Colorimetric Determination of Aromatic Compounds by Hydroxylation

KENNETH S. ALBERT and KENNETH A. CONNORS▲

Abstract □ Aromatic hydroxylation has been developed into a widely applicable analytical method for aromatic compounds. A modified Hamilton hydroxylating system, consisting of hydrogen peroxide, ferric ion, catechol, and glucose, converts aromatic compounds to phenols, which are measured colorimetrically after oxidative coupling with 4-aminoantipyrine. The method is applicable to 10^{-4}–10^{-3} *M* aqueous solutions of many substituted benzene derivatives, linear absorbance–concentration plots being obtained with apparent molar absorptivities in the range of 1–5 × 10^3. The method has been applied to numerous aromatic drugs. Many common tablet excipients do not interfere at normal formulation levels. As one of the few methods available for the detection of the phenyl group, this technique has the advantages and limitations of a general functional group method.

Keyphrases □ Aromatic compounds—hydroxylation, colorimetric determination after oxidative coupling with 4-aminoantipyrine □ Hydroxylation of aromatic compounds—modified Hamilton system (hydrogen peroxide–ferric ion–catechol–glucose), colorimetric determination after coupling with 4-aminoantipyrine □ Phenyl group determination—modified Hamilton hydroxylation, colorimetry after oxidative coupling with 4-aminoantipyrine □ Colorimetry—determination of aromatic compounds, hydroxylation followed by oxidative coupling with 4-aminoantipyrine

A method (Scheme I) for the quantitative determination of aromatic compounds was recently described (1). An aromatic compound is converted to a phenol by the action of the Hamilton hydroxylating system (2–4), consisting of hydrogen peroxide and catalytic amounts of ferric ion and catechol; this system was modified by the incorporation of a cyclodextrin. After hydroxylation, the product phenol is oxidatively coupled with 4-aminoantipyrine for spectrophotometric measurement (5–7). Many benzene derivatives were determined in this manner (1).

Significant improvements have now been made in this aromatic hydroxylation method. The present paper describes the basis for these improvements and the application of the new method to numerous compounds of pharmaceutical interest.

EXPERIMENTAL

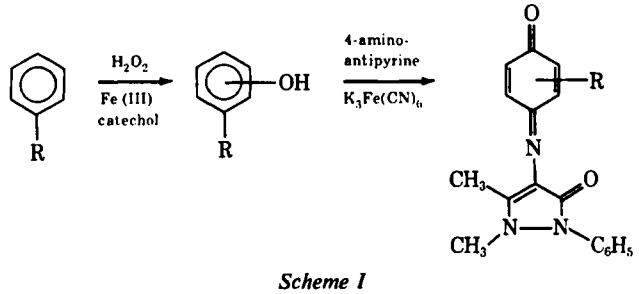
Materials—The following chemicals were used as received: ferric perchlorate hexahydrate[1], potassium ferricyanide[2], ethyl acetate[3], acetone[3], chloroform[3], methanol[3], benzene[3], toluene[3], isopropyl alcohol[3], benzoic acid[3], phenol[3], glucose[3], aniline[3], 30% hydrogen peroxide[3], lactose[3], stearic acid[3], benzaldehyde[3], nitrobenzene[3], hydroquinone[3], sorbitol[4], acetonitrile[4], dioxane[4], talc[5], starch[6], 4-aminoantipyrine[7], *N,N*-dimethylformamide[8], Schardinger α- and β-dextrins[9], L-phenylalanine[10], L-tryptophan[11], quinine sulfate[4], *m*-cresol[8], *p*-cresol[8], *m*-chlorophenol[8], *m*-hydroxyacetophenone[8], chloramphenicol[12], benzocaine[13], phenacetin[12], *d,l*-mandelic acid[12], antipyrine[12], *d,l*-ephedrine hydrochloride[8], benzenesulfonamide[7], sulfanilamide[8], atropine sulfate, and chlorpheniramine maleate. Anisole[7] was found to be gas chromatographically pure (10% SE-30, 165°).

Catechol[13] was recrystallized twice from toluene, m.p. 103–104° [lit. (8) m.p. 104°]; hydrocinnamic acid[8] was recrystallized from petroleum ether, m.p. 47–48° [lit. (9) m.p. 47.5–48°]; benzamide[8] was recrystallized from water, m.p. 125.5–127° [lit. (10) m.p. 125–126°]. The three methoxybenzoic acids were recrystallized from water: *o*-methoxybenzoic acid, m.p. 98.5–100° [lit. (10) m.p. 99–101°]; *m*-methoxybenzoic acid, m.p. 104.5–105° [lit. (10) m.p. 104–105°]; and *p*-methoxybenzoic acid, m.p. 183–184° [lit. (11) m.p. 184°].

N-Methylbenzylamine[8] was distilled at reduced pressure, b.p. 71°/2 mm. Hg [lit. (12) b.p. 78°/14 mm. Hg]; methyl benzoate[8] was distilled, b.p. 199.6° [lit. (13) b.p. 200°]; ethylbenzene[8] was distilled, b.p. 135° [lit. (14) b.p. 135°]; benzyl alcohol[8] was distilled at reduced pressure, b.p. 70°/15 mm. Hg [lit. (15) b.p. 104–105°/20 mm. Hg]; *o*-cresol[8] was distilled, b.p. 191–192° [lit. (16) b.p. 192°]; guaiacol[8] was distilled at reduced pressure, m.p. 28° [lit. (17) m.p. 27.4°]; *p*-methoxyphenol[8] was distilled under reduced pressure, m.p. 53° [lit. (18) m.p. 53°]; and salicylaldehyde[8] was distilled at reduced pressure, b.p. 65–66°/2 mm. Hg [lit. (19) b.p. 197°/760 mm. Hg].

