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Research Article

N-Acetylcysteine is the drug of choice for the treatment of an acetaminophen overdose. It is thought to provide cysteine for glutathione synthesis and possibly to form an adduct directly with the toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine. However, these hypothese have not been tested in vivo, and other mechanisms of action such as reduction of the quinoneimine might be responsible for the clinical efficacy of *N*-acetylcysteine. After the administration to rats of acetaminophen (1 g/kg) intraduodenally (i.d.) and of [^{35}S]-*N*-acetylcysteine (1.2 g/kg i.d.), the specific activity of the *N*-acetylcysteine adduct of acetaminophen (mercapturic acid) isolated from urine and assayed by high pressure liquid chromatography averaged 76±6% of the specific activity of the glutathione-acetaminophen adduct excreted in bile, indicating that virtually all *N*-acetylcysteine-acetaminophen originated from the metabolism of the glutathione-acetaminophen adduct rather than from a direct reaction with the toxic metabolite. *N*-Acetylcysteine promptly reversed the acetaminophen-induced depletion of glutathione by increasing glutathione synthesis from 0.54 to 2.69 μmol/g per h. Exogenous *N*-acetylcysteine did not increase the formation of the*N*-acetylcysteine and glutathione adducts of acetaminophen in fed rats. However, when rats were fasted before the administration of acetaminophen, thereby increasing the stress on the glutathione pool, exogenous *N*-acetylcysteine significantly increased the formation of the acetaminophen-glutathione adduct from 57 to 105 nmol/min per 100 g. Although the excretion of acetaminophen sulfate increased from […]

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ABSTRACT *N*-Acetylcysteine is the drug of choice for the treatment of an acetaminophen overdose. It is thought to provide cysteine for glutathione synthesis and possibly to form an adduct directly with the toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine. However, these hypotheses have not been tested in vivo, and other mechanisms of action such as reduction of the quinoneimine might be responsible for the clinical efficacy of *N*-acetylcysteine. After the administration to rats of acetaminophen (1 g/kg) intraduodenally (i.d.) and of [^{35}S]-*N*-acetylcysteine (1.2 g/kg i.d.), the specific activity of the *N*-acetylcysteine adduct of acetaminophen (mercapturic acid) isolated from urine and assayed by high pressure liquid chromatography averaged 76±6% of the specific activity of the glutathione-acetaminophen adduct excreted in bile, indicating that virtually all *N*-acetylcysteine-acetaminophen originated from the metabolism of the glutathione-acetaminophen adduct rather than from a direct reaction with the toxic metabolite. *N*-Acetylcysteine promptly reversed the acetaminophen-induced depletion of glutathione by increasing glutathione synthesis from 0.54 to 2.69 μmol/g per h. Exogenous *N*-acetylcysteine did not increase the formation of the *N*-acetylcysteine and glutathione adducts of acetaminophen in fed rats. However, when rats were fasted before the administration of acetaminophen, thereby increasing the stress on the glutathione pool, exogenous *N*-acetylcysteine significantly increased the formation of the acetaminophen-glutathione adduct from 57 to 105 nmol/min per 100 g. Although the excretion of acetaminophen sulfate increased from 85±15 to 211±17 μmol/100 g per 24 h after *N*-acetylcysteine, kinetic simulations showed that increased sulfation does not significantly decrease formation of the toxic metabolite. Reduction of the benzoquinoneimine by *N*-acetylcysteine should result in the formation of *N*-acetylcysteine disulfides and glutathione disulfide via thiol-disulfide exchange. Acetaminophen alone depleted intracellular glutathione, and led to a progressive decrease in the biliary excretion of glutathione and glutathione disulfide. *N*-Acetylcysteine alone did not affect the biliary excretion of glutathione disulfide. However, when administered after acetaminophen, *N*-acetylcysteine produced a marked increase in the biliary excretion of glutathione disulfide from 1.2±0.3 nmol/min per 100 g in control animals to 5.7±0.8 nmol/min per 100 g. Animals treated with acetaminophen and *N*-acetylcysteine excreted 2.7±0.8 nmol/min per 100 g of *N*-acetylcysteine disulfides (measured by high performance liquid chromatography) compared to 0.4±0.1 nmol/min per 100 g in rats treated with *N*-acetylcysteine alone. In conclusion, exogenous *N*-acetylcysteine does not form significant amounts of conjugate with the reactive metabolite of acetaminophen in the rat in vivo but increases glutathione synthesis, thus providing more substrate for the detoxification of the reactive metabolite in the early phase of an acetaminophen intoxication when the critical reaction with vital macromolecules occurs.

INTRODUCTION

N-Acetylcysteine and other sulfhydryl donors such as cysteine, methionine, and cysteamine have been shown to be effective antidotes protecting against the hepa-

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totoxicity of acetaminophen both in animal models (1–4) and clinical practice (5, 6). Because of its ease of administration and low toxicity, *N*-acetylcysteine is currently the favorite compound for the treatment of acetaminophen overdose in man (6, 7). Despite its widespread clinical use, however, the mechanism(s) of action of *N*-acetylcysteine in vivo remain to be demonstrated.

N-Acetylcysteine could conceivably protect against the hepatotoxicity of acetaminophen in several ways. First, *N*-acetylcysteine may serve as a precursor for glutathione synthesis. Glutathione plays a critical role in the protection against hepatic necrosis produced by acetaminophen (1). Hepatocellular necrosis occurs only when the hepatic content of glutathione falls below a critical threshold concentration (1). A stimulation of glutathione synthesis following the administration of *N*-acetylcysteine and thus a greater availability of glutathione for the detoxification of the toxic acetaminophen intermediate should protect against liver injury. In isolated hepatocytes, *N*-acetylcysteine is indeed capable of supporting glutathione synthesis when it is the only source of cysteine (8). However, in isolated cells incubated with acetaminophen, very high concentrations of *N*-acetylcysteine or preincubation with the sulfhydryl are required to increase the formation of acetaminophen-glutathione (9). Our recent studies of glutathione kinetics following a toxic dose of acetaminophen further questioned the importance of this mechanism of action (10). The synthesis of glutathione following a large dose of acetaminophen was found to be suppressed, rather than stimulated, as one would expect from a depletion of hepatic glutathione (11). Furthermore, some investigators have reported that *N*-acetylcysteine does not prevent the acetaminophen-induced depletion of glutathione suggesting that the antidote does not support glutathione synthesis in vivo (12).

