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ACETAMINOPHEN-INDUCED HEPATIC GLYCOGEN DEPLETION AND HYPERGLYCEMIA IN MICE*

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Abstract—Two hours following administration of a hepatotoxic dose of acetaminophen (500 mg/kg, i.p.) to mice, liver sections stained with periodic acid Schiff reagent showed centrilobular hepatic glycogen depletion. A chemical assay revealed that following acetaminophen administration (500 mg/kg) hepatic glycogen was depleted by 65% at 1 hr and 80% at 2 hr, whereas glutathione was depleted by 65% at 0.5 hr and 80% at 1.5 hr. Maximal glycogen depletion (85% at 2.5 hr correlated with maximal hyperglycemia (267 mg/100 ml at 2.5 hr). At 4.0 hr following acetaminophen administration, blood glucose levels were not significantly different from saline-treated animals; however, glycogen levels were still maximally depleted. A comparison of the dose–response curves for hepatic glycogen depletion and glutathione depletion showed that acetaminophen (50–500 mg/kg at 2.5 hr) depleted both glycogen and glutathione by similar percentages at each dose. Since acetaminophen (100 mg/kg at 2.5 hr) depleted glutathione and glycogen by approximately 30%, evidence for hepatotoxicity was examined at this dose to determine the potential importance of hepatic necrosis in glycogen depletion. Twenty-four hours following administration of acetaminophen (100 mg/kg) to mice, histological evidence of hepatic necrosis was not detected and serum glutamate pyruvate transaminase (SGPT) levels were not significantly different from saline-treated mice. The potential role of glycogen depletion in altering the acetaminophen-induced hepatotoxicity was examined subsequently. When mice were fasted overnight, hepatic glutathione and glycogen were decreased by 40 and 75%, respectively, and fasted animals showed a dramatic increase in susceptibility to acetaminophen-induced hepatotoxicity as measured by increased SGPT levels. Availability of glucose in the drinking water (5%) overnight resulted in glycogen levels similar to those in fed animals, whereas hepatic glutathione levels were not significantly different from those of fasted animals. Fasted animals and animals given glucose water overnight were equally susceptible to acetaminophen-induced hepatotoxicity, as quantitated by increases in SGPT levels 24 hr after drug administration. The potential role of a reactive metabolite in glycogen depletion was investigated by treating mice with *N*-acetylcysteine to increase detoxification of the reactive metabolite. *N*-Acetylcysteine treatment of mice prevented acetaminophen-induced glycogen depletion.

Large doses of the commonly used analgesic acetaminophen produce a centrilobular hepatic necrosis in man and experimental animals [1–4]. This toxicity is believed to be mediated by a reactive metabolite formed by the cytochrome P-450 mixed-function oxidase system. Evidence has been presented previously that this reactive metabolite is likely to be *N*-acetyl-*p*-benzoquinone imine [5–11]. Following a therapeutic dose, the reactive metabolite is believed to be efficiently detoxified by conjugation with glutathione [12, 13], and 3-(glutathion-*S*-yl)-acetaminophen is formed [14, 15]. This conjugate is further metabolized and excreted in the urine as an acetaminophen mercapturic acid [5]. After a hepatotoxic dose, however, liver glutathione is depleted by greater than 80%, and the reactive metabolite covalently binds to hepatic macromolecules [12, 13]. The amount of covalent binding of this metabolite correlates with severity of the hepatotoxicity [12].

Hepatic glycogen depletion has also been reported to occur following hepatotoxic doses of acetaminophen in mice and rats [4, 16]. Glycogen depletion was detected in histological sections using the glycogen stain, periodic acid Schiff reagent. The relationship between glycogen depletion and other events which occur following hepatotoxic doses of acetaminophen, however, has not been investigated. In this study, we have related acetaminophen metabolism to acetaminophen-induced glycogen depletion. Since starvation decreases liver glycogen [17] as well as liver glutathione [18] and dramatically increases susceptibility to the hepatotoxic effects of acetaminophen [19, 20], we tested the hypothesis that glycogenolysis may be an important determinant in protecting animals against the toxicity of acetaminophen.

MATERIALS AND METHODS

Chemicals. Acetaminophen, 5,5′-dithiobis-(2-nitrobenzoic acid), glutathione, glycogen, transaminase Diagnostic Kit No. 55-10P, and glucose Diagnostic Kit No. 635 were purchased from the Sigma Chemical Co. (St. Louis, MO). Anthrone reagent was obtained from Calbiochem (La Jolla, CA). Thiourea was a product of the Fisher Chemical

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Co. (Pittsburgh, PA). All other chemicals were of the purest grade commercially available.

Animals. Male mice (20 g; F1 offspring of male Balb/C crossed with female C57BL/6J) were obtained from the NCTR Division of Animal Husbandry and maintained on Purina Lab Chow and water *ad lib.* "Fed animals" had free access to the laboratory chow while "fasted animals" received no food after 5.00 p.m. For "fasted animals plus glucose water" the laboratory chow was replaced at 5.00 p.m. with a 5% (w/v) solution of glucose water to which the animals had free access overnight. The dosing began at 9.00 a.m. the next morning. All animals had free access to water. Acetaminophen doses (i.p. and p.o.) were administered as a saturated solution in saline (20 ml/kg), prepared by heating the acetaminophen solution to approximately 60° followed by cooling the solution to 37° in a water bath before injection. If crystals developed in the solution, the heating process was repeated.

