para-Aminophenol (PAP) causes selective injury to rat renal proximal tubules (Newton et al., 1982; Tarloff et al., 1989) and is proposed to account, at least in part, for the nephrotoxicity of acetaminophen (APAP; Newton et al., 1985; Mugford and Tarloff, 1997). PAP cytotoxicity may involve enzymatic or nonenzymatic oxidation of PAP to reactive intermediates. Consistent with the notion of direct cytotoxicity, PAP is genotoxic (Hayward and Lavin, 1985; Eiche et al., 1990), nephrotoxic in the isolated perfused kidney (Davis et al., 1983), and cytoxic to isolated rat renal tubular cells (Klos et al., 1992; Lash and Stahl, 1993) and rabbit proximal tubule suspensions (Lock et al., 1993). However, in situ oxidation to reactive intermediates is inconsistent with the observed selectivity of PAP toxicity, because PAP damages renal but not hepatic cells. Other evidence suggests the involvement of hepatic metabolites of PAP in cytotoxicity. For example, cannulation of the bile duct to prevent hepatic metabolites of PAP from undergoing enterohepatic recirculation partially protected rats against PAP-induced nephrotoxicity (Gartland et al., 1990). Furthermore, pretreating rats with buthionine sulfoximine to deplete GSH protected rats from PAP nephrotoxicity (Garland et al., 1990). Suggesting that nephrotoxic metabolites of PAP generated in liver may include GSH conjugates.

Although glutathione conjugates of PAP (PAP-GSHs) are nephrotoxic in vivo (Fowler et al., 1991, 1994) and cytotoxic in vitro (Klos et al., 1992), it is unclear as to whether sufficient amounts of these conjugates are formed to account for PAP nephrotoxicity. In a previous study examining biliary metabolites of PAP, acetaminophen-O-glucuronide (APAP-Gluc) was identified as the major PAP metabolite appearing in bile with only trace amounts of PAP-GSHs detected (Klos et al., 1992). Therefore, these studies were designed to examine the metabolism of PAP in rat hepatocytes. The results indicate that PAP-GSHs account for over half of the metabolites identified in hepatocytes and quantitatively may contribute to PAP nephrotoxicity.

**Materials and Methods**

**Chemicals.** All chemicals were purchased from commercial sources as analytical or reagent grade and were used without any additional treatment. 1-Aminobenzotriazole (ABT) was obtained from Dr. Bruce Mico (Hoffman-LaRoche, Inc., Nutley, NJ).

**Animals and Hepatocyte Incubations.** Male Sprague-Dawley rats (200–225 g) were purchased from Ace Animals, Inc. (Boytown, PA). All experiments were performed in accordance with the guidelines and practices established by the Institutional Animal Care and Use Committee at the University of the Sciences in Philadelphia. Hepatocytes were prepared using a two-step perfusion and collagenase digestion procedure as previously described (Moldeus et al., 1978; Nyarko and Harvison, 1995). Trypan blue exclusion was used to exclude nonviable hepatocytes.

**Methods.** Hepatocytes were prepared by a modification of the two-step liver perfusion technique of LaRusso and Englesberg (1976). Animals were fasted overnight before perfusion. Male Sprague-Dawley rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL), and their abdomens were opened. The liver was cannulated and perfused with a modified KREBS medium (KREBS et al., 1974) containing 5% CO2/95% O2 at 37°C. The perfusate was added to a Krebs-Henseleit buffer at 37°C for 4–5 h with 2.3 mM PAP under an atmosphere of 5% CO2/95% O2. Aliquots were withdrawn at 0.1 h of incubation and then hourly for 5 h of incubation. Reactions were terminated by the addition of acetonitrile. Hepatocyte viability was unaltered with PAP present in the incubation medium. We found that hepatocytes converted PAP to two major metabolites (PAP-GSH conjugates and PAP-N-acetylcytysteine conjugates) and several minor metabolites [PAP-O-glucuronide, acetaminophen (APAP), APAP-O-glucuronide, APAP-GSH conjugates, and 4-hydroxyformanilide]. Preincubating hepatocytes with 1-aminobenzotriazole, an inhibitor of cytochromes P450, did not alter the pattern of PAP metabolism. In conclusion, we found that PAP was metabolized in hepatocytes predominantly to PAP-GSH conjugates and PAP-N-acetylcytysteine conjugates in sufficient quantities to account for the nephrotoxicity of PAP.

