Biochemical Pharmacology, Vol. 45, No. 6, pp. 1267–1275, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CARBAMAZEPINE METABOLISM TO A REACTIVE INTERMEDIATE BY THE MYELOPEROXIDASE SYSTEM OF ACTIVATED NEUTROPHILS*

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(*Received* 21 *September* 1992; *accepted* 2 *December* 1992)

Abstract—Carbamazepine is an anticonvulsant which is associated with a significant incidence of hypersensitivity reactions including agranulocytosis. We have postulated that many drug hypersensitivity reactions, especially agranulocytosis and lupus, are due to reactive metabolites generated by the myeloperoxidase (MPO) (EC 1.11.1.7) system of neutrophils and monocytes. This led to a study of the metabolism and covalent binding of carbamazepine with $MPO/H_2O_2/Cl^-$ and neutrophils. Metabolism and covalent binding were observed in both systems and the same pathway appeared to be involved; however, the metabolism observed with the MPO system was approximately 500-fold greater than that observed with neutrophils. The metabolites identified were an intermediate aldehyde, 9-acridine carboxaldehyde, acridine, acridone, choloroacridone, and dichloroacridone. We postulate that the first intermediate in the metabolism of carbamazepine is a carbonium ion formed by reaction of hypochlorous acid (HOCl) with the 10,11 double bond. Although we have no direct proof for the proposed carbonium ion, it provides the most likely mechanism for the observed ring contraction. Iminostilbene, a known metabolite of carbamazepine, was also metabolized by a similar pathway leading to ring contraction; however, the rate was much faster and the first step may involve N-chlorination and a nitrenium ion intermediate. These data confirm that carbamazepine is metabolized to reactive intermediates by activated leukocytes. Such metabolites could be responsible for some of the adverse reactions associated with carbamazepine, especially reactions such as agranulocytosis and lupus which involve leukocytes.

Carbamazepine is an iminostilbene derivative which is considered one of the most important drugs used for the treatment of seizure disorders [1]. Of the adverse reactions associated with carbamazepine, 5% can be classified as idiosyncratic or hypersensitivity reactions. These can range from serious skin reactions, such as erythema multiforme [2], to severe haematological disorders, especially agranulocytosis and aplastic anemia [3, 4]. Such reactions are unpredictable and are associated with high mortality rates. More common haematological reactions are transient neutropenia with an incidence of 10% and persistent leukopenia with an incidence of 2% [5]. The incidence of antinuclear antibodies in patients taking carbamazepine has been reported to be 78% [6]; however, the incidence of clinical lupus is much lower, about 0.001% [7].

The mechanism for these reactions is unknown. Evidence suggests that many idiosyncratic reactions are due to reactive metabolites [8]. A good example is halothane hepatitis [9]. Halothane has been shown to be oxidized to a reactive trifluoroacetyl halide by cytochrome P450, and patients with halothane-induced hepatic necrosis have antibodies against trifluoroacetylated hepatic protein [10].

Although the liver is the major site of drug metabolism, the biological half-life of most reactive metabolites is not sufficient to cause toxicity at distant sites. Specifically, a reactive metabolite generated in the bone marrow is more likely to lead to bone marrow toxicity than one produced in the liver.

Leukocytes not only are the major cells involved in the induction of an immune response but also exhibit strong oxidizing properties. These cells, in particular monocytes, neutrophils and neutrophil precursors in the bone marrow, contain the enzyme myeloperoxidase (MPO)¶ which upon activation of the cells combines with hydrogen peroxide (H_2O_2) and chloride to form hypochlorous acid (HOCl) [11]. This system is capable of oxidizing drugs to chemically reactive intermediates. Specifically, procainamide, dapsone, phenytoin, propylthiouracil, sulfamethoxazole, and amodiaquine are metabolized by activated leukocytes to reactive intermediates [12–17]. Evidence pointed to involvement of myeloperoxidase. In general, these drugs are associated with a high incidence of idiosyncratic drug reactions, such as agranulocytosis and lupus.

With this background we investigated the oxidative metabolism of carbamazepine by activated human leukocytes, MPO/H_2O_2 and HOCl to determine if reactive intermediates are formed.

* A preliminary report of this work was presented at the Third North American Meeting of the International Society for the Study of Xenobiotics, San Diego, CA.

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¶ Abbreviations: MPO, myeloperoxidase; HBSS, Hanks' Balanced Salt Solution; EI, electron impact; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; and PMN, polymnorphonuclear leukocytes.

MATERIALS AND METHODS

Materials

Carbamazepine, iminostilbene, acridone and acridine were obtained from the Aldrich Chemical Co., Milwaukee, WI. Albumin (bovine, fraction V) and phorbol 12-myristate 13-acetate (PMA) were obtained from the Sigma Chemical Co., St. Louis, MO. MPO was obtained from Cortex, Los Angeles, CA. [^{14}C]Carbamazepine (labels on carbons 10 and 11, 0.0309 mCi/mg, >98% purity) was obtained from Ciba-Geigy, Basel, Switzerland.

Synthesis of 9-acridine carboxaldehyde and the intermediate aldehyde. 9-Acridine carboxaldehyde was prepared as outlined by Kawashima and Ishiguro [18], using the oxidation of iminostilbene by *m*-chloroperbenzoic acid (0.82 g; 42.0%), m.p. 139–141° (lit. m.p. 138–140°). The identity of the synthetic aldehyde was confirmed by 400 MHz ^{1}H-NMR in [^{2}H]chloroform: ∂ 7.72 (2 H, td), 7.85 (2 H, td), 8.32 (2 H, d), 8.75 (2 H, d), 11.54 (s, CHO). The intermediate aldehyde was generated by reacting HOCl (300 μM) with carbamazepine (100 μM) for 1 min in phosphate-buffered saline (PBS, 0.1 M phosphate, pH 6.0).

The results of the following experiments were calculated from the average of three independent experiments using different days, incubations and donors.

