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ENHANCEMENT OF LIPID PEROXIDATION BY INDOLE-3-ACETIC ACID AND DERIVATIVES: SUBSTITUENT EFFECTS

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The peroxidation of liposomes by a haem peroxidase and hydrogen peroxide in the presence of indole-3-acetic acid and derivatives was investigated. It was found that these compounds can accelerate the lipid peroxidation up to 65 fold and this is attributed to the formation of peroxyl radicals that may react with the lipids, possibly by hydrogen abstraction. The peroxyl radicals are formed by peroxidase-catalyzed oxidation of the enhancers to radical cations which undergo cleavage of the carbon-carbon bond on the side-chain to yield CO_2 and carbon-centred radicals that rapidly add oxygen. In competition with decarboxylation, the radical cations deprotonate reversibly from the N1 position. Rates of decarboxylation, pK_a values and rate of reaction with the peroxidase compound I indicate consistent substituent effects which, however, can not be quantitatively related to the usual Hammett or Brown parameters. Assuming that the rate of decarboxylation of the radical cations taken is a measure of the electron density of the molecule (or radical), it is found that the efficiency of these compounds as enhancers of lipid peroxidation increases with increasing electron density, suggesting that, at least in the model system, the oxidation of the substrates is the limiting step in causing lipid peroxidation.

KEY WORDS: indole-3-acetic acid, horseradish peroxidase, lipid peroxidation.

INTRODUCTION

Some malignant cell lines produce large amounts of hydrogen peroxide[1] as well as haem peroxidases, mainly myeloperoxidase,[2] and have therefore the potential to oxidize a wide range of substances. For example, the myeloperoxidase catalyzed oxidation of vinca alkaloids is thought to be responsible for the resistance of leukaemia cells to these drugs.[3,4] Mammary tumours also exhibit an elevated peroxidase content relative to normal tissue.[5] In addition, tumours are often infiltrated by leucocytes which can be activated by cytokines,[6] administered as drugs or produced by the tumour cells following gene therapy.[7] Once activated, leucocytes can release oxidants including oxygen radicals, hypochlorous acid and peroxidases.[8]

Unfortunately, tumour cells appear to have an unusual ability to endure oxidative stress.[9] The reasons for that unusual resistance are unclear, but the abundance of anti-oxidants or low levels of unsaturated fatty acids in the cell membrane have been suggested as possible causes.[10,11] More recently,[12] the over-expression of the *bcl-2* gene

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has also been shown to make tumour cells resistant to oxidative stress, by a mechanism still unknown.

It is our working hypothesis that the peroxidase activity of some tumours can be exploited to activate adequate drugs to reactive intermediates and damage the malignant cells. To demonstrate this concept of *amplification of oxidative stress*, we have recently[13] used a chemical model in which the enzymatic peroxidation of lipid vesicles (liposomes) was enhanced by indole-3-acetic acid (IAA). This effect was putatively attributed to the reaction of a IAA-derived peroxyl radical with the lipid to initiate the chain of lipid peroxidation. The suggestion was based on the free radical chemistry of indole-3-acetic acid, now established in considerable detail by the use of radiation chemistry techniques.[14–16]

In order to explore further this mechanism and to identify the relevant structural features of potential drugs, we have decided to investigate a series of indole-3-acetic acid derivatives. Using a combination of techniques, we have identified compounds with a high ability to enhance lipid peroxidation induced by a model peroxidase.

EXPERIMENTAL SECTION

Reagents

The indole-3-acetic acid and derivatives (except *N*-methylindole-3-acetic acid) were purchased from Sigma or Aldrich. Peroxidase from horseradish (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) was from Sigma (type VI-A). All other reagents were of analytical quality and used as received. Solutions were prepared daily with water purified with a Millipore Milli-Q system. The indole-3-acetic acid derivatives were dissolved in phosphate buffer (2.5 mmol dm^{-3}) by gentle heating (ca. 40°C) under nitrogen in the dark.

Synthesis of N-methylindole-3-acetic acid

The ethyl ester of indole-3-acetic acid was methylated by a modification of a published method.[17] A mixture of potassium hydroxide and potassium carbonate (2:1) in acetone was used in place of potassium hydroxide and the resulting *N*-methyl ester (73% yield) was then hydrolyzed to give the required acid.

N-*Methylindole-3-acetic acid* Aqueous sodium hydroxide (10%. 10 ml) was added to a solution of the methyl ester (3.2g, 14.7 mmol) in methanol (3 ml) and the mixture was stirred for 15 min at 45°C, cooled to 0°C and concentrated hydrochloric acid was added. The resulting solid was filtered off, the filtrate was evaporated and the residue extracted with ethyl acetate giving a solid which was crystallized from ethyl acetate-petroleum ether to yield *N*-methylindole-3-acetic acid as white prisms (2.4g, 86%); m.p. 286°C (decomp.); ν_{max} 3430, 1710, 1590 cm^{-1}; δ [$(CD_3)_2SO$] 3.62 (2H, s, CH_2), 3.71 (3H, s, NCH_3), 7.16 (1H, s, ArH) and 7.40 (4H, m, ArH); m/z 189 (M^+, 30%), 144 (100), 129 (8) (Found: C 69.64; H, 5.86; N, 7.37. $C_{11}H_{11}NO_2$ requires C, 69.83; H, 5.86; N, 7.40%).

Liposomes

Large multilamelar vesicles were prepared with L-α-phosphatidylcholine from dried egg yolk and cholesterol. The phosphatidylcholine and 5% in weight of cholesterol were

dissolved in diethyl ether (ca. 0.25 mL/mg of phosphatidylcholine). The solutions were evaporated under vacuum at 30°C and further dried under a stream of nitrogen, protected from light. Phosphate buffer 2.5 mmol dm^{-3} at pH 7.4 was added (100 mL of buffer/g of phosphatidylcholine) and briefly sonicated (ca. 10 min) until a homogeneous milky suspension was obtained. These liposomal suspensions were prepared daily and kept under nitrogen in the dark.

