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Identification of Acetaminophen Polymerization Products Catalyzed by Horseradish Peroxidase*

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Horseradish peroxidase catalyzed the H_2O_2-dependent oxidation and polymerization of acetaminophen. Six acetaminophen polymers were isolated from horseradish peroxidase reaction mixtures by semipreparative high pressure liquid chromatography. Chemical structures were determined by a combination of electron impact and chemical ionization mass spectrometry and 500-MHz proton magnetic resonance spectroscopy. Two dimers, three trimers, and one tetramer were identified. The polymers formed primarily through a covalent bond between carbons *ortho* to the hydroxyl group, and to a lesser extent, between the carbon *ortho* to the hydroxyl group and the amino group of another acetaminophen molecule. Greater than 99% of the polymerization reaction products were quenched by the addition of 2.0 mM ascorbate. High acetaminophen concentration favored dimer formation, whereas low acetaminophen concentration favored formation of trimers and tetramers. Since approximately 1 mol of H_2O_2 was consumed per mol of covalent ligand formed between acetaminophen molecules, these products probably result from free radical termination reactions.

Acetaminophen (4′-hydroxyacetanilide) is a widely used analgesic and antipyretic drug. Although therapeutic doses of acetaminophen are reported to be safe, larger doses may result in hepatic necrosis and renal damage in both humans and laboratory animals (1–6). *In vivo* oxidation of acetaminophen to an intermediate(s) that reacts with cellular macromolecules is generally thought to be responsible for cellular toxicity. Enzymatic oxidation of acetaminophen may occur by either a 1- or a 2-electron process to produce free radicals or *N*-acetyl-*p*-benzoquinone imine, respectively. However, the importance of each intermediate in metabolism and toxicity is unknown which is, in part, due to the lack of studies on the chemistry of these intermediates.

Cytochrome P-450 and prostaglandin H synthase are two enzymes that have been shown to catalyze the oxidation of acetaminophen *in vitro*. Cytochrome P-450 is thought to catalyze acetaminophen to *N*-acetyl-*p*-benzoquinone imine which may react with protein or reduced glutathione (4, 6–10). A glutathione conjugate has been isolated from rat bile and identified as 3-(glutathion-*S*-yl)acetaminophen (11). Although the above investigations give credence to the role of cytochrome P-450 in acetaminophen oxidation, it has been suggested that prostaglandin H synthase, primarily located in the inner medulla of kidney and seminal vesicles, may also oxidize acetaminophen to intermediates that bind to protein and react with reduced glutathione to yield 3-(glutathion-*S*-yl)acetaminophen (12–14).

The formation of acetaminophen free radicals catalyzed by horseradish peroxidase has been examined by ESR spectroscopy (15, 16). West *et al.* (16) using fast flow ESR suggested that the acetaminophen phenoxyl free radical was rapidly formed with subsequent formation of melanin-like polymeric products as suggested by the line-broadening ESR signal. In this study, we have characterized a number of polymers formed by peroxidation of acetaminophen by horseradish peroxidase. An abstract describing part of this work has been previously presented (17).

MATERIALS AND METHODS

[*phenyl*-UL-^{14}C]Acetaminophen (1.65 mCi/mmol) was synthesized by Dr. Robert W. Roth of Midwest Research Institute, Kansas City, MO, using a previously described method (18). [^{14}C]Acetaminophen was further purified by silica thin layer chromatography using ethyl acetate as a solvent, followed by reversed phase HPLC[1] on a μBondapak C_{18} semi-preparative column using a solvent which consisted of 87.9% water, 10% methanol, 2% glacial acetic acid, and 0.1% ethyl acetate (v/v). The purity of [^{14}C]acetaminophen was greater than 99% as assayed by HPLC. Horseradish peroxidase (EC 1.11.1.7 (type VI)), acetaminophen, H_2O_2 (30%), and ascorbate were purchased from Sigma. Dimethyl sulfoxide-d_6 was purchased from Merck Isotopes, Inc., Montreal, Quebec. All other reagents and solvents were of the highest grade available.

Metabolism of Acetaminophen—Analytical incubations (1 ml) contained 0.1 M potassium phosphate (pH 7.4), 0–2.0 mM ascorbate, 0–76 nM horseradish peroxidase, 0.1–10.0 mM [^{14}C]acetaminophen or unlabeled acetaminophen, and 0–0.4 mM H_2O_2. Samples were first equilibrated at 25 °C for approximately 2 min, and then the reaction was initiated by adding H_2O_2. Reactions were terminated by adding 1 ml of ice-cold methanol:H_2O (90:10) containing 2.0 mM ascorbate and the incubation mixture placed on ice.

Preparative incubations of 500 ml contained 0.1 M potassium phosphate (pH 7.4), 0.6 μM horseradish peroxidase, 1.0 mM acetaminophen, and 0.2 mM H_2O_2. Reactions were initiated with H_2O_2 and allowed to go to completion without addition of methanol or ascorbate. Samples were evaporated under reduced pressure. Metabolites were then dissolved in 50 ml of methanol and subsequently isolated by HPLC.

Liquid Chromatography—The HRP-mediated polymerization

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; H_2O_2, hydrogen peroxide; EI, electron impact; CI, chemical ionization; MS, mass spectroscopy; NMR, nuclear magnetic resonance; acetaminophen dimer, 4′,4″-dihydroxy-3′,3″-biacetanilide; *N*-acetaminophen dimer, 4′,4″-dihydroxy-*N*,3″-biacetanilide; acetaminophen trimer, 4′,4″,4‴′-trihydroxy-3′,3″:5′,3‴-teracetanilide; *N*″-acetaminophen trimer, 4′,4″,4‴′-trihydroxy-3′,*N*″:3′,3‴-teracetanilide; acetaminophen tetramer, 4′,4″,4‴′,4⁗′-tetrahydroxy-3′,3″:5′,3‴:5⁗′,3⁗′-quarteracetanilide; *N*-acetaminophen trimer, 4′,4″,4‴-trihydroxy-*N*,3″:5′,3‴′-teracetanilide.

