REACTION OF THIOL COMPOUNDS WITH PEROXIDASE AND HYDROGEN PEROXIDE

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Recent studies have indicated that peroxidase might function in the biological synthesis of thyroxine by the thyroid gland. Johnson and Tewkesbury (1) believed that thyroxine might be formed by the oxidative coupling of 2 molecules of diiodotyrosine. Westerfeld and Lowe (2) studied the oxidative condensation of p-cresol by hydrogen peroxide and peroxidase and suggested that peroxidase might be involved in the biological synthesis of thyroxine by a similar mechanism. Keston (3) intimated that hydrogen peroxide may be produced in living cells by the flavoprotein systems and that the hydrogen peroxide and peroxidase accelerated the iodination of protein in vitro. The observation that thiourea inhibited the staining of thyroid tissue by the peroxidase reagent, benzidine-hydrogen peroxide, led Dempsey (4) to propose that the antiperoxidase activity of thiourea might account for its goitrogenic activity. Glock (5) further observed that thiourea and thiouracil inhibited the action of peroxidase and hydrogen peroxide on pyrogallol; however, she found only insignificant amounts of true peroxidase in thyroid gland and considered, therefore, that the goitrogenic activity of thiourea was not due to its antiperoxidase activity. Franklin et al. (6,7) demonstrated that oxidative mechanisms were necessary for the incorporation of iodide into thyroxine by the thyroid gland. This iodination was prevented by thiouracil as well as by oxidative inhibitors. Thiourea has the properties of an antioxidant in the prevention of the discoloration of fruit (8) and in the prevention of peroxide formation in fats (9).

The above observations suggested that the antioxidant properties of the thiol compounds might be related to their antithyroid activities. It was considered further that the antiperoxidase activities of the thiol compounds might be a measure of their antioxidant properties. Accordingly, a number of thiol compounds were tested for their antiperoxidase activities.

Antiperoxidase Activities—Lipmann’s method (10) was used for measuring the inhibitory effect of thiols on horseradish peroxidase. In this method the amount of red dye produced by the action of peroxidase and hydrogen

* An abstract of this investigation has been published (Federation Proc., 5, 501 (1946)).
peroxide on \textit{p}-aminobenzoic acid was measured colorimetrically. It was observed that thiourea inhibited the peroxidase activity by 50 per cent at an average concentration of 0.00043 M; 2-thiouracil was twice as active and 2,6-dithiouracil was 4 times as active as thiourea. A series of thirty-five thiourea derivatives (11) had antiperoxidase activities varying from one-fifth to 6 times that of thiourea, \(o\)-phenetylthiourea having the greatest activity. Sulfanilamide and sulfathiazole were one-tenth as active as thiourea. Cysteine was equally active, while glutathione was 3 times as active as thiourea. These results apparently confirmed the antiperoxidase activities of thiourea and thiouracil, the sulfonamides, and cysteine and glutathione as observed by Glock (5), Lipmann (10), and Balls and Hale (12), respectively. However, further work has now shown that the apparent antiperoxidase activities of thiol compounds are due to their reducing action on the peroxidase system.

Within the series of thirty-five thiourea derivatives there appeared to be a rough correlation of the antiperoxidase activity and the antithyroid potency as assayed by the increase in weight of rat thyroid glands (unpublished experiments). Dithiouracil was more potent than thiouracil and the latter more active than thiourea. The sulfonamides had low antiperoxidase activities and also low antithyroid potencies (13). However, a number of thiol derivatives had high antiperoxidase activities but no antithyroid activities; \textit{e.g.}, cysteine, glutathione, and sodium sulfide.

