Oxidation of Aminopyrine by Hypochlorite to a Reactive Dication: Possible Implications for Aminopyrine-Induced Agranulocytosis

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Aminopyrine is associated with a high incidence of agranulocytosis. It is known to be oxidized by peroxidases and hypochlorous acid to a blue cation radical. It has been proposed that the mechanism by which hypochlorous acid oxidizes aminopyrine to a cation radical involves N-chlorination followed by loss of a chlorine radical. Another possible mechanism is loss of HCl to form an iminium ion and subsequent reaction with another molecule of aminopyrine and a hydrogen ion to form two radical cations. This mechanism would lead to incorporation of a hydrogen from water; however, using a deuterated analog, we found no hydrogen incorporation, thus providing strong evidence against this mechanism. Using a stopped-flow diode array spectrophotometer to study the reaction between aminopyrine and hypochlorous acid, an intermediate with a λ_{max} at ~420 nm was observed in the formation of the cation radical. We propose that this represents a dication formed by the loss of chloride ion from *N*-chloroaminopyrine. This intermediate is very reactive, with a half-life of approximately 15 ms, and in addition to being the precursor of the cation radical, it also appears to react with two molecules of water to form several other products that were observed and are consistent with the proposed dication intermediate. Similar stable products were formed when aminopyrine was oxidized by the combination of myeloperoxidase, hydrogen peroxide, and chloride or activated neutrophils. The reactive dication formed by neutrophil-derived hypochlorous acid could be responsible for aminopyrine-induced agranulocytosis.

Introduction

The analgesic aminopyrine was one of the first drugs to be associated with the induction of agranulocytosis (*1*). It is no longer licensed for use in most countries because of this risk; however, there are "herbal" medications available in North America which contain significant amounts of aminopyrine (*2*). Several studies found strong evidence to suggest that the mechanism of aminopyrine-induced agranulocytosis involves a drug-dependent antineutrophil antibody (*1*, *3*). It is likely that the induction of an antibody would require covalent binding of the drug, or a reactive metabolite of the drug, to neutrophils (*4*). It has been demonstrated that aminopyrine is oxidized to a cation radical by peroxidases such as horseradish peroxidase and prostaglandin synthase (*5*). We have shown that many of the drugs that are associated with agranulocytosis are metabolized to reactive metabolites by activated neutrophils (*6*, *7*). The major neutrophil enzyme involved in such oxidations is myeloperoxidase (MPO).[1] MPO differs from many other peroxidases, which commonly involve a one electron oxidation, because the usual substrate is chloride which undergoes a two electron oxidation to hypochlorite or an activated chlorine bound to MPO that mimics hypochlorite (*8*). Sayo and Saito as well as Kalyanaraman and Sohnle have shown that aminopyrine is oxidized to its cation radical by the combination of MPO or chloroperoxidase, hydrogen peroxide and chloride or simply hypochlorous acid (*9*, *10*, *11*). Sayo and Saito proposed that the mechanism involves N-chlorination followed by loss of a chlorine radical to form the cation radical of aminopyrine (Figure 1). We set out to further test this hypothesis and other possible mechanisms of cation radical formation by hypochlorous acid in search of the species most likely to covalently bind to neutrophils, and therefore possibly responsible for aminopyrine-induced agranulocytosis. Another possible mechanism for formation of the cation radical is the loss of HCl to form an

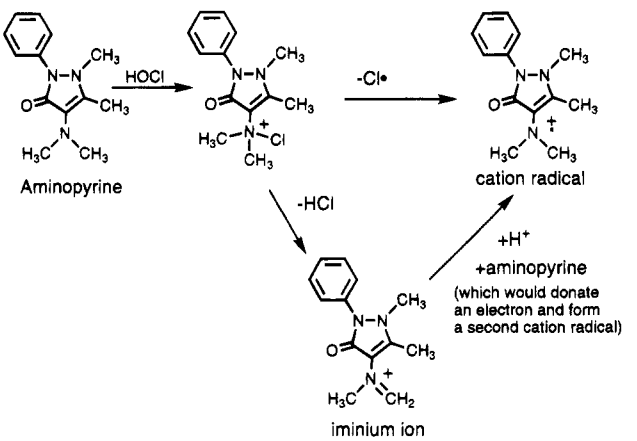

Figure 1. Theoretical mechanisms by which oxidation of aminopyrine by hypochlorite could lead to the observed cation radical.

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[1] Abbreviations: MPO, myeloperoxidase; PMA, phorbol myristate acetate; [AP], concentration of aminopyrine; k_{obs}, observed rate constant.

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iminium ion which could then react with another molecule of aminopyrine and a hydrogen ion to form two cation radicals (Figure 1). The loss of HCl could result in a deuterium isotope effect if this is the rate-limiting step, and it should result in the incorporation of a hydrogen from water in one of the two cation radicals formed. Alternatively, loss of chloride ion would form a dication which could also react with another molecule of aminopyrine to form two cation radicals.

Materials and Methods

Materials. MPO was obtained from Cortex Biochem Inc. (San Leandro, CA). Phorbol myristate acetate (PMA) and aminopyrine were obtained from Sigma Chemical Co. (St. Louis, MO). Aminoantipyrine was obtained from Aldrich Chemical Co. (Milwaukee, WI). Deuterium-labeled methyl iodide was obtained from MSD Isotopes (Montreal, Quebec). Hanks' balanced salt solution was purchased from Gibco Laboratories (Grand Island, NY).