TBA test

The liposome suspensions (1 mL) were diluted in 5 mL of freshly prepared solutions containing the indole-3-acetic acid derivative (0.25 mmol dm^{-3}) and hydrogen peroxide (1 mmol dm^{-3}) in phosphate buffer at pH 7.4 (2.5 mmol dm^{-3}). The reaction was initiated by the addition of horseradish peroxidase (final concentration 10 mg dm^{-3}). The solutions were stirred at room temperature and in the dark and at 10 min intervals, 1 mL aliquots were taken and added to 100 μL of a solution of butylated hydroxytoluene (0.5 g dm^{-3}) in methanol. To this were added 1 mL of thiobarbituric acid (10 g dm^{-3}) in 0.05 mol dm^{-3} sodium hydroxide and 1 mL of aqueous trichloroacetic acid (2.8%). These solutions were heated in a boiling water for 15 min. After cooling, the lipid in suspension was removed by centrifugation and the supernatant was diluted 1:10 in water. The fluorescence was read at 553 nm with excitation at 532 nm with a Perkin-Elmer LS-50B luminescence spectrometer. Excitation and emission spectra were recorded and compared to those obtained with malonaldehyde standards; this confirmed that the measured fluorescence is due to thiobarbituric acid adducts of malonaldehyde. Calibration lines were constructed daily using 1,1,3,3-tetramethoxypropane (malonaldehyde *bis* dimethyl acetal) up to 10 μmol dm^{-3} treated in the same way as the lipid samples.

Stopped-flow

Stopped-flow in the second range was performed with a Hewlett-Packard HP8452A diode array spectrophotometer fitted with a High-Tech SFA-11 fast kinetics accessory. The temperature of the absorption cell (optical path = 1 cm) as well as of the tubes leading the solution to it was kept at 25.0 ± 0.2°C by circulating water. The time resolution of this system is 0.1 s, limited by the response of the spectrophotometer.

HPLC

Analysis of indole-3-acetic acid and derivatives was performed by a HPLC system equipped with a Hichrom Hypersil 5ODS column and a Waters 490 multiwavelength detector, operating at 280 nm. Elution was achieved with a flow of 2 ml min^{-1} of a mixture of 20 mmol dm^{-3} potassium acetate at pH 4.5 and acetonitrile, isocratically or with linear gradients optimized in each case to obtain the best separation.

The formation of carbon dioxide was quantified by conversion to carbonate in alkaline solution followed by high-performance ion chromatography (HPIC) on a Dionex DX-100 chromatograph equipped with a 25 cm Ion Pac ICE-ASI ion-exchange column. The eluent was water + 22.5% acetonitrile and detection was by conductometry.[18]

Pulse radiolysis

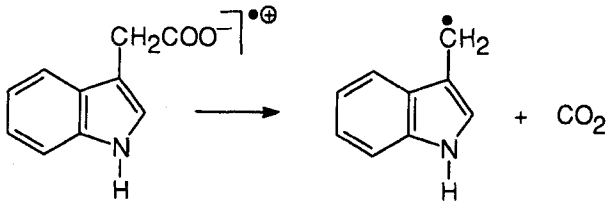
Pulse radiolysis with spectrophotometric detection was performed with a 4 MeV van de Graaff accelerator as described previously.[18] The radiation doses delivered by the 10 or 30 ns pulses were in the range of 1 to 5 Gy, generating ca. 0.5 to 4 μmol dm^{-3} radicals. Dosimetry was performed by measuring the increase of absorption at 472 nm on irradiation of N_2O-saturated KSCN solutions (0.01 mol dm^{-3}) assuming that the product of the yield and extinction coefficient of the $(SCN)_2^-$ radical was 5.06×10^3 Gy^{-1} cm^{-1}. The pulse radiolysis experiments were performed at room temperature (22 ± 2°C).

The one-electron oxidation of indole-3-acetic acid and derivatives (0.2 mmol dm^{-3}) was achieved by irradiation of solutions of potassium bromide (0.05 mol dm^{-3}) in phosphate buffer (2.5 mmol dm^{-3}) saturated with oxygen-free nitrous oxide (N_2O 0.022 mol dm^{-3}, "zero grade" from BOC). Under these conditions, the dibromide radical anion is generated in <1 μs (refs. 19, 20) and reacts with indole-3-acetic acid and derivatives with rate constants of the order of $\sim 5 \times 10^8$ dm^3 mol^{-1} s^{-1} (ref. 15).

RESULTS

Reactivity of the radicals of indole-3-acetic acid and derivatives

Pulse radiolysis of N_2O-saturated potassium bromide solutions containing IAA or derivatives at pH 3 to 4 showed the formation of the respective radical cations, characterized by an absorption band centred at 560–580 nm ($\varepsilon \sim 2000$ dm^{3}mol^{-1}cm^{-1}) and another band in the UV region ($\lambda \sim 340$ nm, $\varepsilon \sim 4000$ dm^{3}mol^{-1}cm^{-1}). Similar results were obtained with all the indoles investigated, extending previous studies.[14,15] All the radical cations investigated, with exception of that of indole-3-propionic acid, decayed exponentially with lifetimes of the order of hundreds of microseconds. Following a previous suggestion[15] recently confirmed,[16] we assign this decay to the cleavage of the carbon-carbon bond in the acetic acid side-chain, yielding carbon dioxide and skatole radicals:

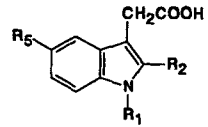


(1)

In order to minimize the second order component arising from the bimolecular decay of the radical cations, the first order rate constants were determined using doses of ~ 1 Gy. The values obtained (Table 1) extend over one order of magnitude, showing a moderate effect of the substituents. In contrast, the decay of the radical cation of indole-3-propionic acid monitored at pH 4 followed second order kinetics, i.e. no evidence was found for a decarboxylation reaction.

