 mM H_2O_2, 0.1 mM $FeCl_3$, 0.5 mM EDTA, 0.1 M phosphate buffer (pH 7.4), 37°C, 30 min.



(2)

to be produced by heating glucose; therefore, one of the mutagenic components in heated glucose responsible for the hydroxylation reaction may be acetol.

Other compounds effective for the hydroxylation reaction are polyphenols and aminophenols (*7*). When these compounds were incubated with dGuo in the presence of H_2O_2 and Fe^{3+}, the formation of 8-OH-dGuo was also observed (Table 2). All of these compounds are known to have mutagenic (quercetin and chlorogenic acid), clastogenic (catechol and hydroquinone), carcinogenic (3-hydroxyanthranilic acid and 3-hydroxykynurenine), or cocarcinogenic (catechol and pyrogallol) activities.

The guanine residue in DNA is also hydroxylated by these treatments. For example, when native calf thymus DNA was reacted with catechol in the presence of H_2O_2 and Fe^{3+}, 8-OH-dGuo was detected by HPLC analysis, after enzymatic hydrolysis of the treated DNA to deoxynucleosides (Fig. 5).

Ionizing radiation is known to induce DNA damage such as strand scission and thymine glycol and 5-hydroxymethyluracil formation by generation of OH radical. We found that X-ray irradiation of native calf thymus DNA produced an 8-hydroxyguanine moiety in the DNA (*8*). The yield of 8-OH-dGuo was increased with X-ray dose (Fig. 6). When an OH radical scavenger, ethanol, was added to the DNA solution at a concentration of 10%, the hydroxylation reaction was inhibited almost completely, indicating that OH radical produced by X-ray is responsible for formation of 8-OH-dGuo (Fig. 6).

The hydroxylation reaction of deoxyguanosine or guanine base in DNA is summarized in Eq. (2). Although this reaction was first discovered by the reaction of IPG and heated glucose, various reducing agents, polyphenols and X-ray were found to be effective for the hydroxylation. In addition, various metals or asbestos also showed the hydroxylating activity (*6,9*).

This hydroxylation reaction seems to proceed via formation of an oxygen radical, such as OH radical, because most of these hydroxylation reactions are inhibited by addition of ethanol or dimethylsulfoxide. It should be also mentioned that the extent of 8-OH-dG formation

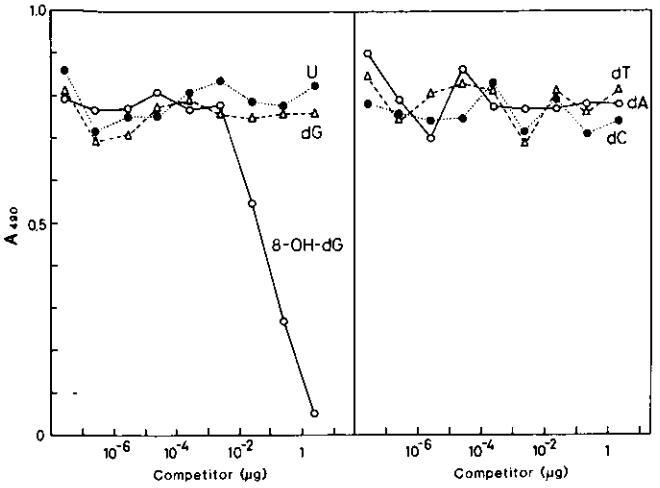


FIGURE 7. Competitive inhibition of the monoclonal antibody binding to 8-OH-Guo-BSA.

in calf thymus DNA by the treatment with catechol–Fe^{3+}–H_2O_2, asbestos–H_2O_2 or X-ray is comparable to the degree of DNA strand scission.

Preparation of Monoclonal Antibodies Specific for 8-OH-Gua

Among various types of DNA damage, thymine glycol and 5-hydroxymethyluracil are known to be repaired by specific glycosylases (*10*). Ames and co-workers reported that thymine glycol and thymidine glycol were detected in human urine (*11*). If 8-OH-Gua or 8-OH-dGuo can be detected in human lymphocytes or urine, it may be useful to monitor DNA damage *in vivo* by oxygen radicals through assay for these derivatives. It is also important to detect 8-OH-Gua in cellular DNA.

For the sensitive and specific detection of 8-OH-Gua, monoclonal antibodies specific for 8-OH-Gua were prepared. 8-OH-Guo was oxidized by periodate and conjugated to bovine serum albumin (BSA). Monoclonal antibodies were obtained after fusion of mouse myeloma cells with spleen cells isolated from mice immunized with 8-OH-Guo-BSA conjugate. For screening of the 8-OH-Gua-specific antibody, an ELISA (enzyme-linked immunosorbent assay) method was used, and a clone producing 8-OH-Gua-specific antibody was obtained. In order to characterize the specificity of this monoclonal antibody, the degree of inhibition using various normal nucleosides and 8-OH-dGuo was examined. As shown in Figure 7, binding of the monoclonal antibody to 8-OH-Guo-BSA complex was inhibited only by 8-OH-dGuo. These results clearly indicate that this monoclonal antibody specifically recognizes 8-OH-Gua. Studies are now in progress to detect 8-OH-Gua residues in DNA from X-irradiated cells using this monoclonal antibody.

Conclusion

A new type of DNA-modifying reaction, namely, the hydroxylation of guanine residues at the C-8 position, was found when mutagens present in heated glucose were identified by analyzing their adducts with guanosine derivatives. Various oxygen radical-producing compounds, most of which are mutagenic and/or carcinogenic, were found to be active in hydroxylation of DNA *in vitro*. Thus it is tempting to speculate that formation of 8-OH-Gua in DNA by oxygen radical-forming agents may be relevant to their mutagenicity and/or carcinogenicity. First, it is important to know whether this reaction also occurs in DNA *in vivo*. It is also of interest to examine whether there are cellular mechanisms for repair of this type of DNA damage and whether 8-OH-Gua in DNA induces mispairing during DNA replication.

In this communication, the formation of 8-OH-Gua by oxygen radical forming agents is described. Isolation of the 8-OH-Guo derivative was facilitated by the use of a new method using Guo derivatives to identify adduct-forming reagents. This method seems to be useful for identification of mutagens, and it is very likely that there are more unidentified mutagens in cooked foods. This procedure is currently being used to screen for and characterize additional mutagens in cooked foods.

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