An Unexpected Side Reaction in the Guaiacol Assay for Peroxidase

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In routine guaiacol assays for thyroid peroxidase and lactoperoxidase employing a newly purchased bottle of guaiacol from Aldrich Chemical Co., we were surprised to find the formation of a blue color instead of the expected amber color classically associated with this assay. This was observed also with horseradish, myeloperoxidase, and cytochrome C peroxidase. The blue color ($A_{650}$ nm) was not formed with guaiacol reagents obtained from two other chemical companies, nor was it seen with a bottle of old Aldrich guaiacol that had been in use in the laboratory for more than 10 years. In the present investigation we provide evidence that formation of the blue color is closely associated with the presence of a low concentration of catechol ($\sim 0.5$ mol%) in the new Aldrich guaiacol reagent. Catechol itself, even in much higher concentration, is a very weak donor for peroxidase, forming a light pink color. The blue color in Aldrich new guaiacol is not formed to the exclusion of 470-nm-absorbing product(s). Formation of the latter is, however, inhibited, and use of Aldrich new guaiacol for assay leads to low values for peroxidase activity. Other dihydroxyphenols (resorcinol and hydroquinone) do not mimic the action of catechol in formation of the blue color. Resorcinol is a very potent inhibitor of peroxidation of guaiacol. Possible schemes are proposed for formation of the products that may be associated with the amber and blue colors.

Guaiacol (o-methoxyphenol) is frequently used for the assay of various peroxidases (1,2). The assay depends upon the formation of an amber color, but the chemical nature of the product remains uncertain (3,4).

In this laboratory we have used guaiacol routinely for many years for the assay of thyroid peroxidase (TPO) and lactoperoxidase (LPO). Assay procedures employing either 33 (5) or 5 mM (6) guaiacol have been employed. The assay with the higher concentration is considerably more sensitive and is based on a procedure initially described by Hosoya et al. (7).

Recently, our attention was called to a paper by Makinen and Tenovuo (8), who reported that the use of aqueous solutions of guaiacol that had been aged for several months led to a marked increase in the sensitivity of the assay for LPO. They attributed this to the formation of peroxidatic compounds and other unidentified products of guaiacol oxidation in aged solution. Although we always prepare a fresh solution of guaiacol for the assay procedure, the observation of Makinen and Tenovuo led us to purchase a new bottle of guaiacol. Our old bottle had been in use for at least 10 years. Both the old and the new bottles were obtained from Aldrich Chemical Co.

When we compared the two bottles for the assay of TPO and LPO, employing 33 mM guaiacol in our routine procedure, we were greatly surprised to discover that the color formed with the new reagent was blue instead of amber. This occurred even though the new Aldrich guaiacol was colorless and was stated by the manufacturer to be $>98\%$ pure. Guaiacol subsequently purchased from two other chemical companies, carrying similar specifications but which were both definitely colored, yielded the usual amber color.

In the present communication, we describe experiments which we believe explain the origin of the unexpected blue color obtained in the guaiacol assay with the new Aldrich guaiacol reagent.

METHODS AND MATERIALS

Peroxidases. Most experiments were performed with bovine LPO, purchased from Sigma (A$_{412}$/A$_{390}$ = 0.90). The enzyme concentration was based on a millimolar extinction coefficient of 114 at 412 nm (9). Some experiments were also performed with porcine TPO, prepared in this laboratory as previously described.

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2 Abbreviations used: TPO, thyroid peroxidase; LPO, lactoperoxidase; HRP, horseradish peroxidase; MPO, myeloperoxidase; CcP, cytochrome C peroxidase; BSA, bovine serum albumin.
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The extinction coefficient of TPO at 412 nm was assumed to be the same as that for LPO. Brief tests were made with horseradish peroxidase (HRP, Sigma), bovine myeloperoxidase (MPO, ExOxEmis Co.), and cytochrome c peroxidase (CcP, kindly provided by Dr. James Erman, North Illinois University).

