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Hydroxyl Radical Mediated Demethylenation of (Methylenedioxy)phenyl Compounds

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The oxidative demethylenation reactions of (methylendioxy)phenyl compounds (MDPs), (methylenedioxy)benzene (MDB), (methylenedioxy)amphetamine (MDA), and (methylenedioxy)methamphetamine (MDMA), were evaluated by using two hydroxyl radical generating systems, the autoxidation of ascorbate in the presence of iron–EDTA and the iron-catalyzed Haber–Weiss reaction conducted by xanthine/xanthine oxidase with iron–EDTA. Reaction products generated when MDB, MDA, and MDMA were incubated with the ascorbate or xanthine oxidase system were catechol, dihydroxyamphetamine (DHA), and dihydroxymethamphetamine (DHMA), respectively. The reaction required the presence of either ascorbic acid or xanthine oxidase. Levels of each catechol increased in proportion to ferric ion concentration and were suppressed by desferrioxamine B methanesulfonate (desferal). Catalase (CAT) inhibited the oxidation by the ascorbate system whereas superoxide dismutase (SOD) had little effect. The addition of hydrogen peroxide to the reaction mixture stimulated the oxidation, but the reaction was not initiated by hydrogen peroxide alone, suggesting that hydrogen peroxide acts as a precursor of hydroxyl radical. SOD and CAT suppressed the demethylenation reactions in the xanthine oxidase system. Hydroxyl radical scavenging agents such as ethanol, benzoate, DMSO, and thiourea effectively inhibited the oxidation by both systems. Urea, which has little effect on hydroxyl radical, was without any effect. These results indicated that hydroxyl radical can effect the cleavage of methylenedioxy group on MDPs.

Introduction

Although xenobiotics are generally oxidatively metabolized by mixed-function oxidases (*1*), interaction of some drugs with hydroxyl radical has also been studied since it is a common byproduct produced in biological systems and is extremely reactive (*2*). For example, it has been found that the dehydrogenation of ethanol to acetaldehyde (*3*), demethylation of DMSO[1] to produce formaldehyde (*4*), denitrosation of *N*-nitrosodimethylamine (*5*), hydroxylation of aniline to *p*-aminophenol (*6*), and generation of ethylene from methional (*7*) can proceed by hydroxyl radical mediated pathways.

(Methylenedioxy)amphetamine (MDA) and (methylenedioxy)methamphetamine (MDMA) are serotonergic neurotoxins that cause a long-term depletion of 5-hydroxytryptamine (5-HT) content in the brain (*8*, *9*). It has been suggested that the neurotoxic effect is due to metabolites (*10*). Recently, we have found in studies with rat liver microsomes that MDMA is demethylenated to DHMA by cytochrome P-450 and that the catechol metabolite was further oxidized to a quinone or semiquinone which reacts with sulfhydryl functions (*11*). The reaction has been reported for brain homogenates also (*12*), but as the levels of cytochrome P-450 in brain are low, the possibility that demethylenation may occur by another pathway was considered. The purpose of the present study was to determine whether hydroxyl radical could promote chemical cleavage of (methylenedioxy)phenyl compounds (MDPs) using (methylenedioxy)benzene (MDB), MDA, and MDMA as substrates (Figure 1). Two hydroxyl radical generating systems were used, the autoxidation of ascorbate in the presence of iron and EDTA (*4*, *13*) and the iron-catalyzed Haber–Weiss reaction in which superoxide is oxidized to oxygen and H_2O_2 reduced to hydroxyl radical (*12*, *14*).

Experimental Procedures

Chemicals. MDB and formaldehyde were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). As the MDB contained small amounts of catechol, the catechol was removed with 1 N NaOH. MDA and MDMA were obtained from the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). DHA was obtained from Merck Sharp and Dohme Laboratories (West Point, PA). DHMA was synthesized according to published procedures (*11*). Catechol, ferric chloride, EDTA, ascorbic acid, xanthine, xanthine oxidase, superoxide dismutase (SOD), catalase (CAT), ethanol, benzoic acid, dimethyl sulfoxide (DMSO), and urea were obtained from Sigma Chemical Co. (St. Louis, MO). Desferal was obtained from CIBA Laboratories (Horsham, Sussex, U.K.). All other chemicals used were of the highest grade available.

