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To cite this article: P. David Josephy (2005) The Molecular Toxicology of Acetaminophen, Drug Metabolism Reviews, 37:4, 581-594, DOI: 10.1080/03602530500205200

To link to this article: http://dx.doi.org/10.1080/03602530500205200

Published online: 09 Oct 2008.

Article views: 454
THE MOLECULAR TOXICOLOGY OF ACETAMINOPHEN

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The history of the development of the analgesic drug acetaminophen is reviewed with an emphasis on the characteristics of its overdose toxicity. The P450-catalyzed oxidation of acetaminophen generates a reactive electrophile that binds covalently to proteins. Involvement of specific P450 enzymes in acetaminophen toxicity can be probed by experiments with knock-out mice. The identification of specific target proteins may help to clarify the mechanism of acetaminophen hepatotoxicity.

Key Words: Acetaminophen; hepatotoxicity; covalent binding; P450

INTRODUCTION

Acetaminophen is one of the world’s most widely used nonprescription medicines. The overdose hepatotoxicity of acetaminophen may have been studied more than any other specific instance of chemical toxicity. However, despite the drug’s familiarity, its simple structure, and years of research, we still know surprisingly little about the molecular mechanisms of acetaminophen action and toxicity.

The structures of acetaminophen (paracetamol; p-acetylaminophenol; APAP; Tylenol™ and some related compounds are shown in Fig. 1. The discovery of acetaminophen involved elements of both good science and good luck (Mann and Plummer, 1991). Two antipyretic (fever-reducing) natural products—the extracts of willow bark (salicylic acid) and cinchona bark (quinine)—were recognized long before the scientific era. However, the former drug had unpleasant side effects, and the latter was scarce. In the late 19th century, an entirely new class of synthetic analgesics and antipyretics was discovered, by chance. The first of these chemicals was acetanilide, acetyaminobenzene. As recounted by Mann and Plummer:

In 1886, two Alsatian interns, Drs. Kahn and Hepp, ordered some naphthalene, a treatment for intestinal parasites, from a nearby pharmacist. The substance they received failed to have the expected effect on the parasites but managed instead to reduce the patient’s fever. Startled, the two doctors requested more of the same. This time, however, they received a substance that got rid of intestinal parasites but did nothing for fever. Upon further analysis, Kahn and Hepp discovered that they had
been the victims of a happy accident; the first material was acetanilide, a coal-tar derivative used in the dye industry, never before given to human beings…. The two doctors approached Hepp’s brother, a chemist at Kalle & Company of Wiesbaden, the company that had supplied the acetanilide, and told him what had happened (Mann and Plummer, 1991).

Kalle & Company began selling acetanilide under the brand name “Antifebrin.” At the same time, another German company, Farbenfabriken Bayer, was accumulating tons of unwanted $p$-aminophenol as a by-product of its synthetic dye production processes. Chemists at Bayer, under the direction of Carl Duisberg, saw an opportunity to make use of this chemical waste. Acetylation would readily convert the amino group to the acetylamino group, to give $p$-ethoxyacetanilide (“phenacetin”). They found this product to be both an antipyretic and an analgesic. Phenacetin was marketed in 1888. Mann and Plummer (1991) observed that this successful project of the conception, development, testing, and marketing of a new chemical entity can be regarded as marking the beginning of the pharmaceutical industry in a recognizably modern form.

Phenacetin was widely used during the influenza epidemic of 1889, and the Bayer company’s new pharmaceutical enterprise1 prospered. Phenacetin remained an important analgesic until concern over its nephrotoxic side effects arose in the 1960s. Heavy use of phenacetin was also associated with increased risk of renal (Gaakeer and De Ruiter, 1979) and bladder cancers, and it was finally withdrawn from sale in 1983.