Guaiacol. Guaiacol reagents from four separate sources were used: (a) A recently purchased reagent (two different bottles) from Aldrich Chemical Co., referred to in text as new Aldrich guaiacol; (b) an old reagent purchased from Aldrich Chemical Co. more than 10 years ago, referred to as old Aldrich guaiacol; (c) a newly purchased reagent from Pfaltz-Bauer Chemical Co.; and (d) a newly purchased reagent from Fluka Chemical Co.

Guaiacol oxidation. This was measured either as ΔA_470 or ΔA_650 in a Cary 219 spectrophotometer. The incubation mixture (2.1 ml total volume) contained either 33 or 4.8 mM guaiacol, 0.48 mg/ml BSA, 7 nM LPO, or 4.8 nM TPO, and 314 μM H_2O_2, in 67 mM phosphate buffer, pH 8.0. BSA was added because we had noticed in previous experiments that its presence in the assay mixture led to better reproducibility among replicate samples. The spectrophotometer was equipped with a magnetic stirrer attachment, and the reaction was started by adding 10 μl of H_2O_2 to the stirred contents of the cuvette with a fine syringe inserted through a port in the sample compartment cover. Results are presented either as reproductions of the actual spectrophotometer recordings (Figs. 1-4) or as ΔA_650 or ΔA_470 in 1 min (Tables 1-3).

Analysis of guaiacol reagents for catechol. For reasons indicated below, it became of interest to analyze the various guaiacol reagents used in this study for the presence of low levels of catechol. This was accomplished by HPLC, using a reverse-phase C_18 ultrasphere column (250 × 4.6 mm, 5 μm, Beckman Instruments) and a mobile phase consisting of 1:1 methanol:water. The flow rate was 1 ml/min, and the uv detector was set at 274 nm. Column peaks were visualized with a chart recorder. Retention times were 6.6 min for guaiacol and 4.4 min for catechol.

RESULTS

Formation of an unexpected blue color in guaiacol assay with a new guaiacol reagent. Most of our experiments were performed with LPO, but the blue color was also observed with other peroxidases (TPO, MPO, HRP, and CcP). The blue solution showed a maximum absorbance at about 650 nm. The amber color that is usually formed in the guaiacol assay displays its maximum absorbance at about 470 nm.

Figure 1 shows spectrophotometric recordings at 650 and 470 nm for our routine guaiacol assay, using the different guaiacol reagents described in the legend. The blue color was observed only with the new Aldrich guaiacol. As shown in Fig. 1A, ΔA_650 with this reagent was much greater than with the other guaiacol reagents. When the color development was followed at 470 nm (the standard wave length used for this assay), the new guaiacol reagent from Aldrich gave much lower results than reagents from two other suppliers (Fig. 1B). The highest results were obtained with an old Aldrich guaiacol reagent that had been in use in our laboratory for more than 10 years. Results similar to those in Fig. 1 for LPO were also obtained with TPO (data not shown). Under the assay conditions used in Fig. 1 (33 mM guaiacol), ΔA_470 in 1 min for the new Aldrich guaiacol was less than half the values obtained with the other guaiacol reagents. Thus, use of the new Aldrich guaiacol for peroxidase assay results in erroneously low values.

Effect of lowering the guaiacol concentration in the assay mixture. The results described in the preceding section were obtained with an assay mixture containing 33 mM guaiacol. We have used this high concentration to increase the sensitivity of the guaiacol assay, as originally suggested by Hosoya et al. (7). However, we and others (6,8) have also used a procedure employing much lower concentrations of guaiacol (~5 mM) for the assay.
FIG. 2. Same as Fig. 1, except that the guaiacol concentration was 4.8 mM instead of 33 mM. Under these conditions, all guaiacol preparations gave an amber color on oxidation with LPO + H₂O₂.

