MECHANISM OF INHIBITION OF ENZYMATIC OXIDATION
OF INDOLE-3-ACETIC ACID BY PHENOLS

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UDC 577.152.1

The effect of natural and synthetic phenolic compounds on peroxidase-catalyzed aerobic oxidation of indole-3-acetic acid (IAA) was studied. Despite the established point of view, substituted phenols are not competitive inhibitors of the enzyme in this reaction. Inhibition by binding of the substrate to the inhibitor is also excluded. To explain the results, we suggest that under our experimental conditions the oxidation of IAA is an enzymatic branched-chain free radical reaction and substituted phenols are traps of free radicals participating in this reaction. The mechanism of inhibition by all the phenol compounds tested appears to be identical.

Key words: horseradish peroxidase, indole-3-acetic acid, branched-chain free radical reaction, inhibition.

Indole-3-acetic acid is a natural phytohormone which regulates many vitally important functions of plants. The level of IAA *in vivo* is determined in particular by its synthesis and enzymatic oxidation catalyzed by IAA-oxidases; many native IAA-oxidases are peroxidases [1]. Enzymatic oxidation of IAA is regulated by natural inhibitors of this reaction, including several phenolic compounds (caffeic, chlorogenic, and gallic acids, etc.) [2]. The mechanism of inhibition by phenols has been the subject of detailed investigations. Phenolic inhibitors were found to delay the beginning of enzymatic oxidation of IAA [3-7]. Originally, it was suggested that the inhibitor reduces IAA free radicals to the molecule concomitant with the formation of an dimer of the inhibitor [8]. However, this hypothesis requires the participation of a cofactor in the reaction, whereas the delay is observed without cofactor. Later, the idea prevailed that inhibition is competitive [3-7, 9] and that the inhibitors are in fact competing substrates and the end of the reaction delay coincides with the end of the oxidation of the inhibitor. This hypothesis also has several drawbacks. In particular, the Lineweaver—Burk plots are reliably nonlinear [5, 10]. Thus, the mechanism of inhibition of enzymatic oxidation of IAA by phenols requires further detailed investigation.

MATERIALS AND METHODS

The following materials were used: horseradish peroxidase (RZ 3.0), indole-3-acetic acid, phosphate buffer, caffeic acid, gallic acid, and rose bengal from Sigma (USA); 6-chloro-4-nitro-2-aminophenol, 3,4-dimethylphenol, o-aminophenol, 4-methoxyphenol, pyrocatechol, and hydroquinone from Reakhim (Russia) of research grade. Anphen, an original water-soluble free radical trap, was synthesized and kindly donated by A. A. Volod'kin (Institute of Chemical Physics, Russian Academy of Sciences, Moscow). All solutions were prepared using tridistilled deionized water.

Unless specifically indicated, standard incubation mixture (3 ml total volume) contained 1 mM IAA, 0.1 µM peroxidase, and 67 mM phosphate buffer (pH 7.4).

The effect of phenolic inhibitors on peroxidase-catalyzed aerobic oxidation of IAA was assayed using a chemiluminescence method having high sensitivity of the kinetics and spectrum of chemiluminescence occurring during the reaction upon the conditions of the reaction [11]. In addition, the spectrophotometric method was used. The formation of IAA oxidation products was assayed by the difference in absorption at 242 and 296 nm (wavelengths at which IAA absorption is equal). The data of the chemiluminescence and spectrophotometric assays were verified using routine colorimetric assay for the determination of IAA concentration by the Salkovsky reagent [12].
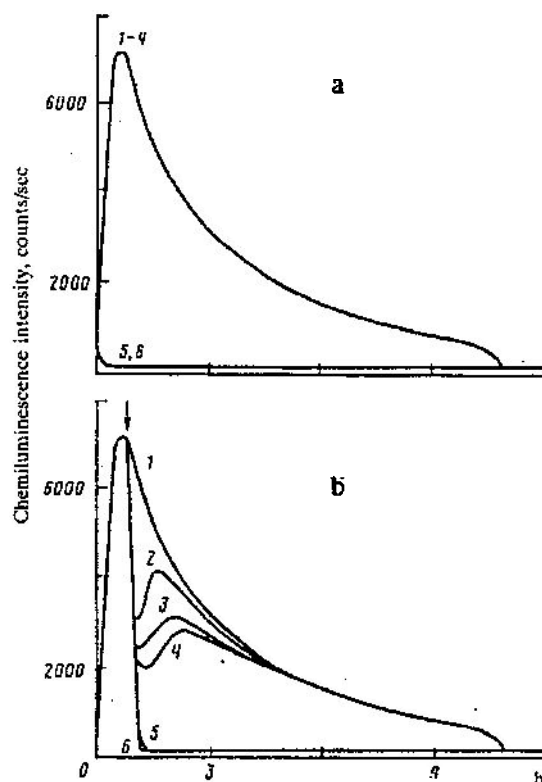
Fig. 1. Effect of caffeic acid on the kinetics of chemiluminescence occurring during peroxidase-catalyzed aerobic oxidation of IAA. a) Peroxidase was added to the mixture of IAA and caffeic acid; concentration of caffeic acid (μM): 1) 0; 2) 0.08; 3) 0.16; 4) 0.24; 5) 0.33; 6) 0.41. b) In 45 min after the reaction was initiated with peroxidase, caffeic acid was added (indicated by arrow); concentration of caffeic acid (μM): 1) 0; 2) 1.3; 3) 2.3; 4) 3.2; 5) 4.0; 6) 4.9.

