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# OXIDATION OF 2-*t*-BUTYL-4-METHOXYPHENOL (BHA) BY HORSERADISH AND MAMMALIAN PEROXIDASE SYSTEMS

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**Abstract**—Di-BHA, 2,2′-dihydroxy-3,3′-di-*t*-butyl-5,5′-dimethoxy-diphenyl, was isolated as the product of the reaction of either commercial horseradish peroxidase or partially purified rat intestine peroxidase (Donor-$H_2O_2$ oxidoreductase, EC 1.11.1.7.) and hydrogen peroxide with 2-*t*-butyl-4-methoxyphenol (BHA). BHA, Di-BHA and other cyclic compounds possessing a hydroxyl group in the ring were found to be competitive inhibitors with respect to guaiacol, and non-competitive inhibitors with respect to hydrogen peroxide in a system containing guaiacol, hydrogen peroxide and peroxidase. A free radical intermediate generated during peroxidatic oxidation of BHA was detected and identified by means of EPR spectroscopy. It was estimated that during one hour incubation the peroxidase activity present in the rat ileum mucosa is able to oxidise 12 μmoles BHA at a saturating concentration. It is suggested that peroxidative oxidation at the intestinal wall may represent a contribution to the inactivation of some phenol derivatives potentially toxic to mammals.

BHA (2-*t*-butyl-4-methoxyphenol), a widely used antioxidant food additive, has been shown to be a powerful perturbing agent for biomembranes and to induce methemoglobin formation in human erythrocytes [1]. In addition, it inhibits growth of some Gram positive bacteria§ and of *Tetrahymena piriformis* [2]. In spite of these high biological activities and of its aptitude to cross membranes [3, 4], BHA exhibits a very low toxicity in mammals when administered orally.‖ It has been recently shown in this laboratory that the $LD_{50}$ of BHA administered intraperitoneally to rats is of two orders of magnitude lower than that reported for this agent given *per os* [5]. As an explanation of this discrepancy, we considered the possibility that an enzymic system present in the intestinal wall of the rat is responsible for the metabolic inactivation of BHA. This compound is known to react with oxidising agents. It is oxidised by alkaline ferricyanide to 2,2′-dihydroxy-3,3′-di-*t*-butyl-5,5′-dimethoxydiphenyl (Di-BHA) [6], while at neutral pH it directly reduces oxidised cytochrome C.‖ Early studies showing that monophenolic compounds are oxidised by peroxidase (Donor-$H_2O_2$ oxidoreductase, EC 1.11.1.7.) [7] suggested that BHA was a substrate of peroxidase. This paper reports how the incubation of BHA with either commercial preparations of horseradish peroxidase or partially purified rat intestine peroxidase and hydrogen peroxide resulted in the formation of an oxidation product of BHA which was identified with the diphenyl derivative (Di-BHA) obtained by ferricyanide oxidation.

## MATERIALS AND METHODS

### *Materials*

*Chemicals.* BHA, 3,5 di-*t*-butyl-4-hydroxytoluene (BHT) and *t*-butyl-hydroquinone were purchased from Fluka A.G. Buchs S.G; 2-methoxyphenol (guaiacol), 4-methoxyphenol, phenol, 2,6-dichloroquinonechlorimide (DCQCI) and $H_2O_2$ from E. Merck, Darmstadt; and horseradish peroxidase from Sigma Biochemicals, St. Louis, MO (165 Units. $mg^{-1}$). Mammalian peroxidase preparations were obtained from rat intestine by two different procedures. That used for EPR experiments was obtained by following up to step 4 (ammonium precipitation and dialysis) the method described by Stelmaszinska and Zgliczinski [8]. The enzyme preparation used for the other studies was obtained by a procedure worked out in this laboratory. All the operations were carried out at 0° to 4°.

Male Sprague–Dawley rats (200–300 body wt) were killed by decapitation and their small intestines were immediately washed with normal saline. The

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§ Unpublished results in this laboratory.

‖ WHO Food Additives Series, No. 5. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents. World Health Organisation, Geneva (1974).

mucosa was separated from the muscle tissue, suspended in 3 vol. of 0.25 M sucrose and homogenized with 6 × 15 sec strokes in a glass homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 800 *g* for 20 min and the resulting supernatant spun down at 20,000 *g* for 10 min. The fraction thus pelleted and containing mitochondria and lysosomes was suspended in 6 vol. of 20 mM sodium phosphate buffer. This was adjusted to pH 11 by adding 1N NaOH and stirred for 4 hr in the presence of 0.1 per cent Triton X 100. The suspension was centrifuged for 1 hr at 38,000 *g* and the supernatant obtained contained almost all the enzyme activity. The supernatant was dialysed overnight against 100 vol. of 0.1 M sodium carbonate–sodium bicarbonate buffer (pH 10.3) and passed through a Sephadex G 200 column equilibrated with the same buffer as was employed as eluant. All of the enzyme activity appeared in the effluent immediately after the void volume. The specific activity of this preparation was about 100 times higher than that exhibited by the crude mucosa homogenate. It was found that in this preparation the enzyme activity corresponding to one rat ileum amounted to 0.8 Units. 1 Unit is defined as the enzyme activity which oxidizes 1 μmole of guaiacol in 1 min at 25°, when guaiacol and $H_2O_2$ are present in the reaction mixture at concentrations of 6.7 and 0.33 mM, respectively.

