

Human DNA Damage Induced by 1,2,4-Benzenetriol, a Benzene Metabolite¹

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ABSTRACT

Reactivities of benzene metabolites (phenol, catechol, hydroquinone, 1,4-benzoquinone, 1,2,4-benzenetriol) and related polyphenols (resorcinol, pyrogallol, phloroglucinol) with DNA were investigated by a DNA sequencing technique using ³²P 5'-end-labeled DNA fragments obtained from human c-Ha-ras-1 protooncogene, and the reaction mechanism was studied by UV-visible and electron-spin resonance spectroscopies. 1,2,4-Benzenetriol caused strong DNA damage even without alkali treatment. Alkali-labile sites induced by 1,2,4-benzenetriol were base residues of guanine and adjacent thymine. Catalase, superoxide dismutase and methional inhibited the DNA damage completely, but sodium formate did not inhibit it. 1,2,4-Benzenetriol-induced DNA damage was inhibited by the addition of a Cu(II)-specific chelating agent, bathocuproine, and was accelerated by the addition of Cu(II). The addition of Fe(III) did not create any significant effects on 1,2,4-benzenetriol-induced DNA damage. Electron-spin resonance studies using spin traps demonstrated that addition of Fe(III) increased hydroxyl radical production during the autoxidation of 1,2,4-benzenetriol, whereas the addition of Cu(II) did not. The results suggest that DNA damage was caused by an unidentified active species which was produced by the autoxidation of 1,2,4-benzenetriol in the presence of Cu(II), rather than by hydroxyl radicals. The possibility that 1,2,4-benzenetriol-induced DNA damage is one of the primary reactions in carcinogenesis induced by benzene is discussed.

INTRODUCTION

Benzene, widely used in the chemical industry, has been shown to cause serious hematological disorders and carcinogenic effects on humans and animals. Extensive epidemiological evidence has been published on the high incidence of leukemias in men occupationally exposed to benzene (1, 2). The administration of benzene to animals has produced leukemia (3), lymphoma and carcinomas of the Zymbal gland, mammary gland and liver (1, 4). Previous studies have demonstrated that benzene induced sister chromatid exchanges in mouse bone marrow (1, 5, 6). However, benzene has not been shown to be mutagenic in bacterial test systems.

It is well accepted that many carcinogens are readily converted to reactive intermediates by drug metabolizing enzymes. A variety of DNA-carcinogen adducts and oxidized bases have been identified in DNA recovered from reactions with carcinogens (7). However, in the case of benzene, little evidence for direct damage to DNA has been presented with the exception of evidence for covalent binding of labeled benzene with DNA (8, 9). Furthermore, the ultimate carcinogenic forms of benzene and the mechanisms of their reactions with DNA remain to be clarified.

Benzene metabolism is considered to be important for the expression of its toxicity (10). Benzene metabolites include phenol, catechol, hydroquinone and 1,2,4-benzenetriol (10-14). Resorcinol, pyrogallol (1,2,3-benzenetriol) and phloroglucinol (1,3,5-benzenetriol) have not been identified as benzene metab-

by using ³²P 5'-end-labeled DNA fragments of defined sequences obtained from protooncogene (c-Ha-ras-1). 1,2,4-Benzenetriol was shown to cause strong DNA damage, and the participation of active oxygen species was investigated.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Bst*II, *Ava*I, *Xba*I, *Pst*I) and T₄ polynucleotide kinase were purchased from Toyobo Co., Osaka, Japan. Calf intestine phosphatase was obtained from Boehringer Mannheim GmbH. [γ -³²P]ATP (6000 Ci/mmol) was supplied by New England Nuclear. Phenol, catechol, hydroquinone, 1,4-benzoquinone, quiny drone, resorcinol, pyrogallol, phloroglucinol, and sodium formate were purchased from Nakarai Chemicals Co., Kyoto, Japan. DTPA, and bathocuproinedisulfonic acid were from Dojin Chemicals Co., Kumamoto, Japan. Desferrioxamine mesylate was from Ciba-Geigy. Agarose was from Takara Shuzo Co., Kyoto, Japan. Acrylamide, bisacrylamide, and piperidine were from Wako Chemicals Co., Osaka, Japan. Hydrazine was from Eastman Organic Chemicals. SOD (3100 unit/mg from bovine blood), catalase (45,000 unit/mg from bovine liver) and methional were from Sigma Chemical Co. 1,2,4-Benzenetriol, DMPO, and dimethyl sulfate were purchased from Aldrich Chemical Co. A stock solution of 0.1 M polyphenol in acetone was kept at 0°C in the dark and used within 4 h of preparation.

