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The Catalytic Effect of Peroxidase on the Reaction between Hydrogen Peroxide and Certain Sulfur Compounds

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Myeloperoxidase catalyzed the reaction between thiourea and hydrogen peroxide. Some of the reaction products were identified and the formation of an unstable intermediate was demonstrated. Myeloperoxidase also catalyzed the oxidation of thiouracil and thiocyanate, but was inactive with true thiol compounds (cysteine, thioglycolic acid, mercaptoethanol). Horse radish peroxidase was inactive with all the aforementioned sulfur compounds. The results were discussed with respect to the metabolism of the sulfur compounds and to the action of thyreostatic compounds.

In connection with research programs on the oxidation of thiol compounds in biological systems and on the biosynthesis of thyroxine, now in progress in our laboratories, the effect of peroxidase plus hydrogen peroxide on certain sulfur compounds has been investigated. Randall [1] previously reported that thiourea and thiouracil were substrates for horse radish peroxidase, but for different reasons a reinvestigation appeared to be necessary. Thus Randall followed the reaction between hydrogen peroxide and the sulfur compound by a manometric method, which was based on a conversion of remaining hydrogen peroxide to oxygen through the action of catalase. However, the inhibitory effect of the sulfur compounds on catalase [2,3] was apparently not considered. This source of error has been avoided in the present work by following the oxidation of the sulfur compounds by spectrophotometry in the ultraviolet region. The present investigation was also carried out with both animal and plant peroxidase and extended to include true thiol compounds (mercaptans).

EXPERIMENTAL

Materials. Crystalline myeloperoxidase (MPO) was a generous gift from Dr. Kjell Agner. Horse radish peroxidase (PZ-65) was a commercial preparation. Salts of formamidine disulfide [4,5] and formamidine sulfinic acid [6] were prepared according to the indicated references.

Methods. The reactions were followed in phosphate buffer of pH 7.4 at room temperature (20°C) in a Beckman DU spectrophotometer. Paper chromatography of the reaction products from thiourea was carried out on Whatman No. 1 filter paper with water saturated phenol or butanol-acetic acid-water (4:1:1) as developers. The spots were visualized with the iodine-azide reagent or with *p*-dimethylaminobenzaldehyde. Thiourea and formamidine disulfide were not separated from each other in these systems (in fact no solvent system was found, which accomplished this separation), both having the R_F values 0.54 in phenol-water and 0.42 in butanol-acetic acid-water. Formamidine sulfinic acid had R_F 0.44 in phenol-water and 0.21 in butanol-acetic acid-water. The presence of formamidine disulfide in the reaction mixtures could, however, be verified through a cyanolysis reaction. The disulfide reacted rapidly at room temperature with cyanide (0.1 M) to give thiocyanate, which could then be demonstrated in the usual manner with ferric ions. No thiocyanate is obtained from thiourea or formamidine sulfinic acid under these conditions. The formation of sulfate from thiourea and thiocyanate was demonstrated by adding barium ions to the acidified sample. The phosphate buffer in the reaction system was in this case replaced by Tris buffer (in order to avoid precipitation of barium phosphate). The time course of the enzymic reactions was the same in phosphate and Tris buffer. Cyanide was demonstrated with the pyridine-barbituric acid reaction [8] after aeration from the acidified sample into 0.1 M NaOH.

RESULTS

The oxidation of thiourea with hydrogen peroxide with and without MPO was first studied. It is known that the products of this reaction are mainly formamidine disulfide and formamidine sulfinic acid; the first being preferen-

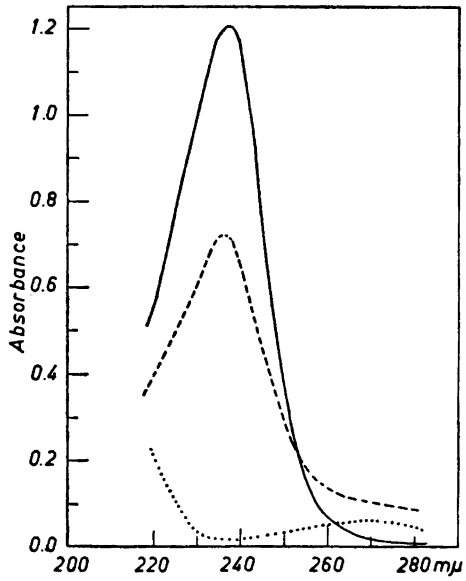

Fig. 1. Ultraviolet absorption curve of thiourea at pH 7.4 ————, formamidine disulfide HCl ------- and formamidine sulfinic acid ····· at 10^{-4}M concentration. (5×10^{-5} M in case of formamidine disulfide calculated as disulfide).