Incubation. The ascorbate system consisted of MDPs (MDB, 2 mM; MDA and MDMA, 1 mM), 10 μM ferric chloride, 20 μM EDTA, and 30 mM potassium phosphate buffer, pH 7.4, in a final volume of 1.0 mL. The reaction was initiated by the addition of ascorbate (final concentration 1 mM). The xanthine oxidase system consisted of the MDPs described above, 0.5 mM xanthine, 10 μM ferric chloride, 20 μM EDTA, and 30 mM potassium phosphate buffer, pH 7.4, in a final volume of 1.0 mL. The reaction was initiated by addition of 0.025 unit of xanthine oxidase. Both reactions were carried out at 37 °C for 10 min and terminated by the addition of 0.5 mL of 7.5% perchloric acid containing 30 mM thiourea (the ascorbic acid system mediated reaction could not be completely stopped by perchloric or trichloroacetic acid). The reaction mixtures were centrifuged at 13500*g* for 5 min, and the supernatants were analyzed by high-performance liquid chromatography–electrochemical detection (HPLC–ECD).

Determination of Metabolites. The catechol products were analyzed by HPLC–ECD using a Biophase ODS (250 × 4.6 mm i.d., 5 μm particle size; Bioanalytical Systems, Inc., West Lafayette,

† Merck postdoctoral fellow (1990–1991).

[1] Abbreviations: MDPs, (methylendioxy)phenyl compounds; MDB, (methylenedioxy)benzene; MDA, (methylenedioxy)amphetamine; MDMA, (methylenedioxy)methamphetamine; DHA, dihydroxyamphetamine; DHMA, dihydroxymethamphetamine; desferal, desferrioxamine B methanesulfonate; SOD, superoxide dismutase; CAT, catalase; DMSO, dimethyl sulfoxide; ECD, electrochemical detection.

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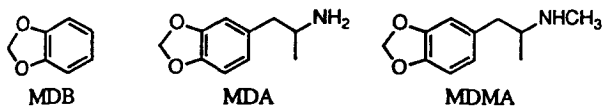

Figure 1. Structures of (methylenedioxy)benzene (MDB), (methylenedioxy)amphetamine (MDA), and (methylenedioxy)-methamphetamine (MDMA).

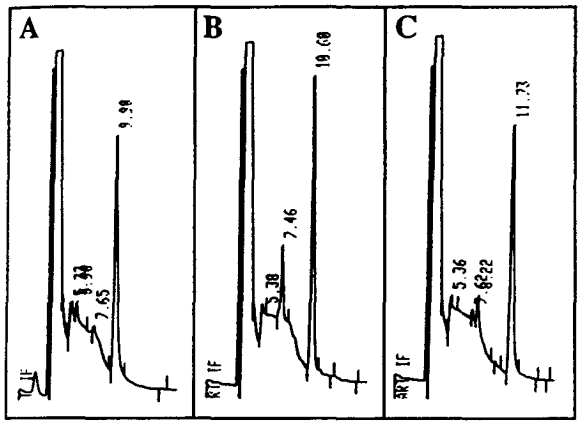

Figure 2. High-performance liquid chromatograms of MDB (A), MDA (B), and MDMA (C) oxidation products obtained from the incubation mixture in the ascorbate system. The chromatographic conditions are described under Experimental Procedures.