Steady-state radiolysis experiment were performed in which the indole-3-acetic acid derivatives were oxidized by Br_2^- at pH 7.4 and the solutions analyzed for the formation of carbon dioxide. Yields of CO_2 in the range of ~ 47% to ≥ 100% were observed (Table I), supporting the mechanism proposed. However, with indole-3-propionic acid

TABLE I
Properties of Indole-3-Acetic and Derivatives



compound	pK_a of *radical cation*[a]	rate of decarboxylation of radical cation /$10^3 s^{-1}$	yield of CO_2 at pH 7.4[c]	rate of reaction with HRP - I /10^{-3} $dm^3 mol^{-1} s^{-1}$	rate of formation of TBARS/nmol dm^{-3} s^{-1}
control	–	–	–	–	0.11 ± 0.03
indole-3-acetic acid $R_1 = R_2 = R_5 = H$	5.09 ± 0.02[b] (5.1)	15.7 ± 0.5	0.47 ± 0.01[b]	1.96 ± 0.05	0.63 ± 0.06
5-methoxyindole-3-acetic acid $R_1 = R_2 = H$; $R_5 = CH_3O$-	5.48 ± 0.04 (5.4)	8.5 ± 0.1	0.56 ± 0.04	18.7 ± 0.3	0.93 ± 0.16
2-methylindole-3-acetic acid $R_1 = R_5 = H$; $R_2 = CH_3$-	6.15 ± 0.02	1.7 ± 0.3	0.74 ± 0.02	370 ± 10	7.2 ± 0.7
2-methyl-5-methoxyindole-3-acetic acid $R_1 = H$; $R_2 = CH_3$-; $R_5 = CH_3O$-	6.16 ± 0.03 (6.2)	2.6 ± 0.3	0.54 ± 0.04	740 ± 40	4.8 ± 1.1
N-methylindole-3-acetic acid $R_1 = CH_3$-; $R_2 = R_5 = H$	–	15.4 ± 0.4	1.40 ± 0.16	1.59 ± 0.05	0.62 ± 0.18
indole-3-propionic acid	not determined	<0.1	<0.1	3.27 ± 0.11	0.13 ± 0.06

a) Values in parenthesis are from ref. 15; b) from ref. 16; c) expressed as a fraction of the $Br_2^{\cdot-}$ reacted.

no evidence could be found for the formation of carbon dioxide. On the basis of the latter result and of the pulse radiolysis observations at pH 4, we conclude that, unlike indole-3-acetic acid and derivatives, indole-3-propionic acid does not decarboxylate upon one-electron oxidation.

In neutral solution, the spectra of the transients obtained on oxidation of IAA and derivatives (with exception of *N*-methylindole-3-acetic acid) were different from those observed in acid solution, showing a blue shift of the visible absorption band on increase of pH. The lifetime of the radical cations increased with increasing pH, until in neutral solution the decay was essentially second order, as previously observed with IAA.[16] Different results were obtained with *N*-methylindole-3-acetic acid (Figure 1): in neutral solution the transient absorption spectrum exhibited a maximum at 570 nm, characteristic of the indole radical cation, and the decay of absorption was fast and first order ($k = 1.5 \times 10^4\ s^{-1}$).

The pH-dependence of the absorption spectra is explained by the deprotonation of the radical cations from N(1) to yield the indolyl radicals, an interpretation confirmed by the disparate behaviour of *N*-methylindole-3-acetic acid. The radical cation of the latter does not have a proton at N(1) and therefore does not deprotonate even in neutral solution.

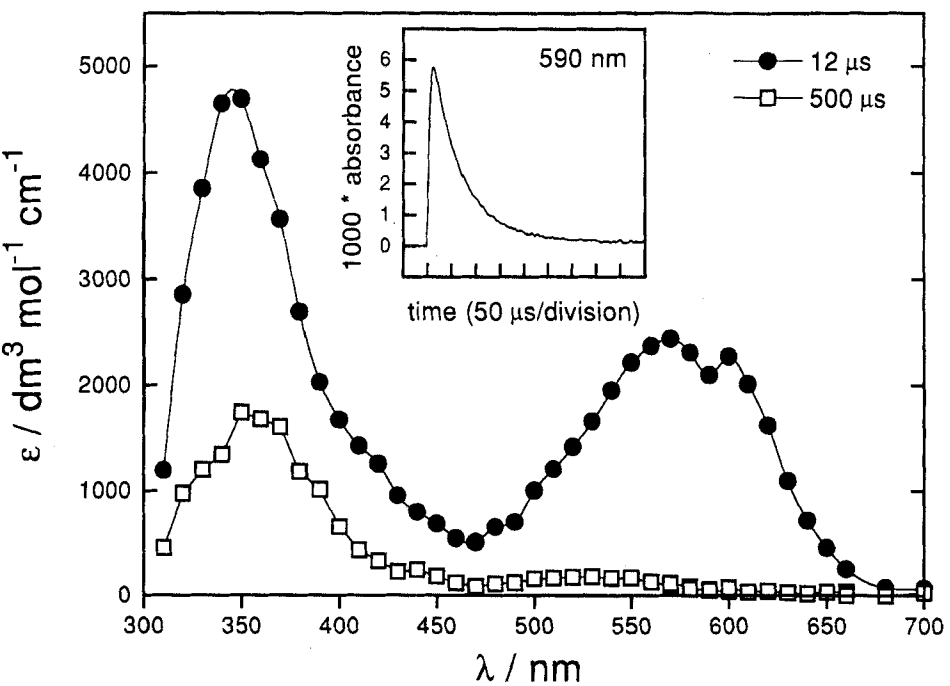
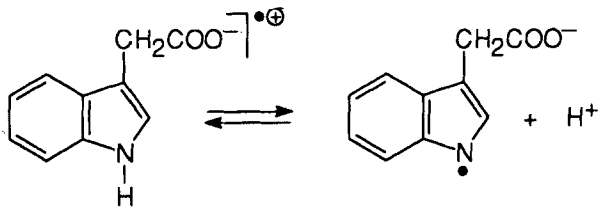