**ACETAMINOPHEN**

$p$-Acetylaminophenol (acetaminophen) was first synthesized in 1888. Even though it was an intermediate in the synthesis of phenacetin, Bayer chemists did not test this compound as a drug, because phenols were generally regarded as too toxic for medicinal use. In 1889, the Swedish physiological chemist Karl Mörner (later the Rector of the Karolinska Institute) discovered that acetaminophen is a urinary metabolite of phenacetin. (We now understand that this biotransformation is a typical P450-catalyzed O-dealkylation reaction.) In 1893, the German physiologist J.F. von

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1A few years later, another Bayer chemist, Felix Hoffmann, acetylated salicylic acid to produce acetylsalicylic acid—Bayer “Aspirin.”
Mering2 tested acetaminophen and showed that it was also an analgesic and antipyretic. However, phenacetin was already a successful product, and acetaminophen was not developed as a commercial drug.

In 1939, a consortium of U.S. proprietary drug manufacturers established the “Institute for the Study of Analgesic and Sedative Drugs,” with the intention of applying modern pharmacological science to the development and testing of analgesics (Mann and Plummer, 1991, p. 186). The Institute organized a symposium to evaluate the evidence about acetaminophen, in 1951. With support from the Institute, Bernard Brodie and colleagues studied the hepatic metabolism of acetanilide in vitro and discovered its enzymatic deacetylation (to give aniline) and aromatic hydroxylation (to give acetaminophen).

Bernard B. Brodie3 (1909–1989) was one of the founders of modern pharmacology and toxicology (Costa et al., 1989). Brodie was raised in Ottawa, Canada, where his father ran a clothing store. He had a “slow start” to his academic career: he dropped out of high school and joined the Canadian army. He enrolled at McGill University in Montreal; it is said that Brodie won the money he needed for tuition by playing poker. In his final undergraduate year, he had an opportunity to do laboratory work for a chemistry professor, W.H. Hatcher, and this experience set him on a research career. He earned a Ph.D. in organic chemistry at New York University and joined their Pharmacology Department. During World War II, he worked on antimalarial drugs, which were desperately needed for the war effort in the Pacific. Brodie’s studies of the metabolism of atabrine (quinacrine) assisted the development of a dosing regimen that minimized toxic side effects of the drug. Later, as head of the Laboratory for Clinical Pharmacology at the U.S. National Institutes of Health (1950–1970), Brodie put together a group of researchers who made pioneering contributions to the study of drug metabolism. His studies of acetanilide-induced methemoglobinemia [in collaboration with Julius Axelrod (Snyder, 2005)] led to the discovery of enzymatic deacetylation (to give aniline) and aromatic hydroxylation (to give acetaminophen), two of the most important P450-catalyzed drug metabolism reactions.

Brodie speculated that acetaminophen might be a useful drug in its own right—as had von Mering, many years earlier. Boréus and Sandberg, at the Karolinska Institute, also obtained encouraging results with acetaminophen (Boréus and Sandberg, 1953). Several chemical producers began selling the compound over the following years, including Squibb Pharmaceuticals in New York and McNeil Laboratories in Pennsylvania, which coined the brand name Tylenol™. (In the remainder of this article, we shall refer to this compound generically as acetaminophen.)

**NSAIDS**

Acetaminophen shares its antipyretic and analgesic properties with a large group of agents known as nonsteroidal anti-inflammatory drugs4 (NSAIDs), including acetylsalicylic acid (ASA), ibuprofen, and naproxen (Paulus and Whitehouse, 1973). However, acetaminophen is not an NSAID: it is not strongly anti-inflammatory. NSAIDs act (at least, primarily) by inhibiting the enzyme cyclooxygenase (prostaglandin synthase), which

2von Mering is also remembered for his discovery that diabetes can be induced in dogs by removing the pancreas.


4The term “nonsteroidal” distinguishes them from the anti-inflammatory steroids, such as hydrocortisone.
catalyzes the first step in the biosynthesis of prostaglandins from arachidonic acid. John Vane shared the 1982 Nobel Prize in Physiology or Medicine for discovering the inhibitory action of ASA on prostaglandin biosynthesis (Vane, 1971). ASA is an irreversible inhibitor: it covalently modifies (by acetylation) a specific serine residue on the cyclooxygenase enzyme (Berg et al., 2001, p. 333).