*Analytical equipment.* Melting points were determined on a Kofler melting point apparatus. $^1$H n.m.r. spectra (deuteriochloroform as solvent) were obtained with a Perkin Elmer R 32 spectrometer; and u.v. spectra with a Cary 14 spectrophotometer. Impact ionization mass spectrometry (i.i.m.s.) was performed by a direct insertion probe on a LKB-2091 apparatus at an accelerating voltage of 3.5 kV, an electron energy of 70 eV, an ionising current of 50 μA and an ion pressure at approximately $1 \times 10^{-7}$ Torr. Source and probe temperatures were 250° and 70°, respectively. ESR spectra were obtained with a Varian E-104 A spectrometer, 100 KHz modulation, operating at a frequency of 9.18 GHz. *g* Values were determined by using peroxylamine disulphonate as a standard. The simulated spectra were obtained according to Komarynsky and Wahl (14). Some of the radical spectra were obtained by using a special electrolytical cell placed in the ESR cavity. The cell was evacuated and the radicals were generated by applying a voltage to platinum parallel electrodes immersed in the solution of the parent compounds. *t*-Butylammonium perchlorate was used as a conductive salt.

*Methods*

*Synthesis of 2,2'-dihydroxy-3,3'-di-t-butyl-5,5'-dimethoxy-diphenyl (Di-BHA).* Di-BHA was obtained by direct oxidation and by peroxidase catalysed oxidation of BHA.

(A). Direct oxidation. A reference sample of Di-BHA was prepared as reported by Hewgill and Hewitt [6]. The product thus obtained, however, was chromatographed through a silica gel column and eluted with chloroform. The fraction containing Di-BHA was evaporated and the product recrystallised from ligroin (m.p. 225–228°, yield 60%).

(B). Peroxidase catalysed oxidation. Horseradish peroxidase (2 mg = 200 Units) was added to 400ml of 10 mM potassium phosphate buffer (pH 7.2) containing BHA (176 mg = 0.976 mmoles) and $H_2O_2$ (16.59 mg = 0.488 mmoles) and kept overnight at room temperature. The reaction mixture was extracted with diethylether. The extract was washed with water, dried ($Na_2SO_4$) and evaporated under vacuum to yield a pink solid which was crystallised from ligroin and washed twice with cold petroleum ether (m.p. 220–226°). The product was finally recrystallised from ligroin to give a white solid (p.p. 224–227°, 20 mg yield). The identity of the products obtained by the two procedures was confirmed by the following criteria: (1) $^1$H n.m.r. (deuteriochloroform), δ 1.4 (9 H,s), 3.75 (3 H,s), 5.0 (1 H,s hydroxyl), 6.6-6.6 (1 H,doublet, 6 and 6' protons), 6.95-6.95 (1 H,doublet, 4 and 4' protons); (2) u.v. λ max 298 nm (Log ε 3.99); (3) i.r. 3540 $cm^{-1}$ (ν O-H); i.i.m.s. (see the Results section).

*Thin layer chromatography.* Thin layer chromatography was performed using Silica Gel G F254 pre-coated plates 5 × 20 cm, 0.25 mm thick (G. Merck, Darmstadt). The chromatograms were usually developed 15 cm at room temperature in the dark with chloroform as eluant. Compounds were detected on plates by visualization under a short wave u.v. lamp, scraped off and eluted with 3 ml methyl alcohol. The recovery of BHA ($R_f = 0.28$) and Di-BHA ($R_f = 0.56$) were almost quantitative.

*Colorimetric determination of BHA and Di-BHA.* A quantitative colorimetric assay method for BHA and Di-BHA was developed by using the phenol reagent 2,6-dichloroquinone-chlorimide (DCQCI) [9]. The 3 ml of the reaction mixture contained (final concentrations): 0.33 mM sodium bicarbonate buffer, pH 9.4; 12.6 M methyl alcohol; 0.26 mM DCQCI. The addition of BHA and Di-BHA (0.01–0.2 μmoles) to the reaction mixture gave rise in a few seconds to the formation of blue chromophores with a maximum absorbance at 590 nm. The extinction coefficients found were 13.35 $cm^2$. $\mu mole^{-1}$ (BHA) and 11.25 $cm^2$. $\mu mole^{-1}$ (Di-BHA).