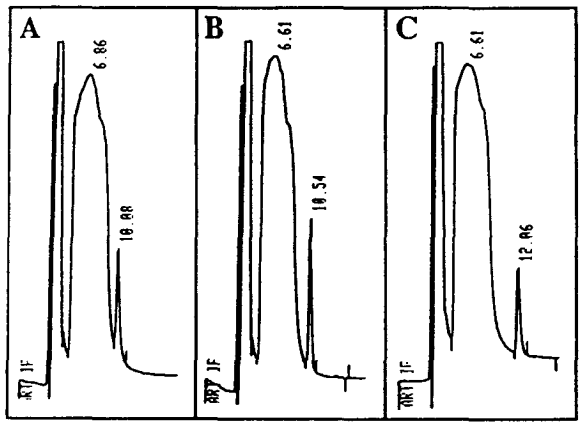

Figure 3. High-performance liquid chromatograms of MDB (A), MDA (B), and MDMA (C) oxidation products obtained from the incubation mixture in the xanthine oxidase system. The chromatographic conditions are described under Experimental Procedures.

IN) column and a glassy carbon working electrode (LC-4, Bioanalytical Systems, Inc.) set at 0.7 V (vs Ag/AgCl reference electrode). The mobile phase consisted of 0.1 M citrate, pH 3.5, containing 1 mM octyl sodium sulfate/acetonitrile/methanol (8:1:1 v/v) at a flow rate of 0.7 mL/min. To confirm catechol production from MDB, 0.1 M citrate buffer, pH 4.0/acetonitrile (4:1 v/v) was also employed as a solvent. The peak height of each metabolite was monitored by a Hewlett Packard 3390A recording integrator. Formaldehyde formation from DMSO was determined by the method of Nash (*15*).

Results

Incubation of MDPs with the two hydroxyl radical generating systems resulting in the formation of electrochemically active products. Figure 2 and 3 show HPLC–ECD product profiles for MDB, MDA, and MDMA oxidation by the ascorbate and xanthine oxidase reaction system. The products (the ascorbate system, 9.9, 10.7, and 11.7 min; the xanthine oxidase system, 10.1, 10.5, and 12.1 min) generated from MDB, MDA, and MDMA were identified as catechol, DHA, and DHMA, respectively, by

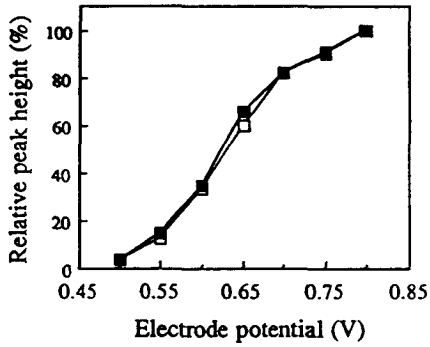

Figure 4. Electrochemical response curves of catechol and a reaction product generated during metabolism of MDB by the ascorbate system: (□) authentic catechol; (■) reaction product.

Table I. Demethylenation of MDB, MDA, and MDMA by Two Hydroxyl Radical Generating Systems[a]

conditions	product formation, nmol/10 min		
	catechol	DHA	DHMA
ascorbate system			
complete	5.78 ± 0.57	9.11 ± 0.29	11.51 ± 0.48
−ascorbate	0	0	0
$-Fe^{3+}$ − EDTA	0.53 ± 0.23	0.65 ± 0.14	0.49 ± 0.11
$-Fe^{3}$ − EDTA + desferal (100 μM)	0	0	0
$+Fe^{3+}$ − desferal[b]	0.34 ± 0.03	0	0
xanthine oxidase system			
complete	4.33 ± 0.04	4.48 ± 0.17	3.21 ± 0.69
−xanthine oxidase	0	0	0
$-Fe^{3+}$ − EDTA	0.06 ± 0.02	0.55 ± 0.06	0.65 ± 0.01
$-Fe^{3+}$ − EDTA + desferal (100 μM)	0	0	0
$+Fe^{3+}$ − desferal[b]	0	0.27 ± 0.02	0.04 ± 0.01

[a] Each MDP concentration was 1 mM, and all incubations were carried out under the conditions described under Experimental Procedures. Each value is the mean ± SD of two to six determinations. [b] Incubation was carried out in the presence of 100 μM desferal instead of EDTA.

