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Melatonin does not React Rapidly with Hydrogen Peroxide

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It has been claimed that melatonin reacts directly with hydrogen peroxide with a very high rate constant ($2.5 \times 10^5 - 2.3 \times 10^6\ M^{-1}\ s^{-1}$). Both these values were derived from inhibition by melatonin of peroxidase-catalyzed oxidation of Phenol Red by hydrogen peroxide, assuming that this inhibition is due to direct scavenging of hydrogen peroxide by melatonin. In this study, we show that this reasoning is illegitimate and melatonin decreases the yield of oxidation of Phenol Red as a competitive substrate. Monitoring changes of concentration of hydrogen peroxide incubated with melatonin using Xylenol Orange points to poor reactivity of melatonin with H_2O_2.

Keywords: Melatonin; Hydrogen peroxide; Antioxidants; Horseradish peroxidase; Phenol Red; Xylenol orange

INTRODUCTION

Numerous studies have been devoted to the antioxidant properties of melatonin. Melatonin has been demonstrated to be an effective antioxidant in various model systems and to scavenge hydroxyl, peroxyl and superoxide radicals, singlet oxygen, peroxynitrite and hydrogen peroxide.[1–7] However, low concentrations of this hormone *in vivo* may raise doubts if the antioxidant properties of melatonin have a significant importance in the body where other antioxidants are present at concentrations higher by orders of magnitude.[8] Answering this question requires precise data on the concentration, distribution and reactivity of melatonin.

Two recent papers claimed high reactivity of melatonin with hydrogen peroxide. The first one reported the value of rate constant of melatonin with hydrogen peroxide to be $2.52 \times 10^5\ M^{-1}\ s^{-1}$,[9] the second put forward an even higher value of $2.3 \times 10^6\ M^{-1}\ s^{-1}$.[10] This high reactivity of melatonin with hydrogen peroxide could provide a new line of explanation of the protective effects of melatonin with respect to oxidative damage caused or mediated by hydrogen peroxide[11,12] and might have physiological importance, especially in extracellular fluids where the catalase activity is low.[1] However, in our opinion there is one problem with these high rate constant values: they are artifactual.

MATERIALS AND METHODS

Sodium phosphates, sodium chloride and Phenol Red were from Polish Chemical Reagents (POCh, Gliwice, Poland) and were of analytical grade. All other reagents were from Sigma (Poznań, Poland).

Concentration of hydrogen peroxide was determined by two methods, one employing Phenol Red and horseradish peroxidase (HRP)[13] strictly as employed by Tan *et al.*,[10] and another, employing Xylenol Orange.[14] In the first method, Phenol Red is oxidized by hydrogen peroxide in the reaction catalyzed by HRP for 5 min. The product of oxidation of Phenol Red has absorption spectrum different from that of the substrate; in particular, absorbance at 610 nm is increased and the magnitude of this increase is proportional to the amount of H_2O_2 present in the medium and consumed by HRP. Routinely, 50 μl of a sample was introduced to 940 μl of 10 mM phosphate-buffered saline, pH 7.4, containing 100 μl ml^{-1}. Phenol Red and 10 μl of HRP was added (final activity: 8.5 U ml^{-1}). After 5-min

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incubation at room temperature, absorbance was measured at 610 nm.

When estimating hydrogen peroxide with Xylenol Orange, 100 μl of a sample was introduced to 1 ml of the working solution obtained by mixing 1 volume of 25 mM Mohr salt in 2.5 M sulfuric acid with 100 volumes of 125 μM Xylenol Orange in 100 mM sorbitol. Absorbance was measured at 560 nm after 30-min incubation at room temperature.

RESULTS

The Dixon plot of the effect of melatonin on the oxidation of Phenol Red points to a competitive inhibition of Phenol Red oxidation by melatonin with an apparent K_i of about 90 μM (Fig. 1). This value is only an estimate since methanol used as the solvent for melatonin had also some (small) contribution to the inhibition of Phenol Red oxidation by HRP; anyhow, such a result suggests that melatonin is a competitive substrate for HRP, with respect to Phenol Red.

