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ACETAMINOPHEN HEPATOTOXICITY

AN ALTERNATIVE MECHANISM

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Abstract—Alcohol-fed hamsters were used to study the mechanism by which acetaminophen initiates hepatotoxicity. Animals maintained on an ethanol-containing diet (Group B) exhibited an increased mortality rate after administration of acetaminophen (400 mg/kg) as compared to control hamsters (Group A). However, in those animals in which the ethanol-containing diet had been replaced by the control diet 24 hr before receiving acetaminophen (Group C), significant protection against acetaminophen toxicity was observed as compared to control animals (Group A). This observation correlates well with the finding that Group C hamsters had higher levels of glutathione and catalase than was found in either Group A or Group B animals. It was also demonstrated that acetaminophen was oxidized by cytochrome P-450, producing acetaminophen free radical and hydrogen peroxide. The free radical in the presence of oxygen was found to generate superoxide and presumably *N*-acetyl-*p*-benzoquinone imine. Microsomal lipid peroxidation was found to be stimulated markedly in the presence of acetaminophen. The role of glutathione in protecting hamsters from acetaminophen-mediated hepatotoxicity is discussed.

The widely prescribed analgesic acetaminophen can cause hepatic necrosis in both humans and experimental animals when high doses of this drug are administered [1, 2]. Evidence has accumulated which demonstrates that acetaminophen has to be bioactivated to a reactive intermediate in order to initiate hepatotoxicity. Using both *in vivo* and *in vitro* experiments, it has been determined that cytochrome P-450 is responsible for the biotransformation of acetaminophen to its toxic intermediate [3–6]. Nevertheless, the nature of this metabolite and the mechanism by which acetaminophen mediates hepatotoxicity remain unclear. It was originally proposed that cytochrome P-450 oxidizes acetaminophen to *N*-hydroxyacetaminophen which loses water giving the hypothetical intermediate *N*-acetyl-*p*-benzoquinone imine (Fig. 1). Once this intermediate is produced, it can be detoxified by reaction with reduced glutathione (GSH) to yield 3-(glutathion-*S*-yl)acetaminophen [7]; however, when GSH is depleted, this quinone imine binds to cellular macromolecules [8]. Based on these findings, it has been proposed that covalent binding is associated with cell death [8]. Recent studies by Hinson *et al.* [9] and Nelson *et al.* [10] cast doubt on aspects of this mechanism. These investigators have demonstrated that, if *N*-acetyl-*p*-benzoquinone imine is the toxic species, it is biosynthesized by a pathway that does not include *N*-hydroxyacetaminophen.

An alternative hypothesis to account for the formation of *N*-acetyl-*p*-benzoquinone imine envisions the epoxidation of acetaminophen followed by ring opening with loss of water (Fig. 1). If such a mechanism were correct, then the addition of heavy oxygen ($^{18}O_2$) to the reaction mixture would lead to the incorporation of ^{18}O into half of the *N*-acetyl-*p*-benzoquinone imine. However, when such a study was conducted, the investigators were unable to detect ^{18}O in any of the acetaminophen metabolites [11, 12].

A third mechanism suggests that cytochrome P-450 initiates a one-electron oxidation of acetaminophen giving acetaminophen free radical [13]. Transfer of an electron from this free radical to oxygen would produce superoxide and *N*-acetyl-*p*-benzoquinone imine (Fig. 1). Support for such a mechanism and for the participation of this reaction pathway in hepatotoxicity comes from the observation