A second potential mechanism of action is direct adduct formation between *N*-acetylcysteine and the reactive intermediate of acetaminophen, thereby preventing covalent binding and cell injury. In microsomal incubations, the reactive metabolite of acetaminophen indeed readily forms adducts with a variety of sulfhydryl anions including the *N*-acetylcysteine anion (13). However, the incubation of isolated mouse hepatocytes with acetaminophen and *N*-acetylcysteine does not result in the formation of the *N*-acetylcysteine adduct of acetaminophen (8, 9). In vivo, the administration of *N*-acetylcysteine only moderately increases the formation of the *N*-acetylcysteine adduct of acetaminophen (mercapturic acid) in overdosed patients (14) and mice (4, 15). Thus it is not clear whether direct adduct formation is an important protective mechanism in vivo.

As a third possibility, *N*-acetylcysteine might protect by increasing the availability of inorganic sulfate, thus increasing the formation of acetaminophen-sulfate and simultaneously decreasing the fraction of acetaminophen metabolized to the toxic intermediate (16).

Fourthly, *N*-acetylcysteine could reduce the reactive intermediate of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine, back to acetaminophen, thereby preventing its reaction with glutathione and vital macromolecules. We have recently demonstrated that the reduction of the reactive metabolite by cysteine or ascorbic acid is a quantitatively important reaction in vitro and prevents covalent binding of the toxic intermediate to microsomal proteins (17).

From data obtained mostly in vitro, therefore, a number of potential mechanisms of action of *N*-acetylcysteine have been demonstrated. However, these mechanistic studies have not evaluated the importance of the potential interactions of *N*-acetylcysteine with the toxic process in vivo. To elucidate the mechanism of action in vivo, we have administered radioactively labeled *N*-acetylcysteine together with acetaminophen to rats to determine the contribution of exogenous *N*-acetylcysteine to the formation of the *N*-acetylcysteine adduct of acetaminophen (mercapturic acid) following a toxic dose of acetaminophen. In addition, we have tested the hypothesis that *N*-acetylcysteine may protect by reducing the toxic metabolite of acetaminophen.

METHODS

[^{35}S]-*N*-Acetyl-L-cysteine was prepared by treating [^{35}S]L-cystine (11.4 mCi/mmol, Amersham Corp., Arlington Heights, IL) with two equivalents of acetic anhydride and reducing the peracetylated disulfide intermediate with zinc and acetic acid according to the method of Sheffner et al. (18). Following the addition of unlabeled compound (Sigma Chemical Co., St. Louis, MO), [^{35}S]-*N*-acetylcysteine was recrystallized from water to a constant specific activity (58.1 dpm/nmol). Radiochemical purity exceeded 99% judging from thin-layer chromatography of the material on Avicel (Analtech Inc., Newark, DE) developed in *n*-butanol/acetic acid/water, 4:1:1 (retardation factor, R_f, 0.61). The aqueous portion of the mobile phase also contained 1 g/liter Na_2EDTA and 0.1 g/liter KCN to inhibit metal-catalyzed oxidation of thiols (18).

In order to test the hypothesis that *N*-acetylcysteine protects in vivo by formation of an adduct with the reactive intermediate of acetaminophen we compared the specific activities of the glutathione adduct excreted in bile and the *N*-acetylcysteine adduct (mercapturic acid) excreted in urine with the specific activity of the administered *N*-acetylcysteine. Male Sprague Dawley rats weighing 250–300 g (Timco Breeding Laboratories, Houston, TX) and having free access to food and water were studied. Under light ether anesthesia, the common bile duct was cannulated with PE-10 polyethylene tubing. A PE-50 polyethylene catheter was placed in the duodenum through a small incision, 0.5 cm

distal from the pylorus and fixed with a pursestring suture. After tying off the urethra, a similar catheter was placed into the bladder and taken out through the same midline abdominal incision as the other two catheters. The animals were then restrained and the experiment was started 1 h after the rats had awakened from the anesthesia. Acetaminophen (Eastman Organic Chemicals, Eastman Kodak Co., Rochester, NY), 1 g/kg dissolved in warm saline at a concentration of 30 mg/ml, was administered through the duodenal cannula, followed 30 min later by 1.2 g/kg of [^{35}S]-*N*-acetyl-L-cysteine (sp act 29 dpm/nmol) dissolved in saline (120 mg/ml) and adjusted to pH 7 with dilute NaOH. After the administration of the *N*-acetylcysteine bile and urine were collected for three 1-h periods.

For the determination of the specific activity of the glutathione-acetaminophen adduct, bile samples were subjected to high-pressure liquid chromatography on a C_{18}-μBondapak column (Water Associates, Milford, MA), with water/methanol/acetic acid 86.5:12.5:1, as solvent (19). Urine samples were analyzed for acetaminophen, the glucuronide and sulfate conjugates of acetaminophen, and the cysteine and the *N*-acetylcysteine (mercapturic acid) adducts of acetaminophen by chromatography on the same column with 10% methanol in 0.05 M sodium acetate, pH 4.4, as the solvent (15). The column effluent was collected in fractions and counted by liquid scintillation spectrometry with quench correction by the channels ratio method. Mass was determined by comparing peak areas of the metabolites of acetaminophen with a standard curve obtained with bile and urine, respectively, of a rat that had received [^{3}H]acetaminophen of known specific activity. The glucuronide, sulfate and sulfhydryl conjugates of acetaminophen were identified by cochromatography with reference standards (20) and were fully characterized enzymatically and by mass spectrometry (15, 19).

In order to test the hypothesis that *N*-acetylcysteine protects in vivo by reducing the metabolite *N*-acetyl-*p*-benzoquinoneimine back to acetaminophen, we measured the biliary excretion of glutathione disulfide and *N*,*N*′-diacetylcystine. Reduction of the intermediate by *N*-acetylcysteine in vivo would result in an increased formation of *N*,*N*′-diacetylcystine, and mixed disulfides of *N*-acetylcysteine and small molecular thiols, which in turn would generate glutathione disulfide by thiol-disulfide exchange mediated by thiol transferases (21). Rats were anesthetized with 50 mg/kg of pentobarbital and the duodenum and the common bile duct were cannulated. The temperature of the animals measured rectally was kept at 37.5±0.5°C with a heating lamp. Bile was collected in preweighed tubes containing 0.1 ml of 4% sulfosalicylic acid to prevent oxidation in vitro of glutathione (22). After three 15-min collection periods that established the base-line excretion of glutathione disulfide, 1 g/kg of acetaminophen (30 mg/ml) was administered intraduodenally. Bile was then collected in 20-min periods between 50 and 170 min after the administration of acetaminophen. One group of animals received 1.2 g/kg of *N*-acetyl-L-cysteine (120 mg/ml) i.d. 80 min after the administration of acetaminophen. The control groups received an equal volume of 0.9% saline instead of acetaminophen or *N*-acetylcysteine. An additional control group received 1 ml/kg of diethyl maleate in corn oil i.p. 80 min prior to the administration of 1.2 g/kg of *N*-acetylcysteine.

