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ANTIOXIDANT PROPERTIES OF THE ALKALOID BOLDINE IN SYSTEMS UNDERGOING LIPID PEROXIDATION AND ENZYME INACTIVATION

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Abstract—Boldine, in low micromolar concentrations, was able to prevent brain homogenate auto-oxidation, the 2,2′-azobis(2-amidinopropane)(AAP)-induced lipid peroxidation of red cell plasma membranes, and the AAP-induced inactivation of lysozyme. These results are indicative of a high reactivity of boldine towards free radicals. The analysis of the boldine effect as a function of incubation times suggests that a metabolite resulting from the interaction of boldine with free radicals also exhibits antioxidant activity, being more efficient than boldine in brain homogenate auto-oxidation and less efficient in lysozyme protection experiments. This behavior may be accounted for in terms of the relative location of the scavengers needed to afford maximal protection.

Active oxygen species and free radical reactions appear to be involved in several biochemical (eicosanoid biosynthesis, ribonucleotide reduction, microsomal mixed-function oxidation) and physiological (inflammation, the bactericidal activity of granulocytes, aging) processes [1, 2]. Additionally, the production of free radicals is recognized broadly as a major event associated with numerous pathological conditions [3], in which peroxidation of cell components might be induced. Such a recognition has prompted the search for safe and effective molecules capable of interfering with the generation of active species and/or hindering the course of the peroxidative cascade. During the last decades, several synthetic and natural compounds have been shown to present antioxidant activity. However, the clinical use of only a few of them seems to be feasible [4]. Among this latter group, the bioflavonoids (+)-cyanidanol-3 [5–9] and silymarin [10, 11], or its constituent isomer silybin [7, 8], have been best studied, although their clinical usefulness remains to be fully established [12, 13].

Boldine is the major alkaloid present in the leaves and bark of boldo (*Peumus boldus* Molina) [14], a widely distributed evergreen tree, native of Chile, the leaves of which are commonly used in traditional medicine for a variety of conditions among which liver complaints are generally mentioned [15]. This substance occurs in boldo bark in unusually high concentrations exceeding 7% (based on dry weight) [14] which, together with the abundance of the source plant, makes it a particularly inexpensive natural product. Although pharmaceutical preparations based on boldo have been used for medicinal purposes since the last century, mainly due to their action on bile secretion, only a few studies have addressed the pharmacology of its main constituent [16, 17]. Boldine, like most alkaloids, is fairly lipophilic and rather insoluble in water, but many of its salts, including the hydrochloride, are water-soluble [18]; its toxicity is remarkably low [16]. The boldine molecule (Fig. 1) exhibits two phenolic groups adjacent to two methoxy moieties, chemical features commonly found in compounds exhibiting high antioxidant activities [19]. In view of these considerations, the present study deals with the possible action of boldine as a substance capable of interfering with free radical-mediated reactions in biological systems. For this purpose, the effect of boldine was assessed in the auto-oxidation of rat brain homogenates [7], in the controlled peroxidation of rat erythrocyte plasma membranes (EPM) induced by the thermolysis of 2,2′-azobis(2-amidinopropane) (AAP) [20], or in the inactivation if lysozyme by AAP [21].

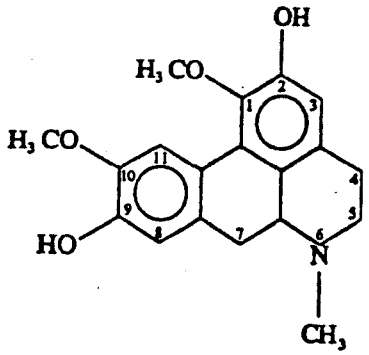

Fig. 1. Structure of boldine, 2,9-dihydroxy-1,10-dimethoxyaporphine.

