This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

Acetaminophen: A Wolf in Sheep's Clothing

by

Jeffrey D. Kirsch

B-180 Medical Laboratories
Free Radical and Radiation Biology Program
The University of Iowa
Iowa City, IA 52242-1181

For 77:222, Spring 2001

2. April 2001

Abbreviations

APAP	*N*-acetyl-*p*-aminophenol, acetaminophen
CYP	cytochrome P450
EPR	electron paramagnetic resonance
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
NAC	*N*-acetylcysteine
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NAPSQI	*N*-acetyl-*p*-benzosemiquinone imine radical
ROS	reactive oxygen species

Table of Contents

1. Abstract 2
2. Introduction 3
3. Structure and Properties 3
4. Redox Chemistry 4
5. Biochemistry 5
6. Summary 8
7. References 9

Abstract

Acetaminophen is a widely used analgesic and antipyretic. Although relatively free of obvious side effects at therapeutic doses, at higher levels it is acutely toxic. Acetaminophen exerts its toxic effects as a consequence of its oxidative metabolism, primarily through the toxic metabolite *N*-acetyl-*p*-benzoquinone imine. The primary immediate result of acetaminophen intoxication is the depletion of glutathione and the covalent modification of proteins. This review will discuss the mechanisms by which acetaminophen is metabolized, and how the resultant metabolites might mediate toxicity.

Introduction

Acetaminophen (APAP) is a widely used over-the-counter analgesic and antipyretic. Acetaminophen exerts its therapeutic effect through its inhibition of cyclooxygenase (COX) in the central nervous system, thereby decreasing the transmission and perception of pain impulses [1]. It is often chosen over aspirin due to its lack of gastrointestinal side effects and apparent nontoxicity at the recommended dosage. However, at higher doses APAP is profoundly toxic, particularly to the liver and kidneys, leading to organ failure, frank necrosis of target organ systems, and death [2].

Much debate has been generated regarding the precise nature of APAP-mediated toxicity, but certain facts have emerged as being critically important. The metabolism of APAP leads to the production of the toxic compound NAPQI, which then simultaneously decreases GSH pools and covalently modifies proteins [2]. Acetaminophen-treated cells and tissues exhibit many of the hallmarks of oxidative stress, including lipid peroxidation, although it is unclear as to whether this is due to actual ROS production or a decrease in antioxidant capacity subsequent to GSH depletion [2]. Since APAP toxicity may well synergize with other oxidative stressors, particularly those that affect the liver (such as alcohol, hepatitis, etc.), understanding the nature of APAP-induced toxicity is of great importance.

Structure and Properties

Structurally, APAP is a substituted phenol with electron-withdrawing groups at the 1 and 4 positions, as shown in Figure 1. Acetaminophen is a colorless solid, with a melting point of 169-170°C. Practically insoluble in cold water and highly nonpolar solvents, APAP is freely soluble in amphipathic solvents such as methanol and ethylene dichloride. Acetaminophen exhibits a substantial UV absorbance, as is typical for compounds with π electron systems,

showing a maximum absorbance at 250 nm (ε = 13,800). In addition to its most common uses as an analgesic and antipyretic, APAP has also been used in the manufacture of azo dyes.