Incubations

Neutrophils. PMN (polymorphonuclear leukocytes) (mostly neutrophils) were prepared from the blood of normal subjects using Ficoll-Paque (Pharmacia LKB, Baie D'Urfe, Quebec) [19]. The viability of the cells was found to be greater than 95% as determined by trypan blue dye exclusion. Carbamazepine, (100 μM) or iminostilbene (10 μM) was incubated with neutrophils (1×10^6–4×10^6 cells/mL) in a final volume of 500 μL Hanks' Balanced Salt Solution (HBSS, Gibco, Mississauga, Ontario). PMA (20 ng in 10 μL dimethyl sulfoxide) was added to activate the cells in some incubations. Cell suspensions were incubated in a shaking water bath at 37° over various times followed by centrifugation at 13,000 *g* for 2 min. The supernatant was removed and aliquots were analyzed by HPLC. Sodium hydroxide (0.01 mL, 1 N) was added to the supernatant to prevent further decomposition of 9-acridine carboxaldehyde to acridine.

Myeloperoxidase/hydrogen peroxide/chloride. Carbamazepine (4 mM solution in ethanol) was added to PBS (0.1 M phosphate, pH 6) to a final concentration of 100 μM. Myeloperoxidase was then added (5 units/mL), and the reaction was initiated with hydrogen peroxide (0.2 mM, Caledon). This was incubated over various times at room temperature. Incubations were also undertaken with various drug, enzyme and hydrogen peroxide concentrations to determine their influence on oxidation.

Sodium hypochlorite. Carbamazepine was added to PBS (0.1 M phosphate, pH 6.0) to a final concentration of 100 μM and the reaction was initiated by adding sodium hypochlorite to a final concentration of 100 μM. The sodium hypochlorite concentration was varied to determine its effect on oxidation. Iminostilbene was also oxidized with hypochlorite using the same conditions. An iodometric method of titration was used to standardize the sodium hypochlorite [20].

A Hewlett-Packard diode array spectrophotometer was used to determine the rate of metabolism of carbamazepine and iminostilbene by HOCl. Immediate scanning of the reaction mixture was performed following addition of the sodium hypochlorite. Reactions were monitored at scans of 2.0-sec intervals for 1 min.

Analytical

Metabolites of carbamazepine were analyzed by HPLC with a Beckman 110B pump (Berkeley, CA), a 4.6 × 15 cm Jones pH stable column packed with 5-μm Spherisorb ODS II (Jones Chromatography Limited, Mid Glamorgan, U.K.), and a Beckman 160 UV detector at a wavelength of 254 nm. The solvent consisted of water, acetonitrile, acetic acid and triethylamine (70:30:0.01:0.03, by vol.), pH 9.0. The flow rate was 1 mL/min. Peak areas were integrated with a Shimadzu Chromatopac C-R6A. Under these conditions the retention times in min were: acridone, 5.1; carbamazepine, 6.7; 9-acridine carboxaldehyde, 14; and acridine, 12.

The amount of metabolite produced by activated neutrophils was less than that produced by MPO/H_2O_2/Cl^-, and a fluorescence detector was used to quantify acridine. Metabolites were analyzed using a 3 × 150 mm Separon SGX CGC column packed with 5-μm silica Separon SGX (Fischer Scientific Ltd., Unionville, Ontario, Canada) and a Kratos FS 970 spectrofluorometer detector at an excitation wavelength of 354 nm and an emission wavelength of 420 nm. The solvent consisted of water, acetonitrile, acetic acid and triethylamine (60:40:0.1:0.05, by vol.) with a pH of 3. Standard curves generated for carbamazepine, acridone, acridine, 9-acridine carboxaldehyde and iminostilbene were linear over a range of concentrations from 0.1 to 100 μM with correlation coefficients >99.

TLC was used to isolate metabolites from the reaction mixture. Silica plates (Whatman LK 5DF, Clifton, NJ, 5 × 20 cm, 250 μm thickness) were developed with a solvent of methylene chloride : ethyl acetate (95:5, v/v).

A Hewlett-Packard 8452A diode array detector was also used to obtain UV spectra of metabolites.

Mass spectra of acridine, acridone, chloroacridone, dichloroacridone and 9-acridine carboxaldehyde were performed using a VG-Analytical ZAB-SE mass spectrometer. Samples were introduced via direct probe, ionized in the electron impact (EI) mode (70 eV), and the source temperature was 200°.

The 9-acridine carboxaldehyde was also analyzed by HPLC/MS performed on a Sciex API III. The HPLC conditions consisted of a 4.6 × 100 mm column packed with 5-μm Ultracarb 20 (Phenomenex, Torrance, CA) and a mobile phase of water/acetonitrile/acetic acid (60:40:1, by vol.) at a flow rate of 1 mL/min. Under these conditions carbamazepine and 9-acridine carboxaldehyde had retention times of 4 and 9 min, respectively.

The intermediate aldehyde was analyzed using similar conditions as the 9-acridine carboxaldehyde

on a Sciex API III, using MS/MS and a heated nebulizer interface.

Covalent binding to neutrophils

PMN were activated with 20 ng/mL of PMA and incubated for various times at 37° in 500 μL HBSS with 1 μCi [^{14}C]carbamazepine (sp. act. 0.0309 mCi/mg). Cells were then collected on GF/F Whatman glass microfiber filters (Whatman, Clifton, NJ) and washed with ethanol until the radioactivity of the washes was below 100 dpm. The filters were placed in 10 mL of scintillation fluid (ACS, Amersham, Oakville, Ontario) and counted in a scintillation counter.