FIGURE 1 Transient absorption spectra of the radical cation of *N*-methylindole-3-acetic acid (solid circles) and of the carbon-centred radical it yields by decarboxylation (open squares). The spectra were recorded by pulse radiolysis of N_2O-saturated solutions of *N*-methylindole-3-acetic acid and potassium bromide in phosphate buffer at pH 7.4, 12 μs and 500 μs, respectively, after pulses of ca. 3.5 Gy.



(2)

The pK_a values of the radical cations of the IAA derivatives (except that of the *N*-methylindole-3-acetic acid radical cation) were determined by monitoring the absorption at 560–580 nm as a function of the pH. This way, we were able to confirm reported values[15] for the pK_a of the radical cations of 5-methoxyindole-3-acetic acid and of 2-methyl-5-methoxyindole-3-acetic acid and to determine the value for 2-methylindole-3-acetic acid (Table 1).

A recent analysis[16] of the decay kinetics of the indole-3-acetic acid radical cation has shown that it undergoes fast elimination of CO_2, but its conjugate base, the indolyl radical, does not. A similar mechanism accounts for the pH dependence of the life-time of the radical cations of the IAA derivatives, indole-3-propionic acid and *N*-methylindole-3-acetic acid excluded. The latter does not deprotonate even in neutral solution and therefore it exhibits a rate of decarboxylation at pH 7 similar to that measured for indole-3-acetic acid at pH 4.

Reactivity of HRP with indole-3-acetic acid and derivatives

Horseradish peroxidase (HRP) oxidizes indole-3-acetic acid both in the presence and in the absence of hydrogen peroxide.[21] Although in the latter case the mechanism is complex,[22] in the presence of H_2O_2 the reaction is probably initiated by the fast two electron oxidation of the enzyme to compound I, HRP-I ($k = 1.8 \times 10^7$ dm^3 mol^{-1} s^{-1}, ref. 23):

$$\text{HRP} + H_2O_2 \rightarrow \text{HRP-I} + H_2O \qquad (3)$$

HRP-I, which is an oxoferryl complex with a haem radical cation, oxidizes indole-3-acetic acid to the respective radical cation.

We have investigated this reaction in phosphate buffer at pH 7.4 by stopped flow, monitoring the absorption in the Soret band of HRP in the time-scale of seconds. Upon mixing of native HRP with H_2O_2 in equal concentrations (final concentration 1.1 μmol dm^{-3}), compound I was formed in <3 s, as shown by the absorption spectrum with a maximum at 400 nm (Figure 2). In the presence of indole-3-acetic acid up to 200 μmol dm^{-3} (added in the H_2O_2 solution), the absorption spectrum recorded after 20–50 s showed a maximum at 418 nm, characteristic of HRP compound II (HRP-II). The increase of absorption at 418 nm followed first order kinetics with rate constant that varied linearly with the IAA concentration (insert of Figure 2).

The spectral changes are interpreted by reduction of compound I to compound II by indole-3-acetic acid:

$$\text{HRP-I} + \text{IAA} \rightarrow \text{HRP-II} + \text{IAA}^{\cdot+} \qquad (4)$$

In equation 4, $IAA^{\cdot+}$ represents the IAA radical cation which, as shown by the pulse radiolysis results, decays in the timescale of milliseconds to species that do not absorb

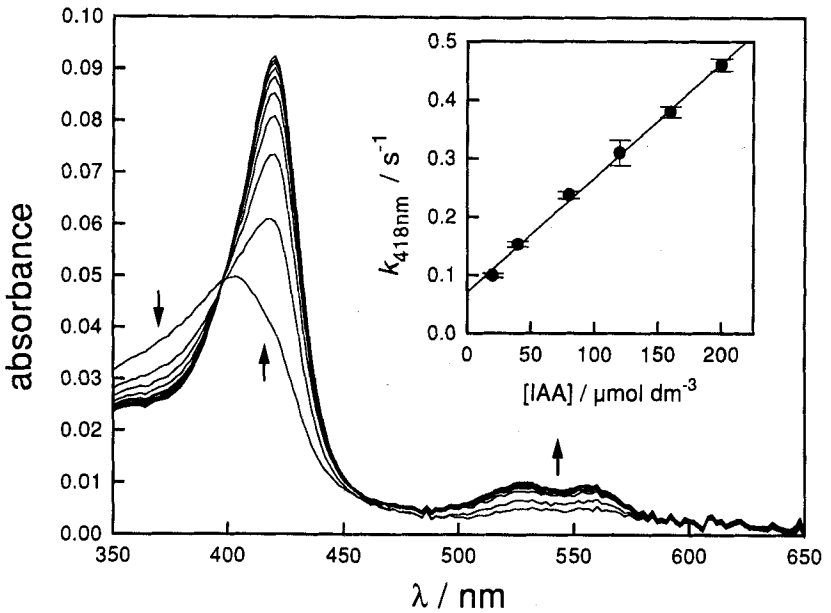


FIGURE 2 Spectral transformations showing the conversion of horseradish peroxidase compound I to compound II by reaction with indole-3-acetic acid (20 μmol dm^{-3}) in phosphate buffer at pH 7.4. The spectra were recorded at 2 s intervals following rapid-mix of solutions of HRP with solutions of indole-3-acetic acid containing hydrogen peroxide. Insert: observed rate at 418 nm as a function of the IAA concentration; the error bars represent the standard deviation of five measurements.

in the visible. The rate of reaction of HRP-I with IAA was determined from the slope of the linear dependence of the observed rate constant on the IAA concentration $k_3 = (1.96 \pm 0.05) \times 10^{-3}$ dm^3 mol^{-1} s^{-1}. Similar results were obtained with indole-3-acetic acid derivatives and the rate constants for their reaction with HRP-I are listed in Table 1.