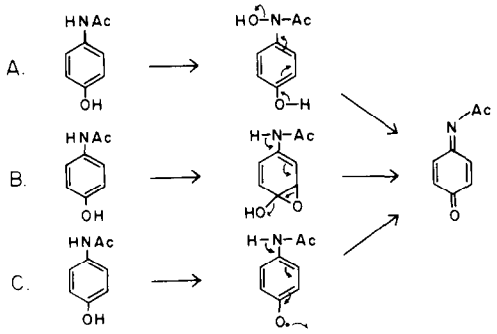

Fig. 1. Proposed mechanisms for the oxidation of acetaminophen to the hypothetical toxic intermediate, *N*-acetyl-*p*-benzoquinone imine. (A) The oxidation of acetaminophen by cytochrome P-450 to form *N*-hydroxyacetaminophen which loses water to give *N*-acetyl-*p*-benzoquinone imine. (B) The oxidation of acetaminophen by cytochrome P-450 to give an epoxide which loses water to yield *N*-acetyl-*p*-benzoquinone imine. (C) The one-electron oxidation of acetaminophen to form acetaminophen free radical. In the presence of oxygen, superoxide and *N*-acetyl-*p*-benzoquinone imine are produced.

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that acetaminophen can be oxidized to a free radical which exhibits all of the electrophilic properties assigned to the hypothetical toxic intermediate [13]. The possibility of a free radical mechanism of hepatotoxicity is further supported by the demonstration that promethazine [14], glutathione [6, 15, 16] and β-dimethylaminoethanol [17], which are known free radical scavengers, afford protection against acetaminophen toxicity.

Recent studies have demonstrated that chronic exposure to ethanol enhances acetaminophen toxicity in humans and experimental animals [18, 19]. It has been suggested that this is due to induction of cytochrome P-450 by ethanol [18, 19]. In this report, we have used the alcoholic animal model to further study the mechanism by which acetaminophen initiates hepatotoxicity. Our observations support the thesis that acetaminophen toxicity is mediated by a free radical mechanism.

MATERIALS AND METHODS

Animals. Random bred male golden Syrian hamsters were purchased from Charles River Laboratories. Animals were housed in groups of four or five in plastic cages containing corn cob bedding. On receipt, hamsters weighed between 70 and 85 g. They were maintained on pelleted rat chow (No. 5001, Ralston Purina Co.) with free access to water until the average weight exceeded 90 g. Upon reaching this weight, the chow and water were removed, and the animals received an ethanol-free liquid diet prepared as described by DeCarli and Lieber [20] except that the water content was decreased so that the diet yielded 2 kcal/ml. After 4 days on the liquid diet, the hamsters were separated into three groups. Group A continued on the liquid diet. Both groups B and C received the liquid diet with ethanol substituted isocalorically for sucrose as follows: day 1, 10% of the calories were provided by ethanol; day 2, 20%; day 3, 30%; day 4, and the next 6 weeks, 37%. Group B then continued to received the 37% ethanol diet up to and following the day of acetaminophen administration, whereas for Group C, the ethanol diet was replaced by the ethanol-free diet beginning 24 hr prior to the injection of acetaminophen. In addition to the liquid diet, all animals received approximately 25 g of cabbage leaves each day. Animals in the control diet group (Group A) increased weight by an average of 50 ± 15.5% (S.D.) compared to an increase of 30 ± 17% (S.D.) in the groups receiving the ethanol containing diet (Groups B and C). Despite the differences in body weight between ethanol-fed and control-fed animals, there were no significant differences in liver weights, which ranged from 3.6 ± 0.2 to 4.2 ± 0.7% (S.D.) of total body weight independent of diet.

Preparation of hepatic microsomes. Hamsters were killed between 9:00 and 10:00 a.m. Animals were stunned with a blow to the head and exsanguinated by cervical laceration. The livers were excised rapidly, and the gall bladders were removed and discarded. The livers were blotted dry and weighed. Hepatic microsomes were prepared according to the procedure of Rauckman *et al.* [21]. The microsomal fraction was analyzed immediately for cytochrome P-450 content and catalase activity. The cytosolic fraction was frozen at −70° and analyzed within 4 days for glutathione and protein content.

