The Eosinophil Peroxidase-Hydrogen Peroxide-Bromide System of Human Eosinophils Generates 5-Bromouracil, a Mutagenic Thymine Analogue†

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ABSTRACT: Eosinophils use eosinophil peroxidase, hydrogen peroxide (H_2O_2), and bromide ion (Br^-) to generate hypobromous acid (HOBr), a brominating intermediate. This potent oxidant may play a role in host defenses against invading parasites and eosinophil-mediated tissue damage. In this study, we explore the possibility that HOBr generated by eosinophil peroxidase might oxidize nucleic acids. When we exposed uracil, uridine, or deoxyuridine to reagent HOBr, each reaction mixture yielded a single major oxidation product that comigrated on reversed-phase HPLC with the corresponding authentic brominated pyrimidine. The eosinophil peroxidase-H_2O_2-Br^- system also converted uracil into a single major oxidation product, and the yield was near-quantitative. Mass spectrometry, HPLC, UV−visible spectroscopy, and NMR spectroscopy identified the product as 5-bromouracil. Eosinophil peroxidase required H_2O_2 and Br^- to produce 5-bromouracil, implicating HOBr as an intermediate in the reaction. Primary and secondary bromamines also brominated uracil, suggesting that long-lived bromamines also might be physiologically relevant brominating intermediates. Human eosinophils used the eosinophil peroxidase-H_2O_2-Br^- system to oxidize uracil. The product was identified as 5-bromouracil by mass spectrometry, HPLC, and UV−visible spectroscopy. Collectively, these results indicate that HOBr generated by eosinophil peroxidase oxidizes uracil to 5-bromouracil. Thymidine phosphorylase, a pyrimidine salvage enzyme, transforms 5-bromouracil to 5-bromodeoxyridine, a mutagenic analogue of thymidine. These findings raise the possibility that halogenated nucleobases generated by eosinophil peroxidase exert cytotoxic and mutagenic effects at eosinophil-rich sites of inflammation.

Activated white blood cells generate oxidants that are central to host defense against microorganisms but may also damage host tissues. Eosinophils, which play a role in immediate hypersensitivity and host defense against parasites, have been implicated in tissue damage during asthma and parasitic infections (*1*, *2*). Their histochemical hallmark is cytoplasmic granules that stain with eosin. During cell activation, these granules release specialized proteins that are key to the eosinophilic cytotoxic armamentarium. Activated eosinophils also generate potentially toxic reactive oxidizing species.

Oxidant production begins with the generation of superoxide by the membrane-bound NADPH oxidase of eosinophils (*3*, *4*). The superoxide then dismutates into hydrogen peroxide (H_2O_2). Eosinophil peroxidase, a major component of the eosin granules secreted by activated cells, uses this H_2O_2 as an oxidizing substrate to generate potent oxidizing species. At plasma halide concentrations ($Cl^- \approx 100$ mM, $Br^- \approx 20-100$ μM, $I^- < 1$ μM; refs *5* and *6*), the major oxidizing product is thought to be hypobromous acid (HOBr) (*7*−*9*).

$$Br^- + H_2O_2 + H^+ \rightarrow HOBr + H_2O \qquad (1)$$

Eosinophils have been implicated in cancer, especially during certain chronic infections characterized by a rich infiltrate of these inflammatory cells. One striking example is the strong epidemiological association between bladder infection by *Schistosoma haematobium* and bladder cancer (*10*, *11*). This raises the possibility that oxidants generated during the eosinophilic response to parasitic infection might damage nucleic acids and exert a mutagenic effect on neighboring cells (*12*−*14*). Indeed, amino acid oxidation products generated by eosinophil peroxidase and myeloperoxidase, a heme enzyme related to eosinophil peroxidase, have been detected in inflamed human tissue (*15*−*21*). Moreover, peroxidase-derived chlorinating and nitrating oxidants halogenate and nitrate DNA bases in vitro (*22*, *23*); oxidants generated by peroxidase-dependent and independent pathways also deaminate and hydroxylate DNA (*24*−*27*).

In the current studies, we examined the ability of the eosinophil peroxidase-H_2O_2-Br^- system to brominate the nucleobase uracil. We found that reagent HOBr or eosinophil peroxidase converted uracil to 5-bromouracil, an analogue

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of thymine. Uridine and deoxyuridine also were brominated by HOBr. The 5-bromouracil generated by eosinophil peroxidase was a substrate for thymidine phosphorylase, a pyrimidine salvage enzyme, which transformed 5-bromouracil to the nucleoside 5-bromodeoxyuridine. Moreover, activated eosinophils generated significant amounts of 5-bromouracil in a reaction blocked by catalase and peroxidase inhibitors. These observations raise the possibility that eosinophil peroxidase produces brominating intermediates that generate mutagenic or cytotoxic DNA precursors at sites of inflammation.

EXPERIMENTAL PROCEDURES

Materials. H_2O_2, organic solvents, sodium hypochlorite, and sodium phosphate were obtained from Fisher Chemical Company (St. Louis, MO). BSTFA,[1] MtBSTFA, and silylation-grade acetonitrile were from Regis Technologies, Inc. (Morton Grove, IL). Thymidine phosphorylase (*Escherichia coli*) was from Worthington Biochemical Corporation (Lakewood, NJ). All other materials were purchased from Sigma Chemical Company (St. Louis, MO), except where indicated.