Not only melatonin but also serotonin, tryptophan and tyrosine inhibited the HRP-catalyzed oxidation of Phenol Red (Fig. 2).

Since the method of determination of reaction of melatonin with H_2O_2 using HRP is biased with the intrinsic error of assaying Phenol Red oxidation without removal of a competitive substrate, we studied the time course of the reaction between melatonin and hydrogen peroxide, applying another method of estimation of hydrogen peroxide concentration, independent of HRP. We monitored the concentration of hydrogen peroxide with Xylenol Orange and found no detectable decrease of hydrogen peroxide concentration during 5-h incubation of 3 μM hydrogen peroxide with 0.5 mM melatonin which evidences a very slow reaction rate. Only when HRP was present in the medium, H_2O_2 concentration decreased rapidly. HRP alone, in the absence of melatonin, also significantly accelerated the decay of hydrogen peroxide (Fig. 3).

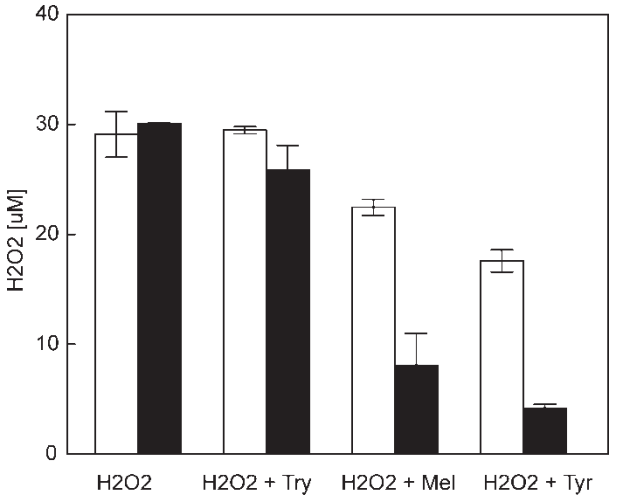

FIGURE 2 Percent inhibition by tryptophan (Try), melatonin (Mel) and tyrosine (Tyr) of HRP-catalyzed Phenol Red oxidation by hydrogen peroxide. Thirty μM H_2O_2, 8.5 U ml^{-1} of HRP, 0.5 mM indole derivatives. Filled bars, indoles preincubated with H_2O_2 and HRP for 5 min before addition of Phenol Red and subsequent 5-min incubation; empty bars, without preincubation. Reaction rate constants of the indoles with HRP Compounds 1 (k_1) and 2 (k_2).[19]

	Try	Mel	Tyr
k_1 ($M^{-1}s^{-1}$)	5.1×10^1	1.3×10^4	5.0×10^4
k_2 ($M^{-1}s^{-1}$)	2.0	5.2×10^2	1.1×10^3

We found an initial decrease of hydrogen peroxide concentration in the "zero time" samples added with melatonin and assayed immediately for H_2O_2 content. This effect was dependent on the concentration of melatonin (Fig. 4). However, Trolox showed a similar dose-dependent zero-time decrease of hydrogen peroxide concentration (Fig. 5) although this compound did not show any detectable reaction with hydrogen peroxide over 180 min as judging from the lack of detectable changes in hydrogen peroxide absorbance (240 nm) during incubation with Trolox (not shown).

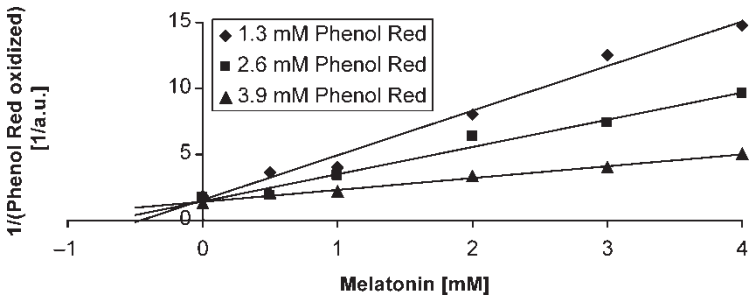

FIGURE 1 Dixon plot of the dependence of Phenol Red oxidation on the concentration of melatonin. Fifty or 150 μM Phenol Red was incubated with, various concentrations of melatonin (0.5–4 mM) and, the increase of absorbance at 610 nm was measured.

