Metabolism of Procainamide to a Hydroxylamine by Human Neutrophils and Mononuclear Leukocytes

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The chronic use of procainamide is associated with a high incidence of drug-induced lupus and also agranulocytosis. We have previously demonstrated that procainamide is metabolized in the liver to reactive hydroxylamine (PAHA) and nitroso (nitroso-PA) metabolites which covalently bind to protein and are toxic to lymphocytes. We proposed that these metabolites were responsible for the toxicities of procainamide. However, PAHA and nitroso-PA do not appear to escape the liver in significant concentrations. In this paper we describe the metabolism of procainamide to a reactive hydroxylamine by neutrophils and mononuclear leukocytes. Such metabolism only occurs if the cells have been stimulated to have a respiratory burst. These observations have obvious possible implications for the mechanism of procainamide-induced agranulocytosis (formation of a reactive metabolite by neutrophils) and procainamide-induced lupus (formation of a reactive metabolite by monocytes). The metabolism of drugs to reactive metabolites by monocytes may be a general mechanism for hypersensitivity reactions because monocytes play a key role in the processing of antigen and stimulation of antibody synthesis.

Procainamide is an effective antiarrhythmic agent, but its chronic use is limited by a high incidence of a lupus-like syndrome (*1*). Procainamide can also cause agranulocytosis, and the incidence of agranulocytosis appears to be higher with the new sustained release forms (*2*).

We previously demonstrated that procainamide is metabolized by rat and human hepatic microsomes to reactive hydroxylamine (PAHA)[1] and nitroso (nitroso-PA) metabolites (*3*). We speculated that these metabolites could be responsible for procainamide-induced lupus because they bind to histone protein (*4*) (histone protein is the major antigen to which the autoantibodies in procainamide-induced lupus bind) and because they are toxic to lymphocytes (*5*). It is also reasonable to speculate that these metabolites could be responsible for procainamide-induced agranulocytosis. Chloramphenicol is a major cause of aplastic anemia and agranulocytosis, and it has been shown that its nitroso metabolite is very toxic to bone marrow cells (*6*, *7*). Likewise, the hydroxylamine of dapsone (another aromatic amine associated with agranulocytosis) is also toxic to bone marrow cells (*8*).

The major difficulty with the hypothesis that these reactive metabolites are responsible for procainamide-induced lupus and agranulocytosis is that the metabolites do not seem to get out of the liver where they are formed. This has been shown for nitrosochloramphenicol (*9*). We also were unable to detect PAHA or nitroso-PA in the effluent of a rat liver perfused with procainamide (*3*). When the liver was perfused with PAHA instead of procainamide, most of it was reduced back to procainamide and some appeared to bind to hepatic protein.

This led us to speculate that procainamide might be metabolized to PAHA and nitroso-PA in other tissues. In early experiments with mononuclear leukocytes we were unable to detect any metabolism of procainamide (unpublished observation). Then we found that dapsone was metabolized to a hydroxylamine by neutrophils, but only if the cells were stimulated to produce a "respiratory burst" (*10*, *11*). In this process myeloperoxidase is released and hydrogen peroxide is generated. We, therefore, returned to procainamide to see if it is metabolized by leukocytes when they have been activated.

Materials and Methods

Leukocyte Isolation. After informed consent, blood was obtained from normal subjects by venipuncture and withdrawal into a heparinized syringe. The leukocytes were isolated by using a standard procedure (*12*). Briefly, dextran (2 mL of 6% w/v in normal saline for every 10 mL of blood, MW 500 000, Sigma Chemical Co., St. Louis, MO) was added to the blood and mixed. The mixture was allowed to stand for 45 min and the plasma layer removed and layered on top of sodium diatrizoate–Ficoll (LSM, 2 mL for every 5 mL of plasma, Litton Bionetics, Charleston, SC). This was centrifuged at 500*g* for 30 min. The polymorphonuclear leukocytes (PMN) which are mostly neutrophils were obtained from the pellet. The erythrocytes in the pellet were lysed with a buffered ammonium chloride solution (0.15 M ammonium chloride, 0.01 M potassium bicarbonate). Mononuclear leukocytes (MNL), which are a mixture of lymphocytes and monocytes, were obtained from the plasma/LSM interphase. By use of a cell sorter (Colter Epics 5, Hialeah FL: using forward angle and orthogonal light scatter of a 5-W argon laser), no PMN were detected in the MNL layer. The viability of the leukocytes was greater than 95% as determined by trypan–blue dye exclusion.

