Acetaminophen Binds to Mouse Hepatic and Renal DNA at Human Therapeutic Doses

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Alkylation of DNA by acetaminophen metabolites has been reported previously, but has received little attention, and the biological impact of this alkylation is essentially unknown. In the present study, apparent covalent binding of acetaminophen metabolites to DNA in male ICR mice was observed at levels of 2.0 ± 0.4 to 18.5 ± 5.5 pmol of acetaminophen/mg of DNA in liver and 0.6 ± 0.1 to 26.9 ± 2.6 pmol of acetaminophen/mg of DNA in kidney with doses ranging from 10 to 400 mg/kg. Investigations of the reaction of [^{3}H]-*N*-acetyl-*p*-benzoquinone imine (NAPQI) or [*ring*-^{14}C]NAPQI with DNA *in vitro* yielded low levels of DNA alkylation. Greater apparent binding of [^{3}H]NAPQI to DNA occurred in reactions containing nuclear proteins, such as by using chromatin or whole nuclei. The binding of NAPQI to purified DNA also was enhanced by the presence of 0.1 mM cysteine, but not by 1.0 mM cysteine. Increased binding of NAPQI to DNA in the presence of cysteine or nuclear protein is in contrast to the effects of alternate sulfhydryls on the binding of NAPQI to proteins, which implies that the mechanisms responsible for binding to DNA may be different than the mechanisms that mediate alkylation of protein. The alkylation of DNA by [*ring*-^{14}C]NAPQI was enhanced markedly at buffer pH <4.0, suggesting participation of a protonated form of NAPQI in binding to DNA under these conditions. Acetaminophen binding to DNA also was assessed in metabolic activation systems, including microsomes with cumene hydroperoxide or NADPH, and with horseradish peroxidase (HRP) and H_2O_2. Measurable binding was obtained in all systems, but HRP and H_2O_2 produced binding levels 200-fold greater than was observed with the microsomal systems. The ^{32}P-postlabeling of DNA from acetaminophen-treated mice, and of DNA reacted with acetaminophen, HRP, and H_2O_2, produced unique spots that were not identical. The present data further support the hypothesis that acetaminophen metabolites bind covalently to DNA and demonstrate that this apparent binding is observed in experimental animals *in vivo* at doses that mimic therapeutic doses in humans.

Introduction

Acetaminophen is used extensively as an analgesic and antipyretic and appears to be safe if used in normal therapeutic doses. However, large doses of acetaminophen produce hepatic and/or renal injury in humans and in experimental animals (*1*, *2*). Although the molecular mechanisms that mediate the observed acute toxicities are not universally agreed upon, little or no cell or tissue damage has been observed in the absence of alkylation of cellular proteins by chemically reactive metabolites of acetaminophen (*3*). In turn, substantive protein alkylation is not observed without marked depletion of cellular GSH (*4*, *5*). Because the alkylating metabolites of acetaminophen are produced at all doses, the GSH-dependent threshold to protein binding and toxicity appears to be critical to the safe use of acetaminophen (*4*).

N-Acetyl-*p*-benzoquinone imine (NAPQI)[1] has been proposed as the alkylating intermediate formed during the oxidative metabolism of acetaminophen, and the properties of NAPQI are consistent with many of the properties of the reactive metabolite(s) of acetaminophen (*6*−*9*). However, the observed properties of the reactive metabolite(s) of acetaminophen are not identical to the chemical reactivities of NAPQI (*10*). NAPQI is a soft electrophile that would not be expected to react effectively with the relatively hard nucleophilic sites found in DNA (*11*), but binding of acetaminophen metabolites to DNA *in vivo* might be mediated by mechanisms not involving direct reactions of DNA with NAPQI. One important implication of this hypothesis is that DNA binding by metabolites other than NAPQI would not necessarily be subject to the same GSH-dependent dose threshold, and alkylation of DNA might be observed at doses extending into the therapeutic range. The purpose of the present study was to determine whether measurable levels of binding would be observed at doses below those required to cause acute hepatic necrosis *in vivo*, and to determine the dose−response of binding of acetaminophen metabolites to DNA.

Materials and Methods

Binding of Acetaminophen Metabolites to DNA *in Vivo*. Male ICR mice weighing 18−20 g were purchased from Harlan Sprague-Dawley Inc. (Houston, TX), and maintained in a 12 h light/dark cycle with food and water *ad libitum*. The [^{3}H]-acetaminophen was purchased from Dupont NEN (Boston, MA), purified using a silica Sep-Pak (Millipore, Bedford, MA) by elution with diethyl ether, and assayed for purity by TLC on silica gel plates. The mice were fasted for 18 h prior to administration of [^{3}H]acetaminophen at doses of 10, 50, 100, or

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[1] Abbreviations: NAPQI, *N*-acetyl-*p*-benzoquinone imine; PCI, phenol/chloroform/isoamyl alcohol (25/24/1, v/v); CI, chloroform/isoamyl alcohol (24/1, v/v); CumOOH, cumene hydroperoxide; HRP, horseradish peroxidase.

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400 mg/kg (1.0−1.3 mCi per animal) or equal volumes of saline, ip. Animals were sacrificed 2 h (or 24 h for some of the ^{32}P-postlabeling studies) after dosing while under pentobarbital anesthesia. Tissues were harvested, freeze-clamped, and stored at −70 °C prior to isolation of DNA.