coelution with authentic compounds. These reaction products were not detected when the incubation was carried out in the absence of each substrate. The electrochemical responses due to authentic catechol and the product formed from MDB by the ascorbate system were compared as the oxidation potential of the detector was varied over the range 0.5–0.8 V. As shown in Figure 4, the product peak, (expressed as fraction of its height at 0.8 V) varied in exactly the same way as the catechol standard. When the comparison between authentic DHA or DHMA and the appropriate product was carried out, similar results were also obtained (data not shown). The identical chromatographic and electrochemical behavior of the proposed reaction products with authentic standards supports the conclusion that the MDPs are demethylenated to the corresponding catechols.

The role of the components contained in the ascorbate and xanthine oxidase systems on the oxidation were examined, and the results are shown in Table I. No products were formed in the absence of ascorbate or xanthine oxidase. The addition of desferal (100 μM), an iron chelator, markedly inhibited or blocked the oxidation. Although small amounts of catechols were observed in the absence of added ferric ion and EDTA, their formation was blocked by desferal. The formation of the products increased in proportion to the iron added. For example, the addition of 5, 50, and 100 μM ferric chloride to the incubation mixture, containing 100 μM EDTA, enhanced the production of catechol from MDB by 1.65-, 4.05-, and 5.82-fold, respectively, in 1 mM ascorbate. In the xanthine

Scheme I. Proposed Hydroxyl Radical Mediated Demethylenation of MDP to Catechol Metabolite

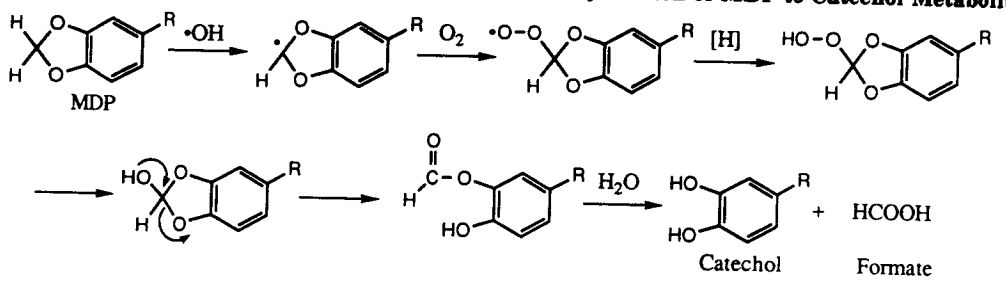

Table II. Effect of SOD, CAT, and Hydrogen Peroxide on MDB, MDA, and MDMA Demethylenation by the Ascorbate System[a]

addition	demethylenation, % of control		
	MDB	MDA	MDMA
none	100	100	100
SOD (10 units)	111 ± 7	107 ± 1	97 ± 2
SOD (50 units)	114 ± 1	91 ± 3	98 ± 1
CAT (20 units)	45 ± 1	46 ± 7	50 ± 1
CAT (100 units)	21 ± 1	15 ± 5	18 ± 1
SOD (10 units) + CAT (20 units)	46 ± 1	53 ± 1	49 ± 2
SOD (50 units) + CAT (100 units)	21 ± 1	15 ± 1	16 ± 2
H_2O_2 (0.5 mM)	520 ± 2	627 ± 18	592 ± 5

[a] MDPs were incubated under the conditions described under Experimental Procedures. Each value is the mean ± SD of two to four determinations.

oxidase system the same concentrations of ferric chloride increased catechol formation by 1.06, 2.18, and 3.23 times, respectively, in 0.5 mM xanthine and 0.025 unit of xanthine oxidase over a control to which iron was not added. Ferric ion could also be replaced with ferrous iron in both systems. Removal of EDTA alone from the ascorbate or xanthine oxidase system caused a slight suppression of the reactions (data not shown). At fixed concentrations of ferric chloride (10 μM) and EDTA (20 μM), the amounts of ascorbate, xanthine, and xanthine oxidase were varied in order to determine optimal conditions for demethylenation reaction. Catechol production depended on ascorbate concentrations. In the xanthine oxidase (0.025 unit) system, the reaction reached a plateau level of 0.5 mM xanthine. When the demethylenation of MDB was compared to demethylation of DMSO, a typical substrate for hydroxyl radical mediated oxidation, at the same concentration (1 mM), catechol production from MDB and formaldehyde production from DMSO were 5.78 and 4.67 nmol/10 min, respectively, in the ascorbic acid system.

