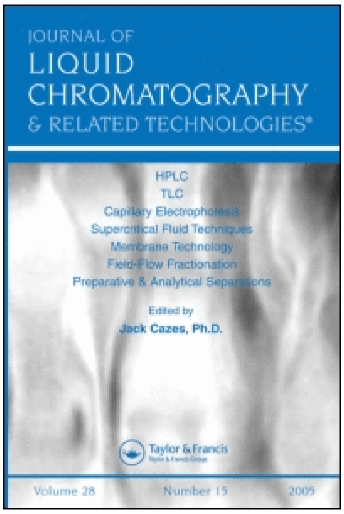

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Electrospray Ionization Mass Spectrometry and HPLC Determination of the Products in the Radiolysis of 5-Bromouracil, its Nucleoside and Nucleotide Derivatives

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ELECTROSPRAY IONIZATION MASS SPECTROMETRY AND HPLC DETERMINATION OF THE PRODUCTS IN THE RADIOLYSIS OF 5-BROMOURACIL, ITS NUCLEOSIDE AND NUCLEOTIDE DERIVATIVES

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ABSTRACT

Radiolysis of 5-bromouracil (BrUr), 5-bromo-2'-deoxyuridine (BrdU) and 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdUMP) has been studied by high performance liquid chromatography and electrospray ionization mass spectrometry. Production of bromide ions and the complementary uracil, 2-deoxyuridine and 2-deoxyuridine-5'-monophosphate has been quantitatively examined. It is concluded that in the presence of hydroxyl radical scavenger, at pH 6 to 10, BrUr, BrdU and BrdUMP quantitatively react with hydrated electron. In the absence of hydroxyl radical (·OH) scavenger in N_2-saturated solutions the yield of bromide ions is about 5.0, indicating that hydroxyl radicals also significantly react with BrUr, BrdU and BrdUMP to eliminate hydrogen bromide. Results from the radiolysis of 1 mM aqueous solutions of BrdU and BrdUMP suggested that abstraction of hydrogen by uracilyl radicals from the sugar moieties is not important. Our mass spectrometry experiments indicated that uracilyl radicals react with the starting substrate to form dimers. The radiolysis of N_2-saturated solutions of nucleosides and nucleotides suggested that the base radicals formed by ·OH addition can react with undamaged reagents to produce dimers.

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INTRODUCTION

Applications of 5-halopyrimidines as radiation sensitizers have been the subject of a large number of investigations because these halopyrimidines are useful tools for studying radiation effects from the model system to whole cells. (1), (2) 5-Bromouracil (BrUr) has been used to localize the site of radiation damage in radiobiological studies by selective attack of hydrated electron (e_{aq}^-) on DNA where it had replaced thymine. (1) In DNA, BrUr can be easily substituted for thymine. Cells (bacterial and mammalian) containing BrUr instead of thymine were deactivated by X-rays considerably faster than those of a control. (3) A similar effect was also observed if BrUr-containing cells were subjected to UV light. (3) In recent years BrUr has been used in clinic. (4), (5) A linear relationship between the cellular radiosensitization factor and the percentage thymidine replacement by 5-bromo-2'-deoxyuridine (BrdU) in human bone marrow cell line was reported. (6)

It is known that the attack of e_{aq}^- on BrUr causes elimination of bromide ion and produces uracilyl radical (Ur·). (1), (7), (8) In the presence of a source of abstractable hydrogen, the uracilyl radical is expected to react to produce uracil (Ur). (1), (9) Improvements in the separation and sensitivity of high performance liquid chromatography (HPLC) methods since the earlier studies now make it possible to investigate the radiolysis of BrUr, and its nucleoside and nucleotide derivatives in more detail. The effort is to provide fundamental data for further exploring the mechanism involved in radiosensitization of DNA by BrdU. We report here the quantitative determination of bromide ion and complementary uracil, 2-deoxyuridine (dU), 2-deoxyuridine-5'-monophosphate (dUMP), produced in the x-ray irradiation of BrUr, BrdU and BrdUMP by HPLC and ion chromatography. Using electrospray ionization mass spectrometry, the products formed in the ^{60}Co γ-radiolysis were identified, which provided valuable information about the reaction mechanisms involved.