Incubations. Leukocytes were suspended in Hanks balanced salt solution (0.5 mL, Gibco Laboratories, Chagrin Falls, OH). Ascorbic acid was added to some incubations to give a final concentration of 1 mM. Phorbol myristate acetate (PMA, 20 ng/5

[1] Abbreviations used in this paper include: PAHA, 4-(hydroxyamino)-*N*-[2-(diethylamino)ethyl]benzamide; nitroso-PA, 4-nitroso-*N*-[2-(diethylamino)ethyl]benzamide; nitro-PA, 4-nitro-*N*-[2-(diethylamino)ethyl]benzamide; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; MNL, mononuclear leukocytes; MPO, myeloperoxidase; MHC II, class II major histocompatability glycoprotein.

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μL of dimethyl sulfoxide, Sigma Chemical Co., St. Louis, MO) followed by procainamide (various concentrations in 10 μL of water, Sigma Chemical Co., St. Louis, MO) was added. The suspension was then incubated at 37 °C in a shaking water bath.

Synthesis of Standards. The synthesis of 4-nitro-*N*-[2-(diethylamino)ethyl]benzamide hydrochloride (nitro-PA), 4-(hydroxyamine)-*N*-[2-(diethylamino)ethyl]benzamide (PAHA), and 4-nitroso-*N*-[2-(diethylamino)ethyl]benzamide (nitroso-PA) were described previously (*3*). Procainamide *N*-oxide was synthesized in the same manner as had been done previously for *N*-acetylprocainamide *N*-oxide, and it also reacted in the same characteristic manner with bisulfite to give procainamide and desethylprocainamide (*13*).

Analytical. After the incubation of procainamide with activated leukocytes was complete, the mixture was centrifuged at 13000*g* for 2 min. The supernatant (15 μL) was injected into the HPLC without further workup. HPLC was performed with a Beckman 110B pump (Berkeley, CA), a Waters C_{18}, 5-mm i.d. Nova-Pak cartridge in a Z-module (Milford, MA), and a Beckman 160 UV absorbance detector at a wavelength of 254 nm. The solvent for procainamide and PAHA consisted of water, acetonitrile, acetic acid, and triethylamine (96:3:1:0.05, v/v) at a flow rate of 1 mL/min. Under these conditions the retention time of procainamide was 5.7 min and that of PAHA was 4.0 min. The solvent system for nitro-PA was the same except the ratio was 79:20:1:0.05 (v/v). With this solvent the retention time of procainamide was 1.8 min and that of nitro-PA was 4.9 min. The peak areas were integrated with a Shimadzu C-R3A Chromatopac (Tokyo, Japan). The standard curves for PAHA and nitro-PA were linear with correlation coefficients of 0.995 and 0.985, respectively.

The identity of the PAHA peak was confirmed by adding NaOH to increase the pH above 12, which converted the PAHA to nitro-PA as determined by HPLC. This is a characteristic reaction of arylhydroxylamines (*3*). The identity of the nitro-PA metabolite was confirmed by GC–MS. The instrument was a Hewlett Packard 5987A GC–MS system. The column was a DB-17 fused silica capillary column (30 m × 0.2 mm i.d., J & W, Folfom, CA). The carrier gas was helium at a pressure of 7.5 psi. The injector temperature was 250 °C. The column temperature started at 60 °C for 2 min and then was increased to 270 °C at a rate of 10 °C/min. The ionization was methane chemical ionization with a methane pressure of 500 mTorr. The instrument was operated in the negative ion mode. The only peak in the spectrum of authentic nitro-PA was at m/z 265. By use of selective ion monitoring at 265 the retention time was 22.3 min. The metabolite also gave only one peak under these conditions at 22.3 min, and a mixture of authentic nitro-PA and metabolite also gave only one peak.