The difference in absorption of the reaction mixture was measured using a Hitachi 557 (Japan) spectrophotometer in dual-wavelength mode in 1-cm quartz cuvettes. Chemiluminescence kinetics were measured using a chemiluminometer constructed by us. Six quartz cuvettes (3 cm in diameter) with reaction mixtures were placed on a rotating barrel driven by a stepper motor. Emission was detected using an FÉU-130 with calibrated spectral sensitivity under photon-counting conditions. The range of sensitivity of was from 200 to 600 nm. The photocathode of the detector was placed under the cuvette, so chemiluminescence was recorded through the bottom of the cuvette. The operation of the chemiluminometer were described in detail previously [11]. An OVS-1 light source was used to illuminate the reaction mixture with visible light.

RESULTS

1. Threshold Effect. A threshold effect was found during the investigation of the effects of natural and synthetic phenolic inhibitors on peroxidase-catalyzed aerobic oxidation of IAA. If the inhibitor concentration is lower than a certain threshold value, then only a temporary decrease in the intensity of chemiluminescence (Fig. 1) and the rate of reaction (Fig. 2) is observed. If the inhibitor concentration exceeds the threshold, then chemiluminescence ceases (Fig. 1) and the reaction terminates (Fig. 2). The reaction terminated by the inhibitor concentration exceeding the threshold is not reinitiated by additional amounts of peroxidase or buffer. The reaction is reinitiated by addition of the substrate solution. Under these conditions, the reaction kinetics are the same as in a reaction mixture at the same concentration of enzyme and substrate which does not contain the inhibitor.

If the inhibitor is added to the enzyme-initiated reaction, then the value of the inhibitor threshold concentration increases concomitant with an increase in the concentration of the enzyme and the substrate (Fig. 3, curves 1). If the enzyme is added after IAA and the inhibitor were mixed, then the inhibitor threshold concentration does not depend on the enzyme
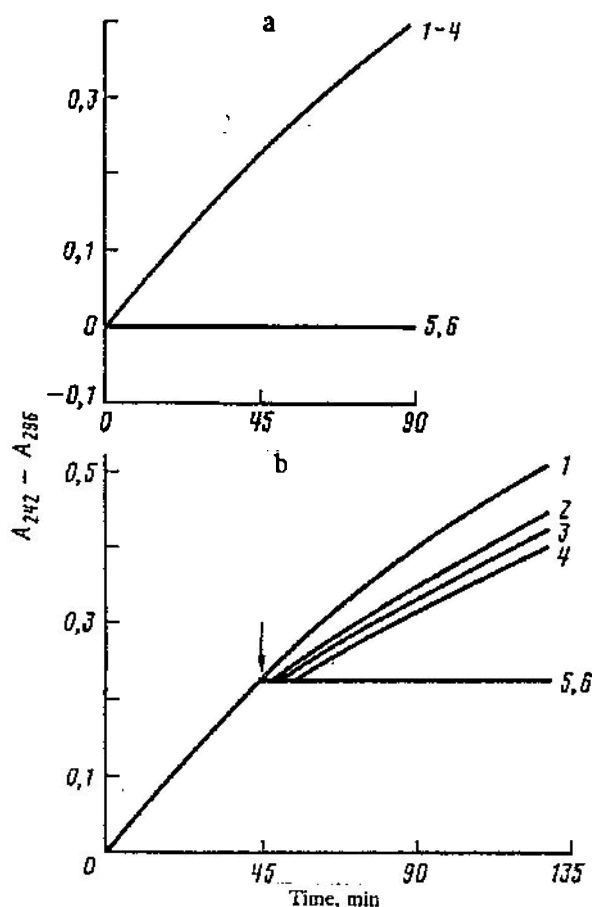
Fig. 2. Effect of caffeic acid on the kinetics of peroxidase-catalyzed aerobic oxidation of IAA. Ordinate axis shows the difference in absorption at 242 and 296 nm. For a and b, see legend to Fig. 1.