Acetaminophen administration. Acetaminophen solution was prepared by dissolving acetaminophen in sterile saline to a concentration of 25 mg/ml. The temperature was brought to 37°, and the solution was injected intraperitoneally at this temperature using pre-warmed syringes to prevent precipitation of acetaminophen. Hamsters were injected with acetaminophen between 5:00 and 7:00 p.m. Each animal was weighed and injected with acetaminophen solution to a final dose of 400 mg/kg body weight. Some animals from each of the three diet groups were injected with normal saline alone in a volume comparable to that injected into animals receiving acetaminophen. There were no deaths found in sixty-eight hamsters injected with saline alone.

Biochemical determinations. Cytochrome P-450 content was measured by the method of Omura and Sato [22]. Both reduced and oxidized glutathione were determined as described by Sies and Summer [23]. Protein was determined by the biuret method using bovine serum albumin as standard [24]. The increased formation of hydrogen peroxide (H_2O_2) in hepatic microsomes was measured using the Nash reagent as described by Hildebrandt *et al.* [25]. Catalase activity was measured as described in the literature [26].

Acetaminophen free radical. The acetaminophen free radical was generated by the action of either horseradish peroxidase (HRP, type II, Sigma Chemical Co.) and hydrogen peroxide or phenobarbital-induced hamster hepatic microsomes and an NADPH-generating system on acetaminophen. In the case of the HRP/H_2O_2 model system, the reaction mixture contained 1 mM acetaminophen, 1 mM H_2O_2, 0.04 units/ml HRP, and sufficient buffer [0.1 M sodium phosphate buffer containing 1 mM diethylenetriaminepentaacetic acid (DETAPAC), pH 5.0] to bring the final volume to 0.5 ml. There was no free radical generated if either HRP or H_2O_2 was omitted from the reaction mixture. In the case of hepatic microsomes generating the acetaminophen free radical, the reaction mixture contained 1 mM acetaminophen, 0.1 ml of microsomal suspension (12 mg/ml) and sufficient buffer containing the NADPH-generating system (pH 7.4) to bring the final volume to 0.5 ml. The reaction was started by the addition of a catalytic amount of NADPH (0.1 mg/ml).

Measurement of superoxide production by acetaminophen free radical. The generation of superoxide was measured during the one-electron oxidation of acetaminophen by the HRP/H_2O_2 model system in the presence of oxygen. The reaction mixture contained 1 mM acetaminophen, 1 mM H_2O_2, 0.04 units/ml HRP, 0.12 mg/ml cytochrome *c* (type VI, Sigma Chemical Co.) and sufficient buffer (0.1 M sodium phosphate buffer, pH 6.5) to bring the final volume to 1 ml. The rate of superoxide production was 5.9 μM/min, as measured by cytochrome *c* reduction, which was inhibited completely by the addition of superoxide dismutase (10 μg/ml). An

extinction coefficient of 20 mM^{-1} cm^{-1} for reduced versus oxidized cytochrome *c* was assumed [27].

Spin trapping of free radicals in the presence of acetaminophen free radical. The spin trapping of free radicals generated during the formation of acetaminophen free radicals in microsomes was undertaken as follows. In a typical experiment, the reaction medium contained 0.1 M *N-tert*-butyl-α-4-pyridylnitrone-1-oxide (4-POBN), 0.1 ml phenobarbital-induced hamster hepatic microsomes, 10 mM acetaminophen and sufficient buffer containing an NADPH-generating system to bring the final volume to 0.5 ml. The reaction was started by the addition of a catalytic amount of NADPH (0.1 mg/ml). The following necessary controls were performed and the rate of spin trapping of lipid peroxyl radicals (LOO·) was determined [28]. The spin trap was incubated with the induced hepatic microsomes in the absence of acetaminophen, and the spin trap was incubated in the absence of either the microsomal preparation or the NADPH-generating system. The HRP/H_2O_2 model system in the presence of hepatic microsomes and the spin trap 4-POBN was also used to determine the rate of lipid peroxyl radical formation. The following necessary controls were undertaken, and the rate of radical trapping (LOO·) was measured. The spin trap was incubated in the absence of acetaminophen and in the absence of either HRP or H_2O_2. The assignment of the free radical spin trapped by 4-POBN was based on previous studies using model systems [28].