Synthesis of Deuterium-Labeled Aminopyrine. Aminoantipyrine (0.1 g, 0.49 mmol) was reacted with deuterium-labeled methyl iodide (0.1836 mL, 2.95 mmol) in methanol (3 mL) at a temperature of 42 °C for 24 h. TLC (silica gel developed with acetone) of the product gave three spots corresponding to unreacted aminoantipyrine, monomethylated species, and aminopyrine with R_fs of 0.33, 0.43, and 0.65, respectively. Purification by silica gel column chromatography using acetone as the mobile phase gave pure deuterated aminopyrine as determined by TLC with a melting point of 106–109.5 °C at a yield of 12%.

Detection of Intermediates of Aminopyrine Oxidation by HOCl and Br_2 Using Stopped-Flow Diode Array Spectroscopy. The reaction of aminopyrine (15 mM in 0.1 M sodium acetate buffer, pH 5.0) with NaOCl (11 mM in 0.1 M sodium acetate buffer, pH 5.0) was studied with a stopped-flow diode array spectrometer (custom-made by Professor A. Queen, University of Manitoba, Manitoba). The spectra were recorded from 310 to 560 nm every 5.4 ms using a tungsten lamp as the light source. In the same manner, oxidation of aminopyrine (15 mM in 0.1 M sodium acetate buffer, pH 5.0) by Br_2 (11 mM in 0.1 M sodium acetate buffer, pH 5.0) was studied.

Stopped-Flow Kinetic Experiments. These were carried out using a High-Tech Scientific (Salisbury, U.K.) apparatus equipped with a Model SF-51 drive unit and a Model SU-40 spectrophotometer. Absorbance–time traces were digitized and processed with a Hewlett-Packard Model 300 computer. The oxidant (HOCl or Br_2, 0.5 mM) dissolved in 0.3 M acetate buffer, pH 5 (0.2 M NaOAc, 0.1 M HOAc), was placed in one syringe of the apparatus, with various concentrations of aminopyrine in the same buffer in the other syringe. The solutions were thermostated at 25 °C before being mixed. Absorbance–time traces were obtained at 565 and 420 nm for time intervals of 0.05–0.5 s. First-order rate constants for the increase in absorbance at 565 nm were obtained by nonlinear least-squares fitting of the absorbance–time data to a single exponential expression, although this approach required that a portion of the initial data be omitted in the experiments with HOCl, particularly at the lower aminopyrine concentrations (see Results). In the experiments with HOCl, first-order rate constants for the rapid initial increase at 420 nm were obtained by fitting to an equation with two exponentials.

Deuterium Isotope Effect on Oxidation of Aminopyrine to the Cation Radical by Hypochlorite. Solutions of aminopyrine and deuterated aminopyrine were prepared with identical concentrations (0.898 mM) in 0.1 M acetate buffer (pH 5) and mixed in the High-Tech stopped-flow apparatus with 0.18 mM HOCl in the same buffer. First-order rate constants for the increase in absorbance at 565 nm were obtained as described above by a fit to a single exponential equation.

Proton Exchange Experiment Using Deuterated Aminopyrine. Deuterated aminopyrine (0.013 mmol in 0.1 M sodium acetate buffer, pH 5.0) was reacted with NaOCl (0.01 mmol) to give a purple color. After 1 min, vitamin C was added to the reaction mixture until the purple color was gone. This solution was extracted with chloroform (3 × 5 mL). The chloroform layer was dried with magnesium sulfate and the solvent evaporated with a stream of nitrogen. The aminopyrine was analyzed for deuterium content by mass spectrometry (VG-Analytical ZAB-SE mass spectrometer, Manchester, U.K., electron impact ionization 70 eV, source temperature 200 °C).

Oxidation by MPO/H_2O_2/Cl^-. The base-line conditions for the incubations were as follows: MPO concentration, 5 units/mL; aminopyrine concentration, 0.1 mM; hydrogen peroxide concentration, 0.2 mM; incubation medium, 0.2 of mL phosphate-buffered saline (pH 6.4); temperature 23 °C; and incubation time 10 min. These conditions were varied to determine their influence on oxidation.

Oxidation by Neutrophils. Blood was drawn from normal volunteers into a heparinized syringe, and polymorphonuclear leukocytes (mostly neutrophils and therefore hereafter referred to as neutrophils) were isolated using the method of Boyum (*12*).

Aminopyrine was added to neutrophils which had been suspended in Hanks' balanced salt solution. The cells were activated by the addition of phorbol myristate acetate (PMA). The suspension was incubated at 37 °C in a shaking water bath for 40 min. The base-line conditions for the incubations were as follows: neutrophil concentration, 6×10^6 cells/mL; aminopyrine concentration, 0.1 mM; PMA, 40 ng/mL of final volume (stock concentration, 2 ng/μL in dimethyl sulfoxide); incubation medium, 500 μL of Hanks' balanced salt solution (pH 7.4); temperature 37 °C; and incubation time 40 min.

HPLC. The incubation mixture from the MPO system was injected onto the HPLC column without further preparation. The incubation mixture with neutrophils was centrifuged at 13000*g* for 1 min to sediment the cells, and the supernatant was injected onto the HPLC column.

For analysis of deuterium-labeled aminopyrine and studies of aminopyrine oxidation using neutrophils, the MPO system, and HOCl, the HPLC system consisted of a Beckman 110B pump (Berkeley, CA), a 4.6 × 150 mm column packed with a 5-μm Spherisorb ODS II (Jones Chromatography, Mid Glamorgan, U.K.), and a Shimadzu C-R3A Chromatopac (Tokyo, Japan) at a wavelength of 240 nm. The solvent for analysis of deuterium-labeled aminopyrine consisted of water/acetonitrile/acetic acid/triethylamine (90:10:1:0.05 v/v) at a flow rate of 1 mL/min. Under these conditions, the retention times of unreacted aminoantipyrine, monomethylated deuterated species, and deuterated aminopyrine were 5.9, 11.8, and 6.9 min, respectively.

