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NUCLEOTIDE MODIFICATION, A RADICAL MECHANISM OF OXIDATIVE TOXICITY

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Reduced nicotinamide adenine dinucleotide (NADH) reacts rapidly with hypochlorite to form five major products separable by reversed-phase high-pressure liquid chromatography (HPLC). The involvement of a free radical mechanism is indicated by an electron spin resonance (ESR) signal as well as unusual pH changes and the uptake of oxygen. The present work suggests that hypochlorite may contribute to the cytotoxic activity of phagocytic cells through its ability to modify important cellular components by means of radicals generated by its reaction with reduced pyridine nucleotides.

KEY WORDS: NADH and hypochlorite, ESR, HPLC, NAD radical, cytotoxicity

INTRODUCTION

Myeloperoxidase, an enzyme present in neutrophils and other phagocytic cells, catalyzes the peroxidation of chloride ion to hypochlorite, a potent microbicidal agent that may also be involved in tumor cytotoxicity and other forms of tissue damage.[1–4] Although hypochlorite is known to react with many biochemical compounds, the exact mechanism by which hypochlorite exerts its cytotoxic effects is still unclear.[5–7] In this report, we present evidence to show that the reaction of hypochlorite with reduced pyridine nucleotides proceeds by a radical mechanism that gives rise to a variety of products. The structure of these products is still unknown, but it is likely that modification at multiple sites has occurred.

MATERIALS AND METHODS

Reaction Conditions – Spectrophotometric, polarographic, and potentiometric measurements were conducted in reaction vessels maintained at 30°C by the circulation of water, and data were recorded with a Model 555 stripchart recorder (Linear Instruments Corp.). Data from ESR and HPLC experiments were collected using a Model 9000 computer (IBM Instruments, Inc.) and stored on disk. See legends to figures for other conditions.

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Chemicals – NADH was obtained preweighed (100 mg; 128 μmol) from Sigma Chemical Co. and dissolved in 1.28 ml of water immediately before use. The water was previously adjusted to pH 8 with a small quantity of 5 M NH_4OH. Sodium hypochlorite was from Clorox Corp. or Baker Chemical Co. Both preparations were approximately 0.7 M NaOCl and identical in reactivity with NADH. Other chemicals and solvents were the best commercial grade available, and solutions were prepared with HPLC-grade water or Pyrex-distilled water that was previously deionized.

RESULTS

Spectrophotometric Changes – The data in Figure 1 shows that the dihydronicotinamide absorption band of NADH (λ_{max} = 340 nm) disappears rapidly upon reaction with NaOCl and is gradually replaced by a broad absorption band in the near ultraviolet having a shoulder at 414 nm (not shown). In part, these changes may reflect polymerization of radicals to yield the soluble brown pigment responsible for the new absorption band. The pigment binds strongly to silica-based chromatographic media. As demonstrated by the absence of NAD^+ as a major product (see below), the initial

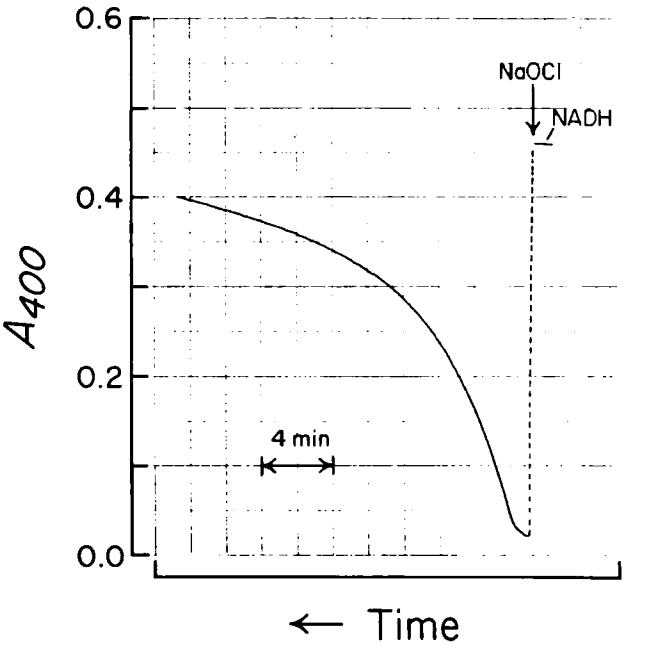

FIGURE 1 Changes in near-ultraviolet absorption during reaction of NADH with hypochlorite. NADH (5 μmol) in 1.0 ml was treated at the time indicated with 20 μl (14 μmol) of NaOCl and the reaction followed at 400 nm in a 10-mm lightpath cuvette at 30°C using a Model 222 spectrophotometer (Gilford Instrument Laboratories, Inc.), equipped with thermospacers. Before addition of NaOCl, the A_{400} was 0.460. The recorder pen was inactivated during the addition.