The tissues were homogenized in 9 volumes of protease buffer [10 mM Tris, pH 8.0, 75 mM NaCl, 25 mM EDTA, 1% SDS, and 0.5 mg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN)] and incubated at 55 °C for 2 h. The resultant digests were extracted with equal volumes of PCI (phenol/chloroform/isoamyl alcohol, 25/24/1, v/v), incubated with 25 μg of RNase per 100 mg of tissue at 37 °C for l h, and extracted again with PCI and then CI (chloroform/isoamyl alcohol, 24/1, v/v), and the DNA was precipitated at −20 °C overnight with addition of 100 μL of 3 M NaOAc and 2 mL of 100% EtOH for each milliter of homogenate (*12*). The DNA was pelleted by centrifugation at 10000*g* for 20 min. The pelleted DNA was suspended in 1 mM unlabeled acetaminophen in 20 mM Tris, pH 8.0, 1 mM EDTA (TE buffer), incubated at room temperature for 3 h, and precipitated as described above. The pelleted DNA was redissolved in TE buffer and precipitated again 3 additional times before quantitation of DNA concentrations and contents of radioactivity were measured. The samples of purified DNA were dissolved in TE buffer, the concentrations estimated by the absorbance at 260 nm, and the purity by $A_{260/280}$ ratios and agarose gel electrophoresis (*12*). The contents of radioactivity were measured by liquid scintillation counting, and the levels of acetaminophen−DNA binding are expressed as picomoles of acetaminophen bound per milligram of DNA.

With each series of measurements, controls for nonspecific binding were run in which tissue homogenates prepared from mice not given radiolabel were mixed with radioactive acetaminophen in amounts equivalent to those observed in homogenates from mice that had been treated with [^{3}H]acetaminophen. For the control samples, radiolabeled acetaminophen was added after the protein digestion, but before organic extraction of the DNA. DNA was extracted and purified, and DNA and radiolabel were quantitated as described previously.

Reactions of [^{3}H]- and [*ring*-^{14}C]NAPQI with DNA *in Vitro*. The [*ring*-^{14}C]acetaminophen was purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled acetaminophen was oxidized with freshly prepared silver oxide in CH_3CN, in the dark for 1 h. The resulting NAPQI concentrations were quantitated by reverse-phase HPLC, as described by Dahlin and Nelson (*8*). The NAPQI contained an average of 27% of unoxidized acetaminophen and was not purified further.

Hepatic nuclei and chromatin were prepared following published procedures (*13*, *14*). Male ICR mice were anesthetized with pentobarbital, and their livers were removed and homogenized in 9 volumes of 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris, pH 7.4 (STE buffer), using seven strokes with each pestle in Dounce homogenizers. The homogenates were centrifuged at 700*g* for 15 min, and the pellets were resuspended in STE buffer and homogenized with the A pestle only. The resultant mixtures were centrifuged at 700*g* for 15 min. The pellets obtained were resuspended as before, overlaid on 2.3 M sucrose, and centrifuged at 37000*g* for 30 min. The nuclei were found in a gelatinous band in the center of the tube. The nuclei were removed and resuspended in 10 mM Tris (pH 7.4). Chromatin was prepared from the nuclei by incubation in 1% Triton X-100 in the 10 mM Tris buffer for 1 h. The mixture was centrifuged at 1000*g* for 15 min, and the chromatin in the supernatant was used.

Aliquots of nuclear or chromatin preparations determined to have 10 and 8 mg of protein, respectively (0.4−0.3 mg of DNA), or 1 mg of salmon testes DNA were incubated with [^{3}H]NAPQI (500 dpm/pmol). Purified salmon testes DNA also was incubated with [^{3}H]NAPQI in the presence of 0, 0.1, or 1.0 mM cysteine at 37 °C for 1 h. The effects of cysteine on binding to DNA *in vitro* also were investigated with [*ring*-^{14}C]NAPQI (4.7 ± 0.7 nmol, 18 dpm/pmol) and calf thymus DNA. DNA was isolated as described above, using three cycles of precipitation and dissolution. Controls for the experiments were conducted by adding [^{3}H]- or [*ring*-^{14}C]acetaminophen to the DNA at the same specific activities as used for the NAPQI, but in 5 times the molar amounts as used in the NAPQI incubations. In these control experiments, no radioactivity above background (less than 30 cpm) was found in DNA extracted from purified DNA, nuclei, or chromatin.

To calf thymus DNA (1 mg) in 100 mM KPO_4 at pH from 1.0 to 7.4 was added 7.2 nmol (1.6×10^4 dpm/nmol) of [*ring*-^{14}C]-NAPQI. After 1 h at 37 °C, the DNA was precipitated, centrifuged, and redissolved in 10 mM acetaminophen. These solutions were incubated for an additional 1 h at 37 °C to minimize nonspecific binding. The DNA was precipitated and dissolved 3 consecutive times and binding estimated as described above.

Binding of [^{3}H]Acetaminophen to DNA with Metabolic Activation *in Vitro*. Exposures of DNA to [^{3}H]acetaminophen *in vitro* were conducted in total volumes of 2 mL of 10 mM potassium phosphate, (pH 7.4), containing 2 mg of calf thymus DNA. The [^{3}H]acetaminophen, with a specific activity of 2000 dpm/pmol, was added to yield a final concentration of 100 μM. Rat liver microsomes (2 mg of protein), 1.5 mM cumene hydroperoxide (CumOOH) or 5 mM NADPH, or 15 units of horseradish peroxidase (HRP) [Sigma type VI-A; activity determined by 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] and 1 mM H_2O_2 were added to samples, as indicated (*15*). After incubation with agitation at 37 °C for 2 h, reactions were terminated by addition of 2 mL of cold acetonitrile. DNA was extracted by protein digestion, extractions, and ethanol precipitation.

^{32}P-Postlabeling. The apparent formation of acetaminophen−DNA adducts, generated *in vivo* and *in vitro*, was assessed by the nuclease P1-enhanced bisphosphate version of the ^{32}P-postlabeling assay, described by Reddy and Randerath (*16*). Briefly, 10 μg of DNA was digested with a mixture of micrococcal nuclease and spleen phosphodiesterase to deoxyribonucleoside 3′-monophosphates. After treatment with nuclease P1 to hydrolyze normal 3′-nucleotides to nucleosides, adducted 3′-nucleotides were converted to 5′-[^{32}P]deoxyribonucleotide 3′,5′-bisphosphates by T4 polynucleotide kinase-catalyzed transfer of [^{32}P]phosphate from [γ-^{32}P]ATP. The labeled digests were then treated with potato apyrase to convert unused γ-^{32}P to [^{32}P]-P_i. Labeled products were purified by multidirectional PEI−cellulose TLC. An initial development was performed with 2.3 M sodium phosphate to remove [^{32}P]phosphate and traces of labeled impurities. The chromatogram was autoradiographed, and the adducts retained at the origin were contact-transferred to fresh PEI−cellulose sheets and mapped by two-dimensional TLC. The solvents used were 3.4 M lithium formate, 6 M urea, pH 3.3 (D3), and 0.49 M sodium phosphate, 4.9 M urea (D4). Under these conditions, the samples produced *in vivo* demonstrated faint spots by autoradiography, but the samples generated *in vitro* showed most of the radioactivity remaining at the origin. All samples were rechromatographed with 4.2 M lithium formate, 7.5 M urea, pH 3.3 (D3), and with 0.7 M sodium phosphate, 7 M urea, pH 6.4 (D4).