Immediate Analyses of Aminopyrine Oxidation Products by Mass Spectrometry in a Flow System. Aminopyrine (0.1 mM in 0.1 M sodium acetate buffer, pH 5.0) and NaOCl (0.1 mM in 0.1 M sodium acetate buffer, pH 5.0) were placed in separate syringes. These solutions were pumped into an Upchurch mixing chamber (Upchurch Scientific, Oak Harbor, WA) with a dead volume of 3.1 μL and from there into a Sciex mass spectrometer (Sciex API III, Sciex, Thornhill, Ontario).

The reaction of aminopyrine (0.5 mM) with Br_2 (0.5 mM, Aldrich Chemical Co. Milwaukee, MI) in water containing 1 μM acetic acid was also studied with the Sciex mass spectrometer.

Analysis of Stable Products of the Oxidation of Aminopyrine by Hypochlorite by LC/MS. NaOCl (0.064 mmol) was reacted with aminopyrine (0.22 mmol) in phosphate buffer at pH 6.0. Similarly, Br_2 (0.064 mmol) was added to aminopyrine (0.22 mmol) in the same buffer. The products of these two reactions were analyzed by LC/MS using an Ultracarb ODS 30 HPLC column (2 × 100 mm, 5 μm, Phenomenex, Torrance, CA) and a solvent of water, acetonitrile, and acetic acid (90:10:1 v/v). This was interfaced with a Sciex API III mass spectrometer using a splitter to reduce the flow to 60 μL/min. Daughter ion spectra were obtained from molecular ions by collisional activation with argon gas. The parent ion spectra of the m/z 203 ion was obtained in order to determine its origin.

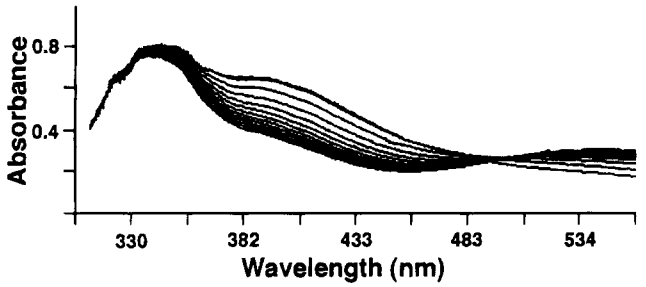

Figure 2. Absorption spectra from the reaction between aminopyrine (15 mM) and NaOCl (11 mM) in 0.1 M sodium acetate buffer (pH 5), with a 5.4 ms interval between spectra. The first spectrum is just below but almost the same as the top spectrum at 420 nm, the next is the top spectrum, and subsequent spectra go down from there.

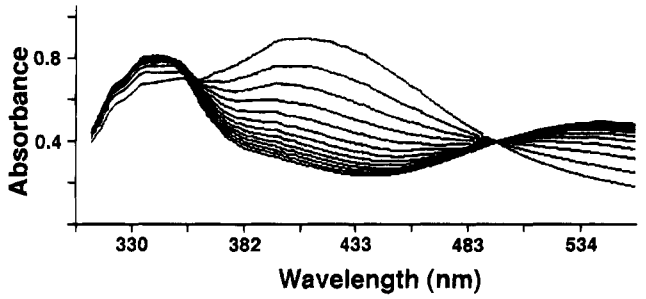

Figure 3. Absorption spectra from the reaction between aminopyrine (15 mM) and Br_2 (11 mM) in 0.1 M sodium acetate buffer (pH 5), with a 5.4 ms interval between spectra. The first spectrum is the top spectrum at 420 nm, and subsequent spectra go down from there.

Results

Stopped-Flow Study: Detection of Intermediate. Repetitive scans (interval between scans = 5.4 ms) for the reaction of aminopyrine with hypochlorous acid are shown in Figure 2. The first scan shows that an intermediate with λ_{max} at 400–440 has formed. The second scan shows a slight increase in this absorbance while subsequent scans show a decrease at this wavelength, with a parallel increase in another species with a λ_{max} near 560 nm, and a reasonable isosbestic point near 500 nm. The species at 560 nm can be identified as the cation radical of aminopyrine (*13*). While this does decay at much longer times (over 10 min), on the time scale of the stopped-flow experiments described in this and the next section, there is no decay.

A similar sequence is seen with the reaction of aminopyrine and bromine, except that the initial formation of the intermediate with λ_{max} near 420 nm is much faster (Figure 3), so that only a decay is observed at this wavelength. This was true even at 20-fold lower concentrations of aminopyrine (data not shown).

Stopped-Flow Study: Kinetics. These experiments were carried out with aminopyrine in at least a 10-fold excess over HOCl or Br_2. Representative traces of the changes in absorbance at 565 nm, the reported λ_{max} of the cation radical (*13*), and 420 nm, the approximate λ_{max} of the initial intermediate, are shown in Figure 4. These curves corroborate and elaborate the observations made in the repetitive scan experiments. The intermediate species that precedes the cation radical is clearly observed with both oxidants. This intermediate is completely formed within the 2 ms dead time of the stopped-flow apparatus with bromine. With HOCl, however, its formation can be observed, although this is still a very rapid process.