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products were routinely analyzed by reversed-phase HPLC. The analytical HPLC system consisted of two model 6000 HPLC pumps, a model 440 UV detector (254 nm), and a model 660 microprocessor from Waters Associates, Inc., a Micromeritics model 725 automatic injector, a model 3390A reporting integrator from Hewlett-Packard, a linear recorder, and a 5-μm C_{18} Ultrasphere ODS reversed-phase column (4.6 × 250 mm) from Altex. A binary solvent system with a flow rate of 1.0 ml/min was used for acetaminophen polymer separation. Solvent A consisted of 87.9% water, 10% methanol, 2% glacial acetic acid, 0.1% ethyl acetate (v/v), and solvent B was 100% methanol. After the HPLC column was equilibrated with 100% solvent A, the sample was automatically injected. Solvent A was maintained at 100% for 10 min, followed by a linear gradient to give 81% A and 19% B in 9 min, and maintained for an additional 26 min. Finally, 100% B was obtained by a linear gradient for 10 min. The total time for sample analysis, including equilibration, elution, and methanol washing time was 85 min. Radioactivity was determined with a Radiomatic FLO-ONE radioactive flow detector using FLO-Scint III with a flow rate of 4.0 ml/min.

The semi-preparative HPLC system, used to isolate metabolites for MS and NMR analysis, was similar to that used for analytical analysis except that 1 ml of methanol containing metabolites from preparative incubation, as described above, was manually injected onto a 10-μm Waters μBondapak-C_{18} column (0.78 × 30 cm), and a Tracor variable wavelength detector set at 254 nm was used to monitor polymer elution. Elution of metabolites was achieved using isocratic conditions. The solvent mixture consisted of 68% water, 30% methanol, 2% glacial acetic acid, and 0.1% ethyl acetate (v/v), and the flow rate was 3 ml/min. Metabolites were isolated by repeated injections and collecting appropriate fractions. Fractions were evaporated, and the residue was suspended in methanol and further purified using the same HPLC system.

Mass Spectrometry—Both EI MS and CI MS were used for analysis of the acetaminophen metabolites. Samples were introduced into the source of a Finnigan 4023 mass spectrophotometer by thermal vaporization off a platinum wire (Vacumetrics probe). CI MS using methane gas was necessary for molecular weight determination of larger polymerization products. Acetaminophen polymers were *O*-acetylated by adding 10 ml of acetic anhydride and 3 μl of pyridine to the dry polymerization sample. Samples were purged with argon and reacted for 24 h. Solvent was removed by evaporation under reduced pressure, and the residue was analyzed by CI MS.

Nuclear Magnetic Resonance Spectroscopy—^{1}H NMR spectra were obtained using a Bruker WM-500 NMR spectrometer. Spectra were obtained at room temperature from solutions of sample in dimethyl sulfoxide-d_6 using tetramethylsilane as an internal standard. ^{1}H NMR spectra were also obtained after samples were derivatized with acetic anhydride.

H_2O_2 and Horseradish Peroxidase Quantitation—Deionized glass distilled water was used to prepare stock solutions of H_2O_2 and horseradish peroxidase. Concentrations were estimated by optical absorbance spectroscopy using an extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm for H_2O_2 (19) and an extinction coefficient of 89.5 × 10^3 M^{-1} cm^{-1} at 403 nm for horseradish peroxidase (20).

Determination of Acetaminophen Free Radical Equivalent—The acetaminophen free radical equivalent was calculated for each polymer by multiplying polymer concentration by 2 $(n - 1)$, where n is the number of acetaminophen molecules/polymer.

RESULTS

Isolation of Horseradish Peroxidase-catalyzed Acetaminophen Metabolites—Fig. 1 shows the HPLC separation of UV-absorbing acetaminophen metabolites from horseradish peroxidase and H_2O_2-catalyzed reaction mixtures. In control reactions which did not contain either horseradish peroxidase or H_2O_2, only compound A (acetaminophen) was present. For the purpose of identification, metabolites were isolated from large reaction mixtures by semi-preparative HPLC.

Structure Characterization of Acetaminophen Metabolites—HPLC peaks (compounds) B through G were analyzed by a combination of MS (Table I) and 500-MHz ^{1}H NMR (Figs. 2 and 3). EI and CI MS were used for molecular weight determinations of the acetaminophen metabolites. As shown in Table I, MS analysis of HPLC compounds B–G indicated

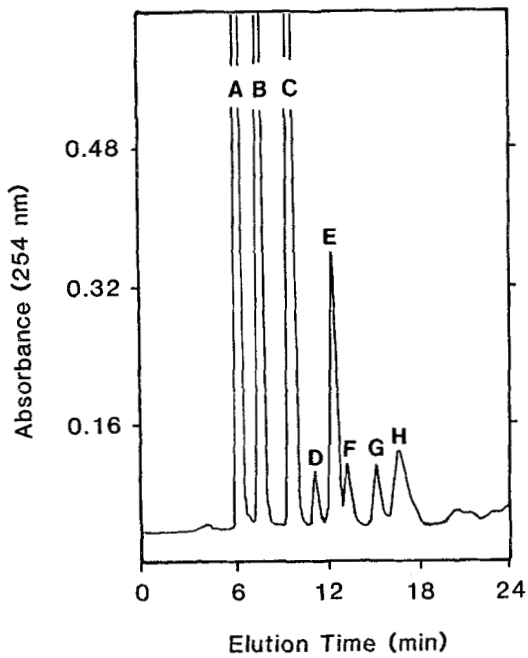

FIG. 1. **Semi-preparative C_{18} reversed-phase HPLC separation of acetaminophen metabolites catalyzed by horseradish peroxidase.** Metabolites were isolated from reaction mixtures containing 1.0 mM acetaminophen (A), 0.6 μM horseradish peroxidase, 0.2 mM H_2O, and 0.1 M potassium phosphate buffer, pH 7.4. HPLC procedures are described under "Materials and Methods."