Total glutathione in bile was measured by the method of Tietze (23). Glutathione disulfide was measured by the same method following derivatization of reduced glutathione with 2-vinylpyridine (24). The addition of *N*-acetylcysteine to bile samples showed that *N*-acetylcysteine does not interfere with the enzymatic glutathione assay.

In similarly prepared animals, the biliary excretion of *N*-acetylcysteine and *N*,*N*′-diacetylcystine was measured between 30 and 45 min following the administration of 1.2 g/kg of *N*-acetylcysteine. In these experiments, the control group received saline instead of 1 g/kg of acetaminophen 80 min before the antidote. An additional control group again received 1 ml/kg of diethyl maleate prior to *N*-acetylcysteine.

N-Acetylcysteine in bile was assayed as the mixed disulfide with thionitrobenzoic acid by high pressure liquid chromatography by a modification of the assay described by Reeve et al. (25) with DL-penicillamine as internal standard (Fig. 1). To 25 μl of bile, 25 μl of 0.2 M phosphate buffer, pH 8.4, were added together with 10 μl of a 5-mM solution of DL-penicillamine followed by 50 μl of a 10-mM solution of 5,5′-dithiobis (2-nitrobenzoic acid). 10 μl of this reaction mixture were injected onto a C_{18} μBondapak column and eluted with 10% methanol in 0.023 M ammonium formate, pH 5.0, at a flow rate of 1.5 ml/min. The column outflow was monitored at 345 nm with a Waters model 450 Variable Wave Length Detector. For the determination of disulfides, the bile samples were incubated for 30 min with dithioerythritol (2 mM final concentration) at room temperature

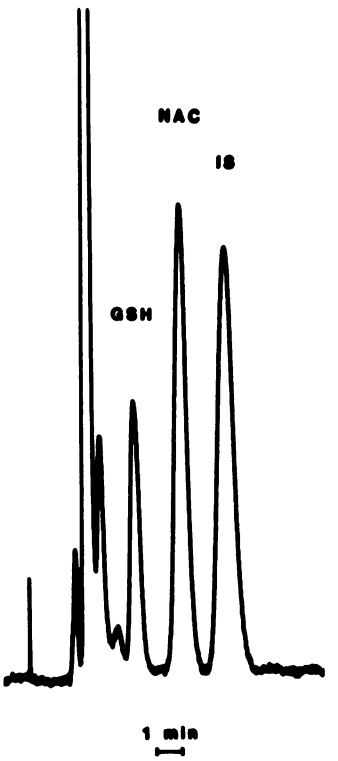

FIGURE 1 High pressure liquid chromatography tracing of 1 μl of bile of a rat that had received 1 g/kg of acetaminophen and 1.2 g/kg of *N*-acetylcysteine. The sulfhydryls are assayed as the mixed disulfides with 5-thio (2-nitrobenzoic acid). For conditions and derivatization procedure, see text. GSH, glutathione, NAC, *N*-acetylcysteine, IS, internal standard, DL-penicillamine. Absorbance units full scale: 0.04.

and the disulfide concentrations were calculated as the difference between the native and reduced samples. With this procedure 98±7% of glutathione disulfide added to bile were recovered as reduced glutathione as determined by the chromatographic assay described above.

The effect of *N*-acetylcysteine on glutathione synthesis and the intrahepatic concentration of glutathione was studied in the same animal model and in animals that had been fasted for 48 h. 30 min after the administration of 1 g/kg of acetaminophen i.d. the glutathione pool was labeled by the intravenous injection of 20 μCi of [^{3}H]glutamic acid [27 Ci/mmol, Amersham Corp.] (26). Bile samples were collected in 30-min periods and the specific activity of the biliary acetaminophen-glutathione was measured as described above. One group of animals received 1.2 g/kg of *N*-acetylcysteine i.d. and one group received saline 80 min after the acetaminophen. Hepatic glutathione was measured before and 80, 140, and 200 min after acetaminophen in similarly treated rats by the method of Tietze (23).

To study the incorporation of [^{35}S]-*N*-acetylcysteine into metabolites of acetaminophen rats were kept in metabolic cages for 24 h after administration of 1 g/kg of acetaminophen (30 mg/ml) followed 30 min later by 1.2 g/kg of [^{35}S]-*N*-acetylcysteine (120 mg/ml) by gavage. Because fed rats are relatively resistant to the hepatotoxic effects of acetaminophen, the animals were fasted for 48 h before the study in order to demonstrate a protective effect of *N*-acetylcysteine. Control animals received 0.9% saline instead of *N*-acetylcysteine. After 24 h, portions of the liver were processed for light microscopy (27) or homogenized in 9 vol of 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA for measurement of nonprotein sulfhydryls by the method of Ellman (28).

The metabolites of acetaminophen in the urine of the rats kept in metabolic cages were quantitated with the sodium acetate/methanol high pressure liquid chromatography system described above and *p*-fluorophenol as internal standard. The necrosis was graded as 0 = absent, 1+ necrosis = <6% of hepatocytes necrotic, 2+ = 6–25%, 3+ = 26–50% and 4+ = >50% of the hepatocytes necrotic as previously described (27). All data are expressed as mean±SE. The Wilcoxon rank sum test was used for statistical analysis.

RESULTS

As shown in Table I, only a very small fraction of the administered *N*-acetylcysteine appeared in the glutathione adduct and the *N*-acetylcysteine adduct of acetaminophen (mercapturic acid) during the first few hours after the toxic dose of acetaminophen when hepatic gluthathione depletion was at its maximum. In rats without biliary fistula, most of the glutathione adduct excreted in bile would eventually appear as the *N*-acetylcysteine adduct (mercapturic acid) in urine. The 2.73% of the administered dose of acetylcysteine excreted in the urine in 24 h as the mercapturic acid thus represent the sum of the adduct formed with glutathione and possibly with *N*-acetylcysteine directly. Both in the early hours and for the entire 24-h collection, the fractional incorporation of *N*-acetylcysteine into acetaminophen-sulfate or excreted free as *N*-acetylcysteine or inorganic sulfate, exceeded the fraction incorporated into the *N*-acetylcysteine adduct of acetaminophen by a factor of at least 5 (Table I). In 24 h, 13.25±1.57% of the administered dose of [^{35}S]-*N*-acetylcysteine was incorporated into the acetaminophen-sulfate fraction, and 2.73±0.22% into the mercapturic acid fraction.