Assays. Hepatic glutathione was assayed as total nonprotein sulfhydryl using Ellman's reagent [13, 21], and hepatic glycogen was assayed as total nonprotein anthrone-positive sugars [17]. For these assays animals were decapitated, and the livers were removed and placed on ice. Subsequently, the livers were weighed and homogenized in 5 vols. of cold 0.1 M sodium phosphate buffer, pH 7.4. One milliliter of each homogenate was thoroughly mixed with 1 ml of 4% (w/v) sulfosalicylic acid and centrifuged at 1000 *g* for 20 min. Aliquots of these supernatant fractions were used to assay for both glutathione and glycogen.

For the assay of glutathione [13, 21], 0.5 ml of the supernatant fractions was thoroughly mixed with 4.5 ml of 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.1 M sodium phosphate pH 8.0. The absorbance of these solutions was measured at 412 nm.

Total hepatic glycogen [17] was determined by removing 0.2 ml of the hepatic supernatant fraction and mixing this aliquot of supernatant with 5 ml of anthrone reagent [0.05% (w/v) anthrone, 1% (w/v) thiourea in 72% (v/v) sulfuric acid]. The color was developed by placing the tubes in a boiling water bath for 15 min. The absorbance of each sample was determined at 620 nm. If the optical density of the solution exceeded 3.0, the sample was diluted with additional anthrone solution.

For determination of serum glutamate-pyruvate transaminase (SGPT) levels and concentrations of blood glucose, blood samples were removed from the retro-orbital sinus. Blood glucose concentrations were determined by the *o*-toluidine spectrophotometric procedure using Sigma Kit No. 635. Controlled experiments revealed that acetaminophen did not interfere with the glucose assay. SGPT levels were determined enzymatically using Sigma Kit No. 55-10P.

Histology. Immediately after sacrifice and liver removal, a liver section was excised and immersed in 10% buffered neutral formalin for 24 hr. Fixed liver was then processed, embedded in paraffin blocks, sectioned at about 5 μm, mounted on glass slides, and stained with hematoxylin and eosin (H & E) and with periodic acid Schiff reagent (PAS). The latter stain was done with and without pretreatment with diastase.

Statistical analysis. The data were analyzed by Student's *t*-test. The minimum number of animals used to determine each mean value was five.

RESULTS

Histological examination of glycogen depletion. Two hours following administration of acetaminophen (500 mg/kg, i.p.) to mice, hepatic glycogen depletion was demonstrated by staining histological sections of the liver with PAS (Fig. 1A). The dark staining areas show the presence of glycogen concentration and the lighter staining areas show glycogen depletion. The acetaminophen-treated liver (Fig. 1A) showed glycogen depletion principally in the centrilobular areas compared to liver from saline-treated mice (Fig. 1B).

Since centrilobular glycogen depletion was prominent at 2 hr following administration of acetaminophen (Fig. 1A), the time course of the acetaminophen-induced central depletion of glycogen was measured. At 1.0 hr following administration of acetaminophen (500 mg/kg, i.p.), a centrilobular depletion of glycogen was evident in three of five acetaminophen-treated animals. At 1.5 and 2 hr following treatment, all the acetaminophen-treated animals had a dramatic glycogen depletion in the centrilobular areas. Furthermore, glycogen depletion extended to the midzonal area in many animals. Liver sections from saline-treated control animals contained a variable amount of glycogen. When glycogen depletion was apparent in these animals, it was characterized by a diffuse depletion in all areas of the liver lobule. The pattern of centrilobular glycogen depletion which characterized the acetaminophen-treated animals was not observed in the saline-treated control animals.

Comparison of glycogen depletion and glutathione depletion. Depletion of hepatic glutathione as a result of detoxification of the reactive metabolite is known to be an early event which occurs following administration of acetaminophen [13]. Since the above findings suggested that glycogen depletion is also an early event following administration of acetaminophen, hepatic glycogen depletion was quantitated by a chemical assay and was compared to hepatic glutathione depletion. A time course for hepatic glutathione depletion and hepatic glycogen depletion (Fig. 2) following acetaminophen administration (500 mg-kg, i.p.) revealed that glutathione depletion occurred before glycogen depletion. Whereas glutathione was depleted by 65% at 0.5 hr, glycogen was depleted by 65% at 1.0 hr. These data support the observation of Fig. 1, that glycogen depletion is an early event following acetaminophen administration.

Since a large dose of acetaminophen (500 mg/kg) was followed, in 2.5 hr, by maximal depletion of glycogen and glutathione, dose–response curves for acetaminophen-induced glutathione and glycogen depletion (Fig. 3) were obtained. These data demonstrate that acetaminophen induced concomitant decreases in both variables over the dose range.