TABLE I

Mass spectral analysis of HPLC compounds B–G

Compounds	*m/z* (per cent relative abundance)[a]
EI	
B	300 (100), 258 (48), 240 (6), 216 (26)
C	449 (76), 407 (100), 389 (24), 365 (52)
D	300 (74), 258 (36), 216 (5)
F	449 (8), 407 (2), 282 (2), 198 (10)
G	449 (65), 407 (100), 389 (5), 365 (26)
CI	
E	599 (10), 581 (38), 557 (27), 539 (44)
Acetylated	
C	576 (59), 534 (100), 492 (34), 343 (76)
E	767 (12), 725 (24), 683 (16), 534 (16)

[a] The data represent a partial list of MS fragmentation patterns and per cent relative abundance. CI MS data are $m/z + 1$.

molecular ions consistent with the formation of two dimers (B and D) (M_r 300), three trimers (C, F, and G) (M_r 449), and one tetramer (E) (M_r 598). EI MS was useful in obtaining molecular ions of all the acetaminophen metabolites except compound E which was analyzed by CI MS using methane as the carrier gas. Compounds C and E were derivatized with acetic anhydride to give *O*-acetylated products and analyzed by CI MS. These data strongly suggested that horseradish peroxidase catalyzed the polymerization of acetaminophen.

The aromatic ^{1}H NMR spectrum of acetaminophen dimer (compound B) is shown in Fig. 2. The protons with resonances at 6.72 ppm (H_5) and 7.30 ppm (H_6) were shown by proton-proton spin coupling constants to be *ortho* to one another ($J_{5,6}$ = 8.6 Hz). The protons with downfield resonances were *meta* to one another ($J_{2,6}$ = 2.6 Hz). Since hydroxyl substitution causes an upfield shift of *ortho* protons by approximately 0.5 ppm (21) the *ortho*-coupled resonance observed at 6.72 ppm was assigned as H_5. The *meta*-coupled resonance at 7.33 ppm was assigned as H_2, and the resonance at 7.30 ppm was

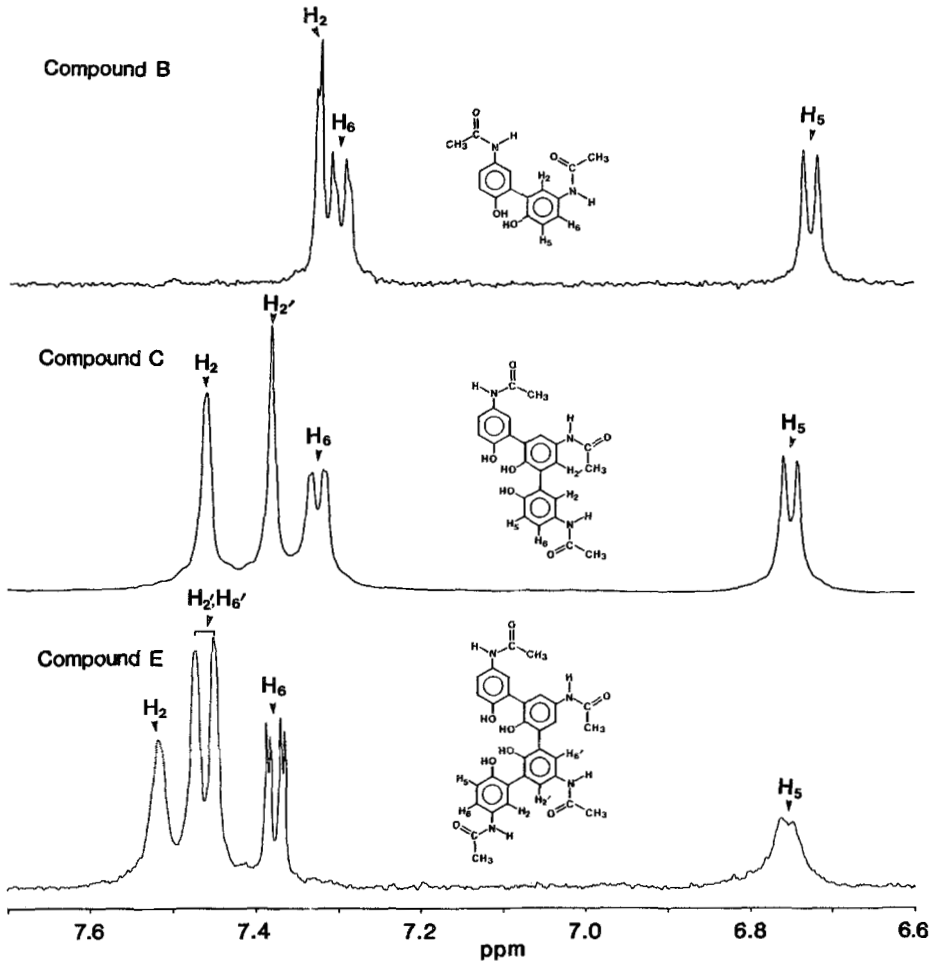

FIG. 2. **Proton NMR spectra of aromatic region of compound B (acetaminophen dimer), compound C (acetaminophen trimer), and compound E (acetaminophen tetramer).** Spectra were determined in dimethyl sulfoxide-d_6 and chemical shift in ppm downfield from trimethylsilane standard. Assignments for nonexchangeable proton resonances for compound B (acetaminophen dimer) are: 1.94 (d,6,NAc), 6.72 (d,2,$J_{5,6}$ = 8.6 Hz, H_5); 7.30 (dd,2,$J_{2,6}$ = 2.6 Hz, H_6), 7.33 (s,2,H_2) ppm. Assignments for compound C (acetaminophen trimer) are: 2.04 (s,6,NAc), 1.94 (s,3,N′Ac), 6.75 (d,2,$J_{5,6}$ = 9.0 Hz, H_5), 7.33 (dd,2,$J_{2,6}$ = 2.6 Hz, H_6), 7.38 (s,2,$H_{2'}$), 7.46 (d,2,H_2) ppm. Assignments for compound E (acetaminophen tetramer) are: 2.02 (s,6,NAc), 2.00 (s,6,N′Ac), 6.75 (d,2,$J_{5,6}$ = 9.0 Hz, H_5), 7.38 (dd,2,$J_{2,6}$ = 2.6 Hz, H_6), 7.45 (d,2,$H_{2'}$ or $H_{6'}$), 7.47 (d,2,$H_{2'}$, or $H_{6'}$), 7.52 (d,2,H_2) ppm.