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MATERIALS AND METHODS

Chemicals. Boldine was extracted from the bark of *P. boldus* and crystallized repeatedly from chloroform [14]. The alkaloid was chromatographically (TLC) pure and its identity was established by IR and NMR spectrometry. The working boldine solution (10 mM) was prepared daily in distilled water, adding 5 N HCl until dissolution of the alkaloid, and adjusted to pH 7 with 5 N NaOH. AAP was obtained from Polysciences (Warrington, PA). The rest of the chemicals used were obtained from the Sigma Chemical Co. (St. Louis, MO). These included lyophilized lysozyme from hen egg white and lyophilized cells of *Micrococcus lysodeikticus* (ATCC 4698), used in the enzyme inactivation studies.

Animals. Male Sprague–Dawley rats weighing 200–250 g were kept on a standard pellet diet *ad. lib.* (Alimentos Concentrados Cistemas M.R., Santiago). Brains and blood samples were obtained from animals anesthetized with nembutal (50 mg/kg, i.p.).

Preparation of rat brain homogenates. Brains were washed in ice-cold 0.15 M NaCl and stripped of the meninges and blood clots. The tissue was homogenized (1:4) in an ice-cold phosphate–saline buffer solution (PSB) containing 140 mM NaCl and 40 mM potassium phosphate buffer, pH 7.4. Tissue homogenates were centrifuged at 1000 *g* for 15 min at 4° and the supernatants obtained were diluted immediately (1:6) with PSB [7] and kept at 0° until used. This preparation was carried out daily with fresh brain tissue.

Preparation of EPM. Blood samples obtained by cardiac puncture using heparinized syringes were centrifuged at 2500 *g* for 10 min at 4° to remove plasma and buffy coats. The cells were washed three times with a cold PSB solution containing 150 mM NaCl and 5 mM sodium phosphate buffer, pH 8.0. EPM, prepared daily by the method of Hanahan and Ekholm [22] and devoid of hemoglobin, were diluted (1:4) with PSB for protein determination [23] and were used for peroxidation studies.

Assays of lipid peroxidation. The lipid peroxidative rate of rat brain homogenates (3 mL) was evaluated from measurements of visible luminescence, formation of thiobarbituric acid reactive substances (TBARS), and oxygen uptake. Light emission was measured at 30° in a Beckman LS-3150P liquid scintillation counter operated in the out-of-coincidence mode [6]. TBARS formation was determined at time zero and after incubation of the samples for up to 60 min at 35°, according to Buege and Aust [24]. Oxygen consumption was measured polarographically [25] at 35° with a Clark-type oxygen electrode.

The peroxidation rate of EPM suspensions (3 mL) was assessed by measurements of oxygen uptake [25] at 37°, upon addition of 10 mM AAP [26].

To assess the response of lipid peroxidation to boldine, the antioxidant capacity (AC) of the alkaloid at each concentration used was evaluated by:

$$AC_{luminescence} = 100\left[1 - \frac{I_{60} - I_0}{I^0_{60} - I^0_0}\right] \quad (1)$$

$$AC_{TBARS} = 100\left[1 - \frac{TBARS_{60} - TBARS_0}{TBARS^0_{60} - TBARS^0_0}\right] \quad (2)$$

$$AC_{oxygen\ uptake} = 100\left[1 - \frac{[O_2]_{20} - [O_2]_0}{[O_2]^0_{20} - [O_2]^0_0}\right] \quad (3)$$

where I, TBARS and $[O_2]$ correspond to light intensities ($I = I_t/I_0$, i.e. light emission at any given time t over that at time zero), TBARS accumulation and oxygen uptake, respectively, determined in the presence or absence (I^0, $TBARS^0$ and $[O_2]^0$) of boldine. Inferior indices indicate the time of measurement in minutes.

Inactivation of lysozyme. Lysozyme activity was determined by monitoring the loss of turbidity at 436 nm, when added to suspensions of lyophilized *M. lysodeikticus*, using the initial $-dE/dt$ derivative (E = extinction) [21]. Experiments were carried out at 45° in the presence of 10 mM AAP, with or without (controls) different boldine concentrations. Boldine by itself did not alter lysozyme activity (data not shown).

