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HYPOCHLORITE-MODIFIED ADENINE NUCLEOTIDES: STRUCTURE, SPIN-TRAPPING, AND FORMATION BY ACTIVATED GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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Adenosine and its nucleotides react with hypochlorite to form unstable products that have been identified as the N^6 chloramine derivatives. These chloramines spontaneously oligomerize, form stable adducts with proteins and nucleic acids, and are converted with loss of chlorine to the original nucleoside or nucleotide by reducing agents. The chloramines are associated with a free radical, and the spin-trapping of adenosine chloramine with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) yielded a mixture of unstable nitroxyl adducts that corresponded to nitrogen-centered radicals from the parent nucleoside. When activated guinea pig polymorphonuclear leukocytes were stimulated with phorbol myristate acetate to produce hypochlorite, they actively incorporated [^{14}C] adenosine into acid-insoluble products by a process that was dependent on oxygen and inhibited by azide and thiols. These findings suggest that adenine nucleotide chloramines are generated by activated phagocytic cells and form ligands with proteins and nucleic acids as observed in model systems. The results imply that nucleotide chloramines are among the cytotoxic and possibly mutagenic factors that are associated with the inflammatory process.

KEY WORDS: Hypochlorous acid, chloramines, adenine nucleotides, nucleotide radicals, spin-trapping, DMPO, polymorphonuclear leukocytes, inflammation injury, cytotoxicity.

INTRODUCTION

Hypochlorous acid is a major end product of oxygen metabolism in activated phagocytes and thus its reaction with cellular constituents is of biochemical and clinical interest.[1-7] The present studies were prompted, in part, by reports of the disappearance of adenine nucleotides in activated neutrophils[8-11] and in tissues[12-15] and bacterial cells[16,17] exposed to hypochlorous acid. In particular, the depletion of ATP could result in cell death or injury and constitute an important mechanism of hypochlorite cytotoxicity. Using adenosine and adenine 5′-monophosphate (AMP) as model compounds, we have isolated the primary hypochlorite-modification product and found that it is the N^6 chloramine derivative. The present discussion will highlight the major findings that led to identification of this chloramine and will further examine the properties of the free radical associated with the chloramine, its ability to form ligands with proteins and nucleic acids, and its occurrence in activated phagocytes. Portions of this work have previously appeared.[18-25]

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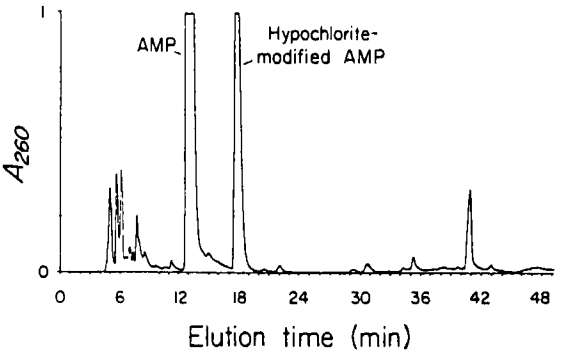


FIGURE 1 Chromatographic separation of major products from the reaction of hypochlorite with AMP (NaOCl/AMP ratio = 0.7). The reaction mixture was stirred for 60 min at ambient temperature and a 10-μl aliquot, equivalent to 1 μmol of AMP, was chromatographed on a 10 by 250 mm Phase-Sep C_{18} column using a Model 9533 liquid chromatograph (IBM Instruments). The mobile phase was 20 mM K_2HPO_4-KH_2PO_4, pH 7, flowing at 1 ml/min. At 20 min, the mobile phase was supplemented with a linear gradient of methanol to 20% in 20 min. The column chamber was maintained at 22°C by the circulation of water, and detection was at 260 nm. From[19] with permission.