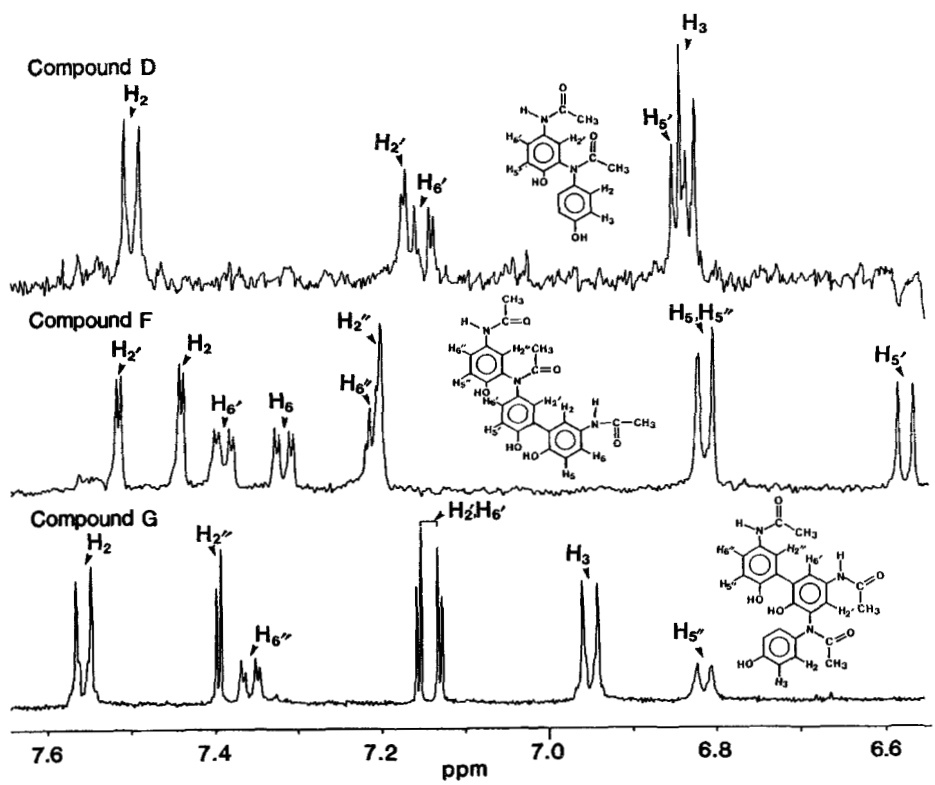

FIG. 3. **Proton NMR spectra of aromatic region of compound D (*N*-acetaminophen dimer), compound F (*N*″-acetaminophen trimer), and compound G (*N*-acetaminophen trimer).** Spectra were determined in dimethyl sulfoxide-d_6 and chemical shift in ppm downfield from trimethylsilane standard. Assignments for nonexchangeable proton resonances for compound D (*N*-acetaminophen dimer) are: 1.94 (s,3,NAc), 2.01 (s,3,N′Ac), 6.82 (d,2,$J_{2,3}$ = 8.8 Hz, H_3), 6.84 (d,1,$J_{5',6'}$ = 8.3 Hz, $H_{5'}$), 7.15 (dd,1,$J_{2',6}$ Hz, $H_{6'}$), 7.17 (d,1,$H_{2'}$), 7.50 (d,2,H_2) ppm. Assignments for compound F (*N*″-acetaminophen trimer) are: 1.95 (s,3,N′Ac), 1.99 (s,3,NAc or N″Ac), 2.01 (s,3,NAc or N″Ac), 6.57 (d,1,$J_{5',6'}$ = 8.6 Hz, $H_{5'}$), 6.81 (ad,2,$J_{5,6}$ = 8.6 Hz, $J_{5'',6''}$ = 8.6 Hz, H_5 and $H_{5''}$), 7.20–7.27 (m,2,$H_{2''}$ and $H_{6''}$), 7.32 (dd,1,$J_{2,6}$ = 2.6 Hz, H_6), 7.34 (dd,1,$J_{2',6'}$ = 2.6 Hz, $H_{6'}$), 7.44 (d,1,H_2), 7.52 (d,1,$H_{2'}$) ppm. Assignments for compound G (*N*-acetaminophen trimer) are: 1.95 (s,3,NAc), 2.00 (s,3,N′Ac or N″Ac), 2.03 (s,3,N′Ac or N″Ac), 6.81 (d,1,$J_{5'',6''}$ = 8.6 Hz, $H_{5''}$), 6.95 (d,2,$J_{2,3}$ = 9.0 Hz, H_3), 7.13 (d,1,$J_{2',6'}$ = 2.6 Hz, $H_{2'}$ or $H_{6'}$), 7.16 (d,1,$H_{2'}$ or $H_{6'}$), 7.36 (dd,1,$J_{2'',6''}$ = 2.4 Hz, $H_{6''}$), 7.40 (d,1,$H_{2''}$), 7.56 (d,2,H_2) ppm.

assigned as H_6 which was consistent with 2 molecules of acetaminophen covalently bound through carbons *ortho* to the hydroxyl substitutions. Nonaromatic resonances present in the NMR spectra of compound B consisted of three singlets with chemical shifts at 1.98 ppm for the acetyl protons, at 4.04 ppm for the hydroxyl proton, and at 9.59 ppm for the amide proton. Conformation of these assignments was obtained by repeating the NMR analysis after compound B was *O*-acetylated with acetic anhydride. A downfield shift of the H_5 resonance was observed after acetylation which confirmed that H_5 was *ortho* to the hydroxyl substitution. NMR and MS analyses of compound B were consistent with the formation of a symmetric acetaminophen dimer with carbon bond formation at the 3-carbon position. This metabolite has been named in accordance with the IUPAC rules of organic nomenclature as 4′,4″′-dihydroxy-3′,3″′-biacetanilide (acetaminophen dimer).