Statistics. Data are presented as means ± SE and were evaluated statistically by one-way analysis of variance with Student−Newman−Keuls (*17*, *18*). Differences are indicated at $p < 0.05$.

Results

Male ICR mice given doses of [^{3}H]acetaminophen of 10, 50, 100, or 400 mg/kg showed levels of apparent binding of radiolabel to hepatic and renal DNA 2 h after dosing that were measurable at the lowest doses and increased with the dose of acetaminophen (Figures 1 and 2). The estimates of binding presented in Figures 1 and 2 are based on several hundred cpm in samples of approximately 0.5 mg of DNA each, whereas our background counts were less than 30 cpm. In control studies, we added radiolabeled acetaminophen to protease-treated tissue homogenates from untreated animals in amounts

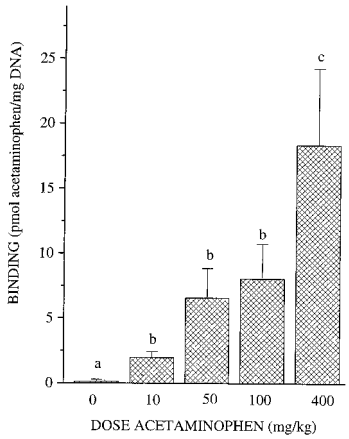

Figure 1. Binding of [^{3}H]acetaminophen to hepatic DNA. Male ICR mice, 23−30 g, were fasted for 18 h and given the indicated doses of [^{3}H]acetaminophen, ip. Tissues were harvested, freeze-clamped, and stored at −70 °C. Ten percent (w/v) homogenates were prepared in protease buffer, and DNA was isolated by organic extraction (see Materials and Methods). The data are means ± SE of $n = 3-7$ animals per group. Experimental groups not sharing a common letter are different (one-way ANOVA, Student−Newman−Keuls, $p < 0.05$).

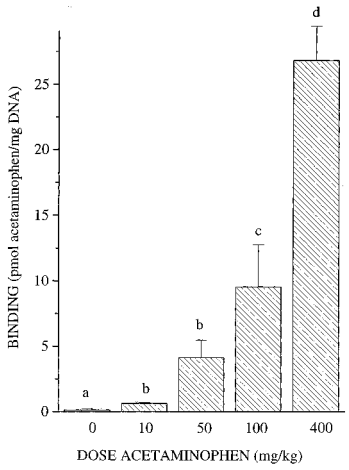

Figure 2. Binding of [^{3}H]acetaminophen to renal DNA. Binding to renal DNA was measured in the same mice described in Figure 1.

equivalent to those observed in homogenates prepared from animals treated with [^{3}H]acetaminophen *in vivo*. In these control experiments, we observed no measurable radioactivity above background (less than 30 cpm), indicating that the methods of DNA isolation we employed in the present studies were effective in removing unbound radioactivity from the DNA.

Addition of [^{3}H]acetaminophen to DNA in buffer produced no measurable alkylation of the DNA. Synthetic [*ring*-^{14}C]NAPQI or [^{3}H]NAPQI added to purified DNA in buffer did produce measurable levels of apparent alkylation of the DNA. However, addition of [^{3}H]NAPQI to isolated hepatic nuclei, or to chromatin isolated from hepatic nuclei, produced greater alkylation of the DNA than when identical amounts of labeled NAPQI were added to equal amounts of purified DNA *in vitro* (Figure 3).

In other experiments, addition of [*ring*-^{14}C]NAPQI to DNA in buffer in the presence of 0.1 mM cysteine also resulted in greater binding to the DNA than was observed without cysteine (Figure 4). Increasing the cysteine concentration to 1 mM resulted in levels of DNA alkylation that were significantly lower than were observed in the presence of 0.1 mM cysteine. The levels of

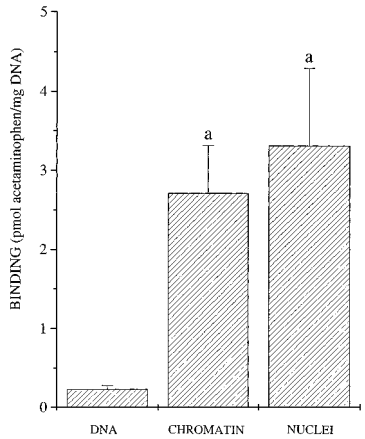

Figure 3. Binding of [^{3}H]NAPQI to DNA *in vitro*. The [^{3}H]-NAPQI was synthesized as described under Materials and Methods and added to solutions containing 1 mg of DNA, as purified DNA alone, or chromatin (8 mg of chromatin protein), or isolated hepatic nuclei (10 mg of protein), containing 0.3−0.4 mg of DNA, and incubated for 1 h at 37 °C. Following incubation, DNA was isolated by homogenization in protease buffer and organic extraction. The measured levels of binding to DNA were greater from the nuclear and chromatin preparations than from the purified DNA. Data are means ± SE, $n = 6$ (one-way ANOVA, Student−Newman−Keuls, $p < 0.05$).