**ACKNOWLEDGMENTS.** This paper is available online at http://www.dmd.org.
to determine viability. The viability of freshly isolated hepatocytes was 91.8 ± 0.8% (n = 3).

For metabolism studies, hepatocytes (5 × 10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) containing 118.1 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO_4, 1.0 mM KH_2PO_4, 3.0 mM glucose, and 23.8 mM NaHCO_3 supplemented with 25 mM HEPES, and 2% BSA (fraction V). PAP (40 μl, 25 mg/ml in methanol) was added to achieve a final concentration of 2.3 mM. Control incubations contained an equivalent amount of methanol.

Hepatocytes were incubated for 5 h at 37°C in a gyrating water bath at 60 rpm and gassed with a mixture of humidified 95% O_2 and 5% CO_2. To inhibit cytochromes P450, hepatocytes were preincubated for 15 min with ABT (4 μM of 1 M ABT in ethanol, final concentration 1 mM) (Nyarok and Harvison, 1995). Aliquots (0.1-ml) of incubation solution were withdrawn at 0.1, 1, 2, 3, 4, and 5 h. Samples were mixed with 0.5 ml of acetonitrile (ACN) to terminate metabolism. Samples were centrifuged at 1000g for 10 min, and the supernatant solutions were analyzed by HPLC.

To verify that hepatocytes contained functional cytochromes P450, we determined ethoxyconumariin O-deethylation activity in the presence and absence of ABT. Hepatocytes (5 × 10^6 cells/ml) were preincubated at 37°C with ABT (final concentration 1 mM) or ethanol for 15 min, following by incubation for 2 h with 800 μM ethoxyconumariin. Cells were pelleted and supernatants were incubated with β-glucuronidase/arylsulfatase to hydrolyze conjugated umbelliferone (Kern et al., 1997). Umbelliferone was detected using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan) as previously described (Aitio, 1978).

Identification and Quantitation of Metabolites. HPLC analysis. Deproteinized samples from hepatocyte incubations were analyzed by HPLC or HPLC/mass spectrometry (MS). HPLC analysis was performed using a system consisting of an LDC ConstaMetric 3000 pump, a SpectroMonitor 3100 detector set at 254 nm, and an LDC Analytical D-2500 Computing Integrator (Milton Roy Co., Riviera Beach, FL). An Alltech Econosil C_18 column, 250 mm × 4.6 mm, 10 μm, was used. The mobile phase consisted of ACN/H_2O (25:75, v/v) at a flow rate of 1 ml/min. The injection volume was 20 μl. Quantitation of PAP metabolites was based on the assumption that PAP and metabolites would have about the same UV absorbivity, as has been demonstrated for APAP and its metabolites (Howie et al., 1977).

HPLC/Thermospray (TSP)/MS analysis. For analyzing synthetic compounds and metabolites from hepatocytes, we used an HPLC/TSP/MS system consisting of a Waters 600 MS pump, a Waters 900 MS diode array detector, and a Delta Nermag R30–10 triple quadrupole mass spectrometer (Delta Nermag Instruments, Argentueil, France) equipped with a Vestec 740C TSP interface (Vestec, Houston, TX). We used a Beekman ODS (250 × 4.6 mm) column, a mobile phase composed of ACN/H_2O (8.5:91.5, v/v) with 0.1 M ammonium acetate (NH_4Ac) at pH 4.7 and a flow rate of 1 ml/min. The injection volume was 20 μl. Quantitation of PAP metabolites was based on the assumption that PAP and metabolites would have about the same UV absorbivity, as has been demonstrated for APAP and its metabolites (Howie et al., 1977).