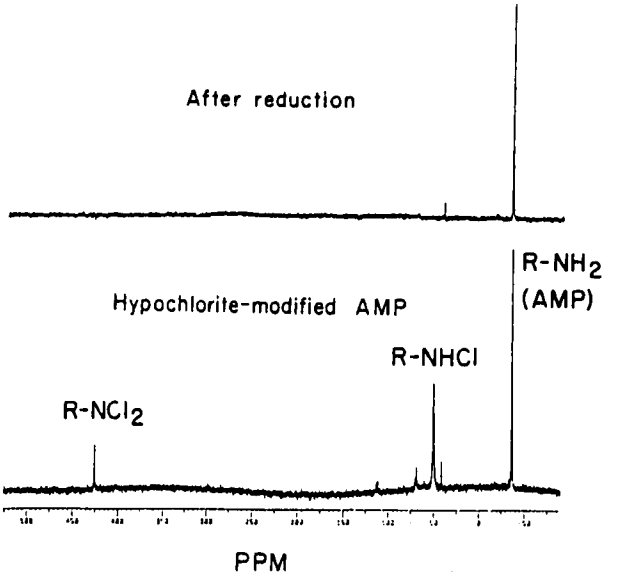


FIGURE 2 ^{15}N NMR spectrum of hypochlorite-modified [$^{15}N^6$] AMP in D_2O before (lower panel) and after (upper panel) reduction with Na dithionite. The pH of the sample was 6.1 before reduction. Spectra were obtained with a Bruker WH-400 spectrometer. R-NHCl and R-NCl_2 represent the mono- and dichloramine of AMP. Unpublished results of C. Bernofsky, A.J. Benesi, A.R. Garber, and H.J. Cohen.

EXPERIMENTAL

^{15}N NMR chemical shifts are δppm relative to an external standard of [^{15}N] formamide, and ^1H NMR chemical shifts are δppm relative to an external standard of

tetramethylsilane (saturated solution) in D_2O. NMR measurements were conducted near 25°C in D_2O, and determinations of pH are uncorrected meter readings. Other experimental details are given in the legends to figures.

RESULTS AND DISCUSSION

Evidence for Chloramine Structure

When AMP is treated with hypochlorite, there is an immediate reaction and formation of numerous products. Figure 1 illustrates the HPLC separation of a typical reaction mixture. There are several polar degradation products of unknown structure that elute prior to the AMP as well as higher-molecular-weight products, oligomers of AMP, with longer retention times (Figure 1). The most significant product is "hypochlorite-modified AMP," which we now know is the chloramine of AMP.

Early in this investigation, we found that if the amino group of AMP was replaced

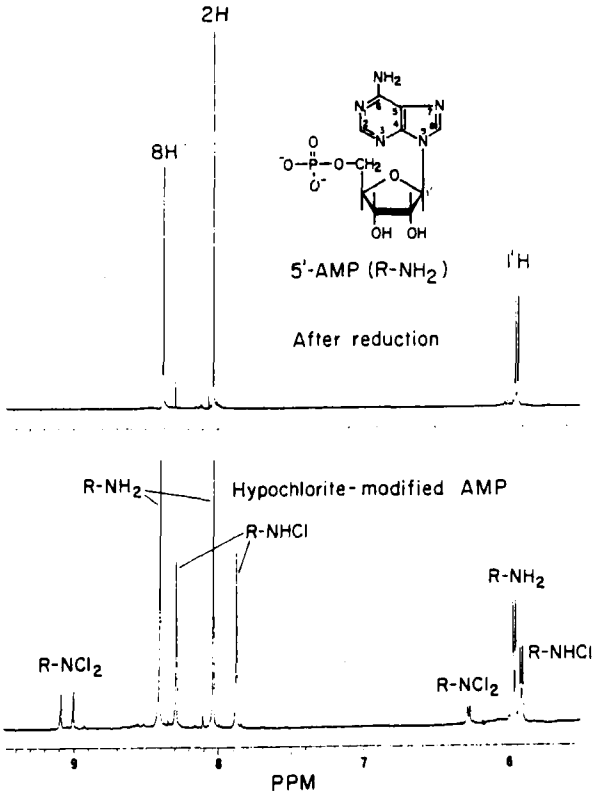


FIGURE 3 ^1H NMR spectrum of hypochlorite-modified AMP in D_2 before (lower panel) and after (upper panel) reduction with Na dithionite. Spectra were obtained with a Bruker AM-300 spectrometer. Only the aromatic and anomeric protons are shown. The preparation contained approximately 36% monochloramine (R-NHCl), 8% dichloramine (R-NCl_2), and 56% AMP (R-NH_2). Upon reduction, the Cl', C2 and C8 protons of the mono- and dichloramine (lower panel) were converted to the corresponding protons of AMP (upper panel). Unpublished results of C. Bernofsky, A.R. Garber, and H.J. Cohen.

by a hydroxyl to give inosine 5'-monophosphate (IMP), there was no comparable reaction with hypochlorite. There was also no reaction if the amino group was dimethylated. However, monomethylation permitted the reaction with hypochlorite. These results suggested the critical involvement of the N^6 amino group in the hypochlorite modification reaction.