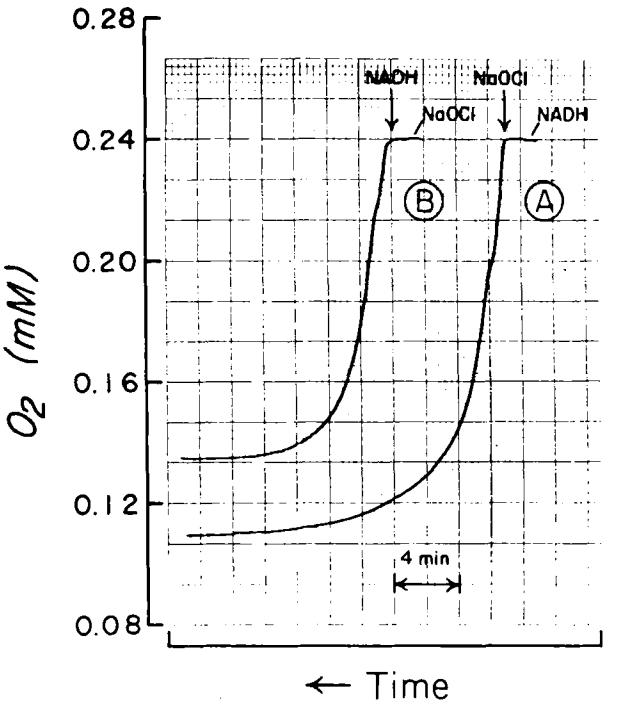

FIGURE 2 Uptake of oxygen during reaction of NADH with hypochlorite. *A*: NADH (10 μmol) in 1.4 ml was treated at the time indicated with 100 μl (25 μmol) of NaOCl and the consumption of oxygen measured at 30°C with a Clark electrode (Yellow Springs Instrument Co.), as previously described.[9] The air-equilibrated solution was assumed to contain 240 μM O_2, and the electrode output was adjusted to an initial reading of 4.5 mV. *B*: Same as above, except started by addition of 100 μl (10 μmol) of NADH to 1.4 ml (25 μmol) of NaOCl.

disappearance of the dihydronicotinamide absorption band does not represent simple oxidation of NADH.

Oxygen Uptake – Figure 2 shows that the early stage of the reaction between NADH and hypochlorite is accompanied by a rapid but limited consumption of oxygen. This uptake probably represents the reaction of oxygen with a transient free radical, presumably NAD· radical,[8] followed by dismutation of the superoxide formed (Eqs. 1–3). In the present instance (Figure 2), the average quantity of oxygen consumed was 0.18 μmol, which corresponds to less than 2% of the NADH initially present.

$$NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^{\cdot-} \quad (1)$$

$$O_2^{\cdot-} + H^+ \rightarrow \tfrac{1}{2}H_2O_2 + \tfrac{1}{2}O_2 \quad (2)$$

$$\text{Sum: } NAD^{\cdot} + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + \tfrac{1}{2}H_2O_2 \quad (3)$$

pH Changes – The reaction of NADH and NaOCl is characterized by a marked burst of hydroxyl ion formation (or proton uptake), followed by a gradual