3,5-*Dimethylacetaminophen.* The synthesis of 3,5-dimethylacetaminophen was undertaken using the methods outlined by Fernando *et al.* [29]. Briefly, the synthesis requires the reaction of 2,6-dimethylphenol with sodium nitrite to yield 2,6-dimethyl-4-nitrosophenol. Catalytic reduction of this compound with platinum and hydrogen in acetic acid/acetic anhydride gave, after purification, 3′,5′-dimethyl-4′-hydroxyacetanilide (3,5-dimethylacetaminophen), m.p. 160–162°.

RESULTS AND DISCUSSION

Half of the animals receiving the ethanol-free diet (Group A) died within 36 hr after a single intraperitoneal injection of acetaminophen (400 mg/kg) (Table 1). This mortality rate is similar to that observed by others [8], suggesting that the liquid diet was not a major determinant of acetaminophen-mediated hepatotoxicity. As expected [18, 19], those animals in which the ethanol-containing diet was continued to the time of injection (Group B) experienced an increase in mortality. Of greater interest were those hamsters in Group C where the ethanol-containing diet had been replaced by control diet 24 hr prior to the injection of acetaminophen. In these animals, there was a striking decrease in mortality when compared both to the animals maintained on the ethanol diet and those receiving no ethanol. The decrease in the death rate among Group C animals suggested that either bioactivation of acetaminophen to its toxic intermediate was depressed or that these hamsters exhibited enhanced protection against the toxic effects of this drug.

Our initial studies were aimed at determining why the Group C animals were less susceptible to acetaminophen toxicity. Histologic examination of the livers of surviving animals, 36 hr after acetaminophen treatment, was conducted to determine whether or not hepatotoxicity was a factor in the observed differences in mortality. As expected, animals in Groups A and B demonstrated massive mid- and centrilobular necrosis with fat deposition in the surrounding hepatocytes. Group C animals exhibited minimal centrilobular necrosis, although some fatty change was present. In control animals fed the alcohol diet but not receiving acetaminophen, neither necrosis nor fatty change was observed. These findings confirmed that acetaminophen induced hepatotoxicity and suggested that the reduced mortality in Group C animals was due to diminished hepatic necrosis.

A number of factors could have been responsible for the differences observed in the animals of Groups A, B, and C. One possibility is alterations in cytochrome P-450 levels. This enzyme is known to be involved in the bioactivation of acetaminophen to its toxic intermediate; diminished mixed function oxidase activity might account for the decreased mortality in Group C. As shown in Table 2, the cytochrome P-450 content of microsomes harvested from Group C animals was no different from that observed in the control group. As predicted [18, 19], there was an increase in cytochrome P-450 in the animals maintained on ethanol (Group B).

The effect of the three dietary regimens on hepatic reduced glutathione (GSH) was measured (Table 3), since it is known that hepatic GSH is an important determinant of acetaminophen-induced hepatotoxicity [8, 15, 16]. The data demonstrate that before acetaminophen administration Group C hamsters exhibited a 70% increase in hepatic GSH levels compared to Group A or Group B animals; however,

Table 1. Death after acetaminophen administration*

Group	N	Died	% Mortality
(A) No ethanol	36	18	50†
(B) Continuous ethanol	45	30	67†‡
(C) Ethanol discontinued 24 hr earlier	27	2	7

* Hamsters received 400 mg/kg acetaminophen (i.p.). There were no deaths among hamsters injected with saline.

† Significantly greater than Group C ($P < 0.01$).

‡ Significantly greater than Group A ($P < 0.01$).