MATERIALS AND METHODS

Irradiation was carried out with a Norelco MG 300 X-ray unit (Philips Electronic Instruments) at absorbed dose rates of 1 Gy min^{-1}. Gamma radiolysis was carried out with ^{60}Co γ-ray source at absorbed dose rates of 4 $x10^2$ Gy min^{-1}.

HPLC analysis of bromide, Ur, dU and dUMP The principal components of the HPLC apparatus are a Waters 6000A pump, Waters U6K injector, an optical and a conductivity detector. For detection of bromide ions, a VYDAC anion chromatography column and Waters 431 conductivity detector were used. Complementary Ur, dU and dUMP were determined using Beckman Ultrasphere ODS Column, VYDAC oligonucleotide column, and Linear 206 PHD optical detector. All quantitative results of HPLC were from comparison of peak area with reference samples run under identical chromatographic conditions.

Mass spectrometry analysis of adducts Products formed in the γ-ray radiolysis of the aqueous solutions of BrUr, BrdU and BrdUMP were analyzed by electrospray ionization mass spectrometry (ESI-MS). The instrumentation used in this study and typical operating conditions have been previously described. (10), (11) Electrospray ion production requires two steps: dispersal of highly charged droplets at near atmospheric pressure, followed by conditions resulting in droplet evaporation. A potential difference of 3-6 kV is applied between the capillary and counter electrode located 0.3-2 cm away, producing highly charged liquid droplets of ~ 1 µm diameter. (12) Positively or negatively charged droplets can be produced depending upon the capillary bias. The negative ion mode requires the presence of an electron scavenger such as oxygen to inhibit electrical discharge. (13) The ESE source is mounted 1.5 cm from the entrance of the quadrupole MS. Highly charged ions are sampled through a 1-mm nozzle orifice and 2-mm skimmer and are efficiently transported through a cryopumped region by the radio frequency (rf) quadrupole lens to a quadrupole mass spectrometer for detection. The mass spectrometer (Extrel Co., Pittsburgh, PA) used for these studies has an effective m/z range of 1700. Scan rates varied between 30 and 2 min over the entire m/z range. With the current data system (Teknivent, St. Louis, MO), peak abundances are collected at integer m/z values. Calibration of the m/z scale for ESE-MS was performed with 5-bromo-2'-deoxyuridine.

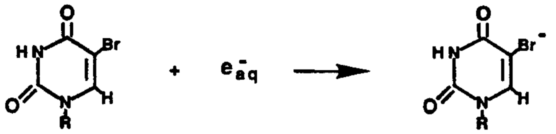
Solutions were prepared in triply distilled water purified by a Millipore Mill-Q Reagent system. The pH was adjusted by addition of Backer Analysis KOH and determined with a Corning 610A pH meter calibrated with VWR buffers. BrUr, BrdU and BrdUMP were from Sigma, 2-propanol, tert-butyl alcohol (t-BuOH) and NaOH from Fisher. The chemicals used in the mobile phases of HPLC were methanol from Burdick & Jackson, acetic acid from Fisher, Na_2HPO_4 from Aldrich and borate/gluconate eluent provided by Waters. Solutions were saturated with nitrogen to purge oxygen. Irradiated samples were covered with aluminum foil to avoid UV exposure and introduced into the HPLC system in a few minutes after completion of irradiation. All experiments were carried out at room temperature (22°C).

RESULTS AND DISCUSSIONS

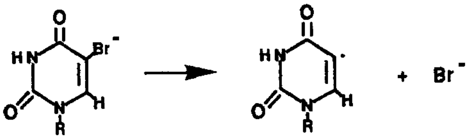
HPLC Analysis

1. Production of Bromide Ions

It is known from studies of the radiation chemistry of BrUr that e_{aq}^- reacts rapidly with BrUr by dissociative electron capture to eliminate bromide ion and produce the uracilyl radical. (1), (7), (8)



(1)



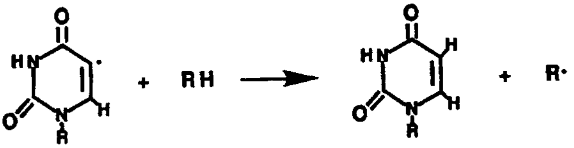
(2)

The production of bromide ion is linear with dose over the range 10 to 100 Gy. The bromide yields of 2.67, 2.71 and 2.45 were determined from the radiolysis of 1 mM BrUr, BrdU and BrdUMP solutions with 0.4 M 2-propanol saturated with N_2 (Table 1).

