Colorimetric Assay for Guaiacol O-Methyltransferase

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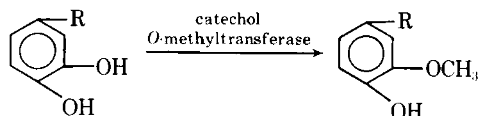
Abstract □ A relatively simple colorimetric assay was developed for guaiacol *O*-methyltransferase. Monomethylated phenolic acid substrates are enzymatically methylated to their corresponding dimethoxyl compounds; *S*-adenosylmethionine serves as the methyl donor. Enzymatic activity is measured by colorimetric assay of the monomethylated (nonreacted) substrate by coupling of the phenolic acid with a diazotized sulfanilic acid. The methoxyl and hydroxyl groups may be in the 3,4- or 4,3-positions on the substrate molecules; however, a side chain (COOH) or a substituted side chain is necessary for enzyme action. The radioactive demonstration of guaiacol *O*-methyltransferase as a separate entity from catechol *O*-methyltransferase was confirmed.

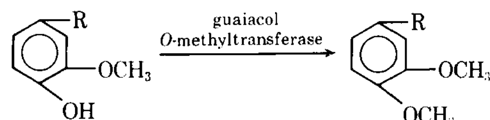
Keyphrases □ Guaiacol *O*-methyltransferase—colorimetric analysis of enzymatic activity □ Colorimetry—analysis, guaiacol *O*-methyltransferase, enzymatic activity □ Enzymatic activity—guaiacol *O*-methyltransferase, colorimetric analysis □ Methylation, enzymatic—guaiacol *O*-methyltransferase, colorimetric analysis

Enzymatic changes essential for the metabolism of the catecholamines have been the subject of numerous investigations. Catechol *O*-methyltransferase (EC 2.1.1.6), the enzyme capable of methylating a number of catechol derivatives, generally methylates at the 3-position, thus converting the hydroxyl to a methoxyl function. However, only one of the two hydroxyl groups is methylated. Recently, Friedhoff *et al.* (1, 2) reported the probable existence of a separate enzyme capable of methylating guaiacol derivatives giving rise to dimethoxy compounds. This guaiacol *O*-methyltransferase is derived from rat livers and capable of *in vitro* methylation. Schemes I and II illustrate the basic reactions for catechol *O*-methyltransferase and guaiacol *O*-methyltransferase, respectively.

As part of an ongoing program for the synthesis of new anticancer compounds, methods were developed to evaluate the inhibition of the new materials on methyltransferase enzymes. Nonradioactive methods were elected for these assays. Since the early investigations on guaiacol *O*-methyltransferase used radioactive substrates or radioactive methyl donors (1, 2), a new, simple, colorimetric assay for guaiacol *O*-methyltransferase has been developed.



Scheme I



Scheme II

EXPERIMENTAL

The assay involves two major steps: (*a*) the enzyme reaction and (*b*) conjugation of the nonreacted guaiacol compound used as the substrate with diazotized sulfanilic acid and colorimetric determination of the conjugate.

Since the enzyme is most reactive at pH 9.0, 1.5 ml of 0.1 *M* tromethamine buffer at pH 9.0 is used. As with catechol *O*-methyltransferase, Mg^{+2} ions are required; therefore, 0.2 ml of 1 *M* $MgCl_2$ is added. The enzyme is a partially purified rat liver extract prepared according to the procedure of Friedhoff (3) using ultracentrifugation, ammonium sulfate precipitation, and dialysis.

The 0.3 ml used per assay contains approximately 15 mg of protein nitrogen, as determined by the method of Oyama and Eagle (4), which is a modification of the protein assay of Lowry *et al.* (5). As the methyl donor, 0.5 ml of 5×10^{-3} *M* *S*-adenosylmethionine is added. As the substrate, 0.1 ml of the guaiacol derivative (1×10^{-2} *M*) is added. For consistent and reproducible results, the substrate is added last. All reagents are added at 0–5°. Ninety minutes at 37° is allowed for the complete enzyme reaction.

Following this enzyme reaction, the mixture is tested for unreacted (nonmethylated) substrate. Low speed centrifugation (800×*g*) for 10 min at 5° removes the agglomerated protein, and 2.3 ml of the supernate is treated with 0.2 ml of the 0.2% diazotized sulfanilic acid, a reagent commonly used in chromatographic studies of the phenolic acids (6–9). The diazotized sulfanilic acid is prepared fresh daily by dissolving in distilled water the commercially available powder[1] (dry stable Pauley's reagent: diazotized sulfanilate trifluoroborate salt).

The conjugation reaction is performed at room temperature; however, temperature does not appear to be critical for this step. The diazotized sulfanilic acid reagent combines with the monomethylated phenol derivatives but not with the dimethylated compounds. The absorbance (*A*) of the conjugate is measured[2] at 520 nm within 1–15 min after the addition of the diazotized sulfanilic acid. The enzyme activity is the ΔA difference between a reaction without enzyme and with enzyme. The reaction without enzyme serves as a control of the reagents in the absence of protein as well as the basis for enzyme calculations.