Table 2. Hepatic microsomal cytochrome P-450 and catalase in hamsters killed before acetaminophen treatment*

Group	Microsomal protein (mg/ml)	Cytochrome P-450 (nmoles/mg protein)	Catalase† (μmoles H_2O_2/min/mg protein)
(A) No ethanol	18.6 ± 1.4	0.79 ± 0.04‡	1.61 ± 0.09§
(B) Continuous ethanol	22.0 ± 1.0	1.14 ± 0.05	1.70 ± 0.09§
(C) Ethanol discontinued 24 hr earlier	20.9 ± 1.4	0.85 ± 0.04‡	2.36 ± 0.10

* Values are the mean ± S.E.; N = 8.
† Forty-eight hours later, catalase specific activity in Group C was identical to that in Group A.
‡ Significantly different from Group B ($P < 0.001$).
§ Significantly different from Group C ($P < 0.001$).

3 hr after acetaminophen, GSH concentrations in all groups were depressed to the same level. These findings suggest that the enhanced GSH levels in the Group C animals were, to some extent, instrumental in the observed decrease in mortality in this group.

The specific activity of catalase was also increased markedly in the Group C animals (Table 2). In other animals (data not shown in Table 2), catalase activity was observed to remain above normal levels until 72 hr after ethanol was removed from the diet. The decreased mortality observed in Group C animals was associated therefore, with both increased GSH levels and increased catalase specific activity. Since catalase and GSH may act to reduce hydrogen peroxide to water [30, 31], we sought evidence that hydrogen peroxide may play a role in acetaminophen-mediated hepatotoxicity.

Incubation of hamster hepatic microsomes with acetaminophen and an NADPH-generating system led to a dose-dependent increase in hydrogen peroxide formation (Fig. 2). There are several pathways that may account for the production of this reactive species. During the oxidation of acetaminophen by cytochrome P-450, we hypothesize that a cytochrome P-450 (ferri)-peroxyl complex is formed which is reduced by acetaminophen giving hydrogen peroxide and acetaminophen free radical (Fig. 3). This free radical in the presence of oxygen produces superoxide which self-dismutes to give additional hydrogen peroxide.

Proof that such a mechanism is feasible comes from our observation that the cytochrome P-450 model, horseradish peroxidase/hydrogen peroxide, which is known to generate acetaminophen free radical [13], leads to the production of superoxide (5.9 μM/min) when incubated with acetaminophen and cytochrome *c* in the absence and presence of superoxide dismutase.

Incubation of hepatic microsomes from phenobarbital-treated hamsters (0.1% sodium phenobarbital in the drinking water for 5 days prior to sacrifice) with acetaminophen in the presence of an NADPH-generating system led to the formation of a free radical species which was identical to that generated by the cytochrome P-450 model system, horseradish peroxidase/hydrogen peroxide (Fig. 4). Based on our previous studies [13], we surmise that the radical species shown in Fig. 4 is acetaminophen free radical.

A mechanism to account for the formation of acetaminophen free radical, *N*-acetyl-*p*-benzoquinone imine, superoxide and hydrogen peroxide is illustrated in Fig. 5. We envision that cytochrome P-450 oxidizes acetaminophen to its free radical and in doing so produces hydrogen peroxide. Acetaminophen free radical in the presence of oxygen generates superoxide and the hypothetical intermediate *N*-acetyl-*p*-benzoquinone imine which we have been unable to isolate from our reaction mixtures.