When we reduced the guaiacol concentration to 5 mM, maintaining the same concentrations of LPO and H₂O₂, we did not observe the blue color with the new Aldrich guaiacol reagent. The same amber color was observed with all the guaiacol reagents. Nevertheless, as shown in Fig. 2A, ΔA₄₅₀ was significantly higher with the new Aldrich guaiacol than with the other guaiacol reagents even under these conditions.

Evidence that contamination of guaiacol with catechol is related to the formation of the blue color. It seemed likely that some contaminant in the new Aldrich guaiacol, not present in the other guaiacol reagents, was responsible for the formation of the blue color. Since catechol (ortho-dihydroxybenzene) is a possible starting material for the synthesis of guaiacol, we suspected that catechol might be the contaminant in question. It was added in varying low concentrations to Pfaltz-Bauer guaiacol, which, as indicated above (Fig. 1), did not by itself form a blue color in the guaiacol assay. The results are presented in Fig. 3, which shows ΔA₄₅₀ for assay mixtures containing 33 mM guaiacol and concentrations of catechol ranging from 47 μM to 1.18 mM. The presence of a low concentration of catechol did indeed lead to the formation of a blue product(s), both with LPO and with TPO. The development of the blue color in the sample containing LPO and 0.14 mM catechol was quan-

FIG. 3. Effect of varying concentrations of catechol added to Pfaltz-Bauer guaiacol on ΔA₄₅₀ in the guaiacol assay system. The incubation system contained 33 mM guaiacol, 0.48 mg/ml BSA, 314 μM H₂O₂, and either 7 nM LPO (A), or 4.8 nM TPO (B). The catechol concentration is indicated alongside each curve. In A, the dashed curve shows results obtained with 0.33 mM catechol in the absence of guaiacol.

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TABLE 1

<table>
<thead>
<tr>
<th>Catechol conc (μM)</th>
<th>Moles of catechol per mole of guaiacol</th>
<th>ΔA₄₅₀ in 1 min</th>
<th>ΔA₆₀₀ in 1 min</th>
<th>Observed color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.516</td>
<td>0.016</td>
<td>Amber</td>
</tr>
<tr>
<td>14.3</td>
<td>0.0029</td>
<td>0.439</td>
<td>0.050</td>
<td>Orange</td>
</tr>
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<td>23.8</td>
<td>0.0048</td>
<td>0.390</td>
<td>0.084</td>
<td>Yellow-orange</td>
</tr>
<tr>
<td>47.6</td>
<td>0.0085</td>
<td>0.253</td>
<td>0.157</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>143</td>
<td>0.029</td>
<td>0.126</td>
<td>0.255</td>
<td>Blue</td>
</tr>
<tr>
<td>238</td>
<td>0.048</td>
<td>0.125</td>
<td>0.264</td>
<td>Blue</td>
</tr>
<tr>
<td>333</td>
<td>0.067</td>
<td>0.120</td>
<td>0.285</td>
<td>Blue</td>
</tr>
</tbody>
</table>
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Note. The incubation system contained 5 mM Pfaltz-Bauer guaiacol, 7 nM LPO, 314 μM H₂O₂, 0.48 mg/ml BSA, and catechol as indicated, in phosphate buffer, pH 8.0. Separate samples were used for the measurement of ΔA₄₅₀ and ΔA₆₀₀. Values obtained with the higher catechol concentrations are corrected for a small blank obtained with the same incubation mixture minus guaiacol.

FIG. 4. Analysis of guaiacol reagents for catechol by HPLC. A, Pfaltz-Bauer guaiacol containing 0.5 mol% of added catechol; B, New Aldrich guaiacol; C, Pfaltz-Bauer guaiacol; D, Fluka guaiacol. Twenty microliters of 0.9 mM guaiacol in methanol was applied to the column.
titatively very similar to that observed with LPO and Aldrich new guaiacol in the absence of added catechol (Fig. 1A). These results support the conclusion that Aldrich new guaiacol contains about 0.4 mol% catechol.