Salicylic acid[8] was recrystallized from methanol–water, m.p. 159–160° [lit. (20) m.p. 158°]; *m*-hydroxybenzoic acid[8] was recrystallized from water, m.p. 200° [lit. (21) m.p. 202°]; *p*-hydroxybenzoic acid[8] was recrystallized from xylene–ethanol, m.p. 213–214° [lit. (22) m.p. 212–213°]; *o*-aminophenol was recrystallized from toluene–methanol, m.p. 174.5–176° [lit. (23) m.p. 171°]; *m*-aminophenol was recrystallized from toluene–methanol, m.p. 122–123° [lit. (24) m.p. 123°]; *o*-nitrophenol[8] was recrystallized from ethanol, m.p. 44.5–45° [lit. (25) m.p. 45°]; *m*-nitrophenol[8] was recrystallized from aqueous hydrochloric acid, m.p. 95–96° [lit. (26) m.p. 95–96°]; *p*-nitrophenol was recrystallized from toluene, m.p. 113–114° [lit. (27) m.p. 114°]; and *m*-hydroxybenzaldehyde[8] was recrystallized twice from water, m.p. 101–103° [lit. (28) m.p. 101–102°].

Acetanilid[5] was recrystallized from water, m.p. 113–114° [lit. (29) m.p. 114°]; acetylsalicylic acid[5] was recrystallized from acetone, m.p. 135–136° [lit. (30) m.p. 135°]; 2-naphthalenesulfonic acid[13] was recrystallized from dioxane–benzene, m.p. 121–123° [lit. (31) m.p. 120–122°]; phenobarbital[14] was recrystallized from water–methanol, m.p. 174–176° [lit. (32) m.p. 174.5–175.5°]; barbital[14] was recrystallized from water, m.p. 188–189.5° [lit. (33) m.p. 190°]; and *d,l*-



Scheme I

[1] G. Frederick Smith Co.
[2] J. T. Baker Chemical Co.
[3] Mallinckrodt.
[4] Fisher Scientific Co.
[5] Merck.
[6] Beaver Dam Wholesale Co.
[7] Aldrich Chemical Co.
[8] Eastman.
[9] Pierce Chemical Co.
[10] Schwartz/Mann.
[11] Nutritional Biochemical Corp.
[12] Ruger Chemical Co.
[13] Matheson, Coleman and Bell.
[14] Z. D. Gilman, Inc.

tropic acid was recrystallized from benzene, m.p. 116–117° [lit. (34) m.p. 116–117°].

Standard buffers were prepared according to Bates (35). Water was purified by passing once-distilled water through an ion-exchange column[15]. Acetate buffer (pH 4.0), in which hydroxylation reactions were run, was prepared to be 5×10^{-3} *M* in total buffer concentration and 0.1 *M* in ionic strength; ionic strength was adjusted with potassium chloride. Borate buffer, used to quench the hydroxylation reaction and as the color development medium, was prepared to be 0.4 *M* in total boric acid and 0.2 *M* in sodium hydroxide. Hydrogen peroxide (3%) was prepared by dilution of the 30% solution and was standardized by titration with potassium permanganate. Aqueous catechol solutions (3×10^{-3} *M*) were stored in the dark. Ferric perchlorate stock solution (1.5×10^{-2} *M*) was stabilized by adding 1 drop of 60% perchloric acid/50 ml. of solution. Ferric perchlorate reagent solution (1.5×10^{-3} *M*) was prepared by diluting the stock solution.

Apparatus—Spectrophotometric measurements were made in spectrophotometers[16] equipped with thermostatted cell compartments. The pH measurements[17] were made at 25.0°. Reaction temperatures were maintained to ±0.1° with constant-temperature water baths. Thermometers were calibrated against thermometers carrying either an NBS or an ASTM certificate.

Procedures—*Analytical Method*—To a 25-ml. volumetric flask are added 1.0 ml. of 3×10^{-3} *M* aqueous catechol, 1.0 ml. of 1.5×10^{-3} *M* ferric perchlorate, 5.0 ml. of 1.8×10^{-2} *M* glucose, and enough of the aromatic sample compound such that its concentration in the diluted solution is in the range of 10^{-4}–10^{-3} *M*. The solution is diluted to the mark with pH 4.0 acetate buffer, and the pH of the final solution should be in the range of 3.5–4.0.

The flask is placed in a 75° water bath, and reaction is initiated by adding 0.1 ml. of 3% hydrogen peroxide solution. After 10 min., a 5.0-ml. aliquot is pipeted into 5.0 ml. of borate buffer. The color is developed by adding 0.1 ml. of 3% (w/v) aqueous 4-aminoantipyrine followed by 0.1 ml. of 10% (w/v) aqueous potassium ferricyanide. The absorbance is measured immediately at the absorption maximum against a reagent blank carried through the same procedure. A standard curve is prepared by subjecting known concentrations, bracketing the unknown concentration, of the same aromatic compound to the procedure.

Chloroform Extraction Method—To achieve greater sensitivity or to circumvent fading of the color with some compounds (discussed later), substances giving a neutral extractable coupling product can be determined as follows. After hydroxylation is complete, 15.0 ml. of the reaction mixture is transferred to a 60-ml. separator containing 10 ml. of borate buffer. To this is added 0.1 ml. of 3% 4-aminoantipyrine, about 4 ml. of chloroform, and then 0.1 ml. of 10% potassium ferricyanide; the mixture is shaken for about 1 min. The chloroform layer is filtered through glass wool into a 5-ml. volumetric flask. Then the separator is rinsed with an additional 0.75 ml. of chloroform, which is added to the first chloroform extract, and this solution is diluted to volume with chloroform. Its absorbance is measured at the absorption maximum against a reagent blank carried through the same procedure.

Optimization Studies—During the development of the proposed method, many system variables were investigated including time, temperature, pH, concentrations and ratios of concentrations of reactants, other colorimetric finishes, other "stabilizers" (see *Discussion*), and interferences. These studies were carried out in much the same manner as described under *Analytical Method*, with appropriate variation in the feature being studied.

RESULTS

Unmodified Hamilton System—When catechol is subjected to the 4-aminoantipyrine colorimetric method, no significant interference is observed at wavelengths used for monohydric phenols. This lack of interference makes the 4-aminoantipyrine method the finish of choice. It is, therefore, possible to carry out the color development directly in the hydroxylation solution.