From the treatment of Balkas, Fendler and Schuler (14) one expects, taking the rate constants typically as 5×10^9 M^{-1} s^{-1}, that the radiation yield of products at millimolar solute concentrations should be ~ 2.7. The yields of bromide in BrUr and BrdU equal the yield of hydrated electrons. The yield of bromide ion in the case of BrdUMP is about 10% lower than that in BrUr and BrdU. Since phosphate group is not expected to reduce the electron affinity of the base moieties, (15) this yield-decreasing effect must be due to electrostatic repulsion between the negatively charged reactants, e_{aq}^- and BrdUMP. The chromatograms of bromide ions produced in the irradiation of 1 mM N_2-saturated solutions of BrdUMP with 0.4 M 2-propanol and t-BuOH are shown in Figures. 1a and 1b. Experiments were carried out in both neutral (pH ~6.4) and basic solutions (pH ~10).

2. Conversion of Complementary Uracil and its Derivative Radical

As shown in reactions 1 and 2, Ur· radical and its nucleoside derivative (dU·) and nucleotide derivative (dUMP·) are formed in the reaction of BrUr, BrdU and BrdUMP with e_{aq}^-. In the presence of a hydrogen donor, such as 2-propanol or t-BuOH, these radicals can be converted to Ur, dU and dUMP, i.e.,



(3)

TABLE 1

YIELD [1] OF BROMIDE IONS IN THE RADIOLYSIS OF BrUr, BrdU AND BrdUMP

Solute	pH	G(Br^-)	pH	G(Br^-)
1 mM BrUr, 2-propanol	6.6	2.67	10.1	5.07
1 mM BrUr, t-BuOH	6.3	2.65	10.2	2.69
0.1 mM BrUr, 2-propanol	6.4	2.20	10.4	3.50
0.1 mM BrUr, t-BuOH	6.3	2.17	10.1	2.20
1 mM BrdU, 2-propanol	6.2	2.71	10.2	5.02
1 mM BrdU, t-BuOH	6.3	2.62	10.3	2.58
1 mM BrdUMP, 2-propanol	6.7	2.45	10.2	4.98
1 mM BrdUMP, t-BuOH	6.5	2.67	10.4	2.40
.16 mM BrdUMP, 2-propanol	6.2	2.09	10.0	3.62
.16 mM BrdUMP, t-BuOH	6.6	2.00	10.1	2.05

1. Yields are given in units of G (molecules/100 eV). Averages of three experiments.

2. In 1 mM solution the concentration of 2-propanol or t-BuOH is 0.4 M. In 0.1 mM solution the concentration of 2-propanol or BuOH is 0.04 M. All solutions were saturated with N_2.

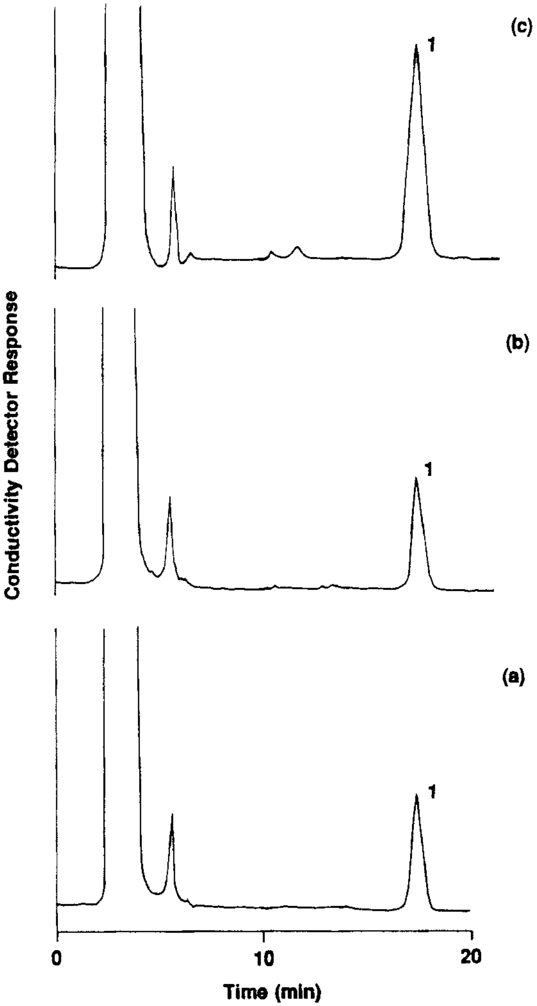


Figure 1 Chromatograms of Bromide Ions Observed in X-Ray Radiolysis of 1 mM BrdUMP Solutions in Neutral pH