Data analysis. Results correspond to the means of at least three separate determinations for which standard errors were less than 10%. Values of the median inhibitory concentration of boldine needed to decrease the peroxidation rate to 50% of that observed in its absence were obtained from double-reciprocal plots. For this purpose, regression analysis of the lines obtained by plotting 1/antioxidant activity versus 1/boldine concentration were carried out on the preprogrammed Monroe-1930 statistic calculator (Ditton Industries, Morristown, NJ).

RESULTS

Rat brain homogenate auto-oxidation. Disrupted brain tissue exhibited a relatively high peroxidative rate with significant visible light emission, TBARS accumulation and oxygen uptake (Fig. 2), as previously established [7, 27–29]. In the concentration range of 10–100 μM, boldine added to this model system effectively inhibited peroxidation, as evidenced by the decreases found in chemiluminescence (Fig. 2A), TBARS formation (Fig. 2B) and oxygen consumption (Fig. 2C). Based on the results obtained at the end of each reaction time and referred to those at time zero, the respective antioxidant capacities of boldine were calculated. Accordingly, IC_{50} values of 25.0, 19.0 and 28.7 μM were obtained by double reciprocal plot analysis (Fig. 2, insets). For the sake of comparison, the effects of propyl gallate, a known inhibitor of rat brain homogenate auto-oxidation [7], and butylated hydroxyanisole (BHA), a commonly used, highly effective antioxidant, were also studied. As can be observed in Fig. 2A, propyl gallate added to a final concentration of 0.5 μM inhibited the luminescent response of this system by about 50%, while BHA exhibited an IC_{50} value of 1.6 μM (data not shown). The antioxidant activities of propyl gallate (Fig. 3) and BHA (data not shown) remained nearly constant through all the period of incubation of brain homogenates. However, boldine was found to display a progressive enhancement of its AC as a