NMR (1H-NMR) analysis. 1H-NMR analyses were performed using a Varian EM390 spectrometer (Varian Associates, Palo Alto, CA). Tetramethylsilane or (trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt was used as the internal standard and CDCl_3, d_6-dimethyl sulfoxide, or D_2O was used as the solvent.

Flow injection analysis electrospray ionization mass spectrometry (FIA/ESI/MS). FIA/ESI/MS analysis was done using a Perkin Elmer Sciex API III Biomolecular Mass Analyzer (Sciex, Ontario, Canada) equipped with a Sciex ESI source.

Synthesis of Proposed PAP Metabolites. 4-Aminophenyl-O-glucuronic acid (PAP-Gluc). PAP-Gluc was synthesized using a method for synthesis of analogous compounds (Focella et al., 1972) with minor modifications. 4-Nitrophenyl glucuronic acid (0.11 g, 0.33 mmol) was dissolved in 1 ml of 10% Na_2CO_3 and 85% Na_2S_2O_4 (0.14 g, 0.70 mmol) was added at 45°C in small portions with stirring. The reaction mixture was heated to 95°C and held for 30 min. After cooling to room temperature, the reaction mixture was lyophilized to obtain a white powder, melting point 250–260°C (decomposed).

HPLC/TSP/MS analysis indicated that the product consisted of two peaks with retention times of 4.84 (84.2%) and 5.56 (15.8%) min. Mass spectrum (4.84-min peak): m/z 303 [M+NH_4]^+, 286 [M+H]^+, 279, 264, 248, 225, 206, 188, 175, 151, 133, 110 (100%).

4-Aminophenol sulfate potassium salt (PAP-O-SO_4K). 4-Nitrophenyl sulfate potassium salt (1.28 g, 5.0 mmol) was dissolved in 8 ml of 10% K_2CO_3 and 85% Na_2S_2O_4 (2.6 g, 12.7 mmol) was added at 45°C in small portions with stirring. The reaction mixture was kept at 95°C for 1 h. After cooling, filtering, and drying, 0.92 g of white crystals were obtained (melting point 265–270°C, decomposed).

HPLC/TSP/MS analysis indicated that the product consisted of four components with peaks at retention times of 4.90 (39%, PAP-O-SO_4K), 5.48 (41%), 6.12 (11%), and 7.87 (9%) min. Mass spectrum (4.90 min peak): m/z 207 [M+NH_4]^+, 191, 190 [M+H]^+, 175, 151, 149, 133 (100%), 121, 110.

4-Hydroxyformanilide (FPAP). We used a method for formylating aniline (Fieser and Jones, 1955) with minor modifications. PAP (5.5 g, 0.05 mol) was mixed with formic acid (5.0 g) and 30 ml of toluene. The mixture was distilled slowly until 10 ml of solvent remained. The residue was washed with 10 ml of petroleum ether and filtered to yield 5.9 g of a grayish-white powder, melting point 128–130°C. Yield: 85.3%.

GC/MS indicated that FPAP with a retention time of 6.9 min accounted for over 99% of the sample and PAP with a retention time of 4.5 min accounted for the remainder. GC/MS (electron impact) m/z 137 [M^+] (100%), 109, 108, 81, 80.

1H-NMR (d_6-dimethyl sulfoxide, tetramethylsilane as internal standard): δ 9.40 (s, 1H, -CHO), 7.80 (s, 1H, OH), 7.05 (d, J = 9Hz, 2H), 6.37 (d, J = 9Hz, 2H).

p-Benzocoumarinimine (PBQI). We followed a previously reported method (Eckert et al., 1990) with minor modifications. PAP (0.22 g, 2 mmol) was dissolved in 40 ml of ACN, and anhydrous magnesium sulfate (1 g, 8.3 mmol) was added. The mixture was stirred for 3 min, lead dioxide (5.0 g, 20 mmol) was added, and the reaction was carried out in the dark for 15 min. Filtration yielded a yellow solution that was kept in the dark for use in preparation of PBQI-thioethers.