Around 1990, molecular cloning revealed the existence of two distinct forms of cyclooxygenase enzyme, called COX-1 and COX-2 (Vane et al., 1998). COX-1 is constitutively expressed in most mammalian tissues. COX-2 is expressed in specific cell types (such as endothelial cells and synoviocytes) and is induced by inflammatory stimuli (Crofford, 1997). ASA is a better inhibitor of COX-1 than of COX-2 (Mitchell et al., 1993). These advances in the understanding of the pharmacology of NSAIDs provoked the idea of designing specific inhibitors of COX-2. Medicinal chemists hoped that such inhibitors would show analgesic activity against chronic inflammatory conditions while presenting reduced risk of the toxic side-effects of earlier NSAIDs (such as the gastrointestinal bleeding sometimes associated with ASA use). COX-2-specific inhibitors (e.g., celecoxib) were developed in the 1990s and proved to be successful therapeutic agents for arthritis, menstrual cramps, and other painful conditions (McMurray and Hardy, 2002). However, concerns over possible cardiovascular toxicity led to the withdrawal from sale of most COX-2 inhibitors in 2004 (Couzin, 2005).

MECHANISM OF THERAPEUTIC ACTION

The coal-tar analgesics, such as acetaminophen, were discovered by accident, and their mechanism of action is still not properly understood. There is considerable overlap of activities between acetaminophen and NSAIDs, and they are treated as competitors in the marketplace for over-the-counter analgesics. Nevertheless, in vitro studies show that acetaminophen is not a highly potent inhibitor of COX-1 or COX-2 (although its inhibitory action may be greater under conditions of low ambient peroxide concentration (Ouellet and Percival, 2001).

In 2002, researchers proposed that a third distinct form of cyclooxygenase, which has been referred to as “COX-3,” is expressed as a splicing variant of the COX-1 gene (Chandrasekharan et al., 2002). Canine COX-3, expressed as a protein in insect cells, proved to be sensitive to inhibition by acetaminophen, and this inhibition was suggested to account for the drug’s analgesic effect. However, further molecular cloning studies (Dinchuk et al., 2003) failed to confirmed the existence of COX-3 in humans, and the possible significance of COX-1 splicing variants remains unclear (Berenbaum, 2004; Schwab et al., 2003). The search for a convincing mechanistic explanation of acetaminophen’s therapeutic activity continues.

ACETAMINOPHEN: HUMAN TOXICITY

Acetaminophen has been used effectively and safely by very large numbers of patients, both children and adults, since its introduction to the consumer market in the 1950s. Despite the drug’s safety when used as indicated, however, acetaminophen toxicity can occur, due to either accidental or deliberate overdose ingestion of the drug (McClain et al., 1999). Postmortem analysis of fatal acetaminophen poisoning cases showed that death results from liver failure associated with hepatic centrilobular necrosis (McJunkin et al., 1976). The damaged and dying hepatocytes release, into the serum,
characteristic liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Measuring the levels of these enzyme activities in the blood provides a reliable clinical measure of hepatotoxicity due to acetaminophen (Singer et al., 1995) or other agents.

Because acetaminophen pills were often found in household medicine cabinets, children occasionally opened the bottles and swallowed the contents, especially before the introduction of “childproof” packaging for hazardous products, in the 1970s. In 1977, the Analgesic Review Panel of the Food and Drug Administration (F.D.A., U.S.A.) recommended that all acetaminophen products carry a warning label stating that overdose can cause liver damage (Mann and Plummer, 1991, p. 205). Unfortunately, acetaminophen continues to be chosen as a poison by suicidal individuals (Gyamlani and Parikh, 2002). In the United Kingdom, about 500 deaths occur annually due to acetaminophen overdose, accounting for about 15% of fatal poisonings (Sheen et al., 2002).

Because of the high incidence of acetaminophen poisoning cases, there has been ongoing research efforts to understand the mechanisms of acetaminophen hepatotoxicity (Bromer and Black, 2003; Rumack, 2002) and to develop therapeutic interventions that can be administered to victims of acetaminophen overdoses, as discussed later.