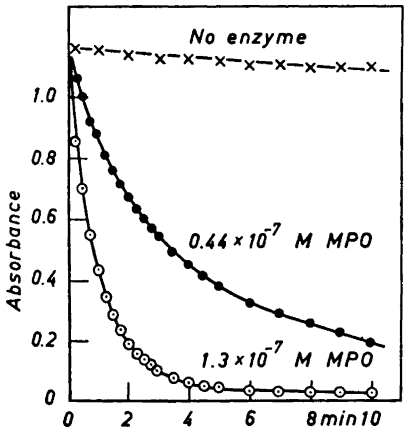

Fig. 2. Reaction of thiourea and hydrogen peroxide followed at 235 mμ. Thiourea 1×10^{-4} M, H_2O_2 2.5×10^{-4} M, phosphate 0.05 M and pH 7.4. Absorbance values corrected for the contribution of MPO.

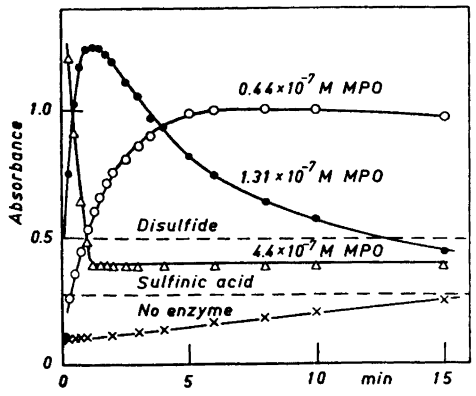

Fig. 3. Thiourea oxidation followed at 270 mμ. Thiourea 5×10^{-4} M, H_2O_2 1.25×10^{-3} M, other conditions as in Fig. 2. Absorbance values corresponding to a complete oxidation of thiourea to formamidine disulfide or sulfinic acid indicated by horisontal lines.

tially formed in acid, the latter in alkaline solution [6]. The spectra of thiourea and the anticipated reaction products * were first compared (Fig. 1) and the reaction was then followed at 235 mμ. A MPO catalyzed reaction was demonstrated (Fig. 2) and the expected decrease in absorbance observed. At 270 mμ, however, the results were different (Fig. 3). With small amounts of enzyme an initial increase in absorbance was obtained, which became more rapid at higher enzyme concentrations. The initial increase in absorbance was then followed by a secondary decrease, and when the enzyme concentration was further increased the initial phase become too rapid to be observed and only the secondary decrease could be followed. As the secondary decrease was faster at higher MPO concentrations, the results indicated that MPO catalyzed not only the formation of a primary reaction product, but also the decomposition of this product. The absorption spectrum of a reaction mixture containing the intermediate showed only a peak at 270 mμ. When the effect of hydrogen peroxide concentration was studied (Fig. 4) it was found that the secondary decrease in absorbance was obtained only with an excess of this compound. Consequently the secondary decomposition of the intermediate must be an oxidation of the latter with hydrogen peroxide. Attempts were now made to identify the reaction products. Paper chromatography (together with the cyanolysis reaction) demonstrated only the presence of formamidine disulfide and formamidine sulfinic acid, but none of these compounds could explain the high absorbance values reached in the enzyme reaction (Fig. 3), and could thus not be the unknown intermediate. Sulfate was also found in the reaction mixtures, but does not absorb light at 270 mμ. The possibility was considered that formamidine sulfonic acid [7], another oxidation product of thiourea, was the intermediate. This sulfonic acid can, however, only be obtained under anhydrous conditions and could also be excluded from being the intermediate by the following experiment. Oxidation of a sulfinic acid with hydrogen peroxide gives the

* Formamidine disulfide is stable only in acid solutions, but at pH 7.4 the spectrum of the compound was essentially unchanged for at least 1 h.