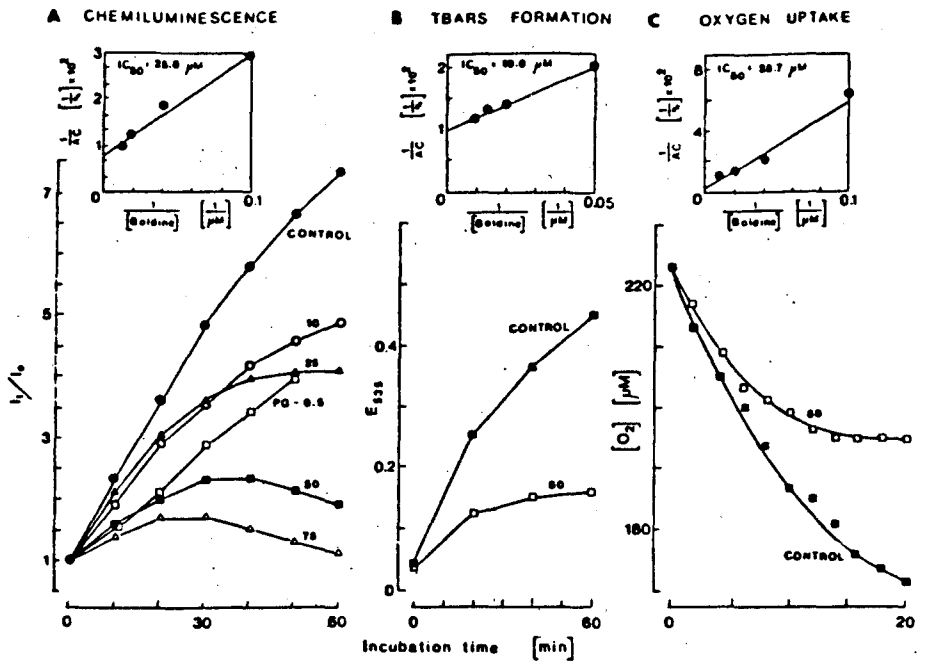


Fig. 2. Effect of boldine on the visible chemiluminescence (A), TBARS formation (B) and oxygen uptake (C) by rat brain homogenates undergoing auto-oxidation. The time course of changes in peroxidation parameters was studied in the absence (controls) or presence of boldine in the 10–100 μM range, as indicated by the numbers adjacent to the traces. Luminescence experiments with 0.5 μM propyl gallate (PG-0.5) were included for comparison. With the different parameters measured, the antioxidant capacity (AC) of boldine at each concentration was evaluated as described in Materials and Methods. Insets: double-reciprocal plots of the AC of boldine as a function of its concentration.

function of the reaction time, a behavior equally observed with each of the peroxidative parameters measured (Fig. 3).

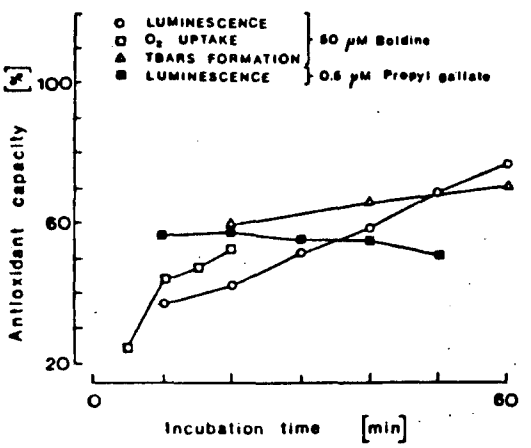
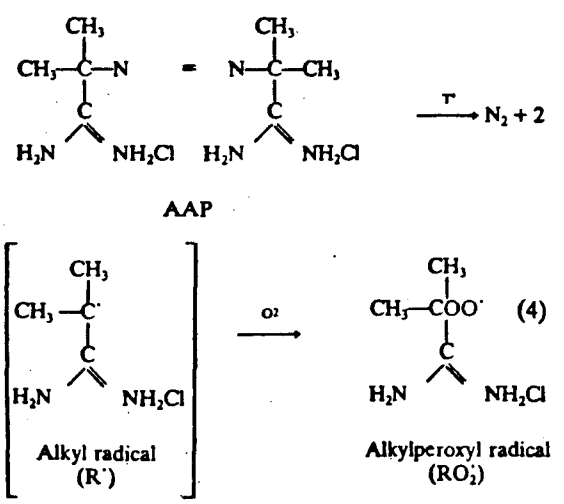


Fig. 3. Antioxidant capacity (AC) of boldine and propyl gallate as a function of the incubation time of rat brain homogenates undergoing auto-oxidation. AC values for 50 μM boldine and 0.5 μM propyl gallate were calculated from the data shown in Fig. 2, at individual times of 10, 20, 30, 40, 50 and 60 min for luminescence, 20, 40 and 60 min for TBARS formation, and 5, 10, 15 and 20 min for oxygen uptake measurements, referred to time zero, using the relationships described in Materials and Methods.

Erythrocyte plasma membrane peroxidation induced by AAP. Peroxidation of EPM was induced by alkylperoxyl radicals generated at a controlled rate by the thermal decomposition of AAP, according to



The lipid peroxidative response can be evidenced by different experimental indices, including oxygen consumption (Fig. 4A) [26, 30]. Upon addition of boldine in the 10–100 μM range, the AAP-induced rate of oxygen consumption by EPM was diminished in a concentration-dependent manner (Fig. 4A). This effect of boldine presented an IC_{50} value of