Evidence for nitroso-PA and procainamide *N*-oxide metabolites was sought with a different HPLC system consisting of a 15-cm, 5-μm, C18, Spheresorb column (Jones Chromatography, Llanbradach, U.K.) and a mobile phase consisting of water, acetonitrile, acetic acid, and triethylamine (80:20:1:0.05, v/v). With a flow rate of 1 mL/min, the retention times of PAHA, procainamide, procainamide *N*-oxide, nitro-PA, and nitroso-PA were 2.8, 3.8, 4.2, 12.7, and 14.1 min, respectively.

Results

Analogous to our results with dapsone, procainamide was oxidized by activated PMN to nitro-PA. In the absence of PMA (which activates the cells) no oxidation occurred. In the presence of ascorbic acid, the PAHA intermediate was detected. This is presumably because the ascorbate prevented oxidation of PAHA to nitro-PA. Although hydrogen peroxide, by itself, did not oxidize procainamide to PAHA, it did oxidize PAHA to nitro-PA, (data not shown). The formation of nitro-PA and PAHA as a function of ascorbate concentration is shown in Figure 1. At a high ascorbate concentration (5 mM) neither of the metabolites were detected, presumably because the ascorbate reduced almost all of the hydrogen peroxide which is necessary for the metabolism. The oxidation of procainamide in the absence of ascorbic acid was roughly

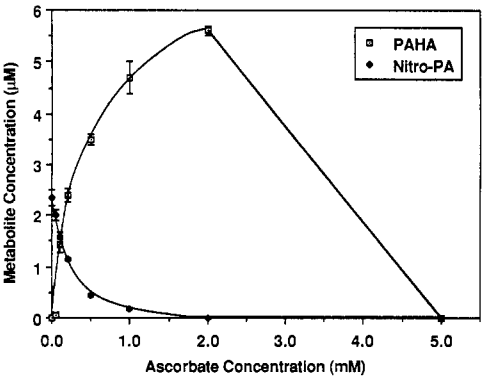


Figure 1. Metabolism of procainamide to PAHA and nitro-PA by activated PMN as a function of ascorbate concentration. Conditions: PMN concentration, 1.5 × 10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; incubation time, 45 min. The values are the mean ±SE from four experiments.

Table I. Effect of Prostaglandin Synthetase Inhibitors on Procainamide Metabolism by Neutrophils

treatment	PAHA, μM[b]	nitro-PA, μM[b]
control[a]	4.7 ± 0.2	1.7 ± 0.1
aspirin (1 mM)	4.6 ± 0.3	1.6 ± 0.1
indomethacin (1 mM)	4.1 ± 0.4	1.6 ± 0.1

[a] Control conditions: PMN concentration, 1.5 × 10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; incubation time, 45 min. The experiments in which PAHA was determined contained 1 mM ascorbic acid. [b] Values are the mean ±SE of four experiments.

Table II. Effect of Prostaglandin Synthetase Inhibitors on Procainamide Metabolism by Mononuclear Leukocytes

treatment	PAHA, μM[b]
control[a]	1.5 ± 0.2
aspirin (1 mM)	1.4 ± 0.2
indomethacin (1 mM)	1.3 ± 0.2

[a] Control conditions: MNL concentration, 6.25 × 10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; ascorbic acid concentration, 1 mM; incubation time, 45 min. [b] Values are the mean ±SE of four experiments.

linear with time, but in the presence of ascorbic acid, although the production of observed metabolite was higher, it started to level off after about 10 min (Figure 2). The production of PAHA and nitro-PA as a function of PMN concentration and procainamide concentration is also shown in Figure 2. The metabolism was inhibited by azide and catalase (Figure 3) but not by inhibitors of prostaglandin synthetase (Table I).