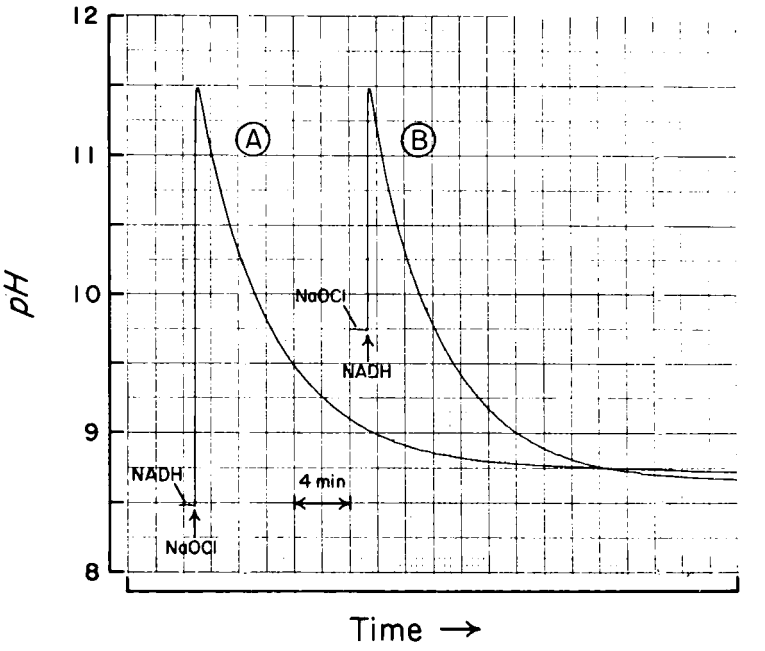

FIGURE 3 Changes in pH during reaction of NADH with hypochorite. *A*: NADH (10 μmol) in 0.9 ml was treated at the time indicated with 100 μl (25 μmol) of NaOCl and the pH determined at 30°C with a Metrohm, Model E-512 pH meter and EA-147 electrode (Brinkmann Instrument Co.). *B*: Same as above, except started by addition of 100 μl (10 μmol) of NADH to 0.9 ml (25 μmol) of NaOCl.

reacidification of the reaction mixture (Figure 3). This sequence occurs even when NaOCl is initally neutralized, but in the latter case, the reacidification causes additional products to be formed from the NADH (not shown).

One possible mechanism of hydroxyl ion formation would entail cleavage of hypochlorite to yield sodoxyl and chlorine radicals (Eq. 4).

$$NaOCl \rightleftharpoons NaO^{\cdot} + Cl^{\cdot} \quad (4)$$

Sodoxyl radicals would react with NADH (Eq. 5) or H_2O (Eq. 6) to generate sodium hydroxide and $NAD^{\cdot}$ or $HO^{\cdot}$ radicals, respectively, and in turn $HO^{\cdot}$ radicals would react with NADH to form additional $NAD^{\cdot}$ radicals (Eq. 7). Additional work is needed to clarify this mechanism.

$$NaO^{\cdot} + NADH \rightarrow NaOH + NAD^{\cdot} \quad (5)$$

$$NaO^{\cdot} + H_2O \rightarrow NaOH + HO^{\cdot} \quad (6)$$

$$HO^{\cdot} + NADH \rightarrow H_2O + NAD^{\cdot} \quad (7)$$

Alternative reactions would be the self-condensation of sodoxyl radicals and chlorine radicals to form, respectively, sodium peroxide (Eq. 8) and chlorine gas (Eq. 9), which may also contribute to product formation. Hydrolysis of the sodium peroxide would give rise to H_2O_2 (Eq. 10), leading in turn to singlet oxygen (Eq. 11).

$$NaO^{\cdot} + NaO^{\cdot} \rightarrow Na_2O_2 \quad (8)$$

$$Cl^{\cdot} + Cl^{\cdot} \rightarrow Cl_2 \quad (9)$$

$$Na_2O_2 + 2H_2O \rightarrow 2NaOH + H_2O_2 \quad (10)$$

$$NaOCl + H_2O_2 \rightarrow {}^1O_2 + H_2O + NaCl \quad (11)$$

One possible fate of the $NAD^{\cdot}$ radical would be reaction with $Cl^{\cdot}$ radical (Eq. 12) or $HO^{\cdot}$ radical (Eq. 13) to yield a substituted derivative of NADH that may be further oxidized to the corresponding derivative of NAD^+. Preliminary NMR studies indicate that there is substitution of the adenine ring as well as the nicotinamide ring, suggesting the formation of more than one type of radical from NADH. Side reactions of the nicotinamide-localized $NAD^{\cdot}$ radical would include oxidation (Eq. 1) and dimerization (Eq. 14), in which the dimer is represented according to the convention described elsewhere.[10]

$$NAD^{\cdot} + Cl^{\cdot} \rightarrow \text{chloro-NADH} \quad (12)$$

$$NAD^{\cdot} + HO^{\cdot} \rightarrow \text{hydroxy-NADH} \quad (13)$$

$$NAD^{\cdot} + NAD^{\cdot} \rightarrow (NADH)_2 \quad (14)$$

Figure 3 shows that there is a gradual decrease of pH following the initial alkaline burst. One explanation for the production of acid would be the alkaline hydrolysis of an NAD^+ analog that would be unstable in alkaline medium and give rise to ADP-ribose and the corresponding modified nicotinamide (Eq. 15), or in the case of substituted adenine, modified ADP-ribose and nicotinamide. This is supported by chromatographic evidence indicating the accumulation of ADP-ribose during prolonged incubation of the reaction mixture (not shown), A second possible mechanism of acid production would be the reaction of chlorine gas with NADH (Eq. 16).