Preparation of ³²P 5'-End-labeled DNA Fragments. DNA fragments were prepared from plasmid pbcNI purchased from American Type Culture Collection, which carries a 6.6 kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-ras-1 protooncogene (15, 16). The plasmid was digested with *Bst*II and *Ava*I, and the resulting DNA fragments were fractionated by electrophoresis on 1 or 2% agarose gels. A ³²P 5'-end-labeled 351 base pairs *Ava*I fragment (*Ava*I* 1294-*Ava*I* 1644) from 1.5 kilobase *Bst*II fragment of pbcNI was obtained by dephosphorylating with calf intestine phosphatase and rephosphorylating with [γ -³²P]ATP and T₄ polynucleotide kinase. The singly labeled 261 base pair fragment (*Ava*I* 1645-*Xba*I 1905) and 341 base pair fragment (*Xba*I 1906-*Ava*I* 2246) were obtained by digesting the ³²P 5'-end-labeled 602 base pair *Ava*I fragment (*Ava*I* 1645-*Ava*I* 2246) from 1.5 kilobase *Bst*II fragment with *Xba*I. The singly labeled 98 base pair fragment (*Ava*I* 2247-*Pst*I 2344) was obtained by digesting the ³²P 5'-end-labeled 435 base pair *Ava*I fragment (*Ava*I* 2247-*Ava*I* 2681) with *Pst*I. The asterisk indicates ³²P-labeling and nucleotide numbering starts with the *Bam*HI site (16).

Detection of DNA Damage Induced by Benzene Metabolites. The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained 2.5 mM benzene and its metabolites and [³²P]DNA fragment (-5000 cpm) in 200 μ l of 20 mM sodium phosphate buffer (pH 7.9) or Tris-HCl buffer (pH 8.0). After incubation for 30 min or 90 min at 37°C, the DNA fragments were precipitated with cold ethanol and dried in a vacuum desiccator, followed by heating at 70°C for 25 min in 1 M piperidine when necessary. The DNA fragments were electrophoresed and the autoradiograms were obtained as previously described (17). The preferred cleavage sites by 1,2,4-benzenetriol were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (18). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides produced by the incubation of DNA fragment with 1,2,4-benzenetriol.

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³The abbreviations used are: DTPA, diethylenetriamine-N,N,N',N',N''-pentaacetic acid; SOD, superoxide dismutase; DMPO, 5,5-dimethylpyrrolidine-N-oxide; DMPO-OH, hydroxyl radical adduct of 5,5-dimethylpyrrolidine-N-oxide; ESR, electron-spin resonance.

UV-visible Spectra Measurements. UV-visible spectra were measured at 37°C with a UV-Vis-NIR recording spectrophotometer (Shimadzu UV-365).

ESR Spectra Measurements. ESR spectra were measured at room temperature using a JES-FE-3XG spectrometer with 100-KHz field modulation according to the method previously described (17, 19). Spectra were recorded with a microwave power of 4 mW and a modulation amplitude of 1.0 G. The magnetic fields were calculated by the splitting of Mn^{2+} in MgO ($\Delta H_{1-4} = 86.9$ G). DMPO was used as a spin trapping reagent. The measurements were carried out after the incubation of 1,2,4-benzenetriol in a buffer solution. $CuCl_2$ or $FeCl_3$ solution was added where indicated.