(a) with 0.4 M 2-propanol; (b) with 0.4 M t-BuOH;
(c) without alcohol.
Irradiation dose: 20 Gray
Detector: Waters 431 Conductivity
Column: VYDAC oligonucleotide column
Mobil Phase: 0.03 M NaH_2PO_4 and 0.3% acetic acid.
Principle peak: (1) Br^-

The rate constant of hydrogen abstraction of Ur· from t-BuOH is 2.3 x 10^7 M^{-1} s^{-1}. (16) The yields of Ur, dU and dUMP are given in Table 2. The chromatograms of dUMP produced in the radiolysis are shown in Figure 2.

Mass Spectrometry Analysis

1. BrUr

In a pulse radiolysis study of BrUr, Edwin and Schuler (16) suggested that Ur· produced in reaction 1 subsequently reacts with the substrate to give a mixture of radical adducts.

$$\text{Ur}\cdot + \text{BrUr} \longrightarrow \text{adducts} \qquad (4)$$

Reaction 4 has a rate constant of ≥ 2 x 10^8 M^{-1} s^{-1}. (16) Apparently, Ur· can react with the starting BrUr to form a dimmer (BrHUr-Ur). Analysis of γ-radiolysis of 20 mM BrUr solution saturated with N_2 was performed using ESI-MS in positive ion mode. One adduct (BrHUr-Ur) was found in Figure 3 (peak B, m/z: 303, 305). Presumably, Ur· reacts with BrUr to first form a radical adduct that subsequently obtains an electron and then protontes to produce BrHUr-Ur. Radiation significantly increases the amount of non-covalent bond dimers of BrUr (peak C, m/z: 381, 383, 385) though these dimers exist in the control sample their concentration is much lower than that in the irradiated sample. The triplets in peak C are due to bromine isotope.

2. BrdU

Products formed in γ-radiolysis of 20 mM BrdU N_2-saturated solution were examined by ESE-MS in positive ion mode. Peaks B (m/z: 455), C (m/z: 535, 537), D (m/z: 551, 553) and E (m/z: 613, 615, 617) were found in the mass spectrum (Figure 4). Peaks B, C, D and E can be contributed to radiation products: dU-dU, BrHdU-dU,

Table 2

YIELD OF Ur, dU AND dUMP IN THE RADIOLYSIS OF BrUr, BrdU AND BrdUMP

Solute [1]	pH	G [2]	G(Br$^-$)/G	pH	G [2]
1 mM BrUr, 2-propanol	6.6	2.14	0.80	10.1	3.95
1 mM BrUr, t-BuOH	6.3	1.78	0.67	10.2	1.80
0.1 mM BrUr, 2-propanol	6.4	1.61	0.73	10.4	2.82
0.1 mM BrUr, t-BuOH	6.3	1.35	0.62	10.1	1.47
1 mM BrdU, 2-propanol	6.2	2.20	0.81	10.2	4.11
1 mM BrdU, t-BuOH	6.3	1.70	0.65	10.3	1.55
1 mM BrdUMP, 2-propanol	6.7	2.01	0.82	10.2	3.95
1 mM BrdUMP, t-BuOH	6.5	1.60	0.60	10.4	1.85
.16 mM BrdUMP, 2-propanol	6.2	1.67	0.80	10.0	2.82
.16 mM BrdUMP, t-BuOH	6.6	1.38	0.69	10.1	1.43

1. In 1 mM solution the concentration of 2-propanol or t-BuOH is 0.4 M. In 0.1 mM solution the concentration of 2-propanol or BuOH is 0.04 M. All solutions were saturated with N_2.