The apparent binding of 9-acridine carboxaldehyde and acridine were determined indirectly by a determination of the decrease in their concentration in solution when cells were added. Specifically, 9-acridine carboxaldehyde or acridine was incubated in various concentrations (20, 10, 5, 1 μM) with leukocytes (2×10^6/mL) for 1 min at room temperature. Samples were then centrifuged at 13,000 *g* for 2 min. The quantity of 9-acridine carboxaldehyde bound to cells was determined by comparison of its concentration (as determined by HPLC) in solution in the absence of cells with its concentration (determined in the same manner) in the supernatant from the cellular incubations.

Covalent binding to protein

[^{14}C]Carbamazepine (1 μCi), MPO (1 U) and bovine serum albumin (200 μg, Sigma) were dissolved in 0.2 mL of phosphate buffer (0.1 M phosphate, pH 6.0) or PBS (0.1 M phosphate, pH 6.0). The reaction was initiated with hydrogen peroxide (final concentration 0.4 mM) and incubated for 45 min. After this time, 2 mL of cold acetone was added to precipitate the protein. The samples were then centrifuged for 3 min at 1200 *g* and the acetone was removed. The protein was then washed repeatedly with acetone until the radioactivity of the washes reached background. Phosphate buffer (200 μL) was added to resuspend the pellet, and a 100-μL aliquot was counted in 10 mL scintillation fluid (ACS, Amersham).

Protein determination was performed on each sample using the Bradford method [21]. The reagents were obtained in a kit from Bio-Rad Laboratories, Richmond, CA.

RESULTS

Identity of metabolites

Carbamazepine was converted by either MPO/H_2O_2/Cl^- or HOCl in a series of reactions to an intermediate aldehyde, 9-acridine carboxaldehyde, acridine, acridone and chloroacridone.

The identity of the intermediate aldehyde was determined by its mass spectrum and its spontaneous conversion to 9-acridine carboxaldehyde. MS/MS of the intermediate aldehyde using the Sciex API III gave a molecular ion at *m/z* 253 and collisional activation of the molecular ion produced fragment ions at *m/z* 208 (100%) and 180 (30%).

The identity of 9-acridine carboxaldehyde formed from carbamazepine by MPO/H_2O_2 was confirmed by comparison of its mass spectra, retention time on HPLC, and UV spectrum with that of synthetic 9-acridine carboxaldehyde. The mass spectrum of 9-acridine carboxaldehyde gave a molecular ion at *m/z* 207 (100%) with fragment ions at *m/z* 179 (90%) and 151 (40%). With HPLC/MS using the API III the base peak was the molecular ion at *m/z* 208 with fragment ions at *m/z* 180 (70%) and 152 (15%). The fragment ions presumably reflect the loss of CO and CO + C_2H_4, respectively. Further evidence for the structure of the 9-acridine carboxaldehyde formed by MPO/H_2O_2 is that it, like the synthetic aldehyde, was readily converted to acridine by acid. This even occurred in HPLC solvent and the half-life of 9-acridine carboxaldehyde in the acidic HPLC solvent (pH of 3.0) was 12 hr (data not shown).

Another complication of using an acidic HPLC solvent for analysis of 9-acridine carboxaldehyde is that two peaks were observed with retention times of 2.5 and 14 min. It appears that the peak at 2.5 min is the hydrate form of the aldehyde. There was "bridging" between the hydrate peak and the free aldehyde peak due to the interconversion of the two forms on the column. A basic solvent and pH stable column were required to prevent conversion of 9-acridine carboxaldehyde to acridine on the column, and it also locked the aldehyde in either the aldehyde or hydrate form so that only one peak was observed. When 9-acridine carboxaldehyde was dissolved in methanol or ethanol, peaks with different retention times were observed on HPLC using a solvent with a pH of 3.0 even though there was no alcohol in the solvent. This indicates that the hemiacetal formed in alcohol hydrolyzed very slowly.

The identities of acridone and acridine formed from carbamazepine by MPO/H_2O_2 were confirmed by comparison of their mass spectra, retention times on HPLC, and UV spectra with those of commercial standards. Acridone gave a molecular ion at *m/z* 195 (100%) and a fragment ion at *m/z* 167 (27%) which could be due to the loss of CO or C_2H_4. The mass spectrum of acridine consisted of a molecular ion at *m/z* 179 (100%) and a fragment ion at *m/z* 151 (13%).

The identities of chloro- and dichloroacridone were based on their mass spectra and the observation that they are also formed by chlorination, either by HOCl or MPO/H_2O_2/Cl^-, of acridone. The positions of the chlorines on the ring are unknown and two isomers of chloroacridone and three isomers of dichloroacridone were observed on HPLC. The mass spectrum of chloroacridone produced a molecular ion at *m/z* 229 (100%) with a chlorine isotope peak at *m/z* 231 (29%) and fragment ions at *m/z* 201 (9%) and 166 (25%). This indicates loss of ethylene and chloroethylene, respectively. The mass spectrum of dichoroacridone was similar with a molecular ion at *m/z* 263 (100%) and chlorine isotope peaks at *m/z* 265 (64%) and 267 (14%). A fragment ion was seen at *m/z* 200 (13%).

In addition to the identified metabolites, a small, unidentified peak with a retention time of 4.1 min was seen when the reaction mixture was immediately