NMR Chemical Shifts and Dismutation We therefore synthesized AMP with an $^{15}N^6$ amino group, reacted it with hypochlorite, and examined the products by ^{15}N NMR. Figure 2 (lower panel) shows the presence of three components in this preparation. The largest resonance at −36.7 ppm belongs to AMP, which is always present in isolated hypochlorite-modified preparations. Downfield from this resonance at 50.3 ppm is a substituted nitrogen that is assigned to the monochloramine, and farther downfield at 425.5 ppm is a highly-substituted nitrogen that is assigned to the dichloramine. Upon reduction with dithionite (Figure 2, upper panel), both the mono- and dichloramine nitrogens are converted to the amine nitrogen of the parent AMP.

The small downfield resonance at 425.5 ppm deserves special attention because it was found to disappear at pH 11 and reappear at pH 7. This behavior was a result of the chloramine dismutation reaction, an important characteristic of this class of compounds.[26] As indicated by Equation (1), monochloramines under acidic conditions undergo a dismutation in which there is transfer of chlorine from one chloramine to another with liberation of the parent amine. This reaction is reversible, and in alkali, the dichloramine gives up a chlorine to the amine to form the monochloramine.

$$2\ \text{R-NHCl} \underset{\text{base}}{\overset{\text{acid}}{\rightleftharpoons}} \text{R-NCl}_2 + \text{R-NH}_2 \qquad (1)$$

Results comparable to the above ^{15}N NMR data are also apparent in the proton NMR spectrum. Figure 3 (lower panel) shows part of a proton NMR spectrum of an AMP chloramine preparation, and three principal components can be seen. The region of 5.8 to 6.4 ppm shows three sets of anomeric protons. The large doublet at 5.96 ppm belongs to AMP, the medium doublet at 5.92 ppm is assigned to the monochloramine, and the small doublet at 6.28 ppm to the dichloramine. When the chloramines were reduced, their anomeric protons were converted to the anomeric proton of AMP (Figure 3, upper panel).

Two aromatic protons are associated with C2 and C8 of the purine ring, and three pairs of these protons were present in the preparation of hypochlorite-modified AMP (Figure 3, lower panel). The large pair at 8.04 ppm and 8.41 ppm belongs to AMP, the medium pair at 7.88 ppm and 8.29 ppm is assigned to the monochloramine, and the small pair at 9.01 ppm and 9.09 ppm to the dichloramine. Upon reduction, the C2 and C8 purine ring protons that were associated with the chloramines were converted to the respective purine ring protons of the parent AMP (Figure 3, upper panel).

The two aromatic protons belonging to the dichloramine at 9.01 and 9.09 ppm are of special interest because they disappeared when the pH was raised to 11 and reappeared when the pH was readjusted to 7. The reappearance of these protons is attributed to reformation of the dichloramine under acid conditions, as required by Equation (1).

Reaction with p-Toluenesulfinic Acid Additional evidence that hypochlorite-modified adenine nucleotides are chloramines comes from a consideration of their reaction

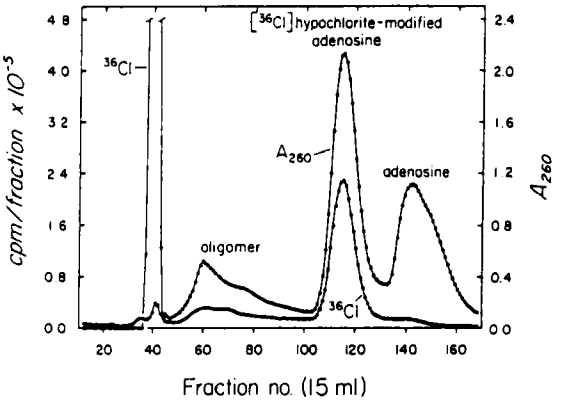


FIGURE 4 Incorporation of ^{36}Cl into hypochlorite-modified adenosine. Adenosine (100 μmol) and K_2HPO_4-KH_2PO_4, pH 7.0 (1 mmol) were stirred for 20 min in a solution containing [^{36}Cl] HOCl (100 μmol, 85μCi), [^{36}Cl] NaCl (17.6 μmol, 15 μCi), and NaOH (100 μmol) in a final volume of 5.1 ml, pH 6.9. The reaction mixture (4.9 ml) was chromatographed on a 5 by 50-cm column of Sephadex G-10 at ambient temperature using a mobile phase of distilled water at 1 ml/min, with continuous detection of conductivity and absorbance at 260 nm. The A_{260} (upper curve) and radioactivity (lower curve) were determined independently in aliquots of collected fractions (15 ml). Unpublished results of C. Bernofsky and B.M.R. Bandara.