GC/MS indicated a single peak with a retention time of 3.04 min. Mass spectrum: m/z 107 [M^+] (100%), 81, 80, 79, 53, 52, 51, 38, 37.

PAP-S-glutathione conjugates (PAP-GSH). A previously reported method (Eckert et al., 1990) was used with modifications. PBQI solution was mixed with a solution containing GSH (0.6 g, 2 mmol), sodium acetate (0.5 g, 6.1 mmol), and sodium hydroxide (0.1 g, 2.5 mmol) in 10 ml of water (pH 11). The mixture was stirred in the dark at room temperature for 10 min. After completion of the reaction, the top layer containing unreacted PBQI and PAP was separated, and the bottom layer containing PAP-GSHs was mixed with an equal amount of ACN to precipitate the solid products. After filtration, the cake was suspended in 20 ml of methanol, filtered, and dried to yield 0.37 g of a greenish-yellow powder, melting point 62–64°C (started to decompose at about 100°C).

GC/MS analysis indicated a mixture of products including mono-, di-, and tri-substituted PAP-GSHs with respective molecular ions (M+H^+) at m/z 415.0, 720.2, and 1025.2. There was degradation during HPLC/TSP/MS analysis with fragments observed at m/z 308 [GSH+H]^+, 290, 274, 264, 256, 230, 212, 195, 179, 147, 130, 129.

PAP-cysteine conjugates (PAP-Cys). A solution of PBQI was mixed with a solution containing cysteine (0.24 g, 2 mmol), sodium acetate (0.5 g, 6.1 mmol), and sodium hydroxide (0.05 g, 1.3 mmol) in 10 ml of water (pH 11).
The mixture was stirred in the dark for 10 min. The top layer containing unreacted PBQI and PAP was discarded, and the bottom layer was mixed with 20 ml of 90% methanol. The mixture was filtered and the cake was suspended in 20 ml of methanol. After filtration and drying, 0.2 g of a brown solid was obtained (melting point 234 – 238°C with decomposition).

FIA/ESI/MS analysis indicated a mixture of mono-, di-, tri-, tetra-, and penta-substituted PAP-Cys with respective molecular ions \([M+H]^+\) at \(m/z\) 229, 348, 586, 317, 467, 585, 586, 348, 586, 271, 144, 166, 187, 152, 198, 222, 237, 279, 585, 205, 205, 121, 151, 144, 166, 187, 222, 240, 234, 238, 308 [GSH + H]^+\) at 2.37, 2.91, 2.35, 2.53, 2.79, 3.84, 5.44, 5.84, 6.63, 7.09.

PAP-S-(N-acetylcysteine) (PAP-NACys) conjugates.

A solution of PBQI was mixed with a solution containing N-acetylcysteine (0.32 g, 2 mmol), sodium acetate (0.5 g, 6.1 mmol), and sodium hydroxide (0.05 g, 1.3 mmol) in Figs. 1, 2, and 3.

FIG. 1. Disappearance of PAP from the incubation medium when hepatocytes (5 \( \times \) 10^6 cells/ml) were incubated in Krebs-Henseleit buffer in the presence (●) or absence (○) of ABT, a suicide substrate inhibitor of cytochromes P450.

Symbols indicate means ± S.E. of three determinations. Means were not significantly different at any time point \((P > .05)\). Inset: Natural logarithmic decline of PAP over 5-h incubation. Linear regression analysis lines are included where the solid line represents hepatocytes incubated with PAP and the dashed line represents hepatocytes incubated with PAP + ABT.

The mixture was stirred in the dark for 10 min. The top layer containing unreacted PBQI and PAP was discarded, and the bottom layer was mixed with 20 ml of 90% methanol. After filtration and drying, 0.2 g of a brown solid was obtained (melting point 234–238°C with decomposition).

FIA/ESI/MS analysis indicated a mixture of mono-, di-, tri-, tetra-, and penta-substituted PAP-Cys with respective molecular ions \([M+H]^+\) at \(m/z\) 229, 348, 467, 586, 707.