After this reaction, further transformations in the Soret band were observed. However, the reaction mechanism was complex. The rate of decay of compound II increased with increasing indole-3-acetic acid concentration in a non-linear fashion. Moreover, the enzyme was not restored to the native (ferric) state. Other authors[21,22,24] have reported complex kinetics of the reaction between horseradish peroxidase and indole-3-acetic acid.

The oxidation of indole-3-acetic acid and its derivatives by catalytic amounts of HRP was also studied by monitoring by HPLC of the concentration of the substrates in the reaction mixture after various periods of incubation in phosphate buffer at room temperature and in the dark (Figure 3). In the case of the more reactive compounds, the reaction is too fast to enable the determination of initial rates. Nevertheless, the results suggest that these are approximately in the same relative order as the reactivity with HRP compound I. However, 2-methylindole-3-acetic acid appears to be exhausted faster than 2-methyl-5-methoxyindole-3-acetic acid, further evidence for the complexity of the reaction mechanism.

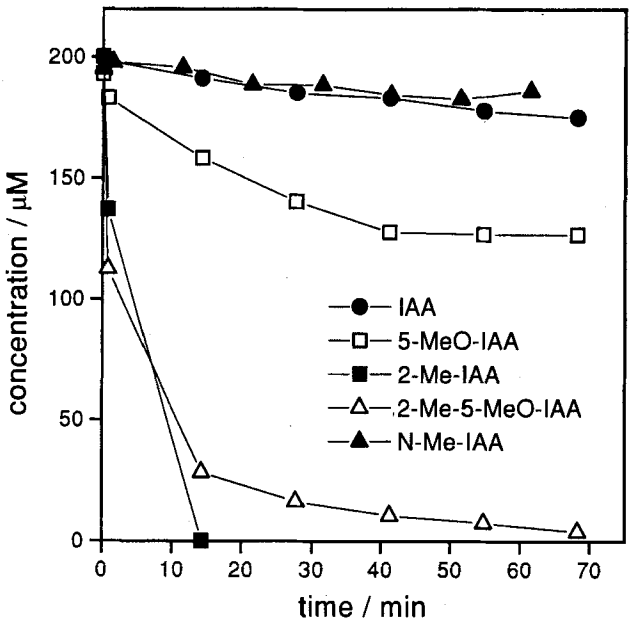


FIGURE 3 Depletion of indole-3-acetic acid and derivatives by 9.8 mg dm^{-3} horseradish peroxidase in the presence of 1 mmol dm^{-3} hydrogen peroxide in phosphate buffer (2.5 mmol dm^{-3}) at pH 7.4, monitored by HPLC.

Enhancement of lipid peroxidation

α-Phosphatidylcholine liposomes were incubated for 30 min with horseradish peroxidase and hydrogen peroxide and the formation of thiobarbituric acid reactive substances (TBARS) tested as described in the experimental section. Peroxidase and hydrogen peroxide alone are very inefficient in causing lipid peroxidation. However, in the presence of IAA and derivatives, TBARS were formed in a time-dependent manner (Figure 4). When the IAA derivatives were incubated in the absence of liposomes no TBARS were formed (data not shown) indicating that they originate from the lipids and not from the indoles.

The initial rates of formation of TBARS in the presence of indole-3-acetic acid derivatives are summarized in Table 1. Different compounds had different abilities to enhance the lipid peroxidation: 2-methylindole-3-acetic acid was over ten times more effective than the unsubstituted compound. In the presence of indole-3-propionic acid, the rate of formation of TBARS was not significantly increased above the control value (HRP, hydrogen peroxide and liposomes).

DISCUSSION

The pulse radiolysis results reported here show that, at pH 3 to 4, the radical cations of indole-3-acetic acid and derivatives decay by a first order process, identified as the cleavage of the carbon-carbon bond in the acetic acid side-chain to yield carbon dioxide and a carbon-centred radical (equation 1). The decay of the radical cation of the leading