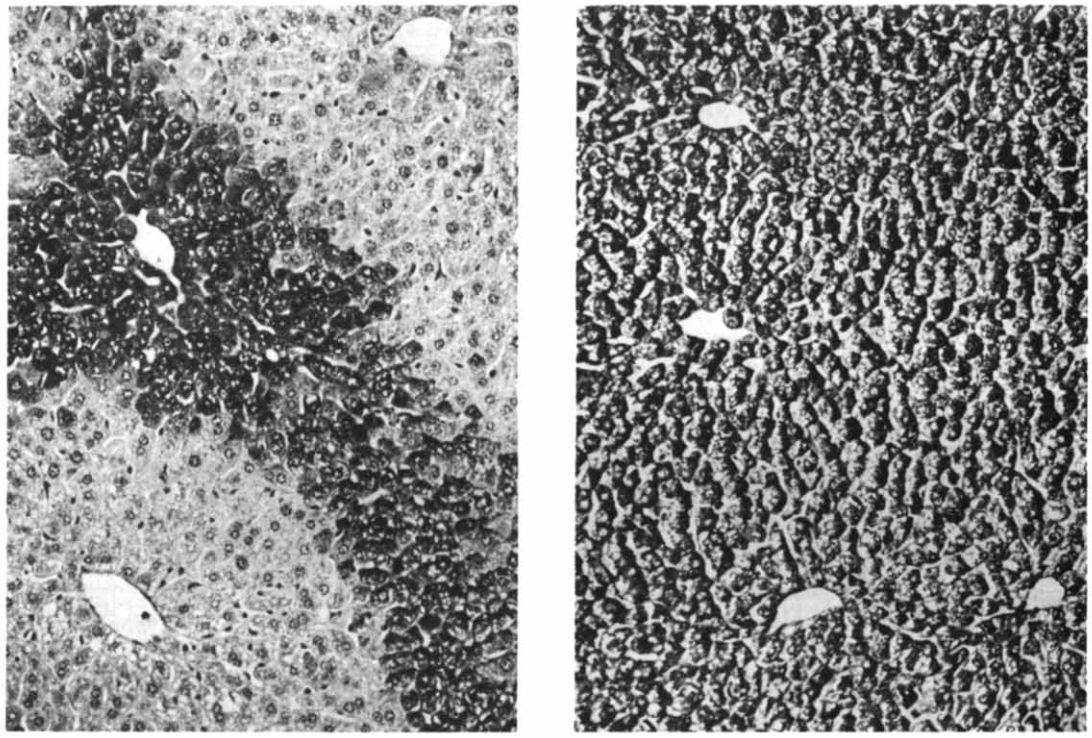

Fig. 1. (A) Photomicrograph of a section of liver from a mouse that had received 500 mg/kg acetaminophen, showing lack of PAS stainable glycogen in centrilobular areas and glycogen staining in periportal region. (B) Photomicrograph of a PAS stained liver section from a mouse that had received saline. Two hours after receiving acetaminophen (500 mg/kg, i.p.) or saline (i.p.) the livers were removed, fixed in neutral buffered formalin, and stained with PAS as described in Materials and Methods.

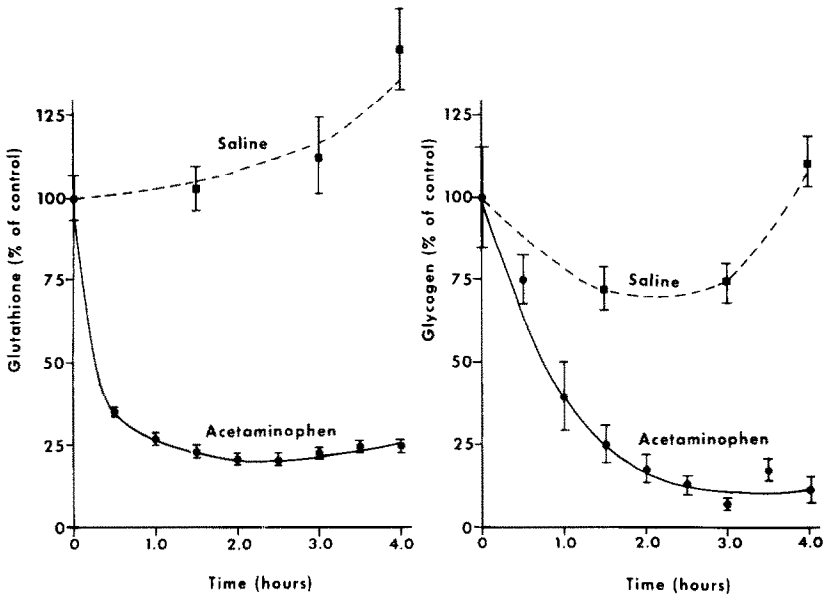

Fig. 2. Time courses of depletion of hepatic glutathione and glycogen following a hepatotoxic dose of acetaminophen. Either acetaminophen (500 mg/kg, i.p.) or saline (i.p.) was injected into mice, and the livers were removed at the indicated time. Glutathione was measured using Ellman's reagent as total nonprotein sulfhydryl [13, 22], and glycogen was measured using anthrone reagent [17] as described in Materials and Methods. Data are expressed as percent mean and S.E.M. N = 5.

Table 1. Dose response of acetaminophen-induced hepatotoxicity*

Treatment	Serum glutamate pyruvate transaminase levels
Saline	144 ± 53
Acetaminophen (100 mg/kg)	122 ± 55†
Acetaminophen (500 mg/kg)	13,370 ± 775‡

* Mice were treated with either saline (i.p.) or acetaminophen (i.p.), and serum glutamate pyruvate transaminase (SGPT) levels were determined 24 hr after dosage as described in Materials and Methods. The data are presented in μmoles per min per ml of plasma as the mean ± S.D., N = 5. Histological examination of mice receiving either saline (i.p.) or acetaminophen (100 mg/kg, i.p.) showed no evidence of hepatic necrosis, whereas mice receiving acetaminophen (500 mg/kg, i.p.) had a centrilobular hepatic necrosis.

† Not significantly different from saline-treated animals.

‡ Significantly different from saline-treated animals, $P < 0.001$. Significantly different from 100 mg/kg acetaminophen-treated animals, $P < 0.001$.

