Methylene blue plus light mediates 8-hydroxy 2'-deoxyguanosine formation in DNA preferentially over strand breakage

J.E.Schneider, S.Price, L.Maidt, J.M.C.Gutteridge* and R.A.Floyd
Molecular Toxicology Research Group, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104, USA

Received September 5, 1989; Revised and Accepted December 20, 1989

ABSTRACT

Methylene blue (MB) plus light, in the presence of oxygen, mediates formation of 8-hydroxyguanine in DNA. The yield of 8-hydroxyguanine may be as much as from 2 to 4% of the guanines present. The results presented here show that treatment of supercoiled plasmid DNA with methylene blue plus light causes single-stranded nicks. However, single-stranded nicking occurs approximately 17-fold less frequently than does formation of 8-hydroxyguanine. The nicking rate is reduced in the presence of Mg ion but is not prevented by inhibitors of the iron-catalyzed Fenton reaction or by scavengers of hydroxyl free radicals. Extensive exposure of DNA to light in the presence of MB produces no detectable thiobarbital reactive material thus implicating that single strand nicking does not occur by hydroxyl free radical attack on deoxyribose. Formation of 8-hydroxyguanine is apparently not dependent upon intercalative binding of MB to DNA, since it is formed in polydeoxyguanylic acid.

INTRODUCTION

We have recently shown that methylene blue (MB) plus light, in the presence of oxygen, forms 8-hydroxyguanine (8-OHGua) in DNA with a yield of approximately 2% of the guanines (1). The 8-OHGua was measured as 8-hydroxy-2-deoxy guanosine (8-OHdG), after enzymatic digestion of the treated DNA, and HPLC electrochemical detection analysis of the mononucleosides (2). DNA damage by MB plus light is very specific for guanine and in combination with subsequent strong alkali treatment to cause strand scission has been used as a guanine specific reagent in Maxam-Gilbert DNA sequencing (3). Since 8-OHdG is formed at high levels by MB plus light and since only guanines are rendered alkaline labile, it seemed possible that 8-OHdG is a unique product of the action of MB plus light on DNA. However, MB plus light causes a small amount of DNA strand nicking in the absence of alkaline treatment ('frank nicks') and this suggests either a lack of specificity in the type of damage occurring at the guanine site or alternatively, that strand nicking may be reflective of the possibility that the 8-OHdG adduct itself perhaps undergoes degradative events. It is also possible that particular sites in supercoiled DNA are incompatible with 8-OHdG or that adjacent 8-OHdGs are incompatible or less stable thus leading to strand breaks. The results presented here shows that strand scissions are relatively rare compared to the level of 8-OHdG formed by MB plus light and that hydroxyl free radicals (·OH) are an unlikely cause of the DNA nicking observed under the conditions used. In addition, the results show that formation of 8-OHdG is not dependent upon the intercalative binding of MB to DNA, and that Mg ions reduce the rate of nicking. MB plus light treatment allows 8-OHdG to be formed in DNA with very high specificity such that use of this treatment makes it possible to study the genotoxic effects of this adduct.

METHODS AND MATERIALS

Methylene blue plus light treatment consisted of exposing 0.1 to 0.2 ml of a solution containing DNA (200 μg/ml) and MB in a 96-well microtiter plate to white light irradiation from a 100 watt tungsram 7 (Sylvania) bulb. The bulb was positioned in a reflective metallic hood, at a distance of 18 cm above the sample. The light passed through a 2 mm thick water layer in a glass or plastic plate above the sample to filter out the infrared radiation. Buffers used are described in the appropriate legends, as are variations in the lighting conditions.

Polydeoxyguanylic acid (polydG) was obtained from Pharmacia. Supercoiled plasmid DNA was prepared from the bluescript plasmid (pBS) (Stratagene) or the pNO1523 plasmid by the (Pharmacia) alkaline SDS method as described in Maniatis (4), except that the supercoiled fraction was banded twice by CsCl density gradient centrifugation. DNA was stored in TE (10 mM Tris, pH 8.0, 1mM EDTA) and precipitated and resuspended in buffer appropriate to the experiment, as indicated.