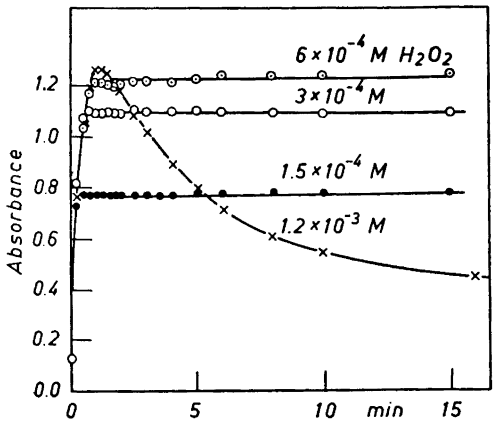

Fig. 4. Effect of hydrogen peroxide concentration on thiourea oxidation. MPO 1.3×10^{-7} M, H_2O_2 as indicated and other conditions as in Fig. 3.

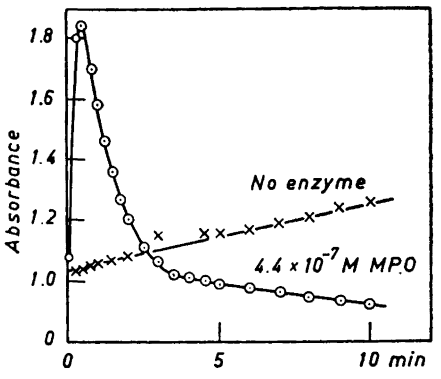

Fig. 5. Oxidation of formamidine disulfide by hydrogen peroxide. Formamidine disulfide 5×10^{-4} M, H_2O_2 2.5×10^{-3} M, phosphate 0.01 M, chloride 2×10^{-3} M and pH 7.4. Followed at 270 mμ.

corresponding sulfonic acid and if formamidine sulfonic acid was the intermediate, strong increase in absorbance should be observed at 270 mμ by treating formamidine sulfinic acid with hydrogen peroxide. This reaction should furthermore be catalyzed by MPO. However, when the experiment was performed only a slow decrease in absorbance was observed and MPO was without effect. On the other hand formamidine disulfide was oxidized by hydrogen peroxide in a MPO catalyzed reaction and an increase and decrease in absorbance, corresponding to the formation and break-down of the intermediate was observed (Fig. 5). Consequently the unknown intermediate must be an oxidation product of the disulfide on an oxidation level between a disulfide and a sulfinic acid. A disulfoxide (R-SO-SO-R) or a sulfenic acid (RSOH) would fulfil these requirements and as both are unstable compounds, which decompose to give a mixture of the corresponding disulfide and sulfinic acid [9], the results obtained by paper chromatography could be explained. As neither a disulfoxide, nor a sulfenic acid has been prepared from thiourea, it was not possible to identify the intermediate further.

Myeloperoxidase was also active with thiouracil (Fig. 6) and with thiocyanate (Fig. 7). The reaction products from thiouracil were not identified, but in the case of thiocyanate the formation of sulfate and cyanide could be demonstrated, and from Fig. 7 the formation of an unstable intermediate is also evident. The catalytic activity of horse radish peroxidase was also studied. Randall [1] had already reported that the plant enzyme catalyzed the oxidation of thiourea and thiouracil, but somewhat unexpectedly we found that the plant enzyme was completely inactive with both these compounds. (The enzyme was tried in concentrations up to 10^{-6} M or about 1 000-fold higher than those used by Randall). It might be added, that a difference in

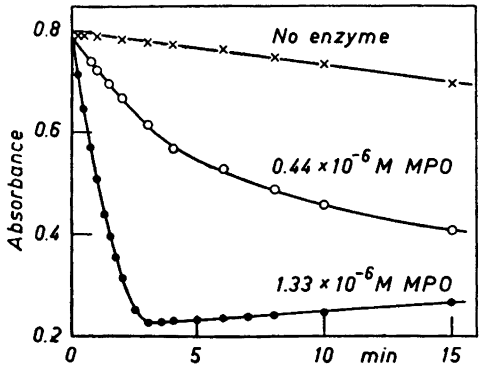

Fig. 6. Oxidation of thiouracil by hydrogen peroxide. Thiouracil 1×10^{-4} M, H_2O_2 2.5×10^{-4} M, reaction followed at 300 mμ. Other conditions as in Fig. 2.

substrate specificity has previously been observed between the animal and plant peroxidase [10,11].