Comparison of the effects of saline (i.p.)-treated mice to acetaminophen (100 and 500 mg/kg, i.p.) on hepatotoxicity in mice (as measured by 24-hr increases in SGPT values) revealed that 100 mg/kg acetaminophen was not hepatotoxic, whereas 500 mg/kg acetaminophen was very hepatotoxic (Table 1). While 100 mg/kg acetaminophen depleted glycogen levels by approximately 25%, this same dose did not produce hepatic necrosis.

Since intraperitoneal doses of saline also lead to a transitory decrease in glycogen (Fig. 2), the effects of oral doses of acetaminophen and saline on glutathione and glycogen depletion were also examined (Table 2). Following a hepatotoxic dose of acetaminophen (500 mg/kg orally), glutathione and glycogen were depleted significantly, whereas orally administered saline did not significantly decrease glycogen levels compared to zero time controls (Table 2A).

To confirm that a dose of acetaminophen which does not produce hepatic necrosis may produce significant glycogen depletion, the effect of oral administration of acetaminophen (100 mg/kg) on hepatic glutathione and glycogen was examined. Neither glutathione nor glycogen levels were decreased significantly by saline treatment (Table 2B); however, orally administered acetaminophen (100 mg/kg) produced significant glutathione and glycogen depletion (Table 2B).

The above results show that glycogen depletion is apparently not the result of hepatic necrosis resulting from acetaminophen treatment. These data also indicate that acetaminophen-induced glycogen depletion is not dependent upon the route of administration.

Hyperglycemic effect of acetaminophen. Since glycogen may be metabolized to glucose, which results in hyperglycemia, the effect of acetaminophen on blood glucose levels was examined. Following administration of a hepatotoxic dose of acetaminophen (500 mg/kg, i.p.), blood glucose was increased significantly (Fig. 4). A maximum blood glucose con-

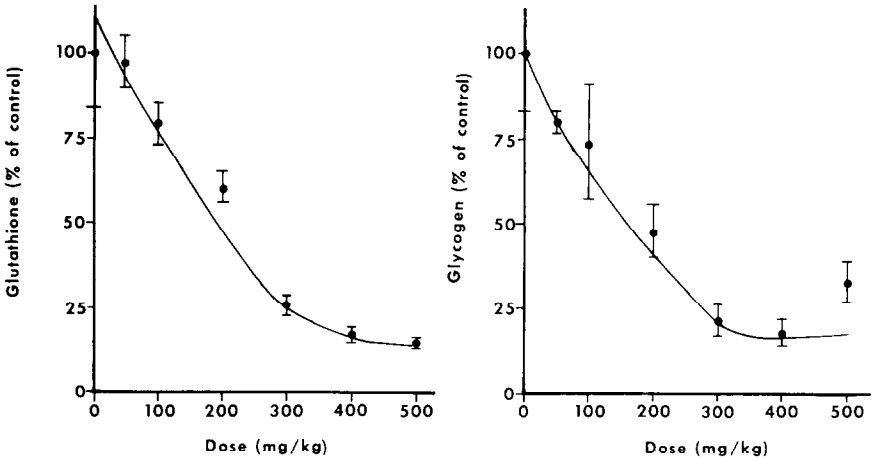

Fig. 3. Acetaminophen dose–response curves for depletion of hepatic glutathione and glycogen. Either saline or various doses of acetaminophen in the same volume of saline were injected into mice, and the livers were removed after 2.5 hr. Total hepatic glutathione and glycogen levels were determined as described in Materials and Methods. Data are expressed as percent mean and S.E.M. N = 8.

Table 2. Effect of oral administration of acetaminophen on hepatic glutathione and glycogen levels*

Treatment	A. High dose Glutathione (0 hr)	Glutathione (3.0 hr)	Glycogen (0 hr)	Glycogen (3.0 hr)
Acetaminophen (500 mg/kg)	100 ± 6	15 ± 4†	100 ± 16	24 ± 3†
Saline	100 ± 7	83 ± 8‡	100 ± 15	88 ± 11§
Treatment	**B. Low dose Glutathione (0 hr)**	**Glutathione (2.5 hr)**	**Glycogen (0 hr)**	**Glycogen (2.5 hr)**
Acetaminophen (100 mg/kg)	100 ± 15	66 ± 10¶	100 ± 22	73 ± 14‖
Saline	100 ± 13	96 ± 14**	100 ± 17	86 ± 14**

* Data are expressed as mean (percent of control) ± S.D., N = 8.

† Significantly different from 0 hr, acetaminophen-treated, $P < 0.001$. Significantly different from 3.0 hr, saline-treated animals, $P < 0.001$.

‡ Significantly different from 0 hr $P < 0.001$.

§ Not significantly different from 0 hr.

¶ Significantly different from 0 hr, $P < 0.001$. Significantly different from 2.5 hr, saline-treated animals, $P < 0.001$.

‖ Significantly different from 0 hr, $P < 0.02$. Significantly different from 2.5 hr, saline-treated animals, $P < 0.05$.

** Not significantly different from 0 hr.

centration occurred at approximately 2.5 hr (267 mg/100 ml blood), and blood glucose levels decreased to values that were not significantly different from saline-treated animals in 4.0 hr. Saline-treated animals (i.p.) (Fig. 4) produced only a marginal increase in blood glucose levels which correlated with a slight decrease in hepatic glycogen (Fig. 2).

An oral dose of acetaminophen which does not produce hepatic necrosis was examined for its effect on blood glucose levels. Acetaminophen (100 mg/kg) slightly although significantly ($P > 0.02$) increased blood glucose levels from 114 to 126 mg/100 ml (Table 3). Saline administration did not alter significantly blood glucose levels. These data correlate with the finding that acetaminophen (100 mg/kg) significantly decreased hepatic glycogen (Table 2B).