RESULTS

Damage of ^{32}P -labeled DNA Fragments Induced by Benzene Metabolites and Related Polyphenols. The extent of DNA damage was estimated by gel electrophoretic analysis. Fig. 1 shows an autoradiogram of DNA fragments treated with benzene and its metabolites. The upper band and lower band in the control show double-stranded and single-stranded forms of DNA fragment, respectively. In the case of 1,2,4-benzenetriol, oligonucleotides were clearly detected on the autoradiogram as a result of DNA cleavage even without alkali treatment (Fig. 1, Lane 5), suggesting breakages in the deoxyribose-phosphate backbone by active species. The amount of oligonucleotides increased with alkali treatment, suggesting that the base alterations were induced by 1,2,4-benzenetriol. The DNA cleavage by 1,2,4-benzenetriol increased with time (data not shown). DNA cleavage induced by hydroquinone was much less than that induced by 1,2,4-benzenetriol (Fig. 1, Lane 2), and no cleavage was observed with benzene, phenol, catechol or resorcinol even with alkali treatment.

Little or no cleavage was observed with pyrogallol or phloroglucinol even with alkali treatment (data not shown). The relative reactivities with DNA were as follows: 1,2,4-benzene-

triol $\gg$ hydroquinone $>$ phloroglucinol, pyrogallol, catechol, resorcinol, phenol and benzene. Benzoquinone and quinhydrone (an addition compound of one mol hydroquinone and one mol 1,4-benzoquinone) caused little or no damage to DNA (data not shown).

Effects of Scavengers, Chelating Agents, and Metal Ions on 1,2,4-Benzenetriol-induced DNA Damage. Fig. 2 shows the effects of SOD, catalase, and hydroxyl radical scavengers on 1,2,4-benzenetriol-induced DNA damage. SOD and catalase almost completely inhibited DNA damage (Fig. 2, Lanes 3 and 4), suggesting the involvement of superoxide and hydrogen peroxide. The addition of hydrogen peroxide enhanced DNA damage to a great extent (data not shown). Methional significantly inhibited DNA damage (Fig. 2, Lane 6), whereas sodium formate did not inhibit it (Fig. 2, Lane 5).

Since buffers and reagents are known to be invariably contaminated with trace amounts of metal ions (20), and copper ion is reported to accelerate drug-induced DNA damage (21), the effects of chelating agents and metal ions on 1,2,4-benzenetriol-induced DNA damage was examined. 1,2,4-Benzenetriol-induced DNA damage was inhibited by the addition of a Cu(I)-specific chelating agent, bathocuproine (Fig. 3, Lane 3), and was accelerated by the addition of Cu(II) ion (Fig. 3, Lane 6). On the other hand, a Fe(III)-specific chelating agent, desferrioxamine did not inhibit DNA damage significantly (Fig. 3, Lane 4), and Fe(III) did not accelerate it (Fig. 3, Lane 7). DTPA inhibited DNA damage (Fig. 3, Lane 5), suggesting that DTPA functions as a copper-chelating agent.

Alkali-labile Sites of DNA Fragments Treated with 1,2,4-Benzenetriol. To estimate the site specificity of DNA cleavage by 1,2,4-benzenetriol, ^{32}P 5'-end-labeled DNA fragments treated with 1,2,4-benzenetriol were electrophoresed and the autoradiogram was scanned with a laser densitometer (Fig. 4). The cleavage sites induced by 1,2,4-benzenetriol were determined by utilizing the Maxam-Gilbert procedure (17). The result showed that cleavages at the positions of guanine and adjacent thymine were more frequent than those of other bases. The cleavages occurred broadly at or near the 12th codon of c-Ha-rus-1 protooncogene under the present conditions (Fig. 4).



Fig. 1. Autoradiogram of ^{32}P -labeled DNA fragments incubated with benzene metabolites. Reaction mixture contained ^{32}P -labeled 351 base pair DNA fragment (*Aval** 1294-*Aval** 1644) and 5 mM benzene or its metabolites in 200 μ l of 20 mM Tris-HCl buffer at pH 8.0. Lane 1, control; Lane 2, hydroquinone; Lane 3, catechol; Lane 4, resorcinol; Lane 5, 1,2,4-benzenetriol; Lane 6, benzene; Lane 7, phenol. After incubation for 90 min at 37°C, cold ethanol was added. The precipitated DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing X-ray film to the gel.