With the enhanced production of hydrogen peroxide and superoxide during the oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine, one might anticipate that lipid peroxidation would take place. One of the most sensitive indicators of lipid peroxidation is the spin trapping of lipid peroxyl radicals (LOO·) by the acyclic nitrone, *N-tert-*

Table 3. Hepatic reduced glutathione concentration in alcoholic hamsters after acetaminophen treatment*

Group	GSH (nmoles/mg cytosolic protein)	
	Time after acetaminophen administration	
	0 hr	3 hr
(A) No ethanol	28.5 ± 0.5†	9.9 ± 0.5
(B) Ethanol continued	27.2 ± 1.2†	10.2 ± 0.4
(C) Ethanol discontinued 24 hr earlier	46.0 ± 2.8	9.5 ± 0.5

* Hamsters received 400 mg/kg acetaminophen (i.p.). Values are the mean ± S.E.; N = 8.
† Significantly different from Group C ($P < 0.001$).

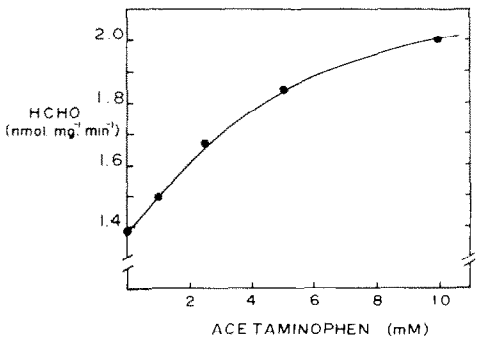

Fig. 2. Dose-dependent production of formaldehyde (a measure of hydrogen peroxide formation) in the presence of acetaminophen, hamster hepatic microsomes, and an NADPH-generating system.

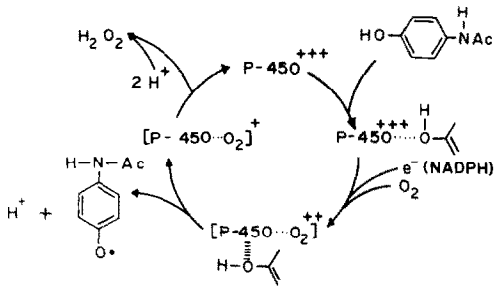

Fig. 3. Mechanism to account for the cytochrome P-450-mediated oxidation of acetaminophen to its free radical with the concomitant production of hydrogen peroxide.

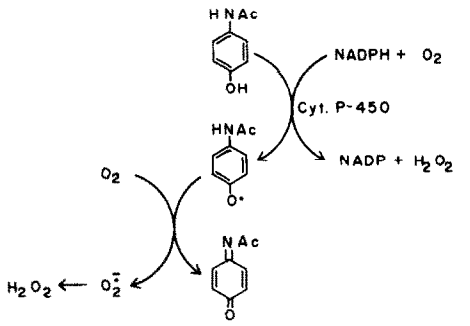

Fig. 5. Hypothetical mechanism to account for the production of superoxide and hydrogen peroxide during the oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine. Acetaminophen is oxidized to acetaminophen free radical along with the production of hydrogen peroxide. In the presence of oxygen, superoxide and hydrogen peroxide (from the dismutation of superoxide) are generated along with *N*-acetyl-*p*-benzoquinone imine.

butyl-α-4-pyridylnitrone 1-oxide (4-POBN) [28, 32]. When phenobarbital-induced hamster microsomes were incubated with acetaminophen, 4-POBN, and NADPH, lipid peroxyl radicals were spin trapped at a rate that was three times faster than was observed when this reaction was conducted in the absence of acetaminophen (Fig. 6). Identical results were observed when HRP/H_2O_2 was substituted for NADPH in the above reaction mixture. These findings clearly demonstrate that, during the oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine, lipid peroxidation occurs. Our observations are supported by the findings of Wendel and Heidinger [33] and Wendel *et al.* [34] who demonstrated that *in vivo* lipid peroxidation takes place after benzpyrene-treated mice have been administered acetaminophen.