The aromatic ^1H NMR spectra of acetaminophen trimer (compound C) and acetaminophen tetramers (compound E) are shown in Fig. 2. These spectra were similar to the acetaminophen dimer (compound B) except that an additional proton resonance was observed in the spectra of the acetaminophen trimer (compound C) at 7.38 ppm ($H_{2'}$) and two additional resonances were observed in the spectra of the acetaminophen tetramer (compound E) at 7.45 and 7.47 ppm ($H_{2'}$ or $H_{6'}$). Protons H_5, H_6, and H_2 were shown to be interrelated by homonuclear decoupling experiments. Also interrelated were protons $H_{2'}$ and $H_{6'}$ of compound E. Assignments of aromatic protons for compounds C and E were based on similar arguments used for compound B. Those arguments were homonuclear decoupling, substituent effects, integral areas, and downfield shift effects on proton resonances *ortho* to hydroxyl substitution after *O*-acetylation. NMR and MS analysis of compounds C and E were consistent with acetaminophen polymerization *ortho* to the hydroxyl substitution. Compound C has been named 4′,4″′,4‴″′-trihydroxy-3′,3‴′:5″′,3‴″′-teracetanilide (acetaminophen trimer), and E has been named 4′,4‴′,4‴″′-tetrahydroxy-3′,3‴′:5‴′,3‴″′:5‴″′,3‴‴″′-quarteracetanilide (acetaminophen tetramer).

The aromatic ^1H NMR spectra of the other acetaminophen dimer (compound D), shown in Fig. 3, consist of five resonances. Two resonances, H_2 and H_3, integrated to 2 protons each as compared to resonances $H_{5'}$, $H_{6'}$, and $H_{2'}$ which integrated to 1 proton each. Homonuclear decoupling demonstrated that the protons with 2 proton resonances (H_2 and H_3) were of the same ring system, and proton-proton spin coupling constants demonstrated that they were *ortho* to one another ($J_{2,3}$ = 8.8 Hz). The three 1-proton resonances were shown to be on the other ring system. $H_{5'}$ and $H_{6'}$ were *ortho* ($J_{5',6'}$ = 8.3 Hz) and $H_{2'}$ and $H_{6'}$ were *meta* ($J_{2',6'}$ = 2.3 Hz). The magnetically nonequivalent acetyl resonances were indicative of dimerization through one of the acetamido nitrogens. Since protons *ortho* to hydroxyl substitution were shifted upfield (21), the doublet resonances at 6.83 and 6.84 ppm were assigned as H_3 and $H_{5'}$, respectively. Further NMR analysis of compound D after *O*-acetylation verified the H_3 and $H_{5'}$ assignments. NMR and MS analysis of compound D was consistent with acetaminophen dimerization through the nitrogen and the carbon *ortho* to the hydroxyl substitution. Compound D was named 4′,4″′-dihydroxy-*N*,3″′-biacetanilide (*N*-acetaminophen dimer).

The aromatic ^1H NMR spectrum of acetaminophen trimer (compound F) shown in Fig. 3 has eight proton resonances. The resonance at 6.81 ppm (H_5 and $H_{5''}$) was an apparent two-proton doublet. The other resonances integrated to 1 proton with respect to the apparent two-proton doublet. Homonuclear decoupling experiments demonstrated that one proton of the apparent two-proton doublet (H_5 and $H_{5''}$) was coupled to each of the outer ring system while the furthest upfield doublet ($H_{5'}$) was coupled to $H_{2'}$ and $H_{6'}$. The furthest upfield doublet resonance was assigned as $H_{5'}$ of the inner ring system which was *ortho* to $H_{6'}$ ($J_{5',6'}$ = 8.6 Hz), and $H_{6'}$ was *meta* to $H_{2'}$ ($J_{2',6'}$ = 2.6 Hz). On the outer ring systems H_6 was *ortho* to H_5 ($J_{5,6}$ = 8.0 Hz) and *meta* to H_2 ($J_{2,6}$ = 2.7 Hz). Likewise, $H_{6''}$ was *ortho* to $H_{5''}$ ($J_{5'',6''}$ = 8.6 Hz). Compound F, shown in Fig. 3, was named 4′,4‴′,4‴″′-trihydroxy-3′,*N*‴:3‴′,3‴″′-teracetanilide (*N*‴-acetaminophen trimer).

The aromatic ^1H NMR spectrum of acetaminophen trimer (compound G) shown in Fig. 3 consists of seven resonances. The doublet resonances at 6.95 and 7.56 ppm integrated to 2 protons each as compared to the other proton resonance which integrated to 1 proton each. The two doublets that integrated to two protons (6.96 and 7.56 ppm) were shown to be H_2 and H_3 by homonuclear decoupling and shown to be *ortho* with a proton-proton spin coupling constant, $J_{2,3}$ = 9.0 Hz. Similarly, the two resonances at 7.13 and 7.16 ppm were shown to be $H_{2'}$ or $H_{6'}$ and *meta* coupled to one another ($J_{2',6'}$ = 2.6 Hz). The remaining resonances at 6.81, 7.36, and 7.40 ppm were shown to be $H_{5''}$, $H_{6''}$, and $H_{2''}$ ($J_{5'',6''}$ = 8.6 Hz; $J_{2'',6''}$ = 2.4 Hz), respectively. Compound G, shown in Fig. 3, was named 4′,4‴′,4‴″′-trihydroxy-*N*-3‴′:5‴′,3‴″′-teracetanilide (*N*-acetaminophen trimer).