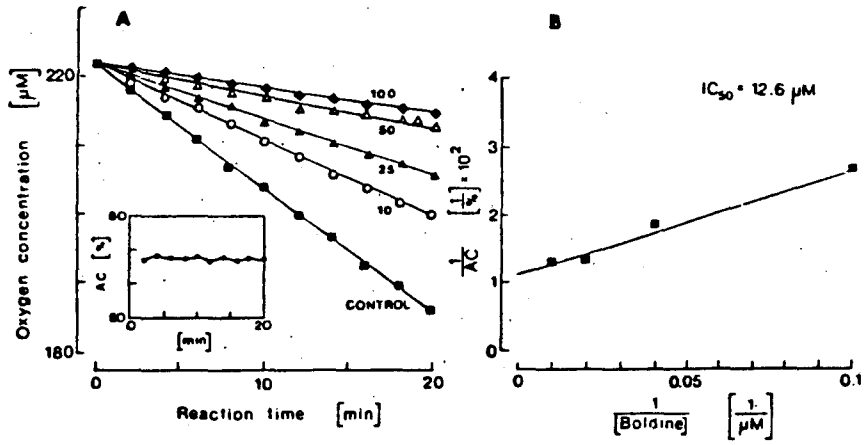


Fig. 4. Effect of boldine on the oxidation of rat erythrocyte plasma membranes (EPM) initiated by 2,2'-azobis-(2-amidinopropane) (AAP). (A) Oxygen uptake initiated by 10 mM AAP, in the absence (control) or presence of boldine, at the micromolar concentrations indicated by the numbers adjacent to the traces. Inset: antioxidant capacity (AC) of boldine at 50 μM, as a function of the reaction time. (B) Double-reciprocal plot of the AC of boldine as a function of its concentration. The AC of boldine was calculated as described in Materials and Methods.

12.6 μM, calculated by double-reciprocal plotting of its AC (Fig. 4B). In this experimental system, the AC of boldine was found to be constant as a function of the reaction time (Fig. 4A, inset).

Lysozyme inactivation induced by AAP. Incubation of lysozyme at 45° with 10 mM AAP led to progressive enzyme inactivation as a function of time, expressed as the remaining activity at a given time (Fig. 5A). Addition of boldine in the 3–30 μM range afforded protection depending upon the alkaloid concentration (Fig. 5A). This effect of boldine was characterized by both an increase in the induction period (T_0) (Fig. 5B) and a decrease in the rate of lysozyme inactivation that follows the lag time (Fig. 5C). T_0 is related to the average number of free radicals trapped by each boldine molecule initially introduced into the system ($\bar{n}$) by

$$T_0 = \bar{n}\,[\text{Boldine}]/R_i \qquad (5)$$

where R_i corresponds to the rate of free radical production [31]. This rate can be obtained by measuring T_0 for a reference inhibitor whose $\bar{n}$ value is known. Employing Trolox as a reference free radical scavenger which exhibits an $\bar{n} = 2$ [31], the estimated $\bar{n}$ value for boldine was found to range from *ca.* 2.5 at low concentrations to *ca.* 1.5 at high concentrations (from data given in Fig. 5B).

DISCUSSION

Data presented in this work reveal the ability of boldine to act as an antioxidant. Such an ability, which represents a novel property of this pharmacologically little known alkaloid, was demonstrated employing three mechanistically different biological systems susceptible to free radical-mediated reactions.

Rat brain homogenates exposed to oxygen spontaneously exhibit lipid peroxidation [7, 27–29] by a mechanism which is independent of superoxide and free hydroxyl radical production [32, 33], and whose initiation step may involve an iron-mediated cleavage of lipid hydroperoxides to yield peroxy or alkoxy radicals [34]. In this system, boldine effectively inhibited the oxidative process as measured by visible chemiluminescence. Since light emission does not always correlate with lipid peroxidation [35], its simultaneous comparison with other peroxidation parameters was required to establish the involvement of such a process. In this respect, the inhibition by boldine of the chemiluminescence associated with the auto-oxidation of brain homogenates was correlated significantly with that of the other lipid peroxidative indexes studied, namely TBARS accumulation ($r = 0.99$; $P < 0.005$) and oxygen uptake ($r = 0.95$; $P < 0.02$). Furthermore, the antioxidant capacity of boldine assessed by these criteria gave estimated values of IC_{50} of the same order of magnitude, ranging from 19.0 to 28.7 μM. Thus, boldine is able to interfere with the peroxidation process with an antioxidant efficiency similar to that of (+)-cyanidanol-3 ($IC_{50} = 20$ μM) [7] and greater than that of silybin ($IC_{50} = ca.$ 400 μM) [7], two known antioxidant bioflavonoids available for clinical use [12, 13].