*Hydrogen peroxide determination.* Hydrogen peroxide was determined colorimetrically by using horseradish peroxidase and *o*-dianisidine following the method of Berndt and Bergmeyer [10].

*Peroxidase assay.* The method was based on that described by Chance and Mahely [11] which utilizes guaiacol as the hydrogen donor. The 3 ml of the incubation mixture contained (final concentrations; the indicated pH values are those at which the maximum oxidation rate of guaiacol was observed): 6.7 mM potassium phosphate buffer, pH 7.0 (horseradish peroxidase) or 66.7 mM sodium borate buffer, pH 8.4 (rat ileum peroxidase); 0.033–0.33 mM $H_2O_2$; 0.33–16 mM guaiacol. Peroxidative oxidation of guaiacol performed at 26° was followed for 2–5 min at 470 nm by means of a double beam Beckman Acta III spectrophotometer equipped with a recorder. The linear part of the curve was used to calculate the initial reaction velocity. BHA and the other tested compounds, dissolved in dimethylsulfoxide at 100 mM concentration, were diluted with the buffer used in the assay to give a final concentration in the cuvette ranging from $10^{-6}$ to $10^{-3}$ M. The nature of the inhibitory effects observed was investigated by

means of double reciprocal plots [12] relating the formation rate of tetraguaiacol, in the presence or in the absence of a fixed concentration of the inhibitor (BHA, 0.033 and 0.066 mM were incubated with the horseradish enzyme and with the intestine enzyme respectively; BHT, 0.05 mM; 4-methoxyphenol, 0.5 mM; Di-BHA, 0.033 mM), to the concentration of guaiacol.

*Capacity of the ileum mucosa peroxidase for converting BHA into Di-BHA.* The reaction was carried out at 37° in the dark. 0.8 Units (3.9 mg of protein) of partially purified peroxidase, that is the activity obtained from one ileum, were suspended in 25.6 ml of a reaction mixture (buffered to pH 8.3 with 0.06 M sodium carbonate and 0.41 M sodium phosphate) which contained (final concentrations): 12.68 μmoles BHA (0.49 mM) and 6.40 μmoles $H_2O_2$ (0.25 mM). The reaction started with the addition of $H_2O_2$. At different intervals 1 ml aliquots of the reaction mixture were extracted with 3 ml methylene chloride. Two millilitres of the extract were evaporated under $N_2$, dissolved into 0.15 ml methylene chloride and placed on TLC plates.

*Protein assay.* Protein content was determined colorimetrically by the method of Lowry *et al.* [12], using bovine serum albumin as standard.

## RESULTS

*Identification of Di-BHA as the reaction product of BHA with horseradish and rat intestine peroxidase.* As indicated in Fig. 1, BHA was found to be a competitive inhibitor with respect to guaiacol and a non-competitive inhibitor with respect to hydrogen peroxide in a system containing guaiacol, hydrogen peroxide and horseradish or rat ileum peroxidase. BHT, phenol, 4-methoxyphenol and Di-BHA behaved like BHA when incubated with the horseradish enzyme. As shown in Table 1, the affinity of the horseradish peroxidase was higher for BHA, BHT and Di-BHA among the phenolic compounds tested. In our experimental conditions the enzyme prepared from the intestinal mucosa showed a lower affinity both for guaiacol and for Di-BHA when compared with the horseradish enzyme. The affinities of both enzymes for BHA and $H_2O_2$, however, were comparable. When guaiacol was omitted in the reaction mixture containing either horseradish or ileum mucosa peroxidase, after the addition of BHA there was an increasing turbidity accompanied by a decrease in the amount of BHA. Thin layer chromatography (TLC) of a methylene chloride extract of the reaction mixture showed that BHA ($R_f = 0.28$) was converted into another compound ($R_f = 0.56$).