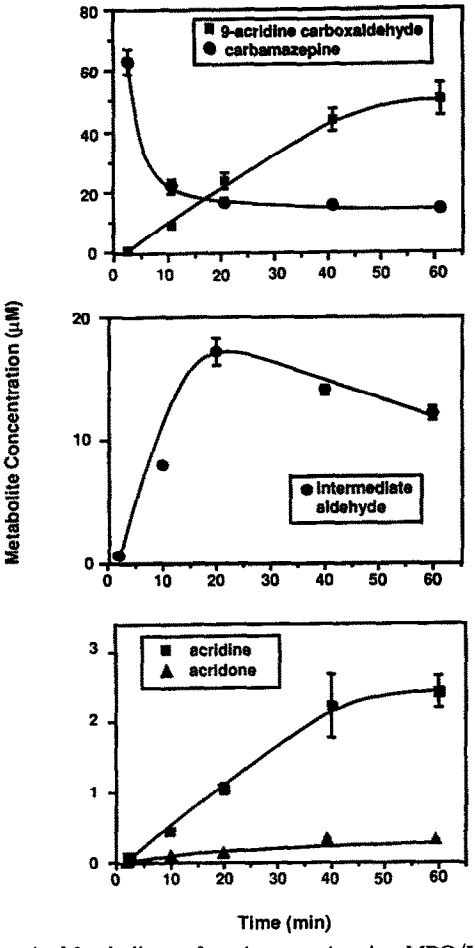

Fig. 1. Metabolism of carbamazepine by MPO/H_2O_2/Cl^- over time. Conditions were: MPO, 5 units/mL; carbamazepine, 100 μM; H_2O_2, 0.4 mM; in 0.2 mL of PBS (pH 6.0), temperature 25°. Values are means ± SEM, N = 3.

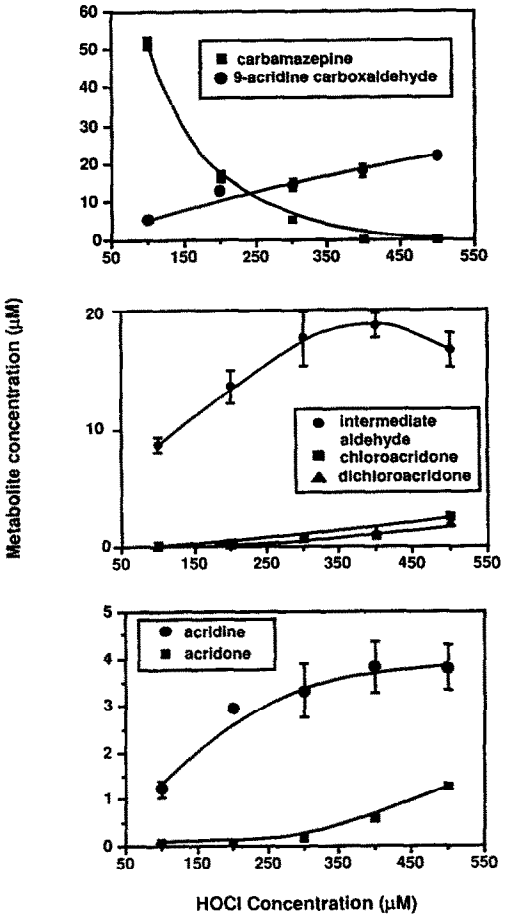

Fig. 2. Carbamazepine metabolism by various concentrations of HOCl. Conditions were: carbamazepine, 100 μM; HOCl, 100–500 μM; in 0.2 mL PBS (pH 6.0); incubation time, 20 min; temperature, 25°. Values are means ± SEM, N = 3.

injected onto the HPLC, but it disappeared after 2 min.

Characterization of pathway

Carbamazepine metabolism by the enzyme system resulted in an initial increase in intermediate aldehyde concentration with a subsequent decrease after 20 min while the other metabolites continued to increase (Fig. 1). A synthetic standard was not available for the intermediate aldehyde or the chloroacridones; therefore, we were not able to determine their exact concentration. Instead, in this and subsequent figures, the relative peak area is reported such that it would represent micromolar concentration if the extinction coefficients of these metabolites were the same as that of 9-acridine carboxaldehyde. Oxidation of the metabolites was investigated to further characterize the sequence of the metabolic pathway. 9-Acridine carboxaldehyde was oxidized to acridine and acridone by HOCl. Acridine was oxidized by HOCl to acridone, chloroacridone and dichloroacridone. Acridone was oxidized to chloroacridone and dichloroacridone. Carbamazepine incubation with increasing concentrations of HOCl resulted in the formation of chloroacridones with retention times of 8 and 10 min on HPLC as well as the dichloroacridone metabolites with retention times of 21, 25 and 26 on the HPLC (Fig. 2). These studies provided further evidence for the sequence proposed in Fig. 3.

Other incubations were performed with various substrate, hydrogen peroxide and MPO concentrations (data not shown). An increase in carbamazepine concentration (up to 100 μM) and MPO concentration (up to 5.0 units/mL) resulted in a subsequent increase in metabolite concentration. An increase in metabolism was also seen with an increase in H_2O_2 concentrations up to 0.4 mM. Higher H_2O_2 concentrations caused a decrease in metabolism, most likely due to inhibition of enzyme activity. In the absence of hydrogen peroxide, MPO or chloride, no metabolites were observed and there