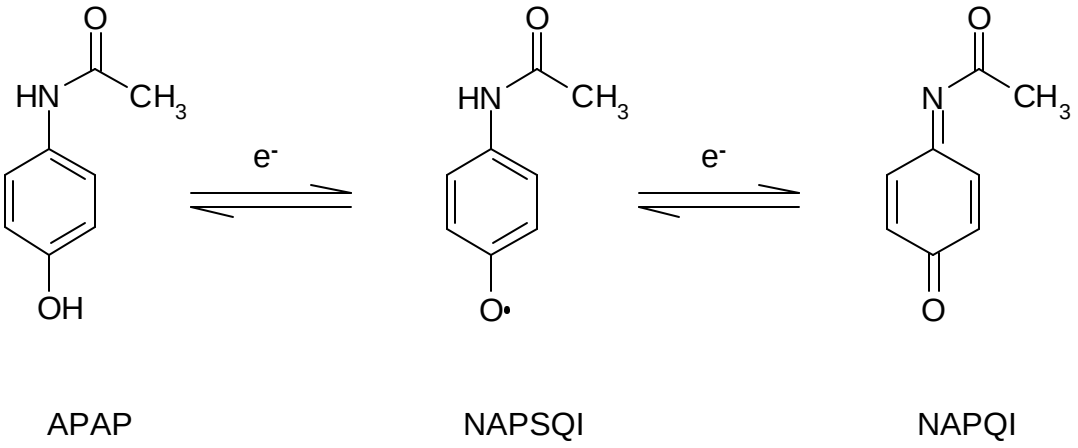

Figure 1. Oxidation of APAP

Redox Chemistry

Acetaminophen can undergo a number of redox changes, the most important biologically being the formation of a highly reactive two-electron oxidation product, the quinone imine (NAPQI), as shown in Figure 1 [3]. The existence of the one-electron oxidation product, the APAP phenoxyl radical (*N*-acetyl-*p*-benzosemiquinone imine; NAPSQI), has been difficult to show directly, owing to its inherent instability [3]. This instability is due, in part, to the electron-withdrawing effect of the acetyl group, which renders the benzene ring electron-poor [3]. Despite the challenges imposed by the nature of the NAPSQI radical, EPR spectra have been obtained by a number of investigators. An example EPR spectrum, from the work of West *et al*., is shown in Figure 2 [4]. The high reactivity of the semiquinone radical leads to the formation of an APAP dimer, as outlined in Figure 3. The dimer depicted in Figure 3 has been identified by Potter *et al*. as the dominant product of one-electron peroxidase-dependent APAP oxidation [5].

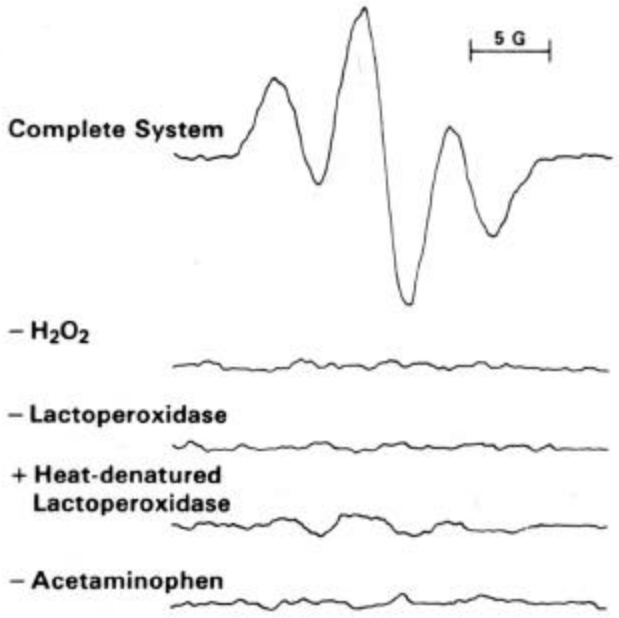

Figure 2. EPR Spectrum of NAPSQI. EPR spectrum of the lactoperoxidase-catalyzed, H_2O_2-dependent oxidation of APAP. Adapted from [4].

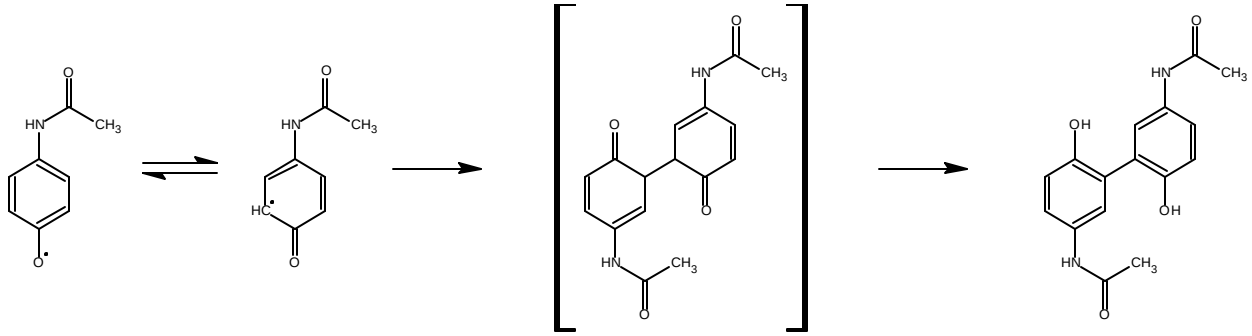

Figure 3. APAP Polymerization. Delocalization of the unpaired electron to the *o*-position leads to a radical coupling reaction between two molecules of NAPSQI, followed by enolization to regenerate the aromatic system. Adapted from [5].