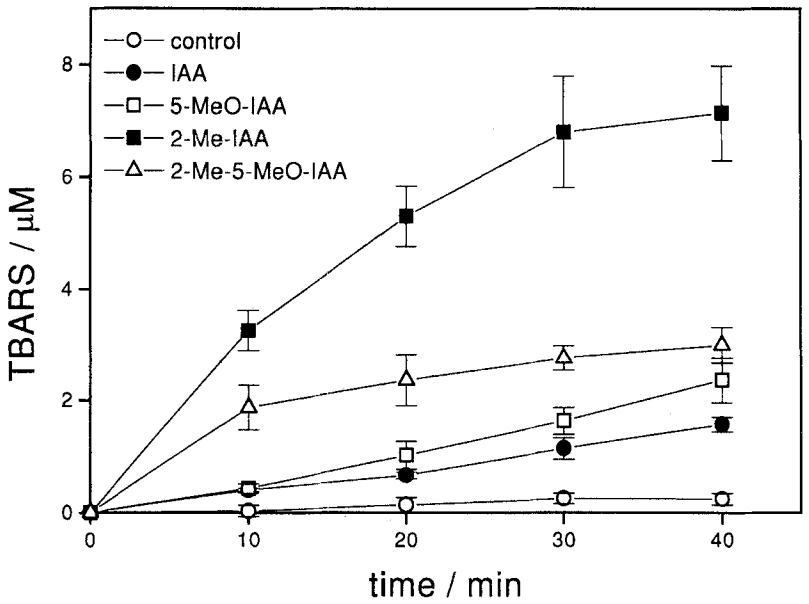


FIGURE 4 Formation of thiobarbituric acid reactive substances (TBARS) on incubation of phosphatidylcholine liposomes with horseradish peroxidase (10 mg dm^{-3}) and 1 mmol dm^{-3} hydrogen peroxide in the absence (control) or in the presence of 0.2 mmol dm^{-3} indole-3-acetic acid derivatives. The error bars show the standard deviation of three independent measurements.

compound indole-3-acetic acid was recently investigated in detail and demonstrated to follow this mechanism.[16] The similar spectroscopic characteristics and kinetic behaviour of the radical cations of substituted indole-3-acetic acid derivatives, as well as the measured formation of carbon dioxide reported here suggest that this mechanism, oxidative decarboxylation, is a general feature of these compounds. Oxidative decarboxylation of other aromatic compounds such as benzoic and phenylacetic acids,[25] flavone-acetic acid[18] and xanthenone-acetic acid[26] has also been reported.

Interestingly, indole-3-propionic acid, which differs from indole-3-acetic acid by having an additional methylene group in the side chain, did not undergo oxidative decarboxylation. The additional methylene group is not expected to prevent the electron transfer between the carboxylate group and the indole moiety, necessary for the decarboxylation reaction. In fact, the rate of through-bond electron transfer can be estimated to be decreased by no more than a factor of ~4 by the additional carbon-carbon bond.[27] We suggest that the stability of the radicals formed upon decarboxylation (equation 1) can account for the disparate behaviour of the radical cations of indole-3-acetic acid and indole-3-propionic acid. In the former case the carbon centred radical is stabilized by the heterocyclic system, but such stabilizing effect is not possible in the case of the radical that would be formed by oxidative decarboxylation of indole-3-propionic acid. Consequently, in the latter case the thermodynamic driving force for elimination of CO_2 is so much lower that the reaction is not observed.

The rates of decarboxylation exhibited by the radical cations of the indole-3-acetic acid derivatives were higher than that of the unsubstituted radical cation, with the possible exception of the *N*-methylated compound in which the rate is approximately equal. In a qualitative way, this observation is explained by the electron donating effect

of the methyl and methoxy substituents which stabilize the radical cations, thereby decreasing the rate of CO_2 elimination. However, a quantitative treatment of the substituent effect is not straightforward: poor correlations are obtained, either with Hammett (σ) or with Brown (σ^+) substituent parameters. Although the number of compounds available is too small to extract definlte conclusions, the data suggest that the effect of the methyl substituent at position 2 is higher than expected. Other authors[15] reported inconsistent substituent effects on the redox and acid-base properties of the radicals of indole-3-acetic acid derivatives (see below).

With other compounds, the decarboxylation of the radical cations suffers competition by diverse reactions. For example, the radical cation of 5-bromoindole-3-acetic acid has been reported[15] to undergo nucleophilic substitution of Br^- by water yielding a 5-hydroxyindolyl radical. A further example is presented by the 5-hydroxyindole-3-acetic acid radical cation which deprotonates from the hydroxyl group to yield a radical with spectrum and reactivity akin of those of a phenoxyl radical (L.P. Candeias, unpublished observations; see also ref. 28).

The decrease of the rate of decay of the radical cations with increasing pH shows that deprotonation from N(1) prevents CO_2 elimination, a conclusion substantiated by the behaviour of the *N*-methylated compound. The pK_a values of the substituted compounds were in all cases higher than those of the radical cations of indole-3-acetic acid, reflecting the electron donating effect of the methyl and methoxy groups. The pK_a values did not correlate with either σ or σ^+ substituent parameters, in agreement with previous observations[15] and in line with the finding for the rates of decarboxylation. It is interesting to note that a moderately good correlation between pK_a and rate of decarboxylation at pH $<< pK_a$ is observed (Figure 5a). In spite of the small number of compounds available, the correlation suggests consistent substituent effects which however, can not be rationalized by the usual σ or σ^+ parameters.

It was observed in an earlier study[16] that although the decarboxylation of indole-3-acetic acid in neutral solution could not be observed in the pulse radiolysis time-scale (≤milliseconds), CO_2 was detected under conditions of low rate of formation of radicals (steady-state irradiation). This was attributed to the reversibility of the deprotonation from N(1) of the radical cation which allows the existence of a small concentration of