Although procainamide oxidation by hydrogen peroxide alone was not detectable, the combination of purified myeloperoxidase (MPO) and hydrogen peroxide oxidized procainamide to PAHA and other reactive metabolites (data not shown). In contrast, horseradish peroxidase and hydrogen peroxide did not oxidize procainamide to PAHA or nitro-PA (data not shown). This will be the subject of a separate paper.

Similar results were obtained with MNL (Figures 4 and 5, Table II) except that no nitro-PA was produced even in the absence of ascorbate. However, ascorbate was added to the MNL incubations because it approximately doubled the yield of PAHA. These reactions are summarized in Scheme I.

Other possible metabolites, such as nitroso-PA and the *N*-oxide of the tertiary amine, were not detected. Nitroso-PA would not be expected because, in the absence of ascorbate, it would be rapidly oxidized to nitro-PA or covalently bind to protein, and, in the presence of ascor-

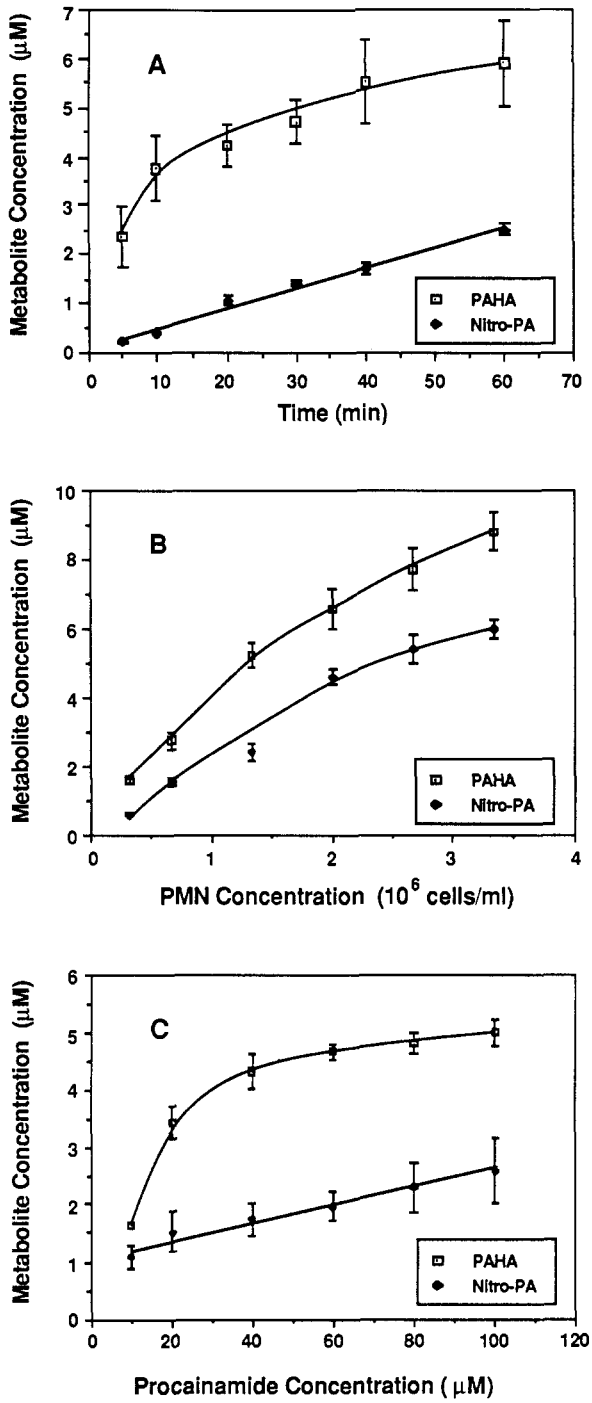


Figure 2. Metabolism of procainamide by PMN in the presence and absence of ascorbic acid: (A) rate; (B) PMN concentration dependence; (C) substrate concentration dependence. Standard conditions: PMN concentration, 1.5×10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; incubation time, 45 min. Ascorbic acid was added to incubations in which PAHA was determined (1 mM) but not to the incubations in which nitro-PA was determined. The values are the mean ±SE from four experiments.

bate, it would be reduced back to PAHA. One might expect to see the *N*-oxide because procainamide is oxidized by hydrogen peroxide to the *N*-oxide. However, this occurs best under strongly alkaline conditions in which the lone pair of electrons on the tertiary amine is available for oxidation.