Effect of N*-acetylcysteine treatment on acetaminophen-induced hepatic glycogen depletion.* Even though glycogen depletion is apparently not dependent upon cell necrosis, the observation that various doses of acetaminophen coordinately depleted hepatic glutathione and glycogen levels (Fig. 3) suggests that these events may be related. The effect of *N*-acetylcysteine treatment on acetaminophen-induced hepatic glycogen depletion in mice was subsequently examined. *N*-Acetylcysteine is known to increase the soluble sulfhydryl content of the hepatocytes and increase detoxification of the reactive metabolite [23, 24]. This compound has thus been used as an antidote to acetaminophen overdose [1, 22].

Table 4 shows that *N*-acetylcysteine treatment (p.o.) alone did not significantly alter hepatic glycogen levels compared to saline-treated (p.o.) mice, whereas acetaminophen treatment (p.o.) results in approximately a 50% decrease in glycogen. *N*-Acetylcysteine treatment significantly prevented this acetaminophen-induced glycogen depletion. The glycogen contents of livers from animals receiving acetaminophen plus *N*-acetylcysteine were not significantly different from animals receiving only saline. Since *N*-acetylcysteine increases detoxifica-

Table 3. Effect of low dose of acetaminophen on blood glucose*

Treatment	Glucose (mg/100 ml, 0 hr)	Glucose (mg/100 ml, 2.5 hr)
Acetaminophen (100 mg/kg)	114 ± 8	126 ± 10†
Saline	105 ± 8	111 ± 11‡

* Acetaminophen (100 mg/kg) and saline were administered by gastric intubation. This dose of acetaminophen did not increase SGPT levels compared to saline-treated controls (Table 1). Data are expressed as mean ± S.D., N = 8.

† Significantly different from 0 hr, $P < 0.02$. Significantly different from saline-treated controls, $P < 0.02$.

‡ Not significantly different from 0 hr.

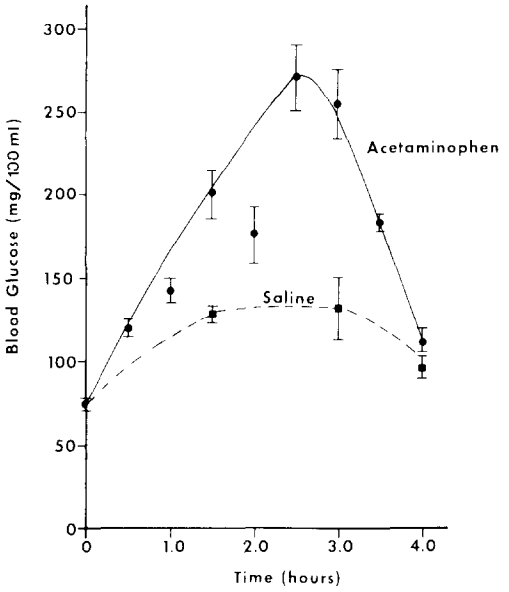

Fig. 4. Effect of acetaminophen on blood glucose levels. Blood samples were taken from the retro-orbital sinuses of the mice used to collect the data presented in Fig. 2 and were analyzed for blood glucose levels as described in Materials and Methods. The data are presented as mean values of mg glucose/100 ml blood ± S.D. N = 5.

Table 4. Effect of *N*-acetylcysteine treatment on acetaminophen-induced hepatic glycogen depletion*

Treatment	Glycogen	Nonprotein sulfhydryl
Saline	100 ± 6	100 ± 8
Acetaminophen	53 ± 3†	42 ± 11†
N-Acetylcysteine	103 ± 5‡	137 ± 5§
Acetaminophen plus *N*-acetylcysteine	92 ± 8‖	146 ± 12§¶

* Mice were treated with either saline (p.o.), acetaminophen (300 mg/kg, p.o.) *N*-acetylcysteine (1200 mg/kg, p.o.) or acetaminophen (300 mg/kg, p.o.) plus *N*-acetylcysteine (1200 mg/kg, p.o.). Acetaminophen and *N*-acetylcysteine were administered in saline. Acetaminophen plus *N*-acetylcysteine was dissolved in the same saline solution for coadministration. Glycogen and nonprotein sulfhydryl were determined 2.5 hr after administration. The data are presented as mean (percent of control) ± S.D. N = 5.

† Significantly different from saline-treated animals, $P < 0.001$.

‡ Not significantly different from saline-treated animals. Significantly different from acetaminophen-treated animals, $P < 0.001$.

§ Significantly different from saline-treated animals, $P < 0.001$. Significantly different from acetaminophen-treated animals, $P < 0.001$.

‖ Not significantly different from saline- or *N*-acetylcysteine-treated animals. Significantly different from acetaminophen-treated animals, $P < 0.001$.

¶ Not significantly different from *N*-acetylcysteine-treated animals.

tion of the reactive metabolite [23, 24], these data suggest that glycogen depletion is related to the formation of the reactive metabolite of acetaminophen.