Methods. Assaying Peroxidase Activity. Analysis of eosinophil peroxidase (ExOxEmis, Little Rock, AR) by nondenaturing polyacrylamide slab-gel electrophoresis and gel system 8 (*28*, *29*) yielded a single band of active material as assessed by peroxidase activity. Glycerol (25% w/v) and CETAB (0.05% w/v) were included in all buffers. Riboflavin (0.024 mg/mL) was used as the polymerization catalyst, and the stacking gel was omitted. Peroxidase activity was visualized by incubating the gel in 400 μM tetramethyl benzidine, 10 mM sodium citrate (pH 5), 10 mM EDTA, 5 mM NaBr, and 200 μM H_2O_2.

Hypobromous Acid. Bromide-free HOBr was prepared by adding silver nitrate to ~80 mM bromine water (1.5:1, mol/mol) (*30*). The precipitate was removed by centrifugation, and 30 mL of the supernatant was distilled under vacuum using a foil-covered microscale distillation apparatus. The distillate was collected in a foil-covered vial at 4 °C. Reagent taurine monobromamine was prepared by adding HOBr to a 100-fold molar excess of taurine. HOBr and taurine bromamine concentrations were determined spectrophotometrically ($\epsilon_{288} = 430\ M^{-1}\ cm^{-1}$; ref *9*).

Oxidation of Pyrimidines by HOBr and Eosinophil Peroxidase. Reactions were performed in buffer A (100 mM NaCl, 100 μM NaBr, 50 mM sodium phosphate buffer, and 100 μM diethylenetriamine pentaacetic acid (DTPA), pH 7) at 37 °C in gastight vials. To inhibit metal-catalyzed oxidation reactions, all buffers were passed over a Chelex (Bio-Rad) column and DTPA was included in the reaction mixture. Reactions were initiated by adding oxidant (H_2O_2, HOBr, or bromamine) from a gastight syringe through the septum while vortexing the sample. They were terminated by adding L-methionine (Calbiochem, San Diego, CA) to a final concentration of 6 mM. The concentration of H_2O_2 was determined spectrophotometrically ($\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$; ref *31*). The pH dependence of product formation was determined using reaction mixtures containing phosphoric acid, monobasic sodium phosphate, and dibasic sodium phosphate (final concentration 50 mM). The pH of the reaction mixture (without L-methionine) was measured at the end of incubation.

Human Eosinophils. Human polymorphonuclear cells were prepared from blood by density gradient centrifugation. Neutrophils were removed by passing the preparation over beads coupled to anti-CD16 antibody (R&D Systems, Minneapolis, MN). Cells (>95% eosinophils and <5% lymphocytes) were suspended in Hank's Balanced Salt Solution (pH 7; phenol red- and bicarbonate-free; Life Technologies, Inc.) supplemented with 100 μM sodium bromide and 100 μM DTPA. Cells were incubated at 37 °C for 60 min and maintained in suspension with intermittent inversion. The reaction was terminated by adding 6 mM L-methionine and removing cells by centrifugation (400*g* for 10 min).

Reversed-Phase HPLC. Supernatants of reaction mixtures were analyzed by reversed-phase HPLC with a C_{18} column (Porasil, 5 μM resin, 4.6 × 250 mm; Beckman, Fullerton, CA) at a flow rate of 1 mL/min with UV detection at 274 nm. Solvent A was 20 mM ammonium formate and solvent B was 20 mM ammonium formate in methanol. For 5-bromouracil analysis, the column was equilibrated and eluted under isocratic conditions with 100% solvent A. For nucleoside analysis, the column was equilibrated and eluted for 10 min with 5% solvent B, followed by a gradient to 50% solvent B over 20 min. For thymidine phosphorylase reaction analyses, the column was equilibrated and eluted by isocratic elution with 10% solvent B. UV−vis spectra for individual HPLC peaks were obtained using a Beckman diode array detector. The yield of 5-bromouracil, 5-bromouridine, and 5-bromodeoxyuridine was quantified by comparing integrated peak areas to standard curves generated with commercially available material. For mass spectrometric analysis, HPLC fractions were collected and concentrated under vacuum. For NMR analysis, concentrated reaction mixtures were fractionated isocratically on a semipreparative C_{18} column (Porasil; 5 μm resin, 10 × 250 mm; Beckman) at a flow rate of 2.5 mL/min with 10% solvent B.

NMR. Reaction products were isolated by HPLC, solubilized in D_2O, and analyzed at 25 °C with a Varian Unity-Plus 500 spectrometer (499.843 MHz for ^1H) equipped with a Nalorac indirect detection probe. ^1H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 in D_2O. Spectra were recorded from eight transients with a 12 s preacquisition delay over a spectral width of 8000 Hz.

Gas Chromatography−Mass Spectrometry (GC/MS). After samples were dried under vacuum, residual water was removed by forming an azeotrope with 50 μL of pyridine and again drying the suspension under vacuum. DNA bases were converted either to trimethylsilyl (TMS) derivatives with excess bis-(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) or to dimethyl-*tert*-butylsilyl (DMTBS) derivatives with excess *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide plus 1% *tert*-butyl-dimethylchlorosilane in acetonitrile (3:1; v/v) at 100 °C for 60 min. Aliquots (1 μL) were analyzed in the positive

[1] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; M^+, molecular ion; MS, mass spectrometry; *m/z*, mass-to-charge ratio; TMS, trimethylsilyl; BSTFA, bis-(trimethylsilyl)-trifluoroacetamide; TMCS, trimethylchlorosilane; DMTBS, dimethyl-*tert*-butylsilyl; MtBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide; t-BDMCS, *tert*-butyl-dimethylchlorosilane; PMA, phorbol myristate acetate.