Table I—Effect of Reagent Age on Yield in Hydroxylation of Anisole[a]

Age of Hamilton System before Addition of Anisole, min.	A_{500}[b]
0	0.461
5	0.429
10	0.317
15	0.230
30	0.205
60	0.146
120	0.068
120[c]	0.461
180	0.012
180[d]	0.036

[a] 5.36×10^{-4} *M* anisole, 5.90×10^{-5} *M* Fe(III), 1.17×10^{-4} *M* catechol, 1.06×10^{-3} *M* H_2O_2, pH 3.9, 25°, 1-hr. reaction time. [b] 4-Aminoantipyrine finish. [c] Additional catechol (1.17×10^{-4} *M*) and H_2O_2 (1.06×10^{-3} *M*) added at this time. [d] Additional H_2O_2 (1.06×10^{-3} *M*) added at this time.

Dependence of Yield on Peroxide–Substrate Ratio—Typical initial reactant concentrations employed by Hamilton *et al.* (3, 4) were catechol, 1.5×10^{-4} *M*; Fe(III), 4×10^{-5} *M*; hydrogen peroxide, 2×10^{-3} *M*; and substrate, 10^{-2} *M*. The reaction was followed by monitoring the loss of peroxide. As an analytical reagent, however, the hydrogen peroxide in the Hamilton system must be in excess of the aromatic substrate (sample). With these more practical conditions, the yield of phenolic product, as reflected by the absorbance from the 4-aminoantipyrine-coupled product, was markedly dependent upon time of reaction and initial ratio of peroxide to substrate. Moreover, when phenol was substituted for an aromatic substrate in such a system, hydroxylation occurred as shown by the absorbance decrease.

Figure 1 shows the time course for the hydroxylation of anisole. At initial ratios of peroxide–anisole much greater than 2, production and then loss (by further hydroxylation) of monohydroxyanisoles are evident. When this ratio is about 2 or less, stable absorbance values are obtained after 1–2 hr.; however, the yield is sharply dependent upon this ratio, being depressed as the ratio is decreased. In a practical analytical situation, this ratio is not known and cannot be controlled. Under such conditions (namely $[H_2O_2]_0$ is constant and $[\text{anisole}]_0$ is variable), the percent yield of phenolic product depends upon the sample concentration, and a Beer's law plot with curvature is observed. The direction of curvature depends upon the $[H_2O_2]_0$–$[\text{substrate}]_0$ ratio, being negative if this ratio is 2 or less and positive for values greater than 2.

Dependence of Yield on pH, Catechol, and Iron—Initial experiments revealed measurable but small differences in yield between studies carried out under nitrogen and in the presence of air. Most subsequent work was done with an air environment.

Studies of yield (in these, as in other experiments unless otherwise noted, it is to be understood that yields are based upon absorbance values of the 4-aminoantipyrine-coupled products) established optimal conditions for several system variables when $[H_2O_2]_0$–$[\text{anisole}]_0$ was held at about 2 so as to give stable yields. The yield was found to be essentially independent of pH in the range of 3.5–4.2. The optimum concentration of catechol is about 1.2×10^{-4} *M*, and the optimum ratio of Fe(III) to catechol is 0.5, although the yield is not very sensitive to this ratio[18]. These optimum conditions were incorporated into the recommended analytical method.

Aging of Hamilton System—Catechol, which is itself an aromatic, should be hydroxylated, possibly altering its catalytic effectiveness as well as depleting peroxide. This anticipated aging phenomenon is demonstrated in Table I, which shows that the time of addition of substrate to a pre-prepared Hamilton system affects the yield of hydroxylated product. When additional catechol plus peroxide is added, the yield returns to its expected value, but addition of peroxide alone does not restore the yield. These observations were used in designing the analytical method, the reaction being initiated by adding peroxide, that is, by completing the Hamilton hydroxylating system.

[15] Continental Deionized Water Service.
[16] Cary models 14 and 16.
[17] Radiometer pH meter model 25 with Corning combination electrode 476051, or Sargent model DR pH meter with Sargent combination electrode S-30072-15.

[18] Data substantiating these and other findings were reported elsewhere (36).

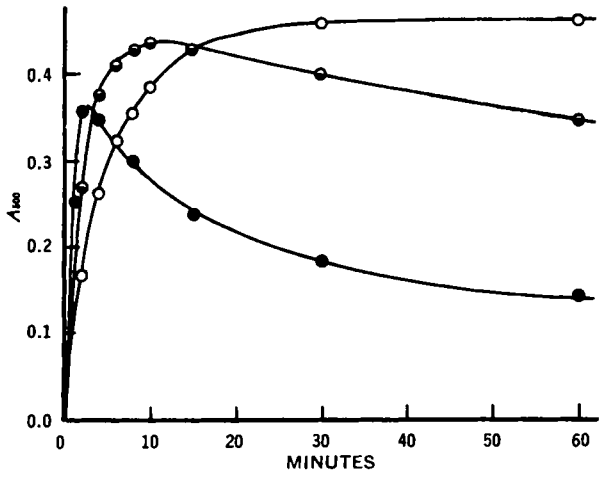


Figure 1—*Change of absorbance with time (4-aminoantipyrine finish) for hydroxylation of anisole at 25° (nitrogen atmosphere). Initial conditions: 5.26 × 10⁻⁴ M anisole, 4.26 × 10⁻⁵ M Fe(III), 1.17 × 10⁻⁴ M catechol, pH 3.9. Key: ○, $[H_2O_2]_0$–$[anisole]_0$ = 2.3; ◒, 4.7; and ●, 23.6.*

Cyclodextrin-Modified Hydroxylating System—Figure 1 shows that under analytical conditions, where the hydrogen peroxide in the Hamilton system is in large excess of the aromatic sample compound, hydroxylation continues beyond the introduction of the first hydroxy group. This phenomenon reduces the attractiveness of the Hamilton system as an analytical reagent.