Catechol by itself (0.33 mM) in the absence of guaiacol produced very little color (dashed curve, Fig. 3A). In a separate experiment with 10 mM catechol (no guaiacol), the solution developed a very light pink color. Formation of the blue color, therefore, seems to depend on a reaction between catechol and guaiacol, probably involving oxidized forms of the two compounds.

Results obtained with the assay employing 5 mM Pfaltz-Bauer guaiacol, with and without added catechol, are shown in Table 1. When the molar ratio of catechol to guaiacol was 0.0048, close to the value estimated for Aldrich new guaiacol (see above), the blue color was not observed. The solution displayed a significant value for \( \Delta A_{470} \), but this was greatly exceeded by the value for \( \Delta A_{650} \). This finding offers an explanation for our observation that Aldrich new guaiacol developed a blue color when assayed at 33 mM but not at 5 mM, if we postulate that the value for \( \Delta A_{650}/\Delta A_{470} \) is not simply dependent on the ratio of catechol to guaiacol but increases as the guaiacol concentration increases. This is consistent with the further observation in Table 1 that a blue color

\begin{table}
\centering
\caption{Comparison of Different Di- and Trihydroxybenzenes on the Guaiacol Assay}
\begin{tabular}{llll}
\hline
Compound added & \( \Delta A_{650} \) in 1 min & \( \Delta A_{470} \) in 1 min & Observed color after 2 min \\
\hline
None & 0.031 & 1.08 & Amber \\
Catechol & 0.68 & 0.44 & Blue \\
Pyrogallol & 0.265 & 0.52 & Green-yellow \\
Hydroquinone & 0.015 & 0.88 & Amber \\
Resorcinol & 0.00 & 0.02 & Slight pink \\
Phloroglucinol & 0.00 & 0.00 & Colorless \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Effect of Aging of Guaiacol Solution on Formation of Peroxidatic Compound(s)}
\begin{tabular}{llllll}
\hline
Source of & Additions before aging & Period of exposure & Exposed to light or dark & \( \Delta A_{470} \) for 1 min & \( \Delta A_{650} \) for 1 min \\
guaiacol & & & & \hline
Aldrich, new bottle & 0 & 0 & light & 0.004 & 0.000 \\
& & 48 & light & 0.280 & 0.428 \\
& & 48 & dark & 0.199 & 0.384 \\
Aldrich, old bottle & 0 & 0 & light & 0.000 & 0.010 \\
& & 48-72 & light & 0.015 & 0.011 \\
Pfaltz-Bauer & 0 & 0 & light & 0.000 & 0.000 \\
& & 72 & light & 0.030 & 0.001 \\
& & 79 & light & 0.435 & 0.744 \\
& & 72 & dark & 0.400 & 0.595 \\
Fluka & 0 & 0 & light & 0.000 & 0.000 \\
& & 72 & light & 0.028 & 0.001 \\
\hline
\end{tabular}
\end{table}

Note. One-tenth molar aqueous solutions at pH 8.0 were allowed to stand in glass flasks in room light or in the dark. The incubation mixture contained 33 mM guaiacol, 0.48 mg/ml BSA, and 7 nM LPO at pH 8.0. The reaction was started with LPO. No H\(_2\)O\(_2\) was added.
developed in the assay employing 5 mM guaiacol when the molar ratio of catechol to guaiacol was raised to 0.029.

Analysis of guaiacol reagents for catechol. An HPLC procedure was developed for detecting low levels of catechol in the different guaiacol reagents (see Methods and Materials). Results are shown in Fig. 4. As shown in Figs. 4C and 4D, no detectable peak for catechol was observed with Pfaltz-Bauer or with Fluka guaiacol. This was true also for Aldrich old guaiacol (results not shown). However, a definite peak for catechol was observed with Aldrich new guaiacol (Fig. 4B). Its magnitude was very similar to that observed with Pfaltz-Bauer guaiacol containing 0.5 mol% added guaiacol (Fig. 4A). These results provide further support for our conclusion that the formation of the blue color in the peroxidase assay with Aldrich new guaiacol involves a side reaction with catechol.