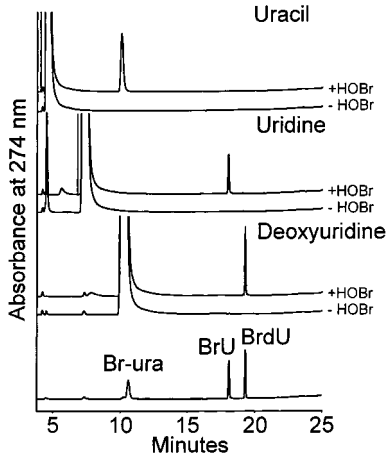


FIGURE 1: HPLC analysis of uracil, uridine, and deoxyuridine oxidized by HOBr. Uracil, uridine, or deoxyuridine (1 mM) was incubated with 50 μM HOBr in buffer A (50 mM sodium phosphate, 100 mM sodium chloride, and 100 μM DTPA, pH 7) for 60 min at 37 °C. Reactions were initiated by adding HOBr and terminated with 6 mM L-methionine. Where indicated, HOBr was omitted from the reaction mixture. The reaction mixtures were analyzed by reversed-phase HPLC with absorbance detection at 274 nm. Elution conditions for separation of nucleobases and nucleosides are described in the Experimental Procedures. The lower panel shows the retention times of authentic 5-bromouracil (Br-ura), 5-bromouridine (BrU), and 5-bromodeoxyuridine (BrdU) subjected to HPLC analysis.

electron ionization mode on either a Hewlett-Packard 5890 Series II gas chromatograph (Santa Clarita, CA) interfaced with a Hewlett-Packard 5972 Series Mass Selective Detector or a Varian Star 3400 CX gas chromatograph (Walnut Creek, CA) interfaced with a Finnegan SSQ 7000 mass spectrometer (San Jose, CA). Each gas chromatograph was equipped with a 12 m DB-1 capillary column (0.2 mm id, 0.33 μm film thickness; J&W Scientific, Folsom, CA). Injector and interface temperatures were 250 and 280 °C, respectively. The initial GC oven temperature was 70 °C for 2 min, followed by a 60 °C/min increase to 180 °C and a final 10 °C/min ramp to 220 °C. Derivatizing agent injections were analyzed between samples to ensure that no residual analyte remained in the injection port.

RESULTS

HOBr Converts Uracil into 5-Bromouracil. We exposed uracil, uridine, and deoxyuridine to HOBr in a physiological buffer (100 mM NaCl, 100 μM NaBr, 50 mM sodium phosphate buffer, 100 μM DTPA, pH 7) containing plasma concentrations of Cl^- and Br^-. After terminating the reaction with methionine (which scavenges HOBr, H_2O_2, and bromamines), we analyzed the mixture by reversed-phase HPLC with absorbance detection at 274 nm. Figure 1 shows representative chromatograms for uracil and the nucleosides exposed to HOBr. The hypohalous acid converted uracil, uridine, and deoxyuridine to single major products that comigrated with authentic 5-bromouracil, 5-bromouridine, and 5-bromodeoxyuridine, respectively. The absorption spectrum of each oxidation product was indistinguishable from that of the corresponding authentic brominated compound. Collectively, these observations suggest that reagent HOBr converts uracil—either free or as the (deoxy)-nucleoside—into 5-bromouracil.

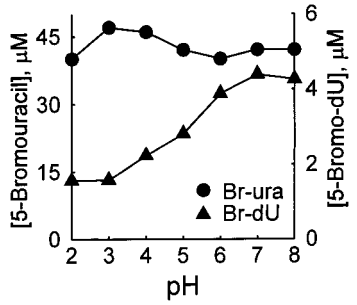


FIGURE 2: Effect of pH on pyrimidine bromination by reagent HOBr. HOBr (50 μM) was added to 1 mM uracil or deoxyuridine in buffer A and incubated for 60 min at 37 °C. The pH was varied by using reaction mixtures containing phosphoric acid, monobasic sodium phosphate, and dibasic sodium phosphate (final concentration 50 mM). The pH of the reaction mixture was determined at the end of the incubation prior to the addition of L-methionine. 5-Bromouracil and 5-bromodeoxyuridine were quantified by reversed-phase HPLC.

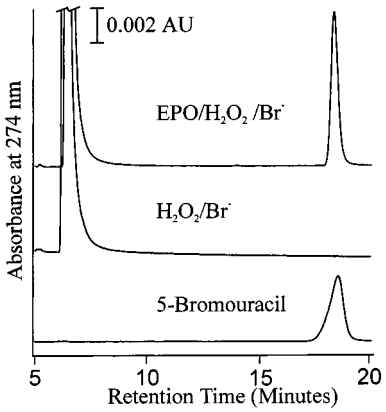


FIGURE 3: HPLC analysis of uracil oxidized by the eosinophil peroxidase-H_2O_2-Br^- system. Uracil (1 mM) was incubated with 3 nM eosinophil peroxidase, 50 μM H_2O_2, and 100 μM NaBr in buffer A (50 mM sodium phosphate, 100 mM sodium chloride, 100 μM DTPA, pH 7) for 60 min at 37 °C. Where indicated, chromatograms depict a reaction in which enzyme was omitted (H_2O_2/Br^-) or 50 μM authentic 5-bromouracil was included in the reaction mixture. Reactions were initiated by adding H_2O_2 and terminated with 6 mM L-methionine.