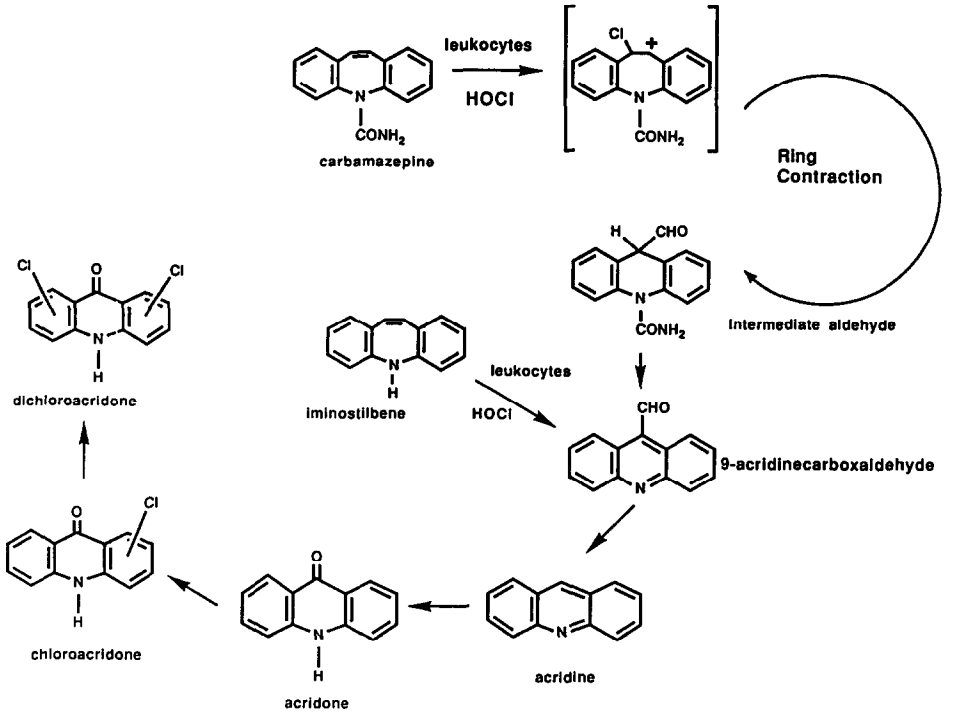

Fig. 3. Proposed pathway of carbamazepine and iminostilbene oxidation by activated leukocytes, MPO/H_2O_2/Cl^- and HOCl.

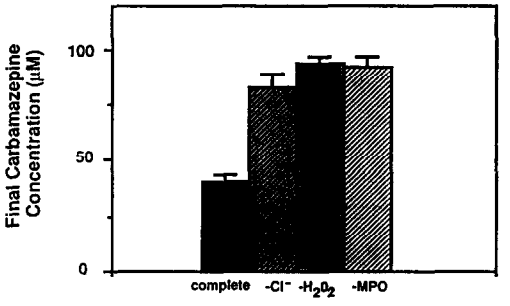

Fig. 4. Metabolism of carbamazepine by MPO/H_2O_2/Cl^- in the absence of MPO, H_2O_2 or Cl^-. Conditions were: MPO, 5 units/mL; carbamazepine, 100 μM; H_2O_2, 0.4 mM; in 0.2 mL of phosphate buffer (pH 6.0); incubation time, 20 min; temperature, 25°. Values are means ± SEM, N = 3.

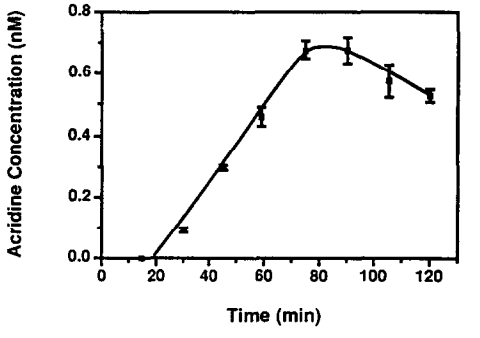

Fig. 5. Metabolism of carbamazepine by neutrophils in the presence of PMA over time. Conditions were: carbamazepine, 100 μM; 2.0×10^6 cells/mL; 20 ng/mL PMA; in 0.5 mL HBSS, temperature 37°. Values are means ± SEM, N = 3.

was no significant decrease in carbamazepine concentration (Fig. 4).

Carbamazepine was also metabolized to acridine by activated neutrophils but the degree of metabolism was less and no other metabolites were detected (Fig. 5). No acridine was detected in the absence of PMA. Acridine did not adhere to neutrophils to a significant degree (data not shown). Varying cell number, carbamazepine concentration and PMA concentration did not increase carbamazepine metabolism significantly (data not shown).

Metabolism of iminostilbene

In contrast to carbamazepine, iminostilbene, a metabolite of carbamazepine [22], was metabolized extensively by activated neutrophils (Fig. 6). The metabolites observed were similar to those derived from carbamazepine metabolism with the absence of the two peaks seen at retention times of 4.1 and 3.3 min. These corresponded to the unidentified peak and the intermediate aldehyde.

Iminostilbene was also oxidized by HOCl and the

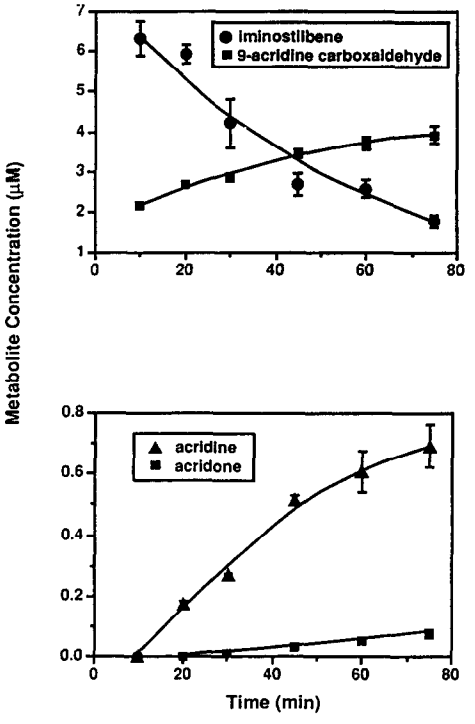

Fig. 6. Metabolism of iminostilbene by neutrophils over time. Conditions were: iminostilbene, 10 μM; 2.0×10^6 cells/mL; in 0.5 mL HBSS, temperature 37°. Values are means ± SEM, N = 3.