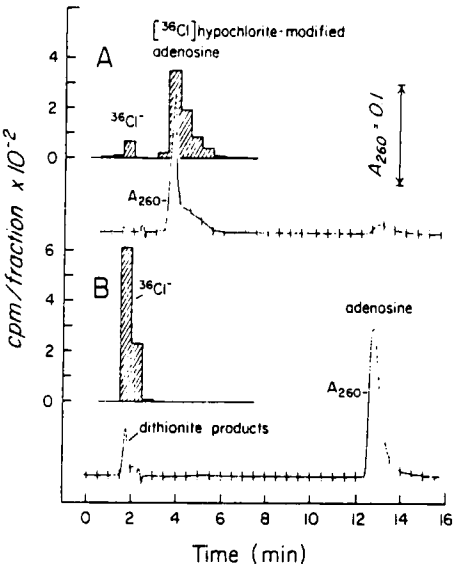


FIGURE 5 HPLC analysis of [^{36}Cl] hypochlorite-modified adenosine. An aliquot (50 μl, 800 cpm) of [^{36}Cl] hypochlorite-modified adenosine from the peak fraction shown in Figure 4 was chromatographed at 24°C on a 4.6 by 250-mm column of Spherisorb ODS2 using a mobile phase of 10% methanol at 1 ml/min. Fractions (0.5 ml) were collected at 0.5-min intervals (vertical marks) into 7-ml glass scintillation vials; radioactivity was determined after mixing with 1.5 ml of water and 3 ml of Aquasol-2. *A*: Untreated. *B*: After treatment with Na dithionite (*ca.* 5 mg/ml) and filtration. Note that the sample had been stored at 4°C for 3 days prior to HPLC analysis and shows evidence (tailing, Panel A) of a second radioactive component. HPLC of the same fraction on the day it was prepared gave a symmetrical peak. Unpublished results of C. Bernofsky and B.M.R. Bandara.

with sulfinic acids. There was no reaction when adenosine was treated with p-toluenesulfinic acid. However, when hypochlorite-modified adenosine was treated with p-toluenesulfinic acid, a tosylate was formed. This tosylate was found to be the 2′-*O*-tosylate of adenosine as determined by mixed melting point and co-chromatography with an authentic sample, mass spectroscopy, and ^{1}H- and ^{13}C-NMR. Natural abundance ^{15}N NMR studies showed that the N^{6} nitrogen was unaffected by the tosylation. It thus appears that the sulfinic acid reacted with adenosine chloramine to liberate adenosine and form the sulfonyl chloride which then reacted with adenosine to give the 2′-*O*-tosylate, the preferred product in this instance.[26a] Because chloramines are known to convert sulfinic acids into sulfonyl chlorides,[27,28] this result is further evidence that hypochlorite-modified adenosine is a chloramine.

Incorporation of Labile Chlorine Direct evidence was obtained for the incorporation of chlorine into adenosine. For the experiment shown in Figure 4, we prepared [^{36}Cl] HOCl, reacted it with adenosine, and separated the products on a column of Sephadex G-10. The peak representing hypochlorite-modified adenosine contained radioactive chlorine (Figure 4). When an aliquot of this peak was subjected to HPLC (Figure 5, upper panel), some of the chloramine had already decomposed, liberating a small portion of adenosine and radioactive chloride. However, after reduction with dithionite (Figure 5, lower panel), all of the chloramine was converted to adenosine with complete release of the radioactive chloride. The incorporation of ^{36}Cl into adenosine and its release with reducing agents is consistent with a chloramine structure.