Discussion

Procainamide was oxidized by activated PMN to nitro-PA. This is a six-electron oxidation and is unlikely to

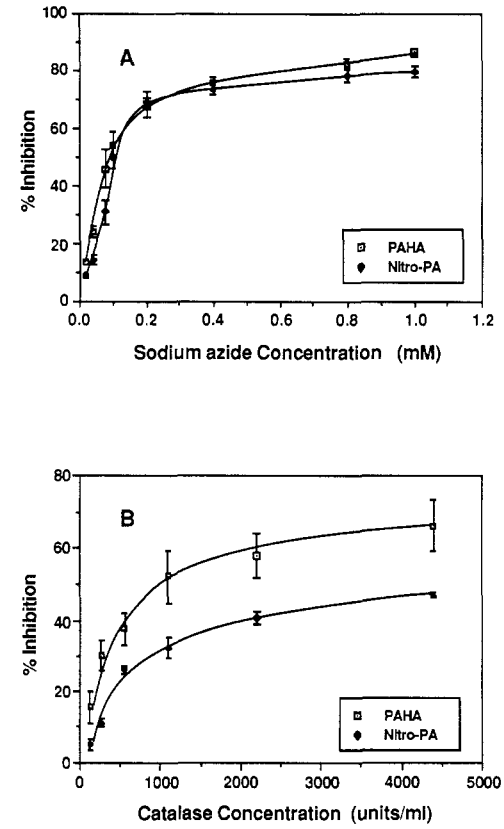
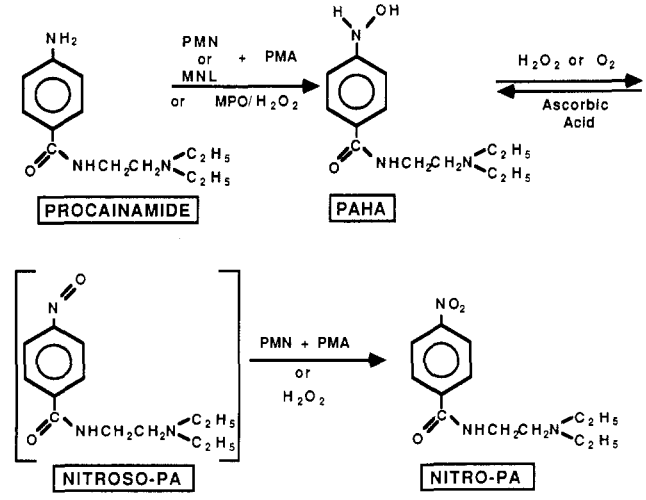


Figure 3. Inhibition of PMN metabolism of procainamide: (A) inhibition by azide; (B) inhibition by catalase. Standard conditions: PMN concentration, 1.5×10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; incubation time, 45 min. Ascorbic acid was added to incubations in which PAHA was determined (1 mM) but not to the incubations in which nitro-PA was determined. The values are the mean ±SE from four experiments.

Scheme I. Summary of Procainamide Metabolism by Leukocytes



occur in one step. The hydroxylamine and nitroso derivatives are the presumed two-electron oxidation intermediates. These intermediates are easily oxidized, and it is not surprising that they would not exist long in the oxidant environment of an activated PMN. We have previously shown that ascorbic acid reduces nitroso-PA to PAHA (*4*). In the present work, ascorbate was able to prevent oxidation of PAHA to nitro-PA without reducing all the hydrogen peroxide which would prevent metabolism of procainamide. Since ascorbic acid and other reductants are