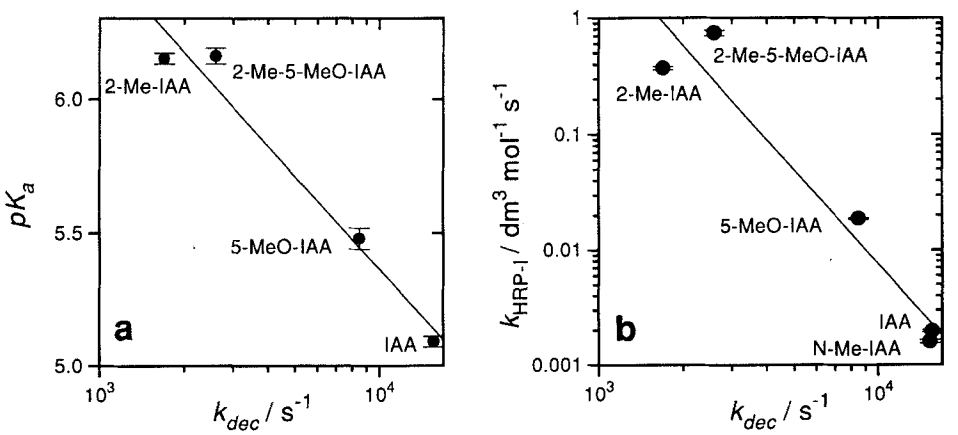


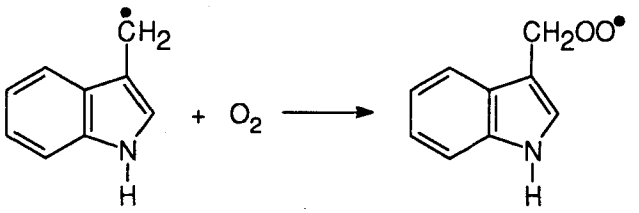
FIGURE 5 Relation between pK_a and rate of decarboxylation of the radical cations of indole-3-acetic acid and derivatives (**a**) and the relation between rate of reaction of horseradish peroxidase compound I with these compounds and the rate of decarboxylation of the respective radical cations (**b**).

radical cation in equilibrium with the indolyl radical and therefore the formation of CO_2, provided the steady-state level of radicals is low enough and therefore their lifetime sufficiently long. Assuming the same mechanism is valid in the case of the substituted derivatives, the apparent rates of decarboxylation at pH 7.4, calculated from the rate of decarboxylation of the radical cations and their pK_a values are of the order of $10^2 s^{-1}$. The radical cation of *N*-methylindole-3-acetic acid is exceptional because it undergoes fast decarboxylation in neutral solution. Accordingly, it produces the highest yield of carbon dioxide.

Indole-3-acetic acid and derivatives were also oxidized by horseradish peroxidase. The detection of the skatole radical as an intermediate[29] lead to the conclusion that indole-3-acetic acid is oxidized by the enzyme active state(s) to the respective radical cation which by decarboxylation yields the skatole radical. A usual paradigm for peroxidase reactions is the modified ping-pong mechanism in which the native enzyme suffers two-electron oxidation by hydrogen peroxide (equation 3) followed by two one-electron reduction steps by reaction with the substrate. Although this mechanism adequately describes the reaction with many oxidizable substances,[30,31] the oxidation of indole-3-acetic acid seems to be more complex.[21,22,24] Our results confirm the complexity of the mechanism: although conversion of compound I to compound II by reaction with substrate did occur, the following reactions were not in agreement with the simple model.

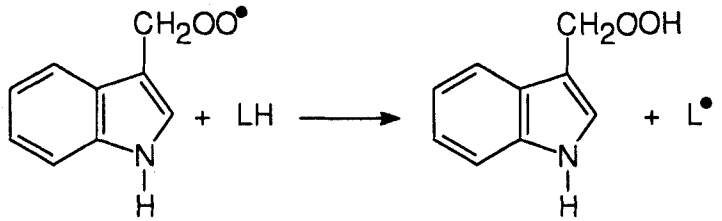
The rates of the reaction of the compounds studied with HRP-I spread over almost three orders of magnitude, evidence for a pronounced substituent effect. The reaction of HRP-I with phenols and aromatic amines has previously been found[30] to be strongly accelerated by electron-donating substituents. However, in the case of indole-3-acetic acid and derivatives the rate of the reaction (k_{HRP-I}), like the pK_a values and rates of decarboxylation of the respective radical cations, could not be quantitatively described using either σ or σ^+ substituent parameters. A correlation was found between k_{HRP-I} and the rate of decarboxylation of the radical cations (Figure 5b), reflecting parallel effects of the substituents on these two reactions, i.e. compounds that are oxidized faster by HRP-I yield radical cations that are more stable with respect to decarboxylation.

The experiments with liposomes show that horseradish peroxidase is a very poor inducer of lipid peroxidation, but indole-3-acetic acid and derivatives can increase its action dramatically (Figure 4). In a previous study,[13] we have proposed that the enhancement of lipid peroxidation was a consequence of the formation of peroxyl radicals following the decarboxylation and addition of oxygen to the skatole radical:



(5)

Peroxyl radicals can react with unsaturated lipids (LH) by hydrogen abstraction, initiating the chain of lipid peroxidation:



(6)

On the basis of the suggested mechanism, it would be predicted that the most efficient enhancers of lipid peroxidation would be the compounds that *a*) are more oxidizable, *b*) yield radicals that decarboxylate faster and *c*) yield peroxyl radicals that are more reactive towards lipids. However, the negative slope in Figure 5b suggests that conditions a) and b) are conflicting: the radical cations of the more oxidizable compounds are thermodynamically more stable and therefore provide a weaker driving force for decarboxylation. The experimental results reveal an inverse correlation between the rate of formation of TBARS observed with the different compounds and the rate of decarboxylation of the respective radical cations (Figure 6), which can be taken as an indication that the oxidizability of the compounds is the determinant factor. Furthermore, the acidity of the radical cations must also be considered, as deprotonation competes with the decarboxylation and therefore with the formation of peroxyl radicals. The case of *N*-methylindole-3-acetic acid provides another argument in favour of the decisive effect of oxidizability. For this compound, the *apparent* rate of decarboxylation *at pH 7* is 2 orders of magnitude higher than that of any of other compounds.