HOBr Brominates Uracil in Near-Quantitative Yield over a Broad pH Range. Conversion of uracil to 5-bromouracil proceeded with an approximately 95% yield (relative to HOBr) over a pH range of 2−8 (Figure 2). Yields of 5-bromouridine and 5-bromodeoxyuridine were smaller, reaching a maximum of ∼10% at pH 7−8.

Eosinophil Peroxidase-H_2O_2-Br^- System Brominates Uracil at Plasma Concentrations of Halide Ions. To determine whether enzymatically generated HOBr can halogenate nucleobases, we exposed uracil to the eosinophil peroxidase-H_2O_2-Br^- system in buffer A and used HPLC to characterize the reaction. As with reagent HOBr, the reaction yielded a new peak of material that migrated on reversed-phase HPLC with a retention time and absorption spectrum consistent with that of 5-bromouracil (Figure 3). When eosinophil peroxidase was incubated with uridine or deoxyuridine, the enzyme generated peaks of material that migrated on reversed-phase HPLC with retention times and absorption spectra consistent with those of 5-bromouridine and 5-bromodeoxyuridine, respectively (data not shown). The yields of these modified nucleosides were 5−10% of the yield obtained for 5-bromouracil.

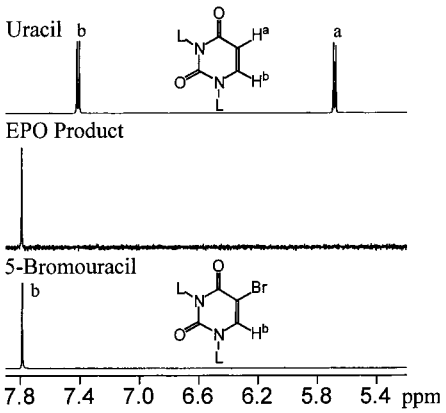


FIGURE 4: ^{1}H NMR spectra of uracil, brominated uracil generated by eosinophil peroxidase (EPO product), and commercially available 5-bromouracil. Protons not observed due to exchange with D_2O are designated (L).

We used electron ionization GC/MS to further characterize the structure of the uracil product generated by eosinophil peroxidase. The TMS derivative of the oxidation product had a GC retention time and electron ionization mass spectrum that were essentially identical to those obtained using authentic 5-bromouracil (data not shown). The compound exhibited a molecular ion ($M^{\bullet+}$) at *m*/*z* 334 and a prominent fragment ion at *m*/*z* 319 consistent with the $^{\bullet}CH_3$ loss typical of TMS derivatives. These ions also exhibited prominent (M + 2) isotope peaks, as expected from the natural isotopic abundance of ^{79}Br and ^{81}Br, strongly suggesting that the product was monobrominated. An ion consistent with loss of bromine radical was observed at *m*/*z* 255 $[M - Br]^+$. As anticipated for compounds lacking bromine, this fragment ion no longer exhibited the prominent (M + 2) isotope pattern.

Eosinophil Peroxidase Brominates Uracil at the C-5 Position. To determine the position of the bromine on the pyrimidine ring, we used HPLC to isolate the product from the eosinophil peroxidase reaction and subjected the purified product to ^{1}H NMR analysis. The product spectrum was essentially identical to that of authentic 5-bromouracil. Significant features included loss of the C-5 proton resonance, a downfield shift in the C-6 proton, and conversion of the C-6 proton resonance from a doublet to a singlet (Figure 4). These findings are consistent with substitution of a bromine atom at the C-5 position.

Reaction Requirements for 5-Bromouracil Production by Eosinophil Peroxidase. We used reversed-phase HPLC to characterize the bromination of uracil by eosinophil peroxidase. The reaction required enzyme and H_2O_2, and it was blocked by catalase, a scavenger of H_2O_2 (Figure 5). Failure to add NaBr did not completely prevent bromination, probably because the NaCl in the buffer was contaminated with Br^- ($\leq$0.01% NaBr; equivalent to 10 μM in buffer A). The heme enzyme inhibitors cyanide, azide, and aminotriazole inhibited product formation. These results demonstrate that bromination of uracil by eosinophil peroxidase requires active enzyme, Br^-, and H_2O_2. Taurine, which can react with HOBr to form bromamines (*9*), had little effect on bromination. Addition of RNase to the reaction inhibited 5-bromouracil formation with an apparent IC_{50} of 0.3 mg/mL (20 μM protein, 3 mM amino acids) for eosinophil peroxidase and 0.5 mg/mL for HOBr. These observations demonstrate

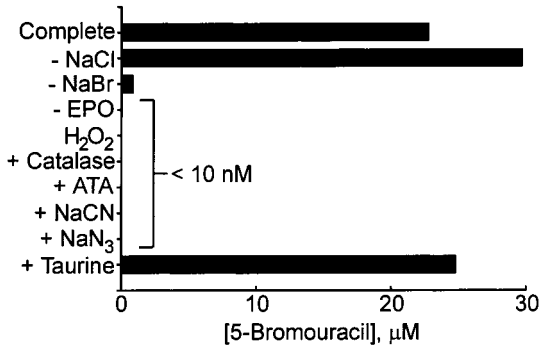


FIGURE 5: 5-Bromouracil generation by the eosinophil peroxidase-H_2O_2-Br^- system. Uracil (1 mM) was incubated with 3 nM eosinophil peroxidase, 50 μM H_2O_2, and 100 μM NaBr in buffer A (50 mM sodium phosphate, 100 mM sodium chloride, and 100 μM DTPA, pH 7) for 60 min at 37 °C. Reaction conditions were varied by adding or removing components as indicated. Catalase, 10 μg/mL; ATA, 10 mM 3-aminotriazole; NaCN, 10 mM sodium cyanide; NaN_3, 10 mM sodium azide; taurine, 10 mM. Bromouracil was quantified by reversed-phase HPLC.