With bromine, the data at the two wavelengths can be satisfactorily fit by a single exponential. For the conditions of Figure 4, these provide $k_{obs} = 58\ s^{-1}$ for the increasing absorbance at 565 nm and $k_{obs} = 56\ s^{-1}$ for the decreasing absorbance at 420 nm; the two numbers are the same within experimental error. For HOCl, the increasing absorbance at 565 nm can also be satisfactorily fit by a single exponential, although this requires that a portion of the data at early times be ignored. For the data in Figure 4, this provides $k_{obs} = 60\ s^{-1}$, with the data from 0 to 0.01 s being deleted in the fitting process. The curve at 420 nm requires fitting to an expression with two exponentials, one corresponding to a rapid rising absorbance, and a second for a slower decrease. For the data of Figure 4, k_{obs} for the rising absorbance is $2.8 \times 10^2\ s^{-1}$, with k_{obs} for the falling absorbance $54\ s^{-1}$. The latter is within experimental error the same as the k_{obs} measured at 565 nm. Moreover, comparing the results for the two oxidants, one sees that the four rate constants, those for the 565 nm increase with each oxidant, and those for the 420 nm decrease with each, are identical. A further observation is that the amount of cation radical as measured by the final OD at 565 nm is within experimental error the same in the two experiments.

A set of experiments was performed with hypochlorite as the oxidant and varying concentrations of the excess aminopyrine. Rate constants are plotted as a function of aminopyrine concentration in Figure 5. These k_{obs} were obtained as described in the previous paragraph, the initial rise at 420 nm from a double exponential fit, and the conversion of the 420 nm species to the cation radical from the increase at 565 nm. The rapid appearance of the 420 nm intermediate proved to be first-order in the aminopyrine concentration with $k_2 = (2.8 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$. The intercept of this plot is not statistically significantly different than zero (-7 ± 34). The formation of the cation radical is also first-order in aminopyrine, but in this case there is a nonzero intercept. Thus k_{obs} for this process follows the expression $k_0 + k_{AP}[AP]$, with $k_0 = 44 \pm 1\ s^{-1}$ and $k_{AP} = (2.07 \pm 0.05) \times 10^3\ M^{-1}\ s^{-1}$. It can be seen that, at low concentrations of aminopyrine, k_{obs} for the initial formation of the 420 nm intermediate actually becomes similar to and even smaller than k_{obs} for the subsequent reactions of this species. Under these circumstances the increasing absorbance at 565 nm deviates significantly from single exponential behavior. It was also noted that the rate constants were sensitive to pH and ionic strength, although these effects were not investigated in detail.

These experiments were carried out with the same concentration of HOCl throughout. Despite this, the total amount of cation radical that formed, as measured by the plateau absorbance at 565 nm, depended on the concentration of aminopyrine. As shown in Figure 6, this dependence was not linear, with the amount of cation radical starting to level off at the higher concentrations of aminopyrine.

Isotope Effect and Deuterium Exchange. In experiments carried out under identical conditions, the mean pseudo-first-order rate constants for the reaction of hypochlorous acid with aminopyrine and its hexadeuterio analog were $21.9 \pm 1.3/s$ (SD, $n = 4$) and $21.7 \pm 0.1/s$ (SD, $n = 4$), respectively. These rate constants are not significantly different, and we conclude that there is no isotope effect associated with the dimethylamino hydrogens.

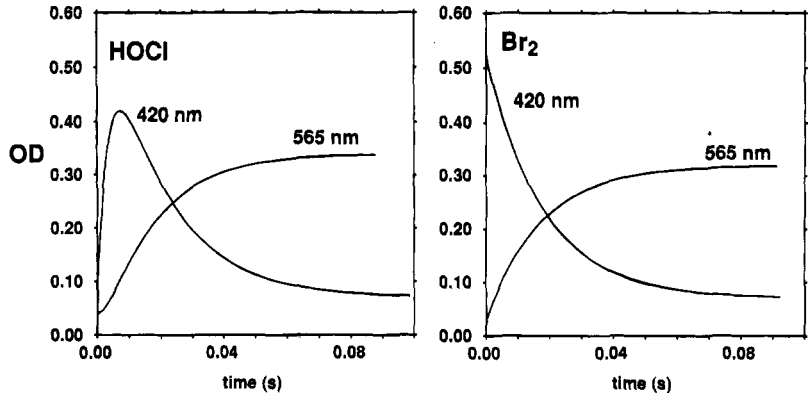

Figure 4. Changes in absorbance at 420 and 565 nm during the oxidation of aminopyrine by HOCl or Br_2. Concentrations after mixing are 0.25 mM HOCl or Br_2 and 10 mM aminopyrine in 0.3 M acetate buffer (pH 5).

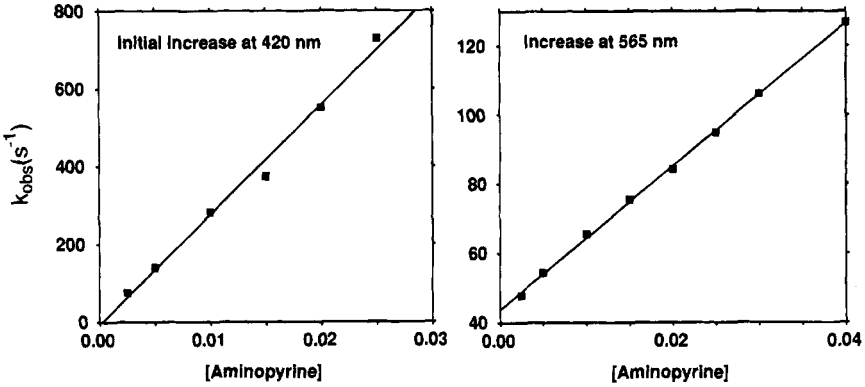

Figure 5. Observed rate constants for the reaction of HOCl (25 mM) with aminopyrine [M] at 25 °C in 0.3 M acetate buffer (pH 5). Lines are the least-squares fit to the data.