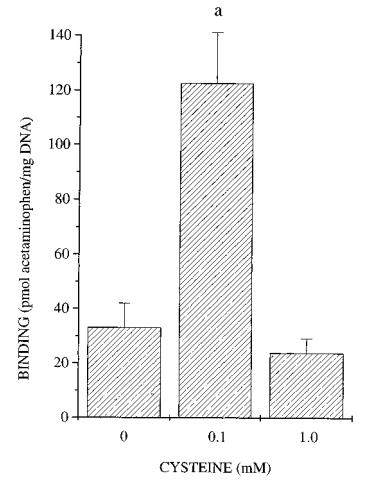

Figure 4. Effects of cysteine upon binding of [*ring*-^{14}C]NAPQI to DNA. The [*ring*-^{14}C]NAPQI was synthesized as described under Materials and Methods, and 4.7 ± 1.1 nmol of [*ring*-^{14}C]-NAPQI/mg of DNA was added to 1 mg of calf thymus DNA in the presence of 0, 0.1, or 1 mM cysteine and incubated at 37 °C for 1 h. Levels of binding of [*ring*-^{14}C]NAPQI to DNA in incubations containing 0.1 mM cysteine were higher than were observed with DNA alone or in the presence of 1.0 mM cysteine. The same pattern of binding also was observed in reactions with [^{3}H]NAPQI (data not shown). Data are means ± SE, $n = 6$, reflecting duplicate incubations in three independent experiments, and were assessed statistically by one-way ANOVA, Student−Newman−Keuls, $p < 0.05$.

binding of labeled NAPQI to DNA alone presented in Figure 4 are noticeably greater than the levels of binding to DNA alone in buffer presented in Figure 3. This difference is attributed to the use of different molar amounts of radiolabeled NAPQI per milligram of DNA in the two series of studies. In the studies described in Figure 4, we measured the concentrations of NAPQI by HPLC using the method described by Dahlin and Nelson (*8*) and in the three independent experiments added 4.7 ± 1.1 nmol of [*ring*-^{14}C]NAPQI per milligram of DNA. In the studies described in Figure 3, absolute concentrations of NAPQI added were not measured, but in each

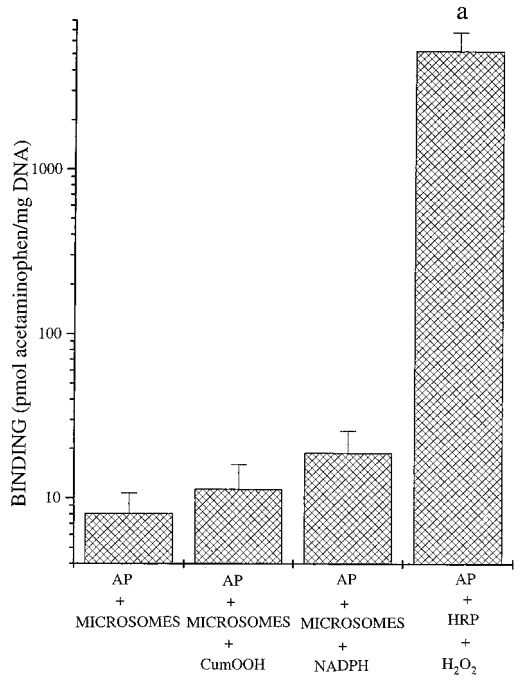

Figure 5. Binding of acetaminophen metabolites to DNA *in vitro*. Calf thymus DNA (2 mg) was incubated with [^{3}H]-acetaminophen in 2 mL of 10 mM KPO_4, in the presence of microsomes (2 mg protein), microsomes + 1.5 mM CumOOH, microsomes + 5 mM NADPH, or 15 units of HRP and 1 mM H_2O_2. The mixtures were shaken at 37 °C for 2 h, at which time the reactions were terminated by addition of 2 mL of cold acetonitrile. DNA was isolated by incubation in protease buffer and organic extraction. Note the log scale. Binding of [^{3}H]-acetaminophen to DNA was greater in the samples incubated in the presence of HRP and H_2O_2 than in the other samples. The data are means ± SE of $n = 4$ (one-way ANOVA, Student−Newman−Keuls, $p < 0.05$).

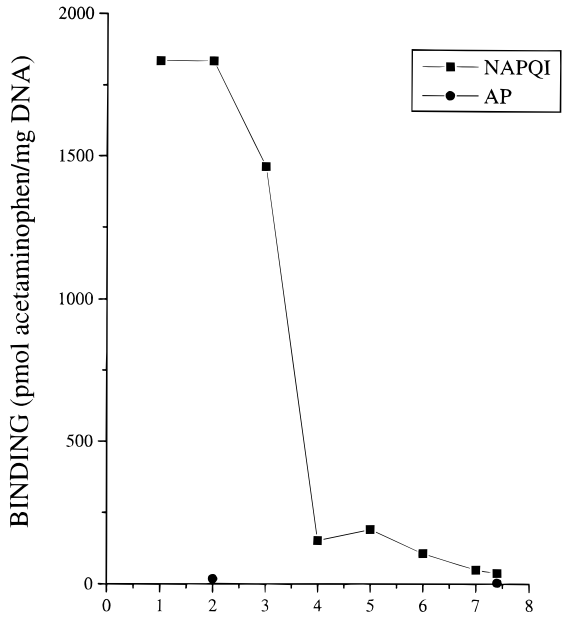

Figure 6. Effects of buffer pH on binding of [*ring*-^{14}C]NAPQI to DNA. The [*ring*-^{14}C]NAPQI was synthesized as described under Materials and Methods, and 4 nmol was added to 1 mg of calf thymus DNA in 100 mM $KHPO_4$ at the pH indicated. Binding to DNA of [*ring*-^{14}C]acetaminophen also was assessed at pH 2.0 and 7.4 using 40 nmol of acetaminophen. After 1 h at 37 °C, DNA was isolated and binding estimated as described under Materials and Methods. Data are means of duplicate determinations.

experiment of both series of studies, equal amounts of NAPQI were added to the three sources or solutions of DNA being compared. In addition, we determined the binding of [^{3}H]NAPQI to DNA in the presence of 0, 0.1, or 1.0 mM cysteine and found that addition of 6.0 nmol of [^{3}H]NAPQI (measured by HPLC as described) gave levels of binding to DNA [16.9 ± 11.4 (mean ± SD, n = 2)] and responses to addition of cysteine (data not shown) that are similar to those obtained with [*ring*-^{14}C]NAPQI and presented in Figure 4.