The hypothesis was conceived that a cyclodextrin (ring compounds of 1,4-linked D-glucose monomers) might have a differential rate effect on the sequential hydroxylation steps as a consequence of its capability of forming inclusion complexes with aromatic compounds (37, 38). Figure 2 shows the time course for the hydroxylation of benzene in the presence and absence of cyclodextrins at an initial peroxide–substrate ratio of 6.75. Evidently the cyclodextrins have a profound stabilizing effect. About 10^{-3} *M* cyclodextrin gives an optimum effect; too high a concentration results in depressed yields. Under the conditions given in Fig. 2, a linear Beer's law plot was obtained for benzene[19] over a concentration range corresponding to initial peroxide–benzene ratios of 3.15–31.5. Incorporation of a cyclodextrin, therefore, provides a satisfactory analytical method.

This method was successfully applied to 15 mono- and disubstituted benzenes, using 1–2-hr. reaction times at 25° (1). Its extension to some drugs and related compounds is shown in Table II. Chlorpheniramine and benzalkonium chloride gave standard curves with marked negative curvature. Benzocaine could not be determined because the product precipitated from solution. The phenobarbital standard plot showed positive curvature. No significant interference (p = 0.05) was observed by tablet excipients in the determination of acetanilid[20]; the mixture contained 35 mg. acetanilid, 100 mg. lactose, 10 mg. starch, 10 mg. talc, and 2 mg. stearic acid.

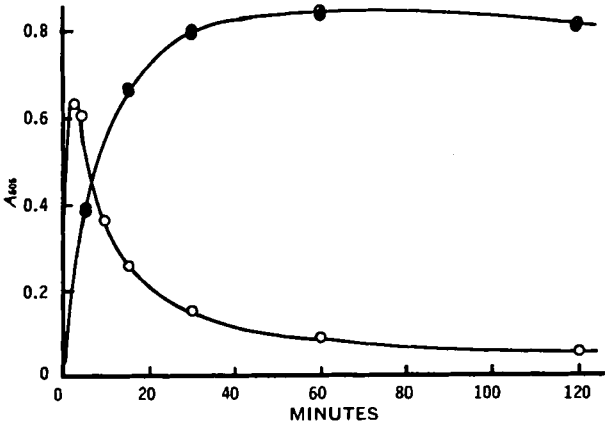


Figure 2—*Change of absorbance with time (4-aminoantipyrine finish) for hydroxylation of benzene at 25°. Initial conditions: 5.38 × 10⁻⁴ M benzene, 1.17 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.63 × 10⁻³ M H_2O_2, pH 3.7. Key: ○, no cyclodextrin; ◑, 1.13 × 10⁻³ M β-cyclodextrin; and ●, 1.26 × 10⁻³ M α-cyclodextrin.*

[19] For this substrate, an apparent molar absorptivity of 4.5 × 10^3 is obtained, corresponding to a 37% yield of phenol.

Table II—Aromatic Compounds Determined by the Cyclodextrin-Modified Hydroxylation Method[a]

Compound	Reaction Time, hr.	Analytical Wavelength, nm.[b]	10^{-3} ϵ_{app}[c]
Acetanilid	2	510	3.9
Phenacetin	1.3	490	2.7
Aspirin	2	490	1.7
Benzenesulfonamide	2	505	1.1
Sulfanilamide	2	435	1.5
Chloramphenicol	2	390	0.7
Mandelic acid	2	500	2.1
L-Phenylalanine	1	495	2.6
Tropic acid	2	500	1.4
Atropine	2	500	2.6
Ephedrine	1	505	2.7
Phenobarbital	2	515	1.1
Antipyrine	2	500	0.9
2-Naphthalenesulfonic acid	2	510	0.6
L-Tryptophan	2	485	0.5
Quinine	2	490	0.5
Physostigmine	2	495	0.2

[a] 1.17 × 10^{-4} *M* catechol, 5.94 × 10^{-5} *M* Fe(III), 3.4 × 10^{-3} *M* H_2O_2, 1.0–1.3 × 10^{-3} *M* β-cyclodextrin, pH 3.6–4.0, 25°. [b] Color developed by the 4-aminoantipyrine method except with chloramphenicol and sulfanilamide, which were measured through the nitrophenolate absorption. [c] Apparent molar absorptivity based upon total sample concentration, regardless of fate, in the final colorimetric solution. For more examples, see *Reference 1.*

Glucose-Modified Hydroxylating System—As a reasonable first step in determining the mechanism of stabilization by cyclodextrins, a time course for hydroxylation was carried out with the incorporation of α-D-glucose instead of cyclodextrin. Figure 3 shows the effect of glucose on the hydroxylation of acetanilid. Evidently, glucose has a stabilizing effect similar to that of cyclodextrin, although higher concentrations of glucose are required. Figure 4 is a Beer's law plot for the hydroxylation of acetanilid, showing that glucose provides a very satisfactory analytical result.

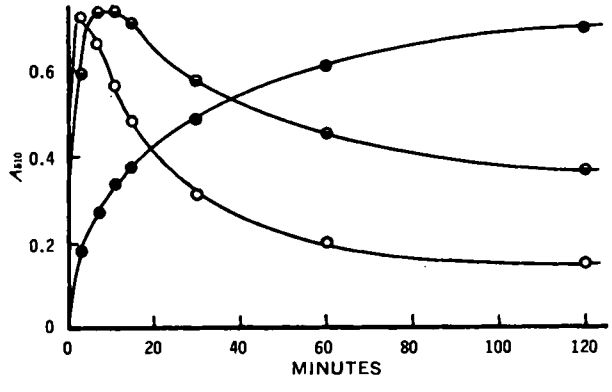


Figure 3—*Time course for hydroxylation of acetanilid (4-aminoantipyrine finish) at 25° in the presence and absence of glucose. Initial conditions: 4.70 × 10⁻⁴ M acetanilid, 1.17 × 10⁻⁴ M catechol, 5.98 × 10⁻⁵ M Fe(III), 4.13 × 10⁻³ M H_2O_2. Key: ○, no glucose; ◒, 1.26 × 10⁻³ M glucose; and ●, 1.26 × 10⁻² M glucose.*

[20] The mixture, contained in a 50-ml. volumetric flask, was shaken with 30 ml. of water for 15 min. After diluting to volume, a 2.0-ml. aliquot of the filtrate was taken for analysis.