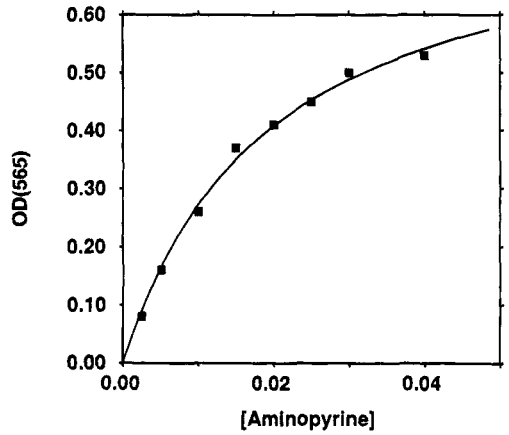

Figure 6. Final optical density of the aminopyrine cation radical at 565 nm as a function of aminopyrine concentration [M]. Conditions are 25 mM HOCl and 0.3 M acetate buffer (pH 5). The optical density was obtained as the plateau absorbance at 565 nm at 0.2–0.5 s in stopped-flow traces such as shown in Figure 3. The curve has been drawn according to the equation presented in the Discussion using the parameters obtained in the fitting procedure.

The molecular ion of hexadeuterated aminopyrine is at m/z 237. An increase in the size of the peak at m/z 236 relative to 237 after formation of the cation radical and reducing it with ascorbate would represent exchange of hydrogen for deuterium. Before reaction the peak at m/z 236 was 1.54 ± 0.12% of the 237 peak, and after the reaction it was 1.44 ± 0.19%, which indicates that no significant hydrogen exchange occurred during the reaction.

Mass Spectral Analysis of the Products of the Reaction of Aminopyrine with HOCl and Br_2 in the Continuous Flow System. The major peaks observed in the mass spectra of the effluent produced by the mixing of aminopyrine and NaOCl were at m/z 203 (2.5%), 221 (5%), 230 (5%), 231 (50%), 232 (100%), 239 (5%), 254 (95%), and 264 (8%). Thus the major ions (232 and 254) were due to the parent drug (the peak at m/z 254 represents the sodium adduct ion of aminopyrine). In order to increase the rate of the reaction and eliminate the sodium adduct ions, Br_2 was utilized instead of NaOCl. The major peaks observed in the mass spectra of the effluent produced by the mixing of aminopyrine and Br_2 were 203 (13%), 221 (100%), 231 (64%), 232 (70%), 239 (58%), 264 (71%), and 266 (78%). The m/z peak of 221 represented the loss of dimethylamine and addition of two hydroxyl groups. Similarly, the m/z peaks of 239 and 203 represented gain or loss of water, respectively, from the 221 ion. Moreover, the m/z peak of 264 represented an oxidation product in which two oxygen atoms had been added to aminopyrine. Similarly, the m/z peak of 266 represented the addition of two hydroxyl groups to aminopyrine.

Daughter Ion and Parent Ion Mass Spectra. Daughter ion spectra of the m/z 203, 221, 239, 264, and 266 ions formed by aminopyrine oxidation were obtained on a Sciex API III mass spectrometer operating in the ion spray mode. The daughter ion spectrum of the m/z 203 ion consisted of fragment ions at m/z 56 (8%), 77 (3%), 83 (3%), 118 (11.5%), 157 (26%), 160 (17.7%), and 185 (22%). The daughter ion spectrum of the m/z 221 ion consisted of fragment ions at m/z 43 (62%), 56 (80%), 65 (7.6%), 77 (6.3%), 92 (22%), 102 (5.4%), and 120 (10%). The daughter ion spectrum of the m/z 239 ion consisted of fragment ions at m/z 43 (100%), 56 (12%), 65 (7.9%), 77 (12.5%), 92 (20%), 101 (12%), 121 (22%), 203 (2%), and 221 (25%). The daughter ion spectrum of the m/z 264

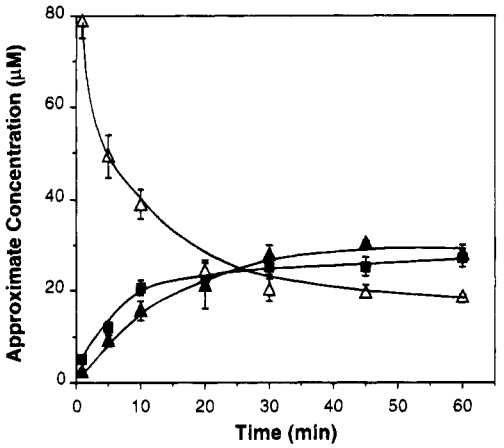

Figure 7. Oxidation of aminopyrine with MPO/H_2O_2/Cl^- as a function of time showing the decrease in the concentration of aminopyrine (△) and the increase in concentrations of the two major products with protonated molecular ions at m/z 248 (▲) and 221 (■) in the mass spectrometer. The concentration of the products is only approximate because standards were not available, and the reported concentrations are based on the assumption that the extinction coefficients of the metabolites are the same as that of aminopyrine at 240 nm. The values represent the mean ± SD from 4 experiments.

ion consisted of fragment ions at m/z 46 (3%) representing protonated dimethylamine, 72 (100%), 92 (4%), 149 (11.4%), 163 (7.1%), 191 (28.5%), and 222 (20%). The daughter ion spectrum of the m/z 266 ion consisted of fragment ions at m/z 46 (100%), 56 (12%), 92 (2%), 203 (2%), and 221 (10%). Parent ion spectrum of the m/z 203 ion demonstrated that the m/z 203 ions were derived from the ions at m/z 221 (20%) and 239 (21.8%).

Evidence for Dimethylamine as a Product of Aminopyrine Oxidation by H-NMR. Aminopyrine (0.05 g) was reacted with Br_2 (2.2 μL) in deuterium oxide (D_2O, MSD Isotopes, Montreal, Quebec) without using any buffer system. The reaction mixture was analyzed by a Gemini-200 H-NMR spectrometer at 200 MHz (Varian, USA). A singlet at δ 2.7 was observed, and the addition of a pure crystal of dimethylamine hydrochloride (Aldrich Chemical Co.) increased the size of this peak.