The apparent binding of DNA to [^{3}H]acetaminophen also could be stimulated *in vitro* by incubation with metabolic activation systems. Labeled acetaminophen incubated with DNA and hepatic microsomes in the presence of NADPH or CumOOH, or with HRP and H_2O_2, also resulted in the apparent alkylation of DNA (Figure 5). The levels of binding observed with the HRP−H_2O_2 system were much (200-fold) greater than the levels of binding produced by the microsomal activation systems, but none of these experimental models were optimized systematically.

We examined the pH profile of binding of [*ring*-^{14}C]-NAPQI to DNA between pH 7.4 and 1.0. Marked increases in binding of [*ring*-^{14}C]NAPQI to DNA were observed at buffer pH below 4.0 (Figure 6). Estimates of binding of [*ring*-^{14}C]acetaminophen to DNA in these studies were below the limits of detection both at pH 2.0 and at pH 7.4.

DNA adduct formation by acetaminophen metabolites also was investigated using the ^{32}P-postlabeling assay of Reddy and Randerath (*16*). Administration of acetaminophen to mice did not lead to readily observable ^{32}P-labeled spots not observed in control animals (Figure 7A), although a faint spot (spot 1) was observed 2 h after dose (Figure 7B). At 24 h after administration of acetaminophen, spot 1 was more readily detected (Figure 7C), and other faint spots (spots 2, 3, and 4) were observed. The unmarked spots in control animals represent I-compounds, which are age-dependent endogenous DNA modifications occurring in rodent tissues (*19*). Incubation of acetaminophen with calf thymus DNA in the presence of HRP and H_2O_2 *in vitro*, and chromatographed under the same conditions, showed most of the radioactivity remaining at the origin (Figure 7D). The samples were rechromatographed with more strongly eluting solvents. The samples generated from acetaminophen administration *in vivo* showed no additional spots (data not shown), but samples generated from HRP and H_2O_2 *in vitro* demonstrated the formation of a spot (spot 5, Figure 7F) that was not observed in control incubations containing acetaminophen and DNA without HRP and H_2O_2 (Figure 7E). The control samples showed three spots, one of which appears to have been enhanced by incubation with HRP and H_2O_2. The spots observed in these studies from the incubations *in vitro* were different chromatographically from the spots observed from DNA of animals exposed to acetaminophen *in vivo*.

Discussion

The formation of the apparent acetaminophen−DNA adducts *in vivo* does not depend upon glutathione depletion, as has been reported previously for the substantial binding of acetaminophen metabolites to protein that accompanies acute cellular necrosis (*4*, *5*). However, acetaminophen binding to protein is observed at doses that do not produce measurable depletion of cellular glutathione, hepatic necrosis, or cell death, which sug-

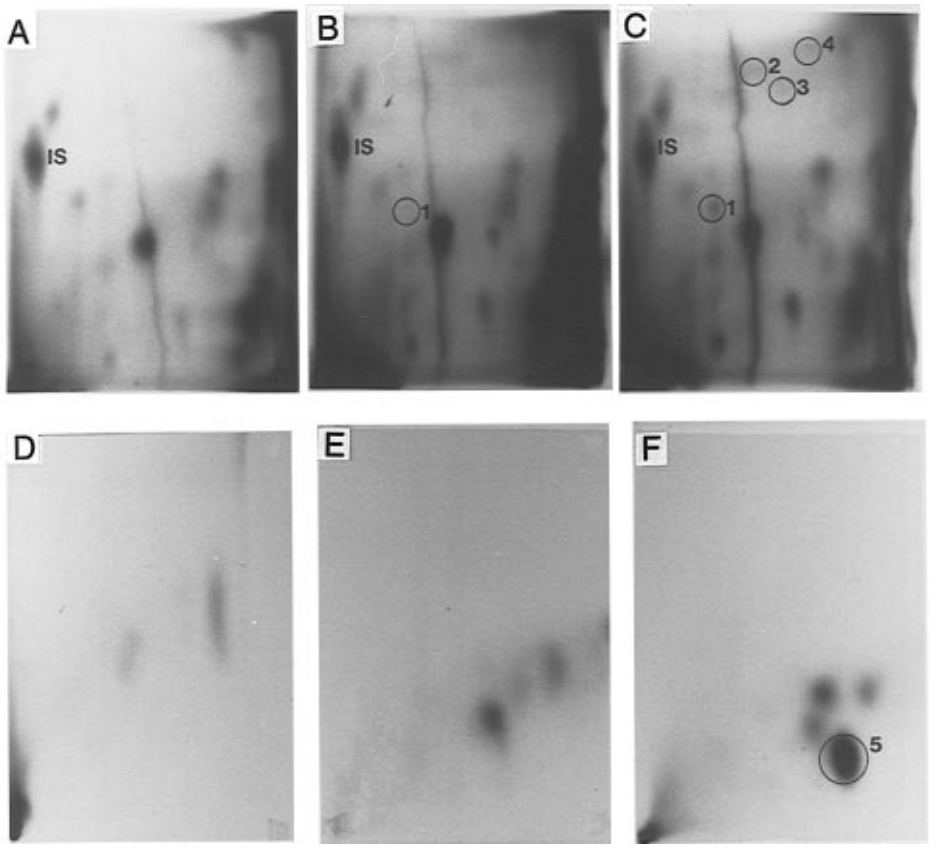

Figure 7. Acetaminophen-induced DNA modifications assessed by ^{32}P-postlabeling. ^{32}P-Postlabeling was carried out using DNA isolated from livers obtained from mice 2 h after administration of saline (A) or 2 h (B) or 24 h (C) after administration of 400 mg/kg of acetaminophen, ip. The lower panels present postlabeling analysis of DNA that was incubated with acetaminophen only (E) or with acetaminophen in the presence of HRP and H_2O_2 (D, F). Panel D represents the postlabeling analysis of the same sample as panel F but under the chromatographic conditions used for the samples in panels A, B, and C. Panels E and F represent control and HRP- and H_2O_2-exposed DNA samples chromatographed with stronger solvent conditions. The solvents used for the 2-dimensional TLC are specified under Materials and Methods. The TLC maps were autoradiographed with Kodak XAR-5 film with intensifying screens for 24 h at −70 °C for the samples from the studies *in vivo*, and 6 h for the samples from the incubation *in vitro*. The internal standard (IS) adduct spot was derived from DNA obtained from mice treated with dibenz[*a*,*j*]acridine. The standard DNA was mixed with sample DNA prior to enzyme digestion to monitor labeling efficiency.