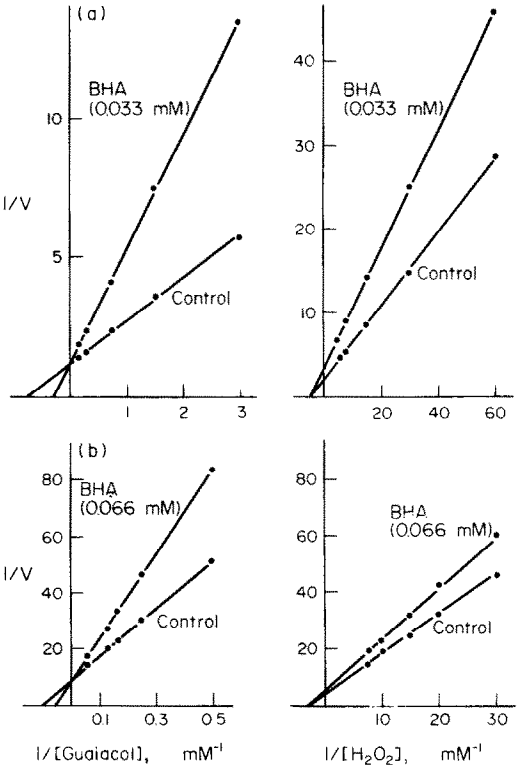


Fig. 1. Kinetic analysis of the inhibition of guaiacol peroxidation by BHA. The reaction was catalysed by commercial horseradish peroxidase (panel a) and by partially-purified rat ileum peroxidase (panel b). The rate of tetraguaiacol formation at 26° was measured in the absence or in the presence of the inhibitor. The points represent the mean values derived from 3 experiments.

Table 1. Affinity of peroxidases for $H_2O_2$, BHA and various related monocyclic compounds*

| Substrate | Peroxidase: Horseradish $K_m$(M) | Horseradish $K_i$(M) | Rat ileum mucosa $K_m$(M) | Rat ileum mucosa $K_i$(M) |
|---|---|---|---|---|
| Guaiacol | $1.2 \times 10^{-3}$ | | $1.0 \times 10^{-2}$ | |
| BHA | | $1.9 \times 10^{-5}$ | | $4.7 \times 10^{-5}$ |
| BHT | | $1.4 \times 10^{-5}$ | | |
| Phenol† | $1.6 \times 10^{-2}$ | | | |
| 4-Methoxyphenol | | $3.1 \times 10^{-4}$ | | |
| Di-BHA | | $2.2 \times 10^{-5}$ | | $1.0 \times 10^{-4}$ |
| $H_2O_2$ | $1.8 \times 10^{-4}$ | | $3.5 \times 10^{-4}$ | |

* Measurements were taken at 25°, at pH values giving maximum guaiacol oxidation rate (pH 7 and 8.4 for horseradish and rat ileum peroxidase, respectively). The substrates were used at 5 different concentrations and kinetic constants were derived from double reciprocal plots [12] of triplicate values. The various $K_i$ and the $K_m$ for $H_2O_2$ were obtained using guaiacol as substrate.

† The peroxidatic oxidation rate of phenol was measured spectrophotometrically by following the formation rate of *ortho-ortho'*-di-phenol [15] at 455 nm.

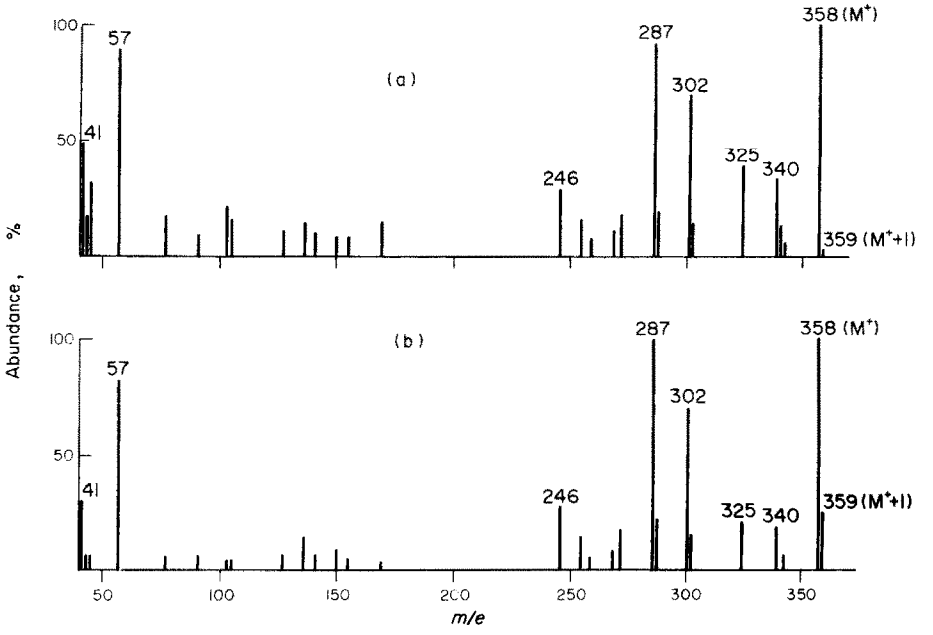


Fig. 2. Mass spectra. Panel a: Di-BHA obtained from BHA by direct oxidation with ferricyanide as described under Methods. Panel b: Di-BHA obtained by intestine peroxidase catalysed oxidation of BHA in the presence of $H_2O_2$.