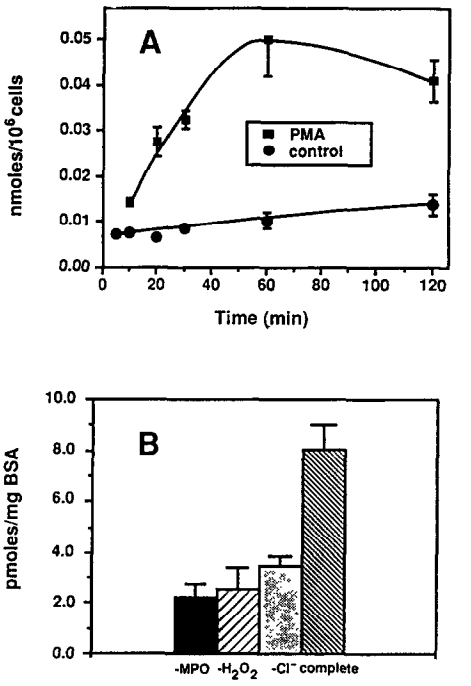

Fig. 7. (A) [^{14}C]Carbamazepine covalent binding to neutrophils in the presence or absence of PMA over time. Conditions were: [^{14}C]carbamazepine, 1 μCi; 2.0×10^6 cells/mL; with or without 20 ng/mL PMA; in 0.5 mL HBSS, temperature 37°. Values are means ± SEM, N = 3. (B) [^{14}C]Carbamazepine covalent binding to albumin in the presence or absence of MPO/H_2O_2/Cl^-. Conditions were: MPO, 5 units/mL; carbamazepine, 1 μCi; H_2O_2, 0.4 mM; albumin, 1 mg/mL; in 0.2 mL of PBS (pH 6.0); incubation time, 20 min; temperature, 25°. Values are means ± SEM, N = 3.

MPO/H_2O_2/Cl^- system to the same metabolites. By following the HOCl oxidation of iminostilbene with a diode array spectrophotometer, the half-life of the reaction was found to be 2 sec as compared to the analogous reaction of carbamazepine under the same conditions which had a half-life of 2 min (data not shown).

Covalent binding

Covalent binding occurred when radiolabeled carbamazepine was incubated with activated neutrophils (Fig. 7A). Very little binding was observed in the absence of cell activation, which strongly suggests that the binding was due to a metabolite rather than carbamazepine. Covalent binding was also observed when radiolabeled carbamazepine was incubated with albumin in the presence of the MPO/H_2O_2/Cl^- system. The absence of significant binding in the absence of enzyme, hydrogen peroxide or chloride (Fig. 7B) again suggests that the binding was due to a metabolite rather than carbamazepine.

We found indirect evidence that 9-acridine carboxaldehyde binds to neutrophils because when neutrophils were added to solutions of varying concentrations of the metabolite there were significant decreases in its concentration, especially at low concentrations of the metabolite (Fig. 8). In contrast, no significant decrease in acridine concentration was observed upon addition of neutrophils (data not shown).

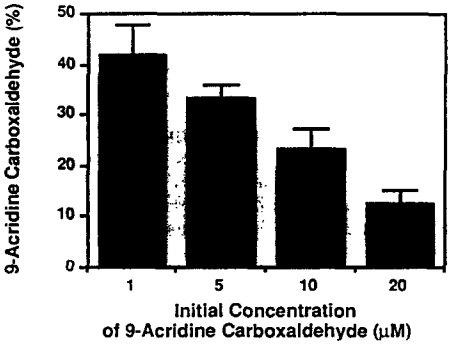

Fig. 8. Apparent binding of 9-acridine carboxaldehyde to neutrophils in the absence of PMA. Conditions were: 9-acridine carboxaldehyde, 1, 5, 10, 20 μM; 2.0×10^6 cells/mL; in 0.5 mL HBSS, temperature 25°. Values are means ± SEM, N = 3.

DISCUSSION

The data presented led to the postulated metabolic scheme shown in Fig. 3. The first intermediate in

this scheme is a carbonium ion formed by reaction of HOCl with the 10, 11 double bond. Although we have no direct proof for the proposed carbonium ion, it provides the most likely mechanism for the observed ring contraction. We were also unable to prove the identity of the intermediate aldehyde because its instability precluded isolation and characterization of synthetic material; however, its mass spectrum, with prominent loss of CO similar to that of 9-acridine carboxaldehyde, and its spontaneous conversion to 9-acridine carboxaldehyde, strongly point to the proposed structure. The intermediate aldehyde has been proposed previously as an intermediate in connection with the hepatic metabolism of carbamazepine [23, 24]. This intermediate aldehyde appears spontaneously to lose CO and NH_3 to give 9-acridine carboxaldehyde, a reaction that is favored by the formation of the highly conjugated acridine system [25]. 9-Acridine carboxaldehyde, in turn, is converted by mild acid to acridine. Although the decarbonylation of pyridine carboxaldehydes is known to occur under strongly acidic conditions [26], the ease of this reaction in mild acid is surprising. In the MPO or HOCl incubations this decarbonylation may involve an oxidative mechanism rather than an acid catalyzed reaction. Acridine and acridone are known metabolites of carbamazepine and may also be formed in the liver [27].

Much less metabolism was observed when carbamazepine was incubated with activated neutrophils than when it was incubated with the principal oxidizing system of neutrophils, i.e. $MPO/H_2O_2/Cl^-$. Furthermore, the only metabolite observed in the cellular system was acridine. Despite these differences, the requirement for PMA in the oxidation by neutrophils, which results in a respiratory burst with degranulation and release of MPO and H_2O_2 as well as the production of the same downstream metabolite by the $MPO/H_2O_2/Cl^-$ system, suggests that the MPO system is involved in the metabolism of carbamazepine by neutrophils. The requirement for chloride suggests that HOCl is the ultimate oxidant. The lesser degree of metabolism in the cellular system may be due to generation of a larger amount of HOCl by the purified enzyme system. Yet, we have observed that incubation of dapsone with neutrophils, as compared to $MPO/H_2O_2/Cl^-$, results in similar degrees of oxidation [13]. Another factor to consider is the relatively slow reaction between HOCl and carbamazepine. Therefore, in the cellular system much of the HOCl generated is likely to react with the cells. HOCl is known to react with other biological material such as protein [28], and this would compete with the reaction of HOCl with carbamazepine. This competition would be less of a factor in the purified enzyme system or with drugs in which reaction of HOCl with the drug is much faster than its reaction with biological material.