Quantitation of the amount of 8-OHdG in DNA was determined as described previously (2). This involved digestion of the DNA to the nucleoside level followed by HPLC with optical and electrochemical detection (2).

* J.M.C.G. was a Greenberg Scholar from July through Dec 1988, permanent address National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts ENG 3QG, UK

Nicking Analysis

Following MB plus light treatment, plasmid DNA samples were mixed with an equal volume of 2× gel loading buffer (2× LB) composed of 30% glycerol, 0.2 mM DETAPAC (diethylenetriamine penta-acetic acid), and 20 μl were then submitted to agarose gel electrophoresis. The gel utilized was 1.0% agarose with the TAE (0.04 M Tris-acetate, pH 8.0, 1 mM EDTA) buffer system described by Maniatis (4). Electrophoresis was performed for 16 hours at 20 volts. Following electrophoresis, gels were stained with ethidium bromide irradiated from below with a U.V. transilluminator box and photographed with Polaroid Type 55 black and white film. The density of the supercoiled (form I) DNA was estimated on the positive print of the photograph with the JAVA video analysis system of Jandel, and expressed as the percent of form I remaining of the form I DNA of untreated DNA in an adjacent lane.

Thiobarbital (TBA) reactivity of DNA and Deoxyribose

Deoxyribose and DNA samples damaged by either a hydroxyl free radical generating system (described below) or a MB plus light reaction were tested for TBA reactivity according to the method of Gutteridge (5). Specifically, 0.2 ml of 25% HCl and 0.2 ml of 1% thiobarbituric acid (TBA) in 50 mM NaOH were added to 2 ml of treated DNA (50 μg/ml), the mixture was then heated at 100°C for 10 minutes and the optical density determined at 532 nm.

Radical generating system

The bleomycin reaction with DNA and the iron reaction with deoxyribose were performed as modified by Gutteridge *et al.* (5,6). The reaction mixture (2 ml) contained 50 μg/ml DNA, 2.5 mM $MgCl_2$, 1 unit of bleomycin, 50 μM sodium phosphate, pH 7.4, 75 μM $FeCl_3$, and 0.2 mM sodium ascorbate (added last to start the reaction). The reaction mixture was incubated at 37°C for 60 minutes and then solutions were added directly to the reaction mixture to test for TBA reactivity of the DNA as described above. Usually, salmon testes DNA (Sigma Chemical Co.) was used, but when plasmid DNA was used, the reaction volumes were scaled down to 0.5 ml per reaction. Deoxyribose was incubated with a ferrous salt or a ferric salt plus reductant (5,13).