2. Yield of Ur, dU and dUMP.

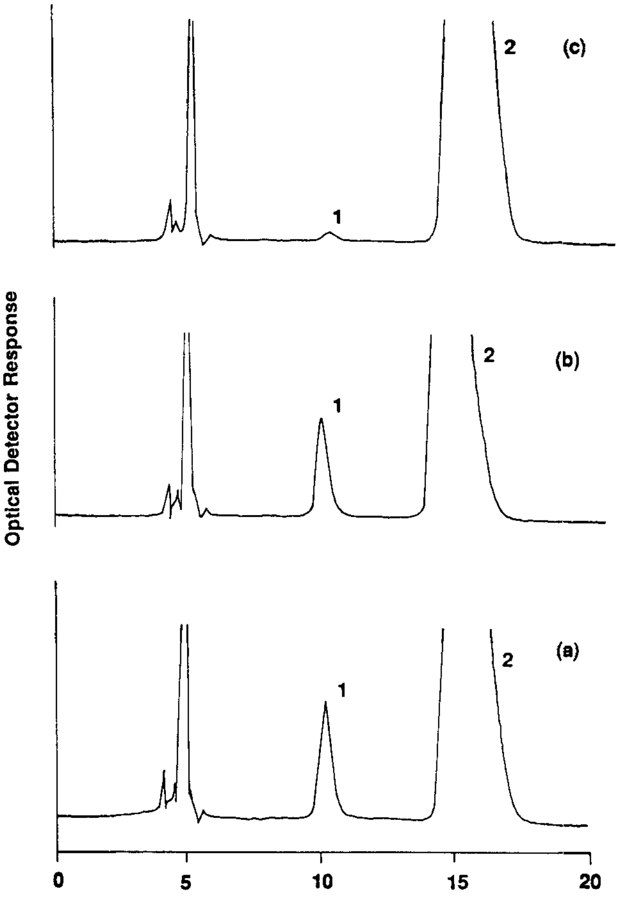


Figure 2 Chromatograms of dUMP Observed in X-Ray Radiolysis of 1 mM BrdUMP Solutions in Neutral pH

(a) with 0.4 M 2-propanol; (b) with 0.4 M t-BuOH;
(c) without alcohol.
Irradiation dose: 20 Gray
Detector: Linear 206 PHD optical at 210 nm
Column: VYDAC 304OL54
Mobil Phase: 0.4 M NaH_2PO_4 and 0.3% acetic acid
Principle peak: (1) dUMP; (2) BrdUMP

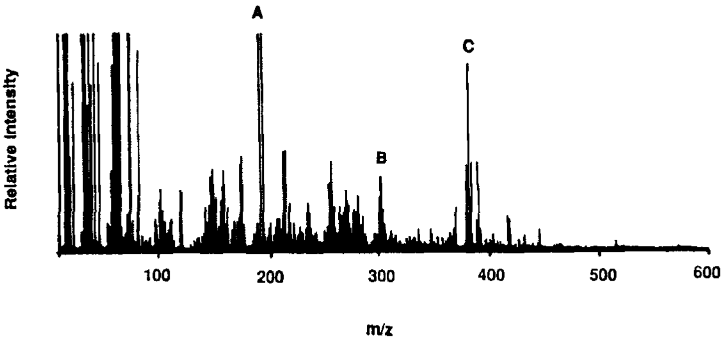
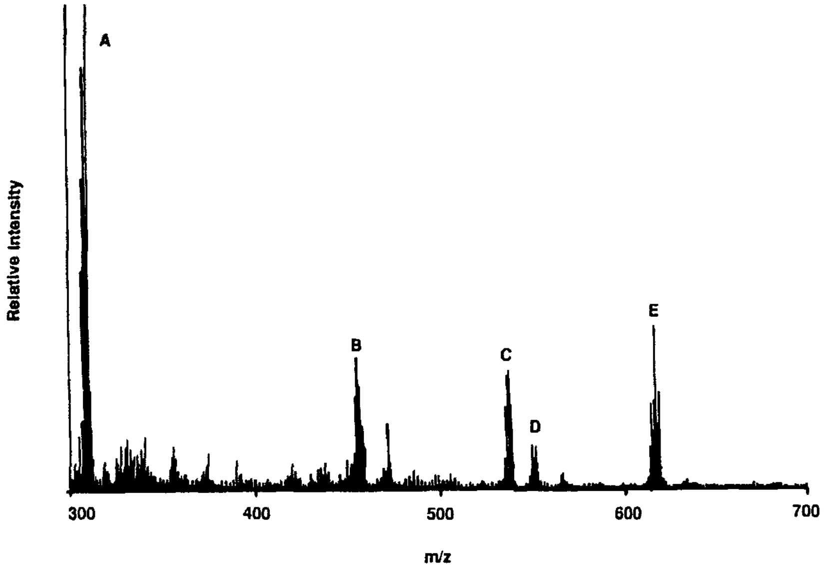