Fig. 2. Effect of SOD, catalase and hydroxyl radical scavengers on DNA damage induced by 1,2,4-benzenetriol. The ^{32}P 5'-end-labeled 341 base pair fragment (*Xba*I 1906-*Ava*I* 2246) in 200 μ l of 20 mM phosphate buffer at pH 7.9 was incubated with 2.5 mM 1,2,4-benzenetriol (Lane 2). 1,2,4-benzenetriol plus 30 units of SOD (Lane 3), 1,2,4-benzenetriol plus 30 units of catalase (Lane 4), 1,2,4-benzenetriol plus 0.1 M sodium formate (Lane 5), and 1,2,4-benzenetriol plus 0.1 M methional (Lane 6) for 30 min at 37°C. Lane 1 shows the electrophoresis pattern of untreated ^{32}P -DNA fragment. The treated DNA fragments were analyzed by the method described in the legend to Fig. 1.

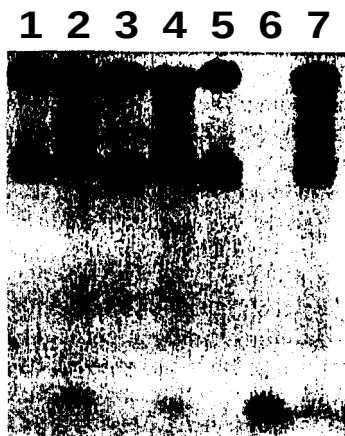


Fig. 3. Effect of chelating agents and metal ions on DNA damage induced by 1,2,4-benzenetriol. The ^{32}P 5'-end-labeled 261 base pair fragment (*Ava*I* 1645-*Xba*I 1905) in 200 μl of 20 mM phosphate buffer at pH 7.9 was incubated with 2.5 mM 1,2,4-benzenetriol (Lane 2), 1,2,4-benzenetriol plus 5 μM bathocuproine (Lane 3), 1,2,4-benzenetriol plus 5 μM desferrioxamine (Lane 4), 1,2,4-benzenetriol plus 5 μM DTPA (Lane 5), 1,2,4-benzenetriol plus 10 μM CuCl_2 (Lane 6), and 1,2,4-benzenetriol plus 10 μM FeCl_3 (Lane 7) for 30 min at 37°C. Lane 1, electrophoresis pattern of untreated [^{32}P]DNA fragment. The treated DNA fragments were analyzed by the method described in Fig. 1. legend.

UV-Visible Spectroscopic Studies on the Autoxidation of 1,2,4-Benzenetriol. Fig. 5 shows changes in the UV-visible spectra of 1,2,4-benzenetriol with time. When a stock solution of 1,2,4-benzenetriol was added to a buffer solution, the solution turned immediately red with an absorption maxima at 482 nm and 267 nm. These absorption maxima can be attributed to 2-hydroxybenzoquinone (22). The conversion from 1,2,4-benzenetriol to 2-hydroxybenzoquinone was inhibited to over 95% by SOD, but was not affected by the addition of Fe(III) or Cu(II) (data not shown). The intensity of absorption at 482 nm began to decrease within about several seconds. Autoxidation of pyrogallol and hydroquinone was slower and simpler than that of 1,2,4-benzenetriol. Under the same conditions, phenol, resorcinol, catechol, and phloroglucinol showed little spontaneous autoxidation.

Production of Free Radicals during the Autoxidation of 1,2,4-Benzenetriol. Spin trapping methods were used to detect free radicals produced during the autoxidation of 1,2,4-benzenetriol. Fig. 6A, shows an ESR spectrum of a spin adduct observed when DMPO was added to a buffer solution of 1,2,4-benzenetriol after incubation at 37°C for 15 min. The 1:2:2:1 pattern of four lines can be interpreted in terms of equivalent hyperfine splitting constants (a_N , a_H) due to both the nitroxide nitrogen atom and the β -hydrogen. The spin adduct ($a_N = a_H = 14.8$ G) can be reasonably assigned to DMPO-OH by reference to the reported constants (17, 19, 23, 24). Catalase completely inhibited the yield of DMPO-OH (Fig. 6B). Fe(III) increased the yield of DMPO-OH (Fig. 6C). On the other hand, when Cu(II) was added, the yield of DMPO-OH did not increase, but a six-line signal with equal intensities ($a_N = 15.7$ G, $a_H = 22.9$ G) could be detected (Fig. 6D). The six-line signal was observed with Cu(II) alone.