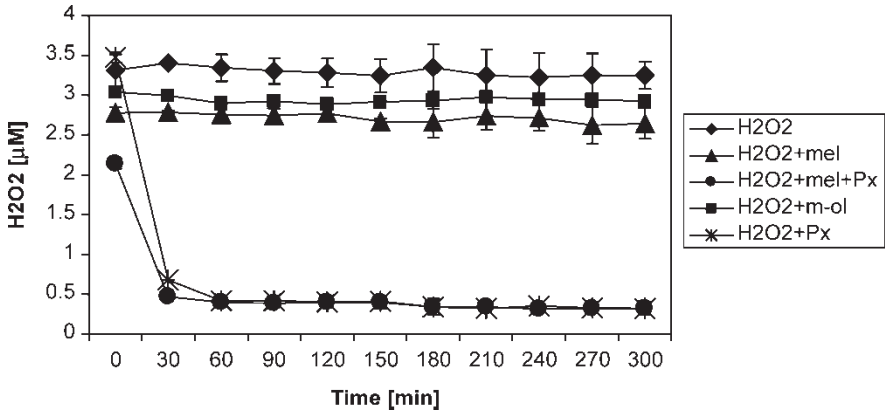

FIGURE 3 Time course of hydrogen peroxide concentration during incubation with melatonin 0.5 mM, methanol 0.125% (v/v; the same as introduced with melatonin), melatonin with HRP ($5\,U/ml^{-1}$), and HRP alone. Incubation at room temperature (21°C). Hydrogen peroxide concentration was measured with Xylenol Orange.

DISCUSSION

The values of reaction rate constants between melatonin and hydrogen peroxide reported[9,10] are incredibly high and are typical for reaction of hydrogen peroxide with peroxidases rather than with low-molecular weight compounds.[15,16] Conspicuously, both studies of the reaction of melatonin with H_2O_2 were obtained in systems containing HRP; they consisted in measurements of inhibition by melatonin of HRP-mediated oxidation of Phenol Red by H_2O_2. Tan *et al*. checked carefully that "different concentrations of melatonin and ethanol" (the latter compound was used as solvent for melatonin) "affect the absorbance at 610 nm at which the product of phenol red oxidation absorbs without H_2O_2". What these Authors apparently omitted is the fact that melatonin (and ethanol) may be substrates for HRP added subsequently to the reaction medium to catalyze the oxidation of Phenol Red by hydrogen peroxide. The present results (Fig. 1) indicate that melatonin is a competitive inhibitor and, therefore, a probable substrate, for HRP. Indeed, it has been demonstrated that melatonin is a substrate for peroxidases including HRP.[17,18]

The following argument speaks in favor of an important role of the reaction of melatonin with HRP in the system employed previously for the determination of rate constant for the reaction of melatonin with H_2O_2. Comparison of the rate constants for the reactions with Compounds I and II of HRP shows that the reactivity of melatonin with both compounds is intermediate between those of serotonin and tryptophan and tyrosine.[19] The same sequence of reactivity was observed for the inhibition of Phenol Red oxidation by these compounds (Fig. 2). Moreover, melatonin may inhibit the HRP-catalyzed oxidation of Phenol Red not only as a competitive substrate. It is an efficient free radical scavenger and may react with a free radical oxidation product of the oxidation of the dye.