Figure 3 Mass Spectra of Radiolysis of BrUr

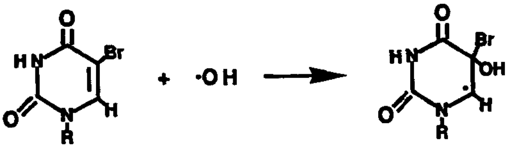
20 mM BrUr irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: BrUr, B: BrHUr-Ur, C: $(BrUr)_2$



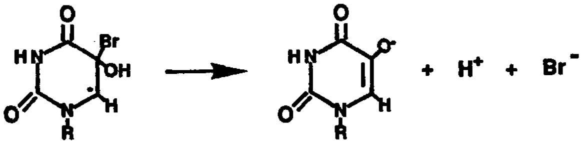
Figures 4 Mass Spectra of Radiolysis of BrdU

20 mM BrdU irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: BrdU, B: dU-dU, C: BrHdU-dU
D: BrHdU-OdU, E: $(BrdU)_2$

BrHdU-OdU and non-covalent dimer ($(BrdU)_2$). In N_2 saturated solution, hydroxyl radicals were produced as well as hydrated electrons, and ·OH can react with BrUr, BrdU and BrdUMP rapidly. (17)

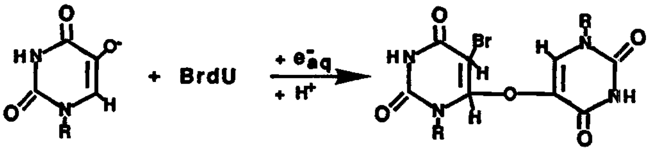


(5)



(6)

The radical produced by oxidative dehydrohalogenation of bromouracil (reaction 6) has been characterized by ESR experiments. (17) BrHdU-OdU must be produced by addition of ·OdU to the starting BrdU to form a radical adduct, dUO-BrdU· which is presumably reduced and protonated to form BrHdU-OdU.



(7)

Peak B can be contributed by the product of the combination reaction of dU·. In addition to radical combination product (dU-dU), radical adduct (BrHdU-OdU) and non-covalent dimer ($(BrdU)_2$), adduct (dU-BrHdU) was also produced in the radiation (peak C). The adduct (BrHdU-dU) must be formed in a similar reaction as in the case of BrUr as discussed above. The list of m/z of MH^+ is given in Table 3.

TABLE 3

m/z OF MH$^+$ AND M$^-$ OF NUCLEOBASES, NUCLEOSIDES, NUCLEOTIDES AND THE PRODUCTS IN RADIOLYSIS

	m/z of MH$^+$	m/z of M$^-$
Br	80, 82	
Ur	113	
BrUr	191, 193	
dU	229	
BrdU	307, 309	
dUMP		307
BrdUMP		385, 387
BrHUr-Ur	303, 305	
$(BrUr)_2$	381, 383, 385	
BrHdU-dU	535, 537	
BrHdU-OdU	551, 553	
dU-dU	455	
$(BrdU)_2$	613, 615, 617	
dUMPO-BrHdUMP		355, 356
dUMPOH		323

3. BrdUMP

Products formed in γ-radiolysis of 20 mM BrdUMP aqueous solution saturated with N_2 were analyzed by ESE-MS in negative ion mode. Three major products, dUMP (peak A, m/z: 307), dUMPOH (peak B, m/z: 323) and dUMPO-BrHdUMP (peak C, m/z: 355, 356) were found in Figure 5. Production of dUMP in this experiment condition is expected. The concentration of BrdUMP is 20 times higher and the radiation dose is 10 times higher (200 x 10^3 rads) than that in HPLC experiments. dUMPOH is possibly formed by the reduction and protonation of a radical (·OdUMP), similar to the one formed in reaction 6, which is a very similar reaction mechanism involved in the oxidation of bromophenols by hydroxyl radical. (18) This radical (·OdUMP) apparently can also react with the starting BrdUMP to form dUMPO-BrHdUMP. The relative importance of these two competitive reactions is not known. Non-covalent dimer, $(BrdUMP)_2$, was not found in the radiolysis, which indicated that phosphate group prevent the formation of the dimer. This must be due to steric hinderance of the phosphate group and electrostatic repulsion between the negatively charged phosphate groups.