DISCUSSION

The present results showed that among benzene metabolites, 1,2,4-benzenetriol caused strong DNA damage, and hydroquinone caused slight DNA damage. Benzene, phenol, catechol, and resorcinol showed no effect. Trace amounts of copper ion were shown to be necessary for the induction of DNA damage by 1,2,4-benzenetriol.

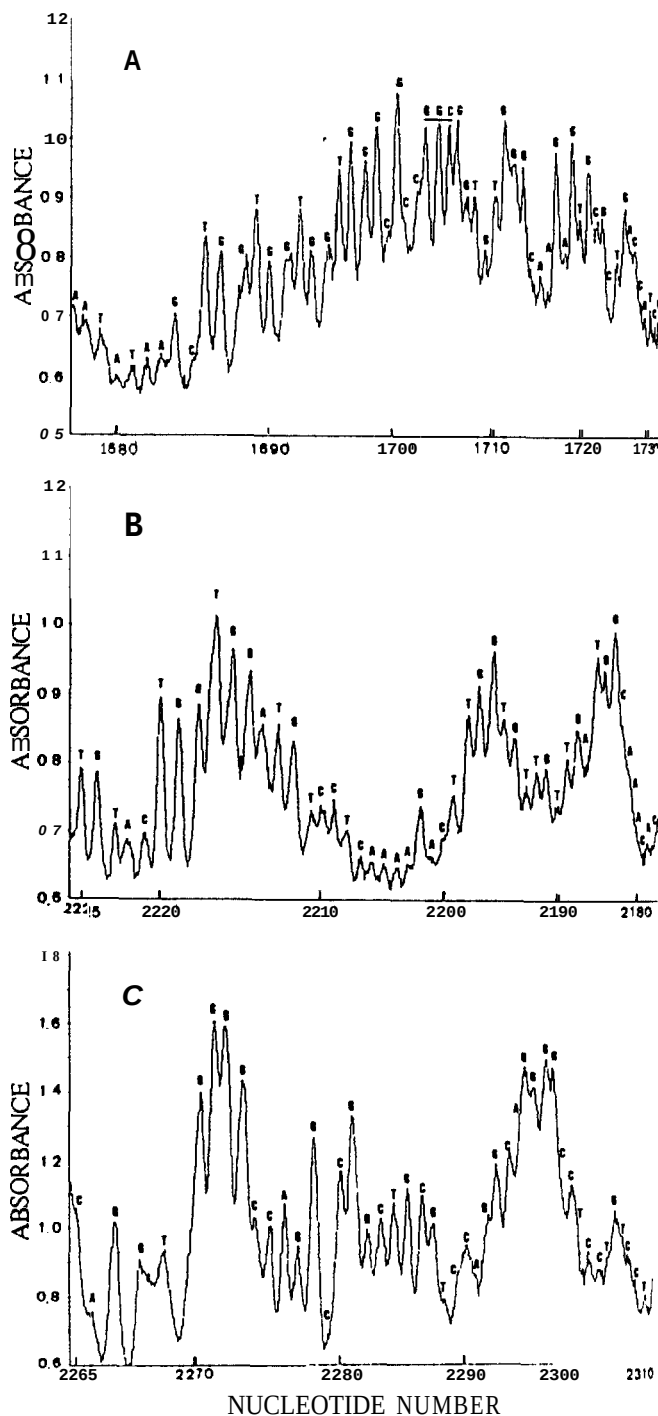


Fig. 4. Alkali-labile sites in ^{32}P -labeled DNA fragments incubated with 1,2,4-benzenetriol. A, ^{32}P 5'-end-labeled 261 base pair fragment (*Ava*I* 1645-*Xba*I 1905) in 200 μl of 20 mM phosphate buffer at pH 7.9 was incubated with 2.5 mM 1,2,4-benzenetriol for 30 min at 37°C. E, ^{32}P 5'-end-labeled 341 base pair fragment (*Xba*I 1906-*Ava*I* 2246) was used. C, ^{32}P 5'-end-labeled 98 base pair fragment (*Ava*I* 2247-*Pst*I 2344) was used. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotides produced by incubation with 1,2,4-benzenetriol were measured by a laser densitometer (LKB 2222 UltraScan XL). The alkali-labile sites of DNA induced by 1,2,4-benzenetriol were determined by direct comparison with the same DNA fragment after undergoing the DNA sequence reaction according to the Maxam-Gilbert procedure (18). Horizontal axis, nucleotide number of human c-Ha-ras-1 protooncogene