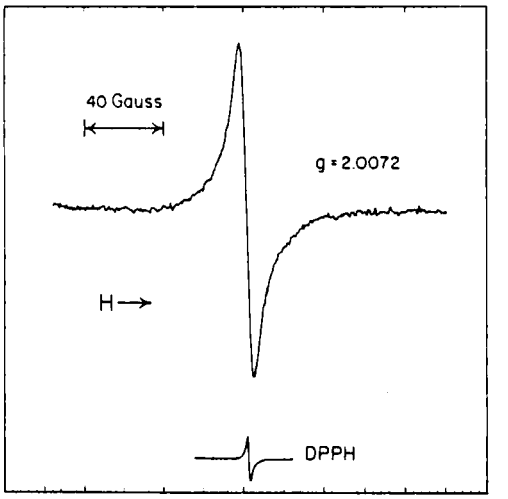
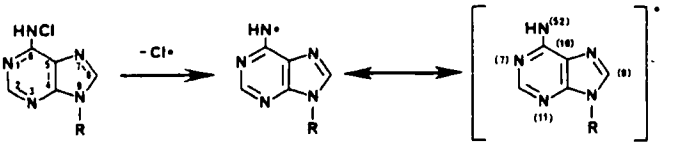


FIGURE 6 Free radical signal from hypochlorite-modified AMP. A lyophylized sample was placed in a 3-mm bore quartz tube and examined at ambient temperature with a Varian, Model E-109 spin resonance spectrometer. Instrument frequency, 8.932 GHz; field modulation, 100 kHz; microwave power, 2 mW; receiver gain, 2.5×10^{4}; time constant, 0.25 sec; scan speed, 50 Gauss/min. The lower signal is from a speck of 2,2-diphenyl-1-picrylhydrazyl (DPPH) taped to the outside of a second quartz tube. Based on a g-value of 2.0036 for the DPPH standard, the g-value of solid hypochlorite-modified AMP was 2.0072. From[19] with permission.



Adenosine chloramine
(Hypochlorite-modified adenosine)

Adenosine radical

FIGURE 7 Formation and delocalization of the free electron in the neutral adenosine radical. Numbers in parentheses indicate electron density within the purine nucleus, as determined by the molecular orbital calculations of Kar and Bernhard,[29] and account for 88% of the free electron distribution. The remaining 12% is shared by the C2, C4, C6, N7 and N9 atoms, which presumably contribute little to the reactivity of the radical. R = ribose. From[25] with permission.

Free-Radical Character and Spin-Trapping

When AMP chloramine was isolated and the solid examined by ESR, it was found to be associated with a free radical having a broad signal with a g-value of 2.0072 (Figure 6). We now believe that this signal arises from the N^6 chloramine derivative as shown in Figure 7. A chlorine atom can apparently dissociate from the chloramine nitrogen to give a nitrogen-centered radical that is stabilized by resonance within the purine nucleus. The distribution of the free electron shown in Figure 7 is taken from the molecular orbital calculations of Kar and Bernhard,[29] which were based on X-ray studies of single crystals.

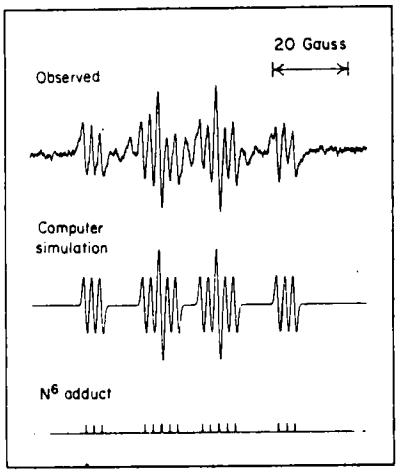


FIGURE 8 *Upper curve*: ESR spectrum of spin-trapped hypochlorite-modified adenosine. Adenosine (150 μl, 3 μmol), was mixed with 80 μl (5 μmol) of Na borate (final pH 8.9), treated for 1 min with 20 μl (1.4 μmol) of HOCl, and reacted with 10 μl (90 μmol) of DMPO. Recording of the spectrum was begun 2 min after mixing and was obtained at ambient temperature in a quartz flat cell with an ER-200D spectrometer (IBM Instruments). Instrument frequency, 9.79 GHz; field modulation, 100 kHz; microwave power, 20 mW; modulation amplitude, 1.00 G; receiver gain, 2.52×10^4; time constant, 0.164 sec; scan speed, 60 G/min. *Middle curve*: Computer simulation of the radical shown above. *Lower curve*: Stick diagram assumes adduct formation at N^6. No signal was obtained from adenosine and DMPO. Unpublished results of C. Bernofsky, B.M.R. Bandara, and O. Hinojosa.