PAP-S-(N-acetylcysteine) (PAP-NACys) conjugates. A solution of PBQI was mixed with a solution containing N-acetylcysteine (0.32 g, 2 mmol), sodium acetate (0.5 g, 6.1 mmol), and sodium hydroxide (0.05 g, 1.3 mmol) in Figs. 2 and 3.

Symbols indicate means ± S.E. of three determinations. Means were not significantly different at any time point \((P > .05)\).

TABLE 1

HPLC/TSP/MS analyses of proposed PAP metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>HPLC/TSP/MS</th>
<th>Molecular Ion</th>
<th>Fragments</th>
<th>RT*min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP-Gluc</td>
<td>285</td>
<td>286</td>
<td>177</td>
<td>130, 147, 198, 238, 308 [GSH + H]^+</td>
<td>1.77</td>
</tr>
<tr>
<td>PAP-Cys(s)</td>
<td>228, 347, 466, 585</td>
<td>348, 586</td>
<td></td>
<td>152, 195, 176, 285</td>
<td>2.30</td>
</tr>
<tr>
<td>PAP-GSH(s)</td>
<td>414, 719, 1024</td>
<td></td>
<td></td>
<td>144, 166, 187</td>
<td>2.53</td>
</tr>
<tr>
<td>APAP-Gluc</td>
<td>327</td>
<td></td>
<td></td>
<td>152, 198, 222, 237, 279</td>
<td>2.91</td>
</tr>
<tr>
<td>PAP-NACys(s)</td>
<td>270, 389, 508</td>
<td>271</td>
<td></td>
<td>146, 152, 187, 222, 240</td>
<td>3.84</td>
</tr>
<tr>
<td>APAP-GSH</td>
<td>456</td>
<td></td>
<td></td>
<td></td>
<td>5.44</td>
</tr>
<tr>
<td>APAP-NACys</td>
<td>312</td>
<td>110</td>
<td></td>
<td></td>
<td>5.84</td>
</tr>
<tr>
<td>PAP</td>
<td>109</td>
<td>110</td>
<td></td>
<td></td>
<td>6.63</td>
</tr>
<tr>
<td>FPAP</td>
<td>137</td>
<td>138</td>
<td></td>
<td></td>
<td>7.09</td>
</tr>
<tr>
<td>N-OH-PAP-O-SO(_3)H</td>
<td>205</td>
<td>205</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>151</td>
<td>152</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Retention time in HPLC chromatogram.

b PAP-Cys, PAP-GSH, and PAP-NACys conjugates included mono- and multi-substituted isomers.

c The compound was not well separated.

TABLE 2

Mass balance of PAP metabolites from hepatocytes

The recovery was calculated based on PAP and its metabolites having equal UV molar absorbance. Data are expressed as means ± S.E. The differences among different time intervals or between treatments (PAP versus PAP + ABT) are not statistically significant.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Number of Samples</th>
<th>Recovery PAP (%)</th>
<th>Recovery PAP + ABT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>3</td>
<td>102.4 ± 2.3</td>
<td>97.4 ± 11.0</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>95.6 ± 5.3</td>
<td>91.9 ± 7.8</td>
</tr>
<tr>
<td>2.0</td>
<td>3</td>
<td>91.2 ± 7.7</td>
<td>92.8 ± 9.3</td>
</tr>
<tr>
<td>3.0</td>
<td>3</td>
<td>96.7 ± 5.3</td>
<td>98.7 ± 10.6</td>
</tr>
<tr>
<td>4.0</td>
<td>3</td>
<td>101.1 ± 7.6</td>
<td>107.9 ± 16.0</td>
</tr>
<tr>
<td>5.0</td>
<td>3</td>
<td>110.9 ± 15.8</td>
<td>140.7 ± 43.5</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>99.6 ± 3.7</td>
<td>104.6 ± 8.2</td>
</tr>
</tbody>
</table>

Symbols indicate means ± S.E. of three determinations. Means were not significantly different at any time point \((P > .05)\).