Biochemistry

Acetaminophen is primarily detoxified by phase 2 xenobiotic-metabolizing enzymes, to primarily form glucuronides and sulfates [2]. Glucuronidation and sulfation generate non-toxic water-soluble compounds that are then excreted in the bile, and are of no toxicologic consequence [2]. A relatively minor percentage of APAP is thought to be metabolized by the

cytochrome P450 (CYP) pathway [2]. The case of CYP-mediated APAP metabolism is rather different, however, in that this pathway generates the highly toxic quinone imine, NAPQI [2, 6]. In addition to the two-electron oxidation catalyzed by the CYP pathway, it has also been reported that various peroxidases are capable of generating the one-electron oxidation product, the benzosemiquinone radical NAPSQI [3, 7-9].

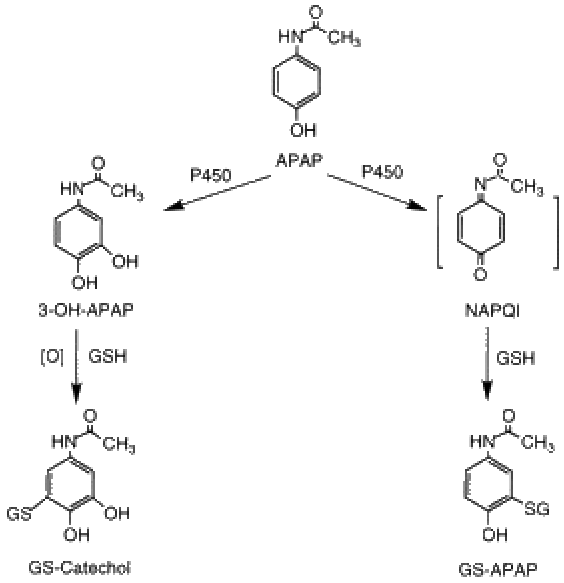

Figure 4. Cytochrome P450 Oxidation of APAP. In an *in vitro* system consisting of CYP enzyme, NADPH, APAP, and GSH, APAP is oxidized *via* the two pathways shown. Adapted from [5].

Cytochromes P-450 (CYP) are a large family of related heme proteins that mediate the oxidative metabolism of a wide range of endogenous substrates and xenobiotics. At least two possible products of CYP-mediated APAP oxygenation are known, NAPQI and 3-OH-APAP, the latter compound being considered non-toxic [7]. A schematic depiction of CYP-mediated APAP oxygenation is shown in Figure 4 [7]. At least three CYP isozymes have been shown to metabolize APAP, namely 2E1, 2A6, and 1A2 . It is not known whether the oxidation of APAP carried out by CYP generates a transient radical species, or if a concerted two-electron oxidation occurs. Interestingly, 2E1 and 2A6 differ significantly in the ratio of NAPQI to 3-OH-APAP

produced. Cytochrome P450 2E1 produces product in the ratio of 6:1, NAPQI vs. 3-OH-APAP, while for 2A6 the ratio is 1:3 [7].

The toxic consequences of NAPQI production are two-fold, namely the conjugation of GSH and the covalent modification of proteins. Although the conjugation of NAPQI to electrophilic amino acids, such as cysteine, is not thought to require enzymatic catalysis, the reaction of NAPQI with GSH can occur with or without the mediation of GSH-conjugating enzymes such as GST [10, 11]. The relatively small amounts of NAPQI produced as a consequence of therapeutic doses of APAP leads to an inconsequential consumption of cellular GSH, but larger doses of APAP can cause the generation of concentrations of NAPQI sufficient to severely deplete GSH [10, 11]. Interestingly, the p isozyme of GST does not appear to play a significant role in the conjugation of NAPQI to GSH, as shown by studies of GST-p knockout mice [10].

No clear consensus in the literature can be found as to the relative importance of these two mechanisms in the mediation of APAP toxicity. However, some studies have shown that GSH replenishment, *via* treatment with GSH prodrugs such as NAC or GSH ester, can protect against hepatic necrosis induced by APAP, independently of the formation of APAP-protein adducts [12-14]. These findings would tend to support GSH depletion as the primary mechanism of APAP toxicity, since both NAC and GSH ester increase the levels of intracellular GSH. NAC does this at least partly by providing substrate for the rate-limiting step of GSH synthesis, the step catalyzed by ?-glutamyl cysteine synthetase, while GSH ester is deesterified following cellular uptake to produce GSH. Although reports of direct oxidative stress induced by APAP can be found, it is unclear if this represents *de novo* formation of ROS or is simply due to a decreased antioxidant capacity subsequent to GSH depletion.

