# Mutagenicity of and Formation of Oxygen Radicals by Trioses and Glyoxal Derivatives†

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Dihydroxyacetone, glyceraldehyde, glyoxal, methyl glyoxal, and glyoxylic acid were found to show mutagenicity on *Salmonella typhimurium* TA 100. The mutagenicities of these substances were inhibited by the addition of S-9 or some free radical scavengers. The alkaline buffered solutions of these mutagenic substances were found to reduce Nitro Blue tetrazolium chloride. DNA was degraded by the addition of these mutagenic substances. It has also been confirmed that free radicals derived from autoxidation of these substances are responsible for their mutagenicity.

In the previous paper,[1] we reported the mutagenicities of trioses and methyl glyoxal on *Salmonella typhimurium*. These substances are known as pyrolysis products of sugars or intermediates in the pathway of glycolysis and they are always contained in living cells. These facts lead us to the assumption that living cells must have some regulating systems to reduce toxicity, in order to protect themselves from mutagenicity.

In the course of these studies, we discovered a factor in rat liver homogenate (S-9) which markedly reduces the mutagenicities of these substances. We also confirmed that some oxygen radical scavengers decreased these mutagenicities and that the ultimate mutagens of these substances are oxygen radicals which are generated by autoxidation. Evidence has also been obtained that DNA is a target molecule of the mutagenicities.

This paper deals with major properties of the mutagenicity and oxygen radicals derived from these mutagenic substances.

## MATERIALS AND METHODS

*Assay method of mutagenicity.* A mutation assay was carried out according to Ames *et al.* with some modification.[2] The histidine-requiring strain of *Salmonella typhimurium* TA 100 was kindly supplied by Dr. B. N. Ames. In the previous paper, we reported that trioses and glyoxal derivatives show mutagenicities without metabolic activation with S-9 mix. A mixture of the test strain, properly diluted trioses or glyoxal derivatives, and a phosphate buffer was incubated at 37°C for 20 min and then poured onto an agar layer. After incubation at 37°C for 48 hr, $His^+$ revertant colonies were counted. The mutagenicity test was at five concentration levels for each sample, but only the results giving the highest potency are shown in this paper.

*Assay of oxygen radicals.* Autoxidation of trioses and glyoxal derivatives was studied by determining the rate of formazan formation ($\Delta A_{560}$/min) by superoxide ($O_2^-$) from Nitro Blue tetrazolium chloride (NBT) by the method described by Kashimura *et al.*[3] The reaction mixture contained various concentrations of the sample, 10 mM NBT, and 100 $\mu$M EDTA, and was equilibrated with air. The change in optical density of the mixture was measured using a Hitachi digital spectrophotometer Model 624 equipped with an automatic recorder.

*Degradation of DNA.* Degradation of calf thymus DNA was monitored by measurement of the decrease in fluorescence of the DNA-ethidium bromide complex ($\lambda_{em}$ 598, $\lambda_{ex}$ 276 nm)[4] using a spectrofluorometer Model FP-550 made by the Japan Spectroscopic Co.

*Materials.* The liver microsomal fraction (S-9) was

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prepared from rats previously treated with PCB as described by Ames *et al.*[5] DL-Glyceraldehyde and dihydroxyacetone were purchased from Wako Chemicals Co. Glyoxal (40% in water) and hydroxyacetone (50% in methanol) were obtained from Nakarai Chemicals Co. Catalase (EC 1.11.1.6, bovine liver) superoxide dismutase (SOD, EC 1.15.1.1, bovine blood) and calf thymus DNA were obtained from Sigma Chemicals Co.

All other reagents used were of guaranteed grade.

## RESULTS AND DISCUSSION

### *Changes of mutagenicities with S-9 and with catalase*

Table I shows both the mutagenicities of trioses and glyoxal derivatives and the reduction of them with S-9 and with catalase.

It was found that all of the three trioses show mutagenicity on *Salmonella typhimurium* TA 100. As for glyoxal derivatives, methyl glyoxal was highly mutagenic while glyoxal and dimethyl glyoxal were moderately and minimally mutagenic, respectively. Glyoxylic acid also showed high mutagenicity.

All of the mutagenicities of trioses and glyoxal derivatives were markedly reduced in the presence of S-9. The mutagenicities of β-propiolactone, propylene oxide and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) also decreased with S-9 but that of glycidol (2,3-epoxy-1-propanol) was not affected.

Similar effects were also seen with catalase but the rates of reduction were lower than those with S-9. In addition, the mutagenicities of AF-2 and glycidol were scarcely affected by catalase.