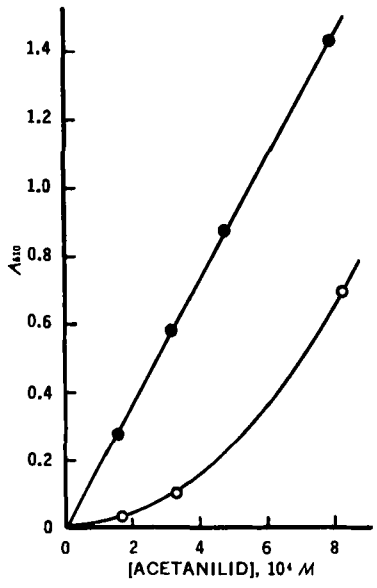


Figure 4—*Concentration–response curves for hydroxylation of acetanilid at 25° in the presence and absence of glucose. Conditions: 1.17 × 10⁻⁴ M catechol, 5.98 × 10⁻⁵ M Fe(III), 4.13 × 10⁻³ M H_2O_2; 2-hr. reaction time. Key: ○, no glucose; and ●, 7 × 10⁻³ M glucose (4-aminoantipyrine finish).*

The effect of temperature on time of hydroxylation is shown in Fig. 5. The hydroxylation reaction reaches a stable yield value after about 10 min. above 70°. At these higher temperatures, yields are slightly lower than at 25°. Moreover, the glucose concentration giving maximum yield, which was established by varying the glucose concentration and measuring the resultant absorbances, is less at higher temperatures and is mildly dependent upon the substrate[18]. At 75° the glucose concentration should be in the range of 2–5 × 10^{-3} *M* for maximal yield and for yields independent of substrate concentration. With such conditions, phenobarbital gave a linear working curve. It was found that yield is independent of catechol concentration in the range of 1–5 × 10^{-4} *M*.

Table III gives analytical results on aromatic compounds subjected to the glucose-modified hydroxylation method as described in the *Experimental* section. Linear working curves were observed for most compounds at concentrations up to 1 × 10^{-3} *M*. Chlorpheniramine showed linear behavior up to 6 × 10^{-4} *M*. Antipyrine gave a standard curve with negative curvature. Many of the Beer's law plots appear to intersect the abscissa close to the origin. This is probably a consequence of the very high peroxide–substrate ratio at low substrate concentration; it is not a serious analytical drawback.

A typical tablet formulation consisting of 65 mg. sodium phenobarbital, 26 mg. lactose, 20 mg. starch, 20 mg. talc, and 0.3 mg. stearic acid gave an average recovery of 98.4 ± 0.6% (four determinations) when compared with a standard phenobarbital solution[21]. When the lactose was increased to 200 mg. in the same formulation, a 10% decrease in response resulted. However, when the phenobarbital was extracted from the tablet formulation prior to hydroxylation, an average recovery of 98.8 ± 1.2% (four determinations) was achieved[22].

Other compounds were found to function effectively as stabilizers, although for analytical purposes they offer no advantages over glucose. The optimum stabilizer concentration depends upon the identity of the stabilizers. These approximate values were observed: lactose, 3 × 10^{-3} *M*; sorbitol, 4 × 10^{-3} *M*; acetonitrile, 0.15 *M*; isopropyl alcohol, 0.01 *M*; dioxane, 10^{-3} *M*; and acetone, 0.1 *M*.

Table III—Aromatic Compounds Determined by the Glucose-Modified Hydroxylation Procedure[a]

Compound	Analytical Wavelength, nm.[b]	$10^{-3}\ \epsilon_{app}$[c]
Benzene	505	2.9
Anisole	505	4.4
Acetanilid	510	3.4
Phenacetin	490	3.1
Benzoic acid	495	1.7
o-Methoxybenzoic acid	493	3.7
m-Methoxybenzoic acid	493	2.8
p-Methoxybenzoic acid	485	5.5
Mandelic acid	500	1.3
Atropine	500	2.1
Ephedrine	505	2.2
Phenobarbital	515	1.3
Chloramphenicol	390	0.8
Chlorpheniramine	500	2.7

[a] 1.17 × 10^{-4} *M* catechol, 5.94 × 10^{-5} *M* Fe(III), 3–4 × 10^{-3} *M* H_2O_2, 3.7 × 10^{-3} *M* glucose, pH 3.6–4.0, 10-min. reaction time, 75°. [b] Color developed by the 4-aminoantipyrine method except with chloramphenicol, which was measured through the nitrophenolate absorption. [c] Apparent molar absorptivity based upon total sample concentration, regardless of fate, in the final colorimetric solution.

Studies on Colorimetric Finish—The stability of the 4-aminoantipyrine-coupled dye has been the subject of several studies (7, 39, 40). Color instability has been associated with the presence of electron-withdrawing groups on the parent phenol (7). In the present work, a series of monosubstituted phenols was subjected to the 4-aminoantipyrine color development and the stability of the color was observed. It has been verified that electron-withdrawing groups enhance instability. Although the rate observations were semiquantitative, it can be stated that significant fading will be observed if the Hammett substituent constant σ is greater than about +0.3. An apparent exception is the amino group; thus, *o*-aminophenol gave an unstable color. It is possible that this group was oxidized to (for example) the *N*-nitroso function.

In the analytical hydroxylation studies with compounds carrying electron-withdrawing groups, the absorbance after 4-aminoantipyrine coupling was measured as a function of time and extrapolated to zero time. With nitro compounds (and with sulfanilamide, which may be oxidized to a nitro compound under hydroxylation conditions), the yellow color produced by the nitrophenolate absorption upon addition of the borate buffer provided a suitable finish.