\textbf{Reducing Power of Thiol Compounds—}Elliott (14) had observed that sulphydryl compounds were capable of reducing the colored dyes formed by the action of peroxidase and hydrogen peroxide on various substrates, such as benzidine, guaiacol, and \textit{p}-phenylenediamine. It was considered, therefore, that the apparent antiperoxidase activities of thiols might be due to their reducing action on the red dye (PABA red) formed by peroxidase and hydrogen peroxide from \textit{p}-aminobenzoic acid. To test the reducing power of the thiols they were added in 0.001 M concentration at pH 7.0 to aliquots of PABA red and benzidine blue. The latter were made by reaction of hydrogen peroxide and peroxidase with \textit{p}-aminobenzoic acid and benzidine and removal of the excess hydrogen peroxide with catalase. Also, the reducing power of thiols was tested against 2,6-dichlorophenol indophenol by measuring the decoloration of the dye in evacuated Thunberg tubes. The thiols were added at 0.001 M concentration to the dye (0.025 per cent) at pH 7.0. The reducing action of the various thiols on the dyes is recorded in Table I.

It was observed that thiourea and \textit{o}-phenethylthiourea did not reduce the red dye, PABA red. However, the other thiols tested were able to reduce the PABA red dye. All of the thiols tested decolorized the benzidine blue. The fact that thiouracil has the power of decolorizing benzidine blue could
partially explain the inhibition of the benzidine blue staining reaction of thyroid tissue by thiouracil, as observed by Dempsey (4). Consequently, thiouracil might not have any action on peroxidase at all but might show an apparent inhibition by reducing the colored compound formed by peroxidase and hydrogen peroxide.

The observation that all of the thiol compounds reduced the dye, 2,6-dichlorophenol indophenol, at pH 7.0, indicated that they had reducing potentials more negative than +0.217 volt (15). Balls and Hale (16) observed that most substrates of peroxidase and hydrogen peroxide were capable of reducing this dye.

**Mechanism of Action of Thiols with Peroxidase and Hydrogen Peroxide**—Since some thiols were found to have the power of decolorizing PABA red and benzidine blue, indicating that their apparent antiperoxidase activities might be due to their reducing powers rather than to a true inhibitory action on peroxidase, it was desired to test the action of thiols on the peroxidase system by a method which did not depend on the formation of a colored complex. The method of Balls and Hale (12) was tried in which the rate of disappearance of hydrogen peroxide in the presence of peroxidase and a suitable substrate is measured. By this method the amount of hydrogen peroxide remaining after it had acted on a substrate for a given time was determined by titrating the iodine liberated from potassium iodide with thiosulfate. It was found that this method could not be used for measuring the effect of thiols on peroxidase, because the thiols reacted with the iodine liberated from potassium iodide by hydrogen peroxide. This fact explains the apparent antiperoxidase activities of cysteine and glutathione observed by Balls and Hale (17). Campbell et al. (18) observed that thiourea was oxidized by iodine to formamidine disulfide hydriodide.

Elliott's (14) semiquantitative gasometric method for measuring the rate of disappearance of hydrogen peroxide was then tried. In this method, manganese dioxide was used to liberate oxygen from hydrogen peroxide and

<table>
<thead>
<tr>
<th>Compound</th>
<th>α-Aminobenzoic acid red</th>
<th>Benzidine blue</th>
<th>2,6-Dichlorophenol indophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiourea</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>o-Phenethylthiourea</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2,6-Dithiouracil</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glutathione</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cysteine</td>
<td>+</td>
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the volume of gas was measured. It was found that thiol compounds react with the manganese dioxide; therefore, this method was not applicable.

A manometric method was developed for determining peroxidase activity by measuring the rate of disappearance of hydrogen peroxide in the presence of peroxidase and a suitable substrate. Catalase was used to liberate oxygen from the hydrogen peroxide remaining after a given time and the volume of oxygen was measured with the Warburg apparatus. The peroxidase, prepared from horseradish by Elliott’s method (14), had 0.55 purpurogallin unit per ml. Catalase was prepared from beef liver by Sumner’s method (19). The reaction vessels contained in the reaction chamber 1.0 ml. of peroxidase, 0.5 ml. of various concentrations of the substrate, $p$-aminobenzoic acid, and 0.5 ml. of 0.1 M phosphate buffer at pH 7.0; in one side arm was placed 1.0 ml. of 0.015 M hydrogen peroxide and in the second side arm, 0.5 ml. of catalase. After equilibration at 38°, the hydrogen peroxide was added to start the reaction. After 5 minutes, catalase was added from the second side arm to stop the reaction and liberate oxygen from the hydrogen peroxide. The volume of oxygen was measured and the amount of hydrogen peroxide calculated as usual. The amount of hydrogen peroxide initially present was determined with a separate vessel in which the substrate was omitted. The rate of peroxidase activity was, therefore, measured by the rate of disappearance of hydrogen peroxide. The effects of thiols on peroxidase activity were studied by measuring the rate of disappearance of hydrogen peroxide in the presence and absence of the thiols.