Conditions of Acetaminophen Polymerization Catalyzed by Horseradish Peroxidase—To examine the mechanism of acetaminophen free radical formation (polymerization) a method for quenching reactions was necessary. Since ascorbate is known to reduce some free radicals and is also a substrate of horseradish peroxidase, the effect of including ascorbate (0–2.0 mM) in the horseradish peroxidase-catalyzed polymerization reaction was examined. As the ascorbate concentration was increased to 0.2 mM the acetaminophen free radical equivalent, as calculated from the total product formation, was decreased by 20% (Fig. 4*A*). As shown in Fig. 4*B*, the decrease in acetaminophen trimer (*C*) and *N*-acetaminophen trimer (*G*) was nearly linear with respect to ascorbate

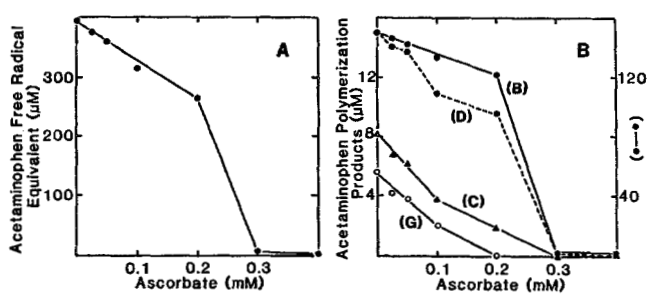

FIG. 4. **Horseradish peroxidase-catalyzed acetaminophen polymerization quenched by ascorbate.** Polymerization products were determined by analytical HPLC. Samples of 1.0 ml final volume contained 10 μmol of acetaminophen, 76 pmol of horseradish peroxidase, 0.1 mmol of potassium phosphate, pH 7.4, and ascorbate (0–0.4 μmol). Samples were equilibrated at 25 °C, and reactions were initiated by the addition of 50 μl of H_2O_2 (0.2 μmol). Ice-cold MeOH (1 ml) was added after 1 min and samples placed on ice or refrigerated until analyzed. *A* shows the total calculated acetaminophen free radical equivalent, which was determined for each polymer by multiplying polymer concentration by 2 ($n - 1$) where n is the number of acetaminophen molecules/polymer. *B* shows the quantitation of each polymerization product. Since the amount of acetaminophen dimer (*B*) was greater than the other products its quantitation is shown on the right *y* axis. The quantitation of the *N*-acetaminophen dimer (*D*), acetaminophen trimer (*C*), and *N*-acetaminophen trimer (*G*) is shown on the left *y* axis.

concentration. At 0.2 mM ascorbate the acetaminophen trimer (*C*) was approximately 22% of control values while the *N*-acetaminophen trimer (*G*) was approximately 1%. The acetaminophen dimers (*B* and *D*) were not substantially quenched by less than 0.2 mM ascorbate. At 0.3 mM ascorbate total polymerization was decreased by approximately 98.5%, and at 0.4 mM ascorbate the decrease was greater than 99%. However, even at ascorbate concentrations as high as 2.0 mM the acetaminophen dimer (*B*) was still observed at approximately 0.2% of control (data not shown). Since greater than 99% of the acetaminophen polymerization was quenched by ascorbate, it was subsequently added to reaction mixtures at a concentration of 2.0 mM to terminate polymerization at the end of incubation.

A time course for acetaminophen polymer product formation revealed that after reactions were initiated by the addition of H_2O_2 (0.2 mM), the polymerization of acetaminophen (1 mM) was rapid (Fig. 5). The *inset* indicates that total product formation was almost complete in the first 0.5 min. Acetaminophen dimer (*B*) and acetaminophen trimer (*C*) were the major polymers formed under these conditions. The *N*-acetaminophen dimer (*D*) was rapidly formed within the first min after initiation of reactions and subsequently decreased, suggesting that it may have been further polymerized.

Examination of the effect of acetaminophen concentration on the horseradish peroxidase-mediated polymerization reaction indicated that it had two major effects. There was an increase in formation of total polymerization products with the increase in acetaminophen concentration (Fig. 6). The total yield of each polymer was, however, concentration dependent. At low concentrations of acetaminophen, formation of large polymers (*C, E, F*) was greater than at high concentrations. The acetaminophen dimer (*B*) was the primary product at acetaminophen concentrations greater than 0.2 mM, but at lower concentrations of acetaminophen the acetaminophen trimer (*C*) was nearly equal to the acetaminophen dimer (*B*) formation. Acetaminophen trimer (*C*) formation was optimal at concentrations of acetaminophen near 0.8 mM whereas *N*″-acetaminophen trimer (*F*) and acetaminophen tetramer (*E*) formation was optimal at approximately 0.6 mM acetaminophen (Fig. 6). In reaction mixtures with the concentration of acetaminophen greater than 1.0 mM a decrease in formation of the large polymers and an increase in formation of both dimers was observed. *N*-Acetaminophen dimer (*D*) was a minor product at low concentrations of acetaminophen, but was a major product, second to that of the acetaminophen dimer (*B*), at high concentrations of acetaminophen. The *N*-acetaminophen trimer (*G*) formation was optimal at 4 mM, and a marginal decrease was observed at higher concentrations of acetaminophen.