Examination of the role of glycogen metabolism in decreasing the hepatotoxic effects of acetaminophen. Since glycogen depletion is apparently related to the formation of the reactive metabolite of acetaminophen, the possibility exists that glycogenolysis affords the animal protection against the toxicity of acetaminophen. An animal model was subsequently devised to examine the role that glycogen metabolism may have in acetaminophen-induced hepatotoxicity. Removal of food from the animals overnight (16 hr-fasted animals) decreased hepatic glutathione and glycogen by approximately 40 and 75%, respectively, as compared to animals that had free access to food (fed animals; Table 5A). When food was removed from animals overnight (16 hr), and glucose water (5%) was made available to these animals (fasted animals plus glucose water), glutathione levels were not significantly different from fasted animals; however, glycogen levels were not appreciably different from fed animals (Table 5A). Thus, we were able to alter glycogen while holding glutathione levels constant.

The hepatotoxic effects of acetaminophen were subsequently examined using this animal model. Fasted animals, and fasted animals plus glucose water, were extremely susceptible to the toxic effects of acetaminophen. Administration of a hepatotoxic dose of acetaminophen (500 mg/kg) to fed animals results in no deaths at 24 hr; however, in fasted animals and fasted animals plus glucose water, this dose produced a high percentage of mortality by 4 hr (data not shown). Similar results were obtained with a 400 mg/kg dose of acetaminophen. An oral acetaminophen dose of 250 mg/kg was not lethal in any of the animal groups, and this dose was used for the comparative hepatotoxicity studies. This acetaminophen dose significantly increased SGPT levels in fasted animals and fasted animals plus glucose water (Table 5B), but did not increase significantly SGPT levels in fed animals. Moreover, SGPT levels of fasted animals, which were low in glycogen and of fasted animals plus glucose water, which were high in glycogen, were not significantly different. Histological examination of livers from these treatment groups revealed marked centrilobular hepatic necrosis in all fasted animals and in all fasted animals plus glucose water, whereas only a very mild centrilobular hepatic necrosis was observed in some, but not all, fed animals. Tabulated histological data on the severity of liver necrosis of acetaminophen-treated fasted animals plus glucose water revealed that the two groups were equally sensitive to the hepatotoxicity. These data suggest that glycogenolysis does not protect the animal against the hepatotoxic effects of acetaminophen.

Since starvation is known to alter drug metabolism [25], the possibility existed that the metabolism of acetaminophen may be different in fasted animals and fasted animals plus glucose water. It could be envisioned that fasted animals plus glucose converted more acetaminophen to a reactive metabolite than fasted animals, but that this effect would be cancelled by a decrease in the severity of the toxicity in the glucose-fed animals due to glycogen metabolism.

Table 5. Role of hepatic glycogen in acetaminophen-induced hepatotoxicity*

A. Effect of fasting and availability of glucose water on hepatic glutathione and glycogen levels

Assay	Fed animals	Fasted animals	Fasted animals + glucose water
Hepatic glutathione	100 ± 9	57 ± 2†	50 ± 7‡
Hepatic glycogen	100 ± 19	23 ± 3†	70 ± 6§

B. Effect of fasting and availability of glucose water on acetaminophen-induced hepatotoxicity

	SGPT		
Treatment	Fed animals	Fasted animals	Fasted animals + glucose water
Acetaminophen (250 mg/kg)	492 ± 273¶	10,166 ± 2,700‖	10,728 ± 2,537**
Saline	302 ± 77	281 ± 31	254 ± 36††

* Fasted animals were without food for 16 hr (from 5:00 p.m. to 9:00 a.m), whereas fasted animals and glucose water had free access to 5% glucose water for the same time interval. Fed animals had free access to laboratory chow. All animals had free access to water. Glutathione and glycogen values are presented as percent mean of fed animals ± S.D. N = 5. SGPT values were determined 24 hr after treatment and are expressed in μmoles per min per ml of plasma as mean ± S.D. N = 5.

† Significantly different from fed animals, $P < 0.001$.

‡ Significantly different from fed animals, $P < 0.001$. Not significantly different from fasted animals.

§ Significantly different from fed animals, $P < 0.01$. Significantly different from fasted animals, $P < 0.001$.

¶ Not significantly different from saline-treated animals.

‖ Significantly different from acetaminophen-treated fed animals, $P < 0.001$. Significantly different from saline-treated fasted animals, $P < 0.001$.

** Significantly different from acetaminophen-treated fed animals, $P < 0.001$. Not significantly different from acetaminophen-treated fasted animals. Significantly different from saline-treated fasted animals plus glucose water, $P < 0.001$.

†† Not significantly different from saline-treated fed or fasted animals.

Table 6. Effect of glucose administration on acetaminophen-induced hepatotoxicity in fasted mice*

Treatment	Serum glutamate-pyruvate transaminase levels
Saline	226 ± 53
Glucose	204 ± 48†
Acetaminophen	13,046 ± 1,706‡
Acetaminophen + glucose	12,890 ± 1,870‡§

* Mice were fasted as described in Materials and Methods and subsequently were treated with either saline (i.p.), glucose (2 g/kg, i.p.), acetaminophen (250 mg/kg, i.p.) or the same doses of acetaminophen plus glucose. In acetaminophen plus glucose-treated animals, glucose was administered immediately after acetaminophen treatment. Levels of SGPT were determined 24 hr after treatment as described in Materials and Methods. The data are presented in μmoles per min per ml of plasma as mean ± S.D. N = 5.

† Not significantly different from saline-treated control.

‡ Significantly different from saline- or glucose-treated control, $P < 0.001$.

§ Not significantly different from acetaminophen-treated animals.