TABLE I

Percent of the Administered Dose of [^{35}S]-N-Acetylcysteine Incorporated into the N-Acetylcysteine Adduct of Acetaminophen (Mercapturic Acid) and Acetaminophen-Sulfate Excreted in Urine and the Glutathione Adduct of Acetaminophen Excreted in Bile during the First 3 h following the Administration of 1 g/kg of Acetaminophen and 1.2 g/kg of [^{35}S]-N-Acetylcysteine

	Urine		Bile
	N-Acetylcysteine-adduct of acetaminophen (mercapturic acid)	Acetaminophen-sulfate	Glutathione-adduct of acetaminophen
h	%	%	%
0–1*	0.008±0.002	0.22±0.05	0.027±0.006
1–2	0.027±0.003	1.35±0.23	0.105±0.016
2–3	0.075±0.013	2.22±0.27	0.278±0.027

* Time after administration of 1.2 g/kg of [^{35}S]-*N*-acetylcysteine i.d. to rats with a biliary fistula and an indwelling bladder catheter. Acetaminophen, 1 g/kg, was administered i.d. 30 min before the *N*-acetylcysteine. The metabolites of acetaminophen were isolated by high pressure liquid chromatography as described in Methods. Mean±SE of six animals.

If the administered *N*-acetylcysteine served as a nucleophile itself in vivo and thus formed an adduct directly with the electrophilic metabolite of acetaminophen, the specific activity of the mercapturic acid excreted in urine would be expected to approach the specific activity of the administered *N*-acetylcysteine. Moreover, the specific activity also would be expected to exceed the specific activity of the glutathione adduct appearing in bile at least initially when the specific activity of the intrahepatic *N*-acetylcysteine must exceed the specific activity of the intrahepatic glutathione. However, the specific activity of the acetaminophen mercapturic acid amounted to only 3.35±0.98 (n = 5), 10.94±0.43, and 17.98±0.84% of the specific activity of the administered *N*-acetylcysteine during the first, second, and third hour after the administration of *N*-acetylcysteine. Furthermore, the specific activity of the acetaminophen mercapturic acid in urine was lower than the specific activity of the glutathione adduct in bile (Fig. 2). This indicates that virtually all of the labeled mercapturic acid in urine originated from labeled acetaminophen-gluta-

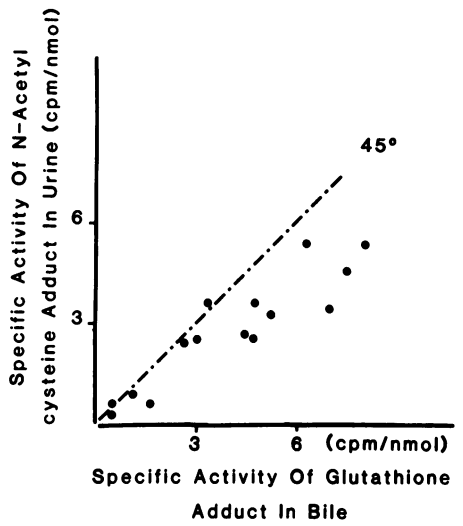

FIGURE 2 Specific activities of the glutathione adduct of acetaminophen excreted in bile and the *N*-acetylcysteine adduct of acetaminophen excreted in urine following the administration of 1 g/kg of acetaminophen and 1.2 g/kg of [^{35}S]-*N*-acetylcysteine 30 min later. Bile and urine were collected simultaneously at hourly intervals for 3 h after the administration of the labeled *N*-acetylcysteine from restrained rats with a biliary fistula and an indwelling bladder catheter. The specific activities were determined as described in Methods. The specific activity of the glutathione adduct equaled or exceeded the specific activity of the *N*-acetylcysteine adduct (mercapturic acid) at all time points indicating that virtually all labeled mercapturic acid originated from labeled acetaminophen-glutathione rather than from a direct reaction of *N*-acetylcysteine with the toxic metabolite of acetaminophen.

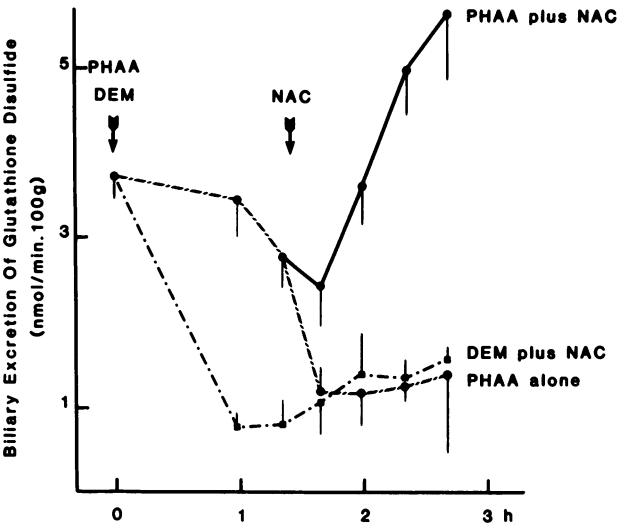

FIGURE 3 Biliary excretion of glutathione disulfide expressed as glutathione equivalents after the administration of 1 g/kg of acetaminophen i.d. or 1 ml/kg diethyl maleate i.p. followed 80 min later by 1.2 g/kg of *N*-acetylcysteine. The effect of acetaminophen alone (PHAA alone) on biliary glutathione disulfide is shown for comparison. The administration of *N*-acetylcysteine 80 min after saline did not affect the excretion of glutathione disulfide (not shown in figure). However, when given 80 min after acetaminophen, *N*-acetylcysteine resulted in a striking increase in the biliary excretion of glutathione disulfide (PHAA plus NAC). No statistically significant increase was observed when *N*-acetylcysteine was administered following diethyl maleate (DEM plus NAC). Mean±SE of four to six rats.

thione rather than from a direct reaction of *N*-acetylcysteine with the toxic metabolite.