ACETAMINOPHEN AND ALCOHOL

Alcohol consumption can sensitize individuals to acetaminophen. Jordan Holtzman (Holtzman, 1995; McClain et al., 1980) describes how he and his colleagues recognized this hazardous interaction:

[W]e observed a series of patients who were hospitalized for acute, severe hepatic failure. These individuals had been drinking heavily and had suddenly stopped their alcohol consumption. They had then taken modest doses of acetaminophen for their hangovers and gone into acute hepatic failure. These individuals had aspartate aminotransferases (AST) and alanine aminotransferases (ALT) in the thousands, whereas the usual patient with alcoholic hepatitis has values in the hundreds. This led Dr. Kromhout, the clinical consultant reviewing the cases, to suspect that there was something unusual about these patients. He discovered that all of them had been on a drinking binge, had suddenly stopped drinking, and had taken acetaminophen for their hangovers…. In light of these clinical observations, we examined the possibility of an interaction between acetaminophen and ethanol consumption in … mice. In this study we demonstrated a nearly 50% decrease in the LD50 for acetaminophen in mice receiving ethanol in their drinking water for 3 weeks. This report … probably represents the only study ever published in the Journal of the American Medical Association which included a probit plot for the LD50 of a drug in mice.

In 1998, the F.D.A. issued a regulation requiring that acetaminophen products carry the following label: “Alcohol Warning: If you consume 3 or more alcoholic drinks every day, ask your doctor whether you should take acetaminophen … Acetaminophen may cause liver damage.”

The mechanism of the alcohol – acetaminophen interaction is not fully understood. Alcohol induces cytochrome P450 2E1 in humans, and this enzyme activates acetaminophen to a toxic metabolite, as discussed below. Other mechanisms are also believed to contribute, such as ethanol-dependent depletion of hepatic glutathione (Zhao et al., 2002).
METABOLISM—CONJUGATION REACTIONS

The phenolic –OH group of acetaminophen is available for metabolic conjugation reactions. At therapeutic doses of the drug, about one-half and one-third of the excreted urinary metabolite are in the forms of acetaminophen O-glucuronide and acetaminophen O-sulfate, respectively (Fig. 2). These conjugations detoxify acetaminophen. Peter Wells and colleagues have shown, in studies with the UDPGT-deficient Gunn rat and other animal models (de Morais and Wells, 1989; de Morais et al., 1992a), that impairment of glucuronidation results in enhanced acetaminophen toxicity. This increased sensitivity may also be obtained in humans with Gilbert’s syndrome, in which glucuronidation activity is reduced (de Morais et al., 1992b).

The sulfation pathway of acetaminophen metabolism becomes saturated at high acetaminophen doses, because of either limited supply of PAPS substrate or limited sulfotransferase enzyme capacity (Liu and Klaassen, 1996). In humans, serum sulfate levels drop after acetaminophen ingestion (Morris and Levy, 1983).

OXIDATION OF PHENACETIN AND ACETAMINOPHEN

Quantitatively, only a few percent of an administered dose of acetaminophen undergoes oxidative metabolism, but this process determines the toxicity of the drug. Before we discuss the oxidation of acetaminophen, it is useful to consider the case of phenacetin and the differences between the oxidation chemistries of these two analgesics. Phenacetin undergoes typical P450-catalyzed oxidation reactions: O-dealkylation to give acetaminophen, as already mentioned; ring-hydroxylation to give 2-hydroxyphenacetin and 3-hydroxyphenacetin (Büch et al., 1967); and N-hydroxylation to give N-hydroxyphenacetin, which accounts for about 0.5% of the urinary excretion of phenacetin in humans (Veronese et al., 1985).

N-Hydroxyphenacetin is undoubtedly a more significant primary metabolite than this percentage indicates: most of the metabolite does not reach the urine. N-Hydroxyphenacetin can be bioactivated to reactive mutagenic intermediates (Nohmi et al., 1987), such as the nitroso compound p-nitrosophenetole (formed by deacetylation and N-oxidation; Fig. 3, upper panel) and reactive
N, O esters formed by sulfation or glucuronidation reactions (Hinson, 1983). These processes probably account for phenacetin’s carcinogenic activity.