concentration but is proportional to the substrate concentration (Fig. 3, curves 2). The addition of the substrate to the mixture of the enzyme and the inhibitor yields analogous results. The threshold effect of phenolic inhibitors on enzymatic oxidation of IAA is observed during the whole course of the reaction. The threshold concentration value depends on the time when inhibitor was added (Fig. 4).

The effect of anphen (a radical trap), which is a nonspecific compound with respect to this reaction, on peroxidase-catalyzed aerobic oxidation of IAA was analogous to the effect of caffeic acid. The value of the inhibition threshold for this compound is significantly higher than that for the other phenolic compounds tested and was ~25 μM if the compound was added 1 min after starting the reaction.

A reaction terminated by the inhibitor concentration exceeding threshold can be reinitiated by illumination with visible light in the presence of rose bengal as a photosensitizer. The rose bengal concentration and illumination dose required for the initiation increase concomitantly with an increase in the inhibitor concentration. After the initiation, the reaction proceeds in the dark following the ordinary kinetics.

A reaction terminated by the inhibitor concentration exceeding threshold can be reinitiated by addition of hydrogen peroxide. The effect of H_2O_2 on chemiluminescence of terminated reaction is presented in Fig. 5. It is evident that the development of chemiluminescence (reinitiation of the reaction) occurs only after the amount of hydrogen peroxide added exceeds a certain threshold value. If the amount of H_2O_2 added is lower than the threshold, then only a weak and transient flash of chemiluminescence is detected and the reaction is not reinitiated. The minimal amount of hydrogen peroxide required for reinitiation of the terminated reaction increases concomitantly with the inhibitor concentration.

2. Spontaneous Reinitiation of Terminated Reaction. As mentioned above, if the inhibitor concentration exceeds a threshold, then the reaction of peroxidase-catalyzed aerobic oxidation of IAA is terminated. This effect is manifested by all the phenolic compounds tested: the only difference between them was in the value of the threshold concentration.
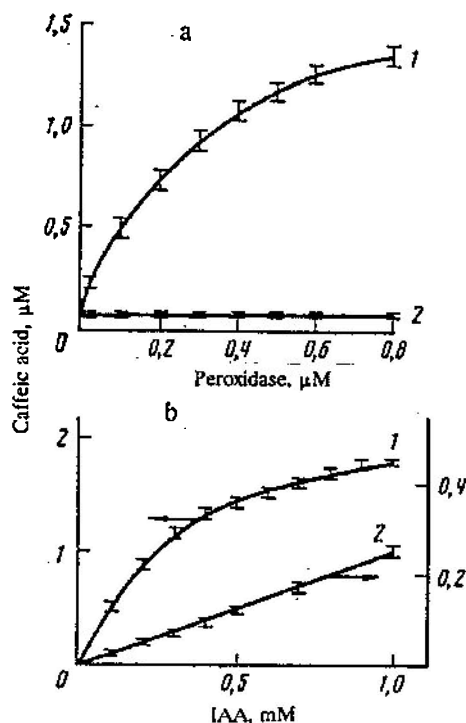
Fig. 3. Minimal concentration of caffeic acid required for termination of peroxidase-catalyzed aerobic oxidation of IAA as a function of: a) peroxidase concentration (IAA concentration was 0.1 mM); b) IAA concentration (peroxidase concentration was 0.1 μM). 1) Caffeic acid was added 3 min after IAA and peroxidase were mixed; 2) caffeic acid was added to IAA before addition of peroxidase.