To test the hypothesis that *N*-acetylcysteine reduces the reactive intermediate of acetaminophen back to acetaminophen and in the process increases the formation of disulfides, we measured the biliary excretion of glutathione and *N*-acetylcysteine disulfides. Increased intracellular concentrations of disulfides result in an increased formation of glutathione disulfide by thiol-disulfide exchange mediated by thiol transferases and a constant fraction of the thus generated glutathione disulfide will be released by the cell (29). As shown in Fig. 3, the administration of acetaminophen led to a progressive decrease in the biliary excretion of glutathione disulfide. The biliary excretion of glutathione (Fig. 4) decreased in parallel, reflecting the progressive intracellular depletion of glutathione. *N*-Acetylcysteine alone did not affect the biliary excretion of glutathione disulfide. However, when the same dose of *N*-acetylcysteine was administered 80 min after 1 g/kg of acetaminophen, the biliary excretion of glutathione disulfide increased promptly (Fig. 3). When *N*-acetylcysteine was administered to rats pretreated with diethyl maleate instead of acetaminophen, the biliary excretion of glutathione disulfide remained low although the hepatic concentration of glutathione increased from 0.43±0.02 to 4.38±0.43 μmol/g and the biliary excretion of reduced glutathione was not significantly different from the animals that had received acetaminophen prior to the *N*-acetylcysteine.

The possibility was further examined that the increased biliary excretion of glutathione disulfide after acetaminophen plus *N*-acetylcysteine, but not after acetaminophen alone, nor after diethyl maleate plus *N*-acetylcysteine, resulted from thiol-disulfide exchange with the *N*-acetylcysteine disulfides formed during reduction of the reactive intermediate of acetaminophen. Rats treated with acetaminophen and *N*-acetylcysteine excreted significantly more *N*-acetylcysteine disulfides in bile (2.68±0.75 nmol/min per 100 g, $n = 4$) than rats treated with *N*-acetylcysteine alone (0.43±0.11 nmol/min per 100 g, $n = 7$) and animals treated with diethyl maleate and *N*-acetylcysteine (0.14±0.02 nmol/min per 100 g). Because the assay is based on the reduction of disulfides by dithioerythritol, these values include *N*,*N*′-diacetylcystine

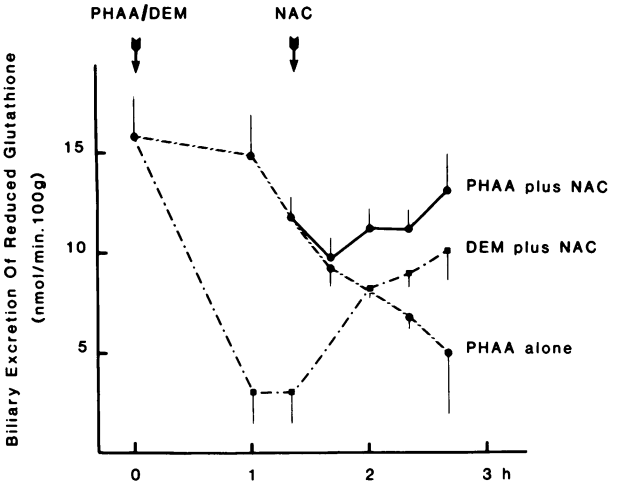

FIGURE 4 Biliary excretion of reduced glutathione following the administration of 1 g/kg of acetaminophen alone, and 1.2 g/kg of *N*-acetylcysteine 80 min after either acetaminophen or diethyl maleate. Acetaminophen (PHAA) resulted in a progressive decrease in the biliary excretion of glutathione reflecting the intrahepatic depletion of glutathione. The administration of *N*-acetylcysteine alone did not significantly alter biliary glutathione (not shown in figure). The biliary excretion of glutathione in the diethyl maleate-treated and acetaminophen-treated groups were not significantly different from 30 min after the administration of *N*-acetylcysteine onward. Mean±SE of four to six rats.

and mixed disulfides of *N*-acetylcysteine with small molecular thiols.

The specific activities of biliary acetaminophen-glutathione after labeling the glutathione pool with [^{3}H]glutamic acid and the hepatic concentration of glutathione following the administration of acetaminophen and *N*-acetylcysteine or saline are shown in Figs. 5 and 6. As expected, acetaminophen depleted the hepatic glutathione, but *N*-acetylcysteine led to a marked and prompt increase in hepatic glutathione (Fig. 6). Under steady-state conditions, the rate of synthesis of glutathione can be calculated from the hepatic concentration and the fractional rate of turnover reflected by the slope of the specific activity-time curve. Between 2 and 3.5 h after acetaminophen, the concentration of glutathione remained quite stable, thus permitting estimation of synthesis from the concentration and the fractional rate of turnover during that time interval. Thus, glutathione synthesis amounted to 0.54 μmol/h per g liver after acetaminophen, which is less than the synthesis in untreated control animals (10). In contrast, glutathione synthesis increased to 2.69 μmol/h per g following *N*-acetylcysteine and was probably even higher during the steep and rapid rise in hepatic glutathione immediately after administration of the antidote.

Because fasting imposes an additional stress on the glutathione pool, we attempted to repeat the same experiment with rats that had fasted for 48 h. The mortality of the fasted, anesthetized rats was high such that only the initial phase of the study could be evaluated. In contrast to the fed rats where *N*-acetylcysteine treatment did not significantly increase the biliary excretion of acetaminophen-glutathione adduct compared to saline-treated controls, the antidote significantly increased the biliary excretion of acetaminophen-glutathione in the fasted animals (Table II). This early increase in the partial clearance by the glutathione-mercapturic acid pathway in fasted animals was not apparent in the 24-h urine collections, although the administration of the antidote markedly increased the formation of acetaminophen sulfate with a concomitant decrease in acetaminophen glucuronide (Table III). The intrahepatic concentration of glutathione 24 h after the administration of acetaminophen

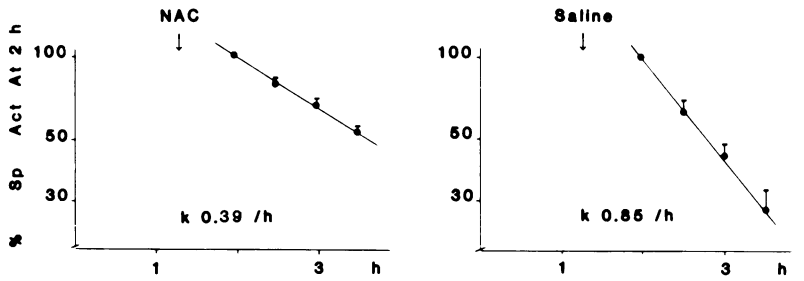

FIGURE 5 Specific activity of the acetaminophen-glutathione adduct excreted in bile after 1 g/kg of acetaminophen (administered at time 0) and 1.2 g/kg of *N*-acetylcysteine or saline, respectively. The glutathione pool was labeled by the intravenous administration of 20 μCi of [^{3}H]glutamic acid. Because of variable doses of radioactivity, the initial specific activities were not identical in all studies. The specific activities are, therefore, expressed as a percentage of the specific activity in the first bile sample. Each point represents mean±SE of four studies in four individual animals. The slope of the two curves represents the fractional rate of glutathione turnover, k.