The spectra were obtained by direct insertion probe on a LKB-2091 mass spectrometer. Electron beam energy 70 eV; ion source temperature 250°. Prior to mass spectrometry the compounds were purified by TLC, using chloroform as eluant, from a methylene chloride extract of the reaction mixture.

This conversion was not observed when either peroxidase or hydrogen peroxide were omitted from the reaction medium. The spectrographic analyses u.v., NMR and i.r. indicate that this compound and synthetic Di-BHA obtained by chemical oxidation of BHA (see Methods) were identical. In addition, as shown in Fig. 2, the i.i.m.s. of the reaction product of BHA with peroxidase and hydrogen peroxide and of Di-BHA chemically synthesized are practically identical, showing an ion at m:e 358 corresponding to the molecular ion ($MH^+$) and fragments ions at m:e 343 ($M^+ - CH_3$), 340 ($M^+ - H_2O$), 302 ($M^+ - t$-butyl), 287 (302 – $CH_3$), m* 254.7 (358→ 302) and 246 (302 – $t$-butyl).

*Capacity of the ileum mucosa peroxidase for converting BHA into Di-BHA.* The capacity of the rat ileum mucosa peroxidase for oxidizing BHA into Di BHA in the presence of $H_2O_2$ was tested. The results obtained are set out in Table 2. After 15 min incubation, about 23 per cent of the BHA was oxidized. It is interesting to note that the amount of BHA which disappeared was nearly twice that of Di-BHA formed and of $H_2O_2$ consumed. The BHA oxidation proceeded until all $H_2O_2$ was consumed and its maximum was achieved after 60 min. From 30 min onwards, however, the stoichiometry of the reaction, (as deduced from the 15-min values) was not respected. As an explanation for the $H_2O_2$ consumption which was higher than expected, the formation of other oxidation products should be considered. In fact, other compounds with an $R_f$ value between those of BHA and Di-BHA became more evident on TLC as the incubation time increased.

*Identification by electron spin resonance spectrometry of a free radical generated from BHA by the two peroxidases.* When BHA was incubated with horseradish peroxidase and hydrogen peroxide, a broad signal was recorded ($g = 2.0042$) typical of a radical with a slow tumbling rate, which suggests that the radical was firmly bound to the enzyme. The radical was released by the addition to the reaction mixture of one volume of methanol and gave a signal

Table 2. Oxidation of BHA into Di-BHA by rat ileum peroxidase in the presence of $H_2O_2$*

| Incubation time (min) | BHA transformed (μmoles) | $H_2O_2$ transformed (μmoles) | Di-BHA formed (μmoles) |
|---|---|---|---|
| 5 | 0.61 | — | 0.59 |
| 15 | 2.97 | 1.85 | 1.43 |
| 30 | 5.08 | 5.50 | 1.90 |
| 60 | 8.92 | 6.48 | 3.53 |
| 120 | 8.51 | 6.48 | 3.80 |

* 0.8 Units of peroxidase (3.9 mg of protein) were incubated at 37° in 25 ml of a medium buffered to pH 8.3 which contained 12.68 μmoles BHA and 6.40 μmoles $H_2O_2$.

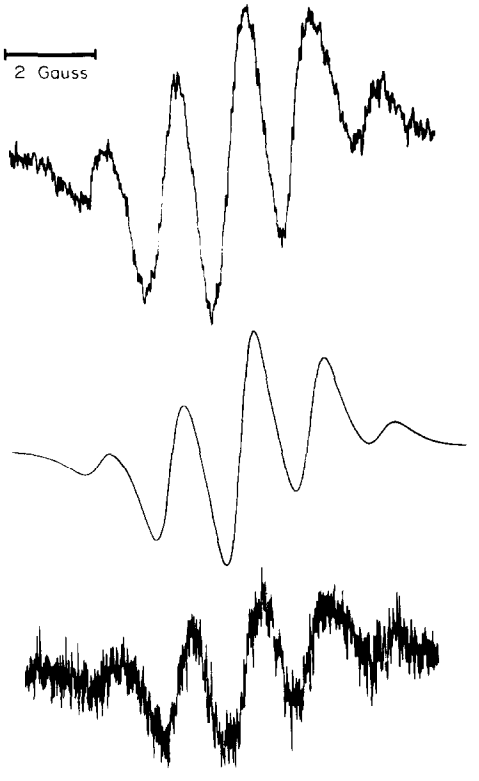


Fig. 3. ESR spectra. Top panel: spectrum obtained after 1 hr incubation at 22° of BHA ($7.5 \times 10^{-3}$M) in the presence of horseradish peroxidase (165U.mg.$^{-1}$ml$^{-1}$), $H_2O_2$ ($10^{-2}$M), Na phosphate buffer pH 7($10^{-2}$M) and dimethylsulfoxide (10 per cent v/v). The spectrum was recorded immediately after the addition of methanol (50:50) to the incubation mixture. Middle panel: simulated spectrum of the Di-BHA radical. Bottom panel: spectrum obtained by applying a voltage of 1.5 V to an electrolytical cell containing a methanol solution of Di-BHA ($7.5 \times 10^{-3}$M). Spectra were obtained with a modulation of 1 Gauss and a microwave power of 10 mW.