Comparisons and Discussions

In Table 1, one can see that the yields of bromide, at neutral pH, obtained from the radiolysis of 1 mM BrUr, BrdU and BrdUMP solutions N_2-saturated in the presence of hydroxyl radical scavenger, 2-propanol or t-BuOH, are about 2.7, showing that the reaction of e_{aq}^- with BrUr, BrdU and BrdUMP is quantitative and the elimination of bromide ion does not depend on the substituted groups. (19) This is not surprising because sugar moiety has very low reactivity with e_{aq}^-. (20) In the presence of alcohol, Ur·, dU· and dUMP· mainly undergo hydrogen abstraction. With 0.4 M 2-propanol in 1 mM solutions of BrUr and BrdU, the yields of Ur, dU and dUMP are about 2.15 and the ratio of the yields of bromide and Ur, dU or dUMP is about 0.80 (Table 2). With t-BuOH

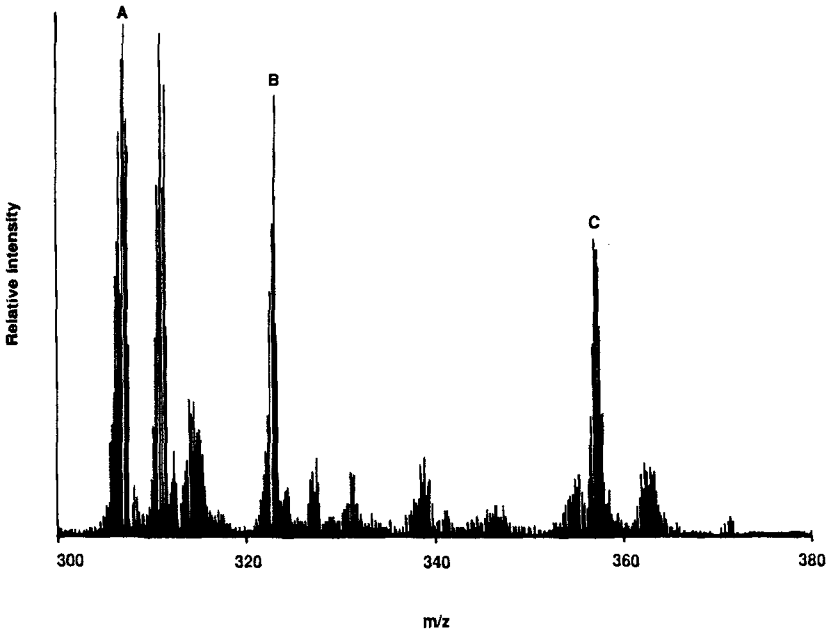


Figure 5 Mass Spectra of Radiolysis of BrdUMP

20 mM BrdUMP irradiated with ^{60}Co at dose 2 x 10^3 Gray.
Principle peaks: A: dUMP, B: dUMPOH, C: dUMPO-BrHdUMP

the yields of Ur, dU and dUMP are lower than those with 2-propanol and the ratio of the yields of bromide and Ur, dU or dUMP is only about 0.65 (Table 2). The chromatograms of dUMP obtained from 1 mM BrdUMP solutions with 2-propanol and t-BuOH were shown in Figures 2a and 2b. Studies of the reaction of halogen substituted benzoic acid indicated that 2-propanol is about an order of magnitude more reactive as a hydrogen donor than t-BuOH in neutral solution. (18) (21). The rate constants for hydrogen abstraction of carboxyphenyl from t-BuOH and 2-propanol are 0.32 and 5.5 x 10^6 M^{-1} s^{-1}. (21) The rate constant for hydrogen abstraction of Ur· from 2-propanol is, unfortunately, not available. From the pseudo-first-order rate constants of reaction 4 and the hydrogen abstraction from t-BuOH, we estimated that less than 5% of Ur· will undergo addition reaction (reaction 4). The yield of uracil formed in the radiolysis is only about 65% of that of bromide ion. The indication is that the rate constant of reaction 4 should be about 3 x 10^9 M^{-1} s^{-1} or that the rate constant of the hydrogen abstraction from t-BuOH should be only about 2.0 x 10^6 M^{-1} s^{-1} which is not unreasonable value when compared with that of the hydrogen abstraction of carboxyphenyl from t-BuOH. Other unknown competition reactions may also be involved with Ur·, dU· and dUMP .