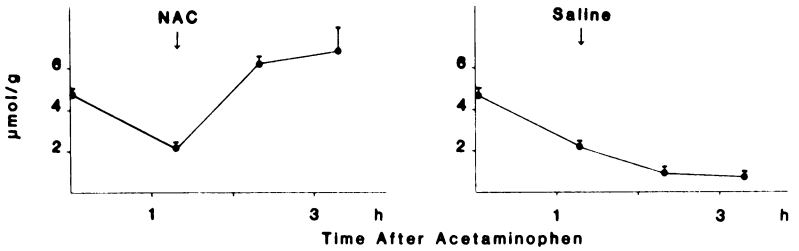

FIGURE 6 Hepatic concentration of glutathione after 1 g/kg of acetaminophen (administered at time 0) and 1.2 g/kg of *N*-acetylcysteine or saline, respectively. Each point represents the mean±SE of four animals. Glutathione was measured by the specific assay of Tietze (23). *N*-Acetylcysteine promptly reverses the acetaminophen-induced depletion of glutathione.

was 6.9±0.6 μmol/g in the animals treated with *N*-acetylcysteine and 6.7±0.8 μmol/g in the rats that received acetaminophen alone. Four of the five animals treated with acetaminophen and *N*-acetylcysteine had no necrosis detectable by light microscopy and one was graded 1+. In contrast, 5 of the 12 animals that received acetaminophen alone had 4+ necrosis, six had 3+ necrosis, and one had a score of 2+.

Simulations

To estimate the effect of an increased availability of sulfate on the formation of the various metabolites of acetaminophen, the generation of the metabolites was simulated using the kinetic model shown in Fig. 7. Estimates of the rate constants for the formation of each metabolite were based on the data of Price and Jollow (30). In one model, glucuronidation was assumed to follow first-order kinetics (20). In a second model, glucuronidation was assumed to be capacity limited and parameter estimates were based on data of Galinsky and Levy (31). Following large doses of acetaminophen, sulfate is rapidly depleted and sulfation is then limited by the mobilization of sulfate, estimated at 50 μmol/h per kg (31). Increasing the availability of sulfate will increase sulfation in a manner consistent with Michaelis-Menten kinetics. In the simulations, the value for the apparent maximal rate of sulfation, V_{max},[1] was therefore gradually increased to reflect an increased availability of sulfate while keeping all other parameters constant. The set of differential equations was integrated using the program INTDIFF of PROPHET (32). The model predicts that within 24 h, 48% of a dose of 1 g/kg of acetaminophen will be metabolized to the glucuronide, 11% to the sulfate and 5% to the reactive metabolite, most of which will appear as the *N*-acetylcysteine adduct, whereas 22% will be eliminated as free acetaminophen and 14% will not have been metabolized as yet (Fig. 8a). Comparable percentages are obtained when glucuronidation is assumed to be capacity limited (Fig. 8b). Similar fractions of administered dose would be expected to appear in urine over the same period of time, and both models are thus quite consistent with our experimentally determined data (Table III). With increasing availability of sulfate (reflected by V_{max} in the model), the formation of acetaminophen-sulfate increases. As expected, the formation of the other metabolites decreases concomitantly (Fig. 8). Although the generation and renal elimination of metabolites formed by first-order processes decreases proportionally, increased sulfation has little effect on the absolute amounts of metabolites formed via relatively minor pathways. Thus, doubling the maximal rate of sulfation decreases the formation of the reactive metabolite only from 5.4 to 5.0% (Fig. 8a) and from 6.1 to 5.6% (Fig. 8b) of the administered dose in the two models, a reduction that is not meaningful toxicologically.

TABLE II
Effect of N-Acetylcysteine on the Biliary Excretion of Acetaminophen-Glutathione in Fed and Fasted Rats°

Fed	ad lib.	NAC	102.4±5.1	nmol/min per 100 g
		saline	106.6±6.4	nmol/min per 100 g
Fasted	for 48 h	NAC	104.5±5.9	nmol/min per 100 g
		saline	56.9±13.4‡	nmol/min per 100 g

° All animals received 1 g/kg of acetaminophen i.d. 80 min later, *N*-acetylcysteine (NAC), 1.2 g/kg, or a corresponding volume of saline was administered by the same route, and bile was collected for 1 h. The biliary excretion of acetaminophen-glutathione was measured by high pressure liquid chromatography.
‡ Significantly different from the three other groups ($P < 0.05$).

DISCUSSION

N-Acetylcysteine is an effective antidote against liver injury produced by acetaminophen in animal models (3, 4, 33) and in clinical practice (5, 7). The successful treatment of an overdose of acetaminophen with *N*-

[1] *Abbreviations used in this paper*: V_{max}, apparent maximal rate of sulfation.

TABLE III

Metabolites of Acetaminophen Excreted in Urine in 24 h (Micromoles/100 g per 24 h)

	n	Acetaminophen-glucuronide	Acetaminophen-sulfate	Cysteine adduct of acetaminophen	Free acetaminophen	Acetaminophen mercapturic acid
Acetaminophen° plus saline	12	325±13	85±15	2±1	152±11	41±2
Acetaminophen‡ plus *N*-acetylcysteine	5	200±19	211±17	4±1	123±17	30±2

° All animals were fasted for 48 h and received 1 g/kg of acetaminophen per os.

‡ *N*-Acetylcysteine, 1.2 g/kg, was administered per os 30 min after 1 g/kg of acetaminophen.

acetylcysteine seemingly confirms the rationale of the therapeutic intervention with sulfhydryls, which were thought to promote glutathione synthesis or to form direct adducts with the reactive intermediate of acetaminophen (1, 2).