Examination of metabolism of acetaminophen to a covalently bound metabolite in microsomal incubation mixtures indicated no differences in the rate of metabolism using microsomes from fed, fasted, and fasted plus glucose water animals (data not shown). This possibility was also examined *in vivo* by determining the effect of glucose administration on acetaminophen-induced hepatotoxicity in fasted mice (Table 6). Since glycogen is metabolized to glucose, administration of glucose may mimic the effect of glycogen depletion. Mice receiving acetaminophen (250 mg/kg) and acetaminophen (250 mg/kg) plus glucose (2 g/kg) were equally susceptible to the hepatotoxic effects of acetaminophen. The finding that glucose administration did not alter the severity of the acetaminophen-induced hepatotoxicity in fasted mice (Table 6) further suggested that carbohydrate metabolism did not appreciably protect the animals against the toxicity.

DISCUSSION

Hepatic glycogen depletion occurs following administration of many hepatotoxins to animals. For

example, carbon tetrachloride [26–29], dimethylnitrosamine [30], bromobenzene [31], and dimethylaminoazobenzene [32, 33] have been shown by histological techniques to produce glycogen depletion. Analogously, acetaminophen has been reported previously (by the use of histological stains) to produce hepatic glycogen depletion [4, 16]. In this study, we have examined the role of glycogen depletion as it may relate to acetaminophen metabolism and glutathione depletion.

At therapeutic doses, acetaminophen is metabolized primarily by sulfation, glucuronidation, and cytochrome P-450 catalyzed conversion to a reactive metabolite. This reactive metabolite has been postulated to be *N*-acetyl-*p*-benzoquinone imine [1, 5, 10, 11] and is believed to be efficiently detoxified by glutathione to form 3-(glutathion-*S*-yl)-acetaminophen [14, 15]. Following a hepatotoxic dose, however, sulfate is depleted [34, 35], glutathione is depleted [13], and glucuronidation becomes the rate-limiting step, suggesting that a greater proportion of the total dose may be metabolized to the reactive metabolite [34]. Under these conditions of glutathione depletion, the reactive metabolite is inefficiently detoxified and covalently binds to cellular nucleophiles [12]. The finding that the amount of covalent binding correlates with the severity of the necrosis suggests that the toxicity is produced by the reactive metabolite. The toxicity, however, cannot necessarily be attributed to covalent binding because the reactive metabolite is not only an electrophile but also is an oxidizing agent and may be reduced by various cellular reducing agents such as ascorbate [1, 9, 36], NADPH [10], and presumably NADH. Depending upon the significance of this oxidation-reduction cycle, and coupled with glutathione depletion, significant alterations in the oxidation-reduction balance within the hepatocyte may occur following a hepatotoxic dose of acetaminophen. Since glycogen mobilization is one of the initial steps in intermediary metabolism leading to the production of reducing equivalents within the hepatocyte, it occurred to us that glycogen depletion may be a manifestation of an initial regulatory step which increases intermediary metabolism, resulting in protection of the hepatocytes. Also, glycogen metabolism may lead to increased UDPGA levels within the hepatocyte and increased glucuronidation of acetaminophen [37]. For these reasons, acetaminophen-induced glycogen depletion has been examined.

In agreement with previous reports, histological examination of livers from animals receiving acetaminophen revealed glycogen depletion which occurred initially in the centrilobular region (Fig. 1A) [4, 16]. Since the centrilobular areas are the site of the developing necrosis and Jollow *et al.* presented evidence that the reactive metabolite is formed in this area [12], centrilobular glycogen depletion suggests a possible relationship between acetaminophen metabolism and glycogenolysis.

A quantitative comparison of acetaminophen-induced total hepatic glycogen depletion and acetaminophen metabolism, as measured by glutathione depletion, revealed that 500 mg/kg acetaminophen produced a 65% depletion of glutathione at 30 min following drug administration, whereas glycogen was depleted 65% at 1 hr (Fig. 2). Thus, glutathione depletion preceded glycogen depletion, and both glutathione and glycogen were decreased maximally at 2.5 hr (Fig. 2). A dose–response curve indicated that various doses of acetaminophen depleted glutathione and glycogen at 2.5 hr by similar percentages (Fig. 3).

Maximal glycogen depletion corresponded to a dramatic increase in blood glucose levels, suggesting that some of the hepatic glycogen, via the intermediate glucose-1-phosphate, was converted to glucose and that the glucose entered the circulation. The factors related to the decrease in blood glucose levels at 4.0 hr from its peak at 2.5 hr (Fig. 2) have not been investigated; however, since glycogen was still depleted maximally at 4.0 hr (Fig. 2), the glucose was not resynthesized into hepatic glycogen.

The clinical implications of acetaminophen-induced hyperglycemia in humans are unclear; however, hyperglycemia is known to occur with parenchymal liver injury [38]. Thomson and Prescott [39] reported hyperglycemia (156 mg/100 ml) and glycosuria in an acetaminophen-overdosed patient. Moreover, the patient had an altered glucose-tolerance curve; however, the role that the acetaminophen may have had in this effect was unclear. Also, the relationship between the hyperglycemia and the liver damage was not established.

The major control mechanism for glycogenolysis in the liver is the concentration of glycogen phosphorylase [40]. This enzyme catalyzes the rate-limiting step in glycogenolysis, and its formation is stimulated, at least in part, by increased levels of cyclic AMP. Various modulators which stimulate cyclic AMP production increase the amount of glycogen phosphorylase in the hepatocyte leading to increased glycogenolysis. Some of these factors are hypoxia, ionic changes, increased levels of epinephrine or glucagon, and alpha adrenergic mechanisms [40–42].