FIG. 2. Formation of PAP-GSHs by rat hepatocytes (5 \( \times \) 10^6 cells/ml) incubated with 2.3 mM PAP in the presence (○) or absence (●) of ABT.

Symbols indicate means ± S.E. of three determinations. Means were not significantly different at any time point \((P > .05)\).
are listed in Table 2, where PAP indicates hepatocytes incubated with 10 ml of water (pH 11). The mixture was stirred in the dark for 10 min. The solvent was evaporated under a reduced pressure to 20% of the original volume. After filtration, the cake was washed with 2 ml of water to yield 0.14 g of a brownish-black powder that started to melt at 50°C with decomposition.

HPLC/TSP/MS indicated that the product was a mixture of mono- and multi-substituted PAP-NACys conjugates. Only the mono-substituted isomer(s) had a clear mass spectrum with a molecular ion at m/z 271 [M+H]+ and fragments at m/z 253, 211, 194, 166, 142, 130. Multi-substituted isomers had fragments at m/z 327, 303, 285, 271, 230, 198, 174, 164, 146, 142, 130, 122.

Data Analysis and Statistical Analyses. All results are expressed as mean ± S.E. of a minimum of three determinations. Data were analyzed by one-way ANOVA followed by a Student-Newman-Keuls test when appropriate. The probability level for statistical significance was P < .05.

Results

Identification of PAP Metabolites by HPLC/MS and GC/MS. We analyzed synthesized postulated metabolites of PAP using HPLC/TSP/MS; results are summarized in Table 1. We observed molecular ions in the spectra for PAP-GHs, PAP-NACys, PAP, FPAP, and APAP. We were unable to detect molecular ions of PAP-GH, APAP-Gluc, or GSH conjugates of APAP (APAP-GH), most likely because these compounds underwent thermal degradation during HPLC/TSP/MS analysis.

Mass Balance of PAP and Metabolites. We assumed that equal molar concentrations of PAP and its metabolites would have approximately the same UV absorptivity at 254 nm. We tested this assumption by calculating the total recovery of PAP and metabolites obtained from hepatocyte samples as determined by HPLC analysis. The results are listed in Table 2, where PAP indicates hepatocytes incubated with PAP alone and PAP+ABT indicates hepatocytes preincubated with ABT. The calculated recovery of PAP and metabolites correlated well with the original concentration of PAP (2.3 mM). There were no significant differences among time intervals or between treatments. The results support the assumption that PAP and its metabolites have approximately equal UV absorbance regardless of the ratio of PAP to its metabolites.

Viability of Rat Hepatocytes. There were no significant differences between viability of hepatocytes incubated in the presence or absence of PAP. Viability was determined by trypan blue exclusion. Initial viability for control and PAP-treated hepatocytes was 91.8 ± 0.85%. Viability in hepatocytes incubated in the presence of PAP declined to 74.9 ± 6.01% after 5 h whereas viability in hepatocytes incubated in the absence of PAP declined to 75.4 ± 4.11% after 5 h.

Hepatocytes metabolized 7-ethoxycoumarin to umbelliferone at a rate of 87.31 ± 9.18 pmol × min⁻¹ × mg of protein⁻¹. Metabolism was significantly decreased to 30.6 ± 2.6% of control (26.86 ± 2.27 pmol × min⁻¹ × mg of protein⁻¹, P = .006) when hepatocytes were preincubated for 15 min with ABT.