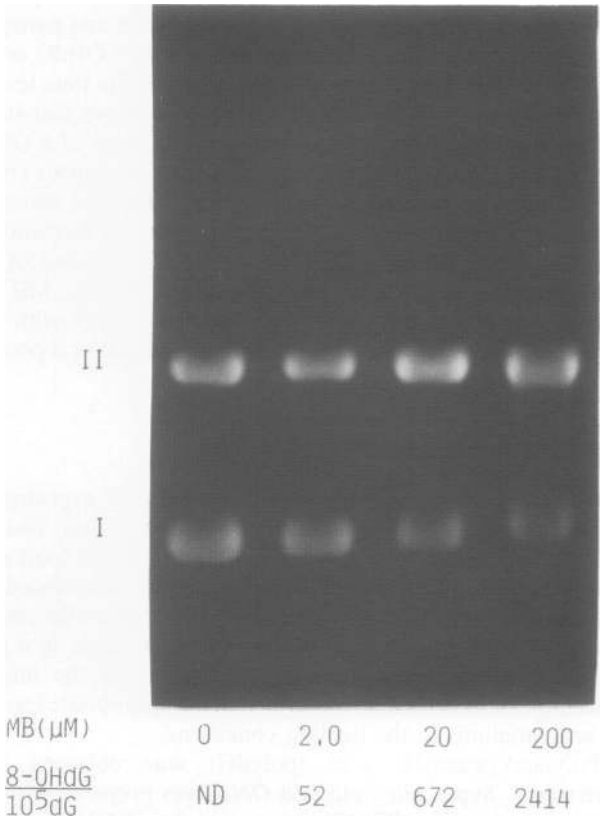

Figure 1. Methylene blue plus light mediated nicking of plasmid DNA and formation of 8-OHdG. Bluescript plasmid (pBS), 200 μg/ml in 10 mM Tris, pH 8.0 and 0.1 mM desferrioxamine was added in 0.2 ml aliquots to microtiter plate wells and either H_2O or MB was added as a 10× solution to give the indicated final concentrations. The microtiter plate was placed in a 37°C water-bath and irradiated and assayed for 8-OHdG as described in the legend for Table 1. In addition, 20 μl of the treated plasmid was removed just prior to ethanol precipitation, mixed with 2× LB, and 20 μl applied to an agarose gel for nicking analysis (described in Methods and Materials). ND denotes a non detectable level of 8-OHdG under the conditions used for the assay as described (11), which in this instance was 16.5 8-OHdG per 10^5 dG. The designations I and II denote supercoiled and open circular (nicked) forms, respectively, of the plasmid DNA molecule. The values for the 8-OHdG levels in the figure are for one representative experiment and are accurate within 30 percent.

RESULTS

A correlation of the amount of nicking with the amount of 8-OHdG formed is shown for plasmid DNA treated with MB plus light in Figures 1 and 2. Nicking of supercoiled DNA, noted as a loss of form I DNA, increased as the concentration of MB used increased. The amount of 8-OHdG formed also increased as MB concentration increased. Visual inspection of Figure 1 shows that whereas 0 and 2 μM MB yields little, if any, nicking, it clearly occurs when 20 and 200 μM MB was used as manifested by both the decreased density of the form I DNA and the increased density of form II DNA. The amounts of 8-OHdG formed shown in Figure 1 are typical of many experiments,

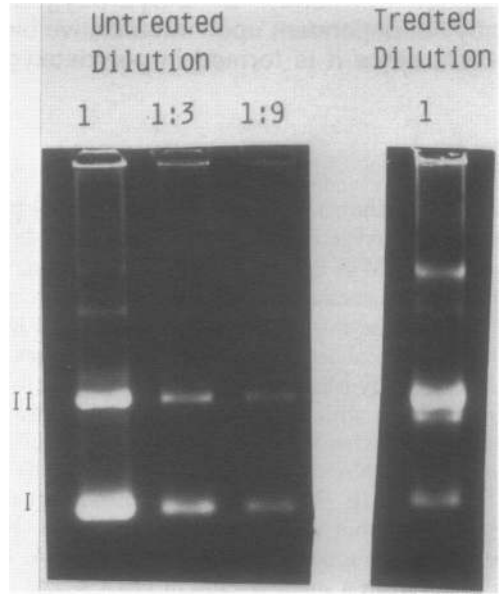

Figure 2. Nicking of supercoiled DNA by methylene blue plus light. The experiment in panel B was treated with the same conditions of MB plus light, as for the experiment described in the legend to Figure 1, followed by gel electrophoresis of 3 dilutions of the undamaged sample to facilitate densitometric analysis of the supercoiled (form 1) DNA band.

although the levels may vary somewhat from experiment to experiment. We have found that 200 μM MB consistently caused formation of 8-OHdG in an amount equivalent to 2 to 4% of the total guanine present. An estimate of the amount of single strand nicking that occurs as a result of MB plus light exposure is shown for one typical experiment in Figure 2. Thus, the amount of nicking under the same conditions that yielded formation of 8-OHdG equal to 2.4% of the dGs in the supercoiled DNA (Fig. 1) caused a loss of 84% of form I (supercoiled) DNA (Fig. 2). Since this plasmid is 2.74 kb long, it is composed of 5.48×10^3 phosphodiester bonds. If 25% of the bases are guanines (G+C=50.3 percent), then a yield of 2.4% 8-OHdG is an average of approximately 31 8-OHdG molecules per plasmid. The average number of nicks per molecule (x) is 1.83, based upon the relationship $x = \ln f$, where f is the surviving fraction (0.16) of supercoiled plasmid molecules (7,8). This relationship assumes a Poisson distribution of target sites on the plasmid (see Discussion). Thus, the ratio of the average number of 8-OHdGs per plasmid molecule to the average number of nicks per molecule is 16.9 for the typical results depicted in Figures 1 and 2. An important assumption is that the 8-OHdG adduct is evenly distributed between supercoiled and relaxed plasmid molecules. This assumption is presently being tested experimentally.