The homolytic loss of chlorine from the chloramine is significant because in aqueous solution chlorine atoms can give rise to dichlorine (Equation (2)), which can hydrate to regenerate hypochlorous acid (Equation (3)). This is important in spin-trapping studies involving 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) because hypochlorous acid can react directly with DMPO to yield 5,5-dimethyl-2-hydroxypyrrolidine-N-oxyl (DMPO–OH)[29a] and 5,5-dimethyl-2-pyrrolidone-N-oxyl (DMPOX).[30] (DMPOX).[30]

$$2\,Cl^{\cdot} \rightarrow Cl_2 \tag{2}$$

$$Cl_2 + H_2O \rightarrow HOCl + HCl \tag{3}$$

Because dissociation of a chlorine atom from adenosine chloramine yields a nitrogen-centered radical whose unpaired electron is delocalized by resonance, one would expect that the reaction of this radical with DMPO would give rise to several adducts and that DMPOX would also be formed. When spin-trapping was conducted at pH 8 in the presence of excess adenosine to absorb the liberated HOCl, we observed mainly the N^6 adduct (Figure 8). When the spin-trapping was conducted in an unbuffered medium at about pH 6, we observed mainly the N3 and N1 adducts, roughly in a 2 to 1 proportion (Figure 9). These assignments are still tentative. In both cases, the observed ESR spectra were eventually replaced by the spectrum of DMPOX, the final product of the reaction of DMPO with hypochlorous acid.[30]

In our early spin-trapping experiments, we used excess AMP chloramine, and DMPOX was the only product that was readily observed.[21] This initially led us to postulate that hypochlorite-modified AMP was a peroxide because peroxides are capable of oxidizing DMPO to DMPOX.[31-34] We now realise that DMPOX arises from hypochlorous acid oxidation of DMPO, and in further studying this reaction in

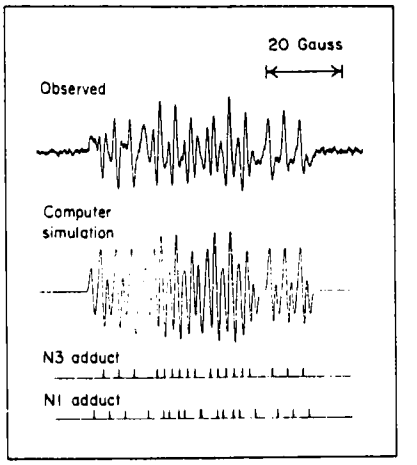


FIGURE 9 *Upper curve:* ESR spectrum of spin-trapped, hypochlorite-modified adenosine. DMPO (5 μl, 45 μmol) in 200 μl of water was added to 5 mg of hypochlorite-modified adenosine (81%) and adenosine (19%), mixed in a vortex, and transfered to a flat ESR cell. Recording of the spectrum was begun 9 min after mixing and obtained as described for Figure 8. *Middle curve*: Computer simulation of the two principal radicals observed. *Lower curves:* Stick diagrams are assumed to have a 2 to 1 ratio and correspond to DMPO adducts at N3 and N1, respectively. Hypochlorite-modified adenosine was prepared and assayed as reported.[22] From[25] with permission.

[^{17}O] H_2O, we have confirmed that ^{17}O is incorporated into DMPOX with the liberation of HCl.[30] Evidence has also been obtained that an N-chloroxy adduct and DMPO–OH are intermediates in this reaction.[29a,30]

Biological Significance

Binding to Proteins and Nucleic Acids The potential relevance of adenine nucleotide radicals and their parent chloramines to biological processes was first indicated by the demonstration that hypochlorite-modified adenine nucleotides formed acid-stable ligands with proteins and nucleic acids.

When [^{14}C] AMP was treated with hypochlorite in the presence of bovine serum albumin and the mixture chromatographed on a column of Sephadex G-25 to separate the unliganded AMP from the protein, the protein was found to contain substantial amounts of radioactivity. No ligand was formed in the absence of hypochlorite.[18] Figure 10 shows that the AMP-to-protein linkage in the isolated protein is stable to rechromatography on Sephadex G-25.