Extraction of the 4-aminoantipyrine dye into chloroform has been proposed to stabilize and to concentrate the color (7, 41). This procedure was also successful when applied to the hydroxylation method, as described in the *Experimental* section.

DISCUSSION

Stabilization Effect—A simplified view of the Hamilton hydroxylating system (Scheme II) incorporates the idea of sequential hydroxylation as revealed by the present data. Only the monohydroxy compounds (and not even all of these) are expected to couple with 4-aminoantipyrine. Since Hamilton and coworkers (2–4) observed that anisole and nitrobenzene are hydroxylated at comparable rates, the reagent is fairly nonselective, and it may be anticipated that $k_1 \approx k_2$. The kinetic behavior is not as simple as Scheme II implies, however, because of the aging effect described earlier. The Hamilton reagent decomposes (by hydroxylation of catechol), and this phenomenon not only alters the catalytic effectiveness of the catechol but also consumes peroxide.

The hypothesis leading to the use of a cyclodextrin as a potential stabilizer invokes a stabilization resulting from inclusion compound formation, the hope being that a phenol will bind more firmly within the cyclodextrin cavity than will the parent aromatic; thus the ratio

$$\text{aromatic compound} \xrightarrow{k_1[H_2O_2]} \text{monohydroxy derivatives} \xrightarrow{k_2[H_2O_2]} \text{dihydroxy derivatives}$$

Scheme II

[21] The mixture, contained in a 50-ml. volumetric flask, was shaken with 30 ml. of water for 15 min., the pH was adjusted to 3.9 with 5% HCl, and the solution was diluted to volume with water. A 4.0-ml. aliquot was taken for analysis.

[22] The mixture, contained in a 60-ml. separator, was shaken with 10 ml. of water for 15 min., acidified with hydrochloric acid, and extracted with one 20-ml. and three 15-ml. portions of chloroform. The chloroform extracts were filtered through glass wool and evaporated to dryness. The residue was dissolved in acetate buffer and quantitatively transferred to a 100-ml. volumetric flask, and the solution was diluted to volume with buffer. An 8.0-ml. aliquot was taken for analysis.

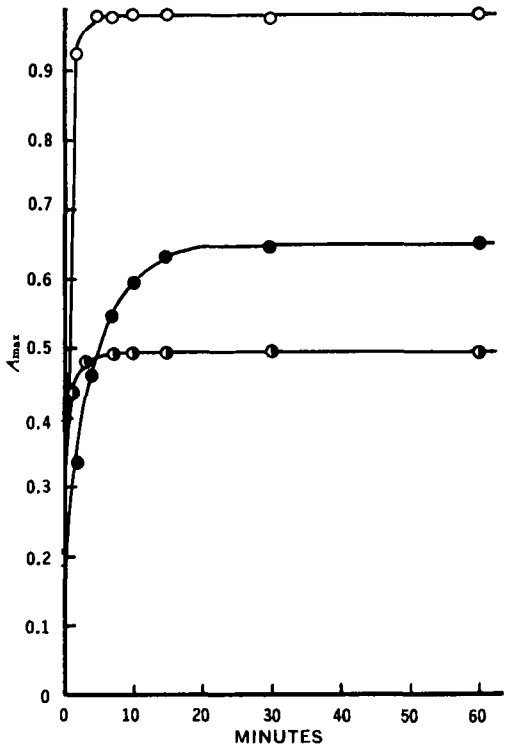


Figure 5—*Effect of temperature on time course for hydroxylation of phenobarbital and acetanilid with a glucose-modified Hamilton system. Conditions: 1.17 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.90 × 10⁻³ M H_2O_2, 7.24 × 10⁻³ M glucose. Key:* ○, *5.94 × 10⁻⁴ M acetanilid, 74°;* ◑, *8.64 × 10⁻⁴ M phenobarbital, 73°; and* ●, *8.64 × 10⁻⁴ M phenobarbital, 55° (4-aminoantipyrine finish).*

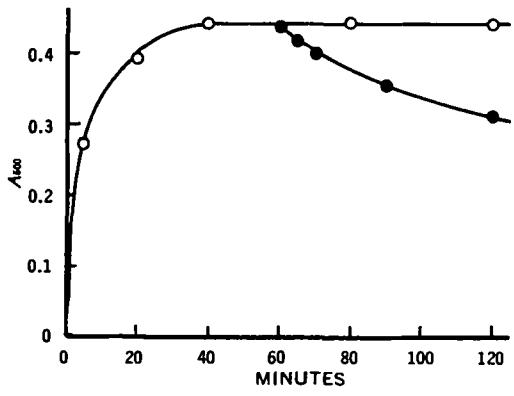


Figure 6—*Effect of addition of Hamilton reagent at the stable state (d[P]/dt = 0) on the time course for hydroxylation in a cyclodextrin-modified system. Conditions: 1.16 × 10⁻⁴ M catechol, 5.94 × 10⁻⁵ M Fe(III), 3.55 × 10⁻³ M H_2O_2, 6.37 × 10⁻⁴ M anisole, 1.08 × 10⁻³ M cyclodextrin, 25°, 4-aminoantipyrine finish. Key:* ○, *normal time course; and* ●, *Hamilton reagent (iron, catechol, and peroxide) added at 60 min.*

k_2/k_1 would be effectively decreased. Although inclusion complexes are certainly formed in many of these solutions, their role in stabilizing the monohydroxy product appears to be a minor one, as evidenced by the similar effects produced by glucose and cyclodextrin. The stabilization effect must, therefore, have a more general basis than the one originally hypothesized for cyclodextrin. The nature of the time course curves suggests this basis. Addition of a stabilizer substance like glucose or cyclodextrin causes behavior that can be duplicated (at least qualitatively) by reducing the peroxide concentration. The kinetic scheme should therefore be modified to provide an additional pathway for depletion of peroxide. In Scheme III, "ox" denotes the actual hydroxylating species, postulated by Hamilton to result from a 1:1:1 complex of hydrogen peroxide, iron, and catechol; S is the aromatic substrate; P is monohydroxylated product; I is the stabilizer or "inhibitor" substance; and P'' is the product of unspecified nature arising from the action of "ox" on I. This kinetic scheme achieves generality with the stipulation that I is any substance other than S or P that can consume peroxide. In the recommended analytical procedure, I is (mainly) glucose, but other solution components can function in the same way; thus, the acetate buffer, catechol, product P', and components of the sample other than S can all serve as I and account for the consumption of peroxide. Stabilization results from the depletion of peroxide.