The mechanism by which acetaminophen stimulates hepatic glycogen depletion is still unclear. However, in an initial analysis five mechanisms seemed plausible: (1) glycogen depletion is a direct result of the parent compound, acetaminophen; (2) glycogen depletion is a result of hepatic necrosis; (3) glycogen depletion is a result of decreased glutathione levels; (4) glycogen depletion is a result of increased levels of extrahepatic modulators of hepatic glycogen metabolism; and (5) glycogen depletion is an indicator of toxicity but does not necessarily lead to necrosis.

The simplest explanation for acetaminophen-induced glycogen depletion is that acetaminophen itself stimulated glycolysis. However, the finding that glycogen depletion could be prevented by *N*-acetylcysteine treatment (Table 4) indicated this mechanism was not correct. Alternatively, it seemed possible that glycogen depletion may be a result of hepatic necrosis. A careful examination of the effects of a low dose of acetaminophen (100 mg/kg) revealed that this dose depleted hepatic glycogen by 30% (Table 2, Fig. 3); however, this acetaminophen dose did not produce histological evidence of hepatic necrosis or increased serum glutamate pyruvate

transaminase levels (Table 1), a sensitive indicator of hepatic necrosis. Thus, acetaminophen-induced glycogen depletion is not the result of the hepatic necrosis.

Available evidence suggests that, at least with other hepatotoxins, hepatic glutathione depletion and hepatic glycogen depletion are separate events. For example, administration of carbon tetrachloride results in hepatic glycogen depletion [28]; however, carbon tetrachloride does not deplete hepatic glutathione [43]. These data indicate that glutathione depletion is not a prerequisite for glycogen depletion with another hepatotoxin. Also, it has been shown using isolated rat hepatocytes that concentrations of diethylmaleate which deplete glutathione do not produce glycogen depletion [44].

A fourth mechanism whereby acetaminophen may be envisioned to produce hepatic glycogen depletion is via an extrahepatic control. For example, increased blood levels of epinephrine or glucagon produce hepatic glycogen depletion [40–42]. Moreover, Rubinstein [28] found that administration of carbon tetrachloride to rats increased blood epinephrine levels. If an extrahepatic control mechanism such as epinephrine is important in acetaminophen-induced glycogen depletion, a number of questions must be answered. What is the relationship between the reactive metabolite and activation by this mechanism? Why was acetaminophen-induced glycogen depletion observed only in the centrilobular areas?

The last of the initially proposed mechanisms is that acetaminophen-induced glycogen depletion is a sensitive indicator of toxicity but does not necessarily lead to necrosis. This mechanism is supported by the *N*-acetylcysteine data (Table 4) which suggest the involvement of a reactive metabolite in the depletion. By this mechanism all the reactive metabolite that is produced following a low dose of acetaminophen such as 100 mg/kg would not be detoxified by glutathione, and some metabolite would interact with other cellular components to activate glycogenolysis. The toxic interaction of the reactive metabolite with cellular components, however, would not be of a magnitude to produce cell necrosis. At a high dose of acetaminophen (500 mg/kg), glycogen depletion would also be initiated but the degree of toxic interaction of the reactive metabolite with cellular components would be much greater and lead to cell necrosis. This mechanism is contrary to the current theory. In essence this theory maintains that, at acetaminophen doses which do not maximally deplete glutathione, the reactive metabolite is very efficiently detoxified; however, at large acetaminophen doses which deplete glutathione maximally, the reactive metabolite covalently binds to protein, and cell necrosis ensues. This theory is based primarily on the correlation of covalent binding with toxicity data, which showed that significant levels of covalently bound metabolite were not detected until necrosis was histologically evident [4, 5, 13].

No matter what the mechanism of glycogen depletion is, available evidence suggests that glycogenolysis is not a regulatory mechanism which substantially decreases the hepatotoxic effects of acetaminophen. This was determined by showing that mice with high and low liver glycogen levels were equally susceptible to the hepatotoxic effects of acetaminophen, whereas animals low in liver glutathione were very sensitive to hepatotoxicity compared to animals high in liver glutathione (Table 5). Thus, glutathione appears to be the most important detoxification mechanism. This conclusion was substantiated by determining that glucose administration did not alter the hepatotoxic effects of acetaminophen (Table 6). Since the dose of glucose chosen (11.1 mmoles/kg) was approximately 9-fold greater than the dose of the glutathione precursor cysteine (1.24 mmoles/kg) which had been shown previously to significantly decrease the hepatotoxic effects of acetaminophen in mice [13], the data add further support to the concept that glutathione is the primary detoxification mechanism. Even though the data in Tables 5 and 6 indicate that glycogenolysis does not lead to as substantial a decrease in the acetaminophen-induced hepatotoxicity as gluthathione does, because of the animal variations in SGPT values (Tables 5 and 6), the data do not completely rule out the possibility that it may play a minor role.

In summary, acetaminophen induced centrilobular hepatic glycogen depletion. The increased glycogenolysis was apparently not a control mechanism that produced substantial protection against the hepatotoxicity. The depletion did not appear to be initiated by the parent compound or by hepatotoxicity. The finding that *N*-acetylcysteine blocked the acetaminophen-induced glycogen depletion suggests that this depletion was a result of reactive metabolite formation. Since acetaminophen doses which did not produce hepatic necrosis did induce glycogen depletion, the depletion may be a sensitive indicator of a toxic event in the cell which does not lead to hepatic necrosis at low doses. At high doses, cumulative events may produce cell necrosis. Alternatively, acetaminophen-induced glycogen depletion may result from an extrahepatic glycogen control mechanism which can be influenced by the reactive metabolite.

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