Comparison of Aminopyrine Oxidation by HOCl, Br_2, MPO/H_2O_2/Cl^- and Activated Neutrophils. Three major stable products were formed from the oxidation of aminopyrine by hypochlorite or bromine. Using LC/MS, they were found to have retention times and protonated molecular ions of 4.7 min (m/z 248), 12.7 min (m/z 203, 221, and 239), and 27.8 min (m/z 264) while that of aminopyrine was 8.0 min (m/z 232). The ions at m/z 203, 221, and 239 coeluted. The parent ion spectrum of the peak at m/z 203 indicated that it came from 221 and 239 and the daughter ion spectrum of the peak at m/z 239, gave a major ion at m/z 221 and a smaller ion at m/z 203. Thus these ions represent different states of hydration of the same product which could be in equilibrium on the HPLC column as well as being formed in the ion source of the mass spectrometer.

The products produced by oxidation of aminopyrine by hypochlorite, MPO/H_2O_2/Cl^-, and activated neutrophils were essentially the same; however, the major product from neutrophils was the product with an M + 1 ion at m/z 264 while the major products from hypochlorite and MPO were those with protonated molecular ions of m/z 221 and 248. Oxidation of aminopyrine by MPO/H_2O_2/Cl^- was monitored by HPLC and is shown as a function of time in Figure 7. A peak with a retention time of 27.8 min and a protonated molecular ion of m/z 264 was also present, but it was too small to adequately show on the scale of this figure. We did not have enough product to determine a standard curve, and therefore the concentrations are only approximate, but it does not appear that the observed products account for all of the aminopyrine that is oxidized. The amount of product decreases with increasing H_2O_2 concentration above 2.5 mM, and the pH optimum is at about 6 (data not shown).

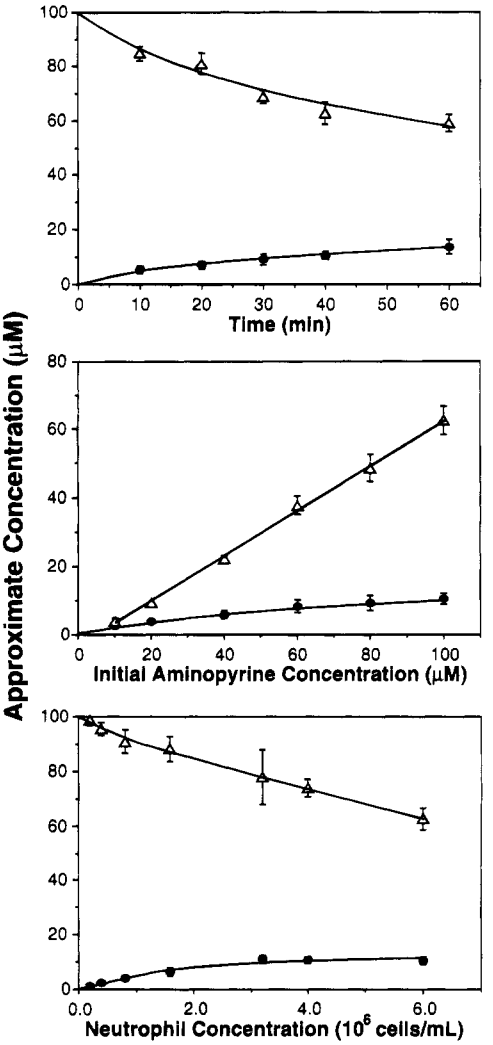

Figure 8. Metabolism of aminopyrine by activated neutrophils as a function of time, initial aminopyrine concentration, and neutrophil concentration showing a decrease in aminopyrine concentration (△) and the production of the major product (●) which has a protonated molecular ion at m/z 264 by mass spectroscopy. Base-line conditions were aminopyrine concentration, 0.1 mM; neutrophil concentration, 6 million cells/mL; and time, 40 min. The concentration of the product is approximate and is based on the assumption that the extinction coefficient of the product is the same as that of aminopyrine at 240 nm. The values represent the mean ± SD from 4 experiments.

The major product formed by oxidation of aminopyrine by activated neutrophils had a retention time of 27.8 min and a protonated molecular ion at m/z 264 with smaller amounts of the products with retention times of 4.7 min (M + 1 at m/z 248) and 12.7 min (M + 1 at m/z 203, 221, 239). Oxidation of aminopyrine to the major product as a function of time and aminopyrine and neutrophil concentration is shown in Figure 8. As before, the concentrations are only approximate because standards were not available to construct a standard curve.

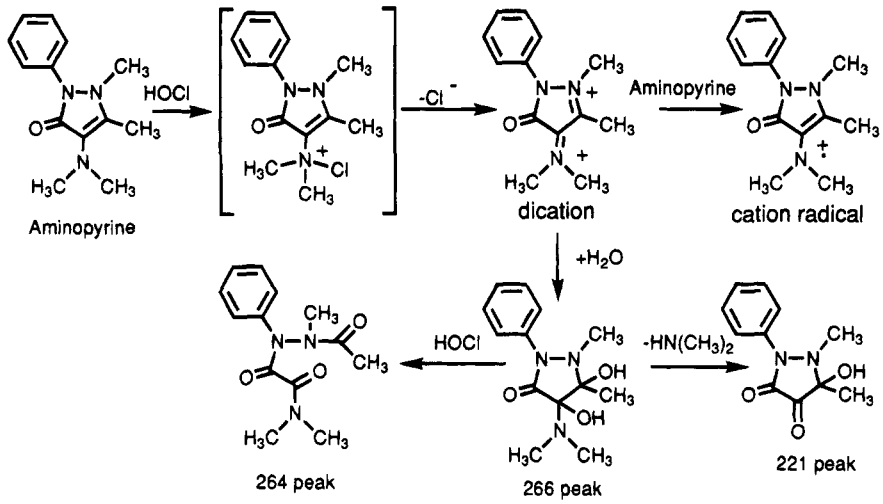

Figure 9. Proposed scheme for the reaction of aminopyrine with hypochlorite to form a dication which in turn either reacts with another molecule of aminopyrine to form two cation radicals or reacts with water to form several of the major products. The designation of 264, 266, and 221 refers to the m/z of the protonated molecular ion of these products.