Data obtained in this work for propyl gallate (Fig. 3) and BHA, as well as those previously reported for prevented antioxidants (i.e. desferrioxamine) [29] or biological fluids (i.e. blood plasma) [7], show that their antioxidant capacities remain constant through all the experimental period studied [7]. Contrary to this finding, the antioxidant capacity of boldine in this system progressively increased as a function of time when assessed by luminescence, TBARS formation or oxygen uptake measurements (Fig. 3). Although the possible cause underlying such behavior is not clear at the present time, it could indicate that a boldine-derived metabolite, resulting from the scavenging action of the alkaloid, is more active than the parent compound. In this regard, it is important to note that boldine has two potentially reactive phenolic groups (Fig. 1) and that

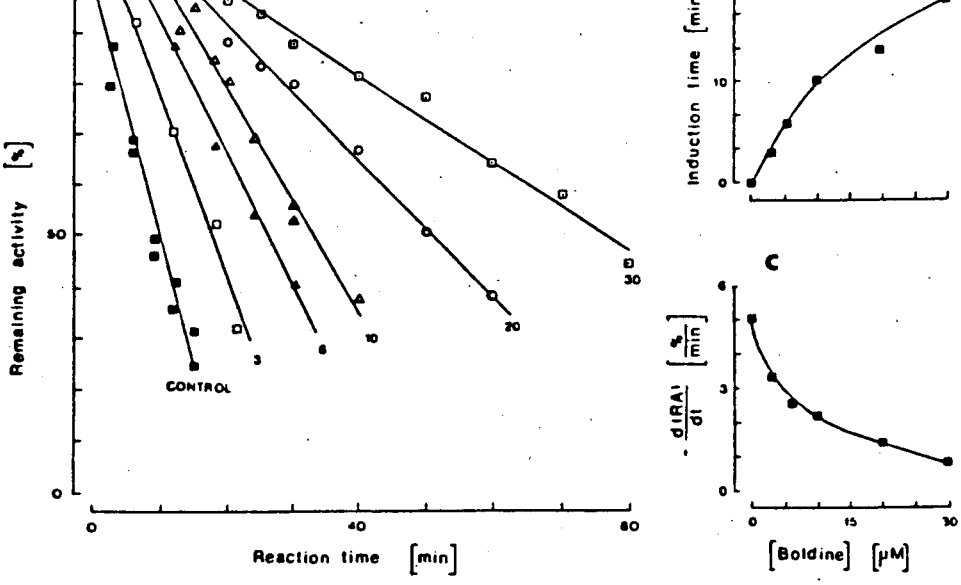


Fig. 5. Effect of boldine on the inactivation of lysozyme initiated by AAP. (A) Time course of changes in enzyme activity studied in the absence (control) or presence of boldine, at micromolar concentrations indicated by the numbers adjacent to the traces. The percentage of the remaining activity (RA) is defined as $100\,(-dE/dt)_{i=}/(-dE/dt)_{i=0}$, where E corresponds to the extinction at 436 nm. One hundred percent activity corresponds to an initial $-dE/dt$ of 0.2 min-1, measured in the absence of AAP. (B) Induction period (T_0) as a function of boldine concentration. (C) Inactivation rate ($-d[RA]/dt$) after T_0 as a function of the initial boldine concentration.

the antioxidant capacity of a given group, assessed in the brain homogenate system, markedly increased with the hydrophobicity of the molecule where it was located.* Thus, the results obtained could be explained in terms of an interaction of boldine with the chain carrying LOO· radicals of the induced lipid peroxidative process, giving rise to a more hydrophobic metabolite still bearing a reactive phenolic group.