$$\text{chloro-NAD}^+ + H_2O \rightarrow \text{ADP-ribose} + \text{chloronicotinamide} + H^+ \quad (15)$$

$$Cl_2 + NADH \rightarrow \text{chloro-NADH} + HCl \quad (16)$$

Free Radical Signal – Figure 4 shows the ESR signal obtained during the reaction of NADH with hypochlorite. The signal was relatively stable, decreasing gradually over several hours. In a separate experiment, removing the sample from the ESR cell and mixing it briefly with air caused the signal intensity to diminish 2.5-fold (not shown), suggesting a reaction with oxygen. From oxygen-uptake data in Figure 2, the NADH present in the ESR experiment would be capable of reacting with 0.62 μmol of O_2 and therefore maintaining an anaerobic state. The ESR results are consistent with the presence of $NAD^{\cdot}$ radical in the reaction mixture.

Chromatography – Reversed-phase HPLC analysis of the reaction mixture indicates that five major products are formed when NADH is treated with hypochlorite (Figure 5). The relative amount of each product is dependent on reaction conditions, but the data shown in Figure 5 is typical. A number of smaller peaks also appear in the chromatogram at retention times later than those shown in Figure 5 and were found to include neither NAD^+ nor NADH. The identity of all products is currently under study.

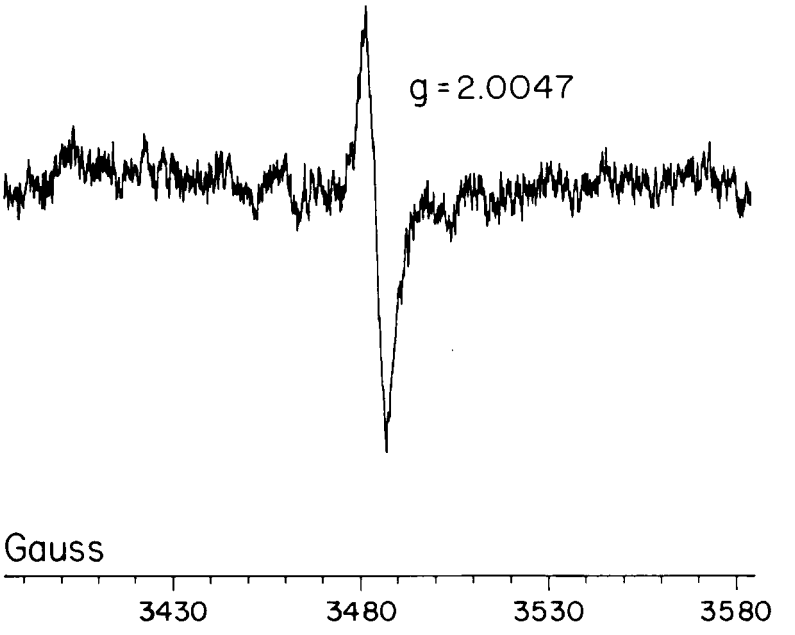

FIGURE 4 Free radical signal produced upon reaction of NADH with hypochlorite. NADH (35 μmol) was mixed with 70 μmol of NaOCl in a final volume of 0.2 ml, transferred to a flat cell, and the acquisition of data begun within 5 min. The ESR spectrum shown is the result of 20 scans taken over a field width of 200 Gauss at 4 Gauss/min and was obtained at 24°C with a Model ER-200D spin resonance spectrometer (IBM Instruments, Inc.), operating at a frequency of 9.7 GHz and employing field modulation of 100 kHz, microwave power of 200 mW, receiver gain of 2.52×10^4, and time constant of 0.082 sec. The g-value indicated is based on a weak pitch/KCl reference of $g = 2.0028$.

DISCUSSION

The generation of hypochlorite by neutrophils and other phagocytic cells (Eq. 17) occurs by the myeloperoxidase-catalyzed peroxidation of chloride[11] and is a key element of the host-defense system, being important for the destruction of invading microorganisms[1] as well as cytotoxic activity toward tumor cells[2,3] and possibly allografts.[12]

$$Cl^- + H_2O_2 \rightarrow OCl^- + H_2O \qquad (17)$$

Several mechanisms have been proposed to explain the cytotoxicity of hypochlorite-generating systems, including oxidation of proteins,[13,14] formation of chloramines,[15,16] peroxidation of fatty acids,[17,18] and degradation of electron transport components.[19,20] However, none of these has been proven to be the principal mechanism.