Nicking experiments shown in Figure 3 were performed in sodium phosphate buffer at pH 7.0. These experiments were conducted in phosphate because Tris buffer scavenges hydroxyl free radicals. The data clearly show that hydroxyl free radicals are very *unlikely* mediators of the nicking of supercoiled DNA in the MB plus light treatment. The DNA used for starting material was significantly nicked due to long-term storage conditions and thus MB plus light treatment eliminated all of the remaining supercoiled (form I) DNA present. Inhibitors of nicking would thus allow form I DNA to survive. Thiourea, which scavenges hydroxyl free radicals (·OH) with a very high rate constant (9,10) does not prevent nicking, nor does mannitol, a well-known ·OH scavenger which also binds iron. Also, glycerol, which we have shown to inhibit DNA nicking in an ascorbate/iron ·OH generating system (11), does not prevent supercoil DNA nicking by MB plus light. Urea was without effect and added as a control for nonspecific ·OH scavenging effects (12). Desferrioxamine (Desferal), an effective iron chelator, catalase, and the combination of the two, failed to protect supercoiled DNA from MB plus light mediated nicking. These results all argue against an iron/H_2O_2-dependent Fenton-type mechanism playing an important role in the nicking process. A direct comparison of the influence of the phosphate and Tris buffer systems on nicking by MB plus light has not been made.

Figure 4 shows the effect of Mg ion on nicking by MB plus light. The nicking is shown to be progressive with time. After 30 min substantial loss of form I (supercoiled) DNA was observed in both the absence and presence of 10 mM $MgCl_2$. The presence of 10 mM $MgCl_2$ has a detectable inhibition effect on the nicking as manifested by a thicker form I band after 30 min than in the paired sample lacking $MgCl_2$.