gests that this low-level binding to protein may be mediated by mechanisms that are different from the mechanisms responsible for the more extensive binding that is associated with acute cell necrosis. Hepatotoxic doses of acetaminophen result in 90% of [^{14}C]-acetaminophen associated with hepatic proteins bound to cysteine residues in the form of 3-cysteinylacetaminophen (70%) or 3-cysteinylhydroquinone (20%) adducts (*20*−*22*). However, the nature of the remaining 10% of bound radiolabel has not been characterized and may arise from mechanisms that are similar to those responsible for DNA alkylation.

The greater extent of alkylation of DNA by NAPQI in the presence of alternative nucleophiles such as the proteins in isolated nuclei or nuclear chromatin (Figure 3) or 0.1 mM cysteine (Figure 4) contrasts with the expected effect of simple competition of two types of nucleophiles for an electrophilic alkylating intermediate. The enhancement of binding of NAPQI to DNA by the presence of nuclear proteins suggested the possibility of formation of acetaminophen−protein−DNA cross-links, but studies with modified electrophoretic mobility shift assays did not support this hypothesis (*23*). The decrease in binding to DNA associated with increasing cysteine concentrations from 0.1 to 1 mM suggests competitive disposition of the intermediates responsible for binding to DNA, but this hypothesis requires further investigation.

Peroxidase-mediated production of the acetaminophen-derived phenoxyl radical has been proposed by several investigators (*24*−*27*), and the levels of binding of acetaminophen to DNA effected by HRP−H_2O_2 are consistent with the hypothesis that radical intermediates are significant contributors to the binding. The phenoxyl radical also may arise from one-electron reduction of NAPQI or related species by cysteine or other reductants, or by comproportionation of NAPQI and acetaminophen (*27*).

Acid catalysis of DNA alkylation has been described for other electrophilic intermediates (*28*−*30*). Kadlubar et al. (*30*) demonstrated increased formation of reactive metabolites of *N*-hydroxy-2-naphthylamine and its *N*-glucuronide by lowering the pH of rat urine from 6.7 to 5.7. Lamm and Pack (*31*) have calculated proton concentrations in microenvironments around DNA, and have predicted effective pH values in some domains to approach 4.0. In our studies, marked increases in alkylation of DNA by [^{14}C]-NAPQI were observed at pH 3.0 or below (Figure 6), which is below the range suggested by the calculations of Lamm and Pack (*31*). The more subtle increase in apparent alkylation of DNA associated with decreasing pH through 4.0, suggested by the data in Figure 6, may reflect a process of greater biological relevance. In contrast, no binding of [^{14}C]-acetaminophen was observed even at pH 2.0.

A major concern in the present studies was the possibility that the apparent alkylation of DNA resulted

from contamination or another artifact, rather than true covalent binding. However, control experiments indicated consistently that contamination of the DNA by unbound radiolabel was unlikely to have contributed significantly to the apparent binding to DNA. Concerns that tritium exchange is a significant contributor to measured levels of binding to DNA are minimized by the studies with [*ring*-^{14}C]NAPQI, which provided results similar to those obtained with [^{3}H]acetaminophen.

Treatment of mice with acetaminophen *in vivo* did not elicit formation of readily detected ^{32}P-postlabeled adducts, although a few unique spots suggestive of adducts were observed, albeit faintly (Figure 7B,C). Detection of ^{32}P-postlabeled adducts has been reported by Hasagawa et al. (*32*) in incubations of calf thymus DNA with NAPQI, but they observed no adducts in hepatic DNA from Fisher-344 rats fed diets containing 0.1−1.5% acetaminophen for 1 week or from rats given 1 g/kg of acetaminophen by gastric intubation 6 h prior to sacrifice.

The intensities of the spots indicated by ^{32}P-postlabel analyses are lower than might otherwise be expected from the levels of the putative acetaminophen−DNA adducts suggested by studies with radiolabeled drug (*33, 34*). The primary concern, that the apparent binding indicated by experiments with radiolabeled drug is artifactual, increasingly appears to be unlikely. Another hypothesis is that the adducts indicated by experiments with labeled acetaminophen are not effective substrates for one or more of the hydrolysis or kinase reactions essential to postlabeling analysis (*35*). In preliminary studies, we observed that omission of the nuclease P1 digestion of DNA samples derived from acetaminophen-treated mice resulted in the detection of additional spots not detected when treatment with nuclease P1 was included, nor were these spots found in samples from animals not treated with acetaminophen, with or without nuclease P1 digestion (data not shown). The compounds responsible for the new spots observed in these experiments required chromatographic conditions than are notably different than those employed for other adducts, and additional separation studies are needed. In other studies, extraction with 1-butanol has been used to increase the sensitivity of detection of adducts derived from highly lipophilic parent compounds **(***36*). However, acetaminophen is much less lipid-soluble than the compounds for which butanol extraction has proved useful, and we have not investigated this variation of the postlabeling method.

The ^{32}P-postlabeled spots we observed from exposures to acetaminophen *in vivo* were different chromatographically from those observed *in vitro*, which indicates that the apparent adducts formed in the two systems are not identical. Stronger solvents were required to elute the adducts generated *in vitro* than were needed for the adducts characterized from exposure to acetaminophen *in vivo*, which indicates that the adducts suggested by the studies *in vivo* are more polar than are the apparent adducts generated *in vitro*. Chromatography of the nucleotide fractions from the DNA samples generated following exposure to acetaminophen *in vivo* under the conditions used for the samples generated *in vitro* did not reveal any new spots or apparent adducts, suggesting that the metabolites responsible for the adducts produced *in vitro* either were not formed *in vivo* or, if formed, were metabolized further. In addition, we may not have identified the appropriate chromatographic conditions for resolution of adducts that might be formed.