Of note is the observation that 9-acridine carboxaldehyde was not detected in the cellular system even though it was present in greater concentrations than acridine in the oxidation of carbamazepine by $MPO/H_2O_2/Cl^-$. To determine the reason for this discrepancy we incubated 9-acridine carboxaldehyde with neutrophils. The concentration in the supernatant was less than in the absence of cells and this had the appearance of saturable binding (Fig. 8). Although this could be due to further metabolism by the cells, significant production of metabolites did not occur without activation of the cells. The observed strong tendency of this aldehyde to form hemiacetals and hydrates suggests that a similar reaction with nucleophilic groups on the cells may be responsible for the observed decrease in 9-acridine carboxaldehyde concentration in the presence of cells. We also attribute the apparent concavity of the first part of the curve in Fig. 5 to the binding of 9-acridine carboxaldehyde. Such binding could decrease the amount of aldehyde available for further metabolism, especially at low concentrations, and this could also contribute to the lesser degree of carbamazepine metabolism observed in the cellular system.

Iminostilbene follows a similar pathway of ring contraction; however, the mechanism could be somewhat different because, in addition to chlorination of the 10,11 double bond, iminostilbene could undergo N-chlorination. N-Chlorination is likely to be more rapid than chlorination of the 10,11 double bond, and *N*-chloroiminostilbene would be expected to lose chloride ion to form a relatively stable nitrenium ion in which the delocalized positive charge could also result in rearrangement leading to 9-acridine carboxaldehyde and hence to acridine, acridone and chloroacridone. In contrast to carbamazepine, metabolism of iminostilbene by neutrophils was quite extensive. Likewise, iminostilene was oxidized by HOCl approximately 60 times faster than carbamazepine. As mentioned earlier, this more rapid reaction of iminostilbene with HOCl could lead to a greater portion of the neutrophil-generated HOCl reacting with the drug rather than with cellular material.

At this point, one can only speculate on the significance of the metabolism of carbamazepine by neutrophils. Although metabolism of carbamazepine is less than that of iminostilbene and is less with neutrophils than with the purified MPO system, metabolism does occur. Covalent binding studies with carbamazepine and activated neutrophils demonstrated that a drug–protein conjugate is formed between a reactive intermediate and activated neutrophils. The requirement of PMA to produce significant binding is strong evidence that binding involves a reactive metabolite formed by reaction of carbamazepine with HOCl. The identity of the intermediate that covalently binds to the activated neutrophils is unknown. The proposed carbonium ion would be reactive, but its half-life may be so short as to preclude significant covalent binding. The apparent binding of the 9-acridine carboxaldehyde to the neutrophils suggests that it may be responsible for the observed covalent binding of carbamazepine. As mentioned earlier, this aldehyde appears to form hydrates and hemiacetals that dissociate more slowly than those of most other aldehydes. It would also likely bind to other cellular nucleophiles such as amino groups. Further metabolism of such an adduct could form more permanent adducts such as an imine or an amide. Since iminostilbene is a known

metabolite of carbamazepine and its metabolism by neutrophils is more rapid than that of carbamazepine, it could also make a contribution to covalent binding of the drug *in vivo*. Although the concentration of iminostilbene in urine is low, it can be assumed that urinary excretion does not represent a major pathway for its elimination and it may be a significant metabolite of carbamazepine.

An arene oxide of carbamazepine has been implicated in generalized carbamazepine-induced hypersensitivity reactions in humans [29, 30]; however, it is less likely to be responsible for toxicity involving the bone marrow because it is unlikely to be formed in bone marrow or get from liver to bone marrow due to its presumed high reactivity. A major route of carbamazepine metabolism is epoxidation of the double bond to form the carbamazepine-10,11-epoxide. This was considered a possible candidate as the cause of carbamazepine-induced adverse reactions [31]; however, this epoxide is relatively unreactive and is known to be equipotent as an anticonvulsant with no higher incidence of adverse reactions than carbamazepine itself [32]. It was also not observed as a metabolite in our studies. The epoxide of iminostilbene has been proposed as a metabolite of carbamazepine but appears to be highly reactive and was difficult to isolate [18]. It is therefore possible for iminostilbene to be involved in carbamazepine-induced haematological disorders, either through the nitrenium ion or epoxide intermediates. The degree to which carbamazepine is metabolized to iminostilbene in the liver could even represent a risk factor for some types of adverse reactions associated with carbamazepine.

In summary, carbamazepine, as well as one of its hepatic metabolites, were metabolized by activated neutrophils through a series of steps that resulted in contraction of the 7-membered ring and eventual loss of the carbon involved. This metabolism was associated with covalent binding of the drug to neutrophils. Metabolism of carbamazepine by neutrophils in the bone marrow, as well as the more mature neutrophil precursors which also contain MPO, may be responsible for some of the adverse reactions associated with carbamazepine, especially those which involve the bone marrow.

Acknowledgements—We wish to thank Nasir Zahid for his assistance and Dr. Henrianna Pang and Mr. Richard Crombie for the Carbohydrate Research Centre, supported by The Medical Research Council of Canada (MT-6499), for performing the mass spectrometry. We are also grateful to Ciba-Geigy, Basel, Switzerland, for donating [^{14}C]-carbamazepine. Mass spectra of the 9-acridine carboxaldehyde were done using a Sciex API III at Sciex Inc., Thornhill, Ontario. The mass spectrometry of the intermediate aldehyde was performed by Luca Matassa at Mann Laboratories, Mississauga, Ontario, Canada. This work was supported by the Medical Research Council of Canada (Grant MA-10036) and a contract with Health and Welfare Canada (2030).