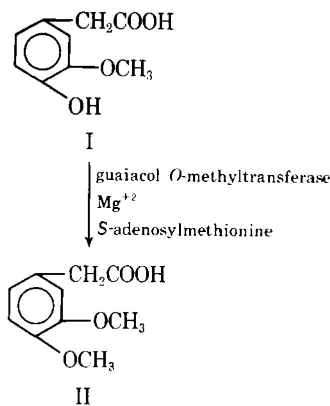
RESULTS AND DISCUSSION

Several guaiacol compounds were tested as substrates for guaiacol *O*-methyltransferase and for their ability to convert the monohydroxyl–monomethoxyl groups to the corresponding dimethoxyl functions.

Scheme III illustrates the enzymatic action of guaiacol *O*-methyltransferase on homovanillic acid (I, 4-hydroxy-3-methoxyphenylacetic acid). It is methylated by transfer of the methyl group from the *S*-adenosylmethionine to position 4 on the ring. Homovanillic acid can be linked to the diazotized reagent, but 3,4-dimethoxyphenylacetic acid (II) cannot.

The enzyme converted 38.3, 30.5, 30.2, and 23.4% of ferulic acid, homovanillyl alcohol, vanillylmandelic acid, and homovanillic acid, respectively, from the monohydroxylated guaiacol derivatives to the dimethoxyl compounds. Guaiacol, the parent compound, was not significantly methylated (1.1%) since it has no side chain. On the other hand, ferulic acid has a double bond in the side chain, homovanillyl alcohol has a primary alcohol side chain, vanillylmandelic acid has a secondary alcohol side chain, and homovanillic acid has a short aliphatic acid side chain.

[1] Calbiochem 28656.
[2] Beckman DU spectrometer.



Scheme III

Vanillic acid and isovanillic acid have the hydroxyl and methoxyl groups reversed, but both were methylated (15.0 and 16.7%, respectively) by guaiacol *O*-methyltransferase. 4-Hydroxy-3-methoxyphenethylamine was also methylated (14.9%). These three compounds are superior substrates to normetanephrine, a phenethanolamine derivative (2.5% methylated). Vanillin (4-hydroxy-3-methoxybenzaldehyde) did not react with the diazo reagent but did yield some color upon standing in the presence of air and enzyme. This vanillin reaction needs additional study.

Based upon the reactions with the various substrates, it appears that the side chain may be an important determinant in the enzyme's specificity and influence the enzyme reaction.

The amount of enzyme used in the guaiacol *O*-methyltransferase reaction determines the amount of methylated product; however, for routine assays, the enzyme activity is standardized to yield the most optimal optical readout. The substrate concentration also is adjusted to give optimal optical readings. No problems of substrate inhibition were encountered in studies using multiple levels of substrate.

The colorimetric assay of guaiacol *O*-methyltransferase was used for several types of studies. A study of the influence of pH on the methylation of homovanillic acid showed the absence of methylation at pH 7.0, the maximum activity at pH 9.0, and somewhat less (47.4%) methylation at pH 9.5. These findings confirm previous data (1, 2) and indicate that catechol *O*-methyltransferase and guaiacol *O*-methyltransferase are two separate enzymes.

Studies by this colorimetric assay on the need for Mg^{+2} and the inhibitory effect of edetic acid, which ties up the Mg^{+2}, also confirmed data from the radioactive guaiacol *O*-methyltransferase assays (2).

Routine assays are currently being run for guaiacol *O*-methyltransferase inhibitors by this colorimetric assay. The enzyme (0.3 ml) and inhibitor (0.5 ml) are mixed and held for 5 min at 0–5°. Then the mixture is incubated to bring about enzyme activity using homovanillic acid as a substrate. The inhibitory effects are determined by comparing the absorbance changes of the combined substrate in the presence and absence of inhibitor according to:

$$\text{percent inhibition} = \frac{\Delta A \text{ enzyme (no inhibitor)} - \Delta A \text{ enzyme and inhibitor}}{\Delta A \text{ enzyme (no inhibitor)}} \times 100 \quad \text{(Eq. 1)}$$

The ED_{50}'s for the inhibitors may be calculated from assays at three inhibitor levels.

A second colorimetric reagent may be used as a modification of the conjugation reaction and its use provides additional information about the influence of substrate structure on the enzyme reaction. Studies conducted with the dichloroquinone chlorimide reagent, which reacts with phenolic acids having a hydroxyl function in position 3 (λ_{max} = 600 nm), demonstrated guaiacol *O*-methyltransferase methylation of isovanillic acid (43.9%) and isovanillin (27.0%) but not of vanillin. No blue color was expected with vanillin.

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