The experiments were also carried out in basic solutions of BrUr, BrdU and BrdUMP (see Table 1). The yields of bromide ions with t-BuOH as a hydroxyl radical scavenger were found to be about 2.67, showing that the reaction of e_{aq}^- with BrUr and BrdU, and the elimination of bromide basically do not depend on the solution pH. This result is similar to that found in the study of the reaction of e_{aq}^- with halogen substituted benzene, phenol and benzoic acid. (18) In basic solutions (pH ~ 10.5) with t-BuOH as a hydrogen donor, the yields of bromide ions and complementary Ur, dU and dUMP are similar to those found in neutral solutions (see Tables 1 and 2). With 2-propanol the yields are significantly higher than that with t-BuOH. The high yield of bromide must be due to that the isopropyl radical anion produced in the radiolysis reduces BrUr, BrdU and BrdUMP to form extra bromide as in the case of the reaction of halobenzonate with isopropyl radical. (18) Chain reactions are involved.

$$(CH_3)_2CHO\!:^- + \cdot OH \longrightarrow (CH_3)_2\dot{C}O\!:^- + H_2O \tag{8}$$

$$(CH_3)_2\dot{C}O\!:^- + BrUr \longrightarrow (CH_3)_2C{=}O + Br^- + Ur\cdot \tag{9}$$

$$(CH_3)_2CHO\!:^- + Ur\cdot \longrightarrow (CH_3)_2\dot{C}O\!:^- + Ur \tag{10}$$

TABLE 4

PRODUCT YIELDS IN THE X-RAY RADIOLYSIS OF BrUr, BrdU AND BrdUMP IN THE ABSENCE OF ALCOHOL

Solute [1]	pH	$G(Br^-)$	G [2]	pH	$G(Br^-)$	G [2]
1 mM BrUr	6.4	4.92	0.48	10.3	4.89	0.51
0.1 mM BrUr	6.2	3.81	0.35			
1 mM BrdU	6.8	5.05	0.47	10.1	5.02	0.45
0.1 mM BrdU	6.4	3.90	0.39			
1 mM BrdUMP	6.5	5.12	0.50	10.4	5.08	0.49
0.1 mM BrdUMP	6.7	3.92	0.42			

1. All solutions were saturated with N_2.
2. Yields of Ur or dU or dUMP.

In the absence of alcohol the yields of Ur, dU and dUMP are very low ($G \leq 0.5$, Table 4). Figure 2c shows the signal of dUMP produced in 1 mM N_2-saturated solutions of BrdUMP. The results indicate that hydrogen abstraction from the sugar moiety is not an important process for dU· and dUMP·. This is expected because the unpaired electron of Ur· is expected to be localized at C_5 in a σ orbital. As result the reactivity of this radical is about as reactive as the phenyl radical. (16), (21) Our mass spectrometry experiments suggest that Ur· and dU· can react with the BrUr and BrdU to form dimers.

In the absence of alcohol, hydroxyl radicals react with BrUr, BrdU and BrdUMP to produce radicals as shown in reactions 5 and 6. Mass spectrometry experiments suggest that radicals produced in the case of BrdU and BrdUMP can react with starting substrates to form stable adducts.

Figure 1c shows the chromatogram of bromide ion obtained from the same irradiated sample as in Figure 2c. The yield of bromide ions was about 5.0 in 1 mM N_2-saturated solutions of BrUr, BrdU and BrdUMP (Table 2). As shown in Table 1, the yield of bromide in the reaction of e_{aq}^- with BrUr, BrdU and BrdUMP is about 2.7, which amounts to 54% of G(Br^-) 5.0. This result indicates that in the absence of hydroxyl scavenger 46% of G(Br^-) 5.0 is contributed from the reactions of hydroxyl radicals and hydrogen atoms with BrUr, BrdU and BrdUMP.

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