In the case of acetaminophen, P450-catalyzed N-oxidation might be expected to form N-hydroxyacetaminophen. However, the presence of a hydroxy rather than an alkoxy substituent para to the amide function makes the chemistry of acetaminophen very different from that of phenacetin. Two distinct two-electron oxidation products of acetaminophen exist: N-hydroxyacetaminophen and its dehydration product, N-acetyl-p-benzoquinone-imine (NAPQI; Fig. 3, lower panel). In fact, NAPQI, not N-hydroxyacetaminophen, is observed as a P450-catalyzed metabolite (Dahlin et al., 1984). Could the N-hydroxy derivative be an intermediate in NAPQI formation? Elimination of water from N-hydroxyacetaminophen would yield NAPQI (Fig. 3). However, when N-hydroxyacetaminophen was synthesized (Gemborys et al., 1978), its half-life (in aqueous solution at 37 °C) was found to be 80 min (Calder et al., 1981), so this is much too slow a process to account for P450-dependent formation of the iminoquinone (Dahlin et al., 1984). A more likely mechanism is one-electron removal from the substrate by P450 to give the cation radical intermediate; deprotonation and transfer of a second electron to the heme group, giving NAPQI, then occurs much more rapidly than oxygen rebound to give the N-hydroxy derivative (Fig. 4).

Several human P450 enzymes can oxidize acetaminophen to the iminoquinone, including forms 1A2, 2A6, 2D6, and 2E1 (Chen et al., 1998; Dong et al., 2000). The connection between P450-mediated acetaminophen metabolism and toxicity was confirmed by studies with knockout mice. Mice lacking P450 1A2 or P450 2E1 were more resistant to acetaminophen than were wild-type animals (increased LD50). Double knockout mice lacking both P450 1A2 and 2E1 enzymes were very much more resistant to the drug, with almost all of the mice surviving a dose of 1.2 g per kilogram body weight; half of that dose was already 100% lethal to the wild-type animals (Kolb et al., 2005; Zaher et al., 1998). Knockout mice devoid of the nuclear receptor CAR do not show induction of P450 1A2,
P450 3A11 (corresponding to human form 3A4), or glutathione transferase Pi in response to acetaminophen, and are also highly resistant to its hepatotoxicity (Zhang et al., 2002).

Peroxidase/hydrogen peroxide systems, which characteristically oxidize organic substrates in one-electron steps, generate the acetaminophen cation radical. The electron spin resonance (ESR) spectrum of the short-lived radical can be detected in solution, using fast-flow systems (Martin and Garner, 1987; Mason and Fischer, 1986). The radical reacts rapidly with itself to form polymeric products.

**REACTIONS OF N-ACETYL-P-BENZOQUINONEIMINE; GLUTATHIONE CONJUGATION**

The quinoneimine oxidation product of acetaminophen, NAPQI, is a reactive electrophile (Corcoran et al., 1980; McGirr et al., 1986; Streeter et al., 1984). The spontaneous reaction of NAPQI with glutathione (GSH) is rapid and yields the adduct 3-(glutathion-S-yl)acetaminophen via Michael addition; a redox reaction also occurs, giving a lesser amount of acetaminophen plus glutathione disulfide (Coles et al., 1988). Glutathione transferases of several classes can catalyze both reactions. At high acetaminophen doses, liver cells become completely depleted of GSH, and the activated acetaminophen binds covalently to cellular proteins (Fig. 5). The major nucleophilic targets in proteins are the thiol groups of cysteine residues (Hoffmann et al., 1985).

The onset of liver damage follows GSH depletion (Savides et al., 1984). Methemoglobinemia occurs with a time-course similar to that of acetaminophen in serum, but this hematological effect is transient and does not cause long-term toxicity (Fig. 6).
hepatic GSH becomes substantially depleted, hepatotoxicity ensues, as indicated by the prolonged rise in liver-derived transaminase and alkaline phosphatase levels in the serum. Also, as mentioned below, intervention to sustain hepatic GSH is an effective treatment for acetaminophen overdose. Most researchers agree that hepatic GSH depletion is the critical trigger for acetaminophen hepatotoxicity (Bessems and Vermeulen, 2001).