Similar observations were made using nucleic acids. When a mixture of [^{14}C] AMP and calf thymus DNA was chromatographed on a column of Sephadex G-25, no radioactivity was specifically associated with the DNA (Figure 11A). However, when the reaction mixture also contained hypochlorite, the DNA was found to have radioactivity specifically associated with it (Figure 11B), indicating that a stable

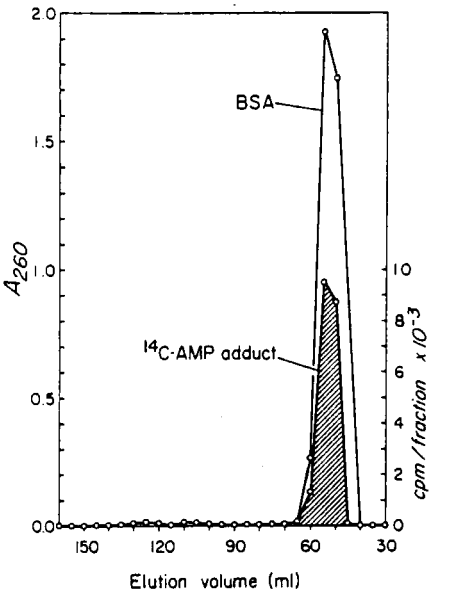


FIGURE 10 Rechromatography of [^{14}C] AMP-protein adduct. To form the adduct, a reaction mixture containing per ml: 2 mg of bovine serum albumin (BSA), 1 μmol (0.4 μCi) of [8-^{14}C] AMP, and 10 μmol of NaOCl in 20 mM KH_2PO_4-K_2HPO_4, pH 6.9, was incubated for 30 min at ambient temperature and chromatographed on a 1.6 by 60-cm column of Sephadex G-25 using a mobile phase of the same buffer at 1 ml/min. The BSA fractions were pooled, lyophylized, reconstituted in a small volume, and 1 ml (20 mg of protein) rechromatographed as shown. From[18] with permission.

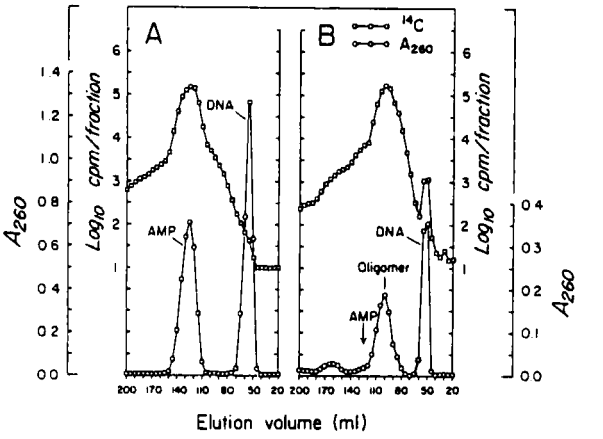


FIGURE 11 Binding of hypochlorite-modified [^{14}C]AMP to DNA. *A* (control): The reaction mixture contained per ml: 1.0 mg of calf thymus DNA, 1 μmol (0.4 μCi) of [8-^{14}C]AMP, 30 μmol of NaCl, and 15.6 μmol of KH_2PO_4-K_2HPO_4, pH 6.9, and was incubated for 30 min at ambient temperature and chromatographed as described for Figure 10. *B*: Same as *A* but containing 10 μmol of NaOCl and 14.8 μmol of KH_2PO_4-K_2HPO_4, pH 6.9. Points are plotted against the cumulative collected volume (abcissa) and represent for each 5-ml fraction the average 260-nm absorbance (circles) and total radioactivity (squares). From[20] with permission.

ligand had been formed. Interestingly, nearly all of the AMP that was not linked to the nucleic acid was oligomerized (Figure 11B), a reaction that implies the involvement of a radical process. Analogous results were obtained with RNA.[20]

Formation by Activated Guinea Pig Polymorphonuclear Leukocytes The above results suggested that, if isolated phagocytes were stimulated to produce hypochlorous acid, there should be intracellular formation of adenine nucleotide chloramines that would be detected by their ability to form stable ligands with proteins and nucleic acids. This idea has proved fruitful.