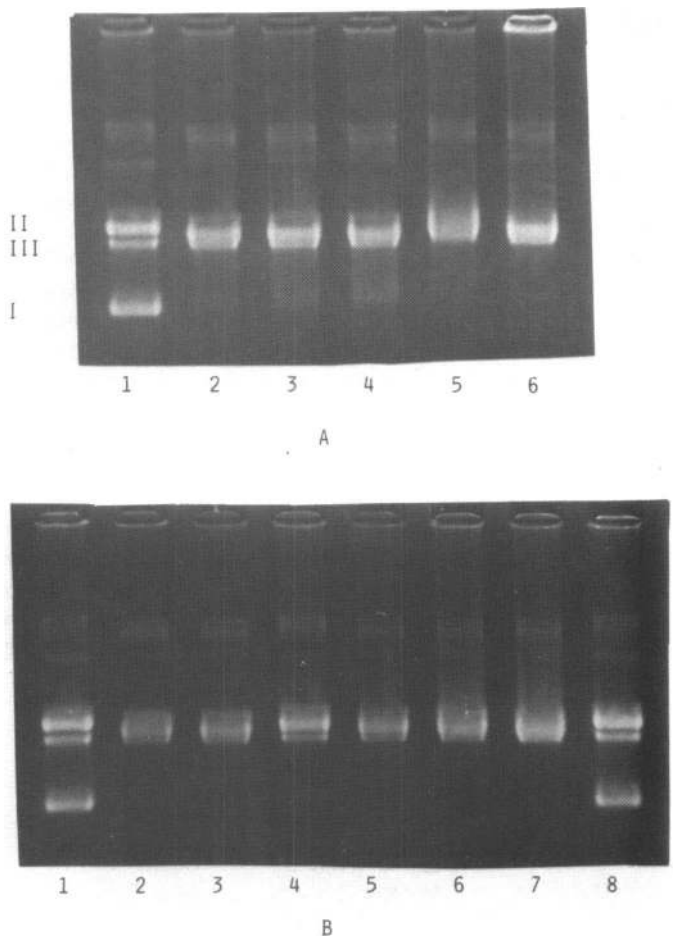

Figure 3. DNA nicking by methylene blue plus light resistance to inhibitors and scavengers of hydroxyl free radicals. DNA (200 μg/ml) in 0.01 M sodium phosphate buffer was irradiated as described in the legend to Table 1 in the presence of 20 μM MB and additions as follows: Panel A, lane 1) no MB, 2) positive control, no addition, 3) 0.1 M mannitol, 4) 10% glycerol, 5) 0.4 mg/ml catalase, 6) 0.4 mg/ml boiled catalase. Panel B conditions were the same as for panel A except that the light was stronger (a No. 1 Superflood BBA 115-120 U Sylvania light bulb). Additions were as follows: 1) and 8) duplicate controls with no MB, 2) no addition, 3) 5 μM urea, 4) 5 mM thiourea, 5) 0.1 mM desferal, 6) 0.1 mM desferal, 0.4 mg/ml catalase, 7) 0.4 mg/ml catalase.

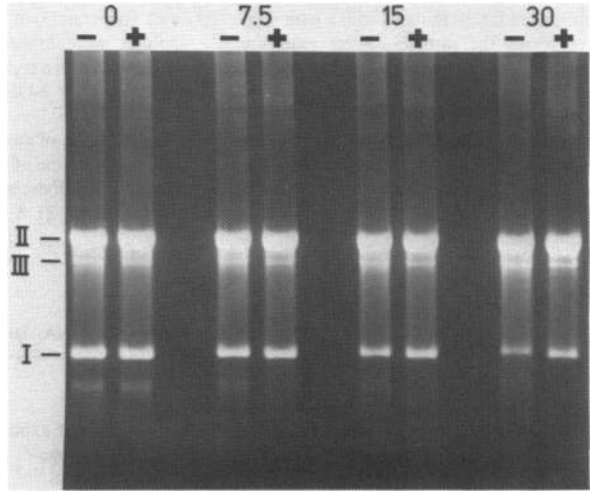

Figure 4. The effect of magnesium on methylene blue plus light mediated nicking of supercoiled plasmid DNA. The reaction mixture contained 10 mM Tris, pH 8.0, 0.1 mM desferal (deferoxamine desylate) and 200 μM MB in 200 μl contained in each of two wells of a 96-well microtiter plate floating in a water bath at 13°C. One of the two reaction mixtures contained 10 mM $MgCl_2$ (designated +). The numbers above each pair of lanes refer to the minutes of exposure to white light of the corresponding samples. Supercoiled DNA is labeled I; nicked, circular DNA, II; and linear DNA, III; the loading buffer used for samples containing no MgCl2 (– lanes) was supplemented with 10 mM $MgCl_2$ to provide the same salt environment for electrophoresis. Electrophoresis was carried out for 18 hours at 25 volts.

The data in Table 1 demonstrate that MB plus light causes formation of 8-OHdG in poly dG. These results effectively rule out the necessity for intercalation of MB into double stranded DNA for mediation of 8-OHdG formation in DNA. Thus, single stranded regions in supercoiled DNA may contribute to nicking associated with 8-OHdG formation.

Gutteridge *et.al.*(10,13) have shown that DNA and deoxyribose when damaged by a hydroxyl free radical system yields products which react with thiobarbituric acid (TBA). Data shown in Table 2 demonstrate that plasmid DNA treated with MB and light for 3 hours did not yield TBA reactive components. Similar results were obtained with the carbohydrate deoxyribose (data not shown). The degree of nicking determined by gel analysis (not shown) was not extensive since the open circular (nicked) form of the DNA was abundant, as was linear form (form III), although form I DNA was eliminated by the 3 hour exposure. The positive control reaction showed that plasmid or salmon testes DNA gave as expected a positive TBA reaction when exposed to the bleomycin/iron/ascorbate system (14).

Thus far, the basis for supercoiled strand nicking during MB plus light treatment is unclear. The above experiments appear to rule out ·OH radicals as the primary cause of the single strand nicking associated with MB plus light treatment.