Discussion

No deuterium isotope effect was observed in the formation of the cation radical from the reaction of aminopyrine with hypochlorous acid. This suggests that the reaction does not involve the rupture of the methyl C–H bond; however, this does not prove that the iminium ion is not an intermediate in the formation of the cation radical since its formation need not be the rate-limiting step. Further experiments found no evidence for hydrogen exchange during the formation of the cation radical. This provides strong evidence against a mechanism for the formation of the cation radical in which an iminium ion intermediate reacts with another molecule of aminopyrine and a proton to form two cation radicals. These results are consistent with the proposal of Sayo and Saito (9) that the cation radical is formed by N-chlorination followed by the loss of a chlorine radical to form the cation radical.

To further probe the mechanism, we repeated in considerably more detail the stopped-flow experiments of Sayo and Saito. These workers had investigated the chemical oxidation of aminopyrine with HOCl and Br_2, following the first-order appearance of cation radical at 565 nm. They reported that with each oxidant the first-order rate constants increase with increasing concentration of aminopyrine, but not in a linear fashion. Our results are in complete agreement with these. The important new observation made in our experiments is that there is an intermediate with λ_{max} near 420 nm that is formed before the cation radical. This intermediate decays with the same rate constant as that associated with appearance of the cation radical, with a good isosbestic point for the interconversion. We therefore conclude that this 420 nm intermediate is the precursor to the cation radical. Examination of the mechanism proposed by Sayo and Saito (Figure 1) reveals that if this mechanism were to be correct, the 420 nm intermediate would have to correspond to the *N*-chloroaminopyrine, since in this mechanism, the *N*-chloro species fragments directly to the cation radical. The first inconsistency is that the *N*-chloro species is expected to have a spectrum and λ_{max} similar to that of parent drug, and is unlikely to absorb at a wavelength as high as 420 nm. A second inconsistency is that the 420 nm intermediate converts to the cation radical in a process that is in part bimolecular, first-order in intermediate and first-order in unreacted aminopyrine. According to the Sayo/Saito mechanism, the *N*-chloro intermediate must proceed to cation radical solely in a unimolecular reaction. The Sayo/Saito mechanism also predicts that the yield of cation radical should be independent of the concentration of aminopyrine, and this is clearly not the case (Figure 6). A final inconsistency is the independence of the rate constant for conversion of the 420 nm intermediate to the cation radical on the nature of the oxidant. In the Sayo and Saito mechanism a significant difference would be expected between bromine and chlorine due to the different stabilities of the two radicals. It can be noted that Sayo and Saito in fact reported a difference in the rate constants for the formation of cation radical for HOCl and bromine, with the latter faster. Analysis of the experimental conditions employed in their study, however, shows that the experiments were carried out with lower concentrations of aminopyrine. With these conditions, conversion of the 420 nm intermediate to cation radical is not entirely rate-limiting in the reaction with HOCl, and the cation radical does form more slowly than in the reaction with bromine carried out under equivalent conditions.

An intermediate that is consistent with the data is a dication. A similar species has been proposed in the oxidation of Wurster's blue (*N,N,N',N'*-tetramethylphenylenediamine) (*14*). In the aminopyrine case the data suggest a mechanism as shown in Figure 9 for HOCl. Reaction of aminopyrine with the oxidant forms *N*-chloroaminopyrine which then loses chloride anion to form a dication, the intermediate with λ_{max} near 420 nm. This dication then reacts with another molecule of aminopyrine to form two cation radicals or with the solvent. We write this latter reaction in Figure 9 as involving two molecules of water forming initially the product with a protonated molecular ion at m/z 266 (molecular weight 265).

In terms of the mechanism of Figure 9, two reaction steps precede the formation of the dication. The kinetic data for the rise at 420 nm show a dependence on aminopyrine concentration (Figure 5), implying that the initial reaction of HOCl and aminopyrine is the slow step, with the *N*-chloroaminopyrine then rapidly losing chloride to give the dication. If this adduct were to be

forming in a very rapid step with its conversion to dication being rate-limiting, rate constants for the appearance at 420 nm would be independent of the aminopyrine concentration in the experiments with this reagent in excess. This scenario would mean that all of the limiting HOCl would be converted to the *N*-chloro adduct in a very rapid step, and the appearance of the dication would occur as the *N*-chloro species undergoes a unimolecular heterolysis, a process that cannot be affected by the excess aminopyrine present. It should be noted that we have no direct evidence for the structure of the proposed *N*-chloroaminopyrine intermediate, and addition of Cl^+ (and with bromine, Br^+) could occur at other locations in the aminopyrine, with subsequent rapid loss of halide to give the same dication.

The kinetics for the further reactions of the dication can be explained by the competing processes involving water and excess aminopyrine, with k_0 representing the reaction with water and the process in k_{AP} representing the reaction with aminopyrine to form the cation radical. It should be noted that, in following the appearance of cation radical, values of k_{obs} still contain the term in k_0 even though this process is "invisible" at this wavelength (*15*).