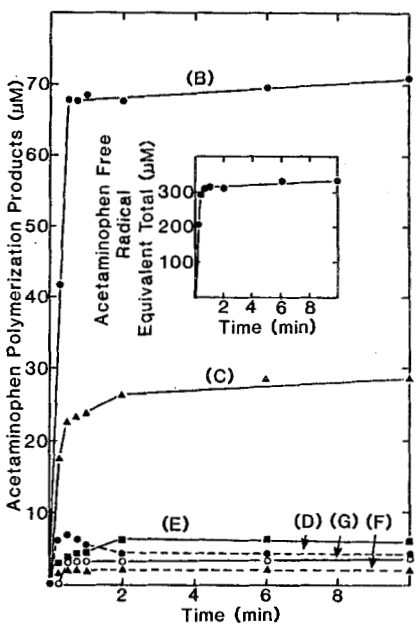

FIG. 5. **Time course for horseradish peroxidase-catalyzed conversion of acetaminophen to polymerization products.** Acetaminophen polymerization products were identified as acetaminophen dimer (*B*), acetaminophen trimer (*C*), *N*-acetaminophen dimer (*D*), acetaminophen tetramer (*E*), *N*″-acetaminophen trimer (*F*), *N*-acetaminophen trimer (*G*). Product analysis and reaction conditions are described in the legend to Fig. 4 except that acetaminophen was 1.0 mM. Samples were incubated for 0–10 min, and reactions were terminated with 1 ml of ice-cold MeOH:H_2O (90:10, v/v) containing 2 μmol of ascorbate. The method of calculation of total acetaminophen free radical equivalent (*inset*) is shown in the legend to Fig. 4.

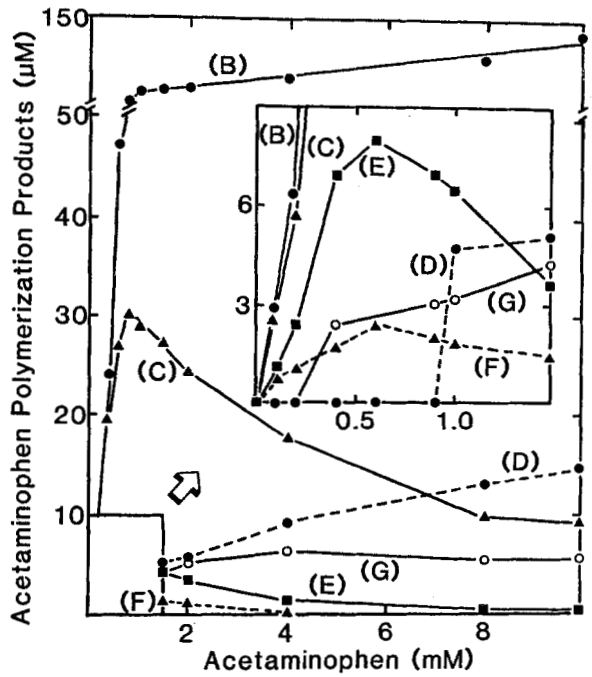

FIG. 6. **Effect of acetaminophen concentration on horseradish peroxidase-catalyzed formation of acetaminophen dimer (*B*), acetaminophen trimer (*C*), *N*-acetaminophen dimer (*D*), acetaminophen tetramer (*E*), *N*″-acetaminophen trimer (*F*), *N*-acetaminophen trimer (*G*).** Reaction conditions were the same as Fig. 4 except reactions were terminated after 5.0 min. The *inset* is an enlargement of the *lower left corner*.

As previously demonstrated in this section, reaction mixtures containing 76 nM horseradish peroxidase resulted in the rapid polymerization of acetaminophen; therefore, the effect of horseradish peroxidase concentration was examined. As shown in Fig. 7, catalytic oxidation of acetaminophen (10 mM) to acetaminophen free radical equivalent was proportional to horseradish peroxidase concentration from 0–8 nM horseradish peroxidase with an apparent first order rate constant of 630 ± 50 s^{-1}. Fig. 7 also shows that acetaminophen dimer (*B*) and *N*-acetaminophen dimer (*D*) were the only polymers detected below 1.0 nM horseradish peroxidase. At 0.1 nM horseradish peroxidase the acetaminophen dimer (*B*) was the only polymer detected. Formation of the two dimers was nearly parallel with increasing concentrations of horseradish peroxidase; formation of acetaminophen dimer (*B*) was approximately 10-fold greater than formation of *N*-aceta-

minophen dimer (*D*). Acetaminophen trimer (*C*) and *N*-acetaminophen trimer (*G*) were observed at horseradish peroxidase concentrations above 2.0 and 4.0 nM, respectively. The results were similar to those observed with ascorbate (Fig. 4), in that the dimers were the first to be formed at low horseradish peroxidase concentration and last to be quenched by ascorbate. At high concentrations of horseradish peroxidase, the concentration of acetaminophen free radical equivalent approached 400 μM, the theoretical amount possible at 200 μM H_2O_2 for polymerization via radical termination.

Stoichiometry of H_2O_2 Consumed per Acetaminophen Free Radical Equivalent in Horseradish Peroxidase-catalyzed Reaction—Although much of the experimental data suggested polymerization might be a termination rather than a propagation reaction, the effect of extended incubations with varying concentrations of H_2O_2 was needed for further conformation. In reactions containing 76 μM horseradish peroxidase the calculated acetaminophen free radical equivalent was approximately twice (1.98 ± 0.17) that of H_2O_2 concentration (Table II).

DISCUSSION

Horseradish peroxidase catalyzes the oxidation of a number of electron-donating hydroxyl- and amino-substituted aromatic compounds. Generally, horseradish peroxidase is thought to be divalently oxidized by H_2O_2 and then reduced by substrates in two 1-electron transfer steps (22–26). Mechanistic studies are often difficult because of complex nonenzymatic free radical reactions. For example, Yamazaki *et al.* (25) demonstrated that ascorbate and hydroquinone were rapidly oxidized by horseradish peroxidase to free radical intermediates which would disproportionate to the 2-electron oxidized compounds. Furthermore, comproportionation reactions were also reported. Other free radical intermediates such as the *p*-aminophenoxy free radical apparently react to form unidentified polymers (27) as suggested by line broadening of ESR spectra.