After termination of the reaction, the behavior of the system depends on inhibitor type. In case of caffeic acid (Figs. 1 and 2), a terminated reaction cannot be reinitiated without the described above effects during the whole course of the observation (36 h). The effect of 3,4-dimethylphenol and o-aminophenol on peroxidase-catalyzed aerobic oxidation of IAA was analogous. If gallic acid, 4-methoxyphenol, pyrocatechol, or hydroquinone was used as the inhibitor, the reaction terminated by the inhibitor concentration exceeding threshold can be spontaneously reinitiated after certain time intervals (Fig. 6). The logarithm of a delay time caused by these inhibitors depends linearly on the inhibitor concentration. This is obvious at low pH when the rate of oxidation of IAA is higher. The delay value does not depend on time of incubation of IAA and inhibitor mixture. The delay value is a non-monotonous function of the peroxidase concentration (Fig. 7).

DISCUSSION

Our experiments indicate that phenolic inhibitors do not affect either the enzyme or the substrate. Indeed, a reaction terminated by the inhibitor concentration exceeding threshold is not reinitiated by addition of the enzyme. Moreover, the threshold inhibitor concentration does not depend on the enzyme concentration if the enzyme is added to the substrate after the inhibitor. These data exclude the possibility that the inhibitor may significantly affect the activity of the enzyme.

The inhibitor concentration exceeding threshold is about 1000-fold lower than the initial substrate concentration. This fact indicates that the substrate is not removed from the reaction due to its binding to the inhibitor, so the inhibitor does not affect the substrate. All further discussion is intended to suggest a model of inhibition which would give a minimal reaction scheme required for the description of the inhibitor threshold effect as well as of the spontaneous reinitiation of a terminated reaction.

To explain the inhibition threshold effect, we had to propose that the inhibitor, which does not affect either the enzyme or the substrate, acts on a third hypothetical compound R. This compound is apparently present in trace amounts in the substrate solution; this is confirmed by the ability of an additional amount of the substrate solution to reinitiate a terminated reaction. The impossibility of reinitiating a reaction by addition of the buffer solution indicates that R is absent in water. Thus,
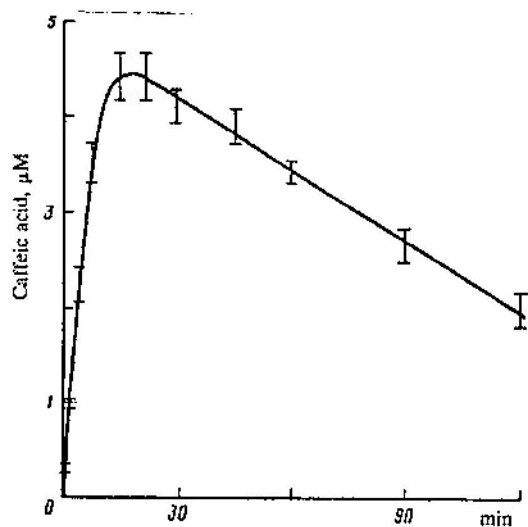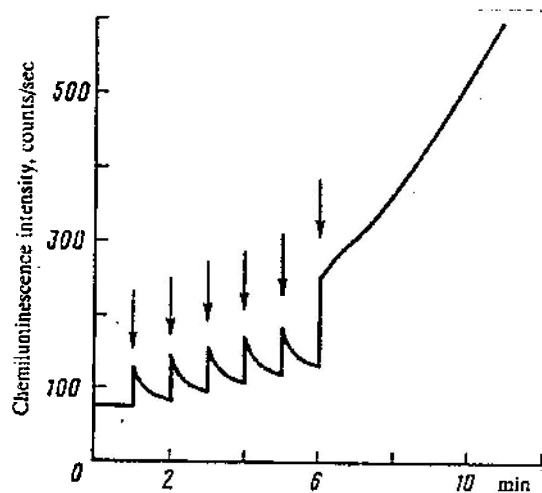
Fig. 4

Fig. 5

Fig. 4. Minimal concentration of caffeic acid required for termination of peroxidase-catalyzed aerobic oxidation of IAA as a function of the time interval between the beginning of the reaction and addition of caffeic acid.