PAP Metabolism in Rat Hepatocytes. PAP was extensively metabolized by rat hepatocytes and less than 5% of the original PAP remained at the end of the incubation. As shown in Fig. 1, PAP metabolism was not significantly altered by preincubating hepatocytes with ABT. Metabolism appeared to follow first-order kinetics with apparent rate constants of 6.81 ± 0.95 × 10⁻¹ h⁻¹ and 5.49 ± 1.74 × 10⁻¹ h⁻¹.
We identified two major metabolites of PAP: PAP-GSH (Fig. 2) accounted for 51.5 ± 5.8% and PAP-NACys (Fig. 3) accounted for 31.0 ± 7.0% of PAP metabolites found in hepatocyte incubations at the end of 5 h. The mass spectra for these metabolites (Fig. 4) corresponded with the mass spectra obtained for synthesized compounds (Table 1). Concentrations of both metabolites rose steadily during the 5-h incubation period (Figs. 2 and 3). We found small amounts of PAP-Gluc (Fig. 5, 8.9 ± 3.2%) and APAP (Fig. 6, 4.2 ± 1.2%), and trace amounts of FPAP (0.9 ± 0.3%), APAP-Gluc (0.1 ± 0.0%), and APAP-GSH (0.1 ± 0.0%). Mass spectral analyses (Fig. 7) corresponded to those of synthesized reference compounds (Table 1). PAP-Gluc was initially present in low concentrations but formation increased toward the end of the 5-h incubation interval (Fig. 5). Formation of APAP increased over the initial 2 h, then remained essentially unchanged during the next 3 h (Fig. 6). Unreacted PAP accounted for 3.1 ± 0.6% of PAP metabolites at the end of 5 h. The metabolism of PAP was not significantly altered in hepatocytes pre-incubated with ABT and we were able to account for all of the PAP initially present in hepatocyte incubations at all time points (Table 2).

**Discussion**

We found that rat hepatocytes readily converted PAP to two major metabolites, PAP-GSH and PAP-NACys conjugates. Minor metabolites included PAP-Gluc, APAP, FPAP, APAP-Gluc, and APAP-GSH conjugates (Fig. 8). A small amount (≤5%) of PAP remained unreacted after a 5-h incubation with hepatocytes. PAP-GSH, PAP-Gluc, APAP, APAP-Gluc, and APAP-GSH conjugates have been observed previously as PAP metabolites (Eckert, 1988; Temellini et al., 1991; Klos et al., 1992) whereas PAP-Cys, PAP-NACys, and FPAP are novel metabolites.

PAP-GSH conjugates are reported to be equitoxic or up to 4-fold more toxic than PAP when administered in vivo (Fowler et al., 1994) or when incubated with renal tubular cells (Fowler et al., 1991; Klos et al., 1992). However, bile contained only trace amounts of PAP-GSH whereas the major metabolite in bile was PAP-Gluc (Klos et al., 1992), raising questions concerning the role of PAP-GSHs in nephrotoxicity. Our data show that hepatocytes converted about 50% of PAP to PAP-GSHs, supporting the idea that PAP-GSHs may mediate the nephrotoxicity of PAP. In addition, we observed depletion of both renal and hepatic GSH after PAP administration in vivo (Shao and Tarloff, 1996), consistent with the concept that PAP undergoes GSH conjugation in liver. The differences observed between our results and those of Klos and coworkers (1992) may relate to differences between Sprague-Dawley and Wistar rats and to differences in experimental procedures.

We anticipated that sulfate conjugates of PAP would be formed by hepatocytes and prepared synthetic sulfate conjugates for mass spectral comparison. However, we did not find peaks corresponding to proposed sulfate conjugates and we were able to account for virtually all of the PAP originally present in our incubations. Thus, we suggest that in our hands, rat hepatocytes do not form sulfate conjugates from PAP. The absence of sulfate conjugates is in contrast to a previous report in which detectable amounts of a sulfate conjugate of PAP were identified during incubations with hepatocytes from Wistar rats.
The pattern of PAP-Gluc formation was interesting in that low concentrations were present during the first 3 h of the incubation and the amount of PAP-Gluc increased appreciably during the last 2 h. Although we did not measure concentrations of GSH or cysteine in our hepatocytes, it is possible that formation of PAP-Gluc increased in response to decreases in hepatocyte thiol concentrations.