Effect of pH on the development of the blue color. As shown in Fig. 5, development of the blue color in the guaiacol assay was greatly affected by pH. This was shown with both Aldrich new guaiacol and with Pfaltz-Bauer guaiacol containing 1 mol% catechol (Fig. 4A). These results provide further support for our conclusion that the formation of the blue color in the peroxidase assay with Aldrich new guaiacol involves a side reaction with catechol.

Addition of pyrogallol (1,2,3-trihydroxybenzene) also resulted in the formation of both 650-nm- and 470-nm-absorbing products, but in this case the absorbance after 1 min was greater at 470 nm than that at 650 nm, and the solution was green-yellow. When the reaction with added pyrogallol was performed in a test tube, it was observed visually that a blue color developed during the first 30 s, but it was transformed to green-yellow as formation of the 470-nm-absorbing product(s) became dominant. Based on the usual guaiacol assay (ΔA in 1 min), pyrogallol inhibited the reaction about 50%.

Hydroquinone (1,4-dihydroxybenzene) was also a mild inhibitor (37%) of the standard guaiacol assay, but it resulted in very little formation of 650-nm-absorbing material.

Resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) are extremely potent inhibitors of the standard guaiacol assay (>98%) and re-
sulted in undetectable formation of 650-nm-absorbing material.

**Effect of aging of guaiacol solutions.** Makinen and Tenovuo (8) reported that aging of aqueous guaiacol solutions over a period of several months led to the spontaneous formation of peroxidatic compound(s) and other unidentified oxidation products of guaiacol. The formation of the peroxidatic compound(s) was reported to be a photochemical process.

In the present study we observed that guaiacol assay results for LPO were somewhat higher with an old bottle of Aldrich guaiacol than with guaiacol newly purchased from two other suppliers (Pfaltz-Bauer and Fluka; see Fig. 1B). It was of interest to determine whether this could be ascribed to the presence of peroxides in the old Aldrich guaiacol. We also tested the effect of short-term aging (48-72 h) on 0.1 M solutions of the various guaiacol reagents used in this study. Our solutions were made up in phosphate buffer, pH 8.0, whereas those of Makinen and Tenovuo were prepared in phosphate buffer, pH 7.0.

Results are shown in Table 3. Freshly prepared solutions of the various guaiacol reagents showed no evidence of peroxidatic compounds, as indicated by the negligible values for \( \Delta A_{470} \) on addition of LPO in the absence of \( H_2O_2 \). This was true also for the old Aldrich guaiacol reagent, indicating that the higher assays observed with this reagent (Fig. 1B) cannot be ascribed to the presence of peroxides. However, when 0.1 M solutions of guaiacol were allowed to stand for 48-72 h, the solution prepared with Aldrich new guaiacol developed a blue color on addition of LPO alone. The solution displayed absorbance at both 650 and 470 nm, and the value for \( \Delta A_{650}/\Delta A_{470} \) was similar to that observed in Fig. 1 with a freshly prepared solution of Aldrich new guaiacol after addition of LPO \( + H_2O_2 \). These observations are most readily explained by the formation of peroxides in the aged solution of Aldrich new guaiacol. The other guaiacol reagents showed low, but seemingly significant, values for \( \Delta A_{470} \) on standing for 72 h, raising the possibility that small amounts of peroxides were formed. In the case of the Aldrich new guaiacol, aging of the solution in the dark resulted in only a mild reduction of \( \Delta A_{450} \) and \( \Delta A_{470} \), indicating that peroxide formation was largely independent of light.