An expression for this effect is easily obtained by applying the steady-state approximation to the intermediate "ox":

$$\frac{d[P]}{dt} = [ak_S - yk_P]\left[\frac{r_t k'[S]_0}{k_{-1}/[S]_0 + ak_S + yk_P + zk_I}\right] \quad \text{(Eq. 1)}$$

where $k' = k_1[\text{Fe(III)}][\text{catechol}]$, $r_0 = [H_2O_2]_0/[S]_0$, $r_t = [H_2O_2]/[S]_0$, $a = [S]/[S]_0$, $y = [P]/[S]_0$, $x = [I]_0/[S]_0$, and $z = [I]/[S]_0$. The condition of a stable state with respect to P means that $d[P]/dt = 0$; this can be met if $r_t = 0$ or if $ak_S = yk_P$. That the former condition holds is indicated by Fig. 6, which shows that the addition of fresh Hamilton reagent at the stable state results in loss of monohydroxylated product.

Equations 2 and 3 are the material balances on substrate and peroxide, where it is assumed that [ox] ≪ $[H_2O_2]$ and that P' is dihydroxylated (hence the stoichiometric factor 2):

$$[S]_0 = [S] + [P] + [P'] \quad \text{(Eq. 2)}$$

$$[H_2O_2]_0 = [H_2O_2] + [P] + 2[P'] + n([I]_0 - [I]) \quad \text{(Eq. 3)}$$

For convenience, n is taken to be unity and $[I]_0$ is adjusted accordingly; that is, the inhibitor concentration is specified on an equivalent rather than a molar basis. Then with the earlier definitions:

$$r_0 = r_t + 2(1 - a) + x - y - z \quad \text{(Eq. 4)}$$

Applying the stable-state condition $r_t = 0$ to Eq. 4 gives Eq. 5 for the yield y of monohydroxylated product:

$$y = 2(1 - a) + x - r_0 - z \quad \text{(Eq. 5)}$$

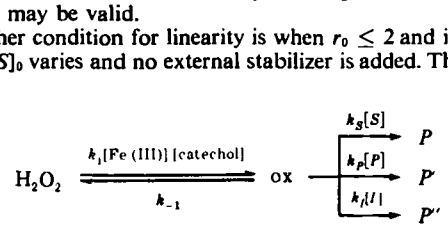
If ϵ_{av} is the weighted average molar absorptivity of the 4-aminoantipyrine-coupled monohydroxy products, then $A = \epsilon_{av}b[P] = \epsilon_{av}by[S]_0$, or:

$$A = \epsilon_{av}b[2(1 - a) + x - r_0 - z][S]_0 \quad \text{(Eq. 6)}$$

Lacking quantitative data on all system components, it is not possible to specify the behavior of a and z; the quantities x and r_0, however, are initial conditions, and it appears that the magnitude and sign of $(x - r_0)$ may control the shape of the concentration–response plot of A against $[S]_0$. When $x \approx r_0$, $d(x - r_0)/d[S]_0 = 0$ and the yield is independent of substrate concentration (with respect to the influences of x and r_0); a linear Beer's law plot is expected. When $x < r_0$, $d(x - r_0)/d[S]_0$ is positive, leading to a positive curvature in the Beer's law plot. When $x > r_0$, negative curvature is predicted.

Some of these predicted phenomena appear to match the results described earlier. There are two conditions under which a linear Beer's law plot is observed. One of these, the typical analytical situation, is when both r_0 and x are large and variable. The suggestion above is that $x \approx r_0$ is the essential condition for a linear response. If the number of oxidizable carbon atoms in the added stabilizer molecule is taken to define the number of equivalents per mole of I, then the optimal range of x values observed is about 20–400 for cyclodextrin, glucose, sorbitol, and lactose, whereas r_0 was 4–40 in these same studies. This overlap of ranges indicates that the approach may be valid.

The other condition for linearity is when $r_0 \leq 2$ and is held constant as $[S]_0$ varies and no external stabilizer is added. Then the role



Scheme III

of I is played by normal system components, including P', so x may remain small and essentially constant. In comparison, when $r_0 \leq 2$ and $[H_2O_2]_0$ is held constant as $[S]_0$ varies, x tends to become larger than r_0 as $[S]_0$ is increased (in the absence of added stabilizer) because of the contribution of P'; negative curvature is seen in the Beer's law plot. When r_0 is large and no stabilizer is added ($r_0 > x$), positive curvature is seen (Fig. 4, open circles).

Analytical Method—The proposed analytical method is applicable to the determination of many aromatic compounds in the range of 10^{-4}–10^{-3} M in aqueous solution. For it to be applicable, the aromatic sample compound must be hydroxylated and the resulting phenol must couple with 4-aminoantipyrine. These are not highly restrictive limitations, although the latter may be important for some samples. For example, pyridine gives no color when subjected to the procedure. Separate experiments showed that *o*- and *p*-hydroxypyridines do not yield a color upon treatment with 4-aminoantipyrine, whereas *m*-hydroxypyridine does give a color. Apparently, pyridine does not produce *m*-hydroxypyridine in significant yield upon treatment with the hydroxylating agent, although the other isomers may be produced. It may usually be expected that a *para*-substituted phenol will not couple with 4-aminoantipyrine unless the substituent is eliminated in the coupling reaction. The sensitivity of the method, which is quantitatively expressed in the apparent molar absorptivities, is therefore a function of the total yield of monohydroxy products, of the positional isomer distribution, and of the molar absorptivities of the 4-aminoantipyrine-coupled dyes of the various isomers.