Of major importance is the role that reduced glutathione (GSH) plays in the prevention of acetaminophen-mediated hepatotoxicity. First, GSH may act as a nucleophile as in the case of its reaction with *N*-acetyl-*p*-benzoquinone imine to give 3-(glutathion-*S*-yl)acetaminophen [7]. Second, GSH is a one-electron reductant in which two molecules of GSH would reduce *N*-acetyl-*p*-benzoquinone imine to yield acetaminophen and oxidized glutathione (GSSG) [35]. Third, GSH is the physiologic co-factor for glutathione peroxidase, the enzyme responsible for the reduction of lipid hydroperoxides and hydrogen peroxide to alcohols and water [36] (Fig. 7). The data presented in Table 4 clearly demonstrate that acetaminophen rapidly depleted the liver of glutathione. Noteworthy is the observation that the ratio of GSH:GSSG dropped from 8.4:1 to 2.6:1 after 3 hr. Since hepatocytes normally maintain a physiologic balance between reduced and oxidized glutathione of approximately 8:1 to 10:1, the change in the GSH:GSSG ratio suggests that GSH may have been converted to GSSG in the presence of acetaminophen. To test this hypothesis, hamsters were treated with diethyl maleate, a chemical known to deplete the liver of GSH. Studies have indicated that

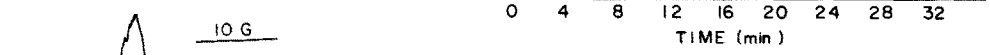

Fig. 4. EPR spectrum of acetaminophen free radical produced using phenobarbital-induced hamster hepatic microsomes and an NADPH-generating system. The microwave power was 20 mW, and the modulation frequency was 100 (k)Hz with an amplitude of 1 G. The sweep time was 12.5 G/min at a response time of 1 sec.

Fig. 6. Rate of spin trapping of lipid peroxyl radicals during the oxidation of acetaminophen by the cytochrome P-450 model system, horseradish peroxidase and hydrogen peroxide in the presence of hamster hepatic microsomes. Identical rate differences were obtained using phenobarbital-induced hepatic microsomes and an NADPH-generating system. Insert shows the spin trapped adduct resulting from the reaction of lipid peroxyl radicals with the spin trap 4-POBN to give 4-POBN-OOL, A_N = 15.8 G and A_H = 2.6 G. To measure the rate of spin trapping of lipid peroxyl radicals, the field set of the spectrometer was placed where indicated in the insert, and the height (which is directly proportional to the concentration) was measured as a function of time. Key: (A) in the absence of acetaminophen, and (B) in the presence of acetaminophen.

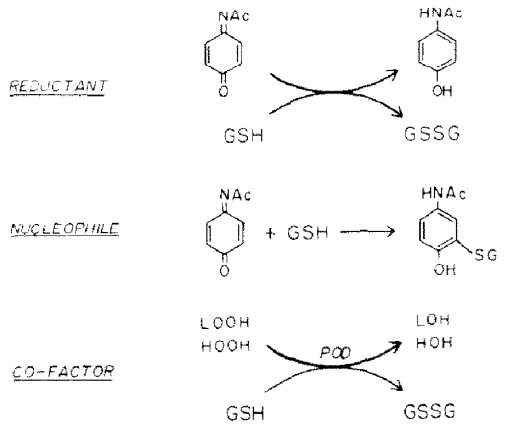

Fig. 7. Various roles reduced glutathione (GSH) can play in the prevention of acetaminophen-mediated hepatotoxicity. GSH can be a one-electron reductant in which two molecules of GSH would reduce *N*-acetyl-*p*-benzoquinone imine to give acetaminophen and oxidized glutathione (GSSG). GSH can act as a nucleophile and yield 3-(glutathion-*S*-yl)acetaminophen from its reaction with *N*-acetyl-*p*-benzoquinone imine. GSH is the physiologic co-factor for glutathione peroxidase (POD) which reduces lipid hydroperoxides and hydrogen peroxide to alcohols and water.