Selvaraj *et al.*[21] reported that, in the presence of H_2O_2 and Cl^-, myeloperoxidase converted NADH and NADPH to products that were no longer active as coenzymes, suggesting that the pyridine nucleotides were chemically altered and not simply oxidized. The incorporation of $^{36}Cl^-$ into organic material indicated that the reduced

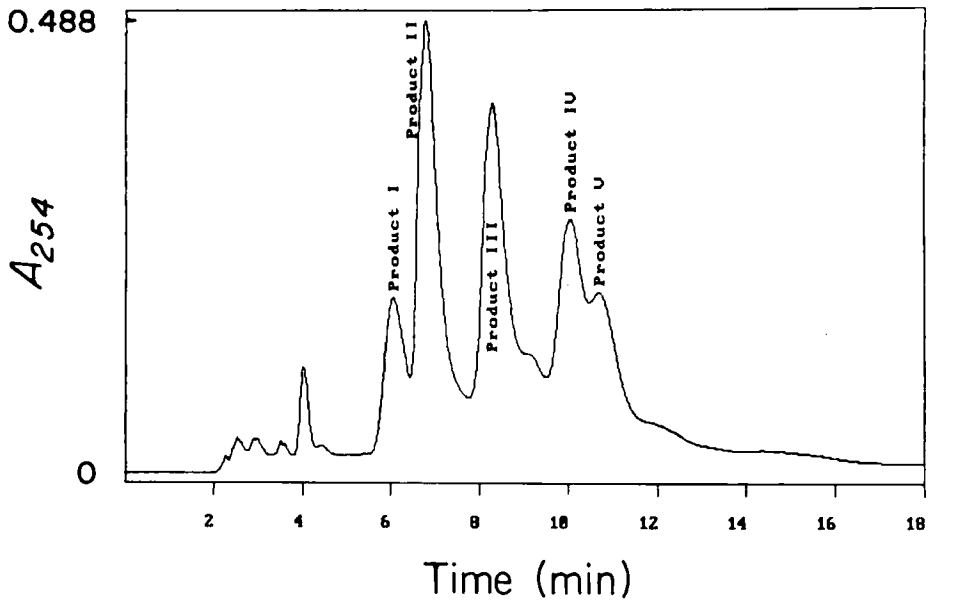

FIGURE 5 Chromatographic separation of major products obtained upon reaction of NADH with hypochlorite. NADH (10 μmol) was incubated at 30°C with 25 μmol of NaOCl for 25 min in a final volume of 1.0 ml and analyzed with a Model 9533 liquid chromatograph (IBM Instruments, Inc.), The reaction mixture was passed through a Gelman, ACRO LC13 filter (0.45 μm), and a 10-μl aliquot chromatographed on an IBM, C_{18} column, 4.6 × 250-mm, using a mobile phase of 0.01 M K_2HPO_4–KH_2PO_4, pH 7.0, at 1 ml/min. The column chamber was maintained at 21°C by circulation of water, and detection was at 254 nm as shown. See text for further details.

pyridine nucleotides had been chlorinated. Under the same conditions, NAD^+ and $NADP^+$ were unaffected.[21]

Virion *et al.*[22] found that treatment of NADH and NADPH with lactoperoxidase in the presence of H_2O_2 and $^{125}I^-$ produced an iodinated product having an ultraviolet absorption maximum near 265 nm. Moreover, NADH and NADPH prevented the iodination of tyrosine and thyroglobulin, which are otherwise readily iodinated by the lactoperoxidase system. NAD^+ and $NADP^+$ were neither iodinated nor did they inhibit iodination of the tyrosine acceptors.[22] Griffin and Haddox[23] reported similar results for the action of chloroperoxidase on NADH in the presence of H_2O_2 and Cl^-, but again, the product was not isolated or further characterized.[23]

The above reports of pyridine nucleotide halogenation by hypohalite-generating systems and the present demonstration of a radical mechanism indicate that the process may contribute to cytotoxicity in two ways. First, inactivation of pyridine nucleotides would bring the central pathways of metabolism to an immediate halt, and second, radicals produced in the process would react with other cellular components and cause additional structural and metabolic damage. Further characterization of this process is in progress.

Acknowledgments

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