Less well studied, but intriguing nonetheless, is the reported one-electron oxidation of APAP by various peroxidases [4]. As shown in Figure 2, lactoperoxidase can catalyze the formation of the NAPSQI radical, thus demonstrating that one-electron oxidation of this substrate can occur with biologically relevant components [4]. Free NAPSQI may go on to form APAP polymers via the mechanism shown in Figure 3 [9]. Alternatively, free NAPSQI could be reduced by ascorbate or GSH [15]. Reduction of NAPSQI by GSH could be of particularly severe consequence, as it was demonstrated that such an interaction could lead to the formation of $GSSG^{?-}$, an extremely reducing radical that can form $O_2^{?-}$ [15].

Summary

The commonly used over-the-counter analgesic and antipyretic APAP can have grave consequences when ingested in excess. Acetaminophen at high doses is extremely toxic, causing liver and kidney failure, and in the most severe cases, death. The metabolism of APAP *via* the CYP system leads to the formation of the toxic metabolite NAPQI, which can lead to the depletion of GSH pools, in addition to the covalent modification of proteins. Although much remains to be settled as to how APAP toxicity is mediated, the importance of GSH homeostasis seems to be central.

Acetaminophen exposure leads to the appearance of oxidative stress, most particularly lipid peroxidation. Whether this is due to de novo ROS production or the loss antioxidants has not been definitively worked out. Since many oxidative stresses are encountered by modern humans, that APAP toxicity could be expected to exacerbate, understanding the mechanism of APAP-mediated toxicity is of general significance.

References

1. Flower RJ, Vane JR. (1972) Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature*. **240**(5381):410-411.

2. Moore M, Thor H, Moore G, Nelson S, Moldeus P, Orrenius S. (1985) The toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca2+. *J Biol Chem*. **260**(24):13035-13040.

3. Mason RP, Fischer V. (1986) Free radicals of acetaminophen: their subsequent reactions and toxicological significance. *Fed Proc*. **45**(10):2493-2499.

4. West PR, Harman LS, Josephy PD, Mason RP. (1984) Acetaminophen: enzymatic formation of a transient phenoxyl free radical. *Biochem Pharmacol*. **33**(18):2933-2936.

5. Potter DW, Hinson JA. (1986) Reactions of N-acetyl-p-benzoquinone imine with reduced glutathione, acetaminophen, and NADPH. *Mol Pharmacol*. **30**(1):33-41.

6. Chen W, Peter RM, McArdle S, Thummel KE, Sigle RO, Nelson SD. (1996) Baculovirus expression and purification of human and rat cytochrome P450 2E1. *Archives of Biochemistry & Biophysics*. **335**(1):123-130.

7. Weis M, Rundgren M, Nelson S, Moldeus P. (1996) Peroxidase-catalyzed oxidation of 3,5-dimethyl acetaminophen causes cell death by selective protein thiol modification in isolated rat hepatocytes. *Chem Biol Interact*. **100**(3):255-265.

8. Potter DW, Hinson JA. (1986) Reactions of glutathione with oxidative intermediates of acetaminophen. *Advances in Experimental Medicine & Biology*. **197**:763-772.

9. Potter DW, Miller DW, Hinson JA. (1985) Identification of acetaminophen polymerization products catalyzed by horseradish peroxidase. *J Biol Chem*. **260**(22):12174-12180.

10. Henderson CJ, Wolf CR, Kitteringham N, Powell H, Otto D, Park BK. (2000) Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione S-transferase Pi. *Proc Natl Acad Sci U S A*. **97**(23):12741-12745.

11. Qiu Y, Benet LZ, Burlingame AL. (1998) Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem*. **273**(28):17940-17953.

12. Rafeiro E, Barr SG, Harrison JJ, Racz WJ. (1994) Effects of N-acetylcysteine and dithiothreitol on glutathione and protein thiol replenishment during acetaminophen-induced toxicity in isolated mouse hepatocytes. *Toxicology*. **93**(2-3):209-224.

13. Ruffmann R, Wendel A. (1991) GSH rescue by N-acetylcysteine. *Klin Wochenschr*. **69**(18):857-862.

14. Ellouk-Achard S, Levresse V, Martin C, Pham-Huy C, Dutertre-Catella H, Thevenin M, Warnet JM, Claude JR. (1995) Ex vivo and in vitro models in acetaminophen hepatotoxicity studies. Relationship between glutathione depletion, oxidative stress and disturbances in calcium homeostasis and energy metabolism. *Archives of Toxicology Supplement*. **17**:209-214.

15. Ramakrishna Rao DN, Fischer V, Mason RP. (1990) Glutathione and ascorbate reduction of the acetaminophen radical formed by peroxidase. Detection of the glutathione disulfide radical anion and the ascorbyl radical. *J Biol Chem*. **265**(2):844-847.