Fig. 5. Effect of hydrogen peroxide addition on the chemiluminescence kinetics of peroxidase-catalyzed aerobic oxidation of IAA terminated by caffeic acid (1.2 μM). Caffeic acid was added to IAA before addition of peroxidase. Arrows indicate the times when hydrogen peroxide (0.05 μM) was added.
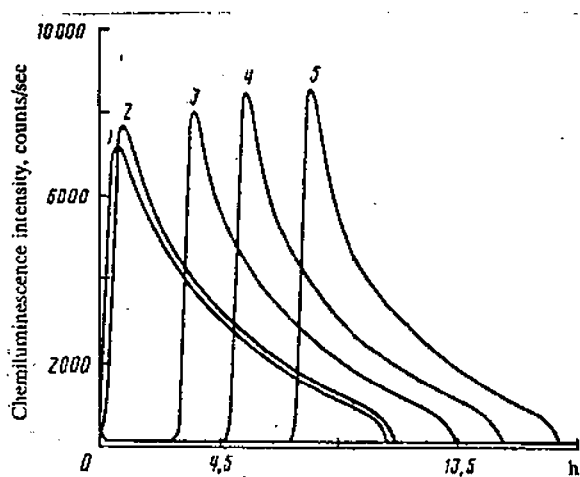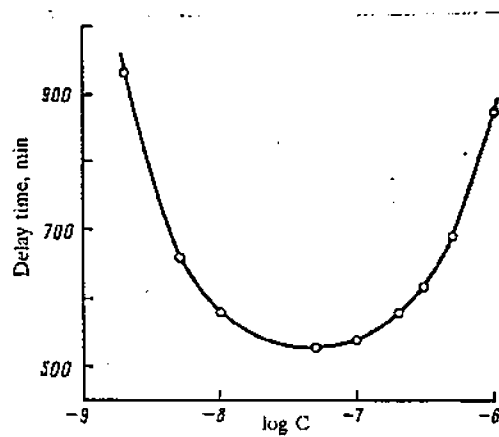

Fig. 6

Fig. 7

Fig. 6. Effect of 6-chloro-4-nitro-2-aminophenol on the kinetics of chemiluminescence during peroxidase-catalyzed aerobic oxidation of IAA. 6-Chloro-4-nitro-2-aminophenol was added to IAA before addition of peroxidase. 6-Chloro-4-nitro-2-aminophenol concentrations (μM): 1) 0; 2) 1.15; 3) 1.32; 4) 1.48; 5) 1.64.

Fig. 7. Effect of peroxidase concentration on delay in the beginning of peroxidase-catalyzed aerobic oxidation of IAA occurring in the presence of 1.8 μM 6-chloro-4-nitro-2-aminophenol. The latter compound was added to IAA before addition of peroxidase. Abscissa axis shows common logarithm of the peroxidase concentration (M).

R is obviously a substrate derivative. Moreover, R is formed during enzymatic oxidation of IAA. This arises from the effect of the time of inhibitor addition (Fig. 4) and of the enzyme and substrate concentrations on inhibition threshold (Fig. 3). Indirect reasons suggest that R is a free radical. Indeed, a reaction terminated by the inhibitor concentration exceeding threshold can by reinitiated by illumination in the presence of rose bengal as a photosensitizer. On illumination, rose bengal readily yields triplet-

excited molecules [13]. These molecules are obviously biradicals, capable of effectively forming free radicals [14]. Moreover, free radicals are formed during peroxidase-catalyzed oxidation of IAA. In particular, the skatole radical involving the carbon atom was identified [15]. Taking into account the suggestion that this hypothetical compound is of free radical nature, we shall use the symbol $R^\bullet$ instead of R.

Because the threshold value of inhibitor concentration was not dependent on the period of storage of the IAA solution, we suggest that $R^\bullet$ is in a quasiequilibrium with the substrate S.

$$S \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \quad R^\bullet \tag{1}$$

Peroxidase-oxidase reactions, including peroxidase-catalyzed aerobic oxidation of IAA, can proceed as an enzymatic branched-chain free radical reaction [16]. We suggest that under our experimental conditions this is the case because of the rapid increase in the threshold inhibitor concentration with the incubation time during the initial stage of the reaction (Fig. 4).