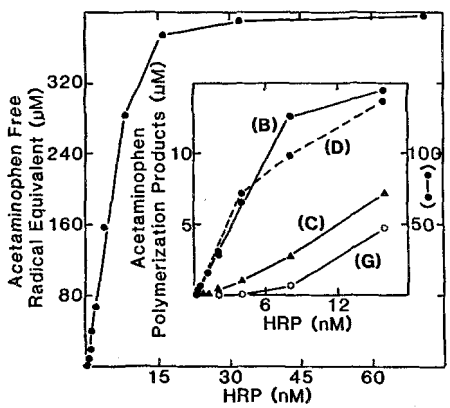

FIG. 7. **Effect of horseradish peroxidase (*HRP*) concentration on catalytic polymerization of acetaminophen.** Acetaminophen concentration was 10 mM, and H_2O_2 concentration was 0.2 mM. Reactions were terminated after 1.0 min as previously described. The method of calculating acetaminophen free radical equivalent is described in the legend to Fig. 4. The *inset* shows the concentration of acetaminophen dimer (*B*) on the right *y* axis, and *N*-acetaminophen dimer (*D*), acetaminophen trimer (*C*), and *N*-acetaminophen trimer (*G*) on the left *y* axis.

ESR line broadening has often been used as evidence for polymerization reactions. Free radicals of large polymers would result in broad ESR signals without fine structure because of restricted tumbling and free movement in solution. Sealy *et al.* (28) have suggested that line broadening may also result when molecules like benzidine are tightly associated in a charge transfer complex. Thus, ESR has been useful in evaluating the formation of primary radicals, while secondary reactions forming larger polymers have been difficult to interpret.

The catalytic oxidation of acetaminophen by horseradish peroxidase to free radical intermediates has been previously examined by ESR spectroscopy (15). Acetaminophen free radicals were detected immediately after the addition of horseradish peroxidase to acetaminophen and H_2O_2; maximal free radical concentration was detected at 16 min, and this slowly declined with a $t_{1/2}$ of 2 h (15). In these studies the formation of acetaminophen polymers was not reported nor was the characteristic ESR line broadening associated with formation of such polymers. In contrast, our studies show that once the free radicals of acetaminophen were formed they quickly polymerized. Furthermore, using similar experimental conditions as those employed by Nelson *et al.* (15) we found that acetaminophen polymerized so rapidly that less than 25% of the original acetaminophen was detected after 5 min. Therefore, it appears that the ESR spectrum may have been the result of polymer radicals rather than the primary acetaminophen radicals. Recently, fast flow ESR results (16) concur that the ESR line-broadened spectrum previously observed (15) was probably due to the melanin-like radical rather than the primary phenoxyl radical.

The horseradish peroxidase-catalyzed oxidation of acetaminophen to acetaminophen polymerization products is consistent with prior formation of free radical intermediates. Six products have been identified, and the amino substituent and the *ortho* hydroxy position were shown to be sites of polymerization. Approximately 1 mol of H_2O_2 was consumed per 2 mol of acetaminophen free radical, and this was consistent with a free radical termination mechanism. The stoichiometry

TABLE II

Stoichiometric determinations of H_2O_2 consumed during acetaminophen polymerization catalyzed by horseradish peroxidase

Assay conditions were the same as described in the legend to Fig. 4 with 76 nM horseradish peroxidase and reacted for 45 min.

H_2O_2	Acetaminophen polymers				Free radical equivalent	Free radical equivalent/H_2O_2
	Dimer	Trimer	*N*-Dimer	*N*-Trimer		
			μM			
24	20.2 ± 1.1		1.8 ± 1.0		44.0	1.83
110	91.8 ± 0.4	3.4 ± 0.2	9.6 ± 0.3		216.4	1.97
221	187.3 ± 13.1	10.9 ± 1.0	17.1 ± 1.1	10.1 ± 2.0	492.8	2.23
331	255.7 ± 10.0	20.4 ± 1.4	19.6 ± 0.8	13.4 ± 1.0	685.8	2.07
442	291.6 ± 6.2	26.2 ± 2.6	21.9 ± 6.3	17.9 ± 2.3	803.4	1.82
					$\bar{X}$ = 1.98 ± 0.17	

studies suggested that the primary acetaminophen free radicals reacted to give dimers which in turn were reoxidized and reacted to give larger acetaminophen polymers. Two possible explanations for polymer free radical formation are: that the polymers were also oxidized by horseradish peroxidase; or that the polymers were oxidized nonenzymatically by the acetaminophen free radical. The former suggestion may be more likely at low concentrations of acetaminophen and high concentrations of H_2O_2; however, in reaction mixtures containing 10 mM acetaminophen and 0.2 mM H_2O_2 (Fig. 4), where the concentration of acetaminophen polymers would be no greater than 2% of the acetaminophen concentration, it seems unlikely that polymer could effectively compete with acetaminophen for the active site of horseradish peroxidase. Whatever the mechanism of polymer radical formation, two dimers are the primary products formed at high acetaminophen or low horseradish peroxidase concentration while trimers and tetramer are major products formed at low acetaminophen or high horseradish peroxidase concentration. Ascorbate quenched the formation of the larger polymers before small polymers.

Unlike the transient free radical of acetaminophen, Fischer and Mason (29) recently showed that the free radical of 3′,5′-dimethyl-4′-hydroxyacetanilide was stable. They suggest that the stability of 3′,5′-dimethyl-4′-hydroxyacetanilide was in part due to the blocking of polymerization. In agreement, our study shows that acetaminophen polymers were primarily formed through a covalent bond between carbons *ortho* to the hydroxyl group. Therefore, substitution *ortho* to the hydroxyl group may be expected to decrease polymerization and increase primary radical concentration.

To gain better understanding of enzymatic and nonenzymatic 1- and 2-electron oxidation mechanisms, we are extending these studies to examine acetaminophen oxidation catalyzed by cytochrome P-450 and prostaglandin H synthase. The nonenzymatic reactions between *N*-acetyl-*p*-benzoquinone imine, reduced glutathione, and acetaminophen are also being examined and compared to enzyme-catalyzed reactions.

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