GSH makes a nucleophilic attack on diethyl maleate and, in this manner, removes GSH [37]. Three hours after the hamsters received diethyl maleate (200 mg/kg, i.p., in olive oil), hepatic glutathione levels dropped from 33.57 to 24.45 nmoles/mg cytosolic protein. Of interest was the finding that the GSH:GSSG ratio was unchanged (a slight increase from 10.4 to 12.3 was observed). This result suggests that the shift in the glutathione ratio 3 hr after acetaminophen treatment was not the result of GSH depletion by a nucleophilic reaction with *N*-acetyl-*p*-benzoquinone imine but was most likely due to conversion of GSH to GSSG.

Recent studies by Fernando *et al.* [29] suggest another approach to separating the action of GSH as a nucleophile from its action as a reductant and a co-factor for glutathione peroxidase. The investigators [29] synthesized 3,5-dimethylacetaminophen and found that this analog of acetaminophen was as hepatotoxic as acetaminophen. By analogy with acetaminophen, they reasoned that the toxic intermediate was *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine. Since GSH can only attack the 3 or the 5 position of *N*-acetyl-*p*-benzoquinone imine [7], depressed levels of GSH in animals receiving 3,5-dimethylacetaminophen would suggest alternative pathways for glutathione protection against 3,5-dimethylacetaminophen-mediated hepatotoxicity. When hamsters received 400 mg/kg of 3,5-dimethylacetaminophen, we found that GSH levels had dropped from 33.51 to 23.52 nmoles/mg cytosolic protein, 3 hr after administration of 3,5-dimethylacetaminophen. While our studies do not allow us to determine if GSH acted as a reductant or a co-factor for glutathione peroxidase, our results clearly demonstrate that GSH protected against the toxic effects of acetaminophen by pathways distinct from covalent binding to *N*-acetyl-*p*-benzoquinone imine.

In conclusion, we have observed that, when hamsters maintained on an alcohol-containing diet are removed from that diet (Group C), they are afforded significant protection against acetaminophen-mediated hepatotoxicity as compared to control animals (Group A). This observation correlates well with the observation that Group C hamsters had higher levels of catalase and reduced glutathione than were found in either Group A or Group B animals. We have also demonstrated that acetaminophen can be oxidized by cytochrome P-450 to acetaminophen free radical which, in the presence of oxygen, produces superoxide and presumably *N*-acetyl-*p*-benzoquinone imine. Although we were unable to identify this quinone imine, it is known that similar free radicals form quinones and superoxide in the presence of oxygen [38]. With the *in situ* formation of both superoxide and hydrogen peroxide, it is not surprising that lipid peroxidation was observed in hamster hepatic microsomes. The role glutathione plays in the protection against acetaminophen-mediated hepatotoxicity was examined. Previous investigators had demonstrated that GSH can make a nucleophilic attack at the 3 position of *N*-acetyl-*p*-benzoquinone imine to yield 3-(glutathion-*S*-yl)acetaminophen [7]. In this report, we have expanded the role of GSH in preventing acetaminophen-mediated hepatotoxicity to that of a reductant and/or a co-factor for glutathione peroxidase. Our findings, however, in no way disprove the current theory that once *N*-acetyl-*p*-benzoquinone imine is formed, binding to macromolecules leads to hepatic necrosis. Nevertheless, our results clearly demonstrate that another mechanism may be operative during acetaminophen-mediated hepatotoxicity.

Table 4. Hepatic reduced and oxidized glutathione levels in hamsters after acetaminophen administration*

Time after acetaminophen treatment (hr)	GSH (nmoles/mg cytosolic protein)	GSSG	GSH:GSSG
0	33.57 ± 1.56	3.99 ± 0.12	8.4:1
1	10.92 ± 0.31	3.28 ± 0.16	3.3:1
3	4.66 ± 0.36	1.79 ± 0.11	2.6:1

* Hamsters (fed Purina Rodent Chow, No. 5001) received 400 mg/kg acetaminophen (i.p.). Values are the mean ± S.E.; N = 8.

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