In the experiment illustrated in Figure 12, isolated guinea pig polymorphonuclear leukocytes were incubated with [^{14}C] adenosine, which permeated the cells and equilibrated with the adenine nucleotides. At various time intervals, a sample of the cell suspension was removed, precipitated with 10% trichloroacetic acid on a glass-fiber filter, and washed extensively with trichloroacetic acid containing non-radioactive adenosine. With cells that were not specifically activated, there was a relatively small incorporation of adenosine into acid-insoluble products (Figure 12). However, when the cells were activated with a phorbol ester, there was a burst of adenosine incorporation into acid-insoluble products (Figure 12).

These results suggested that hypochlorite had converted intracellular adenine nucleotides into chloramines that were binding to macromolecules, and many experiments have corroborated this view. Thus, we demonstrated that the incorporation of adenosine did not represent synthesis of poly (ADP-ribose) or DNA,[23] that it had a requirement for oxygen,[24] and that it was inhibited by azide, a myeloperoxidase inhibitor.[24] Moreover, reducing agents that permeated the cell, such as monothioglycerol, prevented the incorporation of adenosine into acid-insoluble products,[22] and compounds such as guanosine and 2′-deoxyadenosine, which competed for hypochlo-

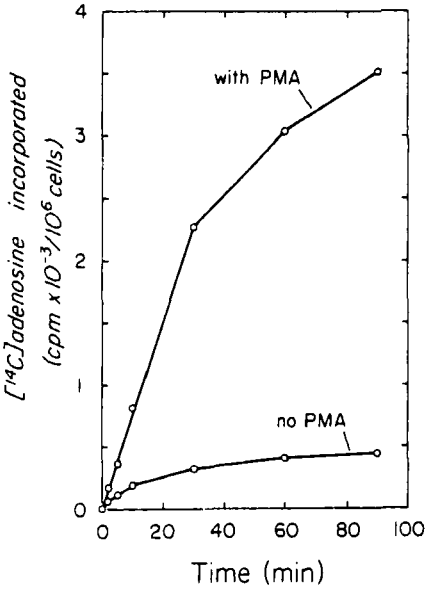


FIGURE 12 Incorporation of [^{14}C] adenosine into acid-insoluble products in isolated, guinea pig polymorphonuclear leukocytes. Reactions were conducted at 37°C with constant shaking in 18 by 65-mm siliconized test tubes and started by addition of 500 μl of cell suspension (7.5×10^7 cells in 91% Eagle's Minimum Essential Medium and 9% heat-inactivated fetal calf serum) to 1 ml of Hank's Balanced Salt Solution, 0.1 ml of fetal calf serum, 20μl (1μCi, 18 nmol) of [8-^{14}C] adenosine, and 2 μl of dimethyl sulfoxide, either neat ("no PMA") or containing 0.2 μg of phorbol 12-myristate 13-acetate ("with PMA"). At the intervals indicated, 50-μl samples were transfered to glass-fiber filter disks, washed exhaustively with cold, 10% trichloroacetic acid containing unlabeled adenosine, dried, and assayed by scintillation counting. Radioactivity was corrected for background and the small amount of [^{14}C] adenosine bound non-specifically to the filters. Data points are the means of 5 separate experiments. From[22] with permission.

rite, inhibited the incorporation of adenosine into acid-insoluble products.[23] Catalase[24] and glutathione,[22] which did not permeate the cells, inhibited the incorporation of adenosine to the extent of 15 to 20%, and this correlated with the amount of adenosine that was found to be incorporated into extracellular proteins.[24] These findings are consistent with the hypochlorite-mediated formation of adenine nucleotide chloramines and the formation of adducts with intracellular macromolecules. Further studies are needed to determine the nature of the ligands that are formed and whether the disappearance of ATP from cells[8-17] correlates with the appearance of modified proteins and nucleic acids.

Modes of Cell Injury In conclusion, our findings indicate that when cells produce or are exposed to hypochlorite, adenine nucleotide chloramines are formed that may be responsible for the loss of ATP and the ensuing death of the cells. Alternatively, those cells that are not killed by this process may be subject to alterations of metabolism that result from the chemical adenylation of functional proteins. Some cells may also undergo neoplastic transformation as a result of modification of their DNA. All of these possibilities are consistent with the types of injury that are known to be associated with the inflammatory process,[35-38] and they indicate the need for further effort toward clarifying the molecular basis of hypochlorous acid-mediated cytotoxicity.

Acknowledgments

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