Since superoxide and hydrogen peroxide, in addition to hydroxyl radical, are generated from the ascorbate and xanthine oxidase systems (*2*, *16*), the effects of scavengers for these active oxygen species on the cleavage of MDPs were examined. Table II shows the oxidation of MDB, MDA, and MDMA by the ascorbic acid system in the presence of SOD and CAT. CAT inhibited catechol, DHA, and DHMA formation whereas SOD had no effect on the demethylenation reactions. The effect of simultaneous addition of SOD and CAT was similar to that of CAT only. The addition of hydrogen peroxide (0.5 mM) resulted in the stimulation of each oxidation but did not promote demethylenation by itself. The xanthine oxidase mediated oxidation was significantly inhibited by both SOD and CAT (Table III). Exogenous hydrogen peroxide did stimulate the xanthine oxidase driven reaction to some extent (Table III).

Table IV shows the effects of hydroxyl radical scavenging agents on the MDB, MDA, and MDMA oxidation by ascorbate system. Ethanol, benzoic acid, DMSO, and

Table III. Effect of SOD, CAT, and Hydrogen Peroxide on MDB, MDA, and MDMA Demethylenation by the Xanthine Oxidase System[a]

addition	demethylenation, % of control		
	MDB	MDA	MDMA
none	100	100	100
SOD (10 units)	43 ± 2	10 ± 1	11 ± 1
SOD (50 units)	39 ± 1	0	0
CAT (20 units)	38 ± 1	54 ± 2	40 ± 1
CAT (100 units)	12 ± 1	13 ± 2	13 ± 1
SOD (10 units) + CAT (20 units)	43 ± 5	7 ± 4	11 ± 1
SOD (50 units) + CAT (100 units)	34 ± 2	0	0
H_2O_2 (0.5 mM)	117 ± 4	113 ± 12	107 ± 8

[a] MDPs were incubated under the conditions described under Experimental Procedures. Each value is the mean ± SD of two to four determinations.

Table IV. Effect of Hydroxyl Radical Scavenging Agents on MDB, MDA, and MDMA Demethylenation by the Ascorbate System[a]

addition	concn, mM	demethylenation, % of control		
		MDB	MDA	MDMA
none		100	100	100
ethanol	10	74 ± 1	60 ± 1	52 ± 1
	100	21 ± 1	14 ± 1	13 ± 1
benzoate	5	62 ± 3	35 ± 1	42 ± 1
	50	21 ± 1	6 ± 1	9 ± 1
DMSO	5	49 ± 1	37 ± 1	37 ± 2
	50	12 ± 2	6 ± 1	8 ± 1
thiourea	1	73 ± 1	60 ± 1	62 ± 3
	10	25 ± 1	11 ± 1	14 ± 1
urea	1	108 ± 2	103 ± 3	100 ± 4
	10	108 ± 1	106 ± 2	101 ± 5

[a] MDPs were incubated under the conditions described under Experimental Procedures. Each value is the mean ± SD of two to four determinations.