with a *g* value of 2.0042 which is equal to the *g* value of the protein-bound radical (Fig. 3, top panel). A comparable spectrum was obtained by simulation assuming that the radical has 4 equivalent protons (Fig. 3, middle panel). When Di-BHA was incubated with $H_2O_2$ and horseradish peroxidase, no signals were recorded, thus suggesting that the radical generation preceded the Di-BHA formation. The ESR spectrum obtained by electrolysis of Di-BHA was identical to that obtained when BHA was oxidized in the presence of horseradish peroxidase (Fig. 3, bottom panel). When BHA was incubated with hydrogen peroxide and a preparation of rat intestine peroxidase no signal was recorded either in the buffer solution or after methanol addition. However, when the water–methanol solution after 3hr incubation was placed in the electrolytical cell under a voltage of 1.5 V, a signal was recorded with a *g* value of 2.0042 (Fig. 4, upper left). This signal was identical to the one obtained when the Di-BHA spectrum was simulated (according to the method of Komarynsky and Wahl [14]). It has been assumed that the radical has a low tumbling rate depending on the high viscosity of the incubation medium and that the structure represented in Fig. 4 (bottom) has 4 equivalent protons, indicated by the heavily-marked hydrogens in the formula. The fact that this signal was not generated spontaneously might indicate that in these reaction conditions the radical has a very short life. In the same conditions, the signal with a *g* value of 2.0046 which had been observed during the electrolysis of BHA (Fig. 4, upper right) was not detected. This suggests that most of the BHA participating in the reaction had disappeared from the incubation mixture.

## DISCUSSION

The results reported here show that BHA is a substrate for horseradish and rat intestine mucosa peroxidase by which in the presence of $H_2O_2$ it is oxidized to Di-BHA. In addition, one indirect piece of evidence was obtained for the similar behaviour of BHT. The structure characterization of Di-BHA leads to the conclusion that the peroxidation process consists in the coupling of two BHA molecules occurring by dehydrogenation in the *ortho* position without the involvement of the OH group. A similar reaction mechanism has been shown to occur for the peroxidative oxidation of phenol [15], tyramine [16], tyrosine [17], *p*-cresol [18] and vanilline [19]. The general scheme by which horseradish peroxidase catalyses the oxidation by hydrogen peroxide of a wide variety of organic compounds was established by the work of Chance [20], George [21, 22] and Yamazaki [23] and is presented in equations (1)–(4).

$$HRP + H_2O_2 \rightarrow HRP\text{-}I \quad (1)$$

$$HRP\text{-}I + AH_2 \rightarrow HRP\text{-}II + AH\cdot \quad (2)$$

$$HRP\text{-}II + AH_2 \rightarrow HRP + AH\cdot \quad (3)$$

$$2\ AH\cdot \rightarrow A + AH_2 \text{ (or AH-AH)}. \quad (4)$$

Here HRP refers to the native enzyme; HRP-I and HRP-II are oxidized forms of the enzyme and containing, respectively, two and one oxidizing equivalents relative to the native enzyme; $AH_2$ is an oxidizable substrate; AH· the free radical interme diate (semiquinone) and A or AH-AH, the oxidation products. According to the above scheme, as shown in Fig. 5, conversion of BHA (1) to Di-BHA (4) would take place via the intermediate (3). The radical (5), which was detected by e.p.r. spectroscopy, would then be generated in the reaction medium from the final product (4). However, an attempt to obtain from a Di-BHA (4) solution the same spectrum as that obtained in the reaction conditions set out in Fig. 2 failed. Therefore, we have to admit that either the actual reaction path involves an interaction of the radical (2) with the parent phenol(1), to give the radical intermediate (5), or the radical (5) is generated from Di-BHA (4) by exchange with the radical (2). Mechanisms of the type (1) + (2)→(5) cannot be excluded from such coupling reactions [24].