FPAP was a unique metabolite of PAP. The results of our experiments support the postulated structure for FPAP but the mechanism of formation is not clear. Yoshida and coworkers observed p-chloroformanilide as a metabolite of chloroaniline formed during metabolism of p-chloronitrobenzene (Yoshida et al., 1991, 1992). They postulated that p-chloroformanilide was a product of thermal degradation of an acidic p-chloroaniline metabolite. S-Triazo[3,4]phthalazine is a minor metabolite of hydralazine that may be formed through formylation and cyclization reactions (Noda et al., 1979; Lacagnin et al., 1986). Alternatively, FPAP may be formed through nonenzymatic combination of PAP with carbon dioxide. Carbon dioxide may combine nonenzymatically with primary and secondary aliphatic amines to form carbamic acids (Straub et al., 1988; Tremine et al., 1989). In turn, carbamic acids may form the corresponding carboxyl-O-glucuronides via UDP-glucuronosyltransferase. Carbamic acids and carboxym-y-O-glucuronides are sensitive to pH and temperature and are readily degraded. Carbon dioxide is a natural component of biological systems as well as a portion of the gas mixture (O2/CO2) used during hepatocyte incubations, and it is possible the FPAP was formed by degradation of PAP-carbamic acid formed nonenzymatically through combination of PAP with carbon dioxide.

Importantly, hepatocyte viability was unaltered with PAP in the incubation medium. This observation is consistent with the known cytotoxicity of PAP for renal tubular epithelial cells (Newton et al., 1982; Evelo et al., 1984) and is in contrast to the notion of autoxidation contributing to PAP cytotoxicity. The lack of toxicity toward rat hepatocytes may be due to different pathways of metabolism in renal tubular cells or enhanced detoxification processes in hepatocytes. However, lack of toxicity in hepatocytes is at variance with the notion that PAP-GSHs are cytotoxic. Because hepatocytes were exposed to significant amounts of PAP-GSH, it is reasonable to expect that some cytotoxicity should occur. Potential explanations for lack of toxicity due to PAP-GSHs include lower concentrations of γ-glutamyl transpeptidase in liver (J. B. Tarloff and S. C. Ring, unpublished observations) and dilution of PAP-GSHs in the incubation medium, possibly preventing these conjugates from reaching toxic concentrations in hepatocytes.

Oxidation of PAP, either enzymatic or nonenzymatic, occurs before conjugation. Several enzymes may mediate oxidative metabolism of PAP, including cytochromes P450 and prostaglandin H synthase (Calder et al., 1979; Josephy et al., 1983). Our data suggest that cytochromes P450 are not involved in PAP metabolism because inclusion of ABT, a suicide substrate inhibitor of cytochrome P450, did not alter the extent or pathways of PAP metabolism. These observations are consistent with our previous results in renal tubules, wherein ABT failed to attenuate cytotoxicity due to hepatic bioactivation of PAP (Shao et al., 1997). The role of cytochromes P450 in PAP-induced nephrotoxicity is unclear. Calder and coworkers (1979) suggested that cytochromes P450 were not involved in PAP nephrotoxicity after observing inconsistent effects with inhibitors and inducers of this enzyme system. In contrast, Klos and coworkers found that inhibition of cytochromes P450 by SKF525A reduced the cytotoxicity of PAP in isolated proximal tubule cells from Wistar rats (Klos et al., 1992). It is possible that PAP is metabolized differently in liver and kidney and that hepatic metabolism of PAP does not require cyto-
chromes P450 whereas renal PAP metabolism is cytochrome P450-dependent.

In conclusion, we found that hepatocytes rapidly metabolized PAP to two major products, PAP-GSH and PAP-NACys. Cytochrome P450-dependent oxidation of PAP was not apparent because a suicide substrate inhibitor of cytochromes P450, ABT, failed to alter the metabolic profile. Quantitatively, PAP-GSH are formed in sufficient amounts to account for the nephrotoxicity of PAP. PAP-GSHs are at least equitoxic, if not more toxic, than PAP itself. Even at relatively high concentrations (2.3 mM), PAP was not cytotoxic to hepatocytes, possibly due to the rapid and efficient metabolism. These studies lend credence to the idea that PAP-GSHs are nephrotoxic.

References