Table V. Effect of Hydroxyl Scavenging Agents on MDB, MDA, and MDMA Demethylenation by the Xanthine Oxidase System[a]

addition	concn, mM	demethylenation, % of control		
		MDB	MDA	MDMA
none		100	100	100
ethanol	10	71 ± 4	47 ± 3	31 ± 1
	100	21 ± 2	14 ± 1	10 ± 1
benzoate	5	58 ± 1	32 ± 2	29 ± 1
	50	22 ± 1	11 ± 1	12 ± 1
DMSO	5	37 ± 3	22 ± 2	21 ± 1
	50	7 ± 1	4 ± 1	7 ± 1
thiourea	1	68 ± 4	45 ± 1	38 ± 4
	10	15 ± 1	3 ± 2	0
urea	1	97 ± 4	90 ± 4	98 ± 1
	10	98 ± 1	90 ± 2	99 ± 5

[a] MDPs were incubated under the conditions described under Experimental Procedures. Each value is the mean ± SD of two to four determinations.

thiourea inhibited all reactions in a concentration-dependent manner. Thiourea dramatically suppressed de-

methylenation of MDPs at 10 mM. But comparable concentrations of urea, which reacts poorly with hydroxyl radical (*4*, *17*), had little effect on the demethylenation. Similar results were obtained from experiments with xanthine oxidase system (Table V).

Discussion

The results presented here indicate that attack by hydroxyl radical, generated by the two typical generating systems, upon MDB, MDA, and MDMA produces the corresponding demethylenated products, catechol, DHA, and DHMA, respectively.

Previously, Marshall and Wilkinson (*18*) had speculated that inhibition of Fenton's reagent mediated epoxidation of aldrin by insecticide synergists such as MDPs appeared to be due to the interaction of the MDPs with hydroxyl radical, and the present data confirm the hypothesis.

Product formation was dependent on substrate concentration and required addition of ascorbate or xanthine oxidase. When ferric ion–EDTA was not added to the incubation mixture of ascorbate or xanthine and xanthine oxidase, limited demethylenation did occur, probably due to the presence of iron in the phosphate buffer used (*19*). The addition of desferal, which inactivates ferric ion (*20*, *21*), to the reaction mixtures caused complete or marked inhibition of product formation (Table I). All reactions were accelerated in proportion to the amount of ferric ion added. These results demonstrated the requirement of iron salts for the active oxygen mediated demethylenation.

We found that CAT suppressed both the ascorbic acid and xanthine oxidase mediated product formation. Although hydrogen peroxide stimulated demethylenation, it did not initiate the reaction. These data suggest that hydrogen peroxide is a precursor to hydroxyl radical. Consistent with this notion, the oxidation of MDB, MDA, and MDMA was inhibited by typical hydroxyl radical scavenging agents such as ethanol, benzoic acid, DMSO, and thiourea. The production of catechol metabolite generated during the interaction of MDP with hydroxyl radical is thought to occur as shown in Scheme I. The ascorbate and xanthine oxidase systems which are based on Fenton (*22*) and iron-catalyzed Haber–Weiss (*23*) reactions require a continuous generation of ferrous from ferric ion to produce hydroxyl radical. In the ascorbate system, ascorbic acid reduces ferric ion. In the xanthine oxidase system, superoxide anion functions as the reducing agent. SOD markedly suppressed the catechol formation in the xanthine oxidase system but had no effect in the ascorbic acid system. The reason for the difference in SOD sensitivity is that superoxide anion is not an essential reducing agent for ferric ion in the ascorbate system.

Klein et al. (*4*) have proposed measuring formaldehyde as a marker for hydroxyl radical reaction with DMSO. The present data show that the rate of the ascorbate system mediated demethylenation of MDB was greater than that of demethylation of DMSO, so that the oxidation of MDPs may be a useful tool in studies of the generation of hydroxyl radical in biological systems.

Lim and Foltz (*12*) reported that demethylenation of MDMA occurs in brain homogenate. The present results raise the possibility that oxidative demethylenation of MDMA in brain could involve participation of not only cytochrome P-450 but locally generated hydroxyl radical as well.

The demethylenated products of MDA and MDMA are highly polar compounds whose access to the CNS from the periphery should be very limited. However, Lim and Foltz (*12*) have shown that the reaction can occur in brain preparations in vitro. The enzymatic nature of the reaction in the brain has not been characterized, and although mediation by cytochrome P450 is likely, the levels of this enzyme are very low. An alternative is an oxygen-mediated pathway such as the one reported here in which a reactive oxygen source is connected to hydroxyl radical which could then cleave the methylenedioxy group.