starting with the *Bam*HI site (15). A, G, T, and C, deoxyadenylate, deoxyguanylate, deoxythymidylate, and deoxycytidylate of DNA, respectively. Underscoring, the 12th codon of human c-Ha-ras-1 protooncogene.

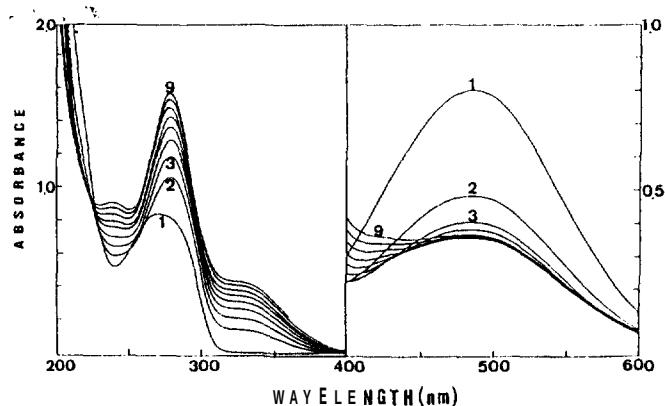


Fig. 5. Changes in UV-visible spectra of a solution of 1,2,4-benzenetriol with time. Visible region (400–600 nm) was recorded using 1-cm quartz cuvetts and UV region (200–400 nm) was done using 1-mm quartz cuvetts at 37°C. To 3.92-ml phosphate buffer (20 mM, pH 7.9), 80 μ l of a stock solution of 0.1 M 1,2,4-benzenetriol in acetone was added. Spectral tracing was recorded every 10 min.

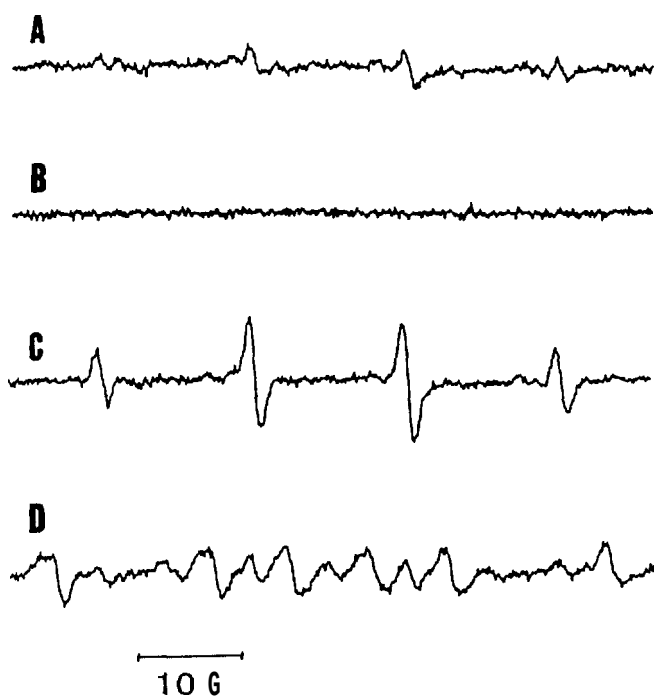


Fig. 6. ESR spectra of the hydroxyl radical spin adduct of DMPO produced during the autoxidation of 1,2,4-benzenetriol. A, sample (100 μ l) contained 2.5 mM 1,2,4-benzenetriol in 20 mM phosphate buffer at pH 7.9; B, catalase (24 units) was added to the sample solution; C, FeCl_3 (10 μ M) was added to the sample solution; D, CuCl_2 (2 mM) was added to the sample solution. After incubation for 15 min at 37°C, 0.3 M DMPO was added, aliquots of the solution were taken up in a calibrated capillary, and ESR spectra were measured at room temperature as described in "Materials and Methods."