In agreement with the studies carried out in rat brain homogenates undergoing auto-oxidation, boldine exhibited marked antioxidant activity ($IC_{50} = 12.6\,\mu M$) in the chemically initiated lipid peroxidation of rat EMP. AAP thermolysis allows uniform and reproducible rates of alkyl radical generation [30], which proceeds independently of the presence of iron to initiate the peroxidative process [26, 30, 36]. In this system, the marked diminution of oxygen uptake by boldine points to the involvement of a free radical-scavenging action of the alkaloid, which may be exerted either on the primary radicals derived from AAP, LOO· radicals involved in the peroxidative chain induced, or both.

The data given in the inset of Fig. 4A show that the antioxidant capacity of boldine in this model system remains almost constant over the entire time span considered. This result, which contrasts with those obtained in the brain homogenate auto-oxidation system (Fig. 2), can be accounted for if boldine is preferentially trapping water-soluble AAP-derived free radicals. In this respect, the ability of boldine to interact with AAP-derived free radicals was assessed by its capacity to diminish the free radical-mediated lysozyme inactivation rate. As can be observed in Fig. 5A, boldine, at concentrations as low as 3 μM, almost completely prevented AAP-mediated lysozyme inactivation at short reaction times. This protective action of boldine could be the result of an efficient trapping mechanism affecting the alkyl peroxyl radicals (ROO·) produced during AAP thermolysis in air-saturated solutions [21] and/or efficient repair of the enzyme after its attack by the alkyl peroxyl radicals [37]. In this experimental system, the presence of induction times can be related to the almost complete consumption of the added antioxidant agent. Since the T_0 values were not linearly correlated with the concentration of boldine in the range studied (Fig. 5B), the average number of the free radicals trapped by each boldine molecule ($\bar{n}$) decreases as a function of the initial concentration of the alkaloid. These observations can be explained if a change in the main fate of the boldine-derived radicals (BO·) with increasing boldine (BOH) concentrations is assumed:

$$BOH + ROO\cdot \rightarrow BO\cdot + ROOH \quad (6)$$

$$BO\cdot + BO\cdot \rightarrow \text{Non-radical products} \quad (7)$$

$$BO\cdot + ROO\cdot \rightarrow \text{Non-radical products} \quad (8)$$

* Lissi EA and Videla LA, unpublished observations.

This simplified mechanism, comprising only processes 6 to 8 under total inhibition conditions, predicts $\bar{n}$ values of about 2 and 1 at low and high boldine concentrations, respectively, in rough agreement with the experimental results. Furthermore, the data presented in Fig. 5C show that, following the induction time (i.e. after total boldine consumption), the enzyme inactivation rate was slower than that observed in the absence of the alkaloid, and that it decreased with the initial boldine concentration. These results can be explained in terms of the formation of a boldine-derived metabolite exhibiting a rather mild free radical scavenging capacity and/or enzyme repair activity that might be ascribed to the second phenolic group of the alkaloid. Due to the lower temperature employed in AAP-induced EPM oxidation (37°) compared to that used in the lysozyme inactivation studies (45°), boldine consumption is likely to be incomplete in the former system. This, and a lower scavenging capacity of the boldine metabolite compared to the parent compound, would explain the almost constant antioxidant capacity of boldine in the chemically initiated peroxidation of EPM.

In conclusion, the studies presented reveal the ability of boldine to act as an efficient antioxidant in biological systems susceptible to free radical-mediated reactions. This antioxidant activity may underlie some of the important therapeutic properties traditionally attributed to boldo and boldine-containing pharmaceutical preparations used in liver ailments. Although the activity measured in the *in vitro* studies reported in the present work appears to be lower than those of synthetic antioxidants such as propyl gallate, the low toxicity of boldine may favor its use for therapeutic purposes [4].

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