When studying the disappearance of hydrogen peroxide incubated with melatonin with Xylenol Orange, a method avoiding the artifact due to the use

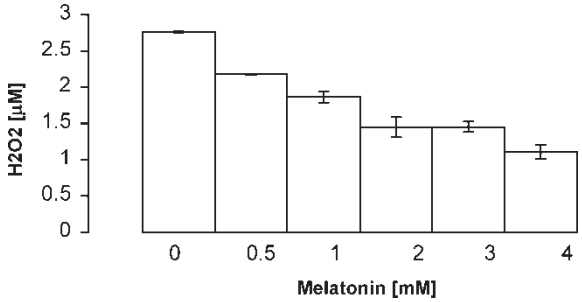

FIGURE 4 Decrease of hydrogen peroxide concentration after addition of different concentrations of melatonin. Hydrogen peroxide concentration was measured with Xylenol Orange immediately after addition of melatonin.

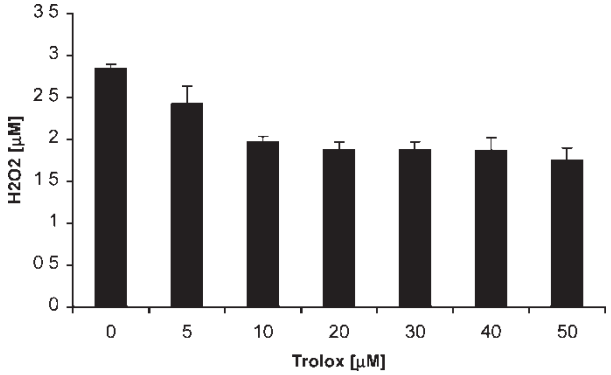

FIGURE 5 Decrease of hydrogen peroxide concentration after addition of different concentrations of Trolox. Hydrogen peroxide concentration was measured with Xylenol Orange immediately after addition of Trolox.

of HRP, we did not find any detectable consumption of H_2O_2 during 5-h incubation, indicating a very slow reaction rate (Fig. 3). Tan *et al.* showed results of time course of the reaction between hydrogen peroxide and melatonin[10] (Fig. 3) demonstrating "rapid reaction phase" and then a "slow reaction" "characterized by a gradual decrease in H_2O_2 content over a several hour period", compatible with a reaction rate constant of about $0.9\,M^{-1}s^{-1}$, as judging from Fig. 5 of their paper. These data would correspond to the present results.

The "rapid reaction phase" observed by Tan *et al.* was terminated within 5 s (the first experimental point taken) what has been ascribed to a rapid reaching equilibrium by this phase of reaction. Repeating the experiments of Tan *et al.* with HRP-catalyzed oxidation of Phenol Red we also found an initial decrease of H_2O_2 concentration in H_2O_2 solutions incubated with melatonin even for the shortest incubation times (not shown). In our opinion, however, the "rapid reaction" does not exist and its appearance is due to the fact that after this short preincubation time, the solutions containing melatonin, H_2O_2 and Phenol Red were added with HRP and allowed to react for 5 min. Therefore, melatonin was present as a competitive substrate (with respect to Phenol Red) in all samples (even those incubated for 5 s only in the absence of HRP) which made an impression of a short reaction completed within this time.

However, employment of another method of determination of hydrogen peroxide with Xylenol Orange also showed an initial decrease of the measured hydrogen peroxide concentration in the presence of melatonin (Fig. 4).

It might confirm the idea of Tan *et al.* of initial rapid reaction of melatonin with hydrogen peroxide. However, the same effect was observed with Trolox (Fig. 5). Therefore, we ascribe this effect rather to re-reduction of the Fe(III)–Xylenol Orange complex by reductants such as melatonin or Trolox. A similar phenomenon has been reported previously for ascorbate.[20]

In our opinion, the direct scavenging of hydrogen peroxide by melatonin is a slow reaction of no physiological significance and the protective effects of melatonin against hydrogen peroxide-generating agents at the organismal level is due rather to the induction of synthesis of hydrogen peroxide-scavenging enzymes[21] than direct action on hydrogen peroxide.

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