Figure 1 shows the dose-response curves of S-9 on the mutagenicities of these mutagens.

The mutagenicities of all of the mutagens used were markedly reduced by increasing the concentrations of S-9. The reduction became complete at concentrations of S-9 higher than 20 μg per plate.

TABLE I. EFFECTS OF S-9 AND CATALASE ON THE MUTAGENICITIES OF TRIOSES AND GLYOXAL DERIVATIVES

Cells of *Salmonella typhimurium* TA 100 were treated at 37°C for 20 min with 0.1 ml of mutagen and 0.25 ml of S-9 or catalase (5 μg/ml) or phosphate buffer (control), then poured onto histidine-deficient agar medium. The plates were incubated at 37°C for 48 hr and $His^+$ revertant colonies induced were counted. The number of spontaneous revertants (120) were subtracted. The numerical values in this table were obtained from the dose-response curves of five concentration levels.

| | Conc/plate | Control | S-9 | Catalase |
|---|---|---|---|---|
| DL-Glyceraldehyde | 100 | 557 | 123 | 273 |
| Dihydroxyacetone | 150 | 663 | 110 | 214 |
| Hydroxyacetone | 500 | 325 | 116 | 183 |
| Glyoxal | 40 | 978 | 115 | 186 |
| Methyl glyoxal | 20 | 1,745 | 102 | 255 |
| Dimethyl glyoxal | 500 | 115 | 87 | 98 |
| Glycol aldehyde | 500 | 132 | 93 | 117 |
| Glyoxylic acid | 200 | 532 | 92 | 230 |
| Glycolic acid | 500 | 53 | 50 | 52 |
| Propylene oxide | 350 | 627 | 104 | 223 |
| β-Propiolactone | 40 | 1,231 | 229 | 565 |
| Glycidol | 500 | 1,130 | 1,210 | 1,118 |
| AF-2 | 0.06 | 1,543 | 137 | 1,485 |

No mutagenicity by oxalic acid, lactic acid, pyruvic acid, glycerol, trimethylene glycol, and acetaldehyde was detected under these conditions.

### *Some properties of the mutagenicity-reducing substance in S-9*

Table II shows some properties of the mutagenicity-reducing substance in the S-9 fraction.

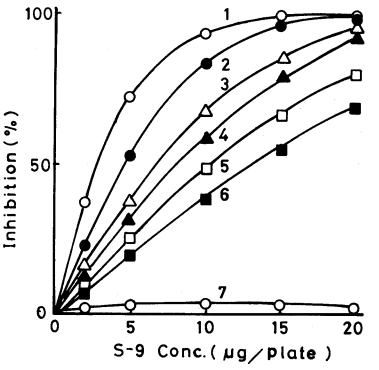


FIG. 1. Effect of S-9 Concentration on the Mutagenicities of These Mutagens.

Concentrations of the mutagens per plate were: for 1, glyceraldehyde, 100 μg; 2, glyoxal, 40 μg; 3, methyl glyoxal, 20 μg; 4, dihydroxyacetone, 150 μg; 5, AF-2, 0.06 μg; 6, β-propiolactone, 40 μg; 7, glycidol, 500 μg.

Other experimental conditions are described in the text and Table I. The numbers of revertant colonies of the controls without S-9 were: for glyceraldehyde, 572; glyoxal, 986; methyl glyoxal, 1,981; dihydroxyacetone, 862; AF-2, 1,465; β-propiolactone, 1,120; glycidol, 1,184.

TABLE II. SOME PROPERTIES OF THE MUTAGENICITY-REDUCING SUBSTANCE IN S-9

S-9 was treated with the various procedures described in this table. After these treatments, the reducing activities on the mutagenicities of some mutagens were determined as described in the text and Table I.

| Treatments of S-9 | Activity (%) | | |
|---|---|---|---|
| | Methyl glyoxal | Glycer-aldehyde | AF-2 |
| No treatment | 100 | 100 | 100 |
| Heated at 70°C for 15 min | 0 | 0 | 0 |
| Pronase digestion | 15 | 12 | 9 |
| Dialysis (activity in bag) | 95 | 98 | 97 |
| Centrifuge ppt | 88 | 91 | 55 |
| Centrifuge sup | 9 | 7 | 38 |

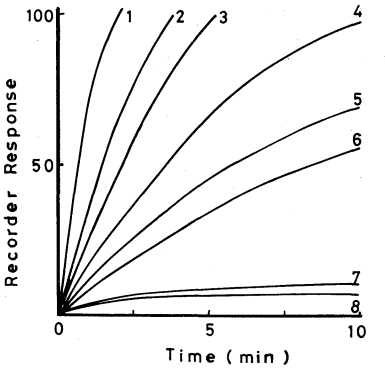


FIG. 2. Rate of Reduction of NBT by Trioses and Glyoxal Derivatives.