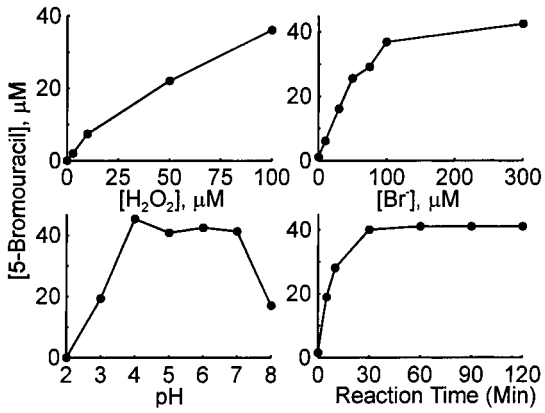


FIGURE 6: Reaction conditions for oxidation of uracil by the eosinophil peroxidase-H_2O_2-Br^- system. The reaction was initiated by adding H_2O_2 (50 nmol) to 1 mL of buffer A containing 1 mM uracil, 3 nM eosinophil peroxidase, and 100 μM NaBr. After a 60 min incubation at 37 °C, the reaction was terminated by adding 6 μmol of L-methionine. Conditions were varied by performing the reaction with the indicated final concentration of H_2O_2, Br^- ions, hydrogen ions, or for the indicated reaction time. 5-Bromouracil was quantified by reversed-phase HPLC.

that bromination of uracil by the eosinophil-H_2O_2-Br^- system proceeds in the presence of an excess of protein-bound amino acids.

Figure 6 shows the effects of varying the reaction conditions. Enzymatic bromination increased with H_2O_2 concentration up to 100 μM. When the concentration of Br^- was increased, the product yield reached a plateau at 100 μM Br^-, the upper range of normal plasma concentrations. Bromination was complete by 30 min, and product yields approached 100% (relative to H_2O_2) over the pH range 4−7.

HOBr, Primary Bromamines, and Secondary Bromamines Generate 5-Bromouracil. Because HOBr can readily react with amines to form bromamines, we compared the abilities of reagent HOBr, the primary bromamine *N*-bromotaurine, and the secondary bromamine *N*-bromosuccinimide to brominate uracil in the presence of 100 mM NaCl and 100 μM NaBr. As shown in Figure 7, the bromination yield and pH dependencies for HOBr, *N*-bromotaurine, and *N*-bromosuccinimide were similar. These observations suggest that bromination by eosinophil peroxidase may be facilitated by

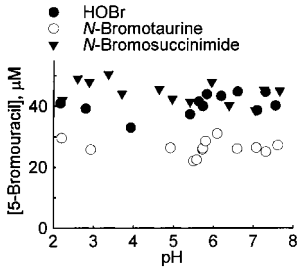


FIGURE 7: pH dependence for generation of 5-bromouracil by HOBr (●), the primary bromamine *N*-bromotaurine (○), and the secondary bromamine *N*-bromosuccinimide (▼). The reaction was initiated by adding oxidant (50 nmol) to 1 mL of buffer A containing 1 mM uracil and 100 μM NaBr in buffer A. After a 60 min incubation at 37 °C, the reactions were terminated with 6 μM L-methionine. The pH was varied using reaction mixtures containing phosphoric acid, monobasic sodium phosphate, and dibasic sodium phosphate (final concentration 50 mM). The pH of the reaction mixture was determined at the end of the incubation prior to addition of L-methionine. 5-Bromouracil was quantified by reversed-phase HPLC.

free and protein-bound amines, which could catalyze 5-bromouracil formation.

Because *N*-bromouracil could be a brominating intermediate, we also investigated the ability of HOBr and bromamines to generate this compound (*22*, *32*). However, we were unable to observe the postulated species in reaction mixtures lacking methionine when we looked for new peaks of material during HPLC analysis.

Thymidine Phosphorylase Converts the 5-Bromouracil Produced by Eosinophil Peroxidase to 5-Bromodeoxyuridine. To determine if the uracil oxidation product generated by eosinophil peroxidase can behave as a thymine analogue, we used thymidine phosphorylase, an enzyme involved in pyrimidine metabolism. In the presence of deoxyribose-1-phosphate, thymidine phosphorylase couples deoxyribose to thymine, generating thymidine. To determine whether brominated uracil can behave similarly, we used reversed-phase HPLC to isolate the 5-bromouracil produced by eosinophil peroxidase. When we substituted the 5-bromouracil for thymine, we obtained a new product peak on reversed-phase HPLC that comigrated with authentic 5-bromodeoxyuridine (Figure 8). This observation suggests that the eosinophil peroxidase product is structurally similar to thymine and could be converted intracellularly to the deoxynucleoside 5-bromodeoxyuridine.