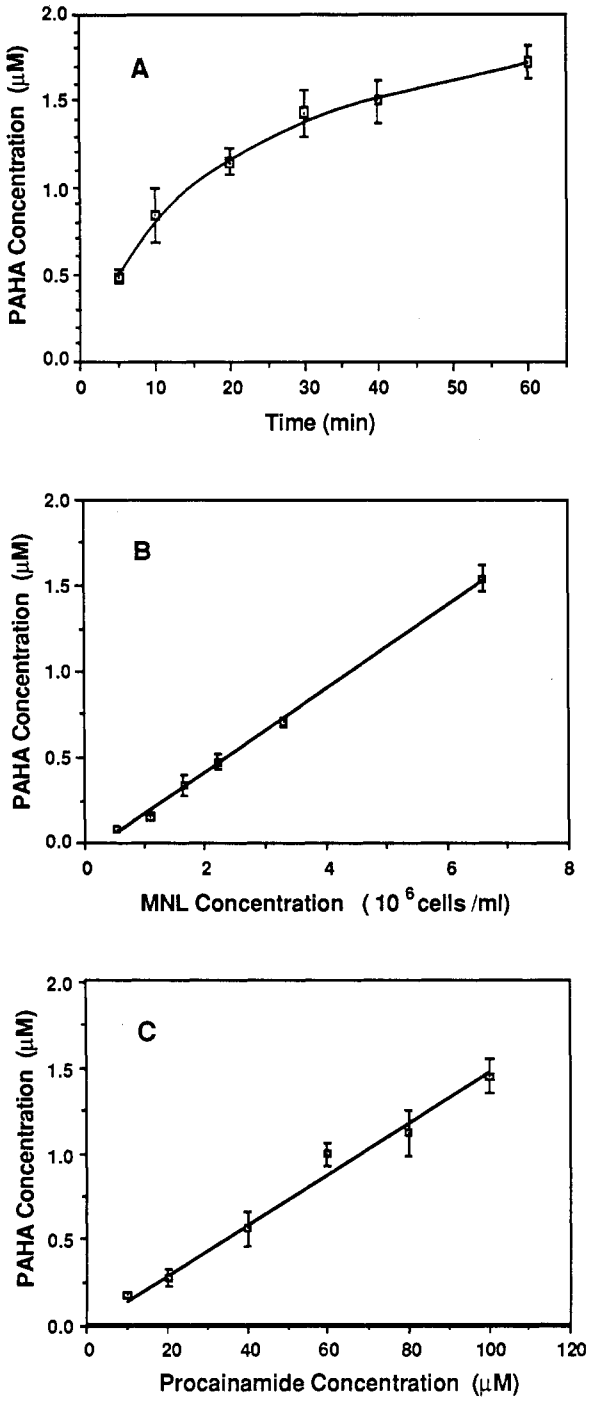


Figure 4. Metabolism of procainamide by MNL: (A) rate; (B) MNL concentration dependence; (C) substrate concentration dependence. Standard conditions: MNL concentration, 6.25×10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; ascorbic acid concentration, 1 mM; incubation time, 45 min. The values are the mean ±SE from four experiments.

present in vivo, the intermediate metabolites may also have a finite lifetime in vivo.

The oxidation of procainamide to PAHA appears to be enzymatic since hydrogen peroxide or superoxide alone did not oxidize procainamide. However, further oxidation of PAHA to nitro-PA does occur with hydrogen peroxide alone. Since no metabolism occurred in the absence of activation of the cells and low concentrations of azide inhibited the metabolism, cytochrome P-450 must not contribute significantly to the metabolism. Lack of inhibition by aspirin and indomethacin rules out prostaglandin synthetase. It is known that MPO and hydrogen