Measurement was made using 20 mg/ml of the sample and 100 $\mu$M of NBT, 10 min after the dissolution of the sample in 0.015 M carbonate buffer (pH 10.4). The reaction mixture was equilibrated with air, and the changes in O.D. at 560 nm were recorded automatically. Samples were: 1, glyoxal; 2, dihydroxyacetone; 3, glyceraldehyde; 4, methyl glyoxal; 5, glycol aldehyde; 6, dimethyl glyoxal; 7, glyoxylic acid; 8, glycolic acid.

The reducing activity was lost completely by heating at 70°C for 10 min. The activity was also lost by protease digestion. It seems that this substance has a large molecular weight judging from the fact that the activity remains in the cellulose bag even after dialysis. On centrifugation at 45,000 × $g$ for 60 min, almost all of the mutagenicity reduction was found only in a precipitate fraction but the activity toward AF-2 was observed in both fractions of precipitate and supernatant fluid. These results suggest that the active substance on glyoxal or trioses consist of protein which is localized in the microsomal fraction.

*Reducibility of NBT*

For the purpose of knowing the relation between the mutagenicities of these mutagens and their reactivity in autoxidation, the reduction of NBT, as a monitored measurement[6,7] at 560 nm of formazan formation, was investigated.

Figure 2 shows the data of the reduction of NBT by various trioses and glyoxal-derivatives.

Glyoxal, dihydroxyacetone and glyceraldehyde which exhibited high mutagenicities, as shown in Table I, also showed high reducing activities on NBT. Methyl glyoxal, hydroxyacetone, glycol aldehyde, and dimethyl glyoxal showed moderate activities while glycolic acid, pyruvic acid, and glyoxylic acid showed no activity on NBT. The rate of reduction was dependent on the concentration of these trioses and glyoxal derivatives (data not shown).

A correlation is seen between mutagenicities and NBT reducing activities, with some exceptions. As for glyoxal derivatives, the order of the mutagenicity was : methyl glyoxal > glyoxal > dimethyl glyoxal (Table I). On the other hand, the order of NBT-reducing activity was: glyoxal > methyl glyoxal > dimethyl glyoxal. Glyoxylic acid was also exceptional, showing a low-NBT reducing activity in spite of its high mutagenicity.

The reductions of NBT by glyoxal and dihydroxyacetone were inhibited by S-9 (93 and 95%), catalase (75 and 78%) and SOD (66 and 61%).

*Effect of some radical scavengers on the mutagenicities*

The effects of superoxide scavengers (Tiron, SOD), singlet oxygen scavengers (DABCO,

TABLE III. EFFECT OF SOME RADICAL SCAVENGERS ON THE MUTAGENICITIES OF METHYL GLYOXAL, DIHYDROXYACETONE, AND GLYOXYLIC ACID

Cells of *Salmonella typhimurium* TA 100 were treated at 37°C for 60 min in the presence of samples with or without various concentrations of scavengers in a total volume of 0.5 ml, then poured on to a histidine-deficient agar medium. The plates were incubated at 37°C for 48 hr and $His^+$ induced revertants (120) was subtracted. The numerical values in this table were obtained from the dose-response curves of five concentration levels.

| | Revertants/plate (inhibition, %) | | |
|---|---|---|---|
| | Methyl glyoxal | Dihydroxy-acetone | Glyoxylic acid |
| None | 1,862 (0) | 785 (0) | 543 (0) |
| Tiron | 1,093 (41.3) | 429 (45.4) | 322 (40.6) |
| DABCO | 1,783 (4.2) | 743 (5.3) | 480 (11.5) |
| Mannitol | 760 (59.2) | 275 (65.0) | 211 (61.2) |
| DMF | 1,743 (6.4) | 728 (7.2) | 487 (10.3) |
| Catalase | 1,020 (45.2) | 325 (58.5) | 258 (52.5) |
| SOD | 963 (48.3) | 383 (51.2) | 268 (50.6) |
| S-9 | 69 (96.2) | 52 (93.4) | 40 (92.7) |
| Catalase +SOD | 257 (86.2) | 147 (81.3) | 47 (91.3) |
| S-9 from normal rats[a] | 138 (92.6) | 45 (94.3) | 56 (89.7) |

[a] The normal liver microsomal fraction which was prepared from normal rats (not treated with PCB) was used.

Abbreviations; Tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid disodium salt; DABCO, 1,4-diazabicyclo-[2.2.2]octane; DMF, 2,5-dimethylfuran.