The analytical data presented here and earlier (1) permit a comparison between the responses of a series of sample compounds subjected to the cyclodextrin-modified reagent at 25° and the glucose-modified reagent at 75°. Although some individual differences appear to be significant (possibly because of the inclusion complexing capability of cyclodextrin), the average molar absorptivity for 13 compounds subjected to the cyclodextrin system was $(2.8 \pm 1.1) \times 10^3$, and it was $(2.7 \pm 1.3) \times 10^3$ for the same compounds with the glucose system. On the average, then, the two analytical systems give the same response, so the glucose-modified reagent, with a 10-min. reaction time at 75°, is preferred for practical reasons.

The most serious limitation to this method of analysis is its susceptibility to interference. The manifestation of this interference is a decreased response (lower absorbance values than anticipated). Its basis is a competition for the available peroxide. In effect, aliphatic components of the sample act as added stabilizers (I in Scheme III) and, since the analytical reagent already contains an optimum stabilizer concentration, they depress the yield of hydroxylated aromatic. This is probably why equimolar concentrations of several organic solvents gave varying degrees of interference, with dioxane being the worst participant (1). If the concentration (that is, its normality with respect to peroxide concentration) is low enough, it will not seriously interfere, but higher concentrations cause a decreased response. This effect was described earlier for a tablet formulation, where it was shown that a simple prior separation eliminated the interference.

REFERENCES

(1) K. A. Connors and K. S. Albert, *Anal. Chem.*, **44**, 879 (1972).

(2) G. A. Hamilton and J. P. Friedman, *J. Amer. Chem. Soc.*, **85**, 1008(1963).

(3) G. A. Hamilton, J. P. Friedman, and P. M. Campbell, *ibid.*, **88**, 5266(1966).

(4) G. A. Hamilton, J. W. Hanifin, and J. P. Friedman, *ibid.*, **88**, 5269(1966).

(5) E. F. Mohler, Jr., and L. N. Jacob, *Anal. Chem.*, **29**, 1369 (1957).

(6) H. O. Friestad, D. E. Ott, and F. A. Gunther, *ibid.*, **41**, 1750(1969).

(7) D. Svobodova and J. Gasparic, *Mikrochim. Acta*, **1971**, 384.

(8) H. D. Dakin, *Org. Syn.*, **1**, 143(1932).

(9) A. W. Ingersoll, *ibid.*, **1**, 304(1932).

(10) J. B. Cohen and H. W. Dudley, *J. Chem. Soc.*, **97**, 1732 (1910).

(11) S. Kanno, *J. Pharm. Soc. Jap.*, **72**, 1193(1954).

(12) N. H. Cromwell, R. D. Babson, and C. E. Harris, *J. Amer. Chem. Soc.*, **65**, 312(1943).

(13) F. W. Hoffman and H. D. Weiss, *ibid.*, **79**, 4759(1956).

(14) F. L. Cline and E. E. Reid, *ibid.*, **49**, 3153(1927).

(15) H. Adkins and R. Billica, *ibid.*, **70**, 695(1948).

(16) R. R. Dreisbach and S. A. Shrader, *Ind. Eng. Chem.*, **41**, 2879(1949).

(17) L. Weschky, Badische Anilin + Soda-Fabrik, German pat. 805,644 (May 25, 1951); through *Chem. Abstr.*, **45**, 9227e(1951).

(18) A. Klemenc, *Monatsh. Chem.*, **35**, 85(1914).

(19) J. C. Duff, *J. Chem. Soc.*, **1941**, 547.

(20) L. C. King, M. McWhirter, and D. M. Barton, *J. Amer. Chem. Soc.*, **67**, 2089(1945).

(21) E. H. Woodruff, *ibid.*, **66**, 1799(1944).

(22) I. A. Pearl, *J. Org. Chem.*, **12**, 85(1947).

(23) C. Graebe, *Chem. Ber.*, **35**, 2747(1902).

(24) S. E. Hunt, J. I. Jones, A. S. Lindsey, D. C. Killoh, and H. S. Turner, *J. Chem. Soc.*, **1958**, 3152.

(25) J. B. Fishman, *J. Amer. Chem. Soc.*, **42**, 2288(1920).

(26) R. H. F. Manske, *Org. Syn.*, **1**, 369(1932).

(27) W. Robertson, *J. Chem. Soc.*, **81**, 1477(1902).

(28) R. B. Woodward and W. E. Doering, *J. Amer. Chem. Soc.*, **67**, 860(1945).

(29) A. I. Vogel, "Practical Organic Chemistry," 3rd ed., Longmans, London, England, 1959, p. 577.

(30) J. T. Carswell, *J. Amer. Pharm. Ass.*, **16**, 306(1927).

(31) L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis," Wiley, New York, N. Y., 1967, p. 712.

(32) F. Yokoyama and M. Pernarowski, *J. Pharm. Sci.*, **50**, 953 (1961).

(33) A. Kofler, *Mikrochim. Acta*, **33**, 4(1947).

(34) M. R. Natarajan and S. Swaminathan, *J. Amer. Chem. Soc.*, **69**, 2560(1947).

(35) R. G. Bates, *J. Res. Nat. Bur. Stand.*, **66A**, 179(1962).

(36) K. S. Albert, Ph.D. dissertation, University of Wisconsin, Madison, Wis., 1972.

(37) D. French, *Advan. Carbohyd. Chem.*, **12**, 189(1957).

(38) J. A. Thoma and L. Stewart, in "Starch: Chemistry and Technology," vol. I, R. L. Whistler and E. F. Parschall, Eds., Academic, New York, N. Y., 1965, p. 209.

(39) M. B. Ettinger, C. C. Ruchhoft, and R. J. Lishka, *Anal. Chem.*, **23**, 1783(1951).

(40) D. Svobodova, J. Gasparic, and L. Novakova, *Collect. Czech. Chem. Commun.*, **35**, 31(1970).

(41) R. J. Lacoste, S. H. Venable, and J. C. Stone, *Anal. Chem.*, **31**, 1246(1959).

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▲ To whom inquiries should be directed.