REFERENCES

1. Smith DB, Mattson RH, Cramer JA, Collins JF, Novelly RA and Craft B, Results of a Nationwide Veterans Administration Cooperative study comparing the efficacy and toxicity of carbamazepine, phenobarbital, phenytoin and primidone. *Epilepsia* **28**: S50–S58, 1987.
2. Alballa S, Fritzler M and Davis P, A case of drug induced lupus due to carbamazepine. *J Rheumatol* **14**: 599–600, 1987.
3. Pisciotta V, Drug-induced agranulocytosis. *Drugs* **15**: 132–143, 1978.
4. Franceschi M, Ciboddo G, Truci G, Borri A and Canal N, Fatal aplastic anemia in a patient treated with carbamazepine. *Epilepsia* **29**: 582–583, 1988.
5. Hart RG and Easton JD, Carbamazepine and hematological monitoring. *Ann Neurol* **11**: 309–312, 1982.
6. Alarcon-Segovia D, Fishbein E, Reyes PA, Dies H and Shwadsky S, Antinuclear antibodies in patients on anticonvulsant therapy. *Clin Exp Immunol* **12**: 39–47, 1972.
7. Jain KK, Systemic lupus erythematosus (SLE)-like syndromes associated with carbamazepine therapy. *Drug Saf* **6**: 350–360, 1991.
8. Park BK, Coleman JW and Kitteringham NR, Drug disposition and drug hypersensitivity. *Biochem Pharmacol* **36**: 581–590, 1987.
9. Kenna JG, Satoh H, Christ DD and Pohl LR, Metabolic basis for a drug hypersensitivity: Antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. *J Pharmacol Exp Ther* **245**: 1103–1109, 1988.
10. Pohl LR, Satoh H, Christ DD and Kenna JG, The immunologic and metabolic basis of drug hypersensitivities. *Annu Rev Pharmacol* **28**: 367–387, 1988.
11. Weiss SJ, Tissue destruction by neutrophils. *N Engl J Med* **320**: 365–376, 1989.
12. Uetrecht JP and Zahid N, N-Chlorination and oxidation of procainamide by myeloperoxidase: Toxicological implications. *Chem Res Toxicol* **4**: 218–222, 1991.
13. Uetrecht J, Zahid N, Shear NH and Biggar WD, Metabolism of dapsone to a hydroxylamine by human neutrophils and mononuclear cells. *J Pharmacol Exp Ther* **245**: 274–279, 1988.
14. Uetrecht J and Zahid N, N-Chlorination of phenytoin by myeloperoxidase to a reactive metabolite. *Chem Res Toxicol* **1**: 148–151, 1988.
15. Waldhauser L and Uetrecht J, Oxidation of propylthiouracil to reactive metabolites by activated neutrophils: Implications for agranulocytosis. *Drug Metab Dispos* **19**: 354–359, 1991.
16. Cribb AE, Miller M, Tesoro A and Speilberg SP, Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. *Mol Pharmacol* **38**: 744–751, 1990.
17. Clarke JB, Maggs JL, Kitteringham NR and Park BK, Immunogenicity of amodiaquine in the rat. *Int Arch Allergy Appl Immunol* **91**: 335–342, 1990.
18. Kawashima K and Ishiguro T, On the reactions of dibenz[*b,f*]oxireno[*d*]azepine derivatives. *Chem Pharm Bull (Tokyo)* **26**: 951–955, 1978.
19. Boyum A, Separation of lymphocytes, granulocytes and monocytes from human blood using iodinated density gradient media. In: *Methods in Enzymology* (Eds. Colowick SP and Kaplan NO), pp. 88–102. Academic Press, Orlando, 1984.
20. Skoog DA and West DM, Applications of oxidation-reduction titrations. *Analytical Chemistry: An Introduction*, pp. 312–317. Saunders College Publishing, New York, 1986.
21. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1972.

22. Csetenyi J, Baker KM, Frigerio A and Morselli PL, Iminostilbene—a metabolite of carbamazepine isolated from rat urine. *J Pharm Pharmacol* **25**: 340–341, 1973.
23. Baker KM, Frigerio A, Morselli PL and Pifferi G, Identification of a rearranged degradation product from carbamazepine-10,11-epoxide. *J Pharm Sci* **62**: 475–476, 1973.
24. Frigerio A, Fanelli R, Biandrate P, Passerini G, Morselli PL and Garattini S, Mass spectrometric characterization of carbamazepine-10,11-epoxide, a carbamazepine metabolite isolated from human urine. *J Pharm Sci* **61**: 1144–1147, 1972.
25. Bellucci G, Berti G, Chiappe C, Lippi A and Marioni F, The metabolism of carbamazepine in humans: Steric course of the enzymatic hydrolysis of the 10,11-epoxide. *J Med Chem* **30**: 768–773, 1987.
26. Mizzoni RH, Pyridine aldehydes and ketones. In: *Pyridine and Its Derivatives, Part IV* (Ed. Lkingsberg E), pp. 125–135. John Wiley, New York, 1964.
27. Beyer KH, Research on the metabolism of the anticonvulsant carbamazepine (Tegretol), particularly regarding the formation of ring-contracted-decomposition-products. *Arch Pharm* (Weinheim) **324**: 397, 1991.
28. Grisham MB, Jefferson MM, Melton DF and Thomas EL., Chlorination of endogenous amines by isolated neutrophils. *J Biol Chem* **259**: 10404–10413, 1984.
29. Riley RJ, Kitteringham NR and Park BK, Structural requirements for bioactivation of anticonvulsants to cytotoxic metabolites *in vitro*. *Br J Clin Pharmacol* **28**: 482–487, 1989.
30. Gerson WT, Fine DG, Spielberg SP and Sensenbrenner LL, Anticonvulsant-induced aplastic anemia: Increased susceptibility to toxic drug metabolites *in vitro*. *Blood* **61**: 889–893, 1983.
31. Eichelbaum M, Tomson T, Tybring G and Bertilsson L, Carbamazepine metabolism in man: Induction and pharmacogenetic aspects. *Clin Pharmacokinet* **10**: 80–90, 1985.
32. Tomson T and Bertilsson L, Potent therapeutic effect of carbamazepine-10,11-epoxide in trigeminal neuralgia. *Arch Neurol* **41**: 598–601, 1984.