The mutagenicity of AF-2 was affected by none of these scavengers except S-9. None of the mutagenicities of β-propiolactone, propylene oxide and glycidol were affected by any of these scavengers.

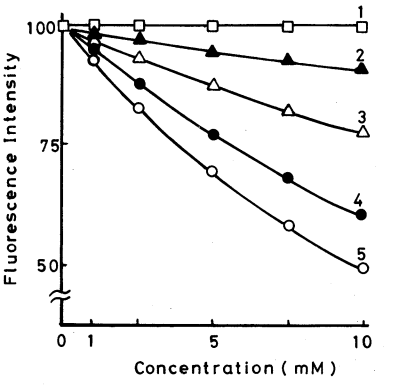


FIG. 3. Decrease of Fluorescence Intensity of the DNA–Ethidium Bromide Complex as a Function of the Concentration of Trioses and Glyoxal Derivatives Used for the Reaction with DNA.

The reaction mixture containing a triose or glyoxal derivative and DNA (50 μg/ml) in 0.05 M Tris-HCl buffer (pH 8.0) was incubated for 20 hr at 37°C. An aliquot was withdrawn, diluted with the buffer, treated with ethidium bromide (1.0 μg), and the intensity of fluorescence immediately measured. A control mixture, containing no triose or glyoxal derivative, was incubated for 20 hr at 37°C before the measurement. □—□, control; ▲—▲, glyceraldehyde; △—△, dihydroxyacetone; ●—●, glyoxal; ○—○, methyl glyoxal.

2,5-dimethylfuran) and hydroxy radical scavenger (mannitol) on the mutagenicities of dihydroxyacetone and glyoxal-derivatives were investigated.

As shown in Table III, the mutagenicities of these mutagens were inhibited by the addition of these scavengers, though the degree of inhibition varied with each scavenger. None of these scavengers alone, however, could inhibit to a greater degree than S-9 did and only the synergy of catalase and SOD could raise the degree of the inhibition to that of S-9. These facts suggest that the mutagenicities of these mutagens were brought not by a single but by plural oxygen radicals generated by autoxidation of these mutagens, and that S-9 may contain plural scavengers (catalase, and SOD for example). By these plural scavengers, the mutagenicities seem to be inhibited to a high degree.

The liver microsomal fraction (S-9) used in these experiments was prepared from rats previously treated with PCB. The normal liver microsomal fraction was also prepared from normal rats (not treated with PCB) by the same method as described in the text. As shown in this table, this fraction also showed reducing activities on the mutagenicities. These results may be worth notice from the viewpoint of a regulating system for reducing toxicity in normal living cells.

*Degradation of DNA*

The degradation of calf thymus DNA by trioses and glyoxal derivatives was monitored by the measurement of the decrease in the

fluorescence of the DNA-ethidium bromide complex.

As shown in Fig. 3, the fluorescent intensities of the DNA-ethidium bromide complex decreased depending on the concentration of these mutagens used, and gave evidence of depolymerization of the DNA. The order of DNA degradation activity by these mutagens was: methyl glyoxal > dihydroxyacetone > glyoxal > glyceraldehyde. And this order coincides with the order of their mutagenicities (Table I).

The reaction was found to be inhibited by catalase and SOD (data not shown). These results also demonstrate that the mutagenic ability of these mutagens depends largely on oxygen radicals generated from these mutagens and causing strand scission of DNA.

Kashimura *et al.*[4,8,9] reported that hexopyranoside-3-uloses, reducing sugar phosphates, and aldopentoses produce oxygen radicals that are able to cleave nucleic acids, and, *in vitro*, to inactivate bacteriophages. In our experiments, the trioses and glyoxal derivatives produced oxygen radicals which cleave nucleic acids and consequently bring about mutagenicity.

The mutagenicity of AF-2 as well as trioses or glyoxal derivatives was decreased by S-9. But AF-2 showed no reducing activity on NBT, nor was its mutagenicity affected by radical scavengers. Bender *et al.*[10] and Taylor *et al.*[11] reported that nitrofuran compounds were metabolized with nitroreductase in the microsomal fraction. It is made clear in this report that the mutagenicities of AF-2 and trioses (or glyoxal derivatives) are decreased by S-9. The effective factor on these two, however, seem to be quite different.

Superoxide is known to (a) undergo disproportionation, yielding hydrogen peroxide, and (b) react with hydrogen peroxide, generating a hydroxyl radical and a singlet oxygen.[12] It is therefore suggested that some of these oxygen-derived species related to the damage of DNA.

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