As for the capacity of the rat intestine mucosa peroxidase for converting BHA into Di-BHA, assuming the oxidation rate at 15 min to be the maximum achievable in our experimental conditions, the enzyme activity present in the intestine mucosa is capable of oxidising 12 $\mu$moles of BHA.hr$^{-1}$ at 37°. This compound was present in the incubation

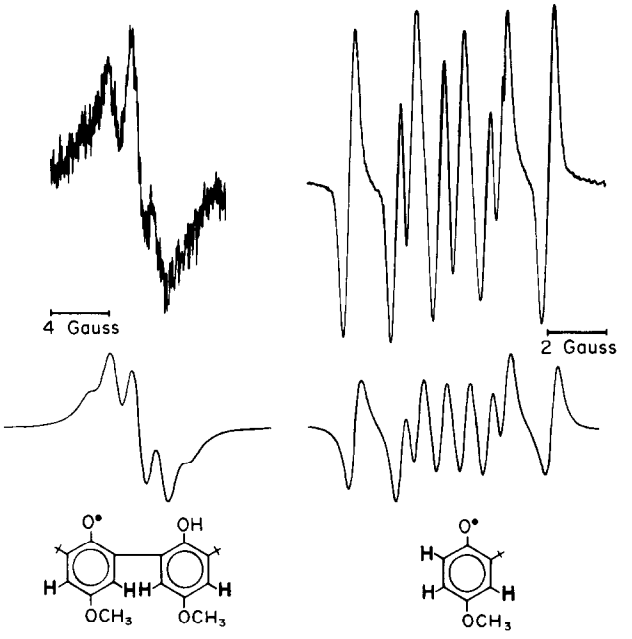


Fig. 4. ESR spectra. Top left panel: BHA ($7.5 \times 10^{-3}$M) was incubated at 22° for 3hr in the presence of rat intestine peroxidase (0.66U.mg$^{-1}$; 1.3mg.ml$^{-1}$) $H_2O_2$ ($4.8 \times 10^{-5}$M), Na phosphate buffer pH 7.3 ($3 \times 10^{-2}$M) and dimethylsulfoxide (10 per cent v/v). The spectrum was obtained by applying a voltage of 1.5 V to an electrolytical cell containing the reaction mixture. Top right panel: spectrum obtained by applying a voltage of 1.75 V to an electrolytical cell containing a methanol solution of BHA ($7.5 \times 10^{-3}$M). Modulation and microwave power as in Fig. 2. Bottom left and right panels: simulated spectra of Di-BHA and BHA radicals, respectively. The probable structure of Di-BHA and BHA radicals are shown; bold-type hydrogens indicate the equivalent protons.

mixture at a concentration fully saturating the enzyme ($10 \times K_m$), while $H_2O_2$ was at half-saturating concentration ($K_m$ concentration). Preliminary data, not reported here, have shown that higher concentrations of $H_2O_2$ inhibited our partially purified enzyme. Although we have no experimental evidence about the peroxidative oxidation rate of BHA *in vivo*, it is conceivable that *in vivo* this reaction proceeds at a similar rate. We should admit, however, that either $H_2O_2$ availability is not limiting or Di-BHA, which has been shown to be a competitive inhibitor of peroxidase, is removed from the enzyme environment. The first problem to be solved, however, is the accessibility of peroxidase to BHA. Data available from the literature show that, in the rat large intestine, peroxidase activity is localised in the mucus-secreting cells of the cryptae [25], while the enzyme purified from hog intestinal mucosa seems

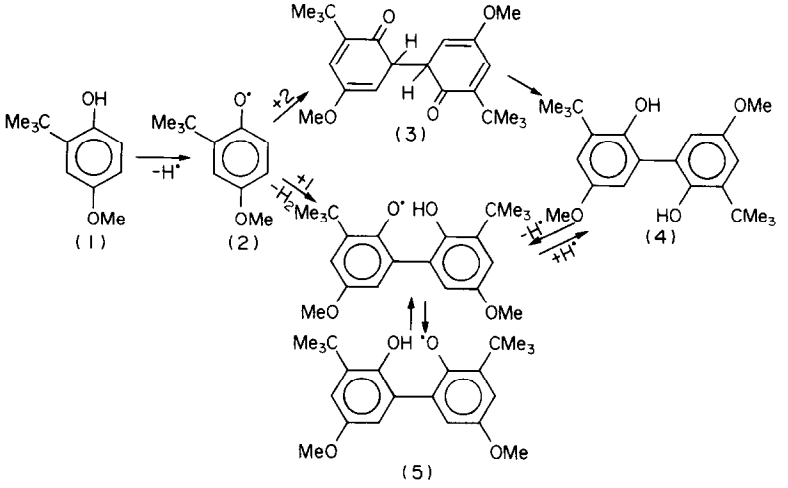


Fig. 5. Oxidation scheme of BHA to Di-BHA by a peroxidase–hydrogen peroxide system (for information, see text). The abstraction of a hydrogen radical is represented only to indicate the stoichiometry of the reaction.

to be identical, as regards its biochemical properties, to that purified from eosinophylic granulocytes [8]. Rat intestinal mucosa peroxidase activity, as recently described by Smith and Castro [26], is much lower in germ-free animals and seems to be related to the number of mucosal granulocytes. A research project in progress in this laboratory is aimed at defining the localization and the biochemical properties of peroxidase in the rat intestinal mucosa.