Evidence of the genotoxicity of acetaminophen, including chromosomal aberrations, induction of single-strand breaks, increases in sister chromatid exchanges, and inhibition of replicative DNA synthesis, has been reported from studies both in animals and in cultured cells, but thus far these studies have been limited to hepatotoxic or cytotoxic doses (*33, 34, 37, 38*). Exposure of experimental animals to hepatotoxic doses of acetaminophen for extended periods has produced increased incidences of tumors in some studies (*39, 40*), whereas no increases were observed in other studies (*41*). Studies in human volunteers have provided evidence that therapeutic doses of acetaminophen cause chromosomal breaks and aberrations, but epidemiological studies have revealed no association of acetaminophen intake with tumor incidence in humans (*42, 43*).

The mixed reports of genotoxicity and carcinogenicity from exposure to acetaminophen suggest that acetaminophen is at most very weakly genotoxic. However, a correlation between cumulative lifetime dose of acetaminophen and end stage renal disease has been reported (*44*). The present evidence of covalent binding to DNA of [^{3}H]acetaminophen at doses that are equivalent to those commonly used therapeutically in humans is interesting in light of this report of chronic toxicity associated with acetaminophen exposure (*44*), and our studies may in part offer an explanation for the correlation between lifetime cumulative dose of acetaminophen and end stage renal disease.

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References

(1) Mitchell, J. R., Jollow, D. J., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. (1973) Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J. Pharmacol. Exp. Ther.* **187**, 185−194.

(2) Black, M. (1984) Acetaminophen hepatotoxicity. *Annu. Rev. Med.* **35**, 577−593.

(3) Smith, C. V., Lauterburg, B. H., and Mitchell, J. R. (1985) Covalent binding and acute lethal injury in vivo: how has the hypothesis survived a decade of critical examination? *Drug Metabolism and Disposition: Considerations in Clinical Pharmacology* (Wilkinson, G., and Rawlins, M. D., Eds.) pp 161−181, MTP Press, London.

(4) Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillette, J. R., and Brodie, B. B. (1973) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* **187**, 211−217.

(5) Smith, C. V., and Mitchell, J. R. (1989) Pharmacological aspects of glutathione in drug metabolism. *Coenzymes and Cofactors* (Dolphin, D., Poulson, R., and Avramovic, O. Eds.) pp 1−44, John Wiley and Sons, New York.

(6) Nelson, S. D. (1990) Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin. Liver Dis.* **10**, 267−278.

(7) Albano, E., Rundgren, M., Harvison, P. J., Nelson, S. D., and Moldeus, P. (1985) Mechanisms of *N*-acetyl-*p*-benzoquinoneimine cytotoxicity. *Mol. Pharmacol.* **28**, 306−311.

(8) Dahlin, D. C., and Nelson, S. D. (1982) Synthesis, decomposition kinetics, and preliminary toxicological studies of pure *N*-acetyl-*p*-benzoquinone imine, a proposed toxic metabolite of acetaminophen. *J. Med. Chem.* **25**, 885−886.

(9) Coles, B., Wilson, I., Wardman, P., Hinson, J. A., Nelson, S. D., and Ketterer, B. (1988) The spontaneous and enzymatic reaction of *N*-acetyl-*p*-benzoquinoneimine with glutathione: A stopped-flow kinetic study. *Arch. Biochem. Biophys.* **264**, 253−260.

(10) Smith, C. V., and Mitchell, J. R. (1985) Acetaminophen hepatotoxicity in vivo is not accompanied by oxidant stress. *Biochem. Biophys. Res. Commun.* **133**, 329−336.