To a first approximation a branched-chain reaction can be described by the following equation.

$$S + R^\bullet \quad \xrightarrow[\text{Peroxidase, } O_2^-]{k_2} \quad 2R^\bullet \tag{2}$$
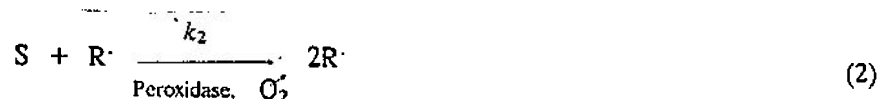
An increase in the rate of the chain reaction would be limited by the bimolecular reaction of radical $R^\bullet$ annihilation.

$$R^\bullet + R^\bullet \xrightarrow{k_3} \tag{3}$$

Because the radical trap anphen and the other tested phenolic compounds affect enzymatic oxidation of IAA identically, we suggest that phenolic inhibitors are essentially traps (quenchers) of free radicals $R^\bullet$:

$$R^\bullet + I \xrightarrow{k_4} P_1 + I^\bullet, \tag{4}$$

where I and $I^\bullet$ are the inhibitor and inhibitor radical, respectively; P_1 is the reaction product. Inhibitor radical, in its turn, can be reduced to form the inhibitor molecule.

$$I^\bullet \xrightarrow{k_5} I \tag{5}$$

Here an electron donor is not specified. Moreover, the reaction of bimolecular irreversible annihilation of $I^\bullet$ is evident.

$$I^\bullet + I^\bullet \xrightarrow{k_6} \tag{6}$$

We suggest that $I^\bullet$ is less reactive than $R^\bullet$ in reaction (2), so $I^\bullet$ cannot sustain the chain reaction.

The reaction (2) is apparently not the only one where the enzyme and radical $R^\bullet$ participate because reaction delay is a non-monotonous function of the peroxidase concentration (Fig. 7). When the enzyme concentration increases, the reaction of enzymatic annihilation of $R^\bullet$ becomes significant:

$$R^\bullet + X \quad \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} \quad R^\bullet X \xrightarrow{k} P_2 + X \tag{7}$$
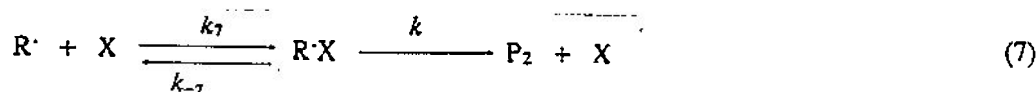
where X is one of the peroxidase forms and P_2 is reaction product.

The system of reactions (1)-(7) is the minimum required for the description of all the results. According to this scheme, the threshold effect is described as follows. The reactions (1)-(4) and (7) indicate the existence of a certain critical concentration

of the inhibitor where the multiplication coefficient of radicals $R^\bullet$ would be equal to unity. If the inhibitor concentration exceeds a critical value or is less than a critical value, then the multiplication coefficient would be less than unity or greater than unity, respectively. In the first case, the reaction is terminated. In the second case, the chain reaction develops as an avalanche until its rate would not be limited by reaction (3). At the same time, the inhibitor concentration rather rapidly tends to zero due to reactions (4) and (6). A given critical inhibitor concentration apparently corresponds to the experimentally measured threshold inhibitor concentration.

The spontaneous reinitiation of the reaction is observed for several inhibitors (Fig. 5) and occurs obviously because even in the terminated reaction the concentration of $R^\bullet$ is not equal to zero due to reaction (1). At the same time, slow and irreversible annihilation of the inhibitor occurs in reactions (4) and (6). After a certain time interval, the inhibitor concentration becomes lower than a critical value. The multiplication coefficient of free radicals $R^\bullet$ becomes greater than unity and the chain reaction develops as an avalanche. The inhibitor concentration relatively rapidly tends to zero and oxidation of IAA occurs according to the usual kinetics.

In the case of caffeic acid (Figs. 1 and 2), 3,4-dimethylphenol, and o-aminophenol, spontaneous reinitiation of a terminated reaction was not observed during the whole course of continuous observation. This is apparently due to the more effective regeneration of the inhibitor (5) than its irreversible annihilation (6). We have to mention that k_6 cannot be rigorously zero, so the spontaneous reinitiation of the reaction terminated by these inhibitors is expected to occur, but outside the time limits of the experiment.

Thus, the analysis of the experiments indicates that all the phenolic inhibitors tested act on peroxidase-catalyzed aerobic oxidation of IAA as radical traps for branched-chain free radical reactions.

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