UV-visible spectroscopic studies suggest that 1,2,4-benzenetriol is autoxidized rapidly in aqueous solution. The initial increase in the absorption maximum at 482 nm seems to correspond with the autoxidation of 1,2,4-benzenetriol to form 2-hydroxybenzoquinone and hydrogen peroxide through the intermediates of semiquinone and superoxide anion. The inhibitory effect of SOD on autoxidation, observed in this and previous studies (25), suggests that the superoxide anion participates as an initiation and chain propagating species during the first step in the autoxidation of 1,2,4-benzenetriol. Cu(II) and Fe(III) did not affect the initial autoxidation.

In an attempt to interpret the mechanism of DNA damage induced by 1,2,4-benzenetriol, we examined what kind of active species are produced and cause DNA damage. Spin trapping

experiments using DMPO detected the production of hydroxyl radicals after the incubation of 1,2,4-benzenetriol in a buffer solution. Cu(II) did not show any effect on the production of hydroxyl radicals, although the addition of Cu(II) accelerated the DNA damage dramatically. Fe(III) accelerated the production of hydroxyl radicals whereas Fe(II) did not show any accelerating effect on 1,2,4-benzenetriol-induced DNA damage. In addition, the yield of hydroxyl radical seems to be much less than the degree of 1,2,4-benzenetriol-induced DNA damage. Methional completely inhibited 1,2,4-benzenetriol-induced DNA damage, whereas sodium formate, another hydroxyl radical scavenger, did not inhibit it. Although methional is a hydroxyl radical scavenger, it can also scavenge other radicals (26). Therefore, it is considered that the species causing DNA damage is an unidentified active species rather than hydroxyl radical. The inhibitory effect of catalase and the increasing effect of hydrogen peroxide on DNA damage suggest that hydrogen peroxide participates in the production of active species. The inhibitory effect of bathocuproine and the accelerating effect of Cu(II) on DNA damage suggest that an inter-conversion of Cu(II) and Cu(I) has an important role in the production of active species.

The DNA sequencing experiments on DNA fragments treated with 1,2,4-benzenetriol revealed that cleavages at the positions of guanine and thymine were more dominant than those of adenine and cytosine. Cleavages of guanine and thymine residues in the guanine-rich region were especially predominant. Our previous paper suggested that hydroxyl radicals caused cleavages at every nucleotide with slightly stronger cleavages occurring at every guanine and thymine position (17). This difference may support the idea that DNA damage induced by 1,2,4-benzenetriol was caused by unidentified active species rather than hydroxyl radicals. Recently it has been demonstrated that mutations activating human c-Ha-ras-1 protooncogene are induced by chemical carcinogens (27). This preliminary experiment suggests that active species produced during the autoxidation of 1,2,4-benzenetriol broadly attacked nucleotides of the 12th codon and its neighboring regions.

The idea that damage to DNA is a critical event in the initiation of carcinogenesis is generally accepted. Recent observations have suggested that some tumor promoters act to produce DNA damage via free radicals (28, 29). The importance of this process in the carcinogenesis of benzene remains to be clarified. The present study shows that 1,2,4-benzenetriol is autoxidized in the presence of copper ion to produce an active species causing DNA damage and may play an important role in benzene-induced carcinogenesis, although the role of hydroquinone cannot be excluded. After benzene exposure, catechol and hydroquinone were shown to be retained in the bone marrow of rats in higher concentrations and for longer periods than phenol (30). It is possible to speculate that catechol is converted to 1,2,4-benzenetriol, resulting in DNA damage. Recently, Pellack-Walker and Blumer have reported that 1,4-benzoquinone may cause DNA damage in cells more efficiently than 1,2,4-benzenetriol (31). In the present results, 1,4-benzoquinone itself did not cause DNA damage. This discrepancy may be explained by the difference in permeability or stability between 1,2,4-benzenetriol and 1,4-benzoquinone, or by the possible activation of 1,4-benzoquinone in cells (32). Further research is necessary to clarify whether or not 1,2,4-benzenetriol induces DNA damage *in vivo* as a critical event in the initiation and/or promotion of carcinogenesis by benzene.

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