Finally the action of peroxidase-hydrogen peroxide on cysteine, thioglycolic acid and mercaptoethanol was studied. The reactions were followed at pH 7.4 and 220—230 mμ, where both the thiol (0.001 M) and hydrogen peroxide (0.002 M) gave a measurable light absorption. The spontaneous reaction was rather fast in case of cysteine and slower with the other compounds, but in no case could a catalytic activity be demonstrated with peroxidase. Both the animal and plant enzyme was tried at concentrations up to 10^{-6} M. Consequently thiol compounds are not substrates for peroxidase.

DISCUSSION

The present work has demonstrated that thiourea, thiouracil and thiocyanate are substrates for animal peroxidase in contrast to true thiol compounds (cysteine, thioglycolic acid and mercaptoethanol). As thiourea, thiouracil and thiocyanate may exist in two tautomeric forms, one containing a thiol group and the other containing sulfur linked to carbon through a double bond, our results suggest that only the last tautomer (with double bonded sulfur) is the one acted upon by the enzyme. Of interest in this connection is also that thiourea, thiouracil and thiocyanate are well known thyreostatic compounds in contrast to true thiols. Peroxidase-hydrogen peroxide has been implicated with the synthesis of thyroxine [12–14], and it is possible that the antithyroid compounds exert their activity by competing with thyroxine precursors for hydrogen peroxide. It has also been suggested [15] that the thyreostatic compounds owe their activity to their reducing properties on iodine. Iodide must first be oxidized to iodine before being incorporated into the tyrosine residues, and it has been shown that the thyreostatic compounds can prevent this conversion of free iodide to organic bound iodine [16]. However, it seems in this case at first difficult to explain why thiols, such as cysteine or glutathione, which also rapidly react with iodine, do not possess antithyroid properties. But another important biochemical distinc-

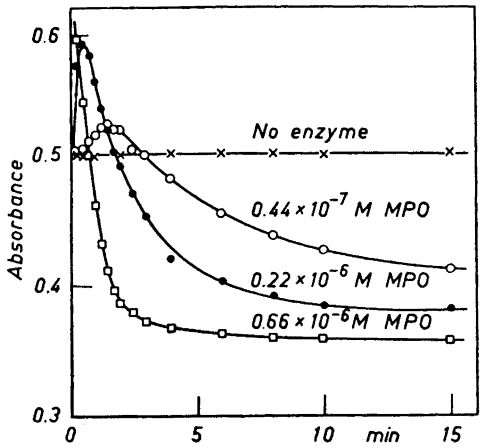

Fig. 7. Oxidation of thiocyanate by hydrogen peroxide. CNS^- 5×10^{-4} M, H_2O_2 1.25×10^{-3} M and other conditions as in Fig. 2 (235 mμ).

tion can be made between the thyreostatic active compounds and the inactive thiols, as the latter are rapidly oxidized through the cytochrome oxidase system [17] in contrast to the thyreostatic compounds. (We have verified that neither thiourea nor thiouracil reduces cytochrome c.) Thus, when thiols are introduced in the body they are probably oxidized before they can reach their presumptive site of action in the thyroid.

Work with ^{35}S-labeled thiourea has shown that the latter is in part oxidized in the body to sulfate, and that the thyroid gland is especially active in this respect [18]. Sulfate was now found to be a reaction product in the peroxidase catalyzed oxidation of thiourea, and as peroxidase is present in the thyroid gland [14] the *in vivo* oxidation of thiourea is probably catalyzed by this enzyme. Of interest in this connection is also that thiocyanate is oxidized *in vivo* to sulfate [19] and to cyanide [20]. The results now obtained strongly suggest that peroxidase is a part also of the "thiocyanate oxidase" [20] system.

When the investigation was started, it was suspected that peroxidase may be responsible for some of the biological effects of ionizing radiations. Hydrogen peroxide is formed when solutions are irradiated and an oxidation of vital thiols or disulfides to sulfinic or sulfonic acids has been suggested as a key reaction in radiation damage [21]. As some well known inhibitors for heme-enzymes, for instance cyanide [22], protect against ionizing radiation, a peroxidase catalyzed destruction of vital thiol or disulfide groups during irradiation was suggested. The results now obtained seem to exclude this possibility.

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