The principles of Lineweaver and Burk (20) were used to design an experiment to examine the nature of reaction between thiourea and the peroxidase system. The rate of reaction of the peroxidase system was determined with various concentrations of the substrate, $p$-aminobenzoic acid. The effect of thiourea at various concentrations was then determined at each substrate concentration.

The reciprocals of the substrate concentrations and the reciprocals of the rates of utilization of hydrogen peroxide were plotted in Fig. 1. The best straight lines were calculated by the method of least squares. The deviations from linearity were not statistically significant. The linear relationship between the reciprocals of the substrate concentration and the rates of utilization of PABA demonstrated the applicability of the Lineweaver and Burk equation to the reaction between PABA and peroxidase and hydrogen peroxide. The effects of thiourea were surprising in that the slopes were decreased with increasing concentrations of thiourea, while the intercepts were increased. The dissociation constants consequently decreased with increasing thiourea concentration.

This experiment was interpreted as indicating that there was no inhibition of peroxidase by thiourea at all, but, on the contrary, an acceleration of the rate of utilization of hydrogen peroxide. It was evident, therefore, that
there was a competition between thiourea and PABA for the available peroxidase and hydrogen peroxide. Thiourea was evidently a substrate for the peroxidase system just as PABA was a substrate.

The reaction between thiols and hydrogen peroxide in the presence and absence of peroxidase was then measured manometrically. The reaction vessels contained the following reactants: 1.0 ml of 0.0075 M thiol, 0.5 ml of 0.1 M phosphate buffer, pH 7.0, 1.0 ml of water or peroxidase, 1.0 ml of 0.015 M hydrogen peroxide, and 0.5 ml of catalase. After equilibration at 38°, the reaction was started by adding hydrogen peroxide from the side arm and stopped at intervals by adding catalase from the second side arm. The moles of hydrogen peroxide used per mole of thiol present were calculated. The data are illustrated in Fig. 2.

The results demonstrated that thiols reacted with hydrogen peroxide at measurable rates and that peroxidase accelerated the reaction. Therefore, the thiols are substrates for the peroxidase-hydrogen peroxide system.

An indication of the extent of the reactions is obtained from the quantities of hydrogen peroxide which reacted with the thiols. 2 moles of thiourea could react with 1 mole of hydrogen peroxide to give a disulfide. The fact that more than 2 moles of hydrogen peroxide were reduced per mole of thiol indicated that the thiols were oxidized beyond the disulfide stage.
DISCUSSION

The antithyroid activities of some thiol compounds cannot be explained by their antiperoxidase activities, as Dempsey suggested (4), because of the following observations: thiol compounds do not inhibit peroxidase, but rather are reducing agents capable of reacting as substrates for the peroxide-peroxidase system, thus competing with other substrates for the available peroxide-peroxidase complex; they are able to reduce the colored dyes formed from p-aminobenzoic acid and benzidine and thus show an apparent inhibition of color production by the peroxidase system; they are able to reduce hydrogen peroxide and thus remove it as a reactant in the peroxidase system.

If it could be demonstrated that hydrogen peroxide, arising from oxidase systems in the cell, is involved in thyroxine synthesis (2, 3), then an explanation of the antithyroid activities of thiol compounds might be that they reduce the hydrogen peroxide as it is formed in the thyroid cell and thus remove it from the reacting system. The reducing power would then be an important chemical characteristic of antithyroid agents.
Thiol compounds do not inhibit peroxidase but, on the contrary, are substrates for the peroxidase-hydrogen peroxide system.

Thiol compounds are oxidized by hydrogen peroxide and the rate is accelerated by peroxidase.

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BIBLIOGRAPHY

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