Table 1. Formation of 8-OHdG in polydeoxyguanylic acid by MB plus light

MB (μM)	8-OHdG* 10^5dG
0	nondetectable
20	258 ± 69
200	947 ± 204

The conditions for exposure of polydG to MB plus light were as such: a volume of 0.1 ml of 200 μg/ml polydG in 10 mM Tris, pH 8.0 and 0.1 mM Desferal (desferrioxamine) was placed in each of 2 wells of a 96 well microtiter plate. Either H_2O (control) or 2 mM methylene blue was added to give a final concentration of 200 μM. The two samples were placed in a 13°C water bath and irradiated for 30 minutes with a white light (100 watt Tungsram 7) in a hood 18 cm above the samples. After exposure, the samples were immediately precipitated with ethanol and 0.3 M sodium acetate, pH 5.2 to remove methylene blue. The DNA was then digested to nucleosides and assayed for 8-OHdG as previously described (18).

* Both dG and 8-OHdG were measured on the same sample by use of an optical detector (254 nm) and an electrochemical detector respectively in line of HPLC eluant (20). The values given are the average of four determinations, and the limit of detectability for the conditions used in this assay were 21 8-OHdG molecules per 10^5 dG.

DISCUSSION

We have shown that MB plus light forms large amounts of 8-OHdG in DNA concurrently where relatively few nicks are formed. Previous work established that MB plus light treatment damages guanines exclusively (3). Thus, we propose that treatment of DNA with MB plus light is a method by which the 8-OHdG adduct may be introduced very specifically into DNA. The relationship of the nicks to the 8-OHdG adduct formed during MB plus light exposure is presently under investigation. It is conceivable that the nicks result from a minor side reaction, such as the production of oxygen free radicals. However since hydroxyl free radical scavengers did not cause changes in the amount of nicks found, it appears that the involvement of hydroxyl free radicals can be ruled out. The assumption that all guanines are equally vulnerable to form 8-OHdG and/or nicks on plasmid molecules, regardless of conformational status (form I or II) of the DNA, is the basis for the 16.9 8-OHdGs per nick value during MB plus light exposure. Since less than 10 percent of the 8-OHdGs are involved in a strand nick, it is conceivable that the hydroxyl moiety is incompatible with the DNA strand structure in specific regions and/or under specific conditions. For instance, adjacent 8-OHdGs or certain structural constraints due to the supercoiled structure may be involved in strand scission. In addition, since 8-OHdG may be relatively unstable, it is conceivable that nicking, in part, reflects further degradation of the adduct, resulting in strand scission. We have shown (unpublished data) that 33% 8-OHdG is lost after storage for 1 week at −20°C and pH 11, but very little loss occurs at pH 4.0, 7.0 or 8.0. Previous work has shown that guanines damaged by MB plus light are alkali labile, and that the frank nicks (independent of alkali treatment) occur at guanine sites (15). Thus, a degradative pathway may result from MB plus light exposure where 8-OHdG is an initial intermediate yielding ultimately a strand break. Thus, we propose that DNA nicking associated with MB plus light exposure results from the formation of 8-OHdG. This hypothesis continues to be tested by searching for perturbations of the MB plus light induced 8-OHdG formation that might alter the ratio of 8-OHdG adducts to nicks.

Damage to DNA by oxygen free radicals, such as hydroxyl free radicals, is known to yield single strand breaks as a major product (16). Single strand breakage has been reported by some (17) to be caused by singlet oxygen but others question the role of singlet oxygen in the process (18,19). Single strand nicks in DNA are regarded as fairly innocuous, since they are quite readily

TABLE 2. Thiobarbital reactivity of DNA damaged by ·OH radical or by MB plus light

Damage conditions	DNA	Time of exposure to damage (minutes)	TBA reactivity	
			O.D.$_{532}$	Fractional increase versus zero time
Bleomycin/Fe/ ascorbate	ST	0	0.055	–
"	ST	20	0.619	11.25
"	ST	60	0.455	11.91
"	ST	0	0.013	
"	P	20	0.089	6.85
"	P	60	0.079	6.08
MB + light	P	180	not detectable	0