In microsomal incubations lacking glutathione and glutathione transferases, *N*-acetylcysteine forms an adduct with the reactive metabolite of acetaminophen (13). The protective effect of *N*-acetylcysteine in vivo might thus conceivably be due to direct conjugation, although no adduct formation has been observed in isolated mouse hepatocytes (8, 9). However, it is evident from our data that the specific activity of the glutathione adduct exceeded the specific activity of the mercapturic acid at all times, indicating that most of the labeled mercapturic acid originated from labeled glutathione. The small differences in the specific activities between the glutathione adduct and the mercapturic acid are probably due to the delay in the appearance of mercapturic acid in urine compared to the appearance of the glutathione adduct in bile. The comparison of the specific activities of the administered *N*-acetylcysteine and the mercapturic acid of acetaminophen appearing in urine also supports the conclusion that most of the labeled mercapturic acid originated from the glutathione adduct. Even shortly after the administration of the antidote, when the total body pool of *N*-acetylcysteine must have the same specific activity as the administered *N*-acetylcysteine, the specific activity of the mercapturic acid still amounted to only 3–18% of the specific activity of the administered *N*-acetylcysteine. At a time when the specific activity of the hepatic glutathione pool is still low, the specific activity of the mercapturic acid in urine should approach the specific activity of the administered *N*-acetylcysteine if substantial direct conjugation of the reactive intermediate of acetaminophen with *N*-acetylcysteine occurs. At a later time, the specific activity of the glutathione pool (and thus of the mercapturic acid excreted in urine) would be expected to approach the specific activity of the administered *N*-acetylcysteine because the amount of cysteine liberated from *N*-acetylcysteine used for glutathione synthesis is so large compared to the endogenous free cysteine pool. This explains why ~20 of the 30 μmol of acetaminophen-mercapturic acid excreted in 24 h contained cysteine originating from the administered *N*-acetylcysteine.

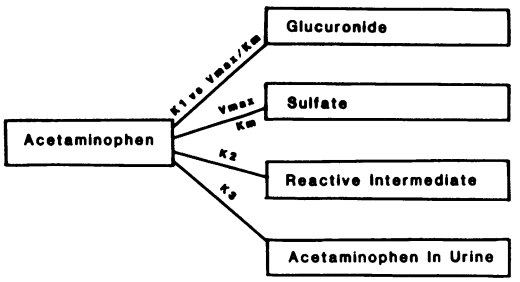

FIGURE 7 Kinetic model and kinetic parameters used to simulate the effect of an increased availability of sulfate on the metabolism of acetaminophen. Estimates of the rate constants for the formation of each metabolite and the rate of urinary excretion of acetaminophen are based on published data (30). The formation of the glucuronide was alternatively assumed to be first-order (k_1 = 0.045 h^{-1}) or capacity-limited (V_{max} 192 μmol/kg per h, k_m 2.7 mmol/kg) (31). The rate constant for the formation of the reactive intermediate, k_2, was assumed to be 0.005 h^{-1}, and the constant for renal elimination of acetaminophen k_3 = 0.02 h^{-1}. Increasing the availability of sulfate will increase sulfation in a manner consistent with Michaelis-Menten kinetics. The V_{max}, was assumed to be 50 μmol/kg per h in the basal state and was gradually increased to simulate an increased availability of sulfate. k_m = 2.15 mmol/kg.

The administration of 1 g/kg of acetaminophen leads to a rapid depletion of hepatic glutathione that reaches a nadir within 1 h. Because glutathione synthesis is regulated by a feedback mechanism (34, 35), this depletion should markedly stimulate glutathione synthesis and thus, increase the requirement for glutathione precursors. As the rate of glutathione synthesis is stimulated, the availability of cysteine, which is present in much lower concentrations than glycine or glutamic acid, may become the rate-limiting step in glutathione synthesis (36). This may be particularly true when the cysteine moiety of glutathione cannot be recovered via the gamma-glutamyl cycle as it prob-

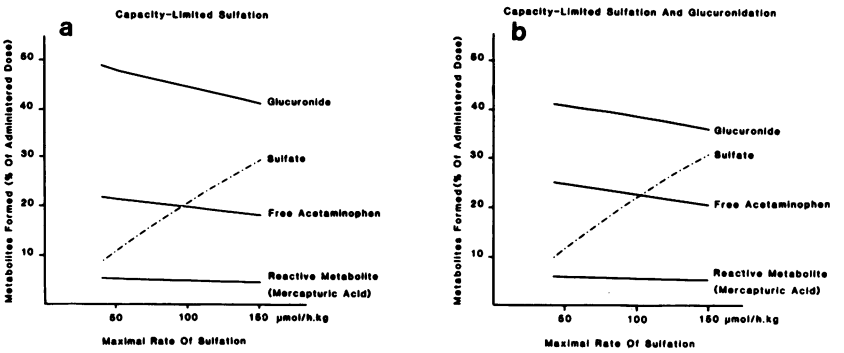

FIGURE 8 Fraction of a dose of 1 g/kg of acetaminophen metabolized to the glucuronide and sulfate conjugate and the reactive metabolite and excreted as free acetaminophen as a function of the availability of sulfate reflected by the V_{max} of the sulfate conjugation. a. Rate of glucuronide formation first order. b. Rate of formation of glucuronide capacity limited. With increasing V_{max} the formation of acetaminophen-sulfate increases and the formation of other metabolites decreases. However, doubling V_{max} from 50 to 100 μmol/kg per h decreases the formation of the reactive metabolite only from 5.4 to 5.0% of the administered dose and from 6.1 to 5.6% if glucuronidation is assumed to be capacity limited. This minimal decrease in the formation of the reactive metabolite resulting from the increased availability of sulfate following the administration of *N*-acetylcysteine cannot account for the protective effect of *N*-acetylcysteine.

ably is during physiologic catabolism of glutathione. The cysteine in the acetaminophen-glutathione adduct is lost as the cysteine conjugate and the mercapturic acid of acetaminophen and thus does not reenter the precursor pathway. Indeed, glutathione synthesis is decreased shortly after a large dose of acetaminophen (10). The increased requirements for sulfate during the metabolism of acetaminophen also compete for the remaining available sulfur amino acids and thus further decrease the synthesis of glutathione. This may explain why after diethyl maleate, which forms a glutathione conjugate but is not sulfated, glutathione synthesis can increase without supplementation of cysteine precursors (11). In our studies, the hepatic synthesis of glutathione promptly increased after *N*-acetylcysteine administration. The kinetic data are supported by the observation that the hepatic concentration of glutathione markedly increased after *N*-acetylcysteine, whereas the loss of glutathione via adduct formation remained unchanged.

N-Acetylcysteine has also been shown to prevent acetaminophen-induced depletion of hepatic glutathione in the mouse (37). This effect was attributed to delayed gastric emptying of acetaminophen resulting in decreased absorption of acetaminophen (37). Such a mechanism can clearly not account for our data because the drug was administered intraduodenally, and the biliary excretion of acetaminophen-glutathione in *N*-acetylcysteine-treated and control animals was either the same in fed rats or even increased in fasted animals.