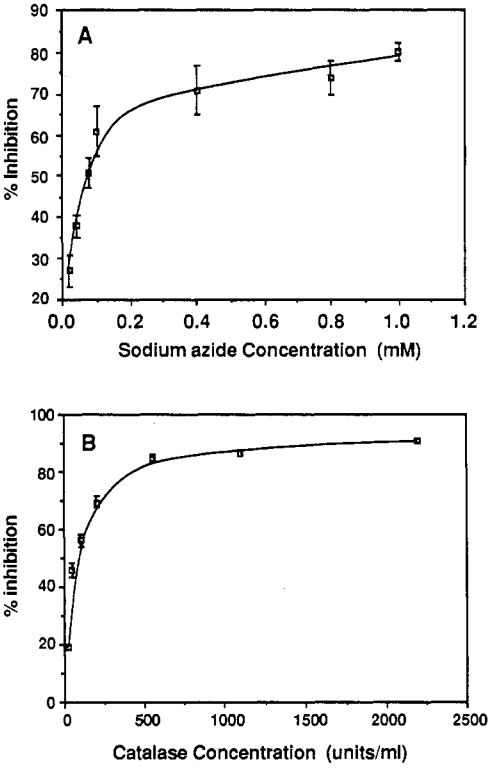


Figure 5. Inhibition of MNL metabolism of procainamide: (A) Inhibition by azide; (B) Inhibition by catalase. Standard conditions: MNL concentration, 6.25×10^6 cells/mL; procainamide concentration, 0.1 mM; PMA concentration, 40 ng/mL; ascorbic acid, 1 mM; incubation time, 45 min. The values are the mean ±SE from four experiments.

peroxide are released when PMN are activated. Since procainamide is also oxidized to PAHA by purified MPO in the presence of hydrogen peroxide, this is the likely explanation for the observed metabolism by PMN. This is supported by the observations that azide (which inhibits MPO) and catalase (which decomposes hydrogen peroxide) inhibit the metabolism. The mechanism appears to involve a two-electron oxidation because horseradish peroxidase (which is said to only catalyze one-electron oxidation) did not catalyze oxidation of procainamide to PAHA.

Procainamide was also metabolized by MNL (which are a mixture of monocytes and lymphocytes). As with PMN, the metabolism appears to be due to MPO since activation of the cells is required and metabolism is inhibited by azide. If this is true, the active cell must be the monocyte since lymphocytes do not contain MPO and are not capable of a respiratory burst. Unlike metabolism by PMN, MNL did not produce detectable concentrations of nitro-PA. PAHA was the only metabolite detected even in the absence of ascorbic acid. This may simply be due to the lesser amount of hydrogen peroxide present, both because monocytes do not produce as much as PMN and because only about $^1/_4$ of the MNL are monocytes.

As with dapsone and sulfadiazine, we propose that procainamide-induced agranulocytosis is due to the chemically reactive PAHA and/or nitroso-PA metabolites formed by activated PMN. The mechanism could involve direct bone marrow toxicity if myeloperoxidase-containing leukocytes were activated in the bone marrow. Alternatively, these reactive metabolites could be formed by activated peripheral neutrophils and bind to their cell membranes, causing the induction of autoantibodies which react with neutrophils. It is likely that a relatively high concentration of reactive metabolites will be present on the neutrophil surface since the hydrogen peroxide gen-

erating system is on the outer surface of the cell membrane, and the myeloperoxidase is released on the surface of the cell or into a phagosome which is formed by invagination of the cell membrane.

Likewise, reactive metabolites of procainamide are likely to bind to the cell membrane of activated monocytes. A key function of the monocyte and other macrophages is to process antigen and to present it, along with the class II major histocompatibility glycoprotein (MHC II), to lymphocytes (*14*). This is an important step in the induction of antibody synthesis. It has been suggested that binding of haptens to the membrane of monocytes may provide a strong stimulus for antibody synthesis (*15*). Furthermore, since it appears as if processed antigen may have to bind to MHC II (*14*), direct binding of a reactive metabolite to MHC II may obviate the need for processing haptenized protein by monocytes. Thus, the formation of reactive metabolites on the surface of activated monocytes where they can react with MHC II or some other membrane structure provides an attractive hypothesis for the mechanism of procainamide-induced lupus. This may represent a general mechanism for the induction of lupus and other hypersensitivity reactions since many of the drugs involved are aromatic amines or hydrazines (*16*). It also appears as if several other types of drugs can be metabolized to reactive metabolites by myeloperoxidase-/H_2O_2 (*17–19*). Some carcinogens can also be activated by this system (*20*).

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