Conditions for the bleomycin reaction are described in Methods and Materials and the MB + light conditions are described in the legend for Table 1. DNA is derived from salmon testes (ST) or plasmid (P) preparations.

repaired by the cell (20). However, if the DNA strand serving as the template for repair contains modified bases, the repair process is apt to mis-repair the strand break by incorporation of non-complementary nucleotides opposite the modified base. Thus, DNA damage with a high ratio of DNA adducts to single strand breaks might be expected to lead to more mis-repair and mutational events, than would events with a low ratio of adducts to strand breaks. We have shown here that oxidative damage to DNA by methylene blue plus light leads to a high ratio of 8-OHdG to strand nicks. This implies that potentially this adduct may be highly mutagenic in the cell if it is not efficiently repaired. In fact, the 8-OHdG adduct has been shown to occur in cells both under conditions that result in tumor formation, and in genomic and mitochondrial DNA during normal metabolism (21). Also, the presence of 8-OHdG in synthetic DNA has been reported to direct the incorporation of non-complementary base pairs by DNA polymerase of *Escherichia coli* (22). The data reported here reveal a method by which this important DNA adduct may be specifically introduced into DNA for use in future studies on the structural and functional effects of 8-OHdG in DNA.

ACKNOWLEDGMENTS

This work was supported in part by monies from NIH Grant No. CA42854.

REFERENCES

1. Floyd, R.A., West, M.S., Eneff, J.L. and Schneider, J.E. (1989) Arch. Biochem. Biophys. **273**, 106–111.
2. Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H. and Rickard, R.C. (1986) Free Rad. Res. Comms. **1**, 163–172.
3. Friedmann, T. and Brown, D.M. (1978) Nucleic Acids Res. **5**, 615–622.
4. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York, N.Y.
5. Gutteridge, John M.C. (1981) FEBS Letters **128**, 343–346.
6. Gutteridge, J.M.C., West, M., Eneff, K. and Floyd, R.A. (1989) Manuscript in preparation.
7. Deng, Ruey-Yun and Fridovich, Irwin (1989) Free Rad. Res. Comms. **6**, 123–129.
8. Kuhnlein, Urs, Penhoet, E.E. and Linn, S. (1976) Proc. Nat. Acad. Sci. USA **73**, 1169–1173.
9. Anbar, M. and Neta, P. (1967) Int. J. Appl. Radiat. Isot. **18**, 493–523.
10. Quinlan, G.J. and Gutteridge, J.M.C. (1987) Biochem. Pharmacol. **36**, 3629–3633.
11. Schneider, J.E., Browning, M.M. and Floyd, R.A. (1988) Free Radical Bio. Med. **5**, 287–295.
12. Gutteridge, John M.C. (1987) Biochem. J. **243**, 709–714.
13. Gutteridge, J.M.C. and Toeg, D. (1982) Int. J. Biochem. **14**, 891–893.
14. Gutteridge, J.M.C. and Hou, Y. (1986) Free Rad. Res. Commun. **2**, 143–151.
15. OhUigin, Colm, McConnell, D.J., Kelly, J.M. and van der Putten, W.J.M. (1987) Nucleic Acids Res. **15**, 7411–7427.
16. Floyd, R.A. and Schneider, J.E. (1989) Hydroxyl free radical damage to DNA. Submitted for publication.
17. DiMascio, P. Wefers, Do-Thi, H., Lafleur, M.V.M. and Sies, H. (1989) Biochem. Biophys. Acta. **1007**, 151–157.
18. Nieuwint, A.W.M., Aubry, J.M., Arwert, F., Kortbek, H., Herzberg, S. and Joenje, H. (1985) Free Rad. Res. Comms. **1**, 1–9.
19. Peak, M.J., Peak, J.G., Foote, C.S. and Krinsky, N.I. (1984) J. Photochem. **25**, 309–315.
20. Blok, J. and Loman, H. (1973) Curr. Top. Radiat. Res. Q. **9**, 165–245.
21. Richter, C., Park, J.W. and Ames, B.N. (1988) Proc. Natl. Acad. Sci. USA **85**, 6465–6467.
22. Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E. and Nishimura, S. (1987) Nature **327**, 77–79.