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Registry No. MDB, 274-09-9; MDA, 4764-17-4; MDMA, 54946-52-0; DHA, 3583-05-9; DHMA, 20521-18-0; catechol, 120-80-9.

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1,3-Dialkyltriazenes: Reactive Intermediates and DNA Alkylation

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The reactions of calf thymus (ct) DNA with 1,3-dimethyltriazene (DMT), *N*-methyl-*N*-nitrosourea (MNU), 1,3-diethyltriazene (DET), *N*-ethyl-*N*-nitrosourea (ENU), and 1-ethyl-3-methyltriazene (MET) were studied as a function of concentration of the alkylating agents, of various buffers, and of ionic strength. The amount of alkylation at the 7- and O^6-positions of guanine increased linearly with dose over a 10-fold concentration range. The slopes of the DMT and MNU curves were identical as were those of DET and ENU. These data suggest that both types of compounds alkylate DNA via a similar intermediate, presumably the corresponding alkanediazonium ion. MET methylates and ethylates DNA, the amount of each product being a function of the competitive formation of the two diazonium ions possible from MET. The MET product ratios could be reproduced by an appropriate mixture of DET and DMT. The alkylation of DNA by DMT and by MET is very sensitive to ionic strength, to the nature of the buffer, and to the identity of the salt used to balance ionic strength. In general, the reaction is favored by low ionic strength, by amine rather than oxy acid buffers, and by doubly charged inert anions. The alkylation of DNA is inversely proportional to the logarithm of the ionic strength over a wide range. The mutagenic activity of triazenes in *Salmonella typhimurium* is correlated very well with the ability of the triazenes to form adducts, particularly O^6-guanine adducts. Thus, symmetrical 1,3-dialkyltriazenes are mutagens in the order of methyl $\gg$ ethyl $>$ butyl = isopropyl, and unsymmetrical 1-alkyl-3-methyltriazenes are mutagens in the order ethyl $>$ butyl $>$ isopropyl. The latter order follows the rate of production of the methanediazonium ion, the most mutagenic of the diazonium ions.

Introduction

Although the first preparation of 1,3-dialkyltriazenes was reported by Dimroth in 1906 (*1*), this class of compounds remained essentially unstudied until a more convenient means of preparation was developed (*2*). The preparation (*3*), hydrolytic chemistry (*4*, *5*), and biological activity (*6*) of a related class of compounds, the 1,3,3-trialkyltriazenes, have also been investigated recently.

The proteolytic decomposition of 1,3-dialkyltriazenes in aqueous buffers has been reported by our laboratory (*7*). The hydrolytic decomposition is initiated by rapid and reversible protonation followed by rate-determining heterolysis to an alkanediazonium ion and an alkylamine. The final products resulting from the reaction of the diazonium ion with water are alkyl alcohols and, in some cases, alkenes. In addition, we (*8*) described kinetics and product distributions of the acid-catalyzed decomposition of unsymmetrical 1,3-dialkyltriazenes. These triazenes exist as a mixture of two rapidly equilibrating tautomeric forms. They decompose in aqueous buffers by a specific acid catalyzed pathway to give rise competitively to a mixture of two alkanediazonium ions. The product distribution is determined largely by the stability of the diazonium ion formed. Comparative rate data for a series of 1-alkyl-3-methyltriazenes show that the species produced in the rate-determining step are the corresponding alkanediazonium ions, rather than the carbocations, even in cases such as benzyl where the carbocation is stabilized by resonance (*8*).

Alkanediazonium ions are the putative electrophilic intermediates responsible for the carcinogenic and cytotoxic properties of several important classes of drugs, including alkyltriazenes (*9*, *10*). Triazenes have been shown to alkylate DNA in vitro and in vivo (*11-14*), and, consistent

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