Especially convincing evidence for the dication mechanism is provided by the dependence of the yield of cation radical on the aminopyrine concentration. At lower concentrations, increasing aminopyrine is expected to increase cation radical as more of the dication is diverted away from the reaction with the solvent. This yield is however expected to level off at higher concentrations, where essentially all of the dication reacts via the cation radical route. In quantitative terms, the yield of cation radical is given by

$$[\text{cation radical}] = 2[\text{HOCl}]_0\left(\frac{k_{AP}[\text{AP}]}{k_0 + k_{AP}[\text{AP}]}\right)$$

where the rate constants k_{AP} and k_0 refer to the reactions of dication with aminopyrine and solvent, [AP] is the aminopyrine concentration, $[\text{HOCl}]_0$ = the initial concentration of the oxidant, and the factor 2 appears since each reaction produces two cation radicals. Rearranging this expression and substituting the Beer's Law equation with ϵ, the extinction coefficient of the cation radical at 565 nm (and unit path length), the following expression results for the optical density at 565 nm (after complete formation of cation radical).

$$\text{OD}(565) = \frac{2\epsilon[\text{HOCl}]_0\left(\frac{k_{AP}}{k_0}\right)[\text{AP}]}{\left(\frac{k_{AP}}{k_0}\right)[\text{AP}] + 1}$$

This equation takes the form

$$y = \frac{abx}{bx + 1}$$

where y = OD(565), x = [AP], $a = 2\epsilon[\text{HOCl}]_0$, and $b = k_{AP}/k_0$. The experimental data were fit to this expression with "*a*" and "*b*" as adjustable parameters. This process provided $k_{AP}/k_0 = 51 \pm 6$, in good agreement with the ratio obtained using the values of the individual rate constants obtained in the kinetic analysis, 47 ± 2. The parameter "*a*" so obtained was 0.85 ± 0.08, leading to a value for the extinction coefficient of $(1.70 \pm 0.14) \times 10^3$ M^{-1} cm^{-1}. This is in reasonable agreement with a literature value of 2.23×10^3 (13).

The mechanism with bromine as oxidant proceeds via the same dication, which partitions in the same way between water and aminopyrine. As is observed, the kinetic behavior for decay of dication and formation of cation radical is independent of whether HOCl or bromine is the oxidant, since the exact nature of this reagent is irrelevant at this reaction stage. The exception to this statement occurs at lower aminopyrine concentrations where with HOCl, but not bromine, the formation of the dication is becoming partly or wholly rate-limiting in the overall reaction. With bromine the initial reaction forming dication is faster than with HOCl, being complete within the 2 ms dead time of the stopped-flow apparatus. In this case we obviously cannot make any comment regarding the nature of the rate-limiting step in the formation of the dication.

We had hoped to directly observe the proposed dication by mass spectroscopy in the flow system since the m/z of an ion with two charges and an odd mass would have a characteristic fractional value, in this case 115.5; however, this ion was not observed. On the basis of the calculated volume of the system and the flow rate, the lag time between the reaction of aminopyrine and hypochlorite and entrance of the products into the mass spectrometer is greater than 1 s. When compared with the approximately 15 ms half-life of the dication observed in the stopped-flow diode array system, it is unlikely that we would be able to obtain a mass spectrum of this intermediate. We attempted to increase the probability of detection by using bromine instead of hypochlorite, by increasing the flow rate to decrease the lag time and by going to a nonaqueous solvent, dimethylethylene glycol, but we were still unable to observe an ion at m/z 115.5.

In terms of our mechanism, the initial product would in turn lead to several of the other observed products. The product with the protonated molecular ion at m/z 266 is only observed in the flow system in which the products of the reaction were introduced directly into the mass spectrometer, presumably because this product hydrolyzes too rapidly to be observed by HPLC. On the basis of the fragmentation pattern we interpret the ion at m/z 221 to represent loss of dimethylamine from the 266 product, and we interpret the ion at m/z 264 to represent a further oxidized ring opened product. In particular, the two largest ions in the daughter ion spectrum of m/z 264 were at m/z 72 and 191, which we interpret as due to rupture of the bond between the two carbonyl groups. This sequence is depicted in Figure 9. The structures drawn for these products represent only one of several tautomers and hydration states which could exist for each product. We interpret the other product with a protonated molecular ion at m/z 248 to represent an *N*-oxide of aminopyrine, but it was never further characterized. Although the same products were formed with hypochlorite, bromine, $MPO/H_2O_2/Cl^-$, or activated neutrophils, the relative amounts of the products were different. The peak with a protonated molecular ion at m/z 221 was the major product formed by all systems except the activated neutrophils in which the major product was the one with the protonated molecular ion at m/z 264. Although we do not know the basis for this difference, it is consistent with previous observations. Specifically, the "264" peak is oxidized relative to the product with the protonated molecular ion at m/z 266

which appears to be the precursor to the "221" peak. This suggests a local high concentration of oxidant although the total amount of oxidant produced by the neutrophils may be relatively small. This is analogous to previous observations of oxidation of arylamines all the way to their nitro analog while the myeloperoxidase system oxidized more of the arylamine but stopped at the hydroxylamine (*16*); however, there are several other possible explanations for this observation.

The major oxidant produced by activated neutrophils is hypochlorous acid, and because similar products were produced by hypochlorous acid to those formed by activated neutrophils, we believe that the same mechanism is involved. The proposed dication is much more reactive than the cation radical and more likely to lead to covalent binding. In addition, the concentration of aminopyrine *in vivo* is likely to be so low as to preclude the sequence leading to the formation of the cation radical shown in Figure 9, although it is possible that some biological molecule could reduce the dication to the cation radical. Binding of the dication to activated neutrophils could lead to an immune response to the altered neutrophils, and this may be responsible for the apparent antineutrophil antibodies detected by other investigators.

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