Another point of discussion is the relevance of this pathway to BHA metabolism. To date, no mention has been made of the peroxidative oxidation of BHA; moreover, the only described metabolic transformation of the BHA isomer here used is the conjugation with glucuronic acid, known to occur in the rat [27].

However, since it has been found that chronic oral treatment with BHA induces biphenyl-4-hydroxylase activity in rat liver [28], it can be suggested that this effect is related to the formation of Di-BHA. We do not yet know whether Di-BHA, once formed in the intestine mucosa, has access to the liver.

All the above questions must be answered before the importance of peroxidatic oxidation in the detoxification processes of BHA is discussed. Preliminary experiments, carried out in this laboratory, however, have indirectly suggested that this metabolic pathway plays a role in the inactivation of BHA. While the BHA $LD_{50}$ i.p. in the rat has been found to be 50 mg (0.28 mmoles) $.kg^{-1}$body wt. [5], i.p. injection of Di-BHA at a dose of 720mg (2 mmoles) $.kg^{-1}$body wt. has only given a 10 per cent lethality.

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## REFERENCES

1. G. P. Sgaragli, L. Della Corte, M. Rizzotti-Conti and A. Giotti, *Biochem. Pharmac.* **26**, 2145 (1977).
2. J. G. Surak, R. L. Bradley, Jr., A. L. Branen and E. Shrago, *Fd Cosmet. Tox.* **14**, 277 (1976).
3. S. M. Metcalfe, *J. Pharm. Pharmac.* **23**, 817 (1971).
4. H. Machleidt, S. Roth and P. Seeman, *Biochim. biophys. Acta* **255**, 178 (1972).
5. G. P. Sgaragli, M. Rizzotti-Conti, G. L. Garetti, B. Magi, R. Puliti, I. Megazzini and L. Della Corte, *2nd Joint Meeting of German and Italian Pharmacologists*. Venice, 4–6 October 1977, Abstracts, p. 213.
6. F. R. Hewgill and D. G. Hewitt, *J. chem. Soc.* (C), 726 (1967).
7. K. G. Paul, in *The Enzymes* (Eds. P. Boyer, H. Lardy and K. Myrbäck) 2nd Edn., Vol. 8, p. 227. Academic Press, New York (1964).
8. T. Stelmaszynska and J. M. Zgliczynski, *Eur. J. Biochem.* **19**, 56 (1971).
9. A. Seher, *Fette. Seifen Anstr Mittel* **61**, 345 (1959).
10. E. Berndt and H. U. Bergmeyer, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), 2nd Edn., Vol. 4, p. 2246. Academic Press, New York (1974).
11. E. Chance and A. C. Mahely, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 764. Academic Press, New York (1955).
12. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1961).
14. M. A. Komarynsky and A. C. Wahl, *J. phys. Chem.* **79**, 695 (1975).
15. D. J. Danner, P. J. Brignac, Jr., D. Arceneau and V. Patel, *Archs Biochem. Biophys.* **156**, 759 (1973).
16. A. J. Gross and I. W. Sizer, *J. biol. Chem.* **234**, 1611 (1959).
17. G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. biophys. Acta* **284**, 34 (1972).
18. W. W. Westerfeld and C. Lowe, *J. biol. Chem.* **145**, 463 (1942).
19. E. Bourquelot and L. Marchadier, *C. r. Acad. Sci.* **138**, 1432 (1904).
20. B. Chance, *Archs Biochem. Biophys.* **41**, 416 (1952).
21. P. George, *Nature, Lond.* **169**, 612 (1952).
22. P. George, *Biochem. J.* **54**, 267 (1952).
23. I. Yamazaki, H. S. Mason and L. Piette, *J. biol. Chem.* **235**, 2444 (1960).
24. M. L. Mihailović and Z. Čeković, in *The Chemistry of the Hydroxyl Group* (Ed. S. Patai), Vol. 1, p. 528. Interscience, New York (1971).
25. M. A. Venkatachalan, M. H. Soltani and H. D. Fahimi, *J. Cell. Biol.* **46**, 168 (1970).
26. J. W. Smith and G. A. Castro, *Am. J. Physiol.* **234**, R72 (1978).
27. D. E. Hathway, *Adv. Fd Res.* **15**, 1 (1966).
28. P. J. Creaven, W. H. Davies and R. T. Williams, *J. Pharm. Pharmac.* **18**, 485 (1966).