Activated Human Eosinophils Generate 5-Bromouracil at Physiologically Plausible Halide Concentrations. To determine whether human eosinophils brominate uracil, we incubated cells in physiological salt solution containing 100 μM NaBr and 1 mM uracil. HPLC analysis detected substantial quantities of 5-bromouracil in the cell-conditioned medium when the eosinophils were stimulated with phorbol ester (Figure 9). The UV−vis absorbance maximum of the product was shifted toward a longer wavelength, which is consistent with conversion of uracil to 5-bromouracil.

To confirm that the oxidation product generated by eosinophils was 5-bromouracil, we used HPLC to isolate the nucleobase from the medium of the cells, converted it to the TMS and DMTBS derivatives, and subjected the derivatives to electron ionization GC/MS. We readily detected peaks of material with GC retention times and mass spectra identical to those of the TMS and DMTBS derivatives of

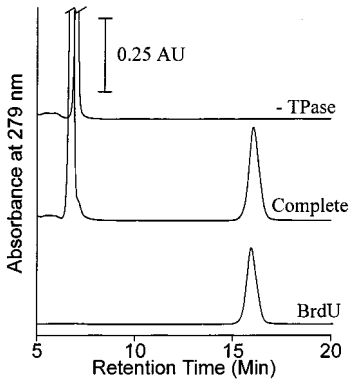


FIGURE 8: Reversed-phase HPLC analysis of 5-bromouracil incubated with thymidine phosphorylase. Uracil was oxidized by eosinophil peroxidase, and the resulting 5-bromouracil was isolated by HPLC and incubated with thymidine phosphorylase (5 units) and deoxribose-1-phosphate (10 mM) for 60 min at 37 °C (−complete). Where indicated, thymidine phosphorylase was omitted (−TPase), or authentic 5-bromodeoxyuridine (BrdU) was analyzed.

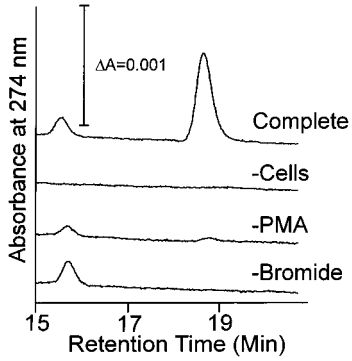


FIGURE 9: Reversed-phase HPLC analysis (A) and UV−vis spectroscopy (B) of uracil oxidized by activated human eosinophils. Eosinophils (5×10^5/mL) in Hank's balanced salt solution (pH 7) containing 1 mM uracil, 100 μM NaBr, and 100 mM NaCl (complete) were stimulated with 200 nM phorbol myristate acetate. The cells were maintained in suspension by intermittent inversion and incubated for 60 min at 37 °C. Reactions were terminated by removing the cells with low speed centrifugation and then adding L-methionine (final concentration 6 mM). Reaction products were analyzed by reversed-phase HPLC. When indicated, eosinophils (−cells), phorbol ester (−PMA), or bromide was omitted from the medium.

5-bromouracil (Figure 10). The full scan positive ion mass spectrum of the TMS derivative of the compound contained the characteristic ^{79}Br and ^{81}Br peaks of the $M^{\bullet+}$ at *m*/*z* 334 and 336. The base peak of the full scan mass spectrum exhibited $[M^{\bullet+} - CH_3^{\bullet}]$ ions characteristic of TMS derivatives of nucleobases at *m*/*z* 316 and 318, respectively. The spectrum of the TMS derivative also contained an ion consistent with loss of $Br^{\bullet}$ at *m*/*z* 255. The base peak of the DMTBS full scan mass spectrum exhibited the prominent $[M^{\bullet+} - \textit{tert}\text{-butyl}^{\bullet}]^+$ ions characteristic of DMTBS derivatives at *m*/*z* 361 and 363.

5-Bromouracil generation by activated eosinophils increased rapidly at plasma concentrations of bromide ion (0−100 μM, Figure 11). The reaction was largely complete 30 min after the cells were stimulated with phorbol ester. 5-Bromouracil formation required activation of the cells with phorbol ester; it was inhibited by heme poisons and catalase, implicating a peroxidase and H_2O_2 in the reaction. The cell-mediated reaction was not affected by millimolar concentrations of taurine, which is consistent with *N*-bromotaurine's