Peroxide formation in the solution of Aldrich new guaiacol appeared to be dependent on its small catechol contamination. This is shown by the observation (Table 3) that 0.1 M Pfaltz-Bauer guaiacol containing 1 mol% of added catechol also displayed evidence of peroxide formation when the solution was aged for 72 h. No evidence for peroxide formation was observed when 1 mol% of catechol was added immediately before addition of LPO. However, a solution of 1 mM catechol at pH 8.0 when aged by itself for 48-72 h, gave evidence of peroxide formation (data not shown). Presumably, therefore, the peroxide in the guaiacol–catechol mixture arose from spontaneous oxidation of the catechol.

Brief treatment (2 min) of the 48-72 h aged solutions with catalase (10 U/ml) completely abolished the color development on addition of LPO (data not shown). This occurred with Aldrich new guaiacol, with Pfaltz-Bauer guaiacol containing 1 mol% of added catechol, and with 1 mM catechol itself. The peroxidatic activity of the aged solutions, therefore, most likely involved formation of \( H_2O_2 \) rather than organic peroxides (11).

**DISCUSSION**

The amber color that is generally produced when guaiacol is oxidized with peroxidase/\( H_2O_2 \) was initially attributed to formation of tetraguaiacol (1,12). However, Booth and Saunders (3,4) reported that tetraguaiacol could not account for the color, and they proposed that the colored product was more likely to be 3,3'-dimethoxybiphenyloquinone. No mechanism was offered for its formation.

Accepting their suggestion for the origin of the amber color, we present in Scheme 1 a possible mechanism for the formation of 3,3'-dimethoxybiphenyloquinone 1. The proposed mechanism is based on the generally accepted view that oxidation of guaiacol with peroxidase/\( H_2O_2 \) initially forms phenoxy radicals (13). Dimerization would lead to formation of 3,3'-dimethoxy-4,4'-dihydroxybiphenyl 2. Further oxidation of 2 affords 1. Only the Z isomer of 1 is shown, but the E isomer would also be expected to be formed under these conditions.

In the presence of catechol, the unsymmetrical biphenyl derivative 3 may also be formed (Scheme 2). It would be very easily oxidized to the corresponding biphenyloquinone 4. This oxidation product would be expected to be amber colored, based on its structural similarity to 1. We suggest below two possible mechanisms for the formation of the blue color from 4.

1. **Free radical mechanism.** In contrast to 1, which should be stable to further oxidation, 4 could react with excess oxidant to form a resonance-stabilized semiquinone radical 5, as shown in Scheme 2. Similar resonance-stabilized radicals are intensely colored—for example the deep blue radical, galvinoxyl (14,15). The fact that only a small concentration of catechol is required for formation of the blue color suggests that it has a high molar extinction coefficient, consistent with a resonance-stabilized radical.

2. **Charge transfer complex.** An alternative possibility, based on a previous study with o-dianisidine, was brought to our attention by a reviewer. Peroxidase-catalyzed oxidation of o-dianisidine 6 (Scheme 3) leads to formation of a transient green color, originally interpreted as deriving from a semiquinone intermediate (16). This reaction was reinvestigated by Claiborne and
Fridovich (17), who concluded that a semiquinone intermediate was not involved. They proposed instead that the transient green color could be attributed to a charge–transfer complex between o-dianisidine and its quinonediamine oxidation product 7 (Scheme 3).

By analogy to these results, it is possible that a similar charge–transfer complex could account for the blue color in the guaiacol reaction (Scheme 4). The unsymmetrical biphenooquinone 4 would be formed in the catechol reaction in the presence of a relatively high concentration of biphenyl 2, formed by the oxidation of guaiacol. The charge–transfer complex 8, formed by reaction between 2 and 4, is more likely than a similar complex between 3 and 4, because 2 would be expected to be present in much higher concentration than 3. It should be noted that neither pure guaiacol nor pure catechol formed the blue color under similar reaction conditions and that the biphenooquinone 1 forms even when the blue color is observed (Table 2).

Additional studies are required to characterize more definitively, not only the origin of the blue color formed by peroxidation of guaiacol containing a small percentage of catechol, but also the origin of the amber color that is generally associated with peroxidation of guaiacol.

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