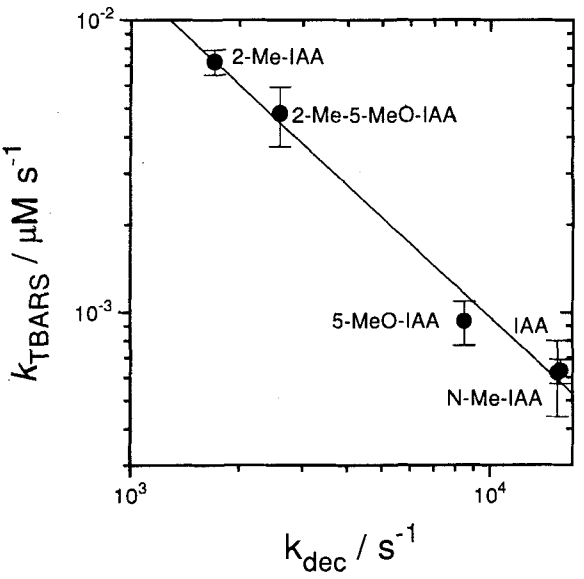


FIGURE 6 Rate of formation of thiobarbituric acid reactive substances (k_{TBARS}) by incubation of phosphatidylcholine liposomes with horseradish peroxidase and hydrogen peroxide in the presence of indole-3-acetic acid derivatives plotted against the rate of decarboxylation of the respective radical cations.

Nevertheless, it is a weak enhancer of lipid peroxidation, which may be attributed to the slow rate at which it is oxidized by HRP.

Presently, the reactivity of the peroxyl radicals derived from indole-3-acetic acid and derivatives is not known, because they absorb so weakly that their direct detection is difficult. Model peroxyl radicals react with reducing agents (e.g. ascorbate) with rate constants that are increased by electron-withdrawing substituents.[32] The enhancement of lipid peroxidation by indole-3-acetic acid and derivatives followed the opposite trend, i.e., compounds with electron-donating groups were more efficient. Unless the substituent effect on reaction of peroxyl radicals with lipids is opposite to that on the reaction with reductants, the reactivity of the peroxyl radicals is not decisive in the enhancement of lipid peroxidation.

Alternative mechanisms to explain the enhancement of lipid peroxidation must be considered. The inverse correlation with the rate of decarboxylation of the radical cations suggests that the lipid peroxidation might be initiated by the radical cations or their conjugated bases, the indolyl radicals. In fact, these radicals have been shown to be strong oxidants.[15,16] However, the radical cation of indole-3-propionic acid did not decarboxylate and this compound did not enhance lipid peroxidation in spite of reacting with HRP compound I slightly faster than indole-3-acetic acid. It must therefore be concluded that the formation of the carbon-centred radicals (and presumably of the peroxyl radicals they are rapidly converted into) is a necessary step in the enhancement of lipid peroxidation.

At the high hydrogen peroxide: enzyme ratio used in the lipid peroxidation experiments ($[H_2O_2]$: [HRP] = 4400), an enhancing effect might be attributed to accelerated cycling of the enzyme. In fact, this mechanism has been suggested to explain the increase of peroxidase-dependent luminol luminescence in the presence of phenols. Contrary to this hypothesis, the peroxidase enhancer *p*-hydroxycinnamic acid (*trans*-3-phenylpropenoic acid) did not increase lipid peroxidation.

The involvement of superoxide radical or singlet oxygen is also unlikely on the basis of the lack of effect of superoxide dismutase[13] or of the singlet oxygen quencher 1,4-diazobicyclo[2.2.2]octane (L.P. Candeias, unpublished observations). It has been shown[33] that electronically excited states are formed during the oxidation of indole-3-acetic acid by HRP and their reaction with lipids to initiate the peroxidation chain reaction is a possibility that cannot be ruled out at present.

SUMMARY AND CONCLUSIONS

We have shown that the peroxidation of phosphatidylcholine liposomes by a haem peroxidase is increased in the presence of indole-3-acetic acid derivatives. It is suggested that the peroxidase oxidizes those compounds to the respective radical cations which, by elimination of CO_2, are converted into carbon-centred radicals. Oxygen adds to the latter in a diffusion controlled reaction to yield peroxyl radicals and these react with the lipid, possibly by hydrogen abstraction, initiating the lipid peroxidation. In agreement with this suggestion, indole-3-propionic acid, which did not decarboxylate upon oxidation, did not enhance the lipid peroxidation.

The rates of decarboxylation of the radical cations of the several indole-3-acetic acid derivatives, determined by pulse radiolysis, are in the range 10^3 to 10^4 s^{-1}. In competition with CO_2 elimination, the radical cations deprotonate with pK_a values in the range 5.1 to 6.2. In consequence, the apparent rates of decarboxylation decrease with increasing pH. An exception to this behaviour is the *N*-methylindole-3-acetic acid radical cation

which can not deprotonate and therefore undergoes fast decarboxylation even at pH 7. The pK_a values of the radical cations are consistent with their rates of decarboxylation, i.e. those with higher electron density are more stable both with respect to CO_2 elimination (lower rates of decarboxylation) and to deprotonation (higher pK_a values). However, the substituent effects can not be quantitatively described in terms of the usual σ or σ^+ parameters. The same is true for the rate of reaction of the indole-3-acetic acid derivatives with the peroxidase compound I, strongly suggesting that the usual substituent parameters do not apply to this class of compounds.

With respect to the enhancement of lipid peroxidation, the electron richer indole-3-acetic acid derivatives are more efficient, in spite of the fact that their radical cations exhibit the lower rates of decarboxylation, suggesting that the oxidation of the substrates may be the limiting step. However, in the presence of antioxidants such as ascorbate, that can react with the radical cations (or with their conjugate bases, the indolyl radicals)[13] the rate of decarboxylation may become decisive. Possible differences between the model peroxidase employed in these studies and oxidants generated by tumour cells (e.g. myeloperoxidase, lactoperoxidase) must also be kept in mind. To elucidate these questions, we are currently performing studies in biological systems.

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