In the face of an increased hepatic concentration of glutathione, one might expect the biliary excretion of acetaminophen-glutathione to increase. However, based on the recent data of Price and Jollow (30), the observed biliary excretion of acetaminophen-glutathione in fed rats corresponds to the estimated rate of formation of the reactive intermediate; thus the formation and excretion of the glutathione adduct is not limited by the availability of glutathione in the fed state. In the fasted state, however, the availability of glutathione becomes the rate-limiting step in the formation of the glutathione adduct (Table II). Because the rate of formation of the reactive metabolite decreases as the concentration of acetaminophen decreases and because the rate of synthesis of glutathione is similar in fed and fasted rats after acetaminophen (10), the availability of glutathione will be insufficient only for the short period of time in which high acetaminophen concentrations lead to a large amount of metabolite. These results are consistent with other animals models wherein *N*-acetylcysteine does not provide protection if administered 2–3 h after a toxic dose of acetaminophen. Since the metabolism of acetaminophen proceeds for many hours following a large dose of acetaminophen, a brief increase produced by *N*-acetylcysteine administration in the formation of the mercapturic acid for only a short, but critical period of time will not be apparent when the sum of the metabolite formed over a 24-h period is evaluated.

In support of the present rat experiments, *N*-acetylcysteine does not significantly increase the excretion of acetaminophen-mercapturic acid in mice either (15). In earlier reports to the contrary (4), the inability

to resolve completely the urinary metabolites of acetaminophen by the chromatographic method used may have been responsible for the minimal increase in mercapturic acid excretion observed after *N*-acetylcysteine administration. In man, the antidote appears to slightly increase the urinary excretion of acetaminophen-mercapturic acid (14). In these studies, however, more acetaminophen was recovered from the patients treated with *N*-acetylcysteine, suggesting that these patients ingested a larger dose of the analgesic, which would explain the increase in acetaminophen-mercapturate.

The extent of cell damage presents a major problem in interpreting data on the formation of metabolites, not only in vitro, but also in vivo, particularly when dealing with models that are very sensitive to the hepatotoxic effect of acetaminophen. Dead cells will no longer be metabolically active. Thus, if *N*-acetylcysteine by maintaining cell viability, results in the formation of more glucuronide, sulfate and sulfhydryl conjugates (8), its protective effect is not necessarily causally related to an increased conjugation of acetaminophen.

The observation that *N*-acetylcysteine and acetaminophen, but neither of the compounds alone, increase the biliary excretion of glutathione disulfide suggests that *N*-acetylcysteine, in addition to increasing glutathione synthesis, reduces a metabolite of acetaminophen. *N*-acetylcysteine did not increase the biliary excretion of disulfides in animals pretreated with diethyl maleate in order to deplete glutathione to a similar degree, as in acetaminophen-treated animals. This indicates a specific interaction of the antidote with a product of acetaminophen metabolism. In vitro, sulfhydryls as well as ascorbic acid reduce the ultimate toxic metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine back to acetaminophen (17, 38), and it is likely that a similar reduction of the benzoquinoneimine occurs with *N*-acetylcysteine in vivo. Indeed, the biliary excretion of *N*-acetylcysteine disulfides was significantly higher in animals pretreated with acetaminophen. Although a similar reduction should theoretically occur with glutathione, the presence of glutathione transferases in vivo will strongly favor the formation of the glutathione adduct of *N*-acetyl-*p*-benzoquinoneimine, i.e., acetaminophen-glutathione, rather than the reductive pathway (17, 39). The quantitative importance of this detoxification pathway cannot be readily assessed. Because of intracellular thiol-disulfide exchange and effective reduction of glutathione disulfide by glutathione reductase, only a small fraction of the generated disulfides will be excreted in bile (40, 41) but the measured increment in biliary disulfides could well account for the decrease in covalent binding of acetaminophen that is associated with the protection by *N*-acetylcysteine (42). If the reductive mechanism contributed significantly to the protective effect of *N*-acetylcysteine one would expect a decreased formation of the glutathione adduct that clearly did not occur. On the other hand, the decrease in the intracellular thiol/disulfide ratio resulting from the reductive pathway will not only lead to an increased formation of glutathione disulfide but also to an increased formation of protein-mixed disulfides that could protect nucleophilic sites on proteins from the electrophilic intermediate of acetaminophen. Further studies will have to assess the quantitative significance of the reductive pathway.

Approximately five times more of the administered radioactivity ended up in the sulfate fraction than in the *N*-acetylcysteine adduct (mercapturic acid) and the excretion of acetaminophen-sulfate markedly increased following *N*-acetylcysteine. The increased partial clearance of acetaminophen via the sulfation pathway would be expected to decrease the fraction of the administered dose passing through the toxifying pathway and may account for the minimal shortening of the half-life observed after relatively low doses of acetaminophen and *N*-acetylcysteine (16). Increased sulfation has, therefore, been proposed to be a possible mechanism of action of *N*-acetylcysteine in preventing the hepatotoxicity of acetaminophen (16). Although high doses of sodium sulfate decrease the mortality from large doses of acetaminophen in mice, we have not been able to demonstrate an actual protective effect against the hepatotoxicity of acetaminophen (Lauterburg and Mitchell, unpublished data). The reason for the lack of a hepatoprotective effect of an increased availability of sulfate becomes evident from our simulation studies. Doubling or even tripling the formation of acetaminophen-sulfate slightly increases the rate of disappearance of acetaminophen but minimally decreases the fraction of a dose of acetaminophen metabolized to the reactive metabolite (Fig. 8). That *N*-acetylcysteine does not protect by increasing the sulfation of acetaminophen is supported by studies in mice where *N*-acetylcysteine prevents acetaminophen-induced hepatic necrosis without increasing the formation of acetaminophen-sulfate (15).

In conclusion, our data demonstrate that exogenous *N*-acetylcysteine does not form significant amounts of conjugate with the reactive metabolite of acetaminophen in the rat in vivo. The antidote provides a source of sulfate, thus stimulating the partial clearance of acetaminophen by the nontoxic sulfation pathway. Our data, in addition, demonstrate that *N*-acetylcysteine reduces the reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine, back to acetaminophen in vivo, although the quantitative significance of this pathway for protection is yet to be determined. Most impor-

tantly, however, *N*-acetylcysteine markedly increases the synthesis of glutathione following a large dose of acetaminophen, thus providing more substrate for the detoxification of the reactive metabolite by adduct formation during the short period of time when the critical cell injury occurs.

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