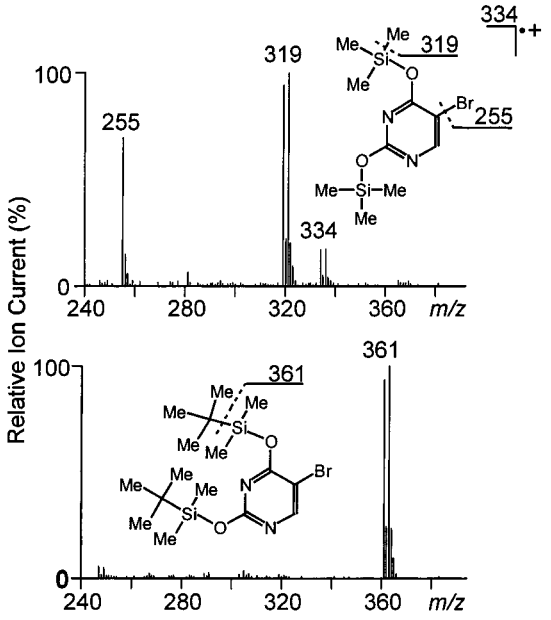


FIGURE 10: Positive ion mass spectra of the TMS (top) and DMTBS (bottom) derivatives of 5-bromouracil generated by activated human eosinophils. The cell product was purified by HPLC, derivatized, and subjected to GC/MS analysis. Note the ~1:1 ratio of ions separated by two mass units characteristic of ^{79}Br and ^{81}Br in the $[M]^{\bullet+}$, $[M - {}^{\bullet}CH_3]^+$, and $[M - t\text{-butyl}^{\bullet}]^+$ ions. Also note the lack of a bromine isotope pattern in the $[M - Br^{\bullet}]^+$ ion. GC retention times and mass spectra were essentially identical to those of the bis-TMS and bis-DMTBS derivatives of authentic 5-bromouracil.

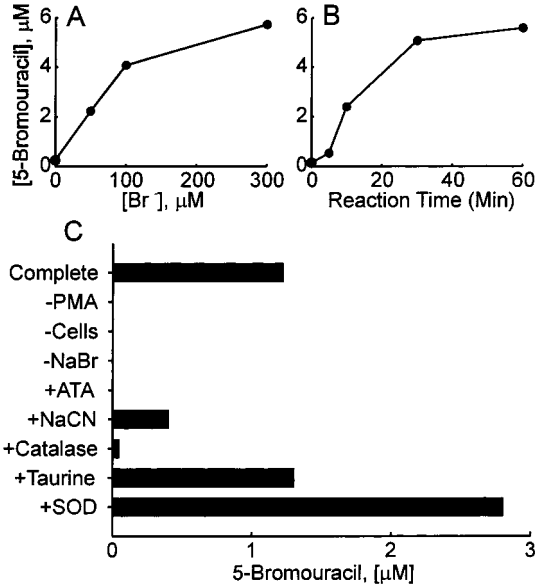


FIGURE 11: Bromide dependence (A), progress curve (B), and reaction requirements (C) for generation of 5-bromouracil by activated human eosinophils. Eosinophils (5×10^5/mL) in Hank's balanced salt solution (pH 7) containing 1 mM uracil and 100 μM NaBr were stimulated with 200 nM phorbol myristate acetate and incubated for 60 min at 37 °C. Reactions were terminated by removing the cells with low speed centrifugation; then 6 mM L-methionine was added. Reaction conditions were varied as indicated. 5-Bromouracil was quantified by reversed-phase HPLC. PMA, phorbol ester; ATA, 10 mM 3-aminotriazole; NaCN, 10 mM sodium cyanide; catalase, 10 μg/mL; taurine, 10 mM; SOD, 10 μg/mL superoxide dismutase. 5-Bromouracil was quantified by reversed-phase HPLC.

ability to brominate uracil (Figure 7). In contrast, superoxide dismutase enhanced the product yield, perhaps by increasing the availability of H_2O_2 or preventing $O_2^{\bullet-}$ from inactivating eosinophil peroxidase (*33*).

DISCUSSION

Our observations indicate that the eosinophil peroxidase-H_2O_2 system of activated human eosinophils can oxidize uracil at plasma concentrations of Cl^- and Br^- by a reaction pathway that involves HOBr or bromamines. Several lines of evidence indicated that 5-bromouracil was the pathway's major product. First, the HPLC retention time and absorption spectrum of the single major uracil oxidation product was identical to that of authentic 5-bromouracil. Uridine and deoxyuridine exposed to reagent HOBr likewise yielded a single major oxidation product that comigrated on reversed-phase HPLC with the corresponding authentic brominated pyrimidine. Second, the retention time on gas chromatography and mass spectrum of two different derivatives of the oxidation product were essentially identical to those of 5-bromouracil. Third, the ^1H NMR spectrum of the reaction product was indistinguishable from that of 5-bromouracil. Fourth, the thymine salvage enzyme thymidine phosphorylase used deoxyribose-1-phosphate to convert the reaction product into a compound that comigrated with 5-bromodeoxyuridine on HPLC. Fifth, eosinophil peroxidase required bromide to generate the product, but the enzyme system could be replaced with reagent HOBr. Collectively, these results indicate that reactive species generated by eosinophil peroxidase brominate uracil at the 5-position.

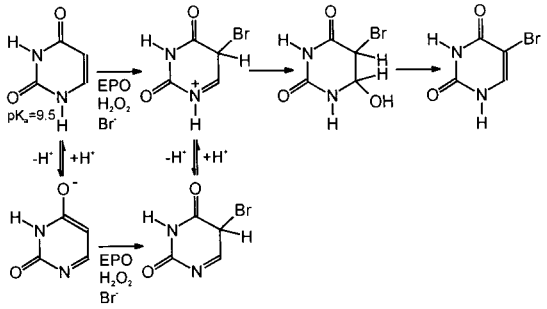
Bromination of uracil by HOBr exhibited a number of important features. It proceeded in near-quantitative yield (relative to oxidant) at neutral pH, suggesting that the reaction could be physiologically relevant. Moreover, both primary and secondary bromamines brominated uracil in high yield over a broad pH range. HOBr reacts rapidly with amine groups, and bromamines are relatively long-lived species, raising the possibility that HOBr-derived oxidants might brominate nucleobases in vivo.