(11) Pearson, R. G., and Songstad, J. (1967) Application of the principle of hard and soft acids and bases to organic chemistry. *J. Am. Chem. Soc.* **89**, 1827−1836.
(12) Anonymous (1992) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, New York.
(13) Nicotera, P., McConkey, D. J., Jones, D. P., and Orrenius, S. (1989) ATP stimulates Ca^{2+} uptake and increases the free Ca^{2+} concentration in isolated rat liver nuclei. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 453−457.
(14) Gajewski, E., Rao, G., Nackerdiem, Z., and Dizdaroglu, M. (1990) Modification of DNA bases in mammalian chromatin by radiation-generated free radicals. *Biochemistry* **29**, 7876−7882.
(15) Pathak, D. N., Pongracz, K., and Bodell, W. J. (1995) Microsomal and peroxidase activation of 4-hydroxytamoxifen to form DNA adducts: comparison with DNA adducts formed in Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis* **16**, 11−15.
(16) Reddy, M. V., and Randerath, K. (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* **7**, 1543−1551.
(17) Zar J. H. (1984) *Biostatistical Analysis. 2*, Prentice-Hall, Englewood Cliffs, NJ.
(18) Norusis, M. J. (1992) *SPSS for windows. Advanced Statistics. Release 5*, SPSS Inc., Chicago.
(19) Randerath, K., Reddy, M. V., and Disher, R. M. (1980) Age- and Tissue-related DNA modifications in untreated rats: detection by ^{32}P-postlabeing assay and possible significance for spontaneous tumor induction and aging. *Carcinogenesis* **9**, 2285−2289.
(20) Streeter, A. J., Dahlin, D. C., Nelson, S. D., and Baillie, T. A. (1984) The covalent binding of acetaminophen to protein. Evidence of cysteine residues as major sites of arylation in vitro. *Chem.-Biol. Interact.* **48**, 349−366.
(21) Hoffmann, K. J., Streeter, A. J., Axworthy, D. B., and Baillie, T. A. (1985) Identification of the major covalent adduct formed in vitro and in vivo between acetaminophen and mouse liver proteins. *Mol. Pharmacol.* **27**, 566−573.
(22) Pascoe, G. A., Calleman, C. J., and Baillie, T. A. (1988) Identification of *S*-(2,5-dihydroxyphenyl)cysteine and *S*-(2,5-dihydroxyphenyl)-*N*-acetylcysteine as urinary metabolites of acetaminophen in the mouse. Evidence for *p*-benzoquinone as a reactive intermediate in acetaminophen metabolism. *Chem.-Biol. Interact.* **68**, 85−98.
(23) Chang, A., McQuinn, T. C., Rogers, L. K., and Smith, C. V. (1994) Acetaminophen metabolite alterations of DNA *in vivo* and *in vitro*. *Pediatr. Res.* **35**, 82A (Abstr.).
(24) Nelson, S. D., Dahlin, D. C., Rauckman, E. J., and Rosen, G. M. (1981) Peroxidase-mediated formation of reactive metabolites of acetaminophen. *Mol. Pharmacol.* **20**, 195−199.
(25) Potter, D. W., Miller, D. W., and Hinson, J. A. (1986) Horseradish peroxidase-catalyzed oxidation of acetaminophen to intermediates that form polymers or conjugate with glutathione. *Mol. Pharmacol.* **29**, 155−162.
(26) Potter, D. W., Miller, D. W., and Hinson, J. A. (1985) Identification of acetaminophen polymerization products catalyzed by horseradish peroxidase. *J. Biol. Chem.* **260**, 12174−12180.
(27) Potter, D. W., and Hinson, J. A. (1987) Mechanisms of acetaminophen oxidation to *N*-acetyl-*p*-benzoquinone imine by horseradish peroxidase and cytochrome P-450. *J. Biol. Chem.* **262**, 966−973.
(28) Turesky, R. J., Lang, N. P., Butler, M. A., Teitel, C. H., and Kadlubar, F. F. (1991) Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carcinogenesis* **12**, 1839−1845.
(29) Kadlubar, F. F., and Beland, F. A. (1985) Chemical properties of ultimate carcinogenic metabolites of arylamines and arylamides. *Polycyclic Hydrocarbons and Carcinogenesis* (Harvey, R. G., Ed.) pp 341−370, American Chemical Society, Washington, D.C.
(30) Kadlubar, F. F., Unruh, L. E., Flammang, T. J., Sparks, D., Mitchum, R. K., and Mulder, G. J. (1981) Alteration of urinary levels of the carcinogen, *N*-hydroxy-2-naphthylamine, and its glucuronide in the rat by control of urinary pH, inhibition of metabolic sulfation, and changes in biliary excretion. *Chem.-Biol. Interact.* **33**, 129−147.
(31) Lamm, G., and Pack, G. R. (1990) Acidic domains around nucleic acids. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9033−9036.
(32) Hasagawa, R., Furukawa, F., Toyoda, K., Jang, J. J., Yamashita, K., Sato, S., Takahashi, M., and Hayashi, Y. (1988) Study for tumor-initiating effect of acetaminophen in two-stage liver carcinogenesis of male F344 rats. *Carcinogenesis* **9**, 755−759.
(33) Honglso, J. K., Smith, C. V., Brunborg, G., Soderlund, E. J., and Holme, J. A. (1994) Genotoxicity of paracetamol in mice and rats. *Mutagenesis* **9**, 93−100.
(34) Dybing, E., Holme, J. A., Gordon, W. P., Soderlund, E. J., Dahlin, D. C., and Nelson, S. D. (1984) Genotoxicity studies with paracetamol. *Mutat. Res.* **138**, 21−32.
(35) Feng, Y., Jiang, W., and Hein, D. W. (1996) 2-Aminofluorene−DNA adduct levels in tumor-target and nontarget organs of rapid and slow acetylator Syrian hamsters congenic at the NAR2 locus. *Toxicol. Appl. Pharmacol.* **141**, 248−255.
(36) Gupta, R. C., and Earley, K. (1988) ^{32}P-adduct assay: compartive recoveries of structually diverse DNA adducts in the various enhancement procedures. *Carcinogenesis* **9**, 1687−1693.
(37) Honglso, J. K., Brogger, A., Bjorge, C., and Holme, J. A. (1991) Increased frequency of sister-chromatid exchange and chromatid breaks in lymphocytes after treatment of human volunteers with therapeutic doses of paracetamol. *Mutat. Res.* **261**, 1−8.
(38) Honglso, J. K., Bjorge, C., Schwarze, P., Brogger, A., Mann, G., Thelander, L., and Holme, J. A. (1990) Paracetamol inhibits replicative DNA synthesis and induces sister chromatid exchange and chromosomal aberrations by inhibition of ribonucleotide reductase. *Mutagenesis* **5**, 475−480.
(39) Flaks, A., and Flaks, B. (1983) Induction of liver cell tumors in IF mice by paracetamol. *Carcinogenesis* **4**, 363−368.
(40) Flaks, B., Flaks, A., and Shaw, A. P. W. (1985) Induction of paracetamol of bladder and liver tumors in the rat. *Acta Pathol. Microbiol. Immunol. Scand.* **A93**, 367−377.
(41) Amo, H., and Matsuyama, M. (1985) Subchronic and chronic effects of feeding of large amounts of acetaminophen in B6C3F1. *Jpn. J. Hyg.* **40**, 567−574.
(42) Kirkland, D. J., Dresp, J. H., Marshall, R. R., Baumeister, M., Gerloff, C., and Gocke, E. (1992) Normal chromosomal aberration frequencies in peripheral lymphocytes of health human volunteers exposed to a maximum daily dose of paracetamol in a double blind trial. *Mutat. Res.* **279**, 181−194.
(43) Kocisova, J., and Sram, R. J. (1990) Mutagenicity studies on paracetamol in human volunteers. III. Cytokinesis block micronucleus method. *Mutat. Res.* **244**, 27−30.
(44) Perneger, T. V., Whelton, P. K., and Klag, M. J. (1994) Risk of kidney failure associated with the use of acetaminophen, aspirin, and nonsteroidal antiinflammatory drugs. *N. Engl. J. Med.* **331**, 1675−1679.

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