COVALENT BINDING TO PROTEINS

James R. Gillette (Estabrook, 2003) was one of the scientists recruited to the Laboratory of Chemical Pharmacology at NIH in the 1950s, by Bernard Brodie. In 1973, Gillette, Brodie, and colleagues published a set of four papers delineating the involvement of covalent binding to protein in the mechanism of acetaminophen hepatotoxicity in mice.
These influential studies demonstrated that radioactivity of $^3$H-labeled or $^{14}$C-carbonyl-labeled acetaminophen became irreversibly bound to hepatic proteins. Binding was observed in all subcellular compartments examined: nuclear, mitochondrial, endoplasmic reticular, and cytosolic. This binding was far greater in liver than in nontarget tissues (e.g., muscle). Pretreatment of mice with P450 inhibitors decreased both the covalent binding and the hepatotoxicity, whereas pretreatment with P450 inducers increased both parameters. Later studies used immunohistochemistry with antibodies against acetaminophen-protein adducts to show that the histological pattern of covalent addition matches that of the toxicity, with damage to the centrilobular rather than the periportal regions (Hinson et al., 1998). Gillette and colleagues also demonstrated the protective role of glutathione. Understanding the significance of GSH for acetaminophen detoxication led directly to improved clinical therapy for acetaminophen poisoning. Administration of N-acetyl-L-cysteine, which replenishes GSH stores (Hazelton et al., 1986) remains the treatment of choice in human overdose cases (Smilkstein et al., 1991).

**PROTEIN BINDING AND HEPATOTOXICITY**

Subsequent evidence has supported Gillette’s hypothesis that covalent binding to protein following GSH depletion causes hepatocellular necrosis and toxicity. As already noted, factors that potentiate or lessen protein binding have corresponding effects on hepatotoxicity. Hinson and colleagues (Roberts et al., 1991) used antiserum to the 3-(cystein-$S$-yl) adduct, formed by reaction of NAPQI with protein cysteine residues, to study acetaminophen toxicity in mice. Patterns of adduct formation correlated with patterns of toxicity, in terms of anatomical distribution within the liver, dose-response, and time-course.

An alternative view is that oxidative stress, rather than protein binding, per se, initiates hepatotoxicity. Certainly, many characteristic features of oxidative stress are observed in acetaminophen hepatotoxicity, including lipid peroxidation, mitochondrial damage and ATP depletion, and formation of nitrotyrosine adducts in proteins, presumably due to formation of superoxide-derived peroxynitrite (Jaeschke et al., 2003). However, these processes may be consequences of damage mediated by protein adduction, rather than direct causes of hepatotoxicity.

Are there specific proteins that represent critical targets for acetaminophen-mediated hepatotoxicity? Ongoing efforts have been directed to the identification of proteins

<table>
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<tr>
<th>Enzyme/protein</th>
<th>Compartment</th>
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<tr>
<td>Aldehyde dehydrogenase</td>
<td>Cytosol, mitochondria</td>
</tr>
<tr>
<td>ATP synthetase $\alpha$-subunit</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>ER</td>
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<tr>
<td>Glutathione peroxidase</td>
<td>Cytosol, mitochondria</td>
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<tr>
<td>Glutathione transferase</td>
<td>Cytosol, mitochondria</td>
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<tr>
<td>Glycine $N$-methyltransferase</td>
<td>Cytosol</td>
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<tr>
<td>3-Hydroxyanthranilicate 3,4-dioxygenase</td>
<td>Cytosol</td>
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<tr>
<td>Tropomyosin 5</td>
<td>Cytoskeleton</td>
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<tr>
<td>Urate oxidase</td>
<td>Peroxisomes</td>
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Adapted from James et al. (2003).
adducted/inactivated by acetaminophen binding, using radiolabeling, immunological, and proteomic methods, including mass spectrometry (Ruepp et al., 2002). A sample of acetaminophen target proteins that have been identified is shown in Table 1.

No single protein or set of proteins, and no specific subcellular compartment, has been found to be a uniquely sensitive target of acetaminophen adduction, although oxidative damage to the mitochondrion may be of particular importance (Coen et al., 2003; Hinson et al., 2004; Reid et al., 2005). Hepatocellular damage likely results from the aggregated effect of damage to multiple proteins and simultaneous inhibition of many enzymes and cellular functions (James et al., 2003).

REFERENCES