It is important to note that eosinophil peroxidase converted uracil into 5-bromouracil in near-quantitative yield (relative to H_2O_2) and that human eosinophils generated micromolar concentrations of 5-bromouracil by a reaction that required H_2O_2, Br^-, and cellular activation. These results suggest that eosinophils might brominate nucleobases at sites of inflammation.

N-Chloropyrimidines have been proposed to mediate chlorination reactions under strongly acidic conditions (*22*, *32*, *34*). A reaction between HOBr and the secondary amine of uracil might similarly produce produce N_1- or N_3-bromouracil, secondary bromamines that are structurally similar to *N*-bromosuccinimide, a widely used brominating reagent. We therefore investigated the ability of HOBr and bromamines to generate *N*-bromouracil, but we were unable to observe the postulated species in reaction mixtures as assessed by HPLC analysis. However, we cannot exclude the possibility that *N*-bromouracil is an intermediate in the bromination of uracil by HOBr because uracil bromamine may be a relatively short-lived species.

Product and kinetic analyses suggest that the bromination of uracil by HOBr involves the initial formation of a bromohydrin (Scheme 1; refs *35* and *36*). The halohydrin

Scheme 1



intermediate then rapidly dehydrates to form 5-bromouracil. For 1-substituted uracils such as the nucleosides uridine and deoxyuridine, the bromohydrin accumulates and dehydrates to the corresponding 5-bromouracil at a much slower rate, which may explain the lower yield of 5-bromouridine and 5-bromodeoxyuridine we observed. The facile generation of 5-bromouracil by eosinophil peroxidase and HOBr at neutral pH may be explained in part by reaction with uracil anion, which is brominated at a nearly diffusion-controlled rate ($k \approx 6 \times 10^9$ M^{-1} s^{-1}) (*36*).

HOBr is the initial brominating oxidant generated by eosinophil peroxidase. However, intermediates such as Br_2 and BrCl are much more effective at brominating aromatic compounds than is HOBr itself. Moreover, Br_2 and BrCl are in equilibrium with HOBr by transhalogenation reactions that require Br^- and Cl^-.

$$HOBr + Br^- + H^+ \rightleftarrows Br_2 + H_2O \quad (2)$$

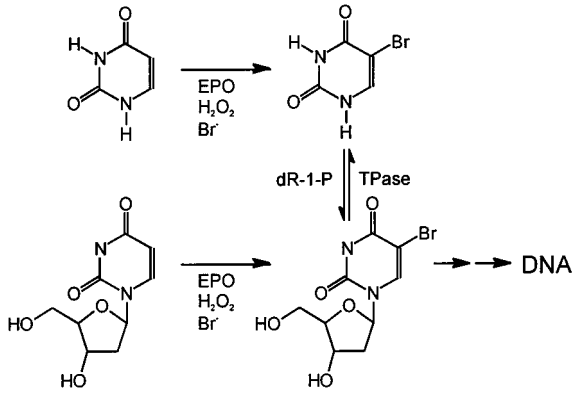
$$HOBr + Cl^- + H^+ \rightleftarrows BrCl + H_2O \quad (3)$$

It is thus possible that stronger oxidants such as Br_2 or BrCl derived from HOBr in the presence of physiologic concentrations of Cl^- are involved in the bromination of uracil.

Mammalian cells can incorporate 5-fluorouracil, 5-chlorouracil, and 5-bromouracil into their DNA (*37*−*39*). The halogenated nucleobase is taken up by cells and converted to the corresponding deoxynucleoside by thymidine phosphorylase in a reaction that requires deoxyribose-1-phosphate (Scheme 2). Purine nucleosides greatly enhance the incorporation of 5-bromouracil into cellular DNA by serving as a source of deoxyribose-1-phosphate (*39*, *40*). Such a situation may occur at sites of inflammation, where cell death and lysis may liberate deoxynucleosides into the extracellular environment. It is noteworthy that deficiencies of folate or other components of the thymidine biosynthetic pathway are associated with an increased risk of cancer (*41*, *42*). It is possible that folate deficiency increases the use of thymine analogues such as 5-bromouracil to replace thymidine in the cell.

Because 5-bromouracil is a thymine analogue that can be taken up by cells and incorporated into DNA and 5-bromouracil is a well-established mutagen that mispairs with guanine (*43*−*45*), our findings suggest a mechanism by which activated eosinophils could damage DNA. In this scenario, brominating agents generated by eosinophils convert extracellular or intracellular uracil to 5-bromouracil. In cells that express pyrimidine salvage enzymes, the 5-bromouracil is converted to 5-bromodeoxyuridine, which is phosphorylated and becomes a substrate for DNA polymerases. In support of this hypothesis, we demonstrated that thymidine phosphorylase converts 5-bromouracil into 5-bromodeoxyuridine.

Scheme 2



Extracellular concentrations of pyrimidines may increase significantly at sites of inflammation as a result of tissue injury, thereby providing high local concentrations of substrate for halogenation by phagocytes. Proliferating cells could incorporate the resulting mutagenic base analogues into DNA during the reparative response to injury; therefore, the mutagenic effects of phagocytes could persist in the inflammatory environment even after cells stopped making oxidants. Indeed, tissue injury, oxidant generation by phagocytes, and tissue repair may synergistically contribute to cytotoxicity and increase the risk of cancer at sites of chronic inflammation. Detection of 5-bromouracil as a free base or a component of DNA at sites of inflammation would strongly support the hypothesis that eosinophils use eosinophil